In vitro expression of long and short ovine prolactin receptors: activation of Jak2/STAT5 pathway is not sufficient to account for prolactin signal transduction to the ovine β -lactoglobulin gene promoter

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ABSTRACT

The recent finding that sheep had long (l-oPRLR) and short (s-oPRLR) prolactin receptors provided new tools to further explore prolactin signaling to target genes. Here we used CHO cells transfected with l-oPRLR or s-oPRLR cDNAs to compare the activation of known key steps of prolactin signaling by the two receptors. We found that prolactin stimulated l-oPRLR tyrosine phosphorylation, although it lacked the last tyrosine residue found in other long prolactin receptors. In addition, l-oPRLR and s-oPRLR both responded to prolactin stimulation by (1) Janus kinase 2 (Jak2) tyrosine phosphorylation, (2) DNA-binding activation of signal transducer and activator of transcription 5

(STAT5), (3) stimulation of transcription from a promoter made of six repeats of STAT5-responsive sequence. However, although it contains STAT5-binding consensus sequences, the ovine β -lactoglobulin promoter (−4000 to +40) was transactivated by l-oPRLR, but not by s-oPRLR. Taken together, our results indicate that activation of Jak2/STAT5 pathway alone is not sufficient to account for prolactin-induced transcription of this milk protein gene, and that sequences of its promoter, other than STAT5-specific sequences, account for the opposite transcriptional activation capabilities of l-oPRLR and s-oPRLR.

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INTRODUCTION

The prolactin receptor (PRLR) belongs to the superfamily of cytokine receptors. In mammals, cDNA cloning provided evidence that there were three kinds of PRLR. Long forms of PRLR have been described in the rat (Shirota *et al.* 1990), mouse (Moore & Oka 1993), human (Boutin *et al.* 1989), rabbit (Edery *et al.* 1989), and ruminant species: bovine (Scott *et al.* 1992), red deer (Jabbour *et al.* 1996) and ovine (Bignon *et al.* 1997). A particular feature of the cytoplasmic domain of ruminant long (l)-PRLR is that it terminates about 40 amino acids before that of the other species. The cDNA for a mutant form with an intermediate size was cloned from rat NB2 cells (Ali *et al.* 1991). Finally, short forms have been described originally in rodents (Boutin *et al.* 1988, Davis & Linzer 1989) and, more recently, in ruminants (Bignon *et al.* 1997, Schuler *et al.* 1997). Comparative genomic

analysis demonstrated that rodents and ruminants used different alternative splicing mechanisms to produce short forms of prolactin receptor (Bignon *et al.* 1997).

In vitro expression of rodent cDNA coding sequences revealed that long and intermediate, but not short forms, had the capacity to transduce the prolactin signal to a milk protein gene promoter (Ali *et al.* 1992, O'Neal & Yu-Lee 1994). During the past few years, key steps of prolactin signaling to target genes have been uncovered. These involve mainly Janus kinase 2 (Jak2) and signal transducer and activator of transcription 5 (STAT5). Jak2 is a quiescent cytoplasmic tyrosine kinase, constitutively associated with long (Dusanter-Fourt *et al.* 1994), intermediate (Campbell *et al.* 1994), and short (Lebrun *et al.* 1995a) prolactin receptors, which becomes transiently tyrosine phosphorylated upon ligand binding. STAT5 is assumed to be the specific substrate of activated Jak2, at least *in vitro*

(Gouilleux *et al.* 1994). By homology with other STATs, the N-terminal half of the molecule is considered as the DNA-binding domain, followed by SH3-like, SH2 and activation domains (Ihle 1996, Moriggl *et al.* 1996, Stoecklin *et al.* 1997). Y694, which is located between SH2 and activation domains of STAT5, has been shown to be the target of Jak2 tyrosine kinase activity (Gouilleux *et al.* 1994). Once Y694 is phosphorylated, STAT5 dimerizes owing to intermolecular pY694/SH2 interactions and translocates into the nucleus where it promotes transcription of target genes. Two STAT5 (a and b) have been cloned which are encoded by separate genes (Liu *et al.* 1995, 1996). Thus, homodimers (a/a or b/b) and heterodimers (a/b) potentially form following prolactin stimulation. Prolactin-induced STAT5a/b heterodimers have been described in NB2 cells (Kirken *et al.* 1997) and in mouse mammary gland (Liu *et al.* 1996) by Western blotting. On the other hand, the facts that COS-7 cells transfected with STAT5a transactivate a β -casein promoter-reporter construct to the same extent as do COS-7 cells transfected with STAT5b (Liu *et al.* 1995, Stoecklin *et al.* 1997), and that β -casein mRNA levels produced by homozygous STAT5a-deficient mice are almost identical to those produced by wild-type mice (Liu *et al.* 1997), indirectly suggest that functional homodimers also form upon prolactin stimulation. STAT5 binding consensus sequence (TTCNNNGAA) has been found in the promoter of most milk protein genes (reviewed in Vilotte & Soulier 1992). Evidence for a critical role of this sequence for target gene responsiveness to prolactin has come from deletion experiments (Schmitt-Ney *et al.* 1992, Demmer *et al.* 1995).

Until recently, short forms of prolactin receptors seemed to be restricted to rodents, and, consequently, *in vitro* functional comparative analyses were performed with rodent prolactin receptors only. The recent finding that ruminants also expressed short forms of prolactin receptor has provided new tools to further our understanding of prolactin signal transduction to milk protein genes.

In the present study, we have taken advantage of particular features of the ovine prolactin receptors. The cytoplasmic domain of the long form of the ovine prolactin receptor (l-oPRLR) is shorter than that of the rat (l-rPRLR) and of the rabbit (rbPRLR) by 38 amino acids, which removes the last tyrosine residue present in l-rPRLR (Y580) and rbPRLR (Y581). The cytoplasmic domain of the short form of the ovine prolactin receptor (s-oPRLR) is also shorter than that of the short

form of the rat prolactin receptor (s-rPRLR), and its C-terminal end shares only little homology with the rat sequence. The effects of these differences on the key steps of prolactin signal transduction described above were investigated in CHO cells transfected with l-oPRLR and s-oPRLR cDNAs. We suggest that our data provide evidence that, in addition to STAT5-binding sequences, other regions of target gene promoters are required for efficient prolactin signaling.

MATERIALS AND METHODS

Ovine prolactin

NIDKK AFP-8277E was provided by the National Hormone and Pituitary Program (Bethesda, MD, USA).

Plasmid constructs

For eukaryotic expression, the full-length coding sequences of long (1.75 kb) and short (0.9 kb) ovine prolactin receptor cDNAs (Bignon *et al.* 1997) were sub-cloned downstream of a glucocorticoid-dependent promoter in pLKneo, an eukaryotic expression vector bearing a resistance gene to G418 (geneticin, Gibco) (Hirt *et al.* 1992). The two constructs were called l-oPRLR-pLKneo and s-oPRLR-pLKneo respectively. To assess their functionality prior to selection of stable CHO cell transfectants, both constructs were checked by CAT assay in transiently transfected CHO cells, as described below. The rbPRLR-pLKneo construct has been described previously (Goupille *et al.* 1997).

Stable CHO cell transfectants

CHO cells were transfected with 10 μ g of either l-oPRLR-pLKneo or s-oPRLR-pLKneo with the calcium phosphate protocol, and transfectants were selected in the presence of 500 μ g/ml G418. Resistant clones were harvested and their capacity to bind ovine prolactin (oPRL) assessed as described below.

¹²⁵I labeling of oPRL

Ten micrograms of oPRL were incubated with 500 μ Ci ¹²⁵I-Na and 3.2 μ g chloramine-T for 1 min at room temperature with gentle shaking. Sodium metabisulfite (3.2 μ g) was then added to stop the reaction, and the iodinated hormone was separated from free iodine by gel filtration on a Sephadex G-25 column (PD10; Pharmacia Biotec, Orsay,

France), equilibrated (and eluted) with 25 mM Tris (pH 7.5), 10 mM MgCl₂ and 0.1% bovine serum albumin (BSA).

Binding experiments

Each stable CHO clone was grown to confluence in three 35 mm diameter plates. Sixteen to twenty-four hours before binding, rich medium (HamF12 supplemented with 10% fetal calf serum) was replaced by GC3 medium (Dulbecco's modified essential medium/HamF12: 50/50, v/v) containing 10⁻⁶ M dexamethasone to induce expression of the receptor from the glucocorticoid-dependent promoter of pLKneo (Hirt *et al.* 1992). The cells were then rinsed twice with phosphate-buffered saline (PBS) and incubated at room temperature in 1 ml PBS containing 0.1% BSA and 5 × 10⁵ c.p.m. ¹²⁵I-oPRL (three plates). Non-specific binding was assessed by saturating all prolactin-binding sites; this was achieved by co-incubating the labeling mixture with 1 µg unlabeled oPRL (one plate). After 4-h incubation, the cells were rinsed twice with 1 ml PBS containing 0.1% BSA, and lysed with 1 ml 3% SDS. The plates were rinsed twice with 0.5 ml water, which was pooled with the lysate. The radioactivity contained in the final 2 ml pool was counted, and the result for each clone was expressed as 'specific binding' for each clone. The specific binding (in c.p.m.) is defined as the mean value of radioactivity recovered from the duplicate plate incubated in the absence of unlabeled oPRL (whole binding) minus the radioactivity recovered from the plate incubated in the presence of unlabeled oPRL (non-specific binding).

Chloramphenicol acetyl transferase (CAT)

β-Galactosidase (β-gal) (Bignon *et al.* 1993, 1995) and luciferase (Sotiropoulos *et al.* 1996) assays have been described already. In brief, CHO cells or clones were seeded in four 60 mm diameter 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 with lipofectamine (Gibco, France), following the manufacturer's instructions, by PCH110, a plasmid encoding the β-gal activity (Pharmacia), and either by pBJ23, a plasmid bearing the (-4000 to +40) ovine β-lactoglobulin promoter-CAT reporter (for CAT assay) (Lesueur *et al.* 1990), or by LHRE-tk-luc, a luciferase reporter plasmid made of six repeats of rat β-casein STAT5-responsive sequence, upstream of a thymidine kinase minimal promoter linked to a luciferase reporter gene (Sotiropoulos *et al.* 1996) and, when indicated, by l-oPRLR-

pLKneo, s-oPRLR-pLKneo or rbPRLR-pLKneo. Transfected cells were then incubated for 24 (luciferase assay) or 72 (CAT assay) h in the presence (two plates) or absence (two plates) of 400 ng/ml oPRL in GC3 medium containing 10⁻⁶ M dexamethasone. The plates were washed with PBS, and the enzymatic activities measured as described (Bignon *et al.* 1993, Sotiropoulos *et al.* 1996). The results are expressed in fold induction, which is the CAT (or luciferase) activity (divided by the β-gal activity to take into account the transfection efficiency) ratio of stimulated to unstimulated cells.

Northern blotting

Northern blotting was essentially as described (Sambrook *et al.* 1989) and ³²P-labeled l-oPRLR cDNA was used as probe.

Western blotting

Cells were grown and starved as for binding experiments, except that five 100 mm diameter dishes were used per experimental point. The cells were incubated in the presence or absence of 500 ng/ml oPRL for 5 min, and then processed as described previously (Daniel *et al.* 1996). Goat anti-rbPRLR antibody 46 (Dusanter-Fourt *et al.* 1984) was used for immunoprecipitation. Anti-Jak2 (UBI) antibody was used for immunoprecipitation and blotting. Anti-phosphotyrosine antibody (UBI) was used for blotting only.

Electromobility shift assay (EMSA)

Electromobility shift assay (EMSA) was performed as described (Pierre *et al.* 1994, Goupille *et al.* 1997). In brief, 3 µg of nuclear extracts of cells that had been stimulated or not by prolactin for 15 min were incubated for 25 min at room temperature with ³²P-labeled double-stranded oligonucleotide GAGAATTCTTAGAATTTAAA. This sequence spans the region -104 to -85 of the rabbit α-S1 casein gene promoter and encompasses STAT5-binding site (in bold). The protein/DNA complex formed was separated from the free probe by electrophoresis in non-denaturing polyacrylamide gel. The result was evaluated by autoradiography of the dried gel after transfer onto DE81 paper. When indicated, a 50-fold excess of unlabeled probe, or 1 µg anti-STAT5a or b (UBI) or 1 µl anti-mammary gland factor (MGF) (Tourkine *et al.* 1995) antibody were incubated with the nuclear extracts for 15 min at room temperature, prior to addition of the labeled probe.

Statistics

Single pairwise comparisons were carried out by Mann–Whitney test.

RESULTS

Prolactin-dependent activation of the ovine β-lactoglobulin promoter transcription by the l-PRLR but not by the s-PRLR

In a first trial, transient transfection was used to assess the functionality of the constructs encoding the receptors. To that end, CHO cells were transiently co-transfected with PCH110 (encoding the β-galactosidase), pBJ23 (the β-lactoglobulin promoter–CAT reporter construct) and plasmids coding for the l-oPRLR, or the s-oPRLR, or the rbPRLR (Lesueur *et al.* 1991). The latter was used as a positive control. The three receptors are featured in Fig. 1A. Activation of the target β-lactoglobulin promoter by prolactin was measured in those cells. As expected, prolactin stimulated transcription of the β-lactoglobulin–CAT construct through the two long receptors (rbPRLR: 7.5 times and l-oPRLR: 5 times), but not through the short receptor (s-oPRLR) (Fig. 1B). These results are in agreement with those previously published on the rat long (l-rPRLR) and short (s-rPRLR) forms of prolactin receptor (Lebrun *et al.* 1995a) and, in two ruminant species, on the red deer (rdPRLR) (Jabbour *et al.* 1996) and bovine (l-bPRLR) (Schuler *et al.* 1997) long forms of prolactin receptor.

FIGURE 1. Functional characterization of l-oPRLR and s-oPRLR coding sequences by CAT assay in transiently transfected CHO cells. (A) Schematic representation of l-oPRLR (557 amino acids), s-oPRLR (272 amino acids) and rbPRLR (592 amino acids). Amino acid 1 is that of the mature protein, after signal peptide removal. The transmembrane domain is represented as a vertically hatched box. Tyrosine residues of the cytoplasmic domains are indicated, and two-headed arrows connect interspecies conserved residues. (B) Prolactin-induced transcription of the ovine β-lactoglobulin promoter. CHO cells were transiently co-transfected with PCH110 (encoding the β-galactosidase), pBJ23 (the β-lactoglobulin–CAT construct) and, as indicated, l-oPRLR–pLKneo, s-oPRLR–pLKneo, or rbPRLR–pLKneo. Prolactin-induced transcription of the CAT gene from the β-lactoglobulin promoter was conducted as described in Materials and Methods. The results are expressed as the mean of two independent experiments performed in duplicate, ± half the difference between them.

Studying prolactin receptor and Jak2 tyrosine phosphorylation requires high levels of expression of the receptor, which cannot be achieved by transient transfection in CHO cells. Therefore, stable CHO clones expressing l-oPRLR or s-oPRLR were selected. Figure 2A shows the binding capacity of some of these clones. Most of the following experiments were performed with l-oPRLR-expressing clone L11 and s-oPRLR-expressing clone S9, because they exhibited the highest binding capacities.

Expression and functionality of the two prolactin receptors were evaluated in those two cellular clones. First, Northern blot analysis of total RNA from L11 and S9 revealed easily detectable levels of expression of both transcripts whose sizes were estimated to be 2.6 and 1.8 kb respectively, in agreement with the size of the inserts (1.75 and 0.9 kb) (Fig. 2B). The stronger signal in the L11 lane was more likely to be due to the use of a probe hybridizing 100% with l-oPRLR transcript and only partially with s-oPRLR transcript, rather than to a lower expression of the short receptor, since S9 had greater prolactin-binding capacity than L11 (Fig. 2A). Second, L11 and S9 were tested in CAT assay for their ability to stimulate transcription of the β -lactoglobulin gene in a prolactin-dependent manner. Figure 2C shows 3.4-fold induction of the basic transcription in L11 cells and no induction in S9 cells.

Prolactin-dependent activation of Jak2 by l- and s-oPRLR

Transcription activation of a given gene by a cell membrane receptor is the ultimate step in a cascade of events, some of which have been described to be common to most members of the cytokine receptor superfamily. The first two steps are the concomitant tyrosine phosphorylation of the receptor and of Janus kinases (Jak1, Jak2, Jak3 or tyrosine kinase 2) (Schindler & Darnell 1995).

We therefore investigated which proteins were tyrosine phosphorylated, in response to prolactin, among those associated with the long or short receptors. With that aim, lysates of L11 and S9 cells, that had been stimulated or not by prolactin, were immunoprecipitated by anti-receptor antibody and blotted with anti-phosphotyrosine antibody. Figure 3A shows that prolactin induced l-oPRLR, but not s-oPRLR, tyrosine phosphorylation. Moreover, a 130 kDa protein was co-immunoprecipitated with l-oPRLR and s-oPRLR, and was tyrosine phosphorylated in either case in a prolactin-dependent manner.

Tyrosine phosphorylation of l-oPRLR was confirmed by a kinetic experiment. Extracts of L11 cells, that had been stimulated for different lengths of time by the same concentration of prolactin, were immunoprecipitated by anti-receptor antibody 46, and blotted with anti-phosphotyrosine antibody. Figure 3B shows that l-oPRLR tyrosine phosphorylation increased up to 5 or 10 min, and then decreased at 30-min incubation.

Jak2, a 130 kDa tyrosine kinase, has been shown to be constitutively associated with the prolactin receptor and to be tyrosine phosphorylated in response to prolactin (Dusanter-Fourt *et al.* 1994). Therefore, it was tempting to speculate that the 130 kDa protein detected in Fig. 3A was Jak2. This was addressed by immunoprecipitating lysates of L11 and S9 cells, which had been stimulated or not by prolactin, with anti-Jak2 antibody, and then blotting with anti-phosphotyrosine antibody. The results shown in Fig. 3C confirmed this hypothesis, and that Jak2 was tyrosine phosphorylated to the same extent by s- and l-oPRLR under prolactin stimulation. Thus, prolactin-induced Jak2 activation did not allow discrimination between l-oPRLR, which transduced the prolactin signal to the ovine β -lactoglobulin gene promoter and s-oPRLR, which did not.

Prolactin-dependent activation of STAT5 by l- and s-PRLR

After Jak2 and receptor tyrosine phosphorylation, the next step of signal transduction common to many members of the cytokine receptor superfamily is the recruitment and activation of STAT5. Once STAT5 has been tyrosine phosphorylated, it dimerizes and translocates to the nucleus where it binds to target promoters to initiate transcription (Gouilleux *et al.* 1994).

Since Jak2 activation by s-oPRLR was identical to that produced by l-oPRLR we next compared the capacity of the two receptors to stimulate the DNA-binding activity of endogenous STAT5 to STAT5-specific double-stranded oligonucleotide, in EMSA. Figure 4A shows that both l-oPRLR (lanes 1 and 2) and s-oPRLR (lanes 5 and 6) elicited such an effect in a prolactin-dependent manner. Moreover, comparable supershift patterns of the DNA/protein complexes were obtained in both instances, with anti-STAT5a and anti-STAT5b antibodies (compare lanes 3 and 4 with lanes 7 and 8). Interestingly, anti-STAT5b antibodies did not supershift the whole complex, the non-supershifted complex was therefore essentially made of STAT5a homodimers (lanes 3 and 7). The same reasoning applied to anti-STAT5a antibody supershifts, with

FIGURE 2. Characterization of CHO clones stably expressing l- and s-oPRLR. (A) Binding capacity of several CHO clones. L3, L11 and L18 are l-oPRLR-expressing clones; S2, S5 and S9 are s-oPRLR-expressing clones. The binding capacity is expressed in c.p.m., as defined in Materials and Methods. (B) Northern blot analysis of S9 and L11 RNA. The same amount (10 μ g) of total RNA of S9 and L11 cells was run in 1% agarose gel, transferred to nylon membrane, and hybridized to 32 P-labeled l-oPRLR cDNA. 28S and 18S indicate the migration of rRNA. The position of s-oPRLR and l-oPRLR mRNAs expressed from pLKneo is denoted by two arrows on the right of the blot. (C) Prolactin-induced transcription of β -lactoglobulin-CAT construct in S9 and L11 CHO clones. S9 and L11 cells were transiently co-transfected with PCH110 (encoding the β -galactosidase) and pBJ23 (the β -lactoglobulin-CAT construct), and transcription of the CAT gene from the lactoglobulin promoter was followed as described in Materials and Methods. The results are expressed as the mean $\pm$ s.e.m. of four independent experiments performed in duplicate, and statistical analysis of the data in Mann-Whitney tests indicate that L11 and S9 responses are significantly different ($P<0.05$).

FIGURE 3. Prolactin-induced tyrosine phosphorylation of l-oPRLR and of Jak2. (A) Extracts of S9 and L11 cells, that had been stimulated or not by prolactin for 5 min, were immunoprecipitated by anti-prolactin receptor antibody 46 (IP: 46), resolved by SDS-PAGE and blotted on nitrocellulose. The blot was revealed with anti-phosphotyrosine antibody (IB: α PY). The position of the molecular mass markers is indicated on the left, and that of l-oPRLR and of the 130 kDa associated protein on the right of the blot. (B) Kinetic of prolactin-induced tyrosine phosphorylation of l-oPRLR. L11 cells were stimulated by prolactin for different lengths of time and then processed as in (A). The duration of prolactin stimulation is indicated at the top of the blot, and migration of the 97 kDa molecular mass marker on the right. (C) Extracts of S9 and L11 cells, that had been stimulated or not by prolactin for 5 min, were immunoprecipitated by anti-Jak2 antibody (IP: α Jak2), resolved by SDS-PAGE and blotted on nitrocellulose. The blot was revealed with anti-phosphotyrosine antibody, and then with anti-Jak2 antibody, as indicated.

a STAT5b homodimer-enriched non-supershifted complex (lanes 4 and 8). The mainly homodimeric nature of STAT5 dimers in the non-supershifted complexes was strengthened by their slight mobility difference (compare lane 3 with lane 4, or lane 7 with lane 8), in agreement with STAT5a being seven amino acids larger than STAT5b (Mui *et al.* 1995). This difference was undetectable in the absence of antibody. Since prolactin-induced STAT5a/b heterodimers have been described in NB2 cells (Kirken *et al.* 1997) and in mouse mammary gland (Liu *et al.* 1996) by Western blotting, the band in lanes 2 and 6 could, in fact, be a mixture of STAT5a homodimers, STAT5b homodimers, and STAT5a/b heterodimers. However, we did not investigate this point, because we were unable to set up an experiment which could demonstrate that heterodimers detected by Western blotting have DNA-binding activity. Thus, l-oPRLR and s-oPRLR appeared to have the same capacity to promote STAT5 DNA binding. Consequently, this step did not provide an explanation for the different transactivation abilities of l-oPRLR and s-oPRLR with respect to transcription of the ovine β -lactoglobulin gene.

In order to check that these results were not unique to L11 and S9, other stable clones were screened by EMSA. Figure 4B shows that nuclear extracts of isolated CHO clones expressing l-oPRLR (L3 and L11) or s-oPRLR (S2, S5 and S9) all produced the same prolactin-induced DNA/protein complex. This complex was specific because it was not any more visible when the reaction mixture was preincubated with an excess of unlabeled DNA, and also because it was supershifted by anti-MGF antibody (which does not discriminate between STAT5a and b). Only quantitative differences (different band intensities) from one clone to another were noticeable which, interestingly, perfectly matched the prolactin-binding capacities of the clones (Fig. 2A). Incidentally, this indicated that the amount of cell surface receptors, irrespective of whether they were short or long, was the limiting step of STAT5 DNA-binding activation. Thus, STAT5 activation by L11 and S9 was not peculiar to L11 and S9, but was a feature of l- and s-oPRLR.

STAT5 DNA-binding activation parallels transcription of a strictly STAT5-dependent promoter

The fact that s-oPRLR could activate STAT5 DNA binding without increasing β -lactoglobulin gene transcription clearly established that prolactin-dependent transcription of this promoter was not

FIGURE 4. Prolactin-dependent activation of STAT5 DNA binding by l- and s-oPRLR. (A) Nuclear extracts of L11 and S9 cells, that had been stimulated or not by prolactin for 15 min, were incubated with ³²P-labeled double-stranded oligonucleotides as described in Materials and Methods, and then used in EMSA. When indicated, 1 μl anti-STAT5a or anti-STAT5b antibody was preincubated with the extracts. In those cases, the relative position of non-shifted STAT5a- and STAT5b-enriched homodimers is indicated on the right of the gel. (B) Nuclear extracts of several l- and s-oPRLR CHO clones, which had been stimulated or not by prolactin for 15 min, were processed as in (A). When indicated, 1 μl anti-MGF antibodies or 1 μg unlabeled DNA were preincubated with the extracts. The DNA/protein and supershifted complexes are indicated by an arrow on both sides or by a thin arrow on the right of the blot respectively.

only STAT5 dependent. We therefore speculated that, conversely, if a promoter was only STAT5 dependent, then it should be transactivated by both s-oPRLR and l-oPRLR, since STAT5 DNA-binding activation was equally mediated by l- and s-oPRLR. Such promoter constructs have been described already (Gao *et al.* 1996, Sotiropoulos *et al.* 1996, Stoecklin *et al.* 1997). One of them is made of six repeats of the rat β-casein STAT5-responsive sequence, located upstream of a thymidine kinase minimal promoter linked to a luciferase reporter gene (LHRE-tk-luc) (Sotiropoulos *et al.* 1996).

To test the above hypothesis, L11, S9 and F3 (a CHO clone stably expressing s-rPRLR (Boutin *et al.* 1988)) cells were transfected with LHRE-tk-luc construct, and prolactin-dependent increase of luciferase activity was measured. Figure 5A shows that LHRE-tk-luc transcription was activated by prolactin to the same extent (three times the level of unstimulated cells) by both l- and s-oPRLR. By contrast, transcription was not activated by prolactin when LHRE-tk-luc plasmid was transfected into F3, which promoted a barely detectable STAT5 DNA-binding activity under prolactin stimulation (Fig. 5B). Note that, in contrast with

FIGURE 5. Prolactin-dependent stimulation of LHRE-tk-luc transcription reflects STAT5 DNA-binding activation. (A) L11, S9 and F3 clones were transfected with PCH110 (encoding the β -galactosidase) and LHRE-tk-luc (a construct containing six STAT5 DNA-binding sequences, upstream of thymidine kinase minimal promoter and luciferase coding sequence), and prolactin-induced increase of luciferase gene transcription was followed as described in Materials and Methods. The results are expressed as in Fig. 1B, and are representative of one (F3) or two (L11 and S9) independent duplicate experiments. (B) Nuclear extracts of S9 and F3 cells, that had been stimulated or not by prolactin for 15 min, were processed as in Fig 4.

the fitting we observed between STAT5 DNA-binding activation and the amount of cell surface l- and s-oPRLR (see above), the different STAT5

DNA-binding activation capacities of S9 and F3 were not due to different levels, nor to different affinities for oPRL of the respective receptors, as confirmed by Scatchard analysis (K_d : 0.2 nM and 0.1 nM, 53 and 43 fmol binding sites/mg membrane proteins, for S9 and F3 respectively (not illustrated)). Thus, STAT5 DNA-binding activation correlated with transcription of a promoter that was only STAT5 dependent.

DISCUSSION

The availability of full-length coding sequences for l- and s-oPRLR provided us with an opportunity to functionally test signal transduction partners (ligand, receptors, target promoter) of the same species (sheep), making this cellular system more physiologically relevant than that we used before (Lesueur *et al.* 1990). In addition, the remaining components of the signaling pathway were endogenous, avoiding ligand-independent activations of Jak2 (Lebrun *et al.* 1995a, Gao *et al.* 1996) or of STAT5 (Moriggl *et al.* 1996) due to overexpression.

In a previous paper, l-oPRLR cytoplasmic domain was shown to end 38 amino acids before that of l-rPRLR and rbPRLR. As a consequence, the last tyrosine residue present in the latter two was missing (Y580, Y581) (Bignon *et al.* 1997). We show here for the first time that a wild-type long form of prolactin receptor, devoid of the last tyrosine residue found in l-rPRLR (Y580) and rbPRLR (Y581), is tyrosine phosphorylated, in a prolactin- and time-dependent manner comparable with that of the two longer receptors (Lebrun *et al.* 1995b, Waters *et al.* 1995). By contrast, and in agreement with data reported on s-rPRLR (Lebrun *et al.* 1995b), s-oPRLR is not tyrosine phosphorylated under the same conditions. The cytoplasmic domain of l-oPRLR contains nine tyrosine residues, and that of rbPRLR only six, five of which are conserved in the ovine sequence (Y237, Y383, Y407, Y488 and Y519, see Fig. 1A). In another paper, we showed that T451, a truncated rbPRLR lacking the last three tyrosines, was not any more tyrosine phosphorylated following prolactin stimulation (Goupille *et al.* 1997). Thus Y488 and Y519, which correspond in l-oPRLR to the first two of these three residues, are good candidates to account for l-oPRLR tyrosine phosphorylation. We anticipate that l-bPRLR (Scott *et al.* 1992) and rdPRLR (Jabbour *et al.* 1996), two ruminant l-PRLR exhibiting the same lack of Y580/Y581 as l-oPRLR, would also be tyrosine phosphorylated under the same conditions. In addition, l-oPRLR transduces the prolactin signal to a milk protein gene promoter,

as do l-bPRLR (Schuler *et al.* 1997), rdPRLR (Jabbour *et al.* 1996) and T451 (Edery *et al.* 1994). Therefore, Y580 phosphorylation appears to be an absolute requirement in the rat (Lebrun *et al.* 1995b), but not in ruminants or the rabbit, for prolactin signal transduction.

Our study also reveals that Jak2, a tyrosine kinase constitutively associated with long (Dusanter-Fourt *et al.* 1994) and NB2 (Campbell *et al.* 1994) prolactin receptors, is similarly tyrosine phosphorylated by l- and s-oPRLR, in response to prolactin. Jak2 was found constitutively associated with s-oPRLR (not shown) as it is with l-oPRLR. This is not surprising since the sequence required for Jak2 recruitment (Lebrun *et al.* 1995a, Gouilleux *et al.* 1997) is present in l- and s-oPRLR. The same result was previously reported for s-rPRLR in 293 cells; however, in that case, the cells had also been transfected with Jak2 construct (Lebrun *et al.* 1995a).

Jak2 activation is assumed to result in STAT5 tyrosine phosphorylation, a prerequisite for DNA binding of target promoters (Gouilleux *et al.* 1994). The long (Wakao *et al.* 1994) and intermediate (NB2) (Pallard *et al.* 1995), but not the short rPRLR (Gouilleux *et al.* 1994) have been shown to induce STAT5 DNA-binding activity. Accordingly, the results we obtained with s-rPRLR (F3) correlate a barely detectable STAT5 DNA-binding activation with the lack of LHRE-tk-luc induction (Fig. 5). This finding could explain, at least in part, the previously reported inability of s-rPRLR to transactivate the ovine β -lactoglobulin promoter/CAT construct (Lesueur *et al.* 1991). Conversely, the definite STAT5 DNA-binding activation by l- and s-oPRLR correlates with effective LHRE-tk-luc transactivation. Therefore, the inability of s-oPRLR to transactivate the ovine β -lactoglobulin gene promoter is not due to an inability of this receptor to promote STAT5 binding to the STAT5-responsive elements of this promoter. This also indicates that, when STAT5-responsive element is multimerized and used away from its native promoter, a negative transcriptional control is lost. In other words, our results strongly suggest that EMSA and experiments using LHRE-tk-luc provide exactly the same information. These artificial promoters should therefore be used with caution, particularly as to how far the results obtained apply to natural promoters, under the pretext that the latter contains the same consensus sequence. A similar discrepancy has been reported in other systems. For instance, STAT5-dependent inhibition of interferon regulatory factor I (IRF-1) transcription under prolactin stimulation is lost when three interferon γ activation sequence elements of IRF-1 promoter (it normally contains only

one) are located upstream of a minimal thymidine kinase promoter (Luo & Yu-Lee 1997). Another example is the loss of virus-specific induction of interferon β (INF β) promoter when six copies of the positive regulatory domain IV element of INF β promoter (which also contains only one normally) are clustered in a synthetic reporter (Thanos & Maniatis 1995).

Finally, the fact that a short prolactin receptor is able to activate STAT5 is in favor of STAT5 recruitment being mediated, at least in part, by Jak2.

Nevertheless, Jak2/STAT5 activation alone is not sufficient to account for enhanced β -lactoglobulin gene transcription by prolactin, since s-oPRLR is unable to transactivate the ovine β -lactoglobulin promoter, although it exhibits the same capacity as l-oPRLR to activate Jak2, STAT5 DNA binding and LHRE-tk-luc transcription. Similarly, binding of growth hormone and erythropoietin to their respective receptor in transfected COS-7 cells does not transactivate a (−344 to −1) β -casein promoter-luciferase construct, whereas it does activate STAT5 DNA binding (Gouilleux *et al.* 1995).

The above data could be summarized as follows: under prolactin stimulation, transcription of the original ovine β -lactoglobulin promoter is enhanced by l-oPRLR only, although immunoblots and EMSA respectively reveal an identical activation of Jak2 and of STAT5 DNA binding by l- and s-oPRLR. However, s-oPRLR recovers the same transcriptional efficiency as l-oPRLR when STAT5-binding DNA sequences are used in another context. Therefore, sequences of the native promoter other than STAT5-specific sequences account for the different transcription activation efficiency of the ovine β -lactoglobulin promoter by l- and s-oPRLR.

By contrast, the following potential explanations of the observed data have been disqualified. (1) STAT5 heterodimerization with other STATs has not been reported thus far. (2) In some instances, cytokine-induced modulation of STAT serine phosphorylation has been described to enhance STAT-dependent transcription (Wen *et al.* 1995, Lüttiken *et al.* 1995, Pircher *et al.* 1997). However, a possible prolactin-induced modulation of STAT5 serine phosphorylation could not explain the opposite signaling capacity of l- and s-oPRLR because this difference was not observed when LHRE-tk-luc was used as reporter (see Fig. 5). (3) The glucocorticoid receptor has been shown to enhance STAT5-dependent transactivation of the ovine β -casein promoter through a protein/protein interaction (Stoecklin *et al.* 1997). However,

identical DNA/protein complexes were detected in EMSA following prolactin stimulation of l- or s-oPRLR (Fig. 4), although the experiments were performed in all cases in the presence of dexamethasone. The same reasoning applies to the prolactin-induced LHRE-tk-luc transactivation (Fig. 5).

The next step of this study is therefore the identification of transcription factors and of their target DNA sequences in the ovine β -lactoglobulin gene promoter, and how the cytoplasmic domain of l-oPRLR, but not of s-oPRLR, either of growth hormone or erythropoietin receptors (Gouilleux *et al.* 1995) activates them. In this regard, s-oPRLR/l-oPRLR couple is a promising tool to characterize transcription factors utilized by l-oPRLR and not by s-oPRLR (activators), or transcription factors used by s-oPRLR and not by l-oPRLR (inhibitors).

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