Identification of Two Specific Prolactin Binding Sites on Nb_2 Rat Lymphoma Cells (43680)

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Abstract. **Several laboratories have earlier identified and characterized a specific prolactin binding site (receptor) on Nb_2 cells which has a Kd of about 2×10^{-10} *M* and a binding capacity of about 12,000 receptor sites per cell. Using [I^{125}]-labeled human growth hormone (hGH), we have confirmed the results of these earlier reports. In addition, a second, "high-affinity" receptor site for prolactin has been identified. This site has a Kd of about 2×10^{-12} *M* and there are an average of 67 sites per cell. Binding of [I^{125}]-hGH to the high-affinity receptor site is saturated by 1 min after incubation at 37°C, whereas saturation of the lower affinity site requires a 1–3 hr incubation at 37°C. Specificity of prolactin binding to both the high- and low-affinity "receptors" was established by the fact that several nonlactogenic hormones, including bovine growth hormone and insulin, do not displace [I^{125}]-hGH from the receptor sites. When Nb_2 cells were incubated for 1 hr at 0°C with 100 pg/ml [I^{125}]-hGH, no binding to the high-affinity receptor was detected, thus demonstrating the temperature dependence of binding to this receptor. The kinetics of binding of hGH to the high-affinity binding site correlates well with the dose-response effects of lactogenic hormones on mitogenesis in the Nb_2 cells. In addition, the rapid binding of hGH to the high-affinity receptor may be related to specific effects of lactogenic-hormones which occur within seconds or minutes after adding these hormones to Nb_2 cells.**

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Lactogenic hormones including prolactin, the placental lactogens, and human growth hormone are potent mitogens in a T lymphoma cell line (Nb_2) derived from an estrogenized Noble rat (1, 2). Receptors for the lactogenic hormones in the Nb_2 cells have been isolated and characterized (3). The receptor consists of a 393 amino acid peptide of which the intracellular, extracellular, and intramembrane portions of the molecule have been identified. Binding studies employing several lactogenic hormones indicate that there are about 12,000 "low-affinity" prolactin receptors per Nb_2 cell and the Kd for binding is about $1–2 \times 10^{-10}$ *M* (2). Interestingly, the concentration of lactogenic hormones that causes a half-maximal mitogenic response in the Nb_2 cells is about $1–6 \times 10^{-12}$ *M*, one to two orders of magnitude less than the Kd for binding to the prolactin receptor (1, 2).

The disparity of the above numbers led us to carry out the present studies in which we reexamined the binding characteristics of lactogenic hormones in the Nb_2 cells using a high specific activity [I^{125}]-labeled hGH preparation. The intent was to determine if we could identify a "high-affinity" prolactin receptor. A precedent for the possible existence of both high- and low-affinity receptors for prolactin is the nerve growth factor (NGF)-receptor system where the two types of receptors have been shown to exist (4). Since both NGF and lactogenic hormones have receptors that are members of the cytokine family of receptors, we reasoned that high-affinity receptors for prolactin may also be present in cells.

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Methods

The Nb_2 node lymphoma cells were provided by Dr. C. T. Beer of the Cancer Control Agency of British Columbia (Vancouver, British Columbia, Canada). Ovine prolactin (NIH-P-S-19), bovine growth hormone (NIH-GH-B18), and human growth hormone (NIH-GH-HS216OE) were gifts from the National Hormone and Pituitary Program, University of Maryland. Other materials used in these studies were from the following sources: fetal calf serum and Fisher's medium from Gibco Laboratories, Grand Island, NY; horse serum, bovine serum albumin (BSH, fraction V) and phosphate buffered saline (PBS) from Sigma Chemical Co., St. Louis, MO; porcine insulin, penicillin, and streptomycin from Eli Lilly Co., Indianapolis, IN; sodium-[I^{125}] from Amersham Corp., Arlington Heights, IL.

The Nb_2 cells were maintained as suspension culture in 25-cm^2 culture flasks containing "growth medium" (Fisher's medium supplemented with 10% fetal calf serum, 10% horse serum, 1×10^{-4} *M* 2-mercaptoethanol, 50,000 IU/liter penicillin, and 50,000 μg/liter streptomycin). The cells were incubated at 37°C in the presence of a 95% air–5% CO_2 gas mixture. Fresh medium was added every 72 hr. Twenty-four hours before beginning an assay the cells were collected by centrifugation at 300xg. The cells were then resuspended and cultured for 24 hr in "stationary medium" (same components as growth medium except for the deletion of fetal calf serum and the reduction of horse serum from 10% to 3%). The cells were next collected by centrifugation at 300xg, washed once with PBS, and then suspended in fresh PBS and used for binding studies. [I^{125}]-hGH at the specified activity and concentration was added to the cells after which the cells (about 1×10^6/ml) were incubated at 37°C for appropriate times. Nonspecific binding was determined by the inclusion of 10 μg/ml oPRL in the binding reaction mixture. Specific binding was determined by subtracting the nonspecific from the total binding. Nonspecific binding constituted about 20%–40% of the total binding in all experiments. After incubation of the cells with [I^{125}]-hGH, the quantity of bound radiolabeled hormone was determined as follows: 0.5 ml of the cells were layered on 3.5 ml PBS in 4.5 ml polypropylene tubes. This and all subsequent steps were carried out at 0°–4°C. The cells were collected by centrifugation at 300xg for 10 min, washed with 4 ml PBS, and again collected by centrifugation for 10 min at 300xg. Radioactivity in the final cell pellet was then determined by scintillation counting. Numbers of cells in the reaction mixture was determined using a Coulter counter.

High (720 μCi/μg) and low (178 μCi/μg) specific activity [I^{125}]-hGH preparations were made by the lactoperoxidase method of Tower *et al.* (5).

Statistical comparisons were made using ANOVA followed by Scheffe's test where applicable.

Results

Panels A and B of Figure 1 show the extent of hGH binding to Nb_2 cells as the hGH concentration was increased from 0.5 pg/ml to 12,000 pg/ml. A Scatchard plot of these data is shown in Panel C of Figure 1. This plot was generated using Lotus 1–2–3; binding affinities and capacities were determined by further analyzing the data using Enzifitter (Elsevier Biosoft). Clearly, two specific binding sites exist on the Nb_2 cells: a "low-affinity" site with a Kd of 1.8×10^{-10} *M* and a capacity of 3,600 sites/cell, and a second "high-

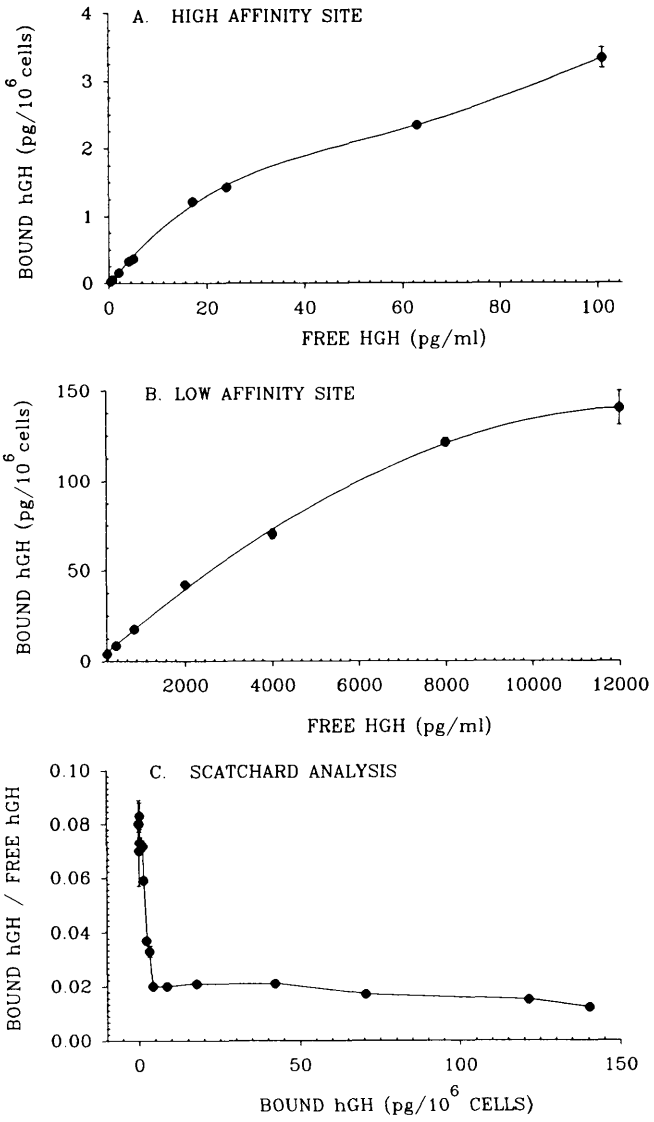

Figure 1. Effect of hGH concentration on binding to lactogenic hormone binding sites in Nb_2 cells. Nb_2 cells (1×10^6/ml) were incubated with [I^{125}]-hGH at the concentrations indicated ±10 μg/ml oPRL for 90 min at 37°C. The extent of specific binding was then determined. Panel A, high-affinity binding, Panel B, low-affinity binding, Panel C: Scatchard plot of binding to both sites. Numbers in the figures represent the mean ± SE of four observations.

affinity" site with a Kd of 2×10^{-12} *M* with an average of 67 binding sites per cell.

The time-courses for the binding of [I^{125}]-hGH to the two Nb_2 cell binding sites are shown in Figure 2. A 1–2 hr incubation period at 37°C is required for a maximal binding to the "low-affinity" site (Panel B) whereas saturation of the "high-affinity" site occurs within minutes after addition of the hormone to the cells (Panel A).

Specificity of [I^{125}]-hGH binding to high- and low-affinity lactogenic hormone binding sites on the Nb_2 cells was established by the experiments shown in Tables I and II. At concentrations of 0.1 and 1 μg/ml, insulin, bGH, and BSA did not displace 10 ng/ml [I^{125}]-hGH from the low-affinity binding site. Similarly at 10 μg/ml, insulin did not displace the [I^{125}]-hGH. However, at 10 μg/ml, both the bGH and BSA preparations had small effects on the displacement of [I^{125}]-hGH, perhaps due to a minor contamination of these preparations with lactogenic hormones. Both oPRL and hGH displaced [I^{125}]-hGH from the low-affinity binding site in a concentration-dependent fashion, with oPRL being more effective than hGH. Similar results were reported earlier (2). Table II shows specificity studies regarding the high-affinity lactogenic hormone binding sites. Like with the low-affinity binding site, insulin, bGH, and BSA at concentrations of 0.1 and 1 μg/ml did not displace [I^{125}]-hGH. Similarly, 10 μg/ml insulin had no effect whereas 10 μg/ml bGH or BSA caused a partial displacement. Both oPRL and hGH caused a concentration-dependent displacement of [I^{125}]-hGH from the high-affinity lactogenic hormone binding site.

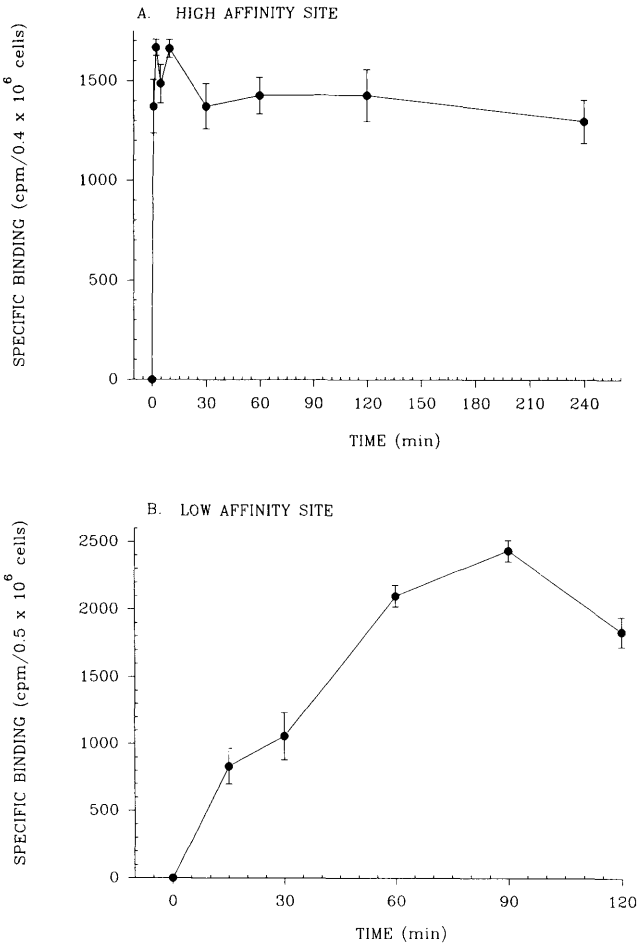

Figure 2. Time-course of [I^{125}]-hGH binding to high- and low-affinity lactogenic hormone binding sites. Nb_2 cells were incubated with 100 pg/ml (Panel A) or 10 ng/ml (Panel B) [I^{125}]-hGH with or without 10 μg/ml oPRL for the times indicated. The extent of specific binding was then determined. Numbers in the figures represent the mean ±SE of four observations.

In further studies (data not presented) the temperature dependence of [I^{125}]-hGH binding to the high-affinity lactogenic hormone binding site was determined. After a 1-hr incubation of Nb_2 cells at 0°C with 100 pg/ml [I^{125}]-hGH, no specific [I^{125}]-hGH binding was found associated with the cells. Thus, like with the low-affinity receptor (2), binding to the high-affinity receptor is temperature dependent.

Discussion

These studies clearly show that Nb_2 cells contain both high- and low-affinity binding sites for lactogenic hormones. The low-affinity site has previously been extensively characterized. It is a glycosylated protein containing 393 amino acids in a single peptide chain (3). Interestingly, when the cDNA for the 393 amino acid Nb_2 cell receptor was transfected and expressed in CHO cells, prolactin stimulated the expression of a reporter gene (CAT) which was fused to the 5′-flanking sequence of milk protein genes (8). The functional role of the low-affinity receptor in the stimulation of differentiative processes is therefore clear. In recent cross-linking studies carried out in our laboratory (6), the molecular weight of the major lactogenic hormone binding protein present on Nb_2 cells was determined to be about 70 kD; these results were confirmed by Rui *et al.* (7). To a lesser extent, the radiolabeled lactogenic hormones were also found cross-linked to larger molecular weight proteins which have not yet been characterized. The discrepancy in the number of low-affinity binding sites identified in the present study vs that in an earlier report (2) (3,600 vs 12,000 sites per cell) may be due to a number of experimental variables. These could include possible real differences in the numbers of binding sites in cells that are propagated in different laboratories under somewhat different conditions. Or the difference could be created by technical differences employed to quantitate the numbers of binding sites.

The lactogenic hormones have been shown to have a number of effects on Nb_2 cells which occur within minutes after hormone addition. These effects include an increased Ca^{++} uptake (9), an activation of Na^+/H^+ exchange (10), an increased rate of RNA syn-

Table I. Specificity of Low-Affinity Lactogenic Hormone Binding in Nb_2 Cells*

Addition	Concentration of addition (μg/ml)			
	0	0.1	1	10
oPRL	100 ± 3%**	120 ± 4.2	6.3 ± 4.8	0 ± 9.7
hGH	100 ± 3	87 ± 4.5	59.3 ± 11.7	24.0 ± 5.1
Insulin	100 ± 3	100 ± 3.4	82.2 ± 12.6	100 ± 4.1
bGH	100 ± 3	100 ± 4.9	95.7 ± 2.9	72.3 ± 4.7
BSA	100 ± 3	100 ± 6.2	100 ± 5.4	78.7 ± 5.5

* Nb_2 cells were incubated at 37°C for 1 hr with 10 ng/ml [I^{125}]-hGH with or without the additions indicated in the table. The percent of specific binding that occurs in the absence of the additions was then calculated.
** Numbers in the table represent the mean ± SE of four observations.

Table II. Specificity of High-Affinity Binding in Nb_2 Cells

Addition	Concentration of addition (ng/ml)*				
	0	10	100	1000	10,000
oPRL	100 ± 1.3%**	50.6 ± 0.9	7.2 ± 4.5	3.8 ± 3.1	1.2 ± 5.7
hGH	100 ± 1.3	39.0 ± 2.2	17.2 ± 2.0	0.2 ± 4.4	0 ± 5.7
Insulin	100 ± 1.3	—	100 ± 1.6	100 ± 1.0	98.9 ± 0.9
bGH	100 ± 1.3	—	97.5 ± 0.6	99.2 ± 3.3	51.6 ± 1.5
BSA	100 ± 1.3	—	94.0 ± 1.9	100 ± 2.2	89.8 ± 2.7

* Nb_2 cells were incubated at 37°C for 1 hr with 100 pg/ml [I^{125}]-hGH with or without the additions indicated in the table. The percent of specific binding that occurs in the absence of the additions was then calculated.
** Numbers in the table represent the mean ± SE of four observations.

thesis (11), and an increased accumulation of mRNAs for actin, c-myc, interferon regulatory factor I, and ornithine decarboxylase (12, 13). In more recent studies (6), we have reported that lactogenic hormones stimulated tyrosine kinase activity within seconds after hormone addition to Nb_2 cells. Several proteins are phosphorylated in response to the prolactin stimulation to tyrosine kinase; however, the earliest phosphorylated protein is one with a molecular weight of 121 kDa. This protein has been shown to be immunoprecipitated with antibodies to the low-affinity Nb_2 cell prolactin receptor (7), thus suggesting that the plasma membrane prolactin "receptor" is in fact a receptor complex composed of more than one protein molecule. The immunoprecipitated receptor complex was also reported to contain tyrosine kinase activity.

By kinetic analysis, the results of our studies demonstrate that existence of a second, high-affinity lactogenic hormone "receptor" in Nb_2 cells. The binding to this receptor is saturated within minutes after hormone addition, thus correlating well with the early responses that occur in these cells. In addition, the Kd of binding to the high-affinity receptor site correlates well with the dose-response curves for the stimulation of mitogenesis (2), as well as specific molecular processes in the Nb_2 cells (11). The likely functional role of the high-affinity binding site in the Nb_2 cells is thus suggested.

The molecular identity of the high-affinity binding site is presently not known and remains to be determined. Of possible relevance is the NGF-receptor system which contains both high- and low-affinity receptor sites. NGF binds with low affinity to both a 42 kDa receptor and to the trk protooncogene protein (4). When both the 42 kDa receptor and trk are concurrently expressed in cells, the high-affinity binding site appears. Occupancy of the high-affinity binding site is required for NGF to express biological responses in cells. Since NGF and lactogenic hormone receptors are structurally similar members of the cytokine family of receptors, it is an intriguing speculation that the lactogenic hormone receptor system may function in a manner similar to the NGF receptor system.

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1. Gout PW, Beer CT, Noble RL. Prolactin-stimulated growth of cell cultures established from malignant Nb rat lymphomas. Canc Res **40**:2433–2436, 1980.
2. Shiu RP, Elsholtz HP, Tanaka T, Friesen HG, Gout PW, Beer CT, Noble RL. Receptor-mediated mitogenic action of prolactin in a rat lymphoma cell line. Endocrinology **113**:159–165, 1983.
3. Ali S, Pellegrini I, Kelly P. A prolactin-dependent immune cell line (Nb_2) expresses a mutant form of prolactin receptor. J Biol Chem **266**:20110–20117, 1991.
4. Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV. High-affinity NGF binding requires coexpression of the trk prot-oncogenes and the low-affinity NGF receptor. Nature **350**:678–683, 1991.
5. Tower BB, Clark BR, Rubin RT. Preparation of I^{125} polypeptide hormones for radioimmunoassay using glucose oxidase with lactoperoxidase. Life Sci **21**:959–966, 1977.

6. Rillema JA, Campbell GS, Lawson DM, Carter-Su C. Evidence for a rapid stimulation of tyrosine kinase activity by prolactin in Nb_2 rat lymphoma cells. Endocrinology **131**:973–975, 1992.
7. Rui H, Djeu JY, Evans GA, Kelly PA, Farrar WL. Prolactin receptor triggering. J Biol Chem **267**:24076–24081, 1992.
8. Ali S, Edery M, Pellegrini I, Lesueur L, Paly J, Djiane J, Kelly PA. The Nb_2 form of prolactin receptor is able to activate a milk protein gene promoter. Mol Endocrin **6**:1242–1248, 1992.
9. Buckley AR, Montgomery DW, Kipler R, Putnam CW, Zukoski CF, Gout PW, Beer CT, Russell DM. Prolactin stimulation of ornithine decarboxylase and mitogenesis in Nb_2 node lymphoma cells: The role of protein kinase C and calcium mobilization. Immunopharmacology **12**:37–51, 1986.
10. Too CKL, Cragoe EJ, Friesen HG. Amiloride-sensitive Na^+/K^+ exchange in rat Nb_2 node lymphoma cells. Stimulation by prolactin and other mitogens. Endocrinology **121**:1512–1520, 1987.
11. Rillema JA, Tarrant TM, Linebaugh BM. Studies on the mechanism by which prolactin regulates protein, RNA and DNA synthesis in Nb_2 node lymphoma cells. Biochim Biophys Acta **1014**:78–82, 1989.
12. Fleming WH, Murphy PR, Murphy LT, Hatton TW, Matusik RJ, Friesen HG. Human growth hormone induces and maintains c-myc gene expression in Nb_2 lymphoma cells. Endocrinology **117**:2547–2553, 1985.
13. Lu-Li LY. Prolactin stimulates transcription of growth-related genes in Nb_2 T lymphoma cells. Molec Cell Endocrinol **68**:21–28, 1990.