Monoclonal antibody detection of prolactin-binding subunits in the rabbit mammary gland

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The structure of prolactin (PRL) receptor in the rabbit mammary gland was examined using a receptor-specific monoclonal antibody (MAb). The PRL receptor preparation used was purified by making use of a PRL-affinity column. MAb inhibited the binding of PRL to the receptor, in a dose-dependent manner and completely at a high concentration. Using the receptor directly labelled by ^{125}I , the preparation was incubated with MAbs and the immune complex was collected by Pansorbin and examined by SDS/polyacrylamide-gel electrophoresis. The autoradiography showed that three species with apparent M_r values of 77 000, 41 000 and 25 000 specifically reacted with MAbs. The pattern changed little in the presence or absence of dithiothreitol. Western blot analysis showed that two species (M_r 77 000 and 41 000) reacted with MAb. Affinity labelling of the receptor with labelled PRL revealed three bands with M_r values of 96 000, 60 000 and 43 000 on SDS gels. The high- M_r complex ($M_r > 200\,000$) was always present at the top of the gel. These results show that the mammary gland contains at least three PRL-binding subunits. The differences in M_r before and after PRL binding were close to the M_r of PRL. This would suggest that each PRL binding subunit reacts with one PRL molecule.

INTRODUCTION

Prolactin (PRL) plays a key role in regulation functions of the mammary gland. Like other peptide hormones, PRL binds to its specific receptors on the membrane of target cells. Both polyclonal and monoclonal antibodies raised against PRL receptors act as an agonist or an antagonist of PRL in the mammary gland (Shiu & Friesen, 1976; Djiane *et al.*, 1981, 1985). Curvilinear Scatchard plots of monoclonal antibody (MAb) binding to PRL receptors were reported (Kato *et al.*, 1985a; Sakai & Murakami, 1987). We showed that PRL receptors in the rabbit mammary gland can be fractionated, by ion-exchange chromatography, into two different species both of which have high PRL binding specificity and sensitivity (Sakai *et al.*, 1986). It is, therefore, important to characterize the structural properties of the PRL receptor for the further study of PRL action in the PRL target organ, mammary gland.

To examine the molecular structure of the PRL receptor, a combination involving cross-linking of labelled PRL to its receptor and SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) has been used (for review, see Kelly *et al.*, 1985). With these methods, however, M_r estimation of the receptor may be influenced by factors such as the number of PRL molecules in the complex and the configuration of both the receptor and PRL molecule after PRL binding. We prepared a MAb specific to the rabbit mammary gland PRL receptor and characterized the binding specificity to the receptor (Sakai & Murakami, 1987). We used the two receptor-specific MAbs to examine directly the species and the M_r of the PRL binding subunit and the results were

compared to those of the cross-linked PRL–receptor complex, to estimate the number of PRLs in the receptor complex.

MATERIALS AND METHODS

Hormones and other materials

Ovine PRL (NIH-P-S15) was kindly supplied by the NIADDK, Bethesda, MD, U.S.A. Nitrocellulose membrane filters (BA85) were obtained from Schleicher & Schuell GmbH, Dassel, Germany. Disuccinimidyl suberate (DSS) was obtained from Pierce. Pansorbin was purchased from Calbiochem. The Immun-Blot Assay Kit with goat anti-(mouse IgG)–alkaline phosphatase conjugates, Protein A MAPS II kit and SDS/PAGE M_r marker standards were purchased from Bio-Rad. The mouse monoclonal typing kit was obtained from Serotec, Bicester, U.K. Mouse normal immunoglobulin G (nIgG) was obtained from Sigma. All other chemicals were of analytical grade.

Solubilization and affinity-purification of PRL receptors

Mammary glands from mid-lactating New Zealand White rabbits were used. Solubilization and affinity-purification of the PRL receptors were as described (Sakai *et al.*, 1985). In brief, the microsomes (100 000 g, 60 min, pellets), collected by differential centrifugation, were solubilized with 7.5 mM-3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (Chaps). PRL receptors in the Chaps extracts were purified by a PRL-affinity column. The column was washed successively with 0.1 M-borate buffer (pH 7.4) containing 1 mM-

Abbreviations used: PRL, prolactin; MAb, monoclonal antibody; PAGE, polyacrylamide-gel electrophoresis; DSS, disuccinimidyl suberate; Chaps, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate.

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Chaps, 4 M-urea and borate buffer/Chaps. The receptors were eluted with 4 M-MgCl₂. The eluates were dialysed against 10 mM-borate buffer (pH 7.4) containing 0.1 mM-Chaps and lyophilized. The protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Iodination of PRL and PRL receptors

PRL (5 µg) was iodinated using lactoperoxidase and H₂O₂ (Sakai *et al.*, 1975); the specific radioactivity was 75–85 µCi/µg. Affinity-purified receptors were iodinated in a manner similar to that for PRL; 16 µg of protein was used for iodination, and the specific radioactivity was 11–25 µCi/µg.

MAb production

Using affinity-purified PRL receptors as an antigen, MAb was produced and purified using a Bio-Rad Protein A MAPS II kit (Sakai & Murakami, 1987). The subclass of MAb was determined using a Serotec mouse monoclonal typing kit. The MAb concentration was determined by the method of Bradford (1976) with bovine γ-globulin as standard.

Inhibition of PRL binding by MAb

For particulate or soluble receptors, the receptors were incubated with 30 000 c.p.m. of ¹²⁵I-PRL in the presence of various concentrations of unlabelled MAb for 16 h at room temperature in a total volume of 0.5 ml. The binding buffer consisted of 25 mM-Tris/HCl (pH 7.4), 10 mM-MgCl₂, 1 mM-phenylmethanesulphonyl fluoride, and 0.2% bovine serum albumin. After incubation, the particulate receptors were precipitated by centrifugation, as described (Sakai *et al.*, 1985). For soluble receptors, 5 mM-Chaps was included in the reaction mixture, and the receptors with carrier γ-globulin were precipitated by poly(ethylene glycol) (Sakai *et al.*, 1986). The radioactivity in the precipitants was measured in a gamma counter. Nonspecific binding was determined by the presence of a 1000-fold excess of unlabelled PRL. The difference between the radioactivity in the presence and absence of excess PRL was taken as specific binding. Each determination was performed in triplicate.

Immunoprecipitation by Pansorbin

The assay was carried out in a 1.5 ml microcentrifuge tube. Radiolabelled receptors (1.5 × 10⁶ c.p.m.) were preincubated with Pansorbin for 15 min at room temperature. Pansorbin was removed by 3 min of centrifugation at 12 000 g. The receptors were then incubated overnight at room temperature with 20 µg of MAbs or normal IgG in 0.5 ml of 25 mM-Tris/HCl (pH 7.4) containing 0.15 M-NaCl, 0.5% Triton X-100 and 0.1% bovine serum albumin. At the end of the incubation, 50 µl of Pansorbin which had been pre-treated with the unlabelled purified receptors was added to the reaction mixture. After 15 min, the tubes were centrifuged for 3 min. The pellets were washed once with incubation buffer and three times with 50 mM-Tris/HCl (pH 6.7). The proteins were released by boiling (100 °C, 5 min) in SDS sample buffer [62.5 mM-Tris/HCl (pH 6.7), 2% SDS, 10% glycerol, 0.01% Bromophenol Blue], with or without 100 mM-dithiothreitol. After removal of the insoluble materials, the samples were analysed by SDS/PAGE. The gels were dried at 70 °C under vacuum and used for autoradiography. In the present experiments,

electrophoresis was performed on 10% polyacrylamide slab gels (1 mm thickness) according to the method of Laemmli (1970). *M_r* determination was made by comparing the migration distance with those of SDS/PAGE *M_r* marker standards.

Immunoblotting

Affinity-purified receptors (40 µg) were analysed by SDS/PAGE under nonreducing conditions. After SDS/PAGE, proteins were transferred electrophoretically onto nitrocellulose membrane filters at 5 °C. The transfer buffer consisted of 25 mM-Tris, 192 mM-glycine, pH 8.3, and 20% (v/v) methanol (Towbin *et al.*, 1979). SDS/PAGE *M_r* markers were stained with Amido Black 10B. PRL receptors were detected using a Bio-Rad Immun-Blot assay kit according to the manufacturer's instructions. After blocking with gelatin, the membrane was incubated with MAb or normal IgG (10 µg/ml) overnight at room temperature. In control experiments, the blotted membrane was preincubated overnight with PRL (1 µg/ml) and used for MAb binding. The membrane was incubated with goat anti-(mouse IgG)-alkaline phosphatase conjugates for 1 h followed by the substrate for 3 h.

Cross-linking of the receptor with labelled PRL

Affinity-purified receptors were incubated with ¹²⁵I-PRL (1.5 × 10⁶ c.p.m.) in the presence or absence of excess unlabelled PRL for 16 h at room temperature, as described above. After removing free ¹²⁵I-PRL, cross-linking was performed on the PRL-receptor complex using 0.75 mM-DSS for 15 min on ice (Sakai & Ike, 1987). The samples were analysed by SDS/PAGE and autoradiography.

RESULTS

Two clones (C3, F10) positive in inhibiting ¹²⁵I-PRL binding to rabbit mammary microsome were used. The MAb in ascites fluids was precipitated by 50% saturation of (NH₄)₂SO₄ and further purified to the IgG form using a Protein A affinity column. The *M_r* and the purity of IgG, estimated by SDS/PAGE, were 150 000 and 95%, respectively. Typing analysis showed that the subclass of C3 or F10 is IgG_{2b} or IgG₁, respectively. When MAb was incubated with ¹²⁵I-PRL, the poly(ethylene glycol)-precipitated MAb did not contain any radioactivity of labelled PRL, showing that both MAbs do not cross-react with PRL. The PRL receptors were purified approx. 130-fold from the Chaps extracts by a PRL-affinity column, and the recovery of the PRL binding activity was less than 5%. The receptor concentration was 60.0 fmol/µg.

As shown in Fig. 1, both C3 and F10 inhibited ¹²⁵I-PRL binding to the microsomal, Chaps solubilized-, or affinity-purified receptors, in a dose-dependent manner. The displacement curves shifted to right or left depending on the receptors, either the particulate or soluble form. At high concentrations, both MAbs completely inhibited the binding of ¹²⁵I-PRL to its receptors. C3 was more potent in inhibiting activity of PRL binding to microsomes, but was less potent for the affinity-purified receptors than F10. The binding of ¹²⁵I-PRL to the receptors was not interfered with by the presence of normal IgG.

To examine the molecular structure of the affinity-

Fig. 1. Competition curves of specific binding of labelled PRL by unlabelled MAB

PRL receptors in the microsome ($\square$), the Chaps extract ($\circ$), and the affinity-purified fraction ($\bullet$) were incubated with ^{125}I -PRL (30000 c.p.m.) in the presence of increasing concentrations of unlabelled C3 (a) or F10 (b). Results were compared to specific binding of ^{125}I -PRL; radioactivity of specific (and nonspecific) binding to the microsome, the Chaps extract, and the purified receptors were $14.8 \pm 0.5\%$ ($1.1 \pm 0.1\%$), $17.6 \pm 1.6\%$ ($3.7 \pm 0.1\%$), and $13.9 \pm 0.4\%$ ($5.7 \pm 0.6\%$) of total radioactivity added, respectively. Values are the mean $\pm$ S.D. of three different experiments.

Table 1. Effect of preincubation with prolactin on the binding of monoclonal antibody to the receptor

The radioiodinated receptor preparation was preincubated in the presence (+) or absence (–) of prolactin (1 μg) for 16 h at room temperature. Values are means $\pm$ S.D. for $n = 6$.

Antibody	Prolactin	Radioactivity (c.p.m.)
C3	–	$63\,580 \pm 7140$
	+	$11\,890 \pm 1460$
F10	–	$40\,950 \pm 6790$
	+	$12\,910 \pm 2290$
Normal IgG	–	$9\,720 \pm 3160$
	+	$11\,240 \pm 2630$

Fig. 2. Immunoprecipitation of purified PRL receptors

Radioiodinated receptors were incubated with MAB and the receptor-antibody complexes were precipitated by Pansorbin. The samples were denatured in the presence (a; left four lanes) or absence (b; right three lanes) of dithiothreitol. a: lane 1, iodinated receptors; lane 2, normal IgG; lane 3, C3; lane 4, F10. b: lane 1, normal IgG; lane 2, C3; lane 3, F10. The radioactivity per lane was 26000 c.p.m., 4000 c.p.m., 12000 c.p.m. and 11000 c.p.m. for iodinated receptors, normal IgG, C3 and F10 respectively.

purified receptors, radioiodinated receptors were incubated with MAB and the immune complex was collected by Pansorbin. Table 1 shows the amounts of the Pansorbin-adsorbed radioactivity in the presence and absence of PRL. Both C3 and F10 precipitated 4–6-fold higher radioactivity than did normal IgG. The binding of MABs to the receptor was inhibited by preincubation with PRL. The immune complex was denatured with SDS in the presence or absence of dithiothreitol and subjected to SDS/PAGE (Fig. 2). The lane of labelled PRL receptors has at least nine bands. Among them, three species specifically cross-reacted with MABs. Under reducing conditions, autoradiograms in Fig. 2(a) show that four species with M_r values of 77000, 50000, 41000 and 25000 reacted with MABs. Since a species with an M_r of 50000 was also precipitated by the presence of normal IgG in the control, the other three bands of M_r 77000, 41000 and 25000 were specific to MABs. The M_r 41000 species was dominant. F10 produced the same autoradiogram pattern of MAB-binding species as did C3. Another high- M_r species ($M_r > 200\,000$) was observed at the top of the gel. This band may represent an aggregated form. The intensity of the band changed greatly depending on the presence or absence of dithiothreitol, with no change in the autoradiogram pattern. Under nonreducing conditions the relative mobility of the MAB-specific band changed little (Fig. 2b). An M_r -41000 species was dominant. In case of preincubation with PRL, the appearance of MAB-specific bands was completely inhibited.

After separation by SDS/PAGE under nonreducing conditions, the affinity-purified receptors were transferred onto a nitrocellulose membrane by electrophoresis. The receptor-incorporated membrane was in-

Fig. 3. Western blot analysis of the purified receptors

Affinity-purified receptors were separated by SDS/PAGE under nonreducing conditions and transferred onto a nitrocellulose filter. The membrane filter was incubated with F10 (10 µg/ml) in the presence or absence of PRL (1 µg/ml). Immunoreactive bands were visualized as described in the Materials and methods section. Lane 1, F10 alone; lane 2, F10 and PRL; lane 3, preincubation with PRL (16 h).

cubated with F10 followed by goat anti-(mouse IgG) serum (Fig. 3). Immunoblots revealed a faint band with M_r 77 000 and a dark band with M_r 41 000 (lane 1). The band of the high- M_r species was relatively diffuse, as compared with that of the low- M_r species. The results also show that the high- M_r form ($M_r > 200\,000$) of binding species was observed at the top of the gel. The intensity of the bands decreased by incubation together with PRL (lane 2). By preincubation of the membrane with PRL, all bands disappeared from the receptor-incorporated membrane (lane 3). When replacing with C3 or normal IgG, no band appeared in the immunoblots.

To examine the species and their M_r of the PRL-receptor complex, the affinity-purified receptors were cross-linked with ^{125}I -PRL using DSS, and were analysed by SDS/PAGE and autoradiography (Fig. 4). Cross-linking labelled PRL to the PRL receptors revealed three bands of M_r 99 000, 60 000 and 43 000. The 60 000- M_r complex was the most heavily labelled species. The high- M_r complex ($M_r > 200\,000$) was present at the top of the gel. The appearance of the bands was prevented by incubation in the presence of excess unlabelled PRL.

Fig. 4. Cross-linking of ^{125}I -PRL to purified receptors

Purified receptors were incubated with 1.5×10^5 c.p.m. of ^{125}I -PRL in the presence (right) or absence (left) of 1 µg of unlabelled PRL for 16 h at room temperature. Cross-linking was performed on the PRL-receptor complex using 0.75 mM-DSS. The samples were analysed by SDS/PAGE under reducing conditions.

Table 2. M_r of the prolactin-binding subunit

The M_r of the PRL-receptor complex (Complex) was obtained from Fig. 4 ($n = 3$). The M_r of the PRL receptor (Receptor) was from pools of Figs. 2 and 3 ($n = 9$). Values are means $\pm$ s.d.

Species	Complex (A)	Receptor (B)	Difference (A-B)
I	> 200 000	> 200 000	—
II	99 000 $\pm$ 5800	77 000 $\pm$ 5200	22 000
III	60 000 $\pm$ 3500	41 000 $\pm$ 1300	19 000
IV	43 000 $\pm$ 800	25 000 $\pm$ 800*	18 000

* M_r estimated from Fig. 2.

The apparent M_r of the PRL-receptor complex and the receptor are summarized in Table 2. These increases in the M_r after PRL binding were close to the M_r of one PRL molecule. In the present experiments, the M_r of the high- M_r aggregate present at the top of the gel could not be estimated.

DISCUSSION

Both MAbs (C3 and F10) inhibited the binding of PRL to the receptors in the particulate and the soluble fractions, in a dose-dependent manner. Our data also show that F10 has a higher inhibiting activity than C3 for the purified receptors. Katoh *et al.* (1985a) reported curvilinear Scatchard plots of MAb binding to the microsome PRL receptors. We also observed similar Scatchard plots of MAb binding to the Chaps-solubilized PRL receptors (Sakai & Murakami, 1987), thereby suggesting that the rabbit mammary gland contains two classes (a high or a low affinity for MAb binding) of binding sites, as suggested (Waters *et al.*, 1984; Sakai *et al.*, 1986; Sakai & Ike, 1987).

The appearance of the MAb-specific band was completely inhibited by preincubation with PRL. Immunoblot analysis showed that the mammary gland contains two species of the MAb binding protein. The M_r -41000 species was comparable to that in the rat liver (Katoh *et al.*, 1987). The high- M_r species (M_r 77000) also cross-reacted with the MAb. This band was faint and appeared in immunoblots after 16 h incubation with MAb F10. The band of the M_r -25000 species did not appear in immunoblots. Furthermore, no band was detected by using C3. It appears that the denaturation of the PRL receptor with SDS may influence the MAb binding or the configuration of the receptor molecule. To eliminate the effect of SDS on the PRL receptors, we incubated the radioiodinated PRL receptors with MAbs under non-denaturing conditions. The autoradiography showed that the two species with similar M_r obtained by immunoblot analysis reacted specifically with MAbs. In the present experiments, the M_r -25000 species reacted with both MAbs. Necessary *et al.* (1984) reported that iodination of the PRL receptor (M_r 42000) leads to a small fragment with M_r 21000. However, the PRL-binding subunit with a similar M_r was detected in the affinity-labelled PRL-receptor complex. We have shown that a M_r -41000 species did not disaggregate into a M_r -25000 species following the chemical treatments involved in affinity labelling and SDS/PAGE (Sakai & Ike, 1987). It has been reported that the receptor binding activity is quite sensitive to oxidizing agents (Necessary *et al.*, 1984; Mahajan & Ebner, 1986). In the iodination procedure, we used a low concentration of H_2O_2 (88 μ M) to avoid oxidation of the receptor. At this concentration, radioiodinated PRL was biologically and immunologically intact, and had the same mobility as native PRL on disc PAGE (Sakai *et al.*, 1975). The results of the present study showed that the preincubation with PRL inhibited the binding of MAbs to the radio-iodinated receptor. This would suggest that the receptor retains the PRL binding activity.

Cross-linking labelled PRL to the affinity-purified receptors revealed three bands in the autoradiogram, of M_r 99000, 60000 or 43000. By subtracting the estimated M_r of free PRL, the M_r of the PRL receptor was estimated to be 74000, 35000 or 18000. The estimates were close to those obtained using MAbs. The results show that each binding subunit interacts with one PRL molecule. By cross-linking labelled PRL or hGH to its receptors, the M_r was reported to range from 32000 to 45000 in the PRL target organs of various species (Borst & Sayare, 1982; Haeuptle *et al.*, 1983; Hughes *et al.*, 1983; Necessary *et al.*, 1984; Yamada & Donner, 1984;

Katoh *et al.*, 1985b; Ashkenazi *et al.*, 1987). The high- M_r form of the PRL binding subunit (M_r 81000–91000) has been noted in the rat ovary (Bonifacino & Dufau, 1984; Mitani & Dufau, 1986) and in the Leydig cell PRL receptors (Bonifacino & Dufau, 1985). We showed here that the high- M_r PRL-binding subunit (M_r > 200000) could be detected under reducing and non-reducing conditions. These results suggest that the mammary gland contains the size-heterogeneous binding subunits. The relationship between these PRL-binding subunits and their biological significance is under investigation.

In the present experiments, the dominant PRL-binding species is a M_r -41000 receptor in the affinity-purified fraction. The dominant PRL-binding species in the microsomes and the Chaps-solubilized fraction was a M_r -83200 receptor (Sakai & Ike, 1987). The discrepancy cannot be explained as present. But it is probably due to the low recovery of the PRL-binding activity from a PRL-affinity column (less than 5% of the activity applied).

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