Monoclonal Antibodies against Rabbit Mammary Prolactin Receptors

SPECIFIC ANTIBODIES TO THE HORMONE BINDING DOMAIN*

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Masao Katoh‡, Jean Djiane§, and Paul A. Kelly‡

From the ‡Laboratory of Molecular Endocrinology, Royal Victoria Hospital, Montreal, Quebec, Canada H3A 1A1 and the §Laboratoire de Physiologie de la Lactation, Institut National de la Recherche Agronomique, 78350 Jouy-en-Josas, France

Three monoclonal antibodies (M110, A82, and A917) were obtained by fusing myeloma cells and spleen cells from mice immunized with partially purified rabbit mammary gland prolactin (PRL) receptors. All 3 antibodies were capable of complete inhibition of 

Antireceptor antibodies have become an effective tool to help understand the mechanisms involved in the interaction of polypeptide hormones with its cell surface receptors. These antibodies sometimes act paradoxically, that is, in addition to antagonistic effects they may also elicit agonistic actions (1–3). We and others have previously prepared polyclonal anti-PRL receptor antibodies from goats, sheep, and guinea pigs using partially purified receptors as antigen (4–7). These antisera exhibited both PRL-inhibitory and mimicking actions (6, 8–10), as well as cross-reactivity with all the tissues and species examined (7). Using polyclonal antibodies for such studies leaves unresolved two important problems: 1) since the antigen used is a partially purified receptor, the possibility exists that the sera contain antibodies to other membrane components; 2) antireceptor antibodies consist of at least two classes of antibodies, that is, to the hormone binding domain and to other regions of the receptor. These points make it difficult to elucidate the mechanisms involved in hormone-mimicking effects. Recently, antibodies that mimic action have been produced for receptors to luteinizing hormone (11), thyrotropin (3), epidermal growth factor (12, 13), and transferrin (14). We have attempted to prepare the monoclonal antibodies to the PRL receptor in order to clearly distinguish the hormone binding domain and separate it from other portions of the receptor molecule. In this study, we report on the production and characterization of binding site specific antibodies as our initial attempt.

MATERIALS AND METHODS

RESULTS

Production and Characterization of Monoclonal Antibodies—Of 983 hybridomas assayed, 3 were found to be positive in inhibition of 

The abbreviations used are: oPRL, ovine prolactin; hGH, human growth hormone; hPRL, human prolactin; oGH, ovine growth hormone; mAb, monoclonal antibody; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propane sulfonate; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

1 Portions of this paper (including "Materials and Methods," Figs. 1, 3, 5–7, 9, and 11, and Table 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-3004, cite the authors, and include a check or money order for $4.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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mAbs to Rabbit Mammary PRL Receptors

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FIG. 2. Effect of monoclonal antibodies on 125I-oPRL binding to rabbit mammary microsomes. Fifty-two fmol of 125I-oPRL were incubated with 300 µg of microsomes as described under "Materials and Methods," in the absence or presence of various concentrations of either IgG-M110 (O), A82 (□), A917 (▲), oPRL (●), or normal IgG (■). Results are expressed as a percentage of the control binding performed in the absence of IgG or oPRL, which was 20.1 ± 0.8 fmol (mean ± S.E., n = 4). The total binding capacity obtained by Scatchard analysis (mean ± S.E.) of the 4 different microsomal preparations used in this study was 177 ± 28 fmol of PRL/mg of protein.

Inhibition of PRL Binding to Receptors—As shown in Fig. 2, all 3 antibodies were able to completely inhibit 125I-oPRL binding to rabbit mammary microsomal PRL receptors. The ID50 (concentration that caused 50% inhibition of binding) was 0.25 ± 0.07 nM (mean ± S.E., 4 different mammary glands) for M110, 2.49 ± 0.76 nM for A82, and 1.66 ± 0.30 nM for A917. M110 was slightly more potent than even oPRL in inhibiting 125I-oPRL binding. (ID50 = 0.40 ± 0.07 nM, molecular weight of oPRL was taken as 23,000). These antibodies also inhibited the 125I-oPRL binding to CHAPS-solubilized as well as affinity purified receptors (Fig. 3) with somewhat lower ID50 values (0.14–1.34 nM for solubilized and 0.12–0.61 nM for purified receptors) than that for microsomes (0.25–2.5 nM). The potency of inhibition of 125I-oPRL binding was M110 > A917 > A82 for all 3 receptor preparations. The species and tissue specificity of the inhibition of 125I-oPRL binding by monoclonal antibodies is demonstrated in Fig. 4. Binding curves to rabbit kidney, ovary, and adrenal were similar to that for the mammary gland receptor for all 3
mAbs to Rabbit Mammary PRL Receptors

mAbs. Twenty to 40% of the labeled PRL bound to rabbit liver microsomes could not be competed for, even at the highest concentration of 3 antibodies used. For rat liver and ovary and human T-47D cells, none of the antibodies were able to inhibit PRL or hGH binding. For pig mammary gland, M110 and A917 showed comparable curves to that for rabbit mammary gland. A82, in contrast, was 10-100 times less potent.

The incomplete inhibition of PRL binding to rabbit liver can be explained by the experiment depicted in Fig. 5A. As has been previously shown (27), ovine GH caused a partial inhibition of PRL binding, and the mAbs resulted in a more pronounced, but incomplete inhibition. The specificity of the mAbs for the PRL receptor is demonstrated in Fig. 5B. None of the antibodies affected 125I-oGH binding to rabbit liver. If the same concentration of oGH was added to the mAbs, complete inhibition of binding was achieved (Fig. 5A), suggesting that labeled oPRL binds to the GH receptor in rabbit liver (27).

Binding of Monoclonal Antibodies to Receptors—The binding characteristics of radioiodinated mAbs were examined in rabbit mammary microsomal PRL receptors. Association profiles were somewhat different among mAbs as depicted in Fig. 6A. All 3 mAbs had a greater association rate than oPRL. M110 did not reach equilibrium even at 56 h (at least 40-50 h were necessary in other sets of experiments). In contrast, A917 quickly achieved the equilibrium at 1 h and a second slower association was observed at 12-24 h. The association profile of A82 was the most similar to that of oPRL reaching equilibrium at 20-36 h. Dissociation experiments (Fig. 6B) also revealed differences among monoclonal antibodies. M110 and A82 dissociated slowly from mammary receptors suggesting an irreversibility which has been already described for oPRL (28, 29), while 70% of bound 125I-A917 was dissociated at 12 h. Fig. 7A shows the saturable nature of monoclonal antibody binding to rabbit mammary microsomes. The results were plotted according to Scatchard ((18) Fig. 7B). M110 revealed a linear regression line suggesting a single class of binding sites. The affinity constant of M110 binding was about 3 times as great as that for oPRL. In contrast, A917 and A82 showed typical curvilinear Scatchard plots.

Fig. 8. 125I-mAb binding to microsomes from various tissues. Fifty-one to 53 fmol of 125I-oPRL, 125I-hGH for T-47D, (□), 125I-M110, 125I-A82 (■), or 125I-A917 (▲), or 125I-oPRL (●) were incubated for 15 h with 150 μg (rabbit adrenal) or 300 μg (other tissues) of microsomes from tissues indicated. Specific binding was determined as described under "Materials and Methods" and expressed as a percentage of tracer added. Nonspecific binding was 0.6-1.5%, 0.2-1.3%, 1.0-5.4%, and 1.8-6.0% of total tracer added for M110, A82, A917, and oPRL, respectively. Values are mean ± S.D. of three independent experiments.

Tissue or species specificity of 125I-mAb binding (Fig. 8) reflected largely the findings described for binding inhibition (Fig. 4). In rabbit tissues, M110 and A917 showed comparable specific binding to that of oPRL except that A917 binding to liver was notably high. Although A82 binding was similar to oPRL binding in mammary gland, binding to other rabbit tissues examined was markedly lower. For rat and human tissues, in which mAbs did not inhibit 125I-oPRL binding, significant (specific binding 1% of added tracer) was not observed except low binding (4.9%) of A917 to rat liver. M110 bound to pig mammary gland at a similar level to that of oPRL, whereas binding of A82 and A917 were low or insignificant.

In order to investigate whether these monoclonal antibodies bind to the same domain of the receptor molecule to that of the lactogen binding domain, competition experiments for 125I-mAb binding were performed. In Fig. 9, excess lactogen hormones (oPRL, hPRL, and hGH) completely inhibited 125I-M110 and 125I-A82 binding to rabbit mammary microsomes; 125I-A917 binding was reduced by only 50-70%, while nonlactogenic hormones did not alter the binding of mAbs. Competition curves using unlabeled oPRL are shown in Fig. 10. 125I-M110 binding was competed for by oPRL with comparable ID50 values (0.44 ± 0.05 nM, mean ± S.E. of 4 different mAbs to Rabbit Mammary PRL Receptors

FIG. 10. Competition for 125I-mAb binding to rabbit mammary microsomes by oPRL. Fifty-one to 53 fmol of 125I-M110 (○), 125I-A82 (■), or 125I-oPRL (●) were incubated for 15 h with 300 μg of microsomes in the absence or presence of various concentrations of cold oPRL. Control binding in the absence of unlabeled oPRL was 20.5 ± 2.1 fmol, 17.0 ± 2.9 fmol, 15.5 ± 1.3 fmol, and 18.9 ± 1.5 fmol for 125I-M110, 125I-A82, 125I-A917, and 125I-oPRL, respectively. Values are mean ± S.E. of 4 different microsomes.
mAbs to Rabbit Mammary PRL Receptors

![Fig. 12. Interaction between mAbs for binding to rabbit mammary microsomes. Fifty-one to 53 fmol of 125I-M110 (A), 125I-A82 (B), or 125I-A917 (C) was incubated with rabbit mammary microsomes (300 μg) in the absence or presence of various concentrations of mAb (M110, A82, A917, Δ), oPRL (●), or normal IgG (○). Control binding was 21.1 ± 2.4 fmol, 15.0 ± 3.6 fmol, 15.0 ± 1.7 fmol, and 18.5 ± 2.0 fmol for 125I-M110, A82, A917, and oPRL, respectively. Values are mean ± S.E. of three microsomal preparations from different rabbits.](image)

mAbs to Rabbit Mammary PRL Receptors

![Graph showing the interaction between mAbs for binding to rabbit mammary microsomes.](image)
than the hormone binding site but inhibits PRL binding by steric hindrance; or 3) A917 binds to a domain distinct from the hormone binding site and induces a conformational change of the PRL binding domain, rendering it less active for PRL binding.

$^{125}$I-A82 or $^{125}$I-A917 binding was stimulated in the presence of A917 and A82, respectively. This observation can probably be best explained by assuming that 2 mAbs bind different antigenic determinants and induce the conformational changes of the domain to which the counterpart binds, increasing the bindability to antibodies. This observation is consistent with that of Chandler et al. (34) where a monoclonal antibody to nerve growth factor receptor stimulated the nerve growth factor binding.

Polyclonal anti-PRL receptor antibodies prepared by us (7) or by others (5) showed a cross-reactivity with all the species tested, such as rabbit, rat, mouse, pig, and human. However, our monoclonal antibodies revealed strong species specificity. This phenomenon has been commonly observed with many monocular anti-receptor antibodies (35-39), suggesting the existence of structural difference among tissues. Within the same species (rabbit) some differences in immunoreactivity among tissues was observed by $^{125}$I-mAb binding experiments. For instance, M110 bound to all 5 tissues to almost the same extent as oPRL. However, binding of A82 was poor in liver, kidney, adrenal, and ovary. Conversely A917 showed enhanced binding in liver. The binding profile of these 3 mAbs (Fig. 4) suggests the immunological similarity of PRL receptors in kidney, adrenal, and ovary with liver and mammary gland receptors different from these 3 tissues. Recently, Waters et al. (40) reported immunological differences in PRL receptors in rabbit tissues with different polyclonal anti-PRL receptor antibodies using as criteria PRL binding inhibition and immunoprecipitation. In general, the present studies support some tissue specificity, although exact comparisons of the techniques in the two studies are difficult.

The present data demonstrate that monoclonal antibodies which recognize different determinants can be obtained by our screening system, the PRL binding inhibition assay, and that these antibodies will be potentially useful tools for the further characterization of the structure of the receptor and the interaction of ligands with the binding sites. Finally, we have investigated the biological activity of these antibodies both in vitro and in vivo (41).

Acknowledgments—The expert technical assistance of Anne Lauvert and Joseph Zachwieja and the valuable advice of Michelle Bröch is gratefully appreciated. Ovine PRL, hGH, human PRL, ovine growth hormone, ovine follicle-stimulating hormone, and ovine lutezinizing hormone were kindly provided by the National Hormone and Pituitary Program, National Institutes of Health.

REFERENCES
mAbs to Rabbit Mammary PRL Receptors

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Nasue Katoh, Jean Ijima and Paul A. Kelly

SUPPLEMENTARY MATERIAL TO

MONOCULAR ANTIBODIES AGAINST RABBIT MAMMARY PRL RECEPTORS:

SPECIFIC ANTAGONISTS TO THE HORMONE BINDING SITES

MATERIALS AND METHODS

Receptor preparations

Mammary gland, adrenal, ovary, kidney and liver from early lactating rabbits treated with bromocriptine for 24 h. (15), ovary and liver from female rats treated with estradiol 17β (1 mg) once a day for 3 days, lactating pig mammary gland and human breast cancer cells (MCF-7) were used as a source of PRL receptors. Microsomal fractions were prepared as described previously (16).

Receptor purification

Rabbit mammary microsomes were solubilized by 3-[(3-cholamidopropyl)-

dimethylammonio]-1-propane sulfonate (CHAPS) using a differential solubilization method (1 ml and 7.5 ml CHAPS) as previously described (17). Phenylmethyl-
sulfonyl fluoride (PMSF) was added to a final concentration of 1 mM. Solubilized receptors were applied to a Sephacryl S-200 (Pharmacia) column (35 ml bed volume) at room temperature with a flow rate of 20-25 ml/hr. The column was washed with 10 volumes of 25 mM Tris-Cl/140 mM NaCl/5 mM PMSF, pH 7.4 (column buffer) and 3 volumes of Tris-Cl buffer were then eluted with 1 volume of 5% (w/v) sucrose in column buffer. Fractions were monitored for turbidity and for high binding activity by adding 1 ml of cold (4°C) assay buffer and centrifuged at 1300 g for 20 min. pellets were washed with a 120 gamma counter with an efficiency of 60%.

In some experiments (Figs. 4, 6A, 7 and 9), specific binding was estimated. Non-specific binding was determined by including cold hormone or immunoglobulin (IgG) at 1000-10,000 fold excess of tracer) in the incubation mixture. The difference between the incubations with and without excess competitor was taken as specific binding.

Binding assays for purified and affinity-purified receptors were performed similarly except that CHAPS was added to the incubation at a final concentration of 0.5%. Bound hormones were separated using the polyacrylamide gel precipitation method. Five hundred of 0.1% bovine gamma globulin in 0.1 M phosphate buffer, pH 7.4, 1 ml of 10% (w/v) polyacrylamide gel 8000 were added, reversed and the tubes were centrifuged at 2500 g for 15 min.

Binding assays were conducted using duplicates or triplicates tubes and repeated at least twice.

Materials

CHAPS (Sigma-CH-519), N-(3-Cholamidopropyl)-dimethylammonio-1-propane sulfonate (CHAPS), egg (Pharmacia-FPL-1), ovine PRL (Novo-Nordisk-252), ovine GH (Novo-Nordisk-411), ovine follicle stimulating hormone (NIAMDD-oFSH-15) and ovine luteinizing hormone (NIAMDD-oLH-15) were kindly supplied by National Hormone and Pituitary Program. Bovine insulin was obtained from Schering/Plough, Spring Valley, N.Y. (CHAPS), protein A-agarose, mouse IgG, Fc and F(ab)2 were purchased from Sigma, St. Louis, Mo. Protein A-agarose 100 is from Bio-Rad Laborato-

ries, Richmond, Ca. Microsomal fractions of T-47D human breast cancer cell line was kindly provided by Dr. J. F. Page (Centre de recherche du Cancers du la Region Nord, Lille, France).
mAbs to Rabbit Mammary PRL Receptors

Fig. 3 Effect of monoclonal antibodies on $^{125}$I-oPRL binding to solubilized (a) or affinity purified (b) receptors. QNSP solubilized receptors (100 µg binding capacity 240 fmol/mg protein) or affinity purified receptors (0.5 µg binding capacity 78 pmol PRL/mg protein) were incubated with 56 fmol of $^{125}$I-oPRL in the absence or presence of various concentrations of IgG (M100 (○), M110 (△), M112 (□)) or normal IgG (■). Incubation conditions are as described in Materials and Methods. Results were expressed as indicated under the legend (a) Fig. 2. Control binding was 30.1 and 24.8 fmol for solubilized and purified receptors, respectively.

Fig. 5 Effect of monoclonal antibodies on $^{125}$I-oPRL and $^{125}$I-mAbs binding to rat mammary microsomes. Fifty-two fmol of $^{125}$I-oPRL (a) or 53 fmol of $^{125}$I-$\gamma$-PRL (b) was incubated with 300 µg of rat mammary microsomes in the absence or presence of 87 nM oPRL, 91 nM oGH, 375 nM immunoglobulin or combination of oGH and immunoglobulin as indicated. Values are expressed as average percentage of $^{125}$I-oPRL or $^{125}$I-mAbs added to each of 3 different experiments.

Fig. 7 $^{125}$I-mAb binding to rabbit mammary microsomes: steady state experiment (A) Varying concentrations of $^{125}$I-oPRL (○), AR2 (□), AR3 (△) or $^{125}$I-oGH (■) was incubated with 300 µg of microsomes in the absence or presence of excess (1000-10,000-fold) of total IgG or oPRL. Incubation was continued for 7 h at 4°C (AR2, AR3) or 40 h at 2°C (M110). Specific binding was plotted as a function of free tracer. Non-specific binding was 1.2 - 1.3, 2.0 - 2.5, 1.3 - 2.2, 0.9 - 1.2, 0.9 - 2.7, 0.9 - 1.2, and 2.5 - 1.2 of total tracer added for M110, AR2, AR3 and oPRL, respectively.

(A) Specific binding of (A) was analyzed according to the method of Scatchard (18).
**mAbs to Rabbit Mammary PRL Receptors**

**Fig. 9** Competition of $^{125}$-labeled binding by lactogenic vs non-lactogenic hormones. $^{125}$-labeled (M10, A; M12, B; M17, C) 5-50 fmol) was incubated with 300 pg of rabbit mammary microsomes for 1 h in the absence or presence of unlabeled hormone (100 ng) or IGF (123-375 mM) indicated. Results are expressed as mean ± SE of 3 microsomes obtained from different rabbits. Control bindings are 26.2 ± 3.2 fmol for M10, 14.7 ± 1.1 fmol for M12 and 11.6 ± 1.1 fmol for M17.

**Fig. 11** Competition of $^{125}$-labeled binding to microsomes from various tissues by PRL. $^{125}$-labeled (30-50 fmol) was incubated with microsomes from rabbit mammary gland, kidney, adrenal, ovary and rat liver (100 pg, except (M12) adrenal), 150 pg in the absence or presence of excess cold PRL (67 ng/mL) or cold IGF (47 ng/mL). Values are expressed as average percentage ± S.E. of 3 independent experiments.

**Table 1**

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<thead>
<tr>
<th>Effect of preincubation with excess unlabelled PRL on subsequent $^{125}$-labeled binding</th>
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<td>Rabbit mammary microsomes (500 ng) were incubated with or without unlabelled PRL (57 ng/mL) or IGF (375-425 mM) for 12 h. $^{125}$-labeled was then added and further incubated for 12 h. The values are mean ± SE of 3 different microsomes.</td>
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<th>1st incubation</th>
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<td>(fmol)</td>
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<td>PRL</td>
<td>15.8 ± 0.9</td>
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<td>IGF</td>
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<td>M17</td>
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