Identification of Cytoplasmic Motifs Required for Short Prolactin Receptor Internalization

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Cloning of rat prolactin receptor (PRLR) cDNAs revealed the existence of two isoforms, termed short and long according to the length of their cytoplasmic domain. Internalization studies show, first, that PRLR internalization is hormone-dependent and, second, that ligand-receptor complexes of the short PRLR are internalized to a larger extent compared to the long form. In order to identify regions within the cytoplasmic domain of the short PRLR required for efficient internalization, serial truncations of the cytoplasmic tail were performed by inserting a stop codon in place of those encoding residues 282, 273, 262, 253, 244, or 237 (wild type short PRLR contains 291 amino acids). Our data show that two motifs, lying within residues 253–261 and 273–281, are involved in internalization. Both regions contain a consensus feature identified within other receptors as internalization signals, namely a di-leucine peptide (amino acids 259–260) and a tetrapeptide predicted to adopt a β-turn structure (amino acids 276–279). We propose these two motifs are involved in PRLR endocytosis. Finally, we show that α-adaptin, a component of adaptor protein AP-2, coprecipitates with short PRLR complexes upon PRL stimulation, which strongly suggests that PRLR internalization is mediated by the clathrin-coated pits endocytotic pathway.

Receptor-mediated endocytosis is the mechanism by which a variety of nutrients, hormones, and growth factors are specifically and efficiently transported into the cell. During this process, receptors are selectively concentrated in clathrin-coated pits from which they are rapidly internalized and delivered to endosomes (for review, see Ref. 1). Some receptors, such as the transferrin receptor (TR; Ref. 2) and low density lipoprotein receptor (3), are constitutively clustered in coated pits and undergo rapid internalization in the absence of ligand. Others, such as epidermal growth factor receptor (4) and insulin receptor (5), are concentrated in coated pits and internalized only upon ligand binding. Previous studies have established that internalization of recycling receptors is driven by the presence of specific signals in their cytoplasmic domain (for review, see Ref. 1). Endocytotic codes have been identified as short linear arrays of amino acids which, despite their low degree of sequence similarity, are all assumed to fold in a tight β-turn (6, 7). These motifs may be recognized specifically by plasma membrane-associated adaptor proteins, or AP-2, which mediate the recruitment of these receptors into clathrin-coated pits and promote high-efficiency endocytosis (for review, see Refs. 8–10). AP-2 is a heterotetrameric complex containing two proteins of ~100 kDa, termed α- and β-adaptins, and two others of 50 and 16 kDa (11). The specific binding of ligand-dependent as well as constitutively internalized receptors to AP-2 may thus be essential for their endocytosis. However, the mechanism of receptor/AP-2 interaction and the universality of this association with different classes of receptors are not yet fully understood. Recently, different sequence motifs, characterized by the presence of a leucine-leucine or a leucine-isoleucine dipeptide and found in the cytoplasmic domain of several receptors, such as the γ- and δ-chain of the T cell receptor, the CD4 complex or the signal transducer gp130 for the interleukin-6 receptor, have also been identified as important for internalization and/or lysosomal targeting (12–14).

The prolactin receptor (PRLR) belongs to the cytokine receptor superfamily, which includes among others receptors for growth hormone (GH) and several interleukins (15–17). The cloning of PRLR cDNAs in several species has shown the natural occurrence of at least two different isoforms, resulting from alternative RNA splicing and/or lysosomal targeting (18–20). The specific binding of ligand-dependent as well as constitutively internalized receptors to AP-2 may thus be essential for their endocytosis. However, the mechanism of receptor/AP-2 interaction and the universality of this association with different classes of receptors are not yet fully understood. Recently, different sequence motifs, characterized by the presence of a leucine-leucine or a leucine-isoleucine dipeptide and found in the cytoplasmic domain of several receptors, such as the γ- and δ-chain of the T cell receptor, the CD4 complex or the signal transducer gp130 for the interleukin-6 receptor, have also been identified as important for internalization and/or lysosomal targeting (12–14).

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PRLR does not possess any intrinsic tyrosine kinase activity and constitutively associates with JAK2, a member of the Janus kinase family (24). Following PRL stimulation, rapid and transient tyrosine phosphorylation of JAK2 and PRLR is observed and signal transduction pathways are initiated (for review, see Ref. 25). Mutational analyses of PRLR, as well as of several members of the PRL/GH/cytokine receptor superfamily, have shown that the membrane-proximal region, which includes a proline-rich motif termed Box 1 (26), is essential for signal transduction, since it mediates receptor association with Janus kinases (for review, see Refs. 25 and 27).

In vivo and in vitro studies have shown that excess of PRL stimulation induces rapid down-regulation of total binding sites on target cells (28, 29), which could reflect an increase of the level of receptor internalization and subsequent degradation in lysosomes. Moreover, we have shown previously that deletion of the cytoplasmic domain of the PRLR results in accumulation of the receptor at cell surface (30). Taken together, these observations suggest first, that PRLR internalization is a ligand-dependent process and, second, that it requires some features of the cytoplasmic tail. The aim of the present study was thus to examine this phenomenon for both naturally occurring long and short PRLR isoforms, and to identify the region(s) within the cytoplasmic domain of the short isoform required for receptor internalization. Our study demonstrates that two regions, each containing consensuses features previously reported to mediate receptor internalization, are involved in PRLR endocytosis.

**EXPERIMENTAL PROCEDURES**

### Hormones and Antibodies—
Ovine prolactin (oPRL) was kindly provided by the Pituitary Hormones and Antiserum Center of the National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK, Baltimore, MD) and recombinant human growth hormone (hGH) was a gift from Ares-Serono Laboratories (Geneva, Switzerland). Carrier-free 125I-Na was purchased from Amersham. 125I-hGH was prepared using 125I-Na and hGH bound equally well to the PRLR (32). 125I-HGH was used in all binding studies. Anti-PRLR monoclonal antibodies (aPRLR) were purified from ascites fluids using prepacked Protein A columns provided by Bio-Rad. Both antibodies recognize epitopes within the extracellular domain of the PRLR, but outside the ligand binding domain (33). Monoclonal antibody (AC1-M11), specific to α-adaptin (34), was kindly provided by Dr. F. M. S. Robinson (Cambridge University, Cambridge, United Kingdom).

### Construction of PRLR Mutants—
The cDNA encoding the short form of the rat PRLR, referred to as clone F3 (18), was subcloned into the pCI-neo expression vector (a gift from Dr. W. Wood, Genentech), containing the cytomegalovirus early promoter. The cDNA encoding the short form of the rat PRLR, referred to as clone F3 (18), was subcloned into the pCI-neo expression vector (a gift from Dr. W. Wood, Genentech), containing the cytomegalovirus early promoter.

- **To construct PRLR mutants**—The cDNA encoding the short form of the rat PRLR, referred to as clone F3 (18), was subcloned into the bacteriophage M13 and serial truncations of the cytoplasmic domain were performed using oligonucleotide-directed mutagenesis (35). PRLR mutants T236, T243, T252, T261, and T272 (T for truncation) were generated by introducing a stop codon in place of those encoding Tyr236, Lys243, Thr252, and Tyr272, respectively (the numbering of truncated PRLR used in the present study refers to the last amino acid of the protein; T236 mutant is thus equivalent to T237 PRLR previously reported by Rozakis-Adcock and Kelly (30)). Construction of PRLR T281 was performed by polymerase chain reaction, using forward and reverse primers containing Boll and Xbol sites, respectively; the reverse primer inserted a stop codon at position 282. Mutations were assessed by DNA sequencing (36). All mutated short PRLR cDNAs were subsequently subcloned into the pCI-neo expression vector (a gift from Dr. W. Wood, Genentech), containing the cytomegalovirus early promoter.

### Cell Culture and Transfections—
Cell culture media and sera were purchased from Life Technologies, Inc. CHO cells were grown in Ham’s F-12, COS-7 cells in minimal essential medium “with Earle’s salts” and human 293 fibroblasts in DMEM/Nut F-12. These media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 0.1% penicillin and 50 μg of streptomycin/mL. Cells were routinely cultured at 37 °C, 5% CO2.

Transfection and selection of CHO cells stably expressing the rat short PRLR isoform have been described previously (18), and the same procedure was used to obtain CHO clones stably expressing the rat long PRLR isoform. It has been shown that PRLR stably expressed in CHO cells exhibit cellular localization, binding, and functional characteristics similar to those found in wild type PRL-target cells (37, 38).

COS-7 cells were transiently transfected in six-well plates at 70–80% confluence by the DEAE-dextran/chloroquine method using 5 μg/plate of expression plasmid containing either wild type or mutated PRLR cDNA. DNA was precipitated with DEAE-dextran (8 mg/ml final) in DMEM medium containing 100 μM chloroquine. Four hours after addition of DNA precipitates, cells were subjected to a Me2SO shock (10% Me2SO diluted in Hanks’ balanced salt solution (Life Technologies, Inc.), for 2 min. After washes with DMEM, cells were cultured in 2 ml of fresh complete medium at 37 °C.

Transfection of human 293 fibroblasts with each PRLR plasmid (4 μg/P100 Petri dish) was performed using the calcium/phosphate procedure as described previously (39).

**Immunoprecipitation of PRLR Complexes and Western Blot Analysis—**Transfected cells (40 × 106 CHO or 5 × 106 293 cells) were cultured in serum-free medium overnight and stimulated with (or without) 45 nM PRL for 20 min at 37 °C. Cellular proteins were extracted in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 10% glycerol, and 0.5% Triton X-100) for 30 min at 4 °C. Insoluble material was discarded by centrifugation at 15,000 × g for 15 min, and the amount of protein was assessed by the Bradford method. Equal protein amounts were incubated overnight at 4 °C with anti-PRLR U5 (2 μg/ml), and immune complexes were precipitated by addition of 20 μl (50% v/v) anti-mouse IgG agarose beads (Sigma) for 2 h. Immunoprecipitates were washed three times in ice-cold lysis buffer, then boiled in sodium dodecyl sulfate (SDS) sample buffer and subjected to 9% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Finally, proteins were transferred onto a nitrocellulose membrane.

**After overnight incubation with anti-α-adaptin AC1-M11 (1:500) or anti-PRLR U6 (2 μg/ml) primary antibodies, membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG for 1 h. Proteins were revealed using the enhanced chemiluminescence detection system (ECL kit, Amersham). When required, membranes were dehydrified overnight at 4 °C in an acidic solution (0.1 M glycine, 0.1 M NaCl, pH 3) and reprobed using the appropriate antibody.

**Binding and Scatchard Analysis—**Stable CHO clones grown to 100% confluence in six-well plates were incubated overnight at 4 °C with ~100,000 cpm of 125I-hGH in the presence of increasing concentrations of unlabeled oPRL, in a total volume of 1 ml of PBS containing 0.5% fraction V bovine serum albumin (BSA, Sigma). After washing with ice-cold PBS, cells were solubilized with 1 ml of 1% NaOH and cell-associated radioactivity was counted using a β-counter.

For transiently transfected COS-7 cells, hormone binding was performed on crude microsomes. Forty-eight hours after transfection, cells were washed with PBS, scraped, resuspended in binding buffer (25 mM Tris-HCl, 10 mM MgCl2, pH 7.5), and lysed by three freeze/thaw cycles. Cell lysates were centrifuged at 50,000 × g for 5 min, then pellets were resuspended in binding buffer and protein content was estimated using the BCA assay method. This preparation was used for Scatchard analysis. Binding studies were performed using ~50,000 cpm 125I-hGH and increasing amounts (0.1 ng to 3 μg) of unlabeled oPRL, in a final volume of 0.4 ml of binding buffer containing 0.5% BSA. In order to account for variable expression of each PRLR mutant, the amount of protein used in the assay was chosen to fall within 30–50% of maximal binding (0.4–2 μg of microsomal proteins/point). After 15 h at 23 °C, the assays were terminated by addition 3 ml of ice-cold binding buffer. After centrifugation (4000 × g) to remove unbound 125I-hGH, radioactivity associated with membrane pellets was measured. Scatchard analysis was performed using the LIGAND program (40).

**Internalization Studies—**Internalization studies of wild type or mutant forms of the PRLR, expressed either stably in CHO cells or transiently in COS-7 cells, were performed by following the internalization of receptor-bound 125I-hGH. First, cells were washed once in ice-cold HEpes binding buffer (HBB: 25 mM HEpes, pH 7.4, 124 mM NaCl, 4 mM KCl, 1 mM CaCl2, 1.5 mM MgCl2, 2 mM KH2PO4), then 125I-hGH (100–150,000 cpm in 1 ml of HBB, 1% BSA) was added for 6 h at 4 °C. These conditions were determined to achieve sufficient binding while almost totally preventing receptor-mediated 125I-hGH internalization and further processing. After incubation, unbound ligand was removed and cells were carefully washed with ice-cold HBB. Culture plates containing 1 ml HBB, 1% BSA were then incubated at 37 °C, in order to initiate internalization. Time-courses were performed from 0 to 90 (or 180 for CHO cells) min. After various times at 37 °C, culture plates were put on ice (preventing further internalization) and washed four times with ice-cold HBB. Surface-associated (non-internalized) 125I-hGH was de-
Although specific binding to cell surface receptors after 6 h of incubation at 4°C was similar for both forms of receptor expressed in CHO cells were washed with acidic buffer and solubilized to determine intracellular radioactivity. Internalization is expressed as the percentage of intracellular [125I]-hGH with respect to the specific binding. Each point represents the mean of duplicate measurements from 5–10 independent experiments. The S.E. values were <8.5%.

For each experimental condition (i.e. the different receptor mutants), the specific binding was determined as the difference between [125I]-hGH bound in the presence and in the absence of unlabeled oPRL (2 × 10^{-8} M) after 6 h of incubation at 4°C. In all experiments, nonspecific binding was <10% of total bound radioactivity. For each time, internalization values are expressed as the percentage of specific intracellular [125I]-hGH bound with respect to the specific binding.

Secondary Structure Prediction—Secondary structure prediction of the cytoplasmic domain of the rat short PRLR isoform was performed on a PC/Gene (IntelliGenetics Inc., Geneva, Switzerland) using the Chou and Fasman (42) and the Garnier-Osguthorpe-Robson (43) algorithms.

RESULTS

Binding and Internalization Studies of Long and Short PRLR Isoforms—Before internalization studies, binding properties of both long and short PRLR isoforms stably expressed in CHO cells were characterized by Scatchard analysis. For both, Scatchard plots were linear (data not shown), in agreement with the existence of a single class of binding sites. Receptor affinity of short and long PRLR were similar, with $K_a$ (affinity constant) values of $8.5 \times 10^5$ M^{-1} and $10.5 \times 10^5$ M^{-1}, respectively. The binding capacity of each clone used was estimated around 34,000 receptors/cell.

The experimental conditions described under “Experimental Procedures” for monitoring PRLR internalization were carefully selected and were further used for all internalization studies reported in this study. Internalization kinetics of short and long forms of PRLR stably expressed in CHO cells are shown on Fig. 1. Both patterns are parallel, with 50% of maximal internalization achieved after 30 min at 37°C and maximal internalization within 90–120 min. After this period, a progressive decrease of the percentage of internalization is observed for both receptors. Although specific binding to cell surface receptors after 6 h of incubation at 4°C was similar for both forms of receptor (~3–5% of total radioactivity), it clearly appears that the short form complexed with the ligand is internalized to a greater extent than the long form (80% versus 50% of internalization after 120 min).

Western Blot Analysis of Truncated Short PRLR—Truncation mutants of the rat short PRLR were constructed as described under “Experimental Procedures.” Constructs are schematically represented in Fig. 2A. In order to assess accurate truncation at the protein level, we analyzed all mutated PRLR by Western blot (Fig. 2B). For that purpose, plasmids encoding the different PRLR isoforms were transiently transfected into human 293 fibroblasts, a cell line routinely used in our laboratory for its high expression level of cDNAs under cytomegalovirus promoter (39, 44). Receptors were immunoprecipitated using an anti-PRLR mAb U5 and analyzed by Western blot, using mAb U6. As shown on Fig. 2B, truncated PRLR exhibit the expected $M_r$ (in the range of 42,000 to 34,000, from wild type to T236 PRLR). It also appears that truncation of cyto-
plasmic tails parallels an increased level of receptor expression.

**Binding Studies of Truncated Short PRLR**—Since it has been reported previously that truncation of cytoplasmic domain interferes with binding properties of the receptor (30, 45), all PRLR mutants were first characterized by Scatchard analysis. In order to circumvent differential cell surface expression of truncated PRLR, binding experiments were performed on crude microsomes. Mutant T281 exhibits similar affinity compared to wild type PRLR ($K_a = 12 \times 10^9 \text{M}^{-1}$), whereas T272, T261, T252, and T243 PRLR show a ~3-fold increased affinity. In agreement with our previous report (30), removal of almost the entire cytoplasmic domain (T236) leads to a ~5-fold increase in binding affinity of $125^I$-hGH.

**Internalization Studies of Mutants of the Rat PRLR**—To identify the region(s) involved in internalization of short PRLR, wild type and truncated forms of the short PRLR were transiently expressed in COS-7 cells. Cells were preferentially transfected using the DEAE-dextran technique, which achieved higher transfection efficiency compared to calcium phosphate precipitation (data not shown). Three patterns of internalization kinetics were obtained (Fig. 3). The first was observed for wild type and T281 PRLR and consists of a rapid internalization, reaching a maximum after 90 min of 57% and 55%, respectively. This suggests that internalization signal(s) of the short PRLR are still present in truncated T281. The second pattern, observed for T272 and T261, shows an intermediate rate of internalization, reaching maximal value of ~30% after 90 min. This suggests that some, but not all, features involved in efficient internalization have been lost or altered in these truncated PRLR. Finally, the pattern obtained for T252, T243, and T236 showed only basal internalization (10–15% at 90 min), suggesting that additional motifs required for efficient internalization have been removed. Thus two regions appear to be required for complete and efficient endocytosis, within aa 273–281 and aa 253–261.

**Secondary Structure Prediction of the Short PRLR Cytoplasmic Domain**—In order to correlate internalization efficiency of truncated PRLR with the presence of internalization motifs...
proposed to adopt the consensus β-turn fold (see Introduction), we performed a secondary structure prediction of the short PRLR cytoplasmic tail. Using the Chou and Fasman (42) method, three tetrapeptides, corresponding to aa 247–250, 264–267, and 276–279, were predicted to adopt a consensus β-turn conformation (Fig. 3). The predicted β-turn encompassing four residues starting at position 2 of the residue is calculated from the values of turn frequencies (f) and is given by the equation p(t) = f1 × f2 × f3 × f4. Conformational parameters (Pβ, Pα, Pψ) and (Pδ) are the averages of the frequencies of the four residues in the β-turn, α-helix, and β-sheets, respectively. A β-turn is predicted if p(t) > 0.75 × 10⁻⁹, |Pβ| > 1, and |Pδ| < |Pα| > |Pψ|.

| Residues   | Tetrapeptide | p(t) × 10⁻⁹ | |Pβ| | |Pα| | |Pψ| | |Pδ|
|------------|--------------|-------------|------|------|------|------|------|------|
| 247–250    | Val-Pro-Gly-Pro | 2.41 | 1.275 | 0.692 | 0.888 |
| 249–252    | Gly-Pro-Lys-Ile | 1.24 | 1.140 | 0.845 | 0.910 |
| 261–264    | Glu-Thr-Gly-Ser | 1.22 | 1.172 | 0.920 | 0.765 |
| 264–267    | Ser-Pro-Ser-Lys | 4.29 | 1.348 | 0.818 | 0.698 |
| 266–269    | Ser-Lys-Tyr-Lys | 1.49 | 1.143 | 0.945 | 0.925 |
| 276–279    | Leu-Pro-Gly-Gly | 5.30 | 1.308 | 0.730 | 0.838 |
| 285–288    | Asn-Ala-Gly-Glu | 1.49 | 1.130 | 1.042 | 0.71 |

**TABLE I**

*Predicted potential for β-turn formation in the cytoplasmic domain of short form of the rat PRLR*

The method of calculation was as follows. Predicted potential for a β-turn encompassing four residues starting at position i is calculated from the values of turn frequencies (f) and is given by the equation p(t) = f1 × f2 × f3 × f4. Conformational parameters (Pβ, Pα, Pψ) and (Pδ) are the averages of the frequencies of the four residues in the β-turn, α-helix, and β-sheets, respectively. A β-turn is predicted if p(t) > 0.75 × 10⁻⁹, |Pβ| > 1, and |Pδ| < |Pα| > |Pψ|.

Fig. 5. **Co-precipitation of α-adaptin and short PRLR.** CHO cells, stably expressing (F3) or not (−) the wild type short PRLR were stimulated (+) or not (−) for 20 min with 45 nM oPRL at 37°C. Cells were lysed, immunoprecipitated using mAb U5 (2 μg/ml), and subjected to 9% SDS-PAGE. Proteins transferred onto a nitrocellulose membrane were detected using a monoclonal α-adaptin antibody AC1-M11 (1:500) (upper panel). A band corresponding to the expected Mr of α-adaptin (100,000) coprecipitates with oPRL-stimulated PRLR. The membrane was then stripped and reprobed with mAb U6 (2 μg/ml) to visualize the co-precipitation of the short PRLR (lower panel).

**DISCUSSION**

In COS-7 cells expressing exogenous rat short PRL receptor cDNA, approximately two thirds of total receptors are localized in intracellular compartments. In contrast, expression of a cDNA encoding a truncated receptor lacking 55 of 57 cytoplasmic amino acids (T236) results in receptors located almost exclusively at the plasma membrane (30). Moreover, the total number of binding sites observed in cells expressing T236 PRLR is 3 times higher compared to the wild type receptor. These observations suggest that although the cytoplasmic tail of PRLR is 3 times higher compared to the wild type receptor, while no band is observed in nonstimulated or unstimulated cells. These data demonstrate that two regions, encompassing residues 273–281 and 253–261, respectively, contain structural motifs required for efficient endocytosis and subsequent degradation within intracellular compartments.

The aim of the present study was to identify the cytoplasmic features involved in the internalization of the prolactin receptor. As shown on Fig. 1, the long as well as the short PRLR isoforms are extensively internalized after ligand binding, indicating that both receptors contain the structural features required for efficient internalization. Due to the limited number of amino acids in its cytoplasmic tail, we focused on the short form to identify the internalization motif(s) using the mutational approach. In order to avoid internal deletions, which are thought to be more structurally disruptive, we generated a series of incremental truncations from the carboxyl terminus of the cytoplasmic tail. Internalization studies clearly demonstrate that two regions, encompassing residues 273–281 and 253–261, respectively, contain structural motifs required for endocytosis of the short form of the rat PRLR (see “Results”).

Within the 273–281 segment, a tetrapeptide (L²⁷⁶PGG²⁷⁹) is predicted to adopt a β-turn conformation. This secondary structural element has been identified as an internalization signal for several receptors (Refs. 6 and 7; for review, see Ref. 1). Hence, we propose the L²⁷⁶GG tetrapeptide to be one of the features required for efficient endocytosis of the short PRLR. Prolactin is a strong β-turn promoter, especially in position 2 (6, 42), which strengthens the likelihood that this motif adopts such a secondary fold in the PRLR. For some receptors, requirement of an aromatic residue within the β-turn has also been described. For example, an aromatic residue is found in the internalization motifs of the low density lipoprotein receptor (NPXY; see Ref. 6) and the TR (YXXF; see Ref. 7) (for review, see Ref. 1). More remarkably, introduction of a tyrosine in a putative β-turn of the TR has been shown to enhance receptor internalization (46). Although the predicted β-turn of the PRLR...
does not contain such a residue, it may be hypothesized that Phe\textsuperscript{280} , the amino acid directly bordering this motif toward the C terminus, could play a role in the internalization process. Alternatively, since the Chou and Fasman algorithm predicts \( \beta \)-turns with an accuracy of \( \pm 2 \) amino acids, it cannot be ruled out that the actual \( \beta \)-turn within the 273–281 region is shifted by one or two amino acids compared to the prediction and, thereby, includes Phe\textsuperscript{280}. Finally, internalization studies of the GH receptor emphasized the major role of a phenylalanine residue (Phe\textsuperscript{346}; see Ref. 47), while this residue was not predicted to be within a \( \beta \)-turn using the Chou and Fasman method (data not shown). These hypotheses obviously await further investigation of receptor structures.

The second internalization motif lies between amino acids 253 and 261. Despite the absence of any predicted \( \beta \)-turn, this region includes a pair of adjacent leucine residues (L\textsuperscript{259}L\textsuperscript{260}). The di-leucine motif, originally described as a cell sorting signal, was also identified as an internalization motif for membrane proteins like gp130, the interleukin-6 signal transducer also belonging to the cytokine receptor superfamily (14), or the more distantly related CD\textsubscript{3}γ chain (12). Moreover, it was reported that substitution of a di-leucine motif for the tyrosine-containing sequence does not alter internalization of the TR (1), suggesting that both features are functionally equivalent in their interaction with the endocytotic machinery. In view of these observations, we propose that the consensus di-leucine motif is the second feature involved in PRLR internalization. In good agreement, truncation of this conserved motif in the GH receptor also reduces the rate of internalization, although this feature was not identified as a major endocytotic signal (47).

Despite the deletion of the 276–279 \( \beta \)-turn, T272 and T261 PRLR mutants resulted in an intermediate rate of endocytosis (\( \pm 30\% \)). This indicates that, in the context of truncated receptors, the di-leucine motif can function alone to ensure partial internalization. In the wild type receptor, it is likely that the two motifs (\( \beta \)-turn and di-leucine) act in a cooperative fashion. Additive effects of two or more internalization signals have already been reported for other receptors, like the cation-dependent mannose 6-phosphate receptor (48), the insulin receptor (5), or the epidermal growth factor receptor (4). When both signals are removed form the PRLR (T252), receptor internalization is dramatically reduced, but not totally abolished (\( \pm 10\% \)). Although such observations might suggest the presence of a third internalization signal in the membrane-proximal region, this is very unlikely, since further truncation does not affect the residual endocytosis (T243, T236). It has been suggested that this basal rate of endocytosis, which is invariably observed for receptors with extensive deletion of their cytoplasmic tail, results from random entrapment in coated pits (2) or nonspecific bulk flow endocytosis (49).

Box 1, a proline-rich region encompassing residues 245–250 in rat PRLR, is the most highly conserved feature within the cytoplasmic domain of cytokine receptors (26). Interestingly, our study did not identify Box 1 as an internalization signal. Due to the incremental mutational strategy that we used, however, the possible involvement of Box 1 in the endocytosis process could have been missed since deletion of this feature occurs between two truncated receptors (T252 and T243) that are already deleted from the other internalization signals and, therefore, that only display the basal rate of internalization. Thus, a possible cooperative effect of Box 1 with any of the two other signals cannot be ruled out from the present study. However, in the context of another study, we have recently shown that structural disruption of Box 1 by replacement of the four prolines into alanines in the long isofrom (so-called 4 PA PRLR) fails to alter receptor internalization, whereas functional properties of this mutant are totally abolished.\(^2\) Taken together, these data suggest that Box 1 is unlikely to play a major role, if any, in PRLR internalization. This is in perfect agreement with observations recently reported for the closely-related GH receptor (47).

Three isoforms of the PRLR have been described that only differ by their cytoplasmic domain after residue 261 (16). The di-leucine motif (aa 259–260) is conserved in all PRLR isoforms and may thus represent a consensus feature involved in PRLR endocytosis, whatever the isoform. Analysis of stable CHO clones expressing either the short or long PRLR isoform showed that the former is internalized to a larger extent compared to the latter (Fig. 1). Although the precise reason of this effect is unknown, it should be noticed that the second internalization motif proposed in this study, namely the tetrapeptide L\textsuperscript{276}PGG\textsuperscript{279} predicted to fold in a \( \beta \)-turn, is not conserved in the long PRLR isoform. Alternatively, the presence of signals negatively regulating endocytosis, as reported for the low density lipoprotein receptor (50), represents another possibility for explaining the less efficient internalization of the long isoform.

There is some evidence that endocytosis of receptors involves interaction(s) of their cytoplasmic domain with adaptor proteins called AP-2. These interactions are thought to account for the ability of cells to selectively include receptors in clathrin-coated vesicles (8, 51). The current model of the coated pit cycle assumes that the receptor/AP-2 interaction is transient and, at least for non-constitutively internalized receptors, induced by ligand binding. Using the AC1-M11 antibody specific to \( \alpha \)-adaptin (34), one of the AP-2 subunits (see Introduction), we show that this protein co-precipitates with complexes containing the wild type short PRLR. This association is hormone-dependent and occurs at 37 °C, which is reminiscent to the observations reported for the epidermal growth factor receptor (52). To the best of our knowledge, this is the first report describing an interaction between the PRLR and an adaptor protein.

Recently, several in vitro studies have shown that the interaction between cytoplasmic domains of receptors and AP-2 involves specific regions previously identified as endocytic codes, such as leucine-leucine and/or tyrosine-containing motifs (53–55). Although the functional role of AP-2 has been clearly linked to receptor coated pit-mediated internalization, the absolute necessity of AP-2/receptor interactions for efficient endocytosis remains controversial (53, 55) and it has been suggested that clathrin-independent endocytotic pathway might also mediate receptor internalization. Determining whether AP-2 plays a major role in PRLR internalization, whether it interacts directly with the receptor and, if so, it binds to any of the internalization signals identified in this work will be the goal of future studies.

In summary, our study (i) shows that both long and short PRLR isoforms undergo efficient endocytosis, (ii) identifies two regions involved in short PRLR internalization, (iii) proposes a di-leucine peptide and a putative \( \beta \)-turn to be the internalization signals, and (iv) shows an interaction between \( \alpha \)-adaptin and PRLR complexes, suggesting that PRLR internalization might be mediated by the clathrin-coated pits endocytotic pathway.

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