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Evidence for *N*-glycosylation and ubiquitination of the prolactin receptor expressed in a baculovirus-insect cell system

Claire Cahoreau[a,*], Laurence Garnier[a], Jean Djiane[b], Gérard Devauchelle[a], Martine Cerutti[a]

[a]*Laboratoire de Pathologie Comparée, Unité de Biologie Cellulaire et Moléculaire, Institut National de la Recherche Agronomique, CNRS UA 1184, 30380 St-Christol-Lez-Alès, France*

[b]*Unité d'Endocrinologie Moléculaire, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas, France*

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Abstract

The molecular mass of the rabbit prolactin receptor (rbPRLR) deduced from cDNA cloning is 66 kDa. However, the molecular mass of the full-length receptor expressed in the insect Sf9 cells was found to be 94 kDa. In order to explain this discrepancy, we analyzed the possible post-translational modifications of the PRLR. Sf9 cells were infected with recombinant baculoviruses in the presence of tunicamycin, an inhibitor of *N*-glycosylation. Results showed that an additional $\approx$ 9 kDa of the extracellular domain could be attributed to the *N*-glycosylation and another additional $\approx$ 20 kDa covalent modification occurred in the cytoplasmic part of the receptor. Western blot analysis, using anti-ubiquitin antibodies, revealed that the rbPRLR was ubiquitinated in its cytoplasmic domain.

Key words: Baculovirus; Cytoplasmic receptor domain; Prolactin; *N*-Glycosylation; Ubiquitination

1. Introduction

Pituitary prolactin (PRL) is a member of a polypeptide hormone family that includes placental lactogen and growth hormone (GH) [1]. PRL is involved in many biological functions in all vertebrates [2]. To date, two forms of PRL receptor have been cloned in different tissues and species [3–7], and recently a mutant form has been identified in the Nb2 cell line [8]. All these forms contain a common PRL binding extracellular domain (210 amino acids) with three potential N-linked glycosylation sites, a short hydrophobic transmembrane segment (24 amino acids), but differ in the size of their cytoplasmic domains. The PRL receptor shares some significant sequence homologies with the GH receptor. These receptors have been recently classified as members of the hematopoietin cytokin receptor superfamily [9,10].

Although the biological effects of PRL on mammary cells are well documented, only limited information is available on the structure and the mechanism of action of the receptor. PRL receptors have been biochemically characterized from rabbit mammary gland (rbPRLR) with an apparent molecular mass of only 42 kDa [11], although higher forms have been reported [12]. Nevertheless, after cloning of the cDNA, the predicted molecular mass of the rbPRLR was found to be 66 kDa [6]. We have previously reported the successful expression in *Spodoptera frugiperda* (Sf9) insect cells of a full-length form of the rbPRLR using a recombinant baculovirus vector [13]. This receptor bound PRL with a specificity and affinity similar to those reported for the native receptor. However, the purified recombinant receptor had an apparent molecular mass of 94 kDa as determined by SDS-PAGE. This paradoxical size was attributed to the cytoplasmic domain of the PRLR.

The aim of this study was to identify post-translational events that could explain this phenomenon. We studied the effects of tunicamycin, an *N*-glycosylation inhibitor, on the apparent molecular size of PRLR. Since previous sequencing of the purified GH receptor had revealed a covalent association with ubiquitin [14], we have investigated the ubiquitin immunoreactivity of all recombinant forms of the receptor.

2. Materials and methods

2.1. Materials

Bovine and yeast ubiquitins, rabbit polyclonal anti-ubiquitin antibodies, rabbit anti-goat and goat anti-rabbit antibodies, and 3-amino-9-ethyl carbazol were purchased from Sigma. Tunicamycin was obtained from Boehringer-Mannheim, Germany. All other reagents were of analytical grade.

2.2. Viruses and cells

Spodoptera frugiperda cells (Sf9) were maintained at 28°C in TC100 medium supplemented with 10% foetal calf serum. Wild-type *Autographa californica* nuclear polyedrosis virus (AcMNPV) clone 1.2 [15] and recombinant viruses expressing full-length (L), soluble (E), short (S) or cytoplasmic (I_1 and I_2) forms of the rbPRLR were propagated and assayed in confluent monolayers of Sf9 cells as described previously [13].

*Corresponding author. Laboratoire de Pathologie Comparée, Université Montpellier II, CP 101, Place Eugène Bataillon, 34 095 Montpellier Cedex 5, France. Fax: (33) 67 14 46 79. E-mail: cahoreau @crit.univ-montp2.fr.

Abbreviations: PRL, prolactin; GH, growth hormone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Sf, *Spodoptera frugiperda*; AcMNPV, *Autographa californica* nuclear polyhedrosis virus.

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2.3. SDS-PAGE and Western immunoblotting

Cells were infected with each recombinant baculovirus at a multiplicity of 10 pfu/cell and incubated at 28°C for 40 or 52 h. Mock-infected and wild-type baculovirus-infected cells were used as controls. Cells were collected and centrifuged for 5 min at 1000 × *g*. Cell extracts or supernatants were separated on either 12% or 8–12% gradient SDS-PAGE gels under reducing conditions, according to the method of Laemmli [16]. Proteins were transferred to nitrocellulose membranes and incubated with goat anti-rbPRLR polyclonal antibodies, or with anti-ubiquitin polyclonal antibodies. The blots were washed and incubated with rabbit anti-goat or goat anti-rabbit immunoglobulins conjugated to horseradish peroxidase. Finally, after extensive washing, sites of antibody binding were visualized using the enhanced chemiluminescence method (ECL; Amersham) or 3-amino-9-ethyl carbazol.

2.4. Glycosylation

Sf9 cells (1.5×10^6 cells/ml) were infected with S, E, L, I_1, I_2 recombinant baculoviruses. Tunicamycin (final concentration 10 μg/ml) was added to the cell cultures at 7 or 20 h post-infection (pi). As controls, incubations and treatments were performed in the absence of tunicamycin. After 52 h incubation the cells were harvested and the proteins were analyzed by 8–12% SDS-PAGE, and electroblotted on to nitrocellulose. Recombinant proteins were detected by the goat anti-rbPRLR antibodies as described above.

2.5. Antisera to ubiquitin

Anti-ubiquitin serum was a kind gift from Dr. P. Mayeux (Paris). This antiserum was prepared as described by Hershko et al. [17]. Briefly, ubiquitin purified from bovine red blood cells was conjugated to γ-globulin as immunogen with glutaraldehyde. Specific antibodies (P-antiserum) were induced in rabbits by immunization with denatured ubiquitin conjugate. Rabbit anti-ubiquitin antibodies from Sigma were also used (S-antiserum).

3. Results

3.1. N-Glycosylation of rabbit PRL receptor expressed in insect cells

Since post-translational processing of PRL receptor involves carbohydrate modification [18], we investigated the effects of the fungal antibiotic tunicamycin (for a review see [19]) on *N*-glycosylation of rbPRLR expressed in Sf9 cells. Cells were infected with all recombinant baculoviruses, and tunicamycin was added at a concentration of 10 μg/ml at either 7 or 20 h pi. The apparent molecular mass (Fig. 1) of the E soluble form (lanes 1–6), S truncated form (lanes 7–9), and L full-length receptor (lanes 10–12) were of about 27, 45 and 85 kDa, respectively. In respective control experiments, i.e. in absence of tunicamycin, molecular masses of 35, 55 and 94 kDa, respectively, were found. Thus, the carbohydrate moieties of L, E, and S forms isolated from insect cells accounted for 8–10 kDa. Furthermore, there was little or no change in the apparent total amount of recombinant proteins produced relative to the control. Whereas the E form secretion of rbPRLR was suppressed upon addition of tunicamycin (lanes 3 and 5), both I_1 and I_2 cytoplasmic recombinant domains were still secreted into the medium (lanes 13, 15, 17, 19, 21 and 23). In addition, the molecular mass of I_1 and I_2 produced with or without tunicamycin did not change (lanes 14, 16, 18, 20, 22 and 24).

3.2. Detection of ubiquitin and ubiquitin-immunoreactive proteins in Sf9 by immunoblotting with anti-ubiquitin antisera

After 48 h infection of Sf9 cells with wild-type AcMNPV and I_2 recombinant baculovirus, proteins from cell extracts and culture supernatants were separated on a 12% SDS-PAGE and subsequently blotted on to nitrocellulose. The blots were probed with two different anti-ubiquitin antisera: a commercial S-antiserum provided by Sigma (Fig. 2A) or P-antiserum (Fig. 2B);

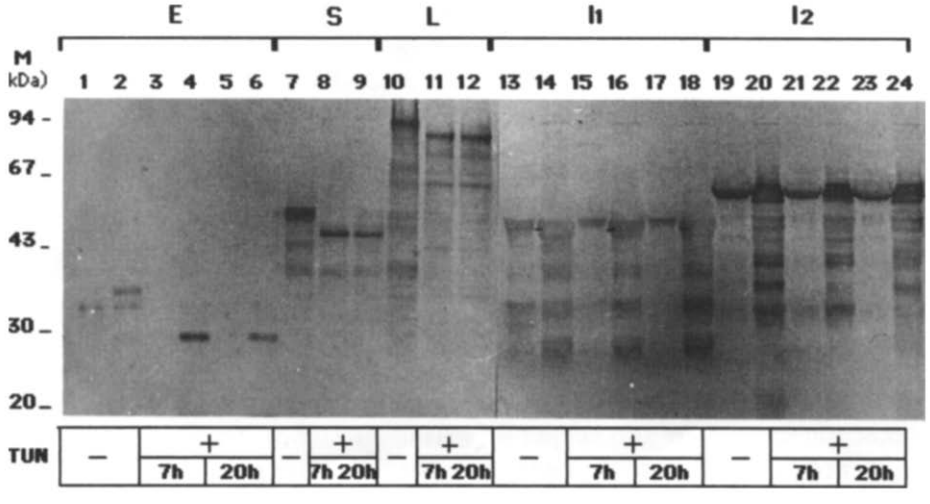

Fig. 1. Effect of tunicamycin on the expression of recombinant proteins. Sf9 cells were infected with S, E, L, I_1, I_2 recombinant baculoviruses and treated with (+) or without (−) tunicamycin (TUN). The medium was replaced 7 or 20 h post-infection with medium containing tunicamycin at 10 μg/ml or with normal medium for control cells. At the end of the incubation period (52 h) the cells were harvested, the proteins were analyzed on a 8–20% gradient SDS-PAGE and subsequently electroblotted on to nitrocellulose membrane. The blots were probed with goat polyclonal anti-rbPRLR antibodies (1:100) and detected with horseradish peroxidase conjugated rabbit anti-goat antibodies (1/1000). Protein-antibody complexes were detected using 3-amino-9-ethyl carbazol at 200 μg/ml as substrate. The lanes contain samples from total cell lysates expressing the recombinant proteins: E (2,4,6), S (7,8,9), L (10,11,12), I_1 (14,16, 8), I_2 (20,22,24); relevant culture media: E (1,3,5), I_1 (13,15,17) and I_2 (19,21,23), a set of molecular mass standards in lane M.

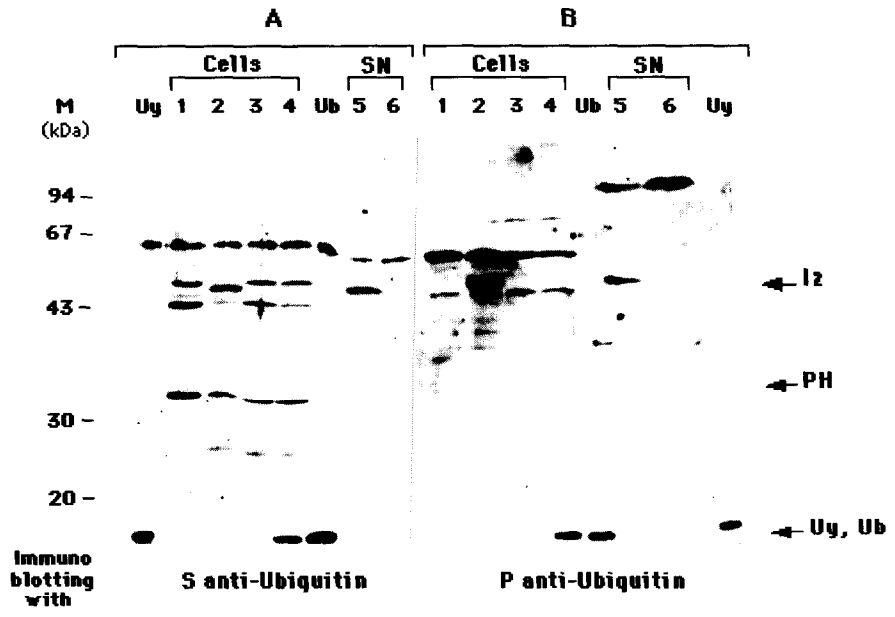

Fig. 2. Detection of ubiquitin and ubiquitin-immunoreactive proteins in Sf9 cells by immunoblotting with anti-ubiquitin antisera. Cellular extracts from uninfected Sf9 cells (lanes 1) or extracts and culture supernatants of cells infected with wild-type baculovirus (lanes 3, 4 and 6) or with I_2 cytoplasmic domain rbPRLR baculovirus (lanes 2 and 5) were prepared and analyzed as described above. Blots were probed with anti-ubiquitin S anti-serum (50 μg/ml) (blot A) and P anti-serum (1/1000) (blot B) and revealed with horseradish peroxidase conjugate goat anti-rabbit antibodies (1/2000). Protein–antibody complexes were detected by an enhanced chemiluminescence (ECL) method. 1 μg of yeast ubiquitin (Uy) and 1 μg of bovine ubiquitin (Ub) were used as controls. Bovine ubiquitin mixted with wild-type infected cell extract was run in lane 4. Molecular mass standards (kDa) are indicated on the left. The arrows indicate the migration of I_2 and polyhedrin (PH) and free ubiquitins (Uy and Ub).

this P-antiserum was developed in rabbit using ubiquitin conjugated to bovine γ-globulin as immunogen, resulting in a high-affinity polyclonal antibody to ubiquitin. Its specificity was established by the addition of 1 μg ubiquitin to extracts of AcMNPV wild-type infected cells. As shown Fig. 2, an additional signal appeared (compare lanes 3 and 4). On the other hand, if P-antiserum was incubated with 1 mg of bovine ubiquitin prior to the incubation of the nitrocellulose sheets, no signal of ubiquitined proteins could be detected (data not shown). Both antibodies recognized, with the same intensity, the ubiquitins from baker's yeast (lanes Uy) and from bovine red blood cells (lanes Ub). The immunoblots showed that the only proteins recognized by both the S- and P-antisera were ubiquitin itself (Uy and Ub) and I_2 recombinant protein (cell extracts lanes 2 and supernatants lanes 5). Thus, the results obtained with S or P anti-ubiquitin antibodies suggested that the I_2 cytoplasmic rbPRLR corresponds to a covalent ubiquitin conjugate. Apart from the ubiquitin and I_2 recombinant protein, a number of other substrates were also ubiquitinated. P-Antiserum was used to detect other ubiquitin-immunoreactive proteins in the following experiment.

3.3. *Immunoreactivity of rbPRLR recombinant proteins with anti-ubiquitin P-antiserum*

Cells were infected with each of the five recombinant baculoviruses. At 48 h, cell extracts or culture supernatants were run on a 12% SDS-PAGE gel and recombinant proteins (L, E, S, I_1, I_2) were detected by Western blot analysis. Non-infected cells and cells infected with wild-type baculovirus were used as controls. Immunoblots using anti-rbPRLR (Fig. 3A) or anti-ubiquitin (Fig. 3B and C) antisera were carried out with the recombinant proteins. All recombinant proteins (I_2, I_1, E, S, L) were specifically revealed using the anti-PRL receptor antiserum (Fig. 3A, lanes 1–5), while only three forms (I_2, I_1 and L) were detected with the antiserum generated against the ubiquitin (Fig. 3B, lanes 1, 2 and 5).

The strongest signal resulted from an interaction with the I_2 cytoplasmic recombinant protein; the immunoreactive band was revealed both in cell extracts (Fig. 3B, lanel) and in the medium (Fig. 3C, lane 1). A signal was also detected with the I_1 intracellular recombinant protein, the presence of a faint immunoreactive band was revealed in the cell extracts (Fig. 3B, lane 2). Consistently, a weak but detectable signal was observed with the full-length receptor (L) recombinant protein (Fig.3B, lane 5). No signal was detected with the E (Fig. 3B and C, lanes 3) and S (Fig. 3B and C, lanes 4) forms of the receptor.

4. Discussion

In contrast with the predicted molecular mass of the fully *N*-glycosylated form of rbPRLR (≈ 75 kDa), we

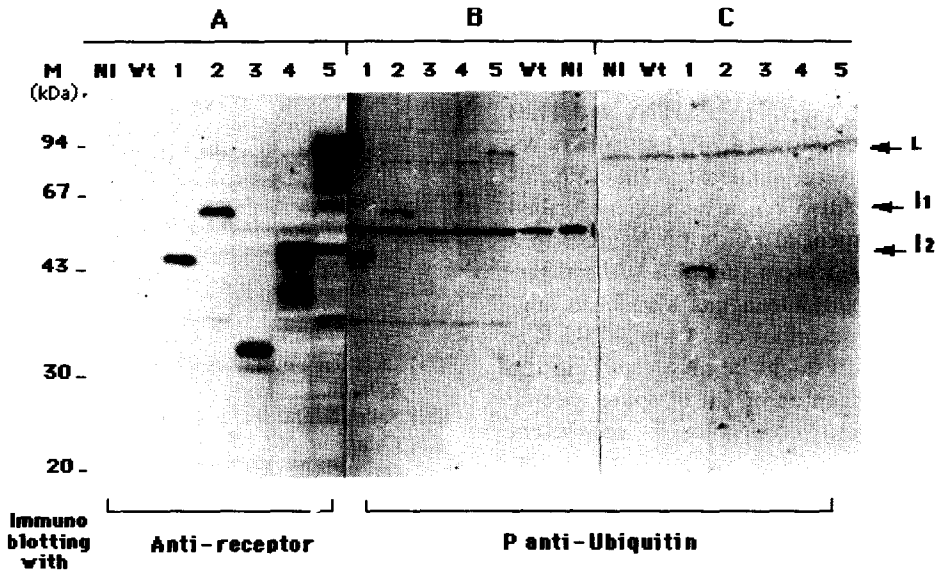

Fig. 3. Immunoreactivity of rbPRLR recombinant proteins with anti-ubiquitin antisera. Cell extracts (A and B) and cell culture supernatants (C) prepared from non infected cells (NI), cells infected with wild-type baculovirus (wt) or with I_2 (lane 1), I_1 (lane 2), E (lane 3), S (lane 4) and L (lane 5) recombinants were analyzed as described above. The blots were probed with (A) goat polyclonal anti-rbPRLR antibody (1:2000), (B and C) rabbit anti-ubiquitin P antiserum (1/1000) and revealed with horseradish peroxidase conjugate (A) rabbit anti-goat antibodies (1/4000) (B and C) goat anti-rabbit antibodies (1/2000). Protein–antibody complexes were detected as described in Fig. 1.

have recently shown that this receptor exhibited a higher molecular mass (94 kDa) when expressed in the baculovirus expression system. Analysis of several deletion mutants [13] showed that while extracytoplasmic domains presented the expected sizes, a 20 kDa additional molecular mass was found for cytoplasmic regions.

Studies with tunicamycin, an inhibitor of *N*-glycosylation, revealed that the extracellular domain of the receptor contains N-linked oligosaccharides while this is not the case for the two intracellular truncated forms (I_1 and I_2). The unglycosylated form of the full-length receptor (94 kDa) protein synthesized in the presence of tunicamycin has a molecular mass of 85 kDa, suggesting the presence of another post-translational modification. As previously reported for human tissue plasminogen activator (t-PA) expressed in insect cells [20], the secretion of the soluble (E) form of the extracellular domain was blocked by addition of tunicamycin. In contrast, the secretion of the I_1 and I_2 cytoplasmic recombinant proteins was unaffected under these conditions. These results suggested that the secretory pathway of cytoplasmic recombinant proteins through the Sf9 cells might be different. Another post-translational modification, such as addition of phosphate groups or ubiquitination, may cause the differences between the apparent and predicted molecular mass. Ubiquitination is a widespread post-translational modification of eukaryotic cellular proteins, which is involved in many normal and pathological biological functions (for reviews see [21–23]).

While antibodies directed against synthetic ubiquitin peptides (Gift of Dr. Muller) did not recognize rbPRLR recombinant proteins (unpublished results), antibodies (P-antiserum) raised against ubiquitin conjugated to bovine γ-globulin had a very strong affinity with the I_2 intracellular domain. The I_1 cytoplasmic domain and the full-length receptor reacted more weakly with these antibodies. The observed differences suggest that the epitope(s) of anti-ubiquitin antibodies are less accessible (likely to be masked) in these forms. From our data we were unable to determine the number of ubiquitin units associated with the receptor, and further experiments will be required to identify the ubiquitination site(s). Directed mutagenesis of lysine residues thought to be implicated in the ubiquitin conjugation would be of great interest.

Sequencing of the purified GH receptor revealed a covalent association with ubiquitin [14]. Interestingly, this ubiquitination was not found on the serum binding protein [24]. The significance of this ubiquitin-association for receptor structure and/or function remains unknown. It has been suggested that ubiquitin could be involved in the rapid receptor turnover observed in vivo or in mechanisms mediating receptor cleavage, and thus generating the binding protein [14,24]. Moreover, Moldrup et al. reported a higher (20 kDa) apparent molecular weight of GH receptor expressed in rat insulinoma cells, and this was attributed to the receptor cytoplasmic part. A covalent modification would occur proximally to the transmembrane domain [25].

The association of ubiquitin to proteins is involved in a great variety of cellular processes [22]. According to the 'tagging' hypothesis, a major function of ubiquitin is to mark proteins for subsequent degradation (for a review see [26]). Thus, ubiquitination might serve as a means to

down-regulate receptors but it is possible that conjugation of ubiquitin to certain proteins also mediates non-proteolytic function(s). Hence, stable ubiquitin acceptors have been reported, including histones H2A and H2B [27], actin [28], the GH receptor [14,24], and others receptors [29,30]. Ligand-induced changes in the ubiquitination of receptors have also recently been described [31,32]. Reversible attachment of ubiquitin residues to a protein for modulation of protein function without targeting the protein for degradation has also been suggested [33]. The role of ubiquitination of the rbPRLR is still unclear. Ubiquitination might be involved (i) in the down-regulation of the receptor or (ii) in some specific protein–protein interactions conferring a 'competent folding' to the receptor .

The ability of the baculovirus expression system to perform the appropriate post-translational modifications to recombinant proteins is well documented [34]. We have demonstrated that the full rbPRLR (i) was targeted to the cell membrane, (ii) bound PRL with a specificity and affinity similar to those reported for the native receptor [13], (iii) was *N*-glycosylated in its extracellular domain, and (iv) was ubiquitinated in its intracellular domain. The major question is whether the ubiquitination we observed in infected Sf9 cells reflects a physiologically relevant association. Thus it is not possible to claim, without direct evidence, that proteins produced in infected insect cells cultures are identical to those found in differentiated tissues. Experiments using functional assays in mammalian cells [35] with mutant forms of the receptor generating ubiquitinated or non-ubiquitinated forms of the receptor, would give a response to this interesting question.

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