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Characterization of lactogen receptors in lactogenic hormone-dependent and independent NB2 lymphoma cell lines

A. Ashkenazi, R. Cohen and A. Gertler

Department of Agricultural Biochemistry, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel

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A comparative study of lactogen receptors in lactogenic hormone-dependent (Nb2-11C) and independent (Nb2-SP) rat lymphoma cell lines revealed no significant differences in their binding and structural characteristics. The affinity for human growth hormone (hGH) and ovine prolactin is identical ($K_d = 1.3 \times 10^{-10}$ M). Exposure to hGH results in a rapid receptor down-regulation. This process is faster in Nb2-SP cells, however, similar receptor levels are reached within 3 h. Affinity labeling revealed 2 major binding species (M_r 75 000 and 29 000) in both cell lines. These observations suggest that the spontaneous proliferation of Nb2-SP cells probably results from an aberration in some post-receptoral event of the mitogenic pathway.

(Nb2 lymphoma cell); Prolactin; Human growth hormone; Lactogen receptor

1. INTRODUCTION

Prolactin (PRL), similar to many polypeptide hormones, initiates its biological action by binding to its specific receptor on the membranes of target cells. Nb2 lymphoma cells, which are absolutely dependent on lactogenic hormones for their proliferation [1,2], have proved to be a most suitable and sensitive in vitro cell model for studying the mechanism of mitogenic action of lactogenic hormones, such as PRL or human growth hormone (hGH) [3–9]. Recently, two cell lines derived from the original Nb2 strain, one of which is lactogen-dependent (Nb2-11C) and another, which is lactogen-independent (Nb2-SP), have been established [4,6]. These cells can be propagated in continuous suspension cultures in medium supplemented with either fetal calf serum that contains lactogenic hormones (Nb2-11C), or lactogen-free horse serum (Nb2-SP). The autonomous proliferation of the Nb2-SP cell line has raised the question of whether the lactogen receptors in these cells have a mitogen-independent, constitutively activated conformation. It has recently been reported [10] that the transforming protein of avian erythroblastosis virus (*erb-B*) is apparently a truncated EGF receptor, lacking the extracellular EGF-binding domain, but not the internal and most of the transmembrane part of the receptor. The lack of the regulatory EGF-binding domain may then be accompanied by a constitutive activation of the effector domain. Here, we compare the binding and structural characteristics of the lactogen receptors in the lactogen-dependent and -independent cell lines, in order to assess the possibility that the receptor itself triggers the mitogenic pathway in the spontaneously proliferating cell line.

Correspondence address: A. Gertler, Dept of Agricultural Biochemistry, Faculty of Agriculture, The Hebrew University of Jerusalem, PO Box 12, Rehovot 76100, Israel

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2. MATERIALS AND METHODS

2.1. *Hormones and reagents*

The sources of all the hormones used in this research were described in our previous paper [5]. Radioiodinated hGH (^{125}I-hGH) was prepared as described in [11]. Carrier-free Na^{125}I was obtained from Amersham (Bucks, England). Fischer's medium for leukemic cells and RPMI 1640 medium were obtained from Gibco (Grand Island, NY). Fetal calf serum (FCS) and lactogen-free horse serum (HS) were obtained from Sera-Lab (Sussex, England). Reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Richmond, CA). M_r standards, dithiothreitol (DTT) and bovine γ-globulin (bgG) from Sigma (St. Louis, MO), and disuccinimidyl suberate (DSS) from Pierce (Rockford, IL).

2.2. *Nb2 lymphoma cells culture*

Two cloned Nb2 lymphoma cell lines, developed in Dr H.G. Friesen's lab (H. Cosby), were employed. Cells were cultured as in [1–4], except that stock cultures of Nb2-11C cells were maintained in RPMI 1640 medium, supplemented with 25 mM Hepes buffer (pH 7.4), instead of Fischer's medium. Synchronization of cells in the G_0/G_1 phase and monitoring of cell proliferation were carried out as described [4].

2.3. *Measurement of binding*

The binding of ^{125}I-hGH was assayed in cell homogenates or in intact cells. Homogenates were prepared from synchronized cells, which were pelleted, resuspended in HS-supplemented Fischer's medium (3.2×10^6 cells/ml) and homogenized in an Ultraturrax (3×15 s at 120 rpm). 0.5 ml aliquots of the homogenate suspension were transferred to Eppendorf tubes and incubated for 16 h at 25°C with 1.5×10^5 cpm ^{125}I-hGH in the presence of increasing concentrations (0–7 nM) of unlabeled hGH or ovine PRL (oPRL). The reaction was terminated by addition of 1 vol. of ice-cold buffer (25 mM Tris-HCl, 10 mM $MgCl_2$, 0.2% bgG, pH 7.5) followed by centrifugation (5 min, $11000 \times g$). The supernatant was removed and the pellets were counted in a Kontron MR-430 gamma counter. The binding to intact cells was measured with synchronized cells, which were pelleted and resuspended in HS-supplemented Fischer's medium (3×10^6 cells/ml). 1 ml aliquots of the cell suspension were transferred to 5 ml plastic vials and incubated for 1 h at 37°C with ^{125}I-hGH (3×10^5 cpm). Non-specific binding was determined in the presence of 1 μg unlabeled hGH. Reaction was terminated by transferring the cells to Eppendorf tubes, pelleting and counting, as described for homogenates.

2.4. *Modulation of receptor binding by hGH*

Synchronized cells (2×10^6/ml) were exposed to various concentrations of hGH at 37°C. Aliquots were removed every 30 min during the first 3 h of exposure, centrifuged (5 min, $500 \times g$) and the medium was decanted. The cell pellets were placed on ice and resuspended in an acid buffer (0.1% acetic acid, 150 mM NaCl, 0.1% BSA, pH 4) for 3 min. This procedure was found to be optimal for the dissociation of cell surface-bound hormone without affecting cell viability (Ashkenazi, A. and Gertler, A., unpublished). After a 5-fold dilution in neutral buffer (25 mM Hepes, 150 mM NaCl, 0.1% BSA, pH 7.4), the cells were pelleted and washed twice in the same buffer. Finally the cells were resuspended in HS-supplemented Fischer's medium and the binding of ^{125}I-hGH was assayed as described.

2.5. *Affinity labeling of lactogen receptors*

Affinity labeling was carried out either with cell homogenates or microsomal fractions, which were prepared from cell homogenates by centrifugation at $500 \times g$ (20 min) followed by centrifugation of the supernatants at $100000 \times g$ (1 h). Approx. 0.5 mg homogenate proteins or 0.2 mg microsomal proteins were incubated with ^{125}I-hGH (1 or 2×10^6 cpm, respectively) in the absence or presence of excess unlabeled hGH (16 h, 25°C). Incubation was terminated by addition of 1 vol. ice-cold Hepes buffer (50 mM, pH 7.5) followed by centrifugation (5 min, $11000 \times g$). The pellets were washed 3 times to remove unbound ^{125}I-hGH and finally resuspended in 0.1 ml of the same buffer. DSS, freshly dissolved in dimethyl sulfoxide, was added to a final concentration of 0.5 mM. The crosslinking reaction was carried out for 15 min at 0°C and stopped by the addition of 0.05 vol. Tris-HCl buffer (2 M, pH 7.5), followed by centrifugation. Pellets were washed 3 times, resuspended in XAR-5 film).

sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.005% pyronin Y, 50 mM DTT), boiled (5 min), and subjected to SDS-PAGE (7.5%) as described by Laemmli [12], followed by autoradiography (Kodak intensifying screen and XAR-5 film).

3. RESULTS AND DISCUSSION

3.1. *Displacement studies*

Human GH and oPRL were equally potent in competing for the binding of ^{125}I-hGH to cell homogenates (fig.1A,C) with identical displacement curves in Nb2-11C and Nb2-SP cells. Scatchard analysis (fig.1B,D) yielded linear curves, indicating a single class of binding sites. The affinity for hGH and oPRL was identical in both cell lines (table 1). It should be noted that this affinity is 10–20-fold higher than those reported for normal target tissues, such as mammary gland or liver [13]. The K_d values are in good agreement with values reported for binding of hPRL to intact cells from the original Nb2 strain [3]. Met^{14}hGH, an antagonist of the mitogenic activity of lactogenic hormones in Nb2-11C cells [5], bound to the lactogen receptors of both cell lines with the same affinity, however, it was devoid of any effect on the lactogen-independent proliferation of Nb2-SP cells (not shown). The binding capacity in Nb2-SP cells was remarkably higher than in Nb2-11C cells (table 1). This difference may result from down-regulation of lactogen receptors in Nb2-11C cells due to the presence of lactogenic hormones in the FCS-supplemented culture medium, followed by an only partial recovery of receptors during synchronization of the cells in HS-supplemented medium.

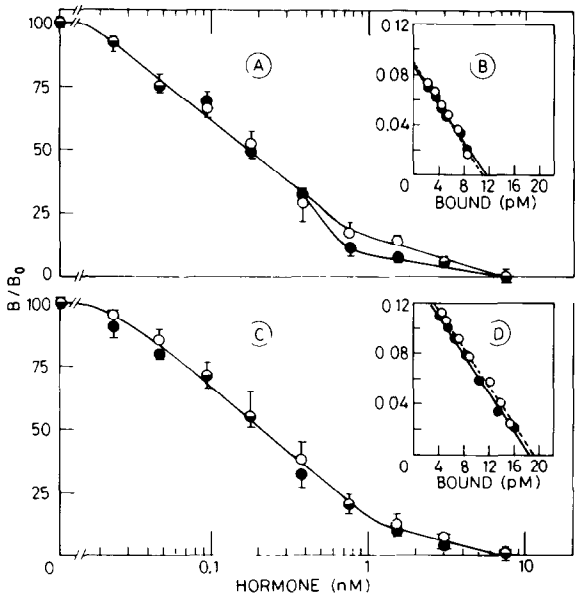

Fig.1. Displacement of the binding of ^{125}I-hGH to cell homogenates by oPRL or hGH. Competitive binding studies were carried out with Nb2-11C (A,B) or Nb2-SP (C,D) cell homogenates, using ^{125}I-hGH as tracer and hGH (○) or oPRL (●) as displacers. Maximal specific binding (B_0) was 7077 ± 880 cpm (A) and 10645 ± 1030 cpm (C). (B,D) Scatchard [14] analyses.

Table 1

Dissociation constants and receptor numbers in Nb2-11C and Nb2-SP cell-homogenates

Cell line	Hormone	K_d[a] (pM)	Receptors/cell[b]
Nb2-11C	oPRL	127 ± 3	2927 ± 208
	hGH	134 ± 14	2867 ± 271
Nb2-SP	oPRL	133 ± 13	4156 ± 180
	hGH	130 ± 13	4577 ± 382

[a] Calculated from Scatchard plots described in fig.1B,D. Values are presented as means ± SE of 3 experiments

[b] As in [a], assuming a hormone:receptor ratio of 1:1 in binding

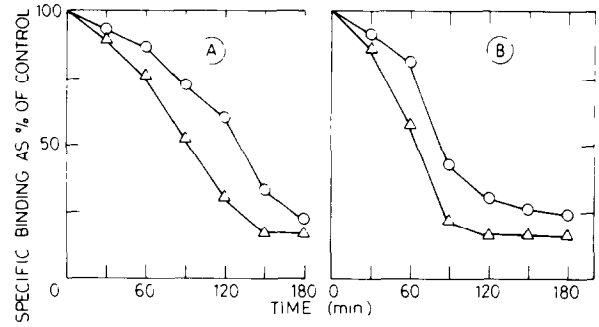

Fig.2. Modulation of receptors by hGH. The binding of ^{125}I-hGH was assayed following exposure of Nb2-11C (○) or Nb2-SP (△) cells to hGH at 10 ng/ml (A) or 100 ng/ml (B). The specific binding at $t = 0$ was 3247 ± 75 cpm and 6597 ± 152 cpm (Nb2-11C and Nb2-SP cells, respectively). In all determinations from 60 to 180 min, the specific binding in Nb2-SP cells was significantly ($P < 0.05$) lower than in Nb2-11C cells.

3.2. *Modulation of receptor binding by hGH*

Exposure of Nb2 cells to hGH results in a rapid decrease in receptor binding, to ~20% of the original levels, within 3 h (fig.2). Scatchard analysis (not shown) revealed that the decrease in binding results from down-regulation of receptors rather than from a change in affinity. Down-regulation was significantly more rapid in Nb2-SP cells, however, it should be noted that the initial receptor levels in these cells were higher. After exposure to hGH, the absolute amounts of ^{125}I-hGH bound/cell were similar in both cell lines. Therefore, the relatively more rapid down-regulation in Nb2-SP cells could result from their initially higher receptor levels. It is most likely that the down-regulation process was accompanied by receptor degradation, since the binding to cell homogenates decreased in a similar manner following exposure of the intact cells to hGH (not shown).

3.3. *Affinity labeling of lactogen receptors*

Lactogen receptors in Nb2-11C and Nb2-SP microsomal fractions were covalently labeled with ^{125}I-hGH (fig.3). Two major, specifically labeled species, corresponding to M_r 97000 and 51000, were observed in both cell lines, indicating an M_r of 75000 and 29000, respectively for the binding species. The intensity of the bands in Nb2-SP cells was higher, due to the higher receptor levels in these cells. Bands of similar M_r, although less intense, were observed when SDS-PAGE was carried out under non-reducing conditions (not shown) indicating a lack of disulfide bonds that stabilize the quaternary structure.

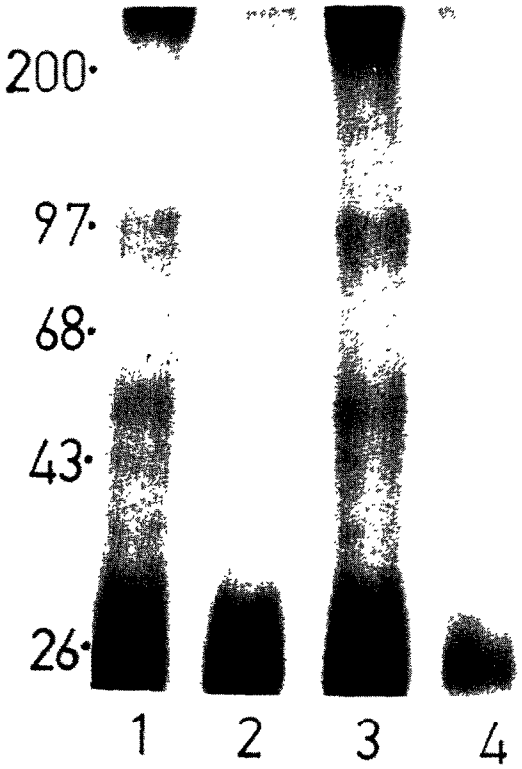

Fig.3. Affinity labeling of microsomal lactogen receptors. Microsomal fractions prepared in the presence of phenylmethylsulfonyl-fluoride (1 mM) and leupeptin (0.1 mM), from Nb2-11C (1,2) or Nb2-SP (3,4) cells were incubated with ^{125}I-hGH in the absence (1,3) or presence (2,4) of hGH. Crosslinking and SDS-PAGE were carried out as described in the text.

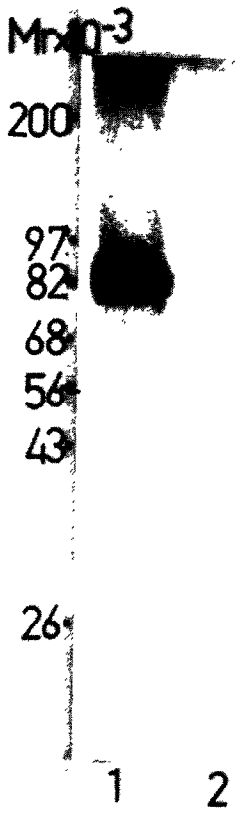

Fig.4. Affinity labeling of lactogen receptors in Nb2-11C cell homogenates. Homogenates were incubated with ^{125}I-hGH (1×10^6 cpm) in the absence (1) or presence (2) of 1 μg oPRL. Crosslinking and SDS-PAGE were carried out as described in the text.

Affinity labeling of lactogen receptors in cell homogenates revealed one major specifically labeled band (M_r 82000) and two faint bands (M_r 68000 and 56000) in Nb2-11C cells (fig.4). The 82 kDa band was also observed in Nb2-SP cells, however, the other species appeared as a diffuse band of 56–68 kDa (not shown). Excess oPRL prevented the labeling of these bands (fig.4, lane 2), whereas recombinant bovine GH had no effect (not shown), thus confirming the identity of the receptors as lactogenic rather than somatotropic binding sites in both cell lines. The differences in the M_r of the species labeled in homogenates and in microsomal fractions could be attributed to proteolytic activity present in the cell homogenates during incubation, originating in the cytosolic fractions. Indeed, when cell homogenates were prepared in the presence of 1 mM phenylmethylsulfonyl fluoride and 0.1 mM leupeptin (not shown), the 97 and 56 kDa bands were the major labeled species; however, they appeared in a diffuse manner. Values of 30–40 kDa have been observed for PRL-binding subunits in various tissues and species [13], and also in cow mammary gland (Ashkenazi, A., Madar, Z. and Gertler, A., submitted). Higher M_r species (M_r 88000–91000) were observed in rat Leydig cells [15] and rat ovary [16,17]. In the former cases the size of the binding species was not affected by reducing agents, as reported here for Nb2 cells. However, in the latter cases [15,16], the high-M_r complex yielded the low-M_r complex under reducing conditions. Therefore, the quaternary structure of the PRL receptor and the nature of its inter-subunit interactions probably vary with the species and/or target tissue.

3.4. *Conclusion*

We have demonstrated that the lactogen receptors in lactogenic hormone-dependent and in autonomously proliferating Nb2 lymphoma cell lines do not seem to differ in their structural and binding properties. These findings suggest that an aberration in a post-receptoral event of the mitogenic pathway, rather than in the receptor itself, is responsible for the spontaneous proliferation of the Nb2-SP cell line. This is further substantiated in the finding that the hGH antagonist Met^{14}hGH, which bound to the lactogen receptors of Nb2-SP cells, did not affect their lactogen-independent proliferation. However, there are a few other possibilities that cannot be precluded, namely that the lactogen receptors of the two cell lines differ in: (i) their cytosolic signal-transducing domain, or (ii) their organization in the cell membrane, in features that do not affect the characteristics that were studied, or that Nb2-SP cells are sensitive to another unidentified mitogen present in the HS-supplemented medium, which signals them to proliferate through a different receptor system.

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