Mitogenic and Binding Properties of Monoclonal Antibodies to the Prolactin Receptor in Nb2 Rat Lymphoma Cells

SELECTIVE ENHANCEMENT BY ANTI-MOUSE IgG*  

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Three monoclonal antibodies (mAbs) (T6, U5, and U6) against prolactin (PRL) receptors in rat liver were studied in the rat lymphoma lactogen-dependent (Nb2-11C) and autonomous (Nb2-SP) cell lines. The mAbs had strong affinity for lactogen receptors (Ka = 12–14 nM−1), similar to that of human growth hormone (hGH) which is a lactogenic hormone. T6 and hGH competed for the same binding site, while U5 and U6 interacted with another epitope. The 125I-hGH-receptor complex could be immunoprecipitated by either U5 or U6, but not by T6. Affinity labeling and immunoblotting revealed that hGH and U6 bind to a protein of 63–65 kDa. T6, U5, and U6 were mitogenic in Nb2-11C cells but their respective potencies were 185–70, and 4700-fold lower than that of hGH. Anti-mouse IgG enhanced the mitogenic effect of all three mAbs and almost completely abolished the differences between them, although their mitogenic activity was still 60–120-fold lower than hGH. Des-13-hGH, a competitive antagonist of hGH which hardly affected the binding of 125I-U5, inhibited the U5-stimulated proliferation of Nb2-11C cells in a noncompetitive manner, indicating that simultaneous binding of both ligands fixed the receptor in a nonactive conformation. A Fab fragment of T6 was not mitogenic, and inhibited the hGH-induced mitogenesis in a competitive manner, but its mitogenicity could be restored by anti-mouse IgG. We suggest that the dimerization or oligomerization of the lactogen receptor in Nb2-11C cells is an obligatory step in the transduction of the mitogenic signal. It may be induced by binding of the mAb to a site, which can be either identical or may even be distinct from that which binds the lactogenic hormone.

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). Recently, two cell lines derived from the original Nb2 strain, one which is lactogen-dependent (Nb2-11C) and another which is lactogen-independent and autonomous (Nb2-SP), have been established (4, 5).

Lactogen receptors in both cell lines are equally potent in binding either oPRL or hGH, as judged by the identical affinity for both hormones in the dependent and autonomous cell lines (6). The number of receptors/cell in the autonomous cell line is, however, ~2-fold higher (6, 7). The molecular mass of the receptor has yet to be fully established and, in different laboratories, varies between 60 and 88 kDa (6, 8, 9). Binding of the hormone to the receptor is an obligatory step for subsequent proliferation. The minimal length of exposure required for commitment to progression through the cell cycle was 4 h (10). Although some initial events that occur subsequent to the exposure of the Nb2-11C cells to lactogenic hormones have been described recently (11–16), the mechanism by which the receptor-mediated hormonal effect is transduced is largely unknown.

Polyclonal antibodies raised against PRL receptors in rabbit mammary gland had biological activity and stimulated DNA, casein, and lactose synthesis in normal rabbit (17) and tumor mammary gland explants (18). It was shown, however, that their monovalent fragments were devoid of any PRL-like activities (19). Similarly one out of three monoclonal antibodies raised against these receptors also exhibited PRL-like activity in explants of rabbit mammary gland (20, 21). Shiu et al. (3) have shown that polyclonal antibodies against rabbit mammary gland PRL receptors were also capable of stimulating Nb2 cells proliferation, while their Fab fragments were devoid mitogenic properties and even inhibited the PRL-simulated proliferation. Due to heterogeneity of these antibodies, calculation of these mitogenic responses on a molecular basis was impossible. To overcome this difficulty, we used recently prepared, specific new mAbs, recognizing various domains of PRL receptors from rat liver. These mAbs showed a cross-reactivity with PRL receptors from other rat tissues, including receptors from Nb2 cells (22). In the present study, we have employed three of these antibodies, directed against at least two different epitopes and quantitated their binding properties, biological activities and enhancement by rabbit anti-mouse IgG as compared to the effects of hGH.
EXPERIMENTAL PROCEDURES

Materials—hGH (hGH-83-8-29H, 2.2 IU/mg) was a gift of Dr. H. G. Friesen (University of Manitoba, Winnipeg, Canada). mAbs directed against rat liver PRL receptor were obtained as described earlier. The Nb2 SP cells were maintained from D. M. D. (Department of Molecular Biology, University of Cambridge, England, United Kingdom). CHAPS, molecular weight standards for gel electrophoresis, Fisher’s medium, RPMI 1640 medium, chloroquine, leupeptin, pepstatin A, PMSF, soybean trypsin inhibitor, BSA, (radioimmunoassay grade), mercuripapain, and normal mouse immunoglobulin were obtained from Sigma, and disuccinimidyl suberate from Pierce Chemical Co. SDS-PAGE reagents and Protein Assay Kit were purchased from Bio-Rad and Tris-HCl from E. Merck (Darmstadt, Germany). Rabbit anti-mouse immunoglobulin (titre 2.8 mg of immunoglobulin/ml) were purchased from Bio-Makor (Rehovot, Israel) and used in cell culture after dialysis against Dulbecco’s phosphate-buffered saline. Pansorbin was purchased from Calbiochem and Superose 12 column from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). All other chemicals were of analytical grade.

Preparation of the Fab Fragment from T6—Mercuripapain (1 mg/ml) was activated by preincubation in 0.1 M Tris-HCl buffer, pH 8.0, containing 2 mM EDTA and 1 mM dithiothreitol for 15 min at 37 °C. Five mg of T6 were dissolved in 0.5 ml of the same buffer and digested for 4 h at 37 °C, using 1:100 enzyme to substrate ratio. The digestion was terminated by addition of iodoacetamide up to 20 mM. Then, 0.2-ml aliquots of the digest were chromatographed on a high performance liquid chromatography Superose 12 column (1 x 30 cm), equilibrated with 50 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl, at 0.5 ml/min. The main peak analyzed by SDS-PAGE revealed existence of a single band of 46 kDa.

Nb2 Lymphoma Cell Culture—Two cloned Nb2 lymphoma cell lines were cultured as described earlier (4, 6) except that Nb2-SP cells were cultured in RPMI-1640 medium supplemented with 5% horse serum. These cells were collected by centrifugation and kept at -20 °C until use. Synchronization of Nb2-11C cells in the G0/G1 phase and monitoring of cell proliferation were carried out as described earlier (4).

Preparation and Solubilization of Nb2-SP Microsomal Fraction—Nb2-SP cells (1010 cells) were homogenized in 250 ml of 25 mM Heps- NaOH, pH 7.5, buffer containing 1 mM PMSF, 50 mM chloroquine, 0.01% pepstatin (w/v), 0.02% soybean trypsin inhibitor (w/v), 0.01% leupeptin (w/v), 1 mM ZnCl2, 1 mM EDTA, 1 mM EGTA, in a Polytron. The homogenate was spun for 20 min at 600 x g. The microsomal fraction was recovered by centrifugation at 100,000 x g for 1 h. The precipitate was suspended in 30 ml of 25 mM Heps-NaOH buffer, pH 7.5, containing 10 mM MgCl2, 0.01% leupeptin (w/v), 0.02% soybean trypsin inhibitor (w/v), and solubilized with either 1% (v/v) Triton X 100 or 0.6% (w/v) CHAPS, by stirring for 30 min at room temperature. Insolubilized material was removed by centrifugation at 150,000 x g for 1 h. The solubilized fraction was stored frozen at -20 °C until used.

Hormone and mAb Iodination—Radioiodination of hGH or mAb (7.5 µg) was carried out in the presence of Chloramine T and 1 mCi of Na121I as described earlier (12). The specific activity was 20-100 Ci/µg of protein.

Binding Determination—The binding of 121I-hGH was assayed in intact cells or homogenates. The binding to intact cells was measured as described earlier (6) except that cells were resuspended in RPMI 1640 medium supplemented with 5% horse serum. The tubes were incubated for 14-16 h at 24 °C with labeled hormone or mAb and without or with excess unlabeled ligand (2 µg/tube). Specific binding was calculated as the difference between the radioactivity bound in the absence and presence of an excess amount of unlabeled ligand.

Cell homogenates were prepared by suspension of the cell pellet in 25 mM Heps-NaOH buffer supplemented with 10 mM MgCl2 and 1 mM PMSF (reaction buffer) and by homogenization in a Polytron. The homogenate was centrifuged at 100,000 x g for 15 s, full speed. Four hundred µl of the homogenate suspension were transferred to Eppendorf tubes with 50 µl of 1% BSA, 50 µl of labeled ligand, 50 µl of reaction buffer or unlabeled ligand. After overnight incubation, 500 µl of cold reaction buffer was added to the reaction mixture. The tubes were centrifuged, the supernatant aspirated and the bound radioactivity counted on a γ counter.

Modulation of Receptor Binding in Intact Cells—Modulation of receptor levels at the surface cells by exposure to hGH or mAb followed by a brief 3-min treatment with an acidic buffer composed of 0.1% acetic acid, 150 mM NaCl, 0.1% BSA, pH 4, at 4 °C were carried out as described earlier (6). Affinity Labeling of Lactogen Receptor in Intact Nb2-SP Cells—Nb2-SP cells were incubated with 121I-hGH in absence or in presence of excess unlabeled hGH at 37 °C. After each incubation time, 50 µl of disuccinimidyl suberate (0.5 mM final concentration), freshly dissolved in dimethyl sulfoxide, was added to 5 ml of a -7.5 x 106 suspension cells in the culture medium. The reaction was carried out for 15 min at 0 °C and was stopped by addition of 500 µl of Tris-HCl buffer (2 M, pH 7.5). The cells were pelleted, washed with saline, and resuspended in 50 µl of reaction buffer. A 15-µl sample buffer, 5-fold concentrate (300 mM Tris-HCl, pH 6.8, 10% SDS (w/v), 25% glycerol (v/v), 0.025% pyronin Y (w/v) was added to the reaction mixtures which were then boiled for 5 min and subjected to 10% SDS-PAGE (25). The gels were fixed, dried, and followed by autoradiography at -70 °C using Kodak XAR-5 film.

Immunoblotting—Solubilized fraction of Nb2-SP cell microsomes was prepared to 7.5% SDS-PAGE analysis by addition of 5-fold concentrate sample buffer. Proteins were electroendoretically transferred onto nitrocellulose membrane (26). The membranes were blocked 2 h with 3% gelatin in 25 mM Tris-HCl buffer, pH 7.5, 500 mM NaCl (Tris-buffered saline) and were incubated overnight in Tris-buffered saline including 1% gelatin with 121I-U6 in absence or presence of excess unlabeled ligand at room temperature. The incubation was followed by extensive rinsing with Tris buffered saline, including 0.1% (v/v) Triton X-100. After drying, the blots were autoradiographed as described previously.

Immunoprecipitation of 121I-hGH-Receptor Complexes—121I-hGH (5 x 106 cpm/ml) and Triton X-100-solubilized receptors (75 µg of protein/ml) were incubated overnight at 24 °C in the presence or absence of excess unlabeled hGH (4 µg/ml), as described previously for binding determination in cells homogenate with the exception of the reaction buffer being supplemented with 0.1% (v/v) Triton X-100. Following centrifugation at 10,000 x g for 5 min to remove any spontaneous precipitations of the receptor, 150 µl of the supernatant were distributed to each tube, and 50 µl of control nlgC or mAb at the indicated amount were added to the tubes, in triplicates and incubated overnight at 4 °C. Five µl of rabbit anti-mouse immunoglobulin was added and incubated for 5 h at 24 °C. Hormone receptor-antibody complexes were finally absorbed to 20 µl of Pansorbin by incubating for 30 min at 24 °C. Following addition of cold reaction buffer, the tubes were centrifuged and counted. The difference in count between the incubations without and with unlabeled hGH was taken as specific precipitation of hormone-receptor complexes.

RESULTS

Binding Experiments—Binding of radiolabeled 121I-hGH or mAbs 121I-T6, 121I-U6, and 121I-U6 to homogenates prepared from Nb2-SP cells was studied in the presence of increasing concentrations of the nonlabeled mAbs or hGH (Fig. 1). As can be seen, the binding of hGH or T6 on one hand and the binding of U5 and U6 on the other, showed mutual competition. U5 and U6 competed for 121I-U6 binding, and hGH inhibited only 50% of the binding of either 121I-U6 or 121I-U6. Partial (~50%) competition was observed by U6 and T6, while U5 and T6 had almost no effect on the binding of either ligand. Dec-13-hGH and a truncated form of hGH which competes for binding with hGH, but has an 85-fold lower affinity toward the receptor (13), exhibited only a slight ability to inhibit 121I-U5 binding. Only ~20% inhibition was observed at concentrations of des-13-hGH, that was 10-fold higher than the concentration of U5 required to achieve the same effect. The association constants for hGH, T6, U6, and U5, were calculated from Scatchard plots, respectively 16, 14, 13, and 12 nM-1. Linear curves indicated the existence of single receptor populations (not shown).

Similar results were also obtained using intact Nb2-SP cells (see Table I, 1st column). As in binding experiments performed with homogenates (see Fig. 1), mAb T6 and hGH showed competitive inhibition of each other but only partially (56 and 37%) competed for 121I-U6 binding. Similarly, U6 displaced only 52 and 65% of bound 121I-T6 and 121I-hGH.
Cells were then collected by centrifugation, washed free of excess 7.4, containing 150 mM NaCl and 0.1% BSA, washed with 25 mM Hepes buffer, pH 7.4, containing 150 mM NaCl and 0.1% BSA, and finally resuspended in RPMI 1640 medium supplemented with 5% horse serum. The medium, washed briefly with 0.1% acetate buffer, pH 4.0, containing 150 mM NaCl and 0.1% IgG, had absolutely no effect on the binding of any ligand in both cell lines. Binding experiments carried out in the presence of 2 fig of the respective lactogens resulted, respectively, in 43 and 75% down-regulation. Since the U5- and UG-receptor complexes do not dissociate completely in pH 2.3-4.5, while at pH 11, when dissociation of the ligand does occur, the cells are killed, and the receptor is degraded, we were unable to assay whether these mAbs were also able to down-regulate the lactogen-dependent Nb-11C cells yielded similar results (not shown).

To evaluate whether initial binding of mAb U6 or hGH affects the subsequent binding of 125I-hGH or 125I-U6, Nb-SP homogenates were preincubated from 0 to 12 h with either U6 (20 nM) or hGH (45 nM). Then, every 2 h the labeled ligands were added and the reaction was terminated 16 h later. In both cases, preincubation had absolutely no effect on the subsequent binding of the ligand (not shown). These competition experiments indicate that three forms of lactogen binding sites may exist on the cell surface and in the cell homogenates, (a) sites that recognize hGH, T6, U5, and U6 but are not able to bind only one of them at a time, (b) sites that bind hGH or T6 but not U5 or U6, (c) sites that bind U5 or U6 but not hGH or T6. The present results do not exclude the possibility that (b) and (c) are in fact identical, but these sites are able to bind simultaneously U5 or U6 and hGH or T6 and therefore U6 or U6 do not compete or compete only partially with either hGH- or T6-specific sites.

**Down-regulation Experiments**—The results of the down-regulation experiments in Nb-SP cells are summarized in Table I. Exposure to hGH resulted in down-regulation of 70-80% of receptors that recognize all four ligands. Exposure to T6 was less effective, despite the fact that the receptor binds both ligands with similar affinity. Only 31-37% of receptors that recognize hGH and/or T6 were down-regulated. However, in another experiment (not shown), in which the T6 concentration was elevated to 27 nM for 3 h, 75% down-regulation occurred. Similarly T6 was also capable of down-regulating lactogen receptors in the Nb-11C cells. A 3-h exposure to 2.7 and 27 nM T6 resulted, respectively, in 43 and 86% down-regulation. Since the U5- and U6-receptor complexes do not dissociate completely in pH 2.3-4.5, while at pH 11, when dissociation of the ligand does occur, the cells are killed, and the receptor is degraded, we were unable to assay whether these mAbs were also able to down-regulate lactogen receptors. It is, however, evident that the proportion of the down-regulated receptors in which 125I-hGH, 125I-T6, or 125I-U6 can be displaced by either hGH, T6, U6, or U5 (see Table I) was roughly equal. A similar proportion of down-regulated receptors also was observed using 125I-U5 as a tracer.

### Table I

| HORMONE OR mAb (nM) | Specific binding \( \times 10^{17} \) | Displacer | Down-regulation Experiments |
|---------------------|-----------------------------|-----------|-----------------------------|
|                     | None                        | hGH       | T6                          |
| 125I-hGH            | 448                         | 96 (79)*  | 294 (34)                    |
| 125I-T6             | 453                         | 101 (78)  | 284 (37)                    |
| U6                  | 218                         | 46 (79)   | 144 (53)                    |
| U5                  | 176                         | 40 (77)   | 135 (24)                    |
| 125I-U5             | 250                         | 69 (72)   | 173 (31)                    |
| 125I-U6             | 263                         | 70 (78)   | 179 (32)                    |
| 125I-T6             | 113                         | 29 (74)   | 83 (27)                     |
| U5                  | 0                           | 0         | 0                           |
| U6                  | 165                         | 40 (77)   | 165 (6)                     |
| 125I-U5             | 115                         | 26 (78)   | 115 (0)                     |
| U6                  | 177                         | 40 (77)   | 165 (7)                     |
| U5                  | 175                         | 38 (79)   | 165 (6)                     |
| 125I-U6             | 206                         | 53 (75)   | 162 (22)                    |
| U6                  | 144                         | 37 (75)   | 109 (25)                    |
| U5                  | 323                         | 61 (82)   | 271 (16)                    |
| 125I-U5             | 326                         | 52 (84)   | 277 (15)                    |

* The respective amounts of 125I-hGH, 125I-T6, 125I-U6, and 125I-U5 (counts/min/tube) were 420,000, 340,000, 390,000, and 120,000.
* The specific binding was calculated after subtracting the nonspecific binding obtained in the presence of 2 μg of the respective nonlabeled ligand.
* The numbers in parentheses represent percent of receptors down-regulated due to the exposure to hGH or T6.

Correspondingly, U6 inhibited 52 and 55% of binding of 125I-T6 and 125I-hGH. Mutual competition was observed also between U6 and U5. Both mAbs behaved similarly toward hGH but there was no competition between U5 and T6. Nonspecific mouse IgG had absolutely no effect on the binding of any ligand in both cell lines. Binding experiments carried out in...
after exposure to hGH. The exposure to a low concentration of T6 barely had any effect. This finding suggests the possibility that hGH and T6 also down-regulate the receptor form that binds simultaneously U6 or U5 and either hGH or T6. The most reasonable interpretation therefore is that the receptor forms (b) and (c), mentioned in the previous paragraph, are indeed identical.

Effect of T6, U5, and U6 on the Proliferation of Nb2-11C Cells—As shown in Fig. 2, all three mAbs exhibited mitogenic activity in Nb2 cells. Surprisingly, despite the fact that the affinity of the mAbs and hGH for the receptor is similar, the molar concentrations of T6, U5, and U6 required for half-maximal effect were much higher than that of hGH. Nonspecific mouse IgG had no mitogenic effect (not shown).

Quantitative analysis of these results was performed by plotting the reciprocal of the number of doublings versus the reciprocal of hGH or mAb concentration. Previous experiments indicated that this plot gives a linear regression with correlation coefficient >0.98 (4). The intersection on the abscissa is equal to -1/concentration of the hormone required for half-maximal proliferation. Using this method, we replotted the results presented in Fig. 2 (not shown). Similarly to hGH, T6, U5, and U6 yielded straight lines and the respective concentrations required for half-maximal proliferation were 0.0027, 0.503, 0.187, and 12.7 nM.

We also found that U6, partially inhibited U5-stimulated mitogenesis (not shown), suggesting that U5 and U6 interact at a very similar site on the lactogen receptor. To investigate whether the mitogenic effect of U5 is mediated through a binding site that recognizes hGH, the effect of des-13-hGH, a competitive antagonist of hGH and oPRL (13, 24), was tested.

As shown in Fig. 3A, a strong dose-dependent inhibition of U5-stimulated proliferation was obtained. Replotting of the results in a double-reciprocal manner (Fig. 3B) revealed that unlike hGH- or oPRL-stimulated proliferation of Nb2-11C cells (see Fig. 1 in Ref. 13) the inhibition was noncompetitive. The K_i value (mean ± S.E.) calculated from two inhibition plots was 830 ± 180 nM.

Previously, we have found that 12-O-tetradecanoylphorbol 13-acetate enhanced the hGH- or oPRL-stimulated mitogenesis of Nb2-11C cells (4). A similar effect, namely a ~20–25% increase in doubling rate, also was observed using concentrations of U5, U6, or T6 required for half-maximal stimulation of the proliferation (data not shown). These findings suggest that the post-receptor effects enhanced by 12-O-tetradeca-

| no. of doubling/72 h | 1.000e-4 | 0.001 | 0.010 | 0.100 | 1.000 | 10.000 |
|---------------------|---------|-------|-------|-------|-------|-------|
| hGH or mAb (nM)    |         |       |       |       |       |       |

Fig. 2. The effect of various concentrations of hGH (△), T6 (○), U5 (●), U6 (△) on the proliferation rate of Nb2-11C lymphoma cells. The doubling rates were calculated after 72 h from the following equation:

\[ \frac{\log \text{no. of cells in presence of the hormone} - \log \text{no. of cells in absence of the hormone}}{\log 2} \]

Fig. 3. A, the effect of various concentrations of U5 on the proliferation of Nb2-11C lymphoma cells in the absence (○) or presence of 600 nM (●) or 2400 nM (△) of des-13-hGH. B, double-reciprocal plot of the data presented in A.
Biological Activity of Anti-PRL Receptor mAbs

Fig. 4. Effect of various concentrations of rabbit anti-mouse IgG (final dilutions 1:1,000 to 1:3,125,000) on Nb2-11C lymphoma cell proliferation stimulated by U6 (A), U5 (B), and T6 (C). The final concentrations of the mAbs were 20 (C), 100 (●), and 500 (▲) pm.

Fig. 5. A, competition of unlabeled hGH (▲), T6 (●), and Fab fragment prepared from T6 (△), with the binding of 125I-hGH. The results are expressed as a ratio of bound/total counts/min. B, the effect of various concentrations of Fab prepared from T6 on the hGH-stimulated proliferation of Nb2-11C lymphoma cells. The respective concentrations of the Fab were: C, none; ●, 130 nM; and △, 390 nM.

Fig. 6. Effect of various concentrations of rabbit anti-mouse IgG (final dilutions 1:200 to 1:125,000) on Nb2-11C lymphoma cell proliferation stimulated by Fab fragment prepared from T6. The final concentrations of the Fab was 2.6 (●), 26 (▲), and 130 (△) nM.

DISCUSSION

Competitive binding experiments in both Nb2-SP and Nb2-11C cell lines (Fig. 1 and Table I) indicate that hGH and T6, on the one hand, and U5 and U6, on the other, compete for an identical or very similar binding site on the lactogen receptor. The competition between hGH and U5 or U6 and hGH can bind simultaneously to lactogen receptors. As shown in Fig. 7, a dose-dependent precipitation was achieved with both U5 and U6, evidently indicating that simultaneous binding of hGH and these Abs occurs. T6 did not precipitate the complex, further indicating that this mAb and hGH compete for the same binding site. No precipitation occurred in the presence of nonspecific mouse IgG.

Determination of the Molecular Weight of hGH and U6 Binding Sites—Cross-linking experiments of 125I-hGH to intact cells, followed by SDS-PAGE and autoradiography, revealed an appearance of a single band of M, 87,000 (Fig. 8). This band appears within as early as 7 min of exposure to 125I-hGH. Its intensity increases through 30 min and then gradually decreases, most likely due to down-regulation of the occupied receptor and degradation of the hormone-receptor complex. Additional minor bands with a lower M, values probably represent partially proteolyzed forms that appear after 15 min. Assuming that one molecule of hGH interacts with one binding site, the M, of the binding unit was estimated to be 65,000. This value is very similar to our previously published results (6) and those of others (8, 9).

Western blot analysis of the membrane fraction solubilized in 0.5% CHAPS (Fig. 9A) or 1% Triton X-100 (Fig. 9B) revealed one major 63-kDa protein band that was specifically recognized by 125I-U6. Similar dose-response interactions with increasing amounts of membrane protein were observed in both cases. Aggregates and a few minor bands with lower M, values, probably representing proteolytic fragments of the receptor, also interacted with 125I-U6.
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Fig. 5. Immunoprecipitation of hormone-receptor complexes with normal mouse IgG or mAbs. After incubation of Triton X-100-solubilized receptor with $^{125}$I-hGH, 150 µl (11 µg of protein and 110,000 cpm) were transferred to Eppendorf tubes. Triplicate tubes were further incubated with addition of 50 µl of nIgG (O), T6 (●), U6 (○), or U5 (△) at the indicated amounts. Hormone-receptor-antibody complexes were precipitated after addition of rabbit anti-mouse IgG antiserum and Pansorbin. The control for specific binding precipitation with polyethylene glycol, performed as described previously (41), was 22,000 cpm.

Fig. 7. Immunoprecipitation of hormone-receptor complexes with normal mouse IgG or mAbs. After incubation of Triton X-100-solubilized receptor with $^{125}$I-hGH, 150 µl (11 µg of protein and 110,000 cpm) were transferred to Eppendorf tubes. Triplicate tubes were further incubated with addition of 50 µl of nIgG (O), T6 (●), U6 (○), or U5 (△) at the indicated amounts. Hormone-receptor-antibody complexes were precipitated after addition of rabbit anti-mouse IgG antiserum and Pansorbin. The control for specific binding precipitation with polyethylene glycol, performed as described previously (41), was 22,000 cpm.

Fig. 8. Affinity labeling of lactogen receptors in intact Nb2-SP cells. Intact cells were incubated for 0-180 min with $^{125}$I-hGH in the absence or presence of 2 µg of unlabeled hGH. After cross-linking with disuccinimidyl suberate, the cells were washed, subjected to SDS-PAGE (7.5%), and autoradiographed.

that U5 and T6 did not compete with each other indicate that these mAbs can bind simultaneously to the receptor existing in both forms and that U5 and U6 bind in a similar but not identical mode.

This hypothesis is further supported by additional experiments that showed: (a) preincubation of homogenates with either hGH or U6 for 12 h prior to addition of the labeled ligand did not affect the binding properties; (b) hGH and T6 down-regulated to similar degrees the receptors in which these ligands did or did not compete with U6 or U5 (Table I); (c) immunoprecipitation studies indicated that the receptor can simultaneously bind hGH and U5 or U6 (Fig. 7); (d) the $M_r$ of the main form that binds hGH in the intact cells (Fig. 8) is almost identical to that which binds U6 in solubilized receptors (Fig. 9); and (e) U6 inhibits partially the U5-stimulated proliferation of Nb2-11C cells.

The physiological importance of these two epitopes is unknown, but the fact that the binding patterns to cell homogenates and intact cells were very similar hints at that both are present on the cell surface.

The biological effect of anti-PRL receptor mAbs was monitored by measuring their effect on the proliferation of Nb2-SP lymphoma cells. As shown in Fig. 2, all three mAbs were mitogenic. Surprisingly, however, despite the fact that the binding association constants of all three mAbs were almost identical and were similar to that of hGH, the respective molar concentrations U5, T6, and U6 required for half-maximal mitogenic effect (Fig. 2) were 70-, 185-, and 4700-fold higher. Simultaneous addition of mAbs and anti-mouse IgG at optimal concentrations enhanced the mitogenic activity of all three mAbs and greatly diminished the differences between the mAbs themselves as well as between the mAbs and hGH. The respective concentrations required for a similar mitogenic response (as calculated from the data presented in Fig. 4) were 59-fold (U5), 60-fold (T6), and 120-fold (U6) higher than that of hGH.

The present results suggest that, similar to epidermal growth factor (27–30), insulin-like growth factor-I (31), or platelet-derived growth factor (32) receptors, dimerization or oligomerization of lactogen receptors in Nb2-11C cells is an obligatory step in transduction of the biological signal. Our present finding that the Fab fragment prepared from T6 became an antagonist of hGH, lost its mitogenic activity but regained it in the presence of an anti-mouse IgG strongly supports this hypothesis. Although definite evidence is still missing, it would appear that binding of hGH (or other lactogenic hormones) may also induce a conformational change that results in dimer formation. Recent reports indicating that anti-hGH antibodies enhanced the somatogen receptor-mediated bioactivity (33) and that the lactogen and somatogen receptors are structurally related (34) support this notion. If this assumption is true, then the fact that the
mitogenic activity of all three mAbs is lower than that of hGH may be related to their impaired ability to induce dimerization of the receptor. Two possible explanations may be offered: (a) the conformational change resulting from binding of hGII shifts the monomer/dimer equilibrium to the right, while the binding of the mAb, has the same effect but to a much lesser degree; (b) mAbs bind but lack the ability to induce dimerization. Only random dimerization that leads to further biological response occurs. The present data are insufficient to select which explanation is the most likely, although selective enhancement of mitogenic activity by anti-mouse IgG does not favor the latter explanation.

Binding of des-13-hGH, a weak antagonist and competitive inhibitor of hGH or oPRL in Nb-11C cells (13), did not prevent simultaneous binding of U5 (Fig. 1) (and most likely also U6) but fixed the receptor in a form that either prevented its dimerization or formed a nonactive dimer. This possibility is in full agreement with the noncompetitive inhibition pattern of the U5-stimulated Nb-11C cells proliferation (Fig. 3).

In an attempt to understand the role of receptor dimerization in transduction of epidermal growth factor stimulated biological signal, an “allosteric oligomerization model” has been proposed (35). According to this model, monomeric receptors are in equilibrium with oligomeric receptors. The binding of the ligand stabilizes the oligomeric state and subsequently leads to phosphorylation of the protein kinase domain by mutual phosphorylation between the neighboring cytosolic domains (36).

A similar hypothesis may apply also to platelet-derived growth factor or insulin-like growth factor-I receptors that have tyrosine kinase in the intracellular domain (37, 38). Cloning of lactogen (34, 39) and somatogen (40) receptors revealed that both are single transmembrane proteins that show regions of high similarity. However both receptors lack sequences associated with protein tyrosine kinase activity or autophosphorylation sites.

Thus, although our present results strongly indicate that dimerization or oligomerization of receptors is an obligatory step in the mitogenic process of Nb-11C lymphoma cells, we have, at present, no indication concerning its mechanism. Elucidation of early events occurring after exposure to hormone or antibody and follow up after receptor trafficking in the stimulated cells may be helpful in resolving this question.

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