Internalization of prolactin receptor and prolactin in transfected cells does not involve nuclear translocation

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SUMMARY

Prolactin (PRL) interacts with a specific, well characterized plasma membrane receptor (PRLR) that is coupled to signal transduction pathways involving Jak2, Fyn, and MAP kinases, and signal transducers and activators of transcription (STAT). Although a few previous studies have indicated nuclear translocation of PRL in IL-2 stimulated T lymphocytes, PRL-dependent Nb2 lymphoma cell lines and 235-1 lactotrophs, the mechanisms of nuclear targeting remain unknown and conflicting results have been reported concerning the putative nuclear translocation of the PRLR. We therefore decided to investigate nuclear translocation of PRLR and PRL in various cell lines transfected with an expression plasmid encoding PRLR, using confocal laser microscopy. We have constructed various cDNAs of the long and short forms of the rat PRLR containing an oligonucleotide encoding a Flag epitope inserted either just before the N-terminal amino acid or in the C-terminal end of the mature receptor (named N-terminal or C-terminal Flag-tagged PRLR). The corresponding receptors function as the PRLR in transfected cells: they are expressed at the plasma membrane and in compartments of the secretory pathway, they bind PRL with normal affinity ($K_d = 4 \times 10^{-10}$ M) and have the same capacity to stimulate the transcriptional activity of a milk protein ($\beta$-casein) gene as wild-type PRLR. In addition, the tagged receptors are much more efficiently immunodetected using anti-Flag antibodies, as compared to anti-PRL antibodies (U5 or U6). Immunofluorescence combined with detailed confocal laser microscopy showed that addition of PRL (0 to 12 hours) to COS-7, CHO and NIH-3T3 transfected fibroblasts induces rapid internalization of the receptor (long form), without any translocation to the nucleus. Using PRL-R tagged both in the N-terminal or C-terminal regions of the mature receptor excludes the possibility of a cleaved fragment which could have been subsequently imported into the nucleus. An absence of nuclear translocation of PRLR was also observed in a 293 cell line stably expressing the receptor, and in physiological targets for PRL, i.e. in Nb2 lymphoma cells expressing the Nb2 form of the receptor or in BGME mammary gland epithelial cells upon over-expression of a Flag-tagged PRLR. Similarly, the short form of the PRLR was not detected in nuclei of transfected COS cells upon PRL treatment. Clearly, our results provide evidence that internalization of the plasma membrane PRLR does not lead to nuclear translocation of the receptor, or part of it, in most fibroblasts and epithelial cells at physiological concentrations of PRL. Also, in co-localization experiments, PRL was internalized without nuclear translocation. Activation of STATs transcription factors and MAP kinases, as well as translocation of these proteins to the nucleus following their phosphorylation, probably remains the intracellular mechanism coupling stimulation to nuclear events.

Key words: Prolactin receptor, Prolactin, Nuclear translocation, Immunocytochemistry, Confocal microscopy

INTRODUCTION

Prolactin (PRL) is known to regulate growth and differentiation in a wide variety of cells (Nicoll and Bern, 1972), through its receptor (PRLR) which belongs to the superfamily of receptors known as hematopoietin/cytokine/GH-PRL receptors (Bazan, 1989). This family includes the receptors for interleukins (IL-2 to IL-15, but not IL-8), G-CSF, GM-CSF, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), erythropoietin (EPO), growth hormone (GH), prolactin (PRL) and thrombopoietin (TPO). The most conserved features of these receptors are in the extracellular, ligand binding domain (four cysteine residues and a Trp-Ser-X-Trp-Ser motif, WSXWS) and in the cytoplasmic membrane-proximal region (Box 1 and Box 2) (Murakami et al., 1991). Several forms of PRLR, differing in the length of their cytoplasmic domains have been described (Kelly et al., 1992): in the rat, these forms consist of a long form of 591 amino acids and a Nb2 form (an isoform lacking 198 amino acids), both of which are able to transmit a lactogenic signal, and a short form of 291 amino acids (Lesueur et al., 1991; Das and Vonderhaar, 1995).

Receptor dimerization (consisting of one molecule of prolactin and two molecules of receptor) appears to be the first step by which PRL mediates its various actions. The activation of the receptor (which does not possess an intrinsic tyrosine kinase) leads to the rapid phosphorylation of an associated cytoplasmic tyrosine kinase, Jak2, that is necessary for the phosphorylation of the PRL-R and signal transduction (for review
see Dusant-Fourt et al., 1994; Lebrun et al., 1994a; Taniguchi, 1995); Box 1 and the adjacent residues upstream of Box 2 in the cytoplasmic proximal region of cytokine receptors are sufficient for Jak kinase activation/activation (Ziemiecki et al., 1994; Lebrun et al., 1994b). Cytoplasmic STAT transcription factors (Darnell et al., 1994), especially STAT5 (or MGF for mammary gland factor) for PRL (Wakao et al., 1994), are one of the major down-stream targets of activated Jak kinase and have been shown recently to translocate into the nucleus.

Although endocytosis of PRL has been demonstrated in a variety of cell types (Josefsberg et al., 1979; Bergeron et al., 1983; Djiane et al., 1988), internalization and intracellular processing of PRL and PRLR remains to be elucidated. A number of studies have shown PRL, and possibly its receptor, to be localized in the nucleus (Clevenger et al., 1990a,b; Rao et al., 1993), accompanied by nuclear actions (Clevenger et al., 1991; Buckley et al., 1988; Rao et al., 1995). We were interested in clarifying the controversy concerning the nuclear translocation of the PRLR, as this could represent a new pathway of cellular signaling. We therefore decided to investigate nuclear translocation in cells transfected with the PRL receptor cDNA. Our laboratory has prepared a number of monoclonal antibodies to the extracellular domain of the PRL receptor that have been used, at relatively high concentrations, for immunodetection of the PRLR. In order to increase the sensitivity of detection, and to be sure that we only measured receptors expressed as a result of the transfected cDNA, we incorporated a Flag epitope either in the N-terminal or in the C-terminal regions of the receptor and used an anti-Flag antibody for immunofluorescence detection. Transfection of these Flag-PRLR cDNA(s) into several cell lines and the use of confocal microscopy allowed the precise localization of the prolactin receptor. We present strong evidence that PRL receptors (long and short forms) are not translocated to the nucleus in epithelial cells and fibroblasts, as well as in Nb2 lymphoma and BMGE bovine mammary epithelial cells, which are known as physiological targets of PRL. In view of these results, we also investigated the internalization of PRL in the same cells that have been transfected with the Flag PRLR cDNA. These results are discussed in relation to previous findings.

**MATERIALS AND METHODS**

**Construction of Flag-tagged prolactin receptors (Flag-PRLR)**

A sequence corresponding to a Flag epitope has been introduced in the cDNA encoding the long form of rat PRL-R, either at the N terminus between the signal peptide and residue 20 or at the C terminus of the receptor. These constructs were generated by polymerase chain reaction (PCR) amplification of PRL-R cDNA in the presence of oligonucleotide primers. We have termed these receptors N-Tagged or C-Tagged PRLR, respectively.

**N-terminal Tag PRLR (long form)**

The sequence corresponding to the Flag epitope has been introduced after the sequence encoding the signal peptide, using 3 successive steps which include deletion of the signal peptide of PRLR, addition of Flag epitope and reintroduction of signal peptide between the initiation methionine and the Flag epitope. The first PCR reaction involves a 5' oligonucleotide primer containing the sequences recognized by EcoRI and the codons for an initiation methionine and the amino acids 20-24 (Gln-Ser-Pro-Pro-Gly; see Shirota et al., 1990 for the abbreviation, following the signal peptide) of wild-type PRLR (5' GTTAA CGAATT CATGCA GCT CACCAC CAGG 3'). The second PCR reaction involves a 5' oligonucleotide primer containing the sequences for the same restriction site EcoRI, a methionine initiation codon, codons for the sequence (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) of the Flag octapeptide and the first amino acids 20-24 previously mentioned (5' GTTAA CGAATT CATGACTCA AGGAC GACGATGAC AGCTCACCAC CAGG 3'). Two PCR reactions were used to reintroduce the coding sequence for the signal peptide between the EcoRI-methionine sequence and the Flag epitope. A common antisense oligonucleotide contained a sequence complementary to the region encoding amino acids 205-210 and a sequence encoding the Nco I restriction site. PCR products were isolated from agarose gels, digested with EcoRI-NcoI and ligated into the Bluescript expression vector and subsequently subcloned into the EcoRI-XbaI sites of the expression vectors pECE (Ellis et al., 1986) or pcDNA3 (Pharmacia).

N-terminal tagged PRLR (short form)

The cDNA encoding the short form of the rat PRL-R and a Flag epitope was generated by subcloning the EcoRI-NcoI fragment into the expression plasmid containing the short form of the PRLR.

**C-terminal tagged PRLR (long form)**

The sequence corresponding to the Flag epitope was introduced into the cDNA encoding the long form of the rat PRL-R before the stop codon. This construct was generated by polymerase chain reaction amplification of PRL-R cDNA in the presence of the following oligonucleotide primers. A sense oligonucleotide contained a sequence of the region encoding amino acids 539-545 and a sequence encoding the XhoI restriction site. The 3' antisense oligonucleotide primer contained the complementary sequence recognized by Xbal, the STOP codon, codons for the sequence of the Flag octapeptide and the last five amino acids, 587-591 (5' AAGCTTTCTAGATCA CTTGTCATCGTGCTCCTTGTAGTGGCA AGGATGTCAT 3'). PCR products were isolated from agarose gels, digested with XhoI-Xbal and ligated into the expression vectors pECE or pcDNA3.

Recombinant plasmids were introduced into Escherichia coli DH5 cells, which were selected for ampicillin resistance. Cultures of bacterial transformants were lysed and plasmid DNA was isolated by standard techniques. Sequence and orientation of the recombinant plasmids were confirmed by dideoxynucleotide analysis (Sanger et al., 1977).

**Cell culture**

COS-7 monkey kidney cells, NIH 3T3 fibroblasts and BMGE bovine mammary gland epithelial cells were grown as monolayers in serum-free Dulbecco’s modified Eagle medium containing 10% (v/v) fetal calf serum (DMEM; Sigma) at 37°C in a 5% CO₂ atmosphere. Chinese hamster ovary (CHO) cells were grown in Ham’s F12/10% FCS. All media were supplemented with glutamine (2.5 mM), penicillin and streptomycin (100 U/ml). A human embryonic kidney cell line (293), transformed by adenovirus (Ad5), was maintained in DMEM /Nut F12 (Gibco) supplemented with 10% (v/v) fetal bovine serum, 100 μg/ml of penicillin and streptomycin and 2.5 mM L-glutamine. Nb2 lymphoma cells, a rat pre-T cell line dependent upon PRL for proliferation, were maintained in RPMI-1640 medium containing 10% fetal calf serum (Sigma), penicillin-streptomycin (50 i.u./ml and 50 μg/ml, respectively) and 2 mM L-glutamine.

For PRL stimulation, confluent cells were incubated in serum-free medium overnight and stimulated at 37°C with 400 ng/ml ovine PRL (oPRL, a gift from NIDDK, NIH, Bethesda, USA).

**Transient and stable transfection**

Transient transfection

cDNAs encoding Flag-tagged and wild-type receptors under the transcriptional control of SV40 early promoter in pECE expression vector
were transiently transfected into CHO cells and NIH 3T3 fibroblasts using the calcium phosphate method (Southern and Berg, 1982; modified by Lesueur et al., 1991), or in COS-7 and BGM cells using the DEAE dextran method, as previously described (Goujon et al., 1994). Experiments (PRL stimulation) were performed 48 hours after transfection.

Generation of a stable transfectant in 293 cells

The plasmid pcDNA3 containing the cDNA encoding the Tagged receptor was introduced in 293 cells using the calcium phosphate method. After 2 days, G418 was added to the medium. Clones which were resistant to G418 Geneticin (Gibco, Grand Island, NY) at 25 µg/ml were selected and cultured in the presence of Geneticin.

β-casein/luciferase assay

The biological activities of the different forms of prolactin receptors were analyzed using a functional bioassay based on the cotransfection of CHO cells with the expression vector pECE containing cDNAs of the various forms of PRLR and with a construct that contains a milk protein (β-casein) gene promoter fused to the reporter gene of the firefly luciferase (plasmid β-casein/luciferase; Lochnan et al., 1995). CHO cells were grown in 60 mm culture dishes to 50-60% confluence. Twelve hours before transfection, cells were incubated overnight with GC3 medium (1:1 mixture of DMEM and Ham’s F12 supplemented with transferin (10 mg/ml), insulin (80 mU/ml), glutamine (2.5 mM), and non essential amino acids). Cells were then transfected by the calcium phosphate procedure with 3 µg of pCH110 (β-galactosidase expression vector; Pharmacia, Bromma, Sweden), 1.5 µg of the fusion gene construct containing the β-casein/luciferase coding sequence and 3 µg of PRLR cDNA/pECE cDNA. After incubation for 4 hours with the calcium phosphate precipitate, cells were subjected to a 14% (v/v) glycerol shock for 2 minutes and incubated in the presence of oPRL (18 nM) and dexamethasone (250 nM, Sigma Chemicals Co, St Louis, MO) or dexamethasone alone for 48 hours. Cells were lysed in lysis buffer (Promega), and centrifuged at 15,000 rpm for 5 minutes. β-casein/luciferase activity was measured in the supernatant as relative light units (Lumat LB 9501, Berthold); to correct for differences in transfection efficiencies between plates, luciferase activity was normalized to the β-galactosidase activity.

Indirect immunofluorescence

Transfected cells were grown as subconfluent monolayer cultures in Labtek chambers. For the experiments, cells were harvested for 3-12 hours in culture medium with 0.1% fetal bovine serum and incubated with oPRL (400 ng/ml) for 10 minutes to 12 hours; cells were washed 3 times with PBS and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 minutes or methanol (−20°C) for 5 minutes. Samples were successively incubated with blocking solution (goat serum diluted 1:40), mouse anti-Flag monoclonal antibody (Kodak; 10 µg IgG/ml) or mouse monoclonal antibody against the extracellular domain of the PRLR (US: 60 µg IgG/ml; Okamura et al., 1989) overnight at 4°C, then washed and further incubated for 45 minutes with a 1:40 dilution of FITC-conjugated goat anti-mouse IgG, as previously described (Perrot-Applanat et al., 1992). Cells were then mounted in 50% glycerol in PBS and observed on a Zeiss microscope. Specificity control experiments included incubation of cells in the absence of primary and/or secondary antibodies, or with IgG control monoclonal antibodies. No immunofluorescence was detected in any of the specificity control experiments.

A detailed confocal laser scanning microscopy was performed using a Bio-Rad MRC 600 (Bio-Rad Laboratories, Palo Alto, CA), as previously described (Perrot-Applanat et al., 1992, 1995), or a Leica TCS4D producing 0.5 µm thick optical sections. Micrographs were sent by Ethernet to a Macintosh computer for processing with Adobe Photoshop 3.0. Images were assembled and printed directly from the computer on a dye sublimation printer (Colorease, Kodak).

Immunofluorescence detection of PRL was performed in the same

PRLR transfected cells incubated with goat serum, rabbit polyclonal antibody against purified sheep PRL (ICN Biomedicals, Inc) and Texas Red-conjugated goat anti-rabbit IgG (Immunootech, Marseille, France). Specificity control experiments included incubation of transfected cells in the absence of primary and/or secondary antibodies, or incubation of non transfected cells with anti-PRLR antibodies. Comparison for immunostaining of PRL and PRLR were made on the same cells.

Binding analysis

125I-hGH was prepared using chloramine T, to a specific activity of 80-140 µCi/µg. The lactogenic hormone human GH binds to the PRLR with the same affinity as oPRL (Djiane et al., 1982). For binding assays, Flag-tagged and wild-type receptor/pECE cDNA were transiently transfected in COS-7 cells on a 100 mm culture dish at 60-70% confluence using the DEAE/dextran-chloroquine procedure, with 5 µg of expression plasmid. Cells were scraped and lysed by 3 freeze-thaw cycles in 25 mM Tris-HCl, pH 7.4, 10 mM MgCl2. After centrifugation at 50,000 g, membranes were incubated with 50,000 cpm 125I-hGH (3×106 cpm/µg hGH) in a total volume of 0.4 ml of the same Tris-HCl buffer containing 0.1% BSA. Incubation was carried out at 23°C for 12 hours; the microscope suspension was washed twice with ice-cold Tris-HCl, 0.1% PBS, and centrifuged at 5,000 rpm for 30 minutes. Radioactivity of the dried pellet was counted in a γ-counter. Specific binding was defined as the difference between total (in the absence of excess unlabeled ligand) and non specific (in the presence of excess ligand, 1 µg/ml) binding. Scatchard analysis was calculated from specific binding obtained in the presence of 125I hGH with increasing concentrations of unlabeled oPRL (0.1 ng to 10 µg).

Internalization assay

COS-7 cells were plated in 6-well plates (350,000 cells/well) and transfected at 60-70% confluence using the DEAE/ dextran-chloroquine procedure. Internalization studies were performed according to the procedure established by Vincent et al. (1997). Briefly, 48 hours after transfection cells were washed with HEPES binding buffer (HBB; 25 mM HEPES containing 125 mM NaCl, 4 mM KCl, 1 mM CaCl2, 1.5 mM MgCl2, 2 mM KH2PO4 and 1% BSA, pH 7.4) and incubated with 125I-hGH (80-100×106 cpm) in the presence or not of unlabelled PRL (2.5 µg/ml) in a 1 ml final volume of HBB for 6 hours at 4°C. Following this incubation, unbound ligand was removed by washing the cells twice with 1 ml of ice-cold HBB. The kinetics of internalization were determined after different periods (0-60 minutes) at 37°C. The cells were washed four times with ice-cold HBB and surface bound 125I-hGH removed by exposure for 3 minutes to an acid wash buffer (150 mM NaCl, 50 mM glycine, pH 2.5). Cells were lysed with 1 ml of 1 M NaOH, and lysates were counted in both the acid wash fraction and the cell lysates using a γ-counter. Specific binding was determined by subtracting the amount of 125I-hGH bound in the presence of unlabelled oPRL. Percentage of internalization was evaluated as the ratio of specific acid-resistant binding and total specific binding. Results represent means (± s.e.m.) of 3 different experiments using duplicate samples.

RESULTS

Functional expression of Flag-tagged prolactin receptors

N-terminal and C-terminal tagged PRLR

Two cDNA encoding the long form of rat PRLR tagged with a Flag epitope (peptide Asp-Tyr-Lys-Asp-Asp-Asp-Lys) inserted either just before the N-terminal amino acid or in the C-terminal end of the mature receptor (named N-terminal or C-terminal Flag-tagged PRLR) were generated by a poly-
A Flag epitope prolactin receptor (Flag PRLR) cDNA was constructed by inserting a sequence coding for a Flag epitope in the PRL cDNA long form, as described in Materials and Methods. For binding studies, COS-7 cells were transfected with a plasmid containing the cDNA encoding the long or Flag-tagged forms of PRLR. $^{125}$I hGH was chosen for binding and internalization studies because of its greater stability as compared to iodinated PRL, and of similar binding affinities of GH and PRL towards PRLR (Djane et al., 1982).

Percentage of binding at the cell surface was determined as the ratio between total $^{125}$I hGH and $^{125}$I hGH bound to intact cells after incubation for 4 hours at 23°C. $K_d$ values were calculated from Scatchard analysis of competitive binding of $^{125}$I hGH to membrane homogenates, as described in Materials and Methods. For determination of transcriptional activity, CHO cells were co-transfected with a plasmid containing the cDNA encoding the long or Flag-tagged forms of PRLR and the fusion gene $\beta$-casein/luciferase. Ovine PRL (400 ng/ml) was added to serum-free medium containing 250 nM dexamethasone during the experiment. The fold induction was calculated as luciferase activity in the presence of PRL divided by the luciferase activity in the absence of PRL. Each value represents the mean ± s.e.m. of duplicates of three independent experiments. Identical results were obtained for PRL binding and $\beta$-casein/luciferase activation of the wild-type PRLR or PRLR tagged either in the N-terminal or in the C-terminal regions.

**Absence of nuclear translocation of PRLR (long form) during ligand-induced internalization**

Exposure of Flag-PRLR cDNA transfected cells, previously incubated at 4°C with iodinated hormone for binding to the membranes, induced rapid internalization of prolactin at 37°C, via receptor mediated uptake (see Materials and Methods), as was seen with the wild-type receptor (Fig. 1). Maximal internalization occurred between 30 and 60 minutes, with a t$_{1/2}$ of ~10 minutes for both receptor constructs.

<table>
<thead>
<tr>
<th>Rat PRLR</th>
<th>Specific PRL binding % Binding</th>
<th>$K_d$ (fM)</th>
<th>$\beta$-Casein/luciferase activity (fold induction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type PRLR</td>
<td>33.3 3$\times$10$^{-10}$ M</td>
<td>8.4±0.2</td>
<td></td>
</tr>
<tr>
<td>N-terminal Flag PRLR</td>
<td>33.8 4$\times$10$^{-10}$ M</td>
<td>8.0±0.3</td>
<td></td>
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**Table 1. Functional expression of wild-type and Flag-tagged PRL receptor evaluated by $^{125}$I hGH binding and transcriptional activity**

N-terminal tagged PRLR

Immunofluorescence microscopy was performed to identify the distribution of PRLR in PRL-stimulated cells. COS-7 and CHO cells transiently transfected with N terminal Flag-PRL PRLR cDNA were analyzed at various time points (10 minutes to 12 hours) after stimulation with oPRL (400 ng/ml). In resting cells, the anti-Flag antibody revealed the presence of PRLR on the cell surface in the absence of hormone. Stimulation with PRL led to the disappearance of immunostaining from the plasma membrane, and presence of PRLR immunostaining in cytoplasmic bright dots and in the juxtanuclear area of some cells. Confocal laser microscopy was used to analyze the possible presence of receptor binding sites within the nucleus. As shown in Fig. 2a-d, immunofluorescence was confined to brights dots within the cytoplasm, with perinuclear accumulation. Such localization probably corresponds to vesicles of the endocytic pathway and to elements present in the juxtanuclear Golgi area. No diffuse staining was observed in cell nuclei. In a few cases, rare spots apparently observed within the nucleus correspond to vesicles aligned along invaginations of the nuclear envelope, as seen using phase contrast microscopy. During the various incubation periods tested (10 minutes to 12 hours) immunofluorescence was never noted within the nucleus of COS-7 or CHO cells.

Similar observations were made with cells either fixed with 4% paraformaldehyde and permeabilized with methanol (–20°C) or fixed and permeabilized with methanol (–20°C). Control cells incubated with preimmune serum or control unrelated IgG antibodies showed extremely low levels of non specific staining (not shown). Also, non transfected cells showed no background staining (data not shown).

Parallel experiments were performed on PRL-stimulated NIH-3T3 transfected cells, using conventional light microscopy and confocal laser microscopy (Figs 3 and 4). As
shown in Fig. 3, immunofluorescence was confined to bright dots within the cytoplasm, during the various periods of PRL incubation (10 minutes to 4 hours). Confocal laser microscopy confirmed the absence of nuclear immunostaining upon PRL stimulation (Fig. 4).

Because these experiments were performed in transiently transfected cells, we also performed similar immunofluorescence experiments on stably transfected cells. Again, when 293 cells stably expressing N-terminal Flag-PRLR were stimulated by PRL for zero to 12 hours, PRLR immunofluorescence disappeared from the plasma membrane and was strongly observed within the cytoplasm, with no apparent fluorescence noted within the nucleus (Fig. 5).

C-terminal tagged PRLR

In order to exclude the cleavage of the cytoplasmic domain of this transmembrane protein, leading to some fragments which might be involved in a nuclear translocation, we inserted a Flag epitope in the C-terminal region of the PRLR long form. Using confocal laser microscopy in transfected COS cells, PRLR was internalized upon exposure of the cells to PRL (0-12 hours), but not translocated to the nucleus (Fig. 2e-h). Perinuclear accumulation of the internalized PRLR was frequently observed, as described for N-terminal Flag PRLR.

From these experiments, we conclude that transfected PRLR (long form) is not translocated to the nucleus of various PRL-stimulated cells (COS-7, CHO and NIH-3T3 cells, 293 cell line). Because these cells are not physiological targets to PRL and could lack components of an intracellular machinery involved in an eventual nuclear translocation of the PRL, we decided to analyze BMGE, a bovine mammary gland epithelial cell line. BMGE cells were transfected with a cDNA encoding the N-terminal Flag PRLR and stimulated with oPRL, as previously described. Again, no nuclear translocation was observed in these physiological targets for PRL upon hyperexpression of a Flag-tagged PRLR (not shown).

Short and Nb2 forms of PRLR do not translocate to the nucleus

PRLR exists in several forms in the rat, differing in the length of their cytoplasmic domains (Kelly et al., 1992; Lesueur et al., 1991; Das and Vonderhaar, 1995): long (591 amino acids), short (291 amino acids, F3) and Nb2 (an isoform of the long PRLR, lacking 198 amino acids); we have thus analyzed PRLR immunostaining at various time points after hormone stimulation in COS-7 cells expressing the short form (F3), or in the Nb2 cell line expressing the Nb2 form of the PRLR.

PRLR short form

COS-7 cells transfected with a cDNA encoding the Flag-PRLR short form revealed PRL immunofluorescence within the cytoplasm after PRL stimulation with no nuclear immunofluorescence (Fig. 6a-b).

PRLR Nb2 form

Similar immunofluorescence studies were performed on rat Nb2 lymphoma cells, a T lymphocyte cell line which requires prolactin for proliferation and has a doubling time of ~20 hours following prolactin stimulation. Again, addition of 20 ng/ml oPRL to either non starved or cells starved overnight stimulated receptor internalization without any significant accumu-
Fig. 3. Indirect immunofluorescence of Flag-PRLR (long form) expressed in NIH-3T3. Cells transfected with the PRLR cDNA were deprived of serum, fixed at 10 minutes (a), 30 minutes (b), 2 hours (c), 3 hours (d), 4 hours (e) after PRL addition and immunostained with anti-Flag antibody, as described in Materials and Methods and in Fig. 2. Again, cytoplasmic staining is observed in all interphase cells, mainly located in vesicles and in the Golgi area. All spots apparently observed in the nucleus (see a,d and e) represent cytoplasmic staining (outside the nucleus), as judged by confocal microscopy (see an example in Fig. 4). ×450.

Fig. 4. Distribution of Flag-PRLR in the same NIH-3T3 fibroblast, observed either by conventional light microscopy (a) or confocal laser microscopy (b). Cells were transfected with N-terminal Flag PRLR, stimulated with PRL for 30 minutes, fixed and immunostained as described in Fig. 3. Confocal laser microscopy was performed using optical sections (0.5 μm) from the top to the bottom of the cell. Note the perinuclear accumulation of PRLR and the complete absence of nuclear staining in the optical section in the center of the nucleus (b). Bar, 10 μm.

tation of fluorescence within the nucleus (Fig. 7a-b). Control Nb2 cells incubated with preimmune serum showed an extremely low level of non specific staining. We thus conclude that, in addition to PRLR long form, PRLR short form transfected in COS-7 cells and endogenous PRLR intermediate form in Nb2 cells do not translocate to the nucleus after ligand stimulation.

Trafficking of prolactin during internalization

In view of the results mentioned above, we decided to investig-
Internalization of prolactin and its receptor

For a review; especially, the organelles involved in receptor-mediated endocytosis (coated pits?), signals of internalization, involvement (or not) of tyrosine kinases, and mechanisms of down-regulation and recycling are unknown. A few studies have suggested the presence of nuclear PRL (Clevenger et al., 1990b; Rao et al., 1993) and attempted to correlate these events with nuclear processes, such as stimulation of protein kinase C and proliferation of IL-2 stimulated lymphocytes (Clevenger et al., 1990a, 1991; Buckley et al., 1988; Rao et al., 1995). However, the mechanism of PRL translocation into the nucleus has never been elucidated, and the presence of nuclear receptors remains controversial (Rao et al. 1995; Clevenger et al., 1990a). We thus decided to investigate putative nuclear addressing during internalization of PRLR and subsequently of PRL.

Using transfection of various cell lines with an expression plasmid encoding rat Flag-tagged PRLR (long and short forms) and detailed confocal microscopy analysis, we have presented strong evidence for rapid internalization of the receptor without any translocation to the nucleus upon hormone stimulation. The Flag-tagged PRLR functions as the wild-type PRLR, as judged by its PRL binding affinity, functional activation of β-casein/luciferase reporter gene and rapid internalization from the plasma membrane upon PRL stimulation. Importantly, the Flag-tagged PRLR is more efficiently immunodetected with monoclonal anti-Flag antibodies (U5 at 60 μg IgG/ml) as primary antibody and fluorescein-conjugated anti-mouse IgG for the secondary antibody. Immunofluorescence using U5 anti-PRLR is reduced compared to immunofluorescence using anti-Flag antibody, with a few cells showing bright spots (arrows). Also, because the Nb2 cells have less cytoplasm than COS-7 cells, the anti-PRLR immunofluorescence within the cytoplasm of Nb2 clone is reduced compared to that in COS-7 cells transfected with PRLR.

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Using transfection of various cell lines with an expression plasmid encoding rat Flag-tagged PRLR (long and short forms) and detailed confocal microscopy analysis, we have presented strong evidence for rapid internalization of the receptor without any translocation to the nucleus upon hormone stimulation. The Flag-tagged PRLR functions as the wild-type PRLR, as judged by its PRL binding affinity, functional activation of β-casein/luciferase reporter gene and rapid internalization from the plasma membrane upon PRL stimulation. Importantly, the Flag-tagged PRLR is more efficiently immunodetected with monoclonal anti-Flag antibodies (U5 at 60 μg IgG/ml) as primary antibody and fluorescein-conjugated anti-mouse IgG for the secondary antibody. Immunofluorescence using U5 anti-PRLR is reduced compared to immunofluorescence using anti-Flag antibody, with a few cells showing bright spots (arrows). Also, because the Nb2 cells have less cytoplasm than COS-7 cells, the anti-PRLR immunofluorescence within the cytoplasm of Nb2 clone is reduced compared to that in COS-7 cells transfected with PRLR.
et al., 1997), exclude the cleavage of some fragments which might be involved in nuclear translocation. Enhanced perinuclear accumulation of PRLR immunostaining was observed in transfected cells, similarly to the perinuclear localization of PRLR observed in PRL-dependent human T lymphocytes (Clevenger et al., 1990a). Our results provide strong evidence that PRLR (long and short forms) are not translocated to the nucleus in epithelial cells (CHO) and fibroblasts (COS-7, 3T3 and 293). Similar results were obtained in transient transfections and in the 293 cell line stably expressing PRLR, suggesting that the targeting of the receptor does not depend upon transient or stable expression in the cell.

In addition, plasma membrane-derived prolactin receptors were not detected in the nuclei of Nb2 cells using a monoclonal anti-PRL antibody (U5). This was also true for PRL-dependent human T lymphocytes (Clevenger et al., 1990a). This conclusion differs from that obtained by Buckley and Rao (Rao et al., 1993) in rat liver and in PRL-dependent Nb2 lymphoma cells, where receptors seems to be constitutively expressed. These differences could be attributed to differences in the cell lines or species used, the specificity of the antibodies used or the nature of the antigen detected (form of the PRL receptor, proteolytic cleavage) or any other technical consideration. First, as previously mentioned, the use of anti-Flag antibody detects PRLR much more efficiently than the anti-PRLR (U5 or U6) which must be used at a relatively high concentration, leading to some background staining, especially when the concentration of the receptor is low. Secondly, the use of confocal laser microscopy allows visualization of the perinuclear localization of PRLR, while this immunostaining could be interpreted as diffuse nuclear staining by conventional light microscopy used in other studies. Consequently, it is possible that the activation of protein kinase C following PRL stimulation could result in PKC associated with the nuclear envelope or other structures outside the nucleus. Finally, the existence of several forms of the rat PRL receptor (Kelly et al., 1992) presents a variety of possibilities for the mode of action of PRL. It can be hypothesized that nuclear PRLR observed mainly in rat Nb2 cells by Rao et al. (1993) could be related to the expression of the Nb2 variant or to the fact that PRL acts as a lymphocyte progression factor. An autocrine mechanism for cell growth stimulation (Davis and Lilner, 1988) could also be involved in breast cancer cells which secrete PRL (Ginsburg and Vonderhaar, 1995). However, we did not detect nuclear immunostaining in PRL stimulated Nb2 cells and in bovine mammary glandular epithelial cells transfected with a cDNA encoding a Flag-tagged PRLR, as is the case in COS-7 and NIH 3T3 cells. Thus, the mitogenic action of PRL does not appear to involve a nuclear prolactin receptor. However, an intracrine mechanism cannot be ruled out at this time.

We therefore decided to investigate nuclear translocation of PRL in COS-7 cells transfected with an expression plasmid encoding PRLR, using confocal laser microscopy. In co-localization experiments, internalized PRL strictly occurred in cells transfected with the hormone receptor, with an enhanced perinuclear accumulation for both PRL and PRLR, most often located in the same vesicles.

That secreted growth factors may play a role in the nucleus has been a controversial topic (Burwen and Jones, 1987; James and Bradshaw, 1984). Early studies indicated that many growth factors were degraded after binding to and entering their target

Fig. 8. Distribution of PRL and Flag-PRLR (long form) expressed in COS-7 cells, as observed by confocal laser microscopy. Cells were transfected with a cDNA encoding the long form of Flag-PRLR, fixed at 30 minutes after oPRL (400 ng/ml) addition, and immunostained using a rabbit anti-PRL antibody (a-d) or a mouse monoclonal anti-Flag antibody (e-h; 10 µg IgG/ml) as primary antibodies, and Texas Red-conjugated anti-rabbit IgG or FITC-conjugated anti-mouse IgG as the secondary antibodies, as described in Materials and Methods. (a-d and e-h) Four optical sections (0.5 µm) from the top (a,e) to the bottom (d,h) of the same cell revealed for PRL (a-d) and PRLR tagged with the Flag epitope inserted at the C-terminal region (e-h). Note the similar pattern and perinuclear accumulation for both PRL and PRLR during internalization (enhanced immunostaining in b-c and f-g); most vesicles appear to contain the two antigens (arrows). Similar observations were made with a N-terminal Flag tagged PRLR.
cells (James and Bradshaw, 1984). But basic fibroblast growth factor (bFGF) and insulin seem clearly to exert some of their effects directly in the nucleus (Miller, 1988; Bouche et al., 1987) where they have been found (Goldfine et al., 1977; Baldin et al., 1990). Several other growth factors have been reported to be internalized and translocated to the nucleus, such as epidermal growth factor (EGF), acid fibroblast growth factor (aFGF), insulin growth factor 1 (IGF-1), nerve growth factor (NGF), platelet-derived growth factor (PDGF, A and B forms), interleukin 1 and interferon γ (Johnson et al., 1980; Savion et al., 1981; Racowicz-Szulcynska, 1993; Yeh et al., 1987; Bader and Wietzerbin, 1994; Grenfell et al., 1989). Studies on protein import into the nucleus have revealed that the information required for targeting proteins of $M_t \geq 50,000$ to the nucleus is encoded in a stretch(es) of basic amino acids, named the nuclear localization signal (NLS) (reviewed by Dingwall and Laskey, 1991). In the case of b-FGF and PDGF (A and B forms), a NLS is capable of targeting a non secreted form of the growth factor to the nucleus (Bouche et al., 1987; Maher et al., 1989); exogenous FGF or PDGF could stimulate nuclear translocation of endogenous FGF or PDGF, via an unknown transduction pathway. However, the mechanism(s) of nuclear localization have not been determined for most other growth factors, and nuclear targeting of each factor needs to be carefully analyzed using new methodologies. Using a transfection approach which permits an enhancement of the immunofluorescence signal of the PRLR by inserting a Flag epitope into the PRLR cDNA long form and confocal laser microscopy, we detected PRL and PRLR in the same cytoplasmic compartment, with no nuclear localization of the PRL hormone.

Evidence for a nuclear translocation of growth factors complexed to their receptors is still lacking. Nuclear binding sites for several growth factors, hormones and neuropeptides have been described (see review by Morel, 1994). The fact that the nuclear transport of some growth factors seems to depend on cell surface receptors (Lobie et al., 1994b; Rakowicz-Szulczynska et al., 1986) suggests a receptor-mediated endocytosis. However, some factors can accumulate in nuclei of growth factor-treated cells in the absence of the receptor, as is the case for insulin (Peralta et al., 1989). It has been suggested that internalized insulin dissociates from its receptor and accumulates in the nucleus without its membrane receptor (Peralta et al., 1989). It is also unlikely that intact cell surface receptors, which contain a transmembrane domain and no nuclear localization signal (NLS) except for IL-1 receptors (Morel, 1994; Curtis et al., 1990), accumulate in the nuclei of target cells as intact receptors. In contrast, it has been reported that growth hormone (GH), and the extracellular binding-protein (GH-BP) are associated with the nucleus, and that the receptor (GHR) is subject to rapid (30 minutes to 1 hour) and ligand-dependent nuclear translocation (Lobie et al., 1991, 1994a). Using mutagenesis of the receptor, the intracellular domain of the receptor appears to be required for nuclear anchorage (Lobie et al., 1994b). It has also been proposed that GH, like PRL, acts on protein kinase C at the nuclear level to regulate transcription, and that GHBP/GHR interacts directly with promoter elements of GH responsive genes, but evidence for this is lacking. Clearly, additional studies are needed to identify the nature of the nuclear binding components for growth factors, the mechanisms for nuclear translocation and to evaluate the importance of these factors in eliciting biological response within the cell.

Our present experiments are not in favor of a nuclear translocation of PRL complexed with its receptor and agree with the mechanism(s) of signal transduction, as extensively described (Dusanie-Fourt et al., 1994; Lebrun et al., 1994a,b; Taniguchi, 1995; Ziemiecki et al., 1994; Darnell et al., 1994; Wakao et al., 1994). Direct effector pathways involving activation of latent transcription factors STATs and MAP kinase, as well as translocation of these factors to the nucleus following their phosphorylation, probably remain the intracellular mechanism coupling PRL receptor stimulation to nuclear events.

We thank G. Gerard (Institut Jacques Monod, University Paris VII) for kind assistance in confocal laser microscopy. This work was supported by the Institut National de la Santé et de la Recherche Médicale and the Centre National de la Recherche Scientifique.

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(Received 25 November 1996 - Accepted 21 February 1997)