Direct evidence that lactogenic hormones induce homodimerization of membrane-anchored prolactin receptor in intact Nb_2-11C rat lymphoma cells

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Abstract The ability of full-size prolactin receptor (PRLR) from Nb_2 rat lymphoma cell line to undergo lactogenic hormone-induced dimerization in intact cells or in a partially purified microsomal fraction was tested. The stoichiometry of ovine placental lactogen (oPL) binding to PRLR was documented by SDS-PAGE of the covalently cross-linked complexes between [^{125}I]oPL and intact Nb_2-11C cells. The molecular masses of the specific bands were 82 and 141 kDa, corresponding to PRLR:oPL and $(PRLR)_2$:oPL complexes. These results provide direct evidence for the occurrence of hormone-induced receptor dimerization in intact cells. Gel-filtration studies revealed that under non-denaturing conditions, the purified receptor forms high-molecular-mass aggregates (190 and 540 kDa) composed of receptor dimers and oligomers. Since this aggregation was not dependent on the presence of lactogenic hormone, it is possible that the receptor in the intact cells may already exist as a non-covalent dimer or oligomer and that hormone-induced dimerization stabilizes the complex or changes its conformation.

Key words: Human growth hormone; Ovine placental lactogen; Prolactin receptor; Nb_2

1. Introduction

Hormone-induced sequential receptor dimerization has been proposed as an initial step in the action of human growth hormone (hGH) [1]. This suggestion was based on chemical and gel-filtration studies [1], on crystallization studies of the 2:1 complex between hGH receptor extracellular domain (hGHR-ECD) and hGH [2], and on indirect experiments with intact hGH-responsive cells [3]. To elucidate whether the same mechanism also applies to prolactin (PRL) action, we prepared PRLR-ECDs from several species and studied their interaction with PRL, hGH, and other lactogenic hormones [4–7]. In contrast to hGHR-ECD, the interaction of PRLR-ECD with lactogenic hormones yielded, in most cases, only a 1:1 complex. Structural 3-dimensional studies of the hGH:hPRLR-ECD complex showed also 1:1 stoichiometry [8]. Only lately, via real-time kinetics studies with a Biacore apparatus, have we been able to provide evidence that, in all cases, lactogenic hormones actually induced PRLR-ECD dimerization [9]. The 2:1 PRLR-ECD:hormone complex was, however, extremely unstable due to the high k_{off} constant, and underwent rapid dissociation to the 1:1 form. Although a few years ago we presented an indirect evidence of receptor dimerization being an initial step in hormone-induced signal transduction in the lactogenic hormone-responsive rat lymphoma Nb_2-11C cell line [10], direct documentation of full-size membrane-embedded PRLR undergoing lactogenic hormone-induced dimerization was lacking. Such evidence is presented here.

2. Materials and methods

2.1. Materials

Ovine (o) PL was prepared by us as described previously [11] and hGH was a gift from Biotechnology General (Rehovot, Israel). Disuccimidyl suberate (DSS) was from Pierce (Rockford, IL, USA). Molecular-mass markers for SDS-PAGE were from Bio-Rad (Hercules, CA, USA). All other reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Nb_2-lymphoma cell proliferation

Nb_2-11C lymphoma cell culture and synchronization procedure were carried out as described previously [12].

2.3. Covalent cross-linking of [^{125}I]oPL to Nb_2-11C cells

Synchronized Nb_2-11C cells were centrifuged and suspended at 10^7 cells/ml in PBS/0.1% (w/v) BSA. To 400 μl of cells in an Eppendorf tube, 50 μl of [^{125}I]oPL (500 000 cpm, 6.2 ng) were added and the cells were incubated at 37°C for 30 min in the presence or absence of 40 μg unlabeled oPL. Freshly prepared DSS (0.25 M in dimethyl sulfoxide) was then added to a final concentration of 0.5 mM and the incubation was continued for an additional 30 min at room temperature. The reaction was stopped by adding 50 μl of 1 M glycine. The cells were pelleted (13 000×*g*, 1 min) at 4°C and washed 3 times in cold PBS. The cell pellet was lysed in 50 μl of lysis solution containing 1% (v/v) Triton X-100 and 1 μM $PhMeSO_2F$ at 4°C for 20 min. Then 12 μl of 5×SDS-PAGE sample buffer was added, the tubes were boiled for 5 min, centrifuged at 13 000×*g* for 5 min and separated by SDS-PAGE on tricine gels [13]. The gels were fixed, dried and autoradiographed at −70°C using Kodak XAR-5 film. Band density was determined by image processing scanner.

2.4. Preparation of a solubilized microsomal fraction from Nb_2 -11C cells

Synchronized Nb_2-11C cells (10^{10} cells) were homogenized with Polytron (3×30 s at maximal speed in 4°C) in 250 ml of 25 mM HEPES–NaOH (pH 7.5) buffer containing 1 mM $PhMeSO_2F$, 50 μM chloroquine, 0.01% (w/v) pepstatin 0.02% (w/v) soybean trypsin inhibitor (SBTI), 0.01% (w/v) leupeptin, 1 mM $ZnCl_2$, 1 mM EDTA and 1 mM EGTA. The homogenate was spun for 20 min at 600×*g* and the microsomal fraction was recovered by subsequent centrifugation at 150 000×*g* for 1 h. The precipitate was suspended in 20 ml of 25 mM HEPES–NaOH buffer, pH 7.5, containing 10 mM $MgCl_2$, 0.01% leupeptin, 1 mM $PhMeSO_2F$, 20 μM chloroquine and 0.01% soybean trypsin inhibitor, and solubilized with 1% Triton X-100, by stirring for 30 min at room temperature. Insolubilized material was removed by centrifugation at 150 000×*g* for 1 h and the soluble fraction was stored at −20°C until use.

2.5. Gel filtration

Aliquots (200 μl) of solubilized microsomal fraction (2.5 mg pro-

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tein/ml) or complex formed by an overnight pre-incubation of [^{125}I]hGH (50×10^4 cpm/tube) with the solubilized microsomal fraction in absence or present of 2 μg hGH/tube were chromatographed at room temperature on a Superose 12 column (1×30 cm) (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM phosphate buffer, pH 7.4, containing 150 mM NaCl and 0.1% Triton X-100. The column was eluted with the same buffer at 0.5 ml/min. Fractions of 0.25 ml were collected during chromatography of the [^{125}I]hGH:soluble receptor complex and counted on a γ-counter. Gel filtration of the solubilized microsomal fraction was performed in an identical manner, except that 0.1 ml (in triplicates) of each 0.5 ml fraction collected was subjected to radioreceptor assay. Each gel filtration experiments was performed twice.

2.6. Radioreceptor assay

Binding of [^{125}I]hGH (5×10^4 cpm/tube) to 100 μl aliquots of fractions collected by gel filtration of the solubilized microsomal fraction was assayed, in triplicates in 25 mM HEPES buffer, pH 7.5, supplemented with 10 mM $MgCl_2$, 0.5 mM $PhMeSO_2F$, 0.1% Triton X-100 and 0.1% BSA. Non-specific binding was determined in the presence of unlabeled hGH (2 μg/tube). The tubes were incubated for 14–16 h at 24°C. Following incubation, 500 μl of 25% polyethylenglycol solution was added to the reaction mixture. The tubes were centrifuged, the supernatant aspirated and the precipitate counted on a γ-counter. Specific binding was calculated as % of total hGH counts. Radioiodination of hGH and oPL was carried out in the presence of chloramine T and 1 mCi Na[^{125}I], as described previously [14].

3. Results

3.1. Determination of the stoichiometry of the full-size PRLR:hGH complex by gel filtration

The first set of experiments was performed using solubilized microsomal fraction prepared from Nb_2 cells synchronized in the absence of lactogenic hormones. Two types of experiments were performed. In the first experiment, molecular masses of the solubilized PRLR:[^{125}I]hGH complex (Fig. 1A) and in the second free receptors in the solubilized fraction (Fig. 1B) were separated by gel filtration on a Superose 12 column and subsequently identified by their capacity to bind iodinated [^{125}I]hGH. The iodinated ligand was found in four of the eluted fractions (Fig. 1A). Two fractions (eluted at 8–9 and 10–11 ml) were judged to contain [^{125}I]hGH:receptor complexes since [^{125}I]hGH was absent when the solubilized receptors were pre-incubated with [^{125}I]hGH in the presence of excess unlabeled hGH. The two other peaks of radioactivity (eluted at 13 and 15 ml) represented dimers and monomers of free [^{125}I]hGH. In the second experiment, fractions eluted following gel filtration of the free receptors revealed two peaks of activity, eluted respectively at 7–8 and 10.5–11.5 ml (Fig. 1B). Their respective molecular masses, as calculated from a standard curve (Fig. 1C), were 540–580 and 190–210 kDa. These fractions were subsequently cross-linked to [^{125}I]hGH and analyzed by SDS-PAGE (4–10% gradient) followed by autoradiography. Several specifically labeled bands were detected within the range of 84–200 kDa (not shown). There was, however, no difference between the two peaks of activity, indicating the higher molecular-mass fraction to be an oligomer of essentially similar components which likely underwent partial proteolysis during the preparation of the solubilized microsomal fraction.

3.2. Determination of the stoichiometry of PRLR:oPL complex in intact Nb2-11C cells by affinity labeling

Since the experiments described in the former paragraph were not conclusive enough to ensure existence of lactogen hormone-induced receptor dimerization this set of experiments was performed in intact cells. Preparation of recombinant oPL [11] and our findings that its complex with rPRLR-ECD are more stable than those formed by hGH prompted us to chose it as a ligand [7,9]. Using autoradiography (Fig. 2), two specific bands with molecular masses of 82 and 141 kDa were identified; scanning of the autoradiograph revealed an approximate 3:1 ratio. These bands most probably correspond to 1:1 and 2:1 hormone/receptor complexes, having

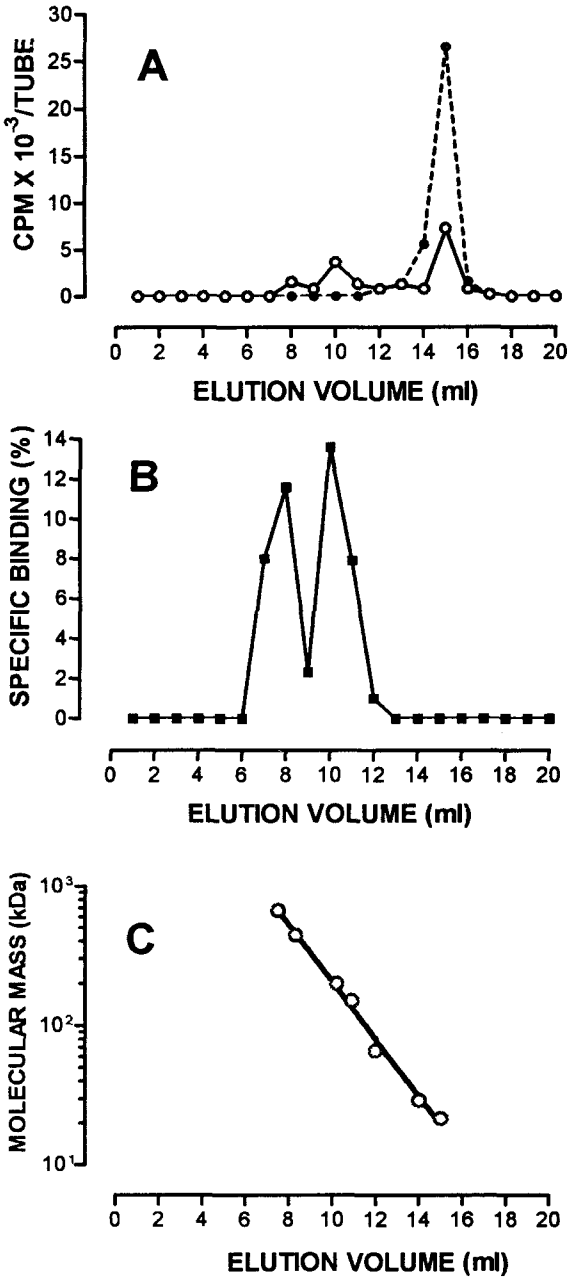

Fig. 1. Superose 12 column chromatography of [^{125}I]hGH:PRL-R complexes (A) or free receptors in the solubilized microsomal fraction (B). Complex formation (A) was performed in the absence (- - -) or in presence (• • •) of excess hGH and the tubes, collected during the chromatography, were counted in a γ-counter. The free receptor eluted from the column (B) was detected by determination of its ability for specific [^{125}I]hGH binding. A standard curve (C) was determined with the following markers: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa) and bovine serum albumin (66 kDa).

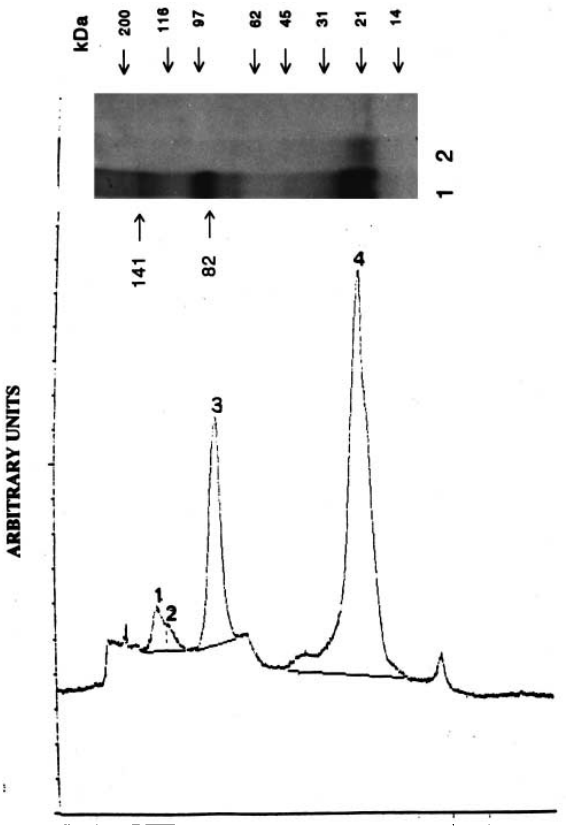

Fig. 2. Affinity labeling of PRLR in intact Nb_2 cells. The cross-linked [^{125}I]oPL:PRLR complexes from intact Nb_2-11C cells prepared in the absence (lane 1) or presence (lane 2) of 40 μg oPL were subjected to tricine-SDS-PAGE (7.5%) in the presence of reducing agent. The gel was then fixed, dried, autoradiographed and scanned.

theoretical masses of 87 and 152 kDa, respectively. The specificity of the binding is further demonstrated in Fig. 2, which shows the binding of [^{125}I]oPL to be fully displaced by the lactogenic hormones such as oPL, hGH and oPRL, but not by the somatogenic oGH. An additional specific band with a molecular mass of 21–25 kDa most likely represents free ligand that was non-covalently associated with the receptor and dissociated due to the SDS treatment.

4. Discussion

The affinity labeling experiments suggest that PRLR anchored in the membrane of intact Nb_2 cells undergoes lactogenic hormone-induced dimerization and support our recent findings which showed that rPRLR-ECD is capable to form 2:1 complex with lactogenic hormones [7,11] although the complex undergoes fast dissociation to a 1:1 form [9]. The finding that the homodimeric complex formed with oPL or bPL is more stable than that formed with hGH may explain why in previous experiments [15], only 1:1 complex could be detected. Our kinetics analysis [9] is consistent with this finding, in that it showed that the k_{on} kinetics constants characterizing the association of the rat PRLR-ECD to oPL sites 1 and 2 are almost identical, whereas the k_{off} values for site 2 were 12-fold those of site 1. This also explains why the relative amount of the 2:1 complex is much lower than that of the 1:1 complex (Figs. 1 and 2).

The gel-filtration experiments (Fig. 3) indicated that the receptor existing in the solubilized microsomal fraction aggregates into large molecular forms. The apparent molecular mass of the lowest of these (190–210 kDa), corrected for the presence of Triton X-100 [16] was 133–147 kDa, close to that obtained by affinity labeling. SDS-PAGE analysis in the presence of reducing agent indicated it to be composed of several species suggesting the occurrence of partial proteolysis during preparation of the microsomal fraction. Since the higher molecular form (540–580 kDa) showed a similar profile when analyzed by SDS-PAGE, it most likely represents a receptor oligomer. It should be noted that formation of these aggregates was not induced by hormone binding, and that almost the same molecular species were yielded by gel filtration of the PRLR-hGH complexes and the free receptor (Fig. 3). One possible explanation of this finding is that in situ, the receptors already exist as dimers or oligomers, and hormone-induced dimerization in fact stabilizes the complex or changes its conformation. This suggestion must however be treated with caution since difficulties concerned with the preparation of the soluble microsomal fraction, such as absolute prevention of partial proteolysis and the use of detergent may lead to an artificial appearance of oligomeric proteins under non-denaturing conditions. The results obtained with the intact cells are more conclusive to show formation of 2:1 receptor/hormone complex. It implies that the hormone-induced receptor dimerization or hormone-induced structural transition of a pre-existing receptor dimer that allows transphosphorylation of the PRLR-associated JAK_2 kinases is an initial event in the transduction of the biological signal [17,18]. Although experiments performed by us [7,9,11] or others [1,2] with soluble recombinant PRLR- or GHR-ECDs and the indirect kinetic

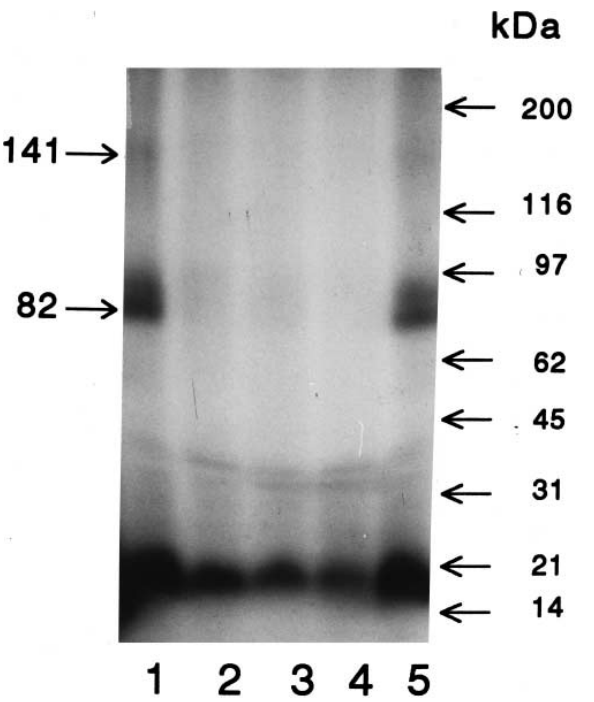

Fig. 3. Affinity labeling of PRLR intact Nb_2 cells. The cross-linked [^{125}I]oPL:PRLR complexes from intact Nb_2-11C cells prepared in the absence (lane 1) or presence of 40 μg oPL, hGH, oPRL or oGH (lanes 2-5, respectively). For other details, see Fig. 1.

experiments [3,10] favor the hormone-induced receptor dimerization model the other possibility cannot be yet excluded.

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