ウサギにおけるプロラクチン受容体の生化学的及び免疫学的解析

村上浩士

(1)

Biochemical and Immunological Characterization of Prolactin Receptors in the Rabbit

Thesis

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ABSTRACT

Prolactin (PRL), a pituitary peptide hormone, has lactogenic activity. PRL binds to its specific receptor on the plasma membrane. Unlike other peptide hormones, signal transduction across the membrane has not been clarified. To address the question that PRL is lactogenic, it is necessary as a first step to characterize PRL receptor from the mammary gland biochemically. Several unclarified problems on PRL receptor are as follows; a) structure of the receptor, b) types of the receptor in one tissue, c) relation of the receptors in one tissue, d) the role of carbohydrate chain of the receptor.

Antireceptor antibodies have been utilized as a tool to investigate the function and structure of the receptor. I produced two types of monoclonal antibodies (MAbs; C3 and F10) recognizing rabbit mammary PRL receptor. The properties of two MAbs are as follows: 1) The binding site of C3 is distinct from that of PRL or F10. C3 seems to recognize tertiary structure of the receptor. 2) The binding site of F10 is very close to that of PRL, but not the same as that of C3. The part of F10 binding site is N-linked carbohydrate chain. F10 seems to recognize the primary structure of PRL receptor. 3) Both MAbs discriminate PRL receptor from growth hormone receptor.

To investigate the tissue-specific heterogeneity of the receptor, PRL was incubated with its receptor in the presence of various concentrations of MAb, and the inhibition curves were compared between the membrane-bound and the solubilized PRL receptors in the rabbit adrenal gland, kidney, liver, mammary gland and ovary. Both MAbs inhibited dose dependently the binding of PRL to its microsome receptor in all tissues examined. However, the inhibition curves obtained by C3 or F10 were significantly different among

tissues. The tissue-specific difference was clearly observed when C3 was used as a competitor. After solubilization, tissue-specific difference was not observed. The results suggested that the heterogeneity is due to the conformational difference of the microsome PRL receptor among the tissues tested.

To clarify the structure of the PRL receptor, receptors in the rabbit mammary microsomes were solubilized with Triton X-100 and were purified twice on a Sepharose column coupled with C3 in the presence of protease inhibitors. Overall recovery was estimated to be of about 30 % and the purity was of over 30 % by Scatchard analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining analysis revealed that purified fraction contained ten species with molecular weight (M_r) of over 200,000, 100,000, 77,000, 63,000, 56,000, 44,000, 41,000, 33,000, 31,000 and 25,000. Among them, seven species with M_r of over 200,000, 100,000, 77,000, 63,000, 56,000, 44,000 and 41,000 were reacted with F10. By cross-linking, the PRL-receptor complex had M_r of over 200,000, 116,000-130,000, 100,000, 82,000, 58,000 and 43,000. These results show that the mammary gland contains at least seven PRL-binding proteins. The difference in M_r before and after PRL binding were close to the M_r of PRL. This would suggest that each PRL-binding proteins react with one PRL molecule.

To investigate the structural relations of PRL-binding proteins, representative receptor with M_r of 77,000 or 41,000 was digested with chymotrypsin, V8 protease or lysilendopeptidase and limited peptide maps were compared. SDS-PAGE showed that all peptide fragments of M_r=41,000 species were coincident with those of M_r=77,000 receptor. Removal of carbohydrate chains by N-glycosidase F, neuraminidase or O-glycan-peptide

hydrolase decreased similarly, M_r of 4,000, 1,000 or 1,000 in both receptors. The results indicated that PRL receptor in the mammary gland is size-heterogeneous, probably due to the difference in peptide length rather than in carbohydrate.

To clarify the role of carbohydrate chain linked to the receptor, effect of deglycosylation of the MAb-purified receptor on the hormone binding was examined. Treatment of the purified receptor with N-glycosidase F, neuraminidase or O-glycan-peptide hydrolase affected little on the affinity and specificity for hormones, and the binding site for PRL. Immunoblot analysis showed that by N-glycosidase F treatment, all PRL binding species did not react with F10. By neuraminidase or O-glycan-peptide hydrolase treatment, the M_r of all the binding species decreased by about 1,000. These findings suggest that these carbohydrates did not participate in the binding of PRL, although PRL receptor contained N- and O-linked carbohydrate chains in addition to sialic acid.

In conclusion, the mammary gland contains at least seven PRL-binding proteins, of which M_r is over 200,000, 100,000, 77,000, 63,000, 56,000, 44,000 and 41,000. Size-heterogeneity is caused by the difference of polypeptide length. Although all the species contain N- and O-linked carbohydrate chains, carbohydrates did not participate in PRL binding. These findings suggest that multiple PRL receptors mediate the signal of the hormone in its distinct way.

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ABBREVIATIONS

hormones

EGF	: epidermal growth factor
FSH	: follicle stimulating hormone
GH	: growth hormone
LH	: luteinizing hormone
PRL	: prolactin
TSH	: thyroid stimulating hormone

prefix to hormones

b	: bovine
h	: human
o	: ovine

others

ATP	: adenosine 5'-triphosphate
BSA	: bovine serum albumin
cAMP	: cyclic adenosine 3': 5'-monophosphate
CB-154	: 2-bromo-α -ergokryptin
cDNA	: complementary deoxyribonucleic acid
cGMP	: cyclic guanosine 3': 5'-monophosphate
Chaps:	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
DSS	: disuccinimidyl suberate
DTT	: dithiothreitol
MAb	: monoclonal antibody
Mr	: relative molecular mass (molecular weight)
mRNA	: messenger ribonucleic acid
nIgG	: normal immunoglobulin G
PMSF	: phenylmethylsulfonyl fluoride
SDS	: sodium dodecyl sulfate
SDS-PAGE	: SDS-polyacrylamide gel electrophoresis
Tris	: tris-(hydroxymethyl)-aminomethane

CHAPTER 1

General Introduction

1. Biological actions of prolactin (PRL)

PRL, a pituitary peptide hormone, exists in a wide variety of vertebrates and has over 85 biological functions, including lactation, reproduction and regulation of water and ion fluxes (Nicoll and Bern, 1972). While mammary glands are the major physiological target organs of PRL in mammals, many other cell types also have regulatory effects of PRL. In the mammary gland, the biological action of PRL includes growth and development of mammary cells, synthesis of milk proteins, fat and antibodies (Meites, 1988). Synthesis of milk protein is affected by a number of other hormones. In the in vitro system of mouse mammary gland, a combination of PRL, insulin and glucocorticoid is known to be essential to induce synthesis of milk proteins. In the rabbit and ewe, PRL alone induces casein synthesis in the explant culture (Houdebine et al., 1985). Furthermore, PRL in synergism with glucocorticoids is involved in the transcription of casein gene which is a major milk protein, but in the absence of PRL this transcription does not occur (Doppler et al., 1989). These observations suggest that PRL plays the major role in the regulation of milk protein synthesis.

2. The site where PRL releases its message

PRL binds to its receptor on the plasma membrane, followed by internalization into the cell (Josefsberg et al., 1979). Like other peptide hormones, PRL and receptor complex seems to be aggregated on the plasma membrane and transferred to Golgi components, followed by degraded in lysosomes.

There are two possibilities where PRL releases its message. One possibility is that PRL releases its message when PRL is degraded in

lysosomes. This is not likely because lysosomotropic agents do not change the casein gene expression (Houdebine and Djiane, 1980). However, internalization may be necessary to regulate the number of cell surface receptor. The other possibility is that PRL releases its message when PRL binds to its receptor. Supporting this possibility, Cuatrecasas (1969) showed that insulin covalently linked to agarose beads, which cannot enter the cell, expressed biological activity of insulin. This phenomenon was also observed in PRL (Turkington, 1970). Anti-receptor antibody which mimic hormonal actions have been reported in a wide variety of hormones, such as insulin (Jacobs et al., 1978), epidermal growth factor (Schreiber et al., 1981), luteinizing hormone releasing hormone (Podesta et al., 1983) and PRL (Djiane et al., 1981, 1985; Rosa et al., 1982; Edery et al., 1983). Using an anti-receptor antibody which mimicked insulin actions, Kahn et al. (1978) demonstrated that the monovalent fragment of this antibody, which was produced by treatment of papain, did not mimic the insulin action, but that by the addition of the anti-Fab antibody the activity of mimicking the action was restored. Similar phenomenon was also observed in PRL (Djiane et al., 1985). These findings suggest that not only a hormone transfers its message to its receptor when a hormone binds to its receptor but also that an aggregation of at least two receptors is important for the transduction of hormonal message.

3. General characteristics of PRL receptor

PRL receptor has two important features; specificity and high affinity for PRL. To investigate specificity and affinity of PRL receptor to PRL, a receptor binding assay is indispensable. In 1973, Shiu et al. developed a receptor binding assay, which made possible to characterize PRL receptor.

Using a crude membrane of rabbit mammary glands as a receptor source, they reported basic properties of PRL receptor as follows (Shiu et al., 1973; Shiu and Friesen, 1974a)

1) PRL receptors bind not only to PRL but also to human growth hormone (hGH) and human placental lactogen, which exhibit lactogenic activity. PRL receptors do not bind to other hormones. This fact represents specificity of PRL receptor for lactogenic hormones.

2) The binding of PRL to its receptors is a saturable and reversible process. Based on this fact, Scatchard plot (1949) can be constructed from competitive binding data. Scatchard analysis showed that apparently a single class of receptor exists and the dissociation constant is in the order of 10^{-9} M. This indicates that PRL receptor can readily binds to the physiological concentration of PRL ($\sim 10^{-9}$ M) in the blood stream.

3) The binding of PRL to its receptors are augmented by the inclusion of magnesium or calcium ion.

4) The receptor binding activity is reduced by the treatment of trypsin or phospholipase C. This observation suggests that protein and phospholipid are involved in the binding activity of PRL receptor.

4. Tissue distribution and subcellular localization of PRL receptor

The existence of PRL receptor has been reported in a wide variety of tissues (review, Kelly et al., 1988); livers, kidneys, pancreata, mammary glands, mammary tumors, adrenal glands, ovaries, testes, epididymides, prostate glands, seminal vesicles, prostate tumors, uteri, smooth chorions, lymphocytes, lymphomas (Nb2), erythrocytes (Bellussi et al., 1987) and lungs (Amit et al., 1987). Biological actions of PRL, however, are unclear in some of

the tissues in which PRL receptors are present. It is conceivable that more biological functions of PRL would be uncovered in many tissues. Thus far, characterization of PRL receptor has been conducted mainly in rabbit mammary glands, rat livers and rat ovaries, since these tissues have high concentrations of PRL receptor.

Like other peptide hormone receptors, PRL receptors are located in the plasma membrane (Shiu and Friesen, 1974a; Shiu and Friesen. 1976). In the rat liver, however, the majority of the receptor was present in Golgi membranes (Bergeron et al., 1978; Posner et al., 1979). Lysosomes also contain PRL receptors (Khan et al., 1981; Ferland et al., 1984). Since rapid turnover times (half life: $t_{1/2}$=40~ 50 min) of PRL receptors in contrast to that ($t_{1/2}$>3 h) of insulin receptor were reported (Baxter, 1985), intracellular PRL receptors may represent newly synthesized or internalized ones. Recently, water soluble PRL receptors located in the cytoplasm have been found (Ymer and Herington, 1986; Ymer et al., 1987). Cytoplasmic receptors do not seem to be a simple cleavage product from the membrane, but, physiological significance of this receptor is unknown at present. Furthermore, a GH binding protein has been found in the serum (Ymer and Herington, 1985). Although a serum binding protein for PRL has not been identified, the existence of a truncated form of PRL receptor is predicted from cDNA analysis in the rat ovary (Zhang et al., 1990). From the analogy of insulin like growth factor-I (Carscieri et al., 1988) and interleukin-4 (Mosley et al., 1989), a serum binding protein for a hormone may function as interfering the access of a hormone to its receptor or delay the clearance of the hormone from the circulation.

5. Regulation of PRL receptors

The development of PRL binding assay enabled investigators to estimate the number of the receptor per cell and its affinity for the hormone. However, when measuring the number of PRL receptor where circulating levels of PRL are high, one must consider that receptor is occupied by PRL. To overcome this problem, two procedures with which one can estimate total binding sites were developed. In the in vitro desaturation procedure, a short exposure of the sample to magnesium chloride released the prebound hormone (Kelly et al., 1979). In the in vivo desaturation procedure, an injection of CB154, a dopamine agonist, to the animal inhibited the release of PRL from the pituitary (Djiane et al., 1977). With these methods, it was demonstrated that the number of the receptor in the liver was greatly influenced by the prebound hormone, but that in the mammary gland was not considerably affected.

Since PRL receptor mediates the hormonal message, it is expected that the sensitivity of the tissue to the hormone is related to the number or affinity of PRL receptor. Indeed, the number in many cases or the affinity of the receptor for the hormone in a few cases vary depending on the physiological state of the animal (Kelly et al., 1974; Djiane et al., 1977; Guillaumot et al., 1984). This finding suggests that some hormones regulate the number or affinity of PRL receptor. In the rabbit mammary gland, the number of the receptor gradually increased until mid-pregnancy, then declined, increased again in late pregnancy and remained high during lactation without any change in the affinity (Djiane et al., 1977; Grisson and Litteleton, 1988). A minor increase in the affinity in the mammary gland in late lactating rabbits was reported (Perry and Jacobs, 1978). In the rodent mammary gland, similar profile of the receptor number were reported except

that during pregnancy the number remained constant (Holcomb et al., 1976; Bohnet et al., 1977; Hayden et al., 1979; Sakai et al., 1981) and fluctuations of the receptor number during estrous cycles are also reported (Guillaumot et al., 1984). Increase in PRL and glucocorticoid and decrease in progesterone are known to be essential to lactogenesis (Houdebine et al., 1985). Treatment of pseudopregnant rabbits with PRL resulted in an increase in the number of PRL receptor, but simultaneous administration of progesterone prevented this increase (Djiane and Durand, 1977). Adrenalectomy reduced the receptor number and subsequent treatment with glucocorticoids restored the receptor number (Sakai and Banerjee, 1979). Ovariectomy increased the receptor number and the simultaneous removal of adrenals prevented this increase and subsequent treatment of glucocorticoids restored the receptor number (Harigaya et al., 1982). Thyroid hormone (T_4), which is involved in the development of mammary glands, also enhanced the receptor number (Marshall et al., 1979). Furthermore, the change of the receptor number correlated with the lactational potential (Sakai et al., 1985a). All these findings suggest that the receptor number in the mammary gland is important in lactogenesis.

In the liver, estrogen (Posner et al., 1974a; Marshall et al., 1978; Norstedt and Mode, 1982), GH (Knazek et al., 1978; Norstedt et al., 1981; Baxter et al., 1984) or PRL (Posner et al., 1974; Djiane and Durand, 1977; Manni et al., 1978; Kelly et al., 1980; Amit et al., 1985) stimulate an increase in the receptor number. In contrast, testosterone decreases lactogenic binding sites (Barkey et al., 1979; Sasaki et al., 1982a). Like the receptor in the mammary gland, the affinity of the receptor for the hormone was unchanged. However, Guillaumot et al. (1988) showed that the affinity vary during estrous cycle.

In granulosa cells, luteinizing hormone (LH) increases the number of PRL receptor (Richards and Midgley, 1976). In testes, LH and follicle stimulating hormone (FSH) stimulate an increase in the receptor number of PRL (Kelly et al., 1980).

In summary, all these observations indicate that the number of PRL receptor is differentially regulated by many hormones, depending on the tissues. Furthermore, the number of the receptor seems to be related to the sensitivity of the tissue.

6. Biochemical characterization of PRL receptors

a) Solubilization

Since PRL receptor is a membrane integral protein, it is required to solubilize receptors for the subsequent biochemical characterization. Many investigators have used Triton X-100, which is a nonionic detergent, and Chaps, which is a zwitterionic detergent. Initially, PRL receptors in the rabbit mammary gland were solubilized from crude membrane fractions by Shiu and Friesen (1974b) using Triton X-100. Because Triton X-100 induced to aggregate PRL but not hGH, hGH was used for binding studies of solubilized receptors. However, hGH binds to both nonprimate GH and PRL receptors (Kelly et al., 1974). If GH receptors exist in the tissue such as a liver (Waters and Friesen, 1979), it is difficult to evaluate the results of hGH binding assays. In 1982, Liscia et al. used 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (Chaps) to solubilize PRL receptors. In contrast to Triton X-100, Chaps did not affect the molecular characteristics of PRL, allowing PRL to be used in the binding assay.

Solubilization of PRL receptors affected both the affinity and the

number of receptors depending on the tissues. In the mammary gland, solubilized receptors have specificity for lactogenic hormones but affinity for the hormone increased 2- to 6-fold, regardless of the detergent used (Shiu and Friesen, 1974b; Waters et al., 1984; Katoh et al., 1985a; Sakai et al., 1986). Like insulin receptor (Harmon et al., 1983), solubilization may remove a membrane regulator which affects the affinity of the receptor. In the ovary (Koppelman and Dufau, 1982) and liver (Alhadi and Vonderhaar, 1982; Gavish et al., 1983), solubilization of the membrane receptors increased the apparent number of the receptor without affecting the affinity with the hormone. The increased receptors which emerge by solubilization were called cryptic receptors. Addition of S-adenosyl-L-methionine, which is a methyl donor, to the membrane from mammary glands resulted in the increased number of the receptor (Bhattacharya and Vonderhaar, 1979). Addition of buthanol, propanol, ethanol (Dave and Witorsch, 1983) or prostaglandin I_2 (Dave and Knazek, 1980) to the membrane from prostate glands also increased the number of the receptors. These reagents affect microviscosity of the membrane. They discussed that increased membrane fluidity lead to the exposure of cryptic PRL receptors.

b) Purification

Since the concentration of PRL receptors are very low, starting materials should be chosen carefully in order to achieve successful purification. Among the tissues which contain PRL receptors, mammary glands, livers and ovaries have relatively high concentrations of the receptor. The binding capacity of crude membrane fractions from bovine mammary glands, pig mammary glands, rabbit mammary glands, rabbit livers, rat livers, mouse livers

or rat ovaries is 20, 30, 150, 200, 1,600, 200 or 800 fmol/mg, respectively. Assuming the molecular weight (M_r) of PRL receptor as 40,000~ 300,000, theoretical binding capacity of purified receptor should be 3~ 25 nmol/mg. Therefore, 2,000~ 1,250,000-fold purification over crude membrane fractions is required to obtain the theoretical purified receptor. In order to achieve high purification, highly specific methods are necessary. Affinity chromatography have commonly been used to purify PRL receptors.

Shiu and Friesen (1974b) initially attempted to purify PRL receptors from rabbit mammary glands. A crude membrane fraction was treated with Triton X-100 and solubilized receptors were purified about 200-fold by affinity chromatography using hGH-coupled agarose. Using magnesium chloride as a dissociating agent, the effective elution of the receptor from affinity adsorbent was achieved. The recovery was about 8 %. In 1979, Waters and Friesen purified the receptor from rabbit liver using almost the same method as that in the rabbit mammary gland. They reported that 20-fold purification over crude membrane fractions was achieved and the recovery was about 23 %. In 1982, Liscia and Vonderhaar purified the receptor from mouse livers. A crude membrane fraction was treated with Chaps and solubilized receptors were purified 120-fold by affinity chromatography using PRL-coupled agarose and the recovery was 4 %. They also use magnesium chloride as a dissociating reagent. The purified receptor appeared a single band of M_r 37,000 on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). In 1983, Haeuptle et al., purified PRL receptor from rabbit mammary glands and livers using different procedures. They utilized biotinylated hGH and streptavidin-agarose. Elution was carried out with magnesium chloride. The purified receptor appeared a single band of M_r=35,000 on SDS-PAGE. In 1984, Necessary

et al. purified PRL receptor from mammary glands by acidic elution. They solubilized crude membrane fractions with Chaps and solubilized receptors were adsorbed on PRL-coupled agarose and eluted at pH 4.2. They achieved 37,500-fold purification and the recovery was about 50 %. The M_r of the purified receptor was 42,000 on SDS-PAGE. Katoh et al. (1985a) utilized the combined method of solubilization with Chaps, PRL-affinity column and elution with magnesium chloride. From rabbit mammary glands, they purified PRL receptor about 660-fold over crude membrane fractions and the recovery was about 9 %. They observed at least nine bands on SDS-PAGE and silver staining. Sakai et al. (1985b) purified PRL receptor from pig mammary glands by the same procedure as that by Katoh et al. (1985a). They achieved 200-fold purification and the recovery was 2 %. In 1986, Mitani and Dufau purified PRL receptor to homogeneity from rat ovaries by two steps of affinity chromatography using concanavalin A-Sepharose and hGH-Sepharose. The binding capacity of purified receptor was 20 nmol/mg and the recovery was about 15 %. The purified receptors were composed of two major bands of M_r=41,000 and 88,000 on SDS-PAGE and silver staining analysis. In 1987, Ashkenazi et al. purified PRL receptors from bovine mammary glands. They utilized the combined method of solubilization with Chaps, hGH-affinity chromatography, and elution with magnesium chloride. They purified receptor about 500-fold and the recovery was 8 %. Berthon et al. (1987) utilized PRL receptor-specific monoclonal antibody (MAb) to purify the receptors from pig mammary glands. They utilized the combined method of solubilization with Triton X-100, MAb-coupled agarose, and elution with magnesium chloride. They purified about 700-fold and the recovery was about 40 %. Okamura et al. (1989a) purified the receptor from rat livers using receptor specific MAb.

They used essentially the same method as used by Berthon et al. (1987). They achieved about 1,100-fold of purification and the recovery was about 50 %.

Although there are many studies on purification of PRL receptor, the recovery was relatively low except that of immunoaffinity chromatography. It is possible that different types of PRL receptor may be lost during the purification procedure.

c) Gel filtration, sucrose gradient centrifugation, isoelectronic focusing and ion-exchange chromatography

PRL receptors have been characterized by several biochemical techniques and its M_r was estimated. In gel filtration analysis, a wide range of M_r has been reported, depending on the tissue, detergent and purity of the receptor.

In the rabbit mammary gland, Shiu and Friesen (1974b) reported that the M_r of PRL receptor is 220,000 using a partially purified receptor and Triton X-100. Haeuptle et al. (1983) reported similar M_r values of 200,000 as a hormone-receptor complex using crude Triton solubilized receptors. Katoh et al. (1984) reported M_r of 133,000 using a partially purified receptor and Triton X-100. In contrast, Necessary et al. (1984) indicated that the M_r of the receptor deduced by gel filtration in the presence of Triton X-100 was overestimated. Using purified receptor, they obtained different M_r values of the receptor depending on the concentration and type of the detergent: 55,000 in 0.5 % Chaps, 17,000 in 1 % Chaps, 180,000 in Triton X-100. When crude receptors in Triton X-100 was used, the M_r of the receptor was 350,000. Supporting this finding, Sakai et al. (1986) reported that the M_r of the receptor was 74,000 in 5 mM Chaps or 37,000 in 7.5 mM Chaps using Chaps-

solubilized membranes. In the bovine mammary gland, M_r of 80,000-85,000 was reported using crude Triton X-100-solubilized receptors (Ashkenazi et al., 1987).

In the rat liver, M_r of 270,000~ 380,000 was reported in the presence of Triton X-100 (Sasaki et al,, 1982b; Amit et al., 1984; Yamada and Donner, 1985). Gavish et al. (1983) reported M_r of 340,000 in the presence of Chaps. In the mouse liver, M_r of 37,000 was reported in the presence of Chaps, irrespective of crude, Chaps-solubilized receptor or purified receptor (Liscia et al., 1982; Liscia and Vonderhaar, 1982).

As discussed above, the M_r of the receptor estimated by gel filtration varied, especially depending on the type of the detergent used. Since Chaps is more effective at breaking protein-protein interaction than is Triton X-100, Chaps disaggregates proteins to a monomeric form (Hjemeland, 1980). In the presence of Chaps, the M_r of the receptor is almost identical in value with those deduced by SDS-PAGE. It is unknown, however, aggregation of the receptor observed in the presence of Triton X-100 is intrinsic nature of the receptor. Furthermore, estimation of M_r by gel filtration is based on the assumption that shape of the sample is almost identical to that of the standard protein and that the extent to which the detergent binds to the protein is similar. It is not so easy to evaluate the M_r of a membrane protein as that of a soluble protein.

Hydrodynamic properties of PRL receptors are also characterized by sucrose gradient centrifugation. In the rat liver, Jaffe (1982) deduced that the M_r of the receptor was 77,800 using Triton X-100 solubilized receptors. Rae-Venter and Dao (1983) reported the similar M_r value (73,000) using the similar procedure. In the rabbit mammary gland, Sakai et al. (1986, 1987)

observed that the Chaps solubilized receptors sedimented at two different positions, the M_r of the receptor was 42,400 or 89,400, respectively. These receptors could be separated by ion-exchange chromatography.

Electric properties of PRL receptors are analyzed by isoelectric focusing and ion-exchange chromatography. Isoelectric focusing has shown that major part of the receptor from non-pregnant or pregnant rat livers had a pI of 7.0~ 8.5 or 4.2~ 4.7, respectively (Sasaki et al., 1982b). In the rabbit tissues, tissue specific difference in the receptor pI ranging from 4.8 to 5.9 are reported (Waters et al., 1984). In the pig mammary gland, two distinct pI values of 5.2~ 5.3 and 5.5~ 5.6 are reported (Sakai et al., 1985b). In the rat ovary, three isoforms with pI 4.0, 5.0 and 5.3 are reported (Mitani and Dufau, 1986). In the mammary gland and ovary, more than two pI values are observed, suggesting that these tissues have different receptors with distinct electric charge. This suggestion was confirmed by Sakai et al. (1986, 1987) who showed that Chaps-solubilized receptors from the mammary gland were separated into two species by ion-exchange chromatography.

d) Cross-linking studies

A combination of cross-linking of ^{125}I-PRL or hGH to its receptor with chemical cross-linking agent and SDS-PAGE analysis is one of the most effective methods to estimate the M_r of PRL-binding species. The M_r of the PRL-binding species is usually deduced by subtracting that of one PRL molecule (M_r=23,000) from that of PRL receptor complex. In the mammary gland, the M_r of the PRL binding species is reported to be 28,000~ 40,000 (Haeuptle et al., 1983; Hughes et al., 1983; Katoh et al., 1985a; Ashkenzi et al., 1987). In addition to the species of M_r=31,000~ 40,000, two other species of M_r=67,000~

91,000 and 20,000~ 25,000 are also found (Bonifacino and Dufau, 1984, 1985; Sakai et al., 1985b; Sakai and Ike, 1987). Species of M_r=20,000~ 25,000 may be a proteolytic product of a larger species or an aggregated form of PRL. Sakai and Ike (1987) showed that both species exist in the membrane fraction and they fractionated Chaps-solubilized receptors into two fractions by ion-exchange chromatography. The subsequent cross-linking studies of fractionated two species showed that one fraction mainly contained a species of M_r=36,800 and the other did a species of M_r=83,200.

In the liver, the M_r of the PRL binding species was estimated to be 32,000~ 45,000 (a small binding species) (Borst and Sayare, 1982; Hughes et al., 1983; Yamada and Donner, 1984, 1985; Bonifacino and Dufau, 1985; Katoh et al., 1985a). When Triton X-100-solubilized receptors were used, a large molecular weight (M_r=80,000~ 87,000) species appeared, but in the membrane fraction this species was not detected (Haldosen and Gustafsson, 1987; Okamura et al., 1989b). Membrane lipids surrounding the receptor may interfere with cross-linking of the labeled hormone to its large molecular weight species (Haldosen and Gustafsson, 1987; Okamura et al., 1989b). Or a large form may be generated by dimerization of a small species (Okamura et al., 1989a).

In the ovary, the membrane fraction contained one PRL binding species (M_r=40,000) and Triton X-100 solubilized fraction contained two PRL binding species (M_r=about 40,000 and 80,000) (Bonifacino and Dufau, 1984; Mitani and Dufau, 1986) like the phenomenon in the liver. They suggest that the M_r=80,000 species may be a cryptic receptor.

In the testis, four PRL binding species (M_r=31,000, 37,000, 81,000, 91,000) are found (Bonifacino and Dufau, 1985). In the kidney, one PRL binding species (M_r=32,000) was found and in the adrenal, two PRL binding species

(M_r=32,000 and 43,000) were reported (Katoh et al., 1985a). In a malignant rat lymphoma (Nb2) cell line (Shiu et al., 1983), a large form of the binding species (M_r=73,000~ 88,000) was identified (Webb and Wallis, 1988).

To summarize, PRL binding species are classified into two species: a small form (M_r=about 40,000) and a large form (M_r=about 80,000). It is known that cross-linking with agents such as disuccinimidyl suberate (DSS) is not only affected by the close proximity and availability of free amino groups but also by a variety of factors (Middaugh et al., 1983). Like the phenomenon observed in Triton X-100 solubilized receptors in the liver, aggregated form of the receptor is also cross-linked. Furthermore, the efficiency of the cross-linking reaction is usually low. Therefore, it is not sufficient to determine the composition of the subunit and complete subunit structure only by covalent cross-linking techniques.

7. Genes for PRL receptor

In 1988, Boutin et al. performed a cDNA cloning for the rat liver PRL receptor. The primary structure deduced from cDNA clone is consisted of 291 amino acids (theoretical M_r=33,368) and has a relatively long extracellular region, a single transmembrane segment, and a short (57 amino acids) cytoplasmic domain. With the rat cDNA as a probe, three clones for cDNA for the receptor in mouse liver were isolated (Davis and Linzer, 1989). Analysis of the cDNA clones suggested that two of these proteins are synthesized as precursors of 303 and 292 amino acids, with common signal sequences, extracellular domains, and transmembrane domains, but these proteins differ in the terminal region. A third protein is a truncated form. With a similar method, a second larger form of the receptor cDNA was isolated in the rabbit

mammary gland (592 amino acids) (Edery et al., 1989) and the human hepatoma (598 amino acids) (Boutin et al., 1989). The second form of the receptor is highly similar to the rat liver receptor except that it has a much longer cytoplasmic domain. Homology research did not reveal any identity with any consensus sequences known to be involved in hormonal signal transduction. Subsequently, the second form of the receptor cDNA clone was isolated in the rat ovary (Zhang et al., 1990). This predicted protein (610 amino acids, theoretical M_r=66,000) has overall similarity with rat liver, rabbit mammary gland and human hepatoma receptor. However, the ovarian receptor contains a unique cytoplasmic domain for 110 amino acids and consensus sequences for both a tyrosine phosphorylation site and ATP/GTP type A binding site. This implies that the ovarian receptor has potential for signal transduction and mitogenic activity. However, Shirota et al. (1990) reported the conflicting data that such a sequence was not present in the cDNA in the rat ovary.

Subsequent homology research showed that a PRL receptor is not only a family of a GH receptor (Leung et al., 1988) but also a family of lymphokine and hematopoietic growth factor receptors (Bazan, 1989). This fact suggests that they are derived from a common ancestor gene. Furthermore, the C-terminal part of the extracellular portion of members of the PRL receptor/GH receptor family contains a type III domain of fibronectin (Patty, 1990), suggesting that this domain is involved in protein-protein interaction.

8. Antibody to PRL receptor

Immunological techniques have been effective in the field of biochemistry and endocrinology. Antibodies can be applied to a wide variety of immunological methods such as radioimmunoassay, immunocytochemistry,

affinity chromatography, immunoblot analysis and immunoprecipitation analysis.

a) Polyclonal antibodies

Antibodies that recognize PRL receptor have been produced by the two methods. One method is that using a partially purified receptor as an antigen, animals are immunized (Shiu and Friesen, 1976; Bohnet et al., 1978; Djiane et al., 1981; Rosa et al., 1982; Edery et al., 1983; Katoh et al., 1984; Waters et al., 1984). This method is usually used to produce antibodies recognizing PRL receptor. The other approach is through the production of antiidiotypic antibodies against the antiligand antibodies to avoid the problems in purification of the receptor. By this approach, Amit et al. (1986) obtained antiidiotypic antibodies against anti-PRL antibodies recognizing the PRL receptor.

Antireceptor antibodies inhibited the binding of ^{125}I-labeled PRL in all tissues of all species tested (Katoh et al., 1984). In the rabbit, Waters et al. (1984) also observed similar but different patterns of inhibition of PRL binding to rabbit tissues by antibodies and suggested that similar but not identical tissue specific PRL receptor exists.

As expected, antireceptor antibodies that inhibited the PRL binding to its receptor blocked the biological action of the hormone (Shiu and Friesen, 1976; Bohnet et al., 1978). In contrast, some antireceptor antibodies mimicked the action of PRL (Djiane et al., 1981; Rosa et al., 1982; Edery et al., 1983; Dusanter-Fourt et al., 1983). However, the results obtained using polyclonal antibodies are difficult to assess. Because the antigens used were not completely pure, the antibodies may have recognized not only receptor but also other unrelated substances. Even if the antigen is pure, more than two

classes of antibodies which recognize different sites of the antigen will be produced.

b) Monoclonal antibodies (MAbs)

MAbs can be produced even if the antigen is not pure (Kohler and Milstein, 1975). Mono-specific property of MAbs has overcome the problems of polyclonal antibodies. Therefore, MAbs are one of the most effective tools in analyzing receptor structure and function.

Katoh et al. (1985b) produced and characterized three MAbs to the PRL receptor with partially purified rabbit mammary gland receptor as antigens. Two of the MAbs acted as antagonists of PRL and the other one acted as an agonist (Djiane et al., 1985). Furthermore, Katoh et al. (1987) produced MAbs to rat hepatic PRL receptor. From the immunoprecipitation and immunoblot experiments, they showed that the M_r of the rat hepatic PRL receptor was 42,000~ 46,000. Similarly, Okamura et al. (1989b) produced MAbs to the rat hepatic PRL receptor and performed immunoblot analysis in a wide variety of tissues of several species of animals. The reported M_r values of the receptor were as follows: 84,000, 51,000 and 42,000 for rat livers; 84,000, 51,000 and 42,000 for rat ovaries; 84,000 and 42,000 for rat prostate glands; 64,000, 52,000 and 42,000 for Nb2 cells; 66,000 and 36,000 for pig mammary glands; and 77,000, 55,000, 45,000 and 36,000 for rabbit mammary glands. Okamura et al. (1989a) also used MAbs to purify rat hepatic PRL receptors.

As has been described, MAbs have been effective tools for investigation of the receptor structure and function. Especially, a combination of SDS-PAGE and immunoblot analysis is useful to estimate the M_r of the receptor. However, the results obtained by immunoblot analysis did not necessarily correspond to those obtained by other methods such as cross-linking studies

and purification and SDS-PAGE analysis. There remains the possibility that MAbs recognize not only the antigen but also immunologically related proteins. It is not sufficient to identify the receptor molecule only by this method.

9. PRL receptor as a glycoprotein

The fact that PRL receptor has carbohydrate chains is first implicated by Costlow and Gallagher (1979), who showed that rat hepatic PRL receptors are retained on and specifically eluted from concanavalin A (Con A)-sepharose. The similar phenomenon were observed in the mouse hepatic receptor (Bhattacharya and Vonderhaar, 1982) and rat ovarian receptor (Mitani and Dufau, 1986). Furthermore, inhibition of PRL binding to its membrane receptor but not to solubilized receptors by lectin is also observed (Costlow and Gallagher, 1979; Bhattacharya and Vonderhaar, 1982). This fact suggests that the receptors have carbohydrate chains distinct from the hormone-binding site. However, purified PRL receptors in the mammary gland (Necessary et al., 1984) and Chaps solubilized mouse liver receptors (Vonderhaar et al., 1985) were not retained on lectin-sepharose. This discrepancy may be caused by the characteristics of the detergent used. The predicted amino acid sequence from cDNA clone from rat liver suggests that at least three potential asparagine glycosylation sites exist (Boutin et al., 1988). Recently, Haldosen et al. (1989) showed that M_r of the carbohydrate moieties of the rat hepatic PRL receptor was 5,000~ 10,000 by glycosidase treatment of the rat hepatic PRL receptor.

10. Second messenger of PRL

In the mammary gland, PRL plays the central role in the functional differentiation. However, signal transduction of postreceptor process is unclear at present and chemical compounds such as cAMP, cGMP, calmodulin, C kinase, phospholipase, and prostaglandins had no effect on synthesis of casein (Matusik and Rosen, 1980; Houdebine et al., 1985). Rillema (1980) proposed a complex model of PRL action involving in cGMP, phospholipase A_2 and polyamines.

In Nb_2 cells, which are dependent on PRL for proliferation (Shiu et al., 1983), G protein may mediate signal transduction by the PRL receptor (Larsen and Dufau, 1988; Barkey et al., 1988; Too et al., 1989). However, significance of G proteins on other tissues is unclear at present.

11. Purpose of this thesis

Unlike signal transduction systems of many other peptide hormones, components of a signal transduction system such as transducer, amplifier, second messenger and internal effector have not been identified for PRL. To address the question that PRL has many functions, it is important to reveal the signal transduction mechanism of PRL. As the first step, biochemical characterization and identification of PRL receptor are necessary. There are several problems that are unclear in the properties of PRL receptors as follows: a) structure of the receptors, b) types of the receptors in one tissue, c) relations of the receptors among tissues, d) the role of carbohydrate of the receptor on PRL binding.

To solve the above questions, I purified and characterized the receptor using MAbs. In Chapter 2, I produced MAbs recognizing PRL receptor in the rabbit mammary gland. First, I substantially purified the PRL receptor using

PRL-affinity chromatography. Using a purified receptor as the antigen, I could obtain four positive clones inhibiting the binding of PRL to mammary microsomes. To use MAbs as probes for further studies, basic properties of two MAbs were examined. In Chapter 2, I discuss that both MAbs recognize not only the receptor in the mammary gland, but also in the liver, adrenal gland, ovary and kidney. In Chapter 3, using two MAbs, I examined the immunological relations of receptors from several rabbit tissues by comparing the inhibition curves of PRL-binding to their microsome and solubilized receptors. In Chapter 4, in order to clarify the species of PRL receptor recognized by the MAbs, I performed immunoprecipitation and immunoblotting analysis using the receptor partially purified by PRL-affinity chromatography. Although I identified three species of PRL binding protein in the partially purified sample, a problem found in Chapter 4 was the low recovery of the receptor during purification. Furthermore, relationships of these PRL-binding species were uncovered. In Chapter 5, by making use of MAb-coupled sepharose, both purity and recovery increased. Using this purified sample, multi-forms of PRL binding protein could be identified. Furthermore, by peptide mapping analysis, I found that two of PRL-binding proteins were highly homologous. In addition, I estimated the $\underline{M}_r$ of the carbohydrate chains of the receptor by three types of glycosidase treatments. In Chapter 6, I studied the effect of deglycosylation of the purified receptor on PRL binding to its receptor. Furthermore, effectiveness of deglycosylation was assessed by immunoblotting.

CHAPTER 2

Production and Characterization of Monoclonal Antibodies Directed to Prolactin Receptor in the Rabbit Mammary Gland

SUMMARY

Prolactin (PRL) receptor in the rabbit mammary gland was purified by making use of a PRL-affinity column. Using purified receptor as an antigen, two monoclonal antibodies (MAbs) (C3 and F10) positive in inhibiting PRL binding to mammary microsome were obtained by the hybridoma technique. Basic properties of both MAbs were characterized as follows;

1) The recognition site of C3 (IgG_{2b}) seems to be near but not exactly the same region of PRL-binding site of the receptor.

2) The recognition site of F10 (IgG_1) seems to be the same as that of PRL.

3) Both MAbs recognize at least two types of PRL receptors in the mammary gland.

4) Both MAbs recognize not only the receptor in the mammary gland, but also that in liver, kidney, adrenal gland and ovary.

INTRODUCTION

Antireceptor antibodies have been utilized as a tool in analyzing structure and function of PRL receptor (review, Kelly et al., 1988). Antibodies recognizing PRL receptor acted as an agonist (Djiane et al., 1981; Rosa et al., 1982; Edery et al., 1983) or antagonist (Shiu and Friesen, 1976; Bohnet et al., 1978) of PRL, suggesting the importance of the PRL receptor as a signal transducer. However, polyclonal antibodies, produced using an incomplete pure antigen, may recognize not only an antigen but also other substances. It is unsuitable to apply a polyclonal antibody as a probe in immunoblot analysis or affinity chromatography. In contrast, mono-specific property of monoclonal antibodies (MAbs) is ideal for a probe in identifying a substance. Furthermore, MAbs can be produced even if the antigen is not pure. PRL or hGH affinity chromatography has been used in purifying the PRL receptor substantially (Shiu and Friesen, 1974b; Katoh et al., 1984, 1985a). The purified sample could be used as an antigen to produce antibodies. By this approach, MAbs directed against the receptor in the rabbit mammary gland and rat liver have been produced (Katoh et al., 1985b, 1987; Okamura et al., 1989b).

In this chapter, I also purified PRL receptor from rabbit mammary gland by PRL affinity chromatography. Using the purified sample as an antigen, MAbs recognizing PRL receptor in the rabbit mammary gland were produced. The characteristics of MAbs binding to mammary receptors were then examined. This is essential for the further characterization of the receptor such as identification and purification.

MATERIALS AND METHODS

Animals

Lactating New Zealand White rabbits were gifts from Dr. Johke, National Institute of Animal Industry. The in vivo desaturation was carried out in order to increase an apparent number of PRL receptors (Djiane et al., 1977). The animals were injected sc with 2 mg of bromocryptine mesylate (CB 154) dissolved in phosphate buffered saline (PBS) at 36, 24, and 12 h before the animals were sacrificed. Mammary glands were removed and processed immediately or stored at -80 ℃ .

Hormones and chemicals

Hormones used (ovine PRL, NIH-P-S 15, 30.5 i.u./mg, hGH, NIH-HS2160E, 1.7 i.u./mg) were gifts from the National Institute of Health, Bethesda, MD, U.S.A. 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (Chaps) were purchased from Wako Pure Chemical Industries, Osaka, Japan. Tris(hydroxymethyl)aminomethane (Tris), mouse immunoglobulin G (nIgG), lactoperoxidase (bovine milk) and CB 154 were from Sigma Chemical Co, St. Louis, MO, U.S.A. Poly(ethylene glycol), phenylmethanesulphonyl fluoride (PMSF) and bovine γ -globulin were from Nacalai Tesque, Inc., Kyoto, Japan. Sodium ^{125}iodine was from New England Nuclear, Boston, Mass, U.S.A. The N-hydroxysuccinimide ester of 3,3'-diaminodipropylaminosuccinyl-agarose (Affi-Gel 10) and Affi-Gel MAPS II kit were from Bio-Rad Laboratories, Richmond, CA, U.S.A. RPMI 1640 medium and fetal calf serum were from GIBCO, Grand Island, NY, U.S.A. HB 102 medium was from Hana Biologicals, Berkeley, CA, U.S.A. All other chemicals were of analytical grade.

Preparation of crude membrane fractions (microsomes)

Microsomes were prepared by the method of Shiu et al. (1973) with a minor modification. Mammary glands obtained from rabbits were rinsed in ice-cold PBS and cut into smaller pieces. The tissue was homogenized in 5 volumes of 0.25 M sucrose with a Polytron mixer on ice for 30 seconds five times. The homogenate was filtered through cheesecloth. The filtrate was centrifuged at 10,000g for 10 min at 4 ℃ . The supernatant was centrifuged at 105,000g for 60 min at 4 ℃ . The pellet (microsome) was suspended with 0.025 M Tris-HCl (pH 7.4), containing 10 mM $MgCl_2$ and 1 mM PMSF (TMP buffer) and stored at -80 ℃ until use.

Solubilization of the microsomes by Chaps

Solubilization of the microsomes with Chaps was carried out as follows (Sakai et al., 1986). The protein concentration of the microsome was adjusted to 10 mg/ml and treated with 1 mM Chaps for 30 min at room temperature with stirring. The sample was centrifuged at 100,000g for 70 min at 4 ℃ and the supernatant was discarded, since this fraction had no PRL binding activity. The pellet was resuspended in TMP buffer containing 7.5 mM Chaps and treated again according to the same procedure described above. After centrifugation, the turbid layer at the top of the supernatant was carefully removed and the remaining supernatant was collected and stored at -80 ℃ until use.

Purification of PRL receptors by PRL affinity chromatography

The preparation of affinity column and the purification of PRL receptors were carried out by the method of Sakai et al. (1985b). Twenty mg of PRL, dissolved in 10 ml of 0.1 M $NaHCO_3$ (pH 8.3), was added to 90 ml of 0.1 M $NaHCO_3$ containing 2 g of Affi-Gel 10 and incubated overnight at 4 ℃ under

constant shaking. The coupling reaction was terminated by the addition of 6 ml of 1 M glycine dissolved in 0.1 M $NaHCO_3$. Unbound PRL was removed by repetitive washing in 8 M urea and 4 M guanidine hydrochloride. Coupling efficiency, which was monitored by ^{125}I-PRL included in the reaction mixture, was about 85 %.

The concentration of Chaps in the Chaps extract was adjusted to 5 mM with TMP buffer. The diluted extract was allowed to run through 40 ml of the Affi-Gel 10 coupled to PRL (column diameter, 3 cm) at room temperature with a flow rate of 35-40 ml/h. The Affi-Gel column was washed with following solutions at maximum flow rate; 10 bed volume of 0.1 M borate buffer (pH 7.4) containing 1 mM Chaps (borate/Chaps), 1 bed volume of 4 M urea in borate/Chaps and 4 bed volume of borate/Chaps. Elution was achieved with 1 bed volume of 4 M $MgCl_2$. The eluates were dialyzed against 10 mM borate buffer (pH 7.4) containing 0.1 mM Chaps and lyophilized.

MAb production

All procedures for MAb production were performed according to the guidelines of the AMBO international training course at Chiba University, Japan (1981). About 1 pmol of affinity-purified PRL receptors was emulsified in a complete Freund's adjuvant. BALB/c mice (6 weeks old, female) were given sc injections of 0.2 ml at 2 week intervals, followed by an iv boost of PRL receptors in normal saline 2 weeks later. Three days postboost, spleens from four mice were pooled and passed through stainless-steel mesh (No. 200) for dispersion into single cells. Spleen cells (10^8 cells) were mixed with 5×10^7 cells of myeloma cells (P3X63 Ag8.653, gifts from Department of Immunology, Faculty of Medicine, University of Tokyo, Japan) and pelleted by

centrifugation at 400g. The cells were then resuspended in 1 ml of pre-warmed (37 ℃) 50 % polyethylene glycol 1500 (BDH Chemical Co., Poole, England) by gentle shaking, followed by the drop-wise addition of 1 ml of serum-free RPMI 1640. The cells were further diluted with RPMI 1640 and centrifuged at 200g for 5 min. The cell pellet was gently resuspended with warm medium containing 10 % fetal calf serum. One hundred $\mu\ell$ of cell suspension was distributed to each well of a 96-well tissue culture plate. Hybrids were cultured in HAT medium (1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine) in RPMI 1640 containing 10 % fetal calf serum for 10 days.

Screening and cloning of hybridomas

After HAT selection of hybridomas, supernatants from the wells containing hybridoma were assayed for anti-PRL receptor activity using the inhibition of ^{125}I-hGH binding to rabbit mammary microsomes as follows. Fifty $\mu\ell$ of culture supernatants were incubated with 50 μg of microsomal receptors in TMP buffer containing 0.2 % BSA (TMPB buffer) in the presence of ^{125}I-hGH (see below, Binding assay section). Four clones positive in inhibiting of ^{125}I-hGH binding to its receptors were obtained. Each hybridoma was cloned twice and grown in serum-free HB102 medium.

Purification and characterization of MAbs

Hybridoma cells (10^7) from two clones (C3 and F10) were injected to BALB/c mice (6 weeks old, female) primed with Pristane. Antibodies were collected from both ascites fluid and supernatants from hybridoma cultures in serum-free medium. Culture supernatants were dialyzed against 10 mM

sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl. The culture supernatants were concentrated from 1,000 ml to 50 ml using a speed-evaporator. Twenty ml of the ascites fluid and 50 ml of the concentrated culture supernatants were fractionated by 50 % saturation of ammonium sulfate and further purified by the Affi-Gel Protein A MAPS II kit. After dialysis against 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, the samples were stored at 4 ℃ in the presence of 0.05 % NaN_3 or stored at -50 ℃ . The final yield of the MAbs (C3 and F10) from the ascites fluid (20 ml) or culture supernatants (1,000 ml) was 60-80 or 8-15 mg, respectively. The subclass of MAb was determined using a Serotec mouse monoclonal typing kit (Serotec, Bicester, UK).

Protein concentration

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard. The sample (0.1 ml) was mixed with 1 ml of 0.01 % $CuSO_4$-$5H_2O$, 0.02 % $Na_2C_4H_4O_6$-$2H_2O$, 2 % Na_2CO_3 and 0.4 % NaOH. After 20 min incubation at room temperature, 0.1 ml of Phenol reagent was added to the mixture. After 20 min incubation, the absorbance was measured with a spectrophotometer at 660 nm.

Iodination of hormones and antibodies

Iodination of PRL or hGH was performed by the method of Sakai et al. (1975). The reactants were added in the following order and amounts at room temperature. (1) 300 μCi of $Na^{125}I$ in 10 μl of 0.1 N NaOH, (2) 25 μl of 0.5 M phosphate buffer (pH 6.9), (3) 5 μl of PRL or hGH (1 mg/ml) in 0.05 M phosphate buffer (pH 7.4), (4) 5 μl of lactoperoxidase (1 mg/ml) in 0.05 M phosphate

buffer (pH 7.4). Five $\mu\ell$ of 0.00025 % hydrogen peroxide was added 5 times to the reaction mixture at a 90-second interval and the reaction was continued for 10 min. The mixture was diluted with 500 $\mu\ell$ of 0.05 M phosphate buffer (pH 7.4) and applied on a Sephadex G-75 column (1× 17 cm), previously washed with 1 ml of 4 % BSA in 0.05 M phosphate buffer (pH 7.4). Fractions of ^{125}I-PRL were collected in tubes containing 0.2 ml of 4 % BSA. Un-reacted Na^{125}I was removed. The radioactivity was determined in an Aloka γ -radiation spectrometer with a counting efficiency of 50 %. MAb was iodinated in a manner similar to that for hormones. Specific activity of iodinated hormone or MAb, calculated by the method of Hunter and Greenwood (1962), was 2.6-3.2 or 1.5-1.9 MBq/μg, respectively.

Binding assay

Binding assay was carried out by the method of Sakai et al. (1986). Microsomes diluted with TMP buffer containing 0.2 % BSA (TMPB buffer) was incubated with 30,000 cpm of ^{125}I-PRL or 100,000 cpm of ^{125}I-MAb for 14-16 h at room temperature. Nonspecific binding was determined in the presence of 1 μg of unlabeled PRL or 15 μg of unlabeled MAb, respectively. The total volume was adjusted to 0.5 ml with TMPB buffer. After incubation, 2.5 ml of TMPB buffer was added and the tubes were centrifuged at 2,300g for 20 min. After discarding the supernatant, the radioactivity in the pellet was determined. Specific binding was determined by the difference between total binding and nonspecific binding.

Scatchard analysis

Microsomes were incubated with 30,000 cpm of ^{125}I-PRL or 100,000 cpm of

^{125}I-MAb in the presence of various amounts of unlabeled PRL or MAb. The dissociation constant and the number of binding sites were calculated according to the procedure of Scatchard (1949).

RESULTS

To prepare the antigen, PRL receptors in the rabbit mammary gland were purified by PRL affinity chromatography. The binding capacity of the purified sample was of 41.4 pmol/mg and the dissociation constant was of 1.2×10^{-10} M. The recovery was below 5 %.

Partially purified receptor was injected into mice and spleen cells were fused to myeloma cells. After HAT selection and limiting dilution of hybridomas, four clones positive in inhibiting ^{125}I-PRL binding to the rabbit mammary microsome were obtained. Two clones (C3 and F10) of them were used and characterized for the further study. MAbs in ascites fluids and culture supernatants were precipitated by 50 % saturation of ammonium sulfate and further purified by a Bio-Rad Affi-Gel Protein A MAPS II kit. The M_r and the purity of IgG, estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and Coomassie blue staining, were 150,000 and greater than 95 %. Typing analysis showed that subclass of C3 or F10 was IgG_{2b} or IgG_1, respectively. The MAbs purified from ascites fluids showed the same character as to those from culture supernatants, by the criteria of anti-PRL receptor activity and the M_r on SDS gels. When MAbs were incubated with ^{125}I-PRL, the PEG-precipitated MAb did not contain any radioactivity of labeled PRL, showing that both MAbs did not cross-react with PRL.

To elucidate the binding characteristics of MAbs, effect of MAbs or PRL

on ^{125}I-PRL binding to mammary microsomes was examined (Fig. 1). Both MAbs inhibited the ^{125}I-PRL binding to its receptor dose dependently, but in non-parallel fashion. At higher concentrations, the MAbs (>2.5 nM) and PRL (> 0.5 nM) completely inhibited the ^{125}I-PRL binding to its receptor. The potency of inhibition of ^{125}I-PRL binding was PRL > C3 > F10, when compared at 50 % inhibition of specific binding. The binding of ^{125}I-PRL to its receptors were not interfered with nIgG.

Effect of MAbs or PRL on ^{125}I-MAb binding to mammary microsomes was examined (Fig 2). As shown in Fig. 2-(a), C3 inhibited the binding of ^{125}I-C3 in a dose-related manner. At higher concentrations (> 500 nM), ^{125}I-C3 binding was completely displaced by unlabeled C3. F10 at lower concentrations (< 100 nM) did not interfere with the binding of ^{125}I-C3. PRL at lower concentrations (< 1 nM) inhibited ^{125}I-C3 binding and at higher concentrations (>1 nM) did not induce further displacement. In Fig 2-(b), PRL and unlabeled F10 displaced ^{125}I-F10 binding with almost equal potency, but C3 inhibited ^{125}I-F10 binding at only higher concentrations (> 100 nM). Scatchard analysis of MAb binding in Fig. 2 was performed (Fig. 3). Both plot lines by C3 and F10 showed typical curves.

Effect of time and temperature on the association of ^{125}I-MAb was examined (Fig. 4). The binding of ^{125}I-C3 occurred rapidly within a short period (1 h) and increased slowly hereafter at 4 , 23 or 37 ℃ . After 16 h incubation, the large amount of ^{125}I-C3 binding was obtained by incubation at 4 ℃ . The binding of ^{125}I-F10 was time and temperature dependent. The binding was almost equilibrated at the end of 8 h incubation either at 23 ℃ or 37 ℃ . The binding was slow and small at 4 ℃ .

Effect of time and temperature on the dissociation of ^{125}I-MAb was

examined (Fig. 5). The dissociation of ^{125}I-C3 or -F10 was time and temperature dependent. After 1 h, 50 % dissociation of ^{125}I-C3 was observed at 37 ℃ . At 23 ℃ , it took 16 h to attain 50 % dissociation of ^{125}I-C3. At 4 ℃ , dissociation of ^{125}I-C3 was small. After 4 h, 40 % dissociation of ^{125}I-F10 was observed at 37 ℃ , but at 23 ℃ or 4 ℃ dissociation was small.

Effect of various reagents on ^{125}I-MAb dissociation at 4 ℃ was examined (Table 1). Dissociation of ^{125}I-C3 was enhanced by various chaotropic reagents or low pH. In particular, substantial dissociation of ^{125}I-C3 was observed in 1 M NaI and 4 M $MgCl_2$. In contrast, dissociation of ^{125}I-F10 was observed only in 4 M $MgCl_2$ but not greatly affected by other chaotropic reagents or low pH.

^{125}I-MAb binding to various tissues was examined (Fig 6). Specific ^{125}I-MAb binding to various tissues was observed in five tissues examined, irrespective of C3 or F10. Mammary gland or adrenal gland had higher binding activity per mg of membrane protein than liver, kidney or ovary did.

DISCUSSION

It is theoretically expected that if an antigen is not pure, MAb can be obtained. In this study, PRL receptor in the rabbit mammary gland was purified using PRL affinity chromatography. The binding capacity obtained was 44.4 pmol/mg protein. Assuming that the Mr of the receptor is 40,000-300,000 and that PRL binds to its receptor in the molar ratio of 1 : 1, the purity was estimated to be of 0.2-1.5 %. In spite of the low purity, four MAbs recognizing PRL receptor were successfully obtained. Two (C3 and F10) of them were characterized and used in this study.

Competitive binding study revealed binding site and specificity of the

two MAbs as follows. F10 seems to recognize PRL-binding region of the receptor, because 1) F10 completely inhibited ^{125}I-PRL binding to its receptor and 2) PRL completely blocked ^{125}I-F10 binding almost equal potency with unlabeled F10. In contrast, the recognition site of C3 seems to be near but not exactly the same region of PRL or F10, because 1) C3 completely inhibited ^{125}I-PRL binding but 2) PRL induced only partial inhibition of ^{125}I-C3 binding and F10 did not affect ^{125}I-C3 binding. The incomplete inhibition of ^{125}I-C3 binding by PRL may be caused by a change in the affinity of PRL receptors rather than the simple competition with PRL for PRL binding sites. Similar incomplete inhibition of ^{125}I-MAb binding by PRL were reported in other MAbs directed to PRL receptors (Katoh et al., 1985b, 1987; Okamura et al., 1989b).

Scatchard analysis of ^{125}I-MAbs (C3 and F10) binding to the microsome or nitrocellulose-immobilized receptor (Sakai and Murakami, 1987) produced a curvilinear line, suggesting that rabbit mammary gland contains at least two types (a high and low affinity for MAb binding) of PRL receptors. Katoh et al. (1985b) reported one linear and two curvilinear Scatchard plots using MAbs specific to the rabbit mammary gland PRL receptor. It is generally accepted that Scatchard plotting of PRL binding to the mammary microsome generates a linear regression line. The mammary gland contains two types of PRL receptors (Sakai and Ike, 1987). Both receptors have the same order of dissociation constant for PRL binding. It seems that MAb binds to each PRL-binding species of protein with greatly different affinity.

Another important finding in this study is that both MAbs recognize PRL receptors in liver, kidney, adrenal gland and ovary in addition to mammary gland. Although the number of MAb binding sites was not calculated, the percentage of ^{125}I-MAb specific binding to various tissues was comparable

to that of ^{125}I-PRL binding (Shiu and Friesen, 1974a). Immunological study suggests that the common structures of PRL receptors were maintained among tissues.

The data on association and dissociation of ^{125}I-MAbs are useful when determining the conditions such as immunoprecipitation, immunoblot and immuno-affinity chromatography. In particular, 1 M NaI will be used as a dissociating reagent of the receptor from C3-affinity matrix.

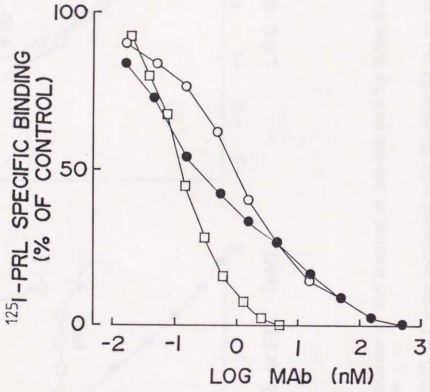

Fig. 1. Displacement curves of the binding of labeled PRL to mammary microsomes by PRL or MAbs

Mammary microsomes (80 μg) were incubated with ^{125}I-PRL (30,000 cpm) in the absence or presence of various concentrations of unlabeled PRL (□), MAb C3 (●) or F10 (○) for 15 h at room temperature. Results were compared to specific binding of ^{125}I-PRL. Radioactivity of specific (and nonspecific) binding were 15.3± 0.7% (1.3± 0.1%) of total radioactivity added. Data are the means of six experiments. Standard deviations were less than 4 % of the mean.

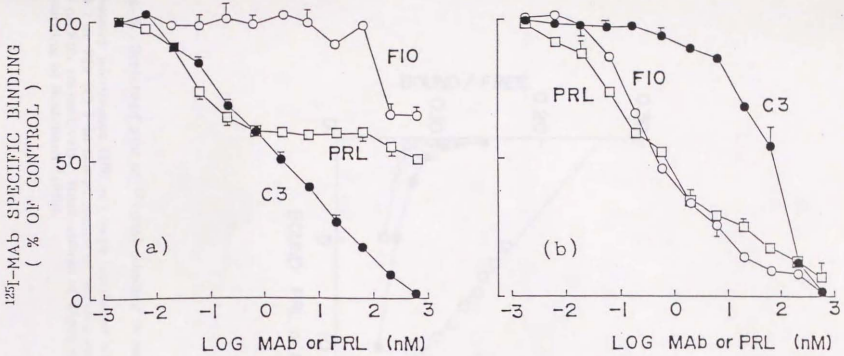

Fig. 2. Displacement curves of the binding of labeled MAb by MAbs with various concentrations

Mammary microsomes (160 μg) were incubated with ^{125}I-MAb C3 (a) or F10 (b) (100,000 cpm) in the absence or presence of various concentrations of unlabeled PRL (□), C3 (●) or F10 (○). Results were compared to specific binding of ^{125}I-MAb: radioactivity of specific (and nonspecific) binding were 12.7% (2.7%) for C3 and 5.2% (4.1%) for F10 of total radioactivity added, respectively. Values are the mean ± S.D. (n=6).

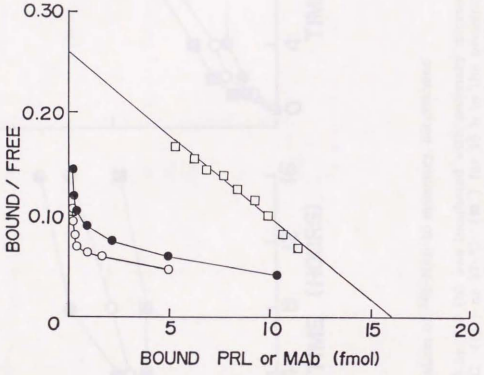

Fig. 3. Scatchard plot of ^{125}I-MAb binding to mammary microsomes

Mammary microsomes (100 μg) were incubated with ^{125}I-PRL (□), ^{125}I-MAb C3 (●) or F10 (○) in the presence of various amounts of unlabeled PRL, MAb C3 or F10, respectively. Mean values of three experiments were plotted according to Scatchard (1949).

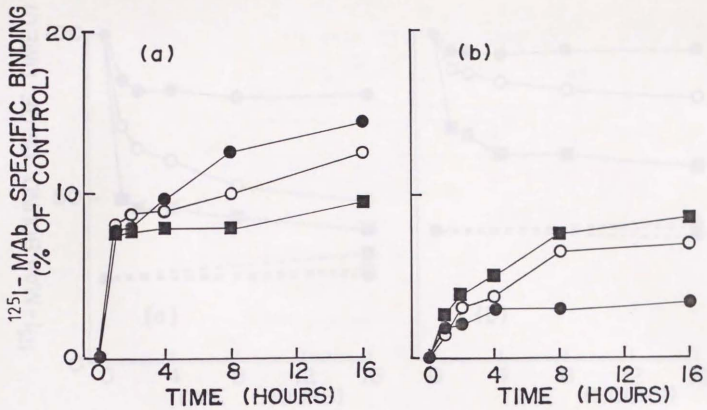

Fig. 4. Association of ^{125}I-MAb to mammary microsomes

^{125}I-MAb C3 (a) or F10 (b) was incubated with mammary microsomes (160 μg) at 4 ℃ (●), 23 ℃ (○) or 37 ℃ (■) for 15 h in the presence or absence of 15 μg of unlabeled MAb. After incubation at various periods, 2.5 ml of assay buffer was added and centrifuged. Values are expressed as a percentage of total radioactivity added.

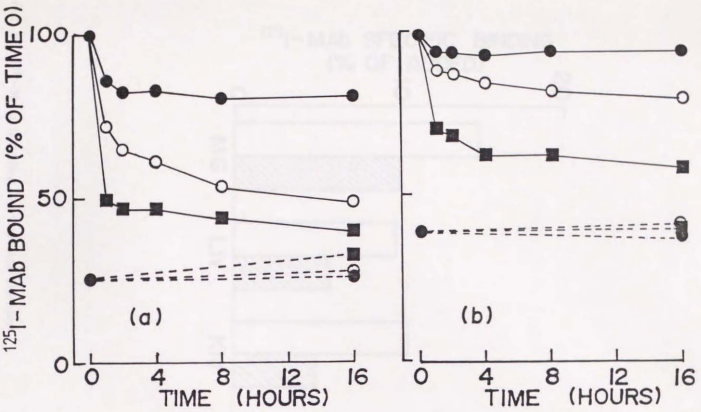

Fig. 5. Dissociation of ^{125}I-MAb from mammary microsomes

After incubation for 15 h at room temperature in the absence (—) or presence (---) of 15 μg of unlabeled MAb, MAb C3 (a) or F10 (b) was allowed to dissociate from receptors by adding 2.5 ml of assay buffer containing 45 μg of MAb at 4 °C (●), 23 °C (○) or 37 °C (■). Dissociation was continued at various times. Values are expressed as a percentage of total binding at time=0.

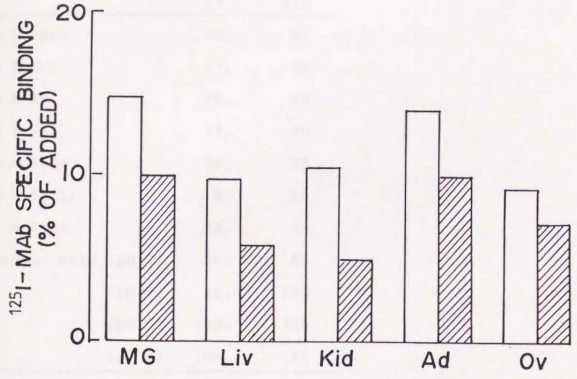

Fig. 6. ^{125}I-MAb binding to various tissues

^{125}I-MAb C3 (□) or F10 (▨) was incubated with microsomes of mammary gland (MG, 160 μg), liver (Liv, 500 μg), kidney (Kid, 1000 μg), adrenal gland (Ad, 160 μg) or ovary (Ov, 400 μg) for 15 h at room temperature in the presence or absence of 15 μg of unlabeled MAb. Values are expressed as a percentage of total radioactivity added.

Table 1. Dissociation of ^{125}I-MAb from mammary microsome with various agents.

	C3	F10
1 M NaCl	50,	84
1 M KCl	53,	87
1 M KF	76,	90
1 M NaI	17,	70
1 M NaBr	38,	76
4 M $MgCl_2$	15,	14
4 M Urea	33,	48
Acetic acid (pH 3)	36,	80
(pH 4)	66,	100
(pH 5)	100,	100
(pH 6)	100,	92

^{125}I-MAb (100,000 cpm) was incubated with mammary microsomes for 15 h at room temperature in the presence or absence of 15 μg of unlabeled MAb. After removal of free MAb by centrifugation, 1 ml of various reagents was added and further incubated at 4 ℃ for 30 min. After incubation, 2 ml of assay buffer was added and centrifuged. Values are expressed as a percentage of control (assay buffer) binding.

CHAPTER 3

Immunological Relations of Prolactin Receptors among Rabbit Tissues

SUMMARY

The heterogeneity of the prolactin (PRL) receptor in the rabbit adrenal gland, kidney, liver, mammary gland and ovary was examined using the different type of monoclonal antibody (MAb, C3 and F10) raised against the rabbit mammary gland PRL receptor. Both MAbs inhibited dose dependently the binding of PRL to its microsome receptor in all tissues examined. However, the inhibition curves obtained by C3 or F10 were significantly different among tissues. The tissue specific difference was clearly observed when a MAb C3 was used as a competitor. After solubilization with a zwitterionic detergent Chaps, PRL receptors were similarly analyzed. Even though microsome PRL receptors showed the heterogeneity to MAb, the heterogeneity was no longer observed after solubilization. The results suggested that the heterogeneity is due to the conformational difference of the microsome PRL receptor among tissues.

INTRODUCTION

Like other peptide hormones, prolactin (PRL) initiates its action by binding to its membrane receptor in the target organ. PRL exerts a wide variety of function on various tissues. The existence of PRL receptors has been reported in adrenal gland, chorion laeve, choriod plexus, epididymis, hypothalamus, kidney, liver, lymphoid tissue, mammary gland, ovary, pituitary, prostate, seminal vesicle, testis, uterus and some tumors (review, Kelly et al. 1988).

Antireceptor antibodies have commonly been used as probes of receptor structure and function (Fraser and Lindstrom, 1984). Using antibodies, tissue specific variations of some hormone receptors have been demonstrated in the PRL receptor (Waters et al., 1984), growth hormone receptor (Barnard et al., 1985; Thomas et al., 1987) and insulin receptor (Caro et al., 1988). The variation appeared to be due to the difference in the structure of receptor present in the microsome fraction, since it has been demonstrated that only one type of growth hormone receptor (Spencer et al., 1988) or insulin receptor (Fujita-Yamaguchi et al., 1979) has been identified. However, the rabbit mammary gland has two species of PRL receptors (Sakai and Ike, 1987). It is unclear at present whether the tissue specific variation is due to the difference in the receptor species or the structure. It is expected that by solubilization of membrane receptor, the receptor restores the original form. This will allow for me to examine the immunological properties of the solubilized receptors. In this study, I examined the binding of PRL to its receptor in the presence of various concentrations of MAb, and the inhibition curves were compared between the membrane-bound and the solubilized PRL receptors in some selected tissues.

MATERIALS AND METHODS

Hormones and other chemicals

Ovine PRL (NIADDK-oPRL-15) and bovine growth hormone (GH) (NIH-GH -B3) were gifts from NIADDK, Bethesda, MD. All other chemicals were of analytical grade.

Iodination of hormones and MAbs

The production of the MAbs (C3 and F10) raised against rabbit mammary gland PRL receptors has been described in Chapter 2. Radioiodination of PRL was performed by the lactoperoxidase method (Sakai et al., 1975). The specific radioactivity of PRL was 2.8-3.1 MBq/μg.

Tissue preparation

Adrenal glands, kidneys, livers, ovaries, and mammary glands were obtained from mid-lactating New Zealand White rabbits. Crude membrane fractions (microsomes) were collected by differential centrifugation and solubilized with 7.5 mM Chaps as described in Chapter 2. Some preparations of the liver were pre-treated with 250 ng/ml of bovine GH for 24 h at 4 ℃ in order to saturate the GH receptor with GH (GH saturated liver).

Binding studies

The conditions of the competitive binding studies have been described in Chapter 2. In brief, microsomes were incubated with ^{125}I-PRL (30,000 cpm) in the presence of various dilutions of MAbs for 16 h at room temperature. The binding buffer consisted of 25 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM phenylmethanesulphonyl fluoride and 0.2 % bovine serum albumin. At the end of incubation, the reaction mixture was diluted with 2.5 ml of the binding buffer and microsomes were precipitated by centrifugation at 2,000g for 20

min. For the solubilized receptors, 5 mM Chaps were included in the reaction mixture. When the reaction was terminated, the reaction mixture was mixed with poly(ethylene glycol). The PRL- or MAb-receptor complexes with carrier γ-globulin were precipitated by centrifugation. Non-specific binding was determined by the presence of 1,000-fold excess of unlabeled PRL.

Statistical analysis

Statistical significance was determined using a two factor analysis of variance (ANOVA). Differences were considered to be significant at $P < 0.05$.

RESULTS

The dissociation constant of the binding of PRL to its receptor was determined by the Scatchard analysis (1949). The dissociation constants to the microsome receptor in all tissues examined varied within the range of 1.1-1.8$\times 10^{-10}$ M. The differences among tissues were small and insignificant. After the solubilization, the dissociation constants were decreased to be 5.0-6.6$\times 10^{-11}$ M. The differences among tissues were insignificant. In the following experiments, the concentration of receptor was adjusted to 10 fmol/tube.

The PRL-binding assay in the presence of various concentrations of MAb was carried out in order to examine the effect of C3 or F10 on the binding of ^{125}I-PRL to the microsome or solubilized receptor. The displacement curves of PRL binding to its receptors by C3 and F10 are shown in Fig.1 and 2, respectively. Both MAbs were able to inhibit the binding of PRL to the receptor dose dependently at lower concentrations and completely at higher concentrations. In the liver, however, both MAbs failed to induce complete displacement of PRL binding. By preincubation of the liver preparations with

bovine GH, both MAbs could inhibit the binding of PRL completely. The effect of C3 on PRL binding to microsome receptor was examined. As shown in Fig.1a, it is apparent that the displacement curves by C3 were different among the tissues ($P<0.05$), whereas those of the adrenal gland and ovary were identical. Fig. 1b shows the effect of C3 on ^{125}I-PRL binding to solubilized receptors in various tissues. In contrast to microsome receptors, the displacement curves were almost parallel. C3 showed the same binding character to the solubilized receptors, while those in the receptors from adrenal gland and kidney were significantly different ($P<0.05$).

The effect of F10 on ^{125}I-PRL binding to microsomal receptors was examined (Fig.2a). The displacement curves by F10 were significantly different among tissues ($P<0.05$) whereas the curves were not different between GH saturated liver and mammary gland or between GH saturated liver and ovary. Fig. 2b shows the effect of F10 on ^{125}I-PRL binding to solubilized receptors. As was the case with C3, the displacement curves by F10 were similar among the tissues although that of kidney was significantly different from those in the other tissues ($P<0.01$).

DISCUSSION

The tissue specific inhibition of a hormone binding by antibodies has been reported in the insulin receptor (Caro et al., 1988), PRL receptor (Waters et al., 1984) or GH receptor (Barnard et al., 1985; Thomas et al., 1987). I obtained the similar results using our MAbs and the microsome PRL receptor, and confirmed the findings of Waters et al. (1984). The effect of C3 on the binding of PRL to its receptor in various tissues was not equivalent to that of F10, suggesting that, in membrane PRL receptor, the structure of a binding

site for C3 was more variable than that for F10. It has been known that the dissociation constant of PRL binding to its receptor was decreased by 2- to 6-fold after solubilization (Shiu and Friesen, 1974b, Waters et al., 1984, Katoh et al., 1985b, Sakai et al., 1986). As shown here, the tissue-specific variation was almost disappeared except for the kidney. The solubilized receptor had the same binding affinity for C3 as well as for F10. From the cDNA analysis, extracellular domain of PRL receptor has the same primary structure between liver and ovary (Boutin et al., 1988; Zhang et al., 1990; Shirota et al., 1990). These facts strongly suggested that the tissue-specific variation was due to the difference in the conformation of the receptor depending on the tissues.

In this study, I showed that if the solubilized receptor had the same characters for MAb binding, the structure of membrane receptor might be varied depending on the difference in tissues. The tissue specific variation may be important in mediating a wide variety of PRL action. To elucidate the functional and structural relations of PRL receptors, efforts are current underway with MAbs.

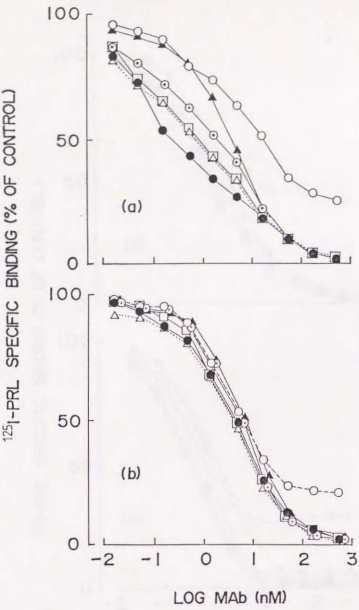

Fig. 1. Competition curves of specific ^{125}I-PRL binding to its receptors by MAb C3

Microsomes (a) or Chaps extracts (b) were incubated with ^{125}I-PRL (30,000 cpm) in the presence of increasing concentrations of C3 and the amount of specific binding of ^{125}I-PRL was determined. The receptor concentration was adjusted to 10 fmol receptor/tube (the amount of protein used were: In microsome form, mammary gland (●), 80 μg; liver (○) and GH saturated liver (◉), 500 μg; adrenal gland (△), 87 μg; ovary (□) 433 μg; kidney (▲), 983 μg. In the Chaps solubilized form, mammary gland 20 μg; liver and GH saturated liver, 250 μg; adrenal gland, 17 μg; ovary, 48 μg and kidney 247 μg). Data are the means of six experiments. Standard deviations were all less than 4 % of the mean and were not shown.

Significant differences (P values, ANOVA) are as follows: Microsomal receptors: mammary vs. GH saturated liver, adrenal, ovary, < 0.05 ; mammary vs. kidney < 0.01; GH saturated liver vs. adrenal, ovary < 0.05; GH saturated liver vs. kidney < 0.01; adrenal vs. ovary not significant (NS); adrenal vs. kidney < 0.01; ovary vs. kidney < 0.01: Solubilized receptors: adrenal vs. kidney < 0.05; mammary vs. GH saturated liver vs. adrenal vs. ovary vs. kidney NS.

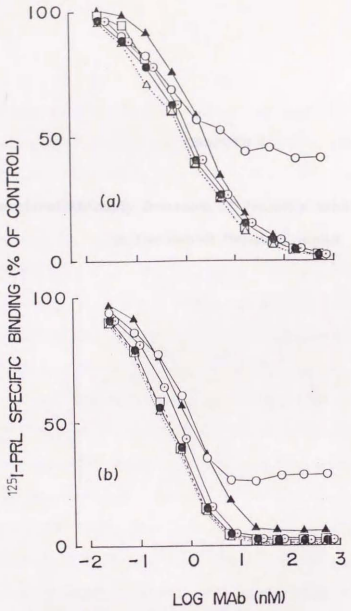

Fig. 2. Competition curves of specific ^{125}I-PRL binding to its receptors by MAb F10

Microsomes (a) or Chaps extract (b) from the various tissues were incubated with ^{125}I-PRL in the absence or presence of increasing concentration of MAb F10. Conditions in these figures are the same as those in Fig. 2. See the footnote in Fig. 2.

Significant differences (P value, ANOVA) are as follows: Microsome: mammary vs. GH saturated liver NS; mammary vs. adrenal, ovary, kidney < 0.01; GH saturated liver vs. adrenal < 0.01, GH saturated liver vs. ovary NS; GH saturated liver vs. kidney < 0.05; adrenal vs. ovary, kidney < 0.01; ovary vs. kidney < 0.05: Solubilized receptors: mammary vs. GH saturated liver vs. adrenal vs. ovary NS; kidney vs. mammary, GH saturated liver, adrenal, ovary < 0.01.

CHAPTER 4

Monoclonal Antibody Detection of Prolactin Binding Proteins in the Rabbit Mammary Gland

SUMMARY

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 higher concentration. Using the receptor directly labeled 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 labeling of the receptor with labeled 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 species. 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 species react with one PRL molecule.

INTRODUCTION

Prolactin (PRL) plays a key role in regulation of the mammary gland function. Like other peptide hormones, PRL binds to its specific receptor on the membrane of target cells. Both polyclonal and monoclonal antibodies raised against PRL receptor act as an agonist or an antagonist of PRL in the mammary gland (Shiu and Friesen, 1976; Djiane et al., 1981, 1985). Curvilinear Scatchard plots of monoclonal antibody (MAb) binding to PRL receptors were reported (Katoh et al., 1985b) and in Chapter 2. Sakai et al. (1986, 1987) 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. 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 labeled 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. I prepared a MAb specific to the rabbit mammary gland PRL receptor and characterized the binding specificity to the receptor, as described in Chapter 2. I used the two receptor-specific MAbs to examine directly the species of the receptor and the M_r of the PRL binding proteins, 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 and Schull 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 and SDS-PAGE M_r standards were purchased from Bio-Rad. 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 described in Chapter 2. In brief, the microsomes (100,000g, 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 Chaps, 4 M urea and borate buffer/Chaps. The receptors were eluted with 4 M $MgCl_2$. The eluates were dialyzed against 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 H_2O_2 as described in

Chapter 2. The specific radioactivity of PRL was 2.8-3.1 MBq/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 0.4-0.9 MBq/μg.

Inhibition of PRL binding by MAb

For particulate or soluble receptors, the receptors were incubated with 30,000 cpm of ^{125}I-PRL in the presence of various concentrations of unlabeled 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 $MgCl_2$, 1 mM phenylmethanesulphonyl fluoride, and 0.2 % bovine serum albumin. After incubation, the particulate receptors were precipitated by centrifugation as described in Chapter 2. For solubilized receptors, 5 mM Chaps were included in a reaction mixture, and the receptors with carrier γ -globulin were precipitated by poly(ethylene glycol) (PEG) as described in Chapter 3. The radioactivity in the precipitants was measured in a gamma counter. Nonspecific binding was determined by the presence of a 1000-fold excess of unlabeled PRL. The difference between the radioactivity in the presence and absence of excess unlabeled 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. Radiolabeled receptors (1.5×10^6 cpm) were preincubated with Pansorbin for 15 min at room temperature. Pansorbin was removed by 3 min of centrifugation at 12,000g. 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 incubation, 50 μl of Pansorbin which had been pre-treated with the unlabeled 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 ℃ , 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 (DTT). After removal of insoluble materials, the samples were analyzed by SDS-PAGE. The gels were stained with 0.2 % Coomassie Brilliant Blue, destained in acetic acid/ methanol/ water (10/25/65), dried at 70 ℃ under vacuum and exposed to Eastman Kodak X-Omat AR-5 films together with Dupon Cronex Lightning Plus intensifying screens at -80 ℃ . 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 analyzed by SDS-PAGE, proteins were transferred electrophoretically at 40 V for 2.5 h onto nitrocellulose membrane filters at 5 ℃ . The transfer buffer consisted of 25 mM Tris, 192 mM glycine, pH 8.3, and 20 % (v/v) methanol (Towbin et al., 1979). The lane containing SDS-PAGE M_r markers was cut out and stained with 1 % Amido Black 10 B dissolved in 7 % acetic acid. PRL receptors were detected using a Bio-Rad Immun-Blot assay kit according to the manufacture'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 labeled PRL

Cross-linking of the receptor with PRL was performed according to the method by Sakai and Ike (1987). Affinity-purified receptors (10 μg) were incubated with ^{125}I-PRL (1.5×10^5 cpm) in the presence or absence of a 1,000-fold excess of unlabeled PRL in the total volume of 0.3 ml for 16 h at room temperature. At the end of incubation, 0.2 ml of 0.25 mg/ml gamma globulin solution dissolved in 50 mM phosphate buffer (pH 7.4) and 0.5 ml of 40 % PEG solution dissolved in distilled water were added and the tubes were centrifuged at 12,000g for 15 min. The tubes were then washed once with a 20 % solution of PEG. The precipitants were dissolved in 0.1 ml of 10 mM phosphate buffer (pH 7.4) containing 7.5 mM Chaps. The cross-linking reagent DSS was dissolved in dimethyl sulfoxide and prepared 7.5 mM solution. The ^{125}I-PRL-receptor complex were then affinity-labeled using DSS at a final concentration of 0.75 mM for 15 min on ice. The samples were denatured in the presence of 2 % SDS and 100 mM DTT for 5 min in boiling water. The samples were analyzed by 10 % SDS-PAGE and autoradiography as described above.

RESULTS

Two different types of MAbs (C3 and F10) positive in inhibiting ^{125}I-PRL binding to rabbit mammary microsome were used. The PRL receptors were

purified approx. 130-fold, and recovery of the PRL binding activity was less than 5 %. The receptor concentration was 60.0 pmol/mg.

As shown in Fig. 1, both C3 and F10 inhibited ^{125}I-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 higher concentrations, both MAbs completely inhibited the binding of ^{125}I-PRL to its receptors. C3 was more potent in inhibiting activity of PRL binding to microsomes, but less potent for the affinity-purified receptors than F10. The binding of ^{125}I-PRL to the receptors was not interfered with by the presence of normal IgG.

To examine the molecular structure of the affinity-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 labeled PRL receptors has at least nine bands. Among them, three species specifically cross-reacted with MAbs. Under reducing conditions, autoradiograms in Fig. 2a show that four species with M_r values of 77,000, 50,000, 41,000 and 25,000 reacted with MAbs. Since a species with a M_r of 50,000 was also precipitated by the presence of normal IgG in the control, the other three species of M_r 77,000, 41,000 and 25,000 were specific to MAbs. The M_r 41,000 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-41,000 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 incubated 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 analyzed by SDS-PAGE and autoradiography (Fig. 4). Cross-linking labeled 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 labeled 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 unlabeled PRL.

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. The data also show that F10 has a higher inhibiting activity than C3 for the purified receptors. Katoh et al. (1985b) reported curvilinear Scatchard plots of MAb binding to the microsome PRL receptors. I also observed similar Scatchard plot (Sakai and Murakami, 1987; Chapter 2), 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 and 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-41,000 species was comparable to that in the rat liver (Katoh et al., 1987). The high-M_r species (M_r 77,000) 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-25,000 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, I 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-25,000 species reacted with both MAbs. Necessary et al. (1984) reported that iodination of the receptor (M_r 42,000) leads to a small fragment with M_r 21,000. However, the PRL-binding species with a similar M_r was detected in the affinity-labeled PRL-receptor complex. Sakai and Ike (1987) have shown that a M_r-41,000 species did not disaggregate into a M_r-25,000 species following the chemical treatments involved in affinity-labeling and SDS-PAGE. It has been reported that the receptor binding activity is quite sensitive to oxidizing agents (Necessary et al., 1984; Mahajan and Ebner, 1986). In the iodination procedure, I used a low concentration of H_2O_2 (88 μM) to avoid oxidation of the receptor. At this concentration, radio-iodinated 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 labeled PRL to the affinity-purified receptors revealed three bands in the autoradiogram of M_r 99,000, 60,000 or 43,000. By subtracting the estimated M_r of free PRL, the M_r of the PRL receptor was estimated to be 74,000, 35,000 or 18,000. The estimates were close to those obtained using MAbs. The results show that each binding species interacts with one PRL molecule. By cross-linking labeled PRL or hGH to its receptors, the M_r was reported to range from 32,000 to 45,000 in the PRL target organs of various species (Borst and Sayare, 1982; Haeuptle et al., 1983; Hughes et

al., 1983; Necessary et al., 1984; Yamada and Donner, 1984; Katoh et al., 1985a; Ashkenazi et al., 1987). The high-M_r form of the PRL binding species (M_r 81,000-91,000) has been noted in the rat ovary (Bonifacino and Dufau, 1984; Mitani and Dufau, 1986) and in the Leydig cell PRL receptors (Bonifacino and Dufau, 1985). I showed here that the high-M_r PRL-binding species (M_r> 200,000) could be detected under reducing and non-reducing conditions. These results suggest that the mammary gland contains the size-heterogeneous binding species. The relationship between these PRL-binding species and their biological significance is under investigation.

In the present experiments, the dominant PRL-binding species is a M_r-41,000 receptor in the affinity-purified fraction. The dominant PRL-binding species in the microsomes and the Chaps-solubilized fraction was a M_r-83,200 species (Sakai and Ike, 1987). The discrepancy cannot be explained at 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).

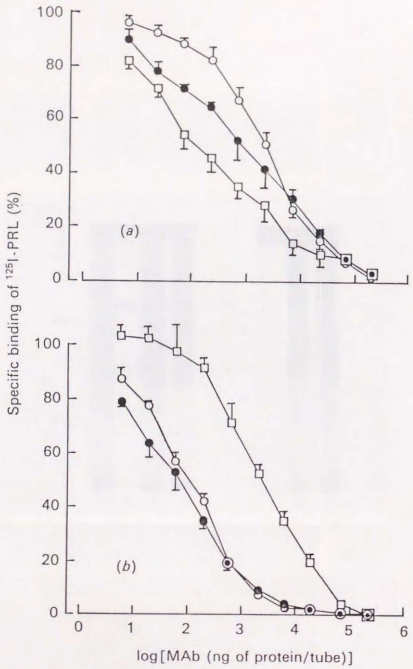

Fig. 1. Competition curves of specific binding of labeled PRL by unlabeled MAb

PRL receptors in the microsome (□), the Chaps extract (○), and the affinity-purified fraction (●) were incubated with ^{125}I-PRL (30,000 cpm) in the presence of increasing concentrations of unlabeled 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± 0.5 % (1.1± 0.1%), 17.6± 1.6% (3.7± 0.1%), and 13.9± 0.4% (5.7± 0.6%) of total radioactivity added, respectively. Values are the mean± S.D. of three different experiments.

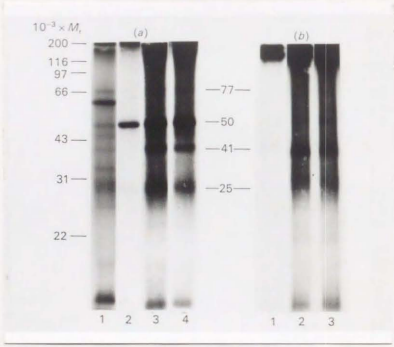

Fig. 2. Immunoprecipitation of purified PRL receptors

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 26,000 cpm for iodinated receptors, normal IgG, C3 and F10, respectively.

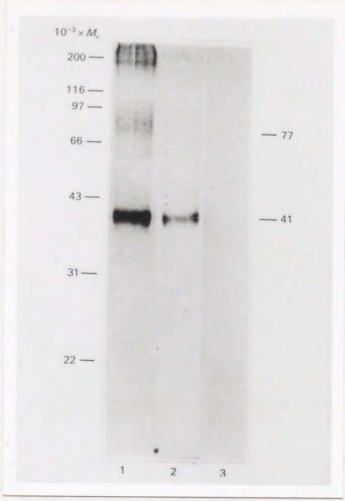

Fig. 3. Western blot analysis of the purified receptors

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).

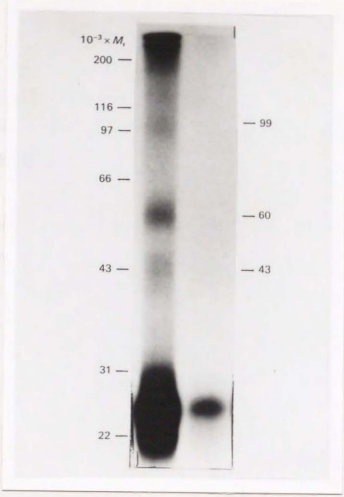

Fig. 4. Cross-linking of ^{125}I-PRL to purified receptors

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

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

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

Antibody	Prolactin	Radioactivity (cpm)
C3	-	63,580± 7,140
	+	11,890± 1,460
F10	-	40,950± 6,790
	+	12,910± 2,290
Normal IgG	-	9,720± 3.160
	+	11,240± 2,630

Table 2. M_r of the prolactin-binding species.

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 Fig. 2 and 3 (n=9). Values are means± S.D.

Species	Complex (A)	Receptor (B)	Difference (A-B)
1	>200,000	>200,000	------
2	99,000± 5,800	77.000± 5,200	22,000
3	60,000± 3,500	41,000± 1,300	19.000
4	43.000± 800	25,000± 800*	18,000

* M_r estimated from Fig. 2.

CHAPTER 5

Multiple Forms of Prolactin Receptors in the Rabbit Mammary Gland

SUMMARY

The Triton-solubilized prolactin (PRL) receptors, obtained from lactating rabbit mammary glands, were purified on an affinity column coupled with a receptor-specific monoclonal antibody (MAb) C3. Overall recovery was of about 30 % from crude membrane fractions. By silver staining, the purified fraction contained ten species of protein. Among them, seven species with molecular weights (M_r) of about 200,000, 100,000, 77,000, 63,000, 56,000, 44,000 and 41,000 were reacted with a receptor specific MAb F10 which reacted with the receptor at the same place where PRL did. These reacting species with F10 appeared to be able to bind to PRL. By cross-linking, almost identical species were appeared on the autoradiogram. Two major receptors with M_r of 77,000 and 41,000 were isolated in order to compare the peptide map and the carbohydrate chains. By digestion with chymotrypsin, V8 protease or lysyl endopeptidase, peptide fragments of the M_r 77,000 receptor included those of the M_r 41,000 receptor, indicating that the M_r 77,000 receptor had additional peptide fragments more than the M_r 41,000 receptor. Deglycosylation of both receptors resulted the decrease in M_r with the same degree, regardless any kinds of glycosidases were used. These results suggested that size-heterogeneous receptors are due to the difference in peptide length rather than in carbohydrate.

INTRODUCTION

Prolactin (PRL) regulates the mammary gland function. The mammary gland has a specific receptor for PRL and its concentration is high during lactation (Bohnet et al., 1974; Holcomb et al., 1976; Djiane et al., 1977; Hayden et al., 1979; Sakai et al., 1981; Grisson and Littleton, 1988). Like other peptide hormones, it is generally believed that PRL initiates its action by binding to its receptor on the membrane of target cells (Shiu and Friesen, 1980).

A heterogeneous population of PRL receptors has been shown in the rabbit mammary gland (Sakai and Ike, 1987; Chapter 4) and rat ovary (Mitani and Dufau, 1986; Buczko et al., 1989). The molecular weight (M_r) value of PRL binding protein is reported to be around 40,000 or 80,000. The shorter and longer form of the PRL receptor cDNA clones have recently been isolated from the mammary gland (Edery et al., 1989), hepatoma cells (Boutin et al., 1989), liver (Boutin et al., 1988; Shirota et al., 1990) and ovary (Zhang et al., 1990). Northern blot analysis suggest that size-heterogeneous mRNAs are present in the rabbit mammary gland (Edery et al., 1989), rat ovary (Zhang et al., 1990), rat liver (Shirota et al., 1990) and human breast cancer (Boutin et al., 1989).

Although cDNA analysis predicted that two receptors are highly homologous, the homology is yet unknown at the peptide level. In the present experiments, I intended to compare between the peptide maps of the higher M_r and lower M_r species. Therefore, it is essential to use the pure receptor preparation for direct comparison. PRL receptor has been purified using an affinity column coupled with human growth hormone (hGH) (Shiu and Friesen, 1974b; Mitani and Dufau, 1986), PRL (Liscia et al., 1982; Katoh et al.,

1985a; Sakai et al., 1985b; Chapter 2) or a receptor-specific antibody (Berthon et al., 1988; Okamura et al., 1989a). I used here a receptor-specific MAb C3 as a receptor-binding ligand to improve the recovery of the receptor. It is known that many proteolytic enzymes are present in the tissue. Different types of protease inhibitors are commonly employed to prevent the proteolytic damage of GH receptors (Smith and Talamantes, 1987; Yamada et al., 1987; Spencer et al., 1988). Purification was carried out in the presence of various types of protease inhibitors.

In this study, PRL-binding proteins and their M_r were examined by immunoblotting using a receptor-specific MAb F10. PRL receptor was further identified by the binding of PRL as a criteria. Two major receptors with M_r=41,000 and 77,000 were separated by SDS-PAGE, electroeluted from the gel and digested with three types of proteolytic enzymes. The maps of the peptide fragments were compared between two receptors in order to examine the homogeneity of the two receptors at the peptide level.

MATERIALS AND METHODS

Materials

Ovine PRL (NIADDK-oPRL-17, 30 I.U./mg), ovine growth hormone (oGH, NIADDK-oGH-15, 1.9 I.U./mg), human growth hormone (hGH, NIADDK-hGH-01), ovine follicle stimulating hormone (oFSH, NIADDK-oFSH-S14), ovine luteinizing hormone (oLH, NIADDK-oLH-S24) were gifts from NIADDK, Bethesda. Porcine insulin was purchased from Novo Industri A/S (Bagsvaerd, Denmark). Disuccinimidyl suberate (DSS) was from Pierce (Rockford, Illinois). Lactoperoxidase, bovine serum albumin (BSA), aprotinin, leupeptin, pepstatin, benzamidine HCl, phenylmethanesulfonyl fluoride (PMSF), and octyl phenoxy

polyethoxyethanol (Triton X-100) and normal mouse IgG were from Sigma (St Louis, MO). Cyanogen bromide (CNBr)-activated Sepharose 4B was from Pharmacia (Uppsala, Sweden). Sodium [^{125}]Iodine was from New England Nuclear (Boston, Mass). Peptide-N-glycosidase F (N-glycosidase F, EC 3.2.2.18), neuraminidase (EC 3.2.1.18), endo-α -N-acetylgalactosaminidase (O-glycan-peptide hydrolase, EC 3.2.1.97) and endoproteinase Glu-C (V8 protease, EC 3.4.21.19.) were from Boehringer Mannheim (Penzberg, Germany). Chymotrypsin (EC 3.4.21.1) was from Worthington (Freehold, NJ). Lysyl endopeptidase (EC 3.4.21.50), ethylenediaminetetraacetic acid (EDTA), and ethyleneglycol bis(2-aminoethylether) tetraacetic acid (EGTA) were from Wako (Osaka, Japan). Polyvinylidene difluoride (PVDF) membrane was from Millipore (Bedford, MA). The reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), immunodetection kit and silver staining kit were from Bio-Rad (Richmond, CA). All other reagents were of analytical grade. Characteristics of MAbs C3 and F10 have been described in Chapter 2,3,4.

Animals and preparation of tissues

All procedures were carried out at 0-4 ℃ except as noted. Mammary glands from mid-lactating New Zealand White rabbits were stored at -80 ℃ until use. Frozen mammary glands were thawed at 4 ℃ , cut into small pieces, and homogenized in five volumes of 50 mM Tris-HCl (pH 7.4), containing 0.3 M sucrose, 10 mM EGTA, 2 mM EDTA, 2 μg /ml aprotinin, 2 μg /ml leupeptin, 1 μg /ml pepstatin, 1 mM PMSF, and 1 mM benzamidine HCl. The homogenate was centrifuged at 14,000g for 20 min. The supernatant was centrifuged at 100,000g for 60 min. The pellet was suspended in 1.5 volumes (based on the wet weight of original mammary gland) of 50 mM Tris-HCl (pH 7.4) containing 1%

Triton X-100, 10 mM EGTA, 2 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF, and 1 mM benzamidine HCl. The suspension was stirred at room temperature for 20 min and centrifuged at 140,000g for 70 min. The extract was stored at -50 ℃ until use.

Protein concentration

Protein concentrations were determined by the dye binding method according to the Bio-Rad instructions using bovine gamma globulin as standard.

Receptor purification

Coupling of normal mouse immunoglobulin (nIgG) or MAb C3 to CNBr-Sepharose 4B was carried out as follows. Twenty five mg of nIgG or 20 mg of MAb was coupled to CNBr-activated Sepharose 4B (for nIgG, 10 ml gel; for MAb, 4 ml gel) by incubating the mixture in 0.1 M bicarbonate buffer (pH 8.3) overnight at 4 ℃. After incubation, the beads were washed with bicarbonate buffer and the remaining active groups were blocked with 0.1 M Tris-HCl buffer (pH 8.0) for 2 h at room temperature, followed by washing the product with three cycles of alternating pH according to the manufacture's instruction (Pharmacia). Finally, the beads were washed with 1 M NaI and equilibrated with 25 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 0.1% TritonX-100 and 1 mM PMSF (Buffer A). A tracer amount of ^{125}I-IgG (about 100,000 cpm) was included in the reaction mixture to determine the coupling efficiency. The coupling efficiency was about 95 %.

All procedures were carried out at 4 ℃. Ten ml of a nIgG Sepharose column was washed and equilibrated with Buffer A. Triton X-100 solubilized

membranes (200 ml, 2.1 g protein) were diluted by 2-fold with Buffer A and applied to the column and recycled 3 times. Flow through fractions were applied to the MAb C3 column. After washing with 100 ml of Buffer A, the bound proteins were eluted with Buffer A containing 1 M NaI. The flow rate of apply, wash or elution was at 15, 15, or 6 ml/h, respectively. The first eluted fractions (150 ml) were immediately dialyzed against 2.5 mM Tris-HCl (pH 7.4) containing 0.01 % TritonX-100, 1 mM benzamidine HCl and 1 mM PMSF (Buffer B). The dialyzed sample was diluted by 2-fold with Buffer A and again applied to the MAb column. After washing with Buffer A, the bound proteins were eluted with 30 ml of Buffer A containing 1 M NaI. The eluted samples were dialyzed against Buffer B and aliquoted and stored at -50 ℃ .

Receptor binding assay

PRL was iodinated by the lactoperoxidase method as described in Chapter 2. Specific activity of iodinated PRL was 2.2-2.8 MBq/μg .

Binding of ^{125}I-PRL to particulate or solubilized receptors was described in Chapter 2. In brief, particulate receptors were incubated with 30,000 cpm of ^{125}I-PRL in 25 mM Tris-HCl (pH 7.4) containing 10 mM $MgCl_2$, 1 mM PMSF and 0.2% BSA (TMPB buffer) for 15 h at room temperature (final volume, 0.5 ml). The reaction mixture was diluted with 2.5 ml of TMPB buffer and microsomes were precipitated by centrifugation at 2,000g for 20 min. For the solubilized receptors, samples were incubated with 20,000 cpm of ^{125}I-PRL in TMPB buffer containing 5 mM 3-[(3-cholamidopropyl)-dimethylammonio]-propanesulfonate (Chaps) for 15 h at room temperature (final volume, 0.5 ml). After incubation, PRL-receptor complex were mixed with 0.5 ml of 0.2% bovine gamma globulin and 1 ml of 32 % polyethyleneglycol, and precipitated by

centrifugation at 2,000 g for 15 min. The radioactivity in the precipitates was counted in a gamma counter. Nonspecific binding was determined in the presence of 1,000-fold excess of unlabeled PRL.

Cross-linking and immunoblotting

PRL receptors in Triton extract (receptor concentration; 100 fmol/ 550 μg protein), first MAb purified fraction (100 fmol/ about 1 μg protein) and second MAb purified fraction (100 fmol/ about 30 ng protein) were incubated with ^{125}I-PRL (3×10^5 cpm) in 0.3 ml of TMPB buffer containing 5 mM Chaps in the presence or absence of unlabeled PRL (10 μg) for 16 h at room temperature. After removing unbound ^{125}I-PRL, cross-linking was performed on the PRL-receptor complex using 0.75 mM DSS for 15 min on ice (Chapter 4). The samples were analyzed by SDS-PAGE (Laemmli, 1970) and autoradiography.

Purified receptors (500 fmol) were separated in a 10 % acrylamide gel and electroblotted onto a PVDF membrane at 30 V for 30 min using semi-dry blotting apparatus according to the instructions (Millipore). The membrane was blocked with 3 % gelatin for 1 h at room temperature and then incubated with MAb F10 or nIgG overnight at room temperature. After the membrane was incubated with goat anti-(mouse IgG)-alkaline phosphatase conjugates, the substrate was added and incubated for 30 min (Chapter 4).

Iodination of PRL receptor

PRL receptor was iodinated by the chloramine T method (Hunter and Greenwood, 1962). Second MAb purified PRL receptor (18 pmol/ about 5 μg protein) was combined with 8.5 MBq of Na^{125}I in 0.5 M phosphate buffer (pH 6.9), then 5 μg of chloramine T was added. After 4 min at room temperature, the

reaction was stopped by the addition of 125 μg of sodium metabisulphate. The reaction mixture was diluted with 25 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 0.1 % TritonX-100 and 10 % glycerol (Buffer C). Free iodine was separated by gel filtration using a Sephadex G-25 column previously equilibrated with Buffer C. Specific radioactivity of PRL receptor was 560-930 kBq/μg.

Electroelution and peptide map analysis

Iodinated receptor (1×10^7 cpm) was subjected to 7.5% SDS-PAGE under reducing conditions. After SDS-PAGE, the gel pieces with M_r of 77,000 and 41,000 were cut out from the gel and electroeluted at 200 V in 2.5 mM Tris and 14.2 mM glycine buffer for 60 min at 4 ℃. Eluted sample was dialyzed against distilled water for 10 h and concentrated. Limited peptide map was performed by the method of Cleaveland et al. (1977) with a minor modification. Electroeluted sample (2,000 cpm) was incubated at 37 ℃ with 1 μg of chymotrypsin, V8 protease or lysyl endopeptidase in 0.125 M Tris-HCl (pH 6.7) containing 0.5% SDS, 10 % glycerol and 0.01 % bromophenol blue. The reaction period was varied from 1 min to 12 h. The reaction mixture was denatured by boiling for 2 min in the presence of 2 % SDS and 5 % 2-mercaptoethanol. The sample was subjected to a 13 % acrylamide gel and analyzed by autoradiography.

Deglycosylation studies

An aliquot (1,000 cpm) of electroeluted sample was reacted for 24 h at 37 ℃ with 1.0 unit of N-glycosidase F in a total volume of 30 μl containing 10 mM phosphate buffer (pH 7.4), 5 mM Chaps, 0.01 % SDS, 0.1 % TritonX-100, 10

mM EDTA. An aliquot (1,000 cpm) of the sample was reacted for 24 h at 37 ℃ with 0.02 U of neuraminidase in a total volume of 30 μl containing 10 mM phosphate buffer (pH 7.4) and 5 mM Chaps. An aliquot (1,000 cpm) of the sample was reacted for 1 h at 37 ℃ with 0.02 U of neuraminidase, and further incubated in the presence of 1.0 mU of O-glycan-peptide hydrolase for another 23 h at the same temperature in a total volume of 30 μl containing 10 mM phosphate buffer (pH 7.4) and 5 mM Chaps. Then samples were electrophoresed and analyzed by autoradiography.

RESULTS

PRL receptor purification

The mammary gland PRL receptors were purified in the presence of a number of protease inhibitors to minimize the proteolytic damage by endogeneous proteases. The solubilized receptors were purified using an immuno-affinity column coupled with a receptor-specific MAb C3. Over 90 % of the original PRL binding activity was absorbed by the column and about 55 % was eluted by NaI from the column (first MAb-purified fraction). Using the same column, the first MAb-purified fraction was further purified (second MAb purified fraction). In the second purification, about 80 % of the applied receptors were recovered. Fig. 1. shows the hormonal specificity and binding affinity for the hormones to the second MAb purified receptor. Competitive binding assay showed that the binding of ^{125}I-PRL to its receptor was inhibited by lactogenic hormones (PRL and hGH). Other peptide hormones tested showed no inhibitory activity. Scatchard plotting of the binding of ^{125}I-PRL generated a linear regression line, suggesting that the purified receptor has a single class of PRL-binding component. As shown in Table 1,

the overall recovery from microsomes was of about 32 %. The receptor concentration in the second MAb-purified fraction was increased by about 20,000-fold as compared to that in microsomes. Assuming the receptor M_r value of 40,000~ 100,000, the purity of the receptors was calculated to be in the range of 8~ 40 %. In Fig. 2, silver stain of the SDS-gel also showed that the purity was of greater than 50 % by densitometer scanning. In this experiment, about 360 pmol (about 120 μg of protein) of purified receptor could be practically obtained from 300 g of fresh mammary tissue.

I used 1 M NaI for elution. At this concentration, the PRL receptor and the MAb did not lose the PRL- and the receptor-binding activity, respectively. The affinity column could be re-used without losing the receptor-binding activity.

Silver staining and immunoblotting analysis

Second MAb purified fractions were subjected to SDS-PAGE under reducing conditions. The proteins and MAb-binding species of protein in this fraction were visualized by silver staining (Fig. 2-1) and by immunostaining (Fig. 2-2), respectively. Ten protein species with M_r of > 200,000, 100,000, 77,000, 63,000, 56,000, 44,000, 41,000, 33,000, 31,000 and 25,000 were detected by silver staining. Among them, the protein with a M_r of > 200,000, 100,000, 77,000, 63,000, 56,000, 44,000 or 41,000 specifically reacted with MAb F10. Proteins with M_r smaller than 41,000 did not react with F10. No bands reacted with nIgG. Although the band intensity varied greatly depending on the kind of protein species, the M_r 41,000 species was most heavily labeled and the smallest one.

Cross-linking of PRL to its receptors

Cross-linking of ^{125}I-PRL to the receptor was performed in order to determine which protein was able to bind to PRL (Fig. 3). Triton X-100 solubilized, first and second MAb purified fractions were used for analysis. Autoradiography showed that cross-linking of Triton X-100 solubilized receptors with ^{125}I-PRL revealed three major bands (M_r=over 200,000, 58,000 and 43,000) and two faint bands (M_r=100,000 and 82,000). By using first or second MAb purified fractions, a broad band with M_r=116,000-130,000 appeared on the autoradiogram. All bands (M_r=> 200,000, 116,000-130,000, 100,000, 82,000, 58,000 and 43,000) were disappeared on the autoradiogram by incubation in the presence of an excess amount of unlabeled PRL. By subtracting the molecular value (M_r=23,000) of labeled PRL on autoradiogram, the M_r of PRL binding proteins were estimated to be 93,000-107,000, 77,000, 69,000, 35,000 and 20,000.

Peptide map analysis

The second MAb purified fractions were radio-iodinated by the chloramine T method. Two major receptors with M_r=77,000 and 41,000 were separated by SDS-PAGE and electroeluted from the gel with recovery of 70-90%. The radioactivity of M_r=77,000 or 41,000 receptor was comprised of 4$\sim$ 10 % or 8$\sim$ 12 % of total radioactivity applied, respectively. The other receptor species was less labeled and could not be used for analysis. The two receptor species were digested with three different types of proteases (chymotrypsin, V8 protease and lysyl endopeptidase) under identical conditions. Both receptors were sensitive to proteolysis. Fig. 4 shows that treatment of M_r=41,000 receptor with chymotrypsin, V8 protease and lysyl endopeptidase resulted in a few peptide fragments, all of which the fragments

were included in those of M_r=77,000 receptor.

Deglycosylation studies

To investigate the M_r of carbohydrate residues contributing to those of two PRL receptors (M_r=77,000 and 41,000), deglycosylation study was performed. The results of treatment of electroeluted ^{125}I-labeled receptor with N-glycosidase F, neuraminidase and O-glycan-peptide hydrolase are shown in Fig. 5. Both receptors were sensitive to N-glycosidase F, neuraminidase and O-glycan-peptide hydrolase. Treatment of M_r=77,000 or 41,000 receptor with N-glycosidase F resulted in a decrease in M_r by 4,000. Treatment of M_r=77,000 or 41,000 receptor with neuraminidase or neuraminidase plus O-glycan-peptide hydrolase resulted in the reduction of M_r by 1,000 or 2,000, respectively.

DISCUSSION

Two types of PRL-binding proteins have been shown in the rabbit mammary gland (Sakai and Ike, 1987; Chapter 4) and rat ovary (Mitani and Dufau, 1986; Buczko et al., 1989). I could demonstrate here that the lactating rabbit mammary gland has at least seven PRL-binding proteins having different M_r. I have purified PRL receptors from rabbit mammary gland using two step immunoaffinity chromatography with a recovery of about 30 % and specific binding capacity of about 3 nmol/mg. Both the recovery and the purity were greater than those obtained by PRL-affinity-chromatography in Chapter 2. In the silver staining analysis, second MAb purified fraction contained at least ten bands. Of ten bands observed, seven bands were specifically reacted with MAb F10. These proteins could participate in the

binding of PRL. Cross-linking studies also showed that multiple forms of PRL receptor exist in the purified fraction. Subtracting the M_r of one PRL molecule from that of PRL-receptor complex, each value of PRL binding proteins were almost comparable to that obtained from immunoblot analysis. Okamura et al. (1989b) also reported that four PRL binding proteins existed in a partially purified fraction of the rabbit mammary gland. This is also supported by Northern blot analysis using a receptor-specific cDNA probe, showing that PRL receptor mRNA is size-heterogeneous observed in the rabbit mammary gland (Edery et al., 1989), rat ovary (Zhang et al., 1990), rat liver (Shirota et al., 1990) and human breast cancer (Boutin et al., 1989). The present results indicated that small M_r receptor was not a degraded product of a large M_r receptor. It has been demonstrated that GH receptor, susceptible protein to proteases (Smith and Talamantes, 1987; Yamada et al., 1988), can be successfully purified to be uniform by the presence of a number of protease inhibitors (Spencer et al., 1989).

The electroeluted M_r=77,000 receptor did not generate the M_r=41,000 receptor and the M_r=41,000 receptor did not aggregate to the M_r=77,000 receptor. The peptide mapping of M_r 41,000 and 77,000 receptor also indicated that the M_r 77,000 receptor is not a dimerous form of the M_r 41,000 receptor. Buczko et al. (1989) also reported that M_r=80,000 receptor did not convert to the lower molecular weight form on SDS-PAGE analysis or reverse phase high performance liquid chromatography. In the rat liver, however, the M_r 42,000 receptor is easily aggregated to form a dimer (M_r=84,000) (Okamura et al., 1989a). The results of limited peptide map in this study show that two PRL receptors (M_r=77,000 and 41,000) are related in primary structure. The sequence analysis of cDNA clones also suggests that the amino acid sequence

of two receptors, especially in extracellular portion, is expected to be highly homologous (Boutin et al., 1988; Zhang et al., 1990; Shirota et al., 1990). These observations suggest that multiple PRL binding proteins are derived from the same gene. Furthermore, the fact that apparently a single class of the receptor revealed by Scatchard analysis confirm the cDNA studies that N-terminal extracellular domain of the two size-heterogeneous cDNAs was identical (Zhang et al., 1990; Shirota et al., 1990). Sakai and Ike (1987) demonstrated that two types of the receptor have similar affinity for PRL. Probably, all multiple forms of the receptors have similar affinity for PRL binding.

PRL receptor has been reported to be a glycoprotein (Costlow and Gallagher, 1979, Haldosen et al., 1989). The present results indicated that both the M_r 77,000 and 41,000 receptor have N-linked and O-linked carbohydrate chains in their molecules. Deglycosylation of both receptors by N-glycosidase F decreased the M_r by about 4,000. The M_r values of both receptor were decreased by about 1,000 after digestion with neuraminidase or O-glycan-peptide hydrolase. It allows to estimate the amount of carbohydrate chain to be about 5,000~ 6,000 in the M_r 77,000 and 41,000 receptor. Furthermore, similar patterns of peptide digestion studies confirmed the above findings. Considering the same molecular weight of carbohydrate residue existing in both PRL receptor, heterogeneity of the PRL receptor can not be explained by difference in glycosylation. It is more likely that heterogeneity is caused by difference in the peptide length.

I presented evidence that multiple forms of PRL receptor exist in the rabbit mammary gland and that heterogeneity is caused by the difference in the polypeptide length. It remains unclear that the size-heterogeneous PRL

receptor is caused by alternative splicing or post-translational modification. Further study is required to elucidate the function of each PRL receptor.

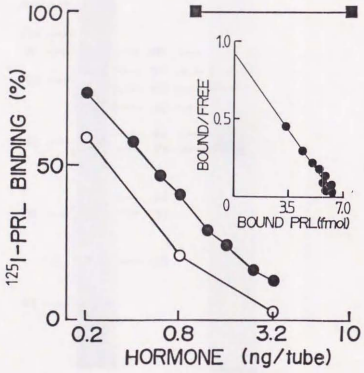

Fig. 1. Competitive displacements of specific ^{125}I-PRL binding to purified receptor by peptide hormones and Scatchard plot

Second MAb purified fractions were incubated with ^{125}I-PRL (20,000 cpm) in the absence or presence of various concentrations of unlabeled hormones for 15 h at room temperature. The concentration of unlabeled hormone used was as follows: 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, 2.4 and 3.2 ng of PRL (●), 0.2, 0.8 and 3.2 ng of hGH (○), 1.0 and 10 ng of other hormones [oGH, oLH, oFSH and insulin (■)]. Values were expressed as a percentage of specific binding; nonspecific binding was 6.5-7.5 % of total radioactivity added. Bound and bound/free values of PRL were plotted as described by Scatchard (1949).

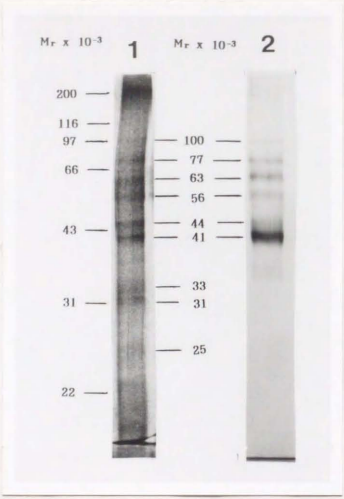

Fig. 2. Silver- and immune-staining of second MAb purified receptor

Second MAb purified receptors (500 fmol, about 170 ng protein) were subjected to SDS-PAGE under reducing conditions. Proteins were visualized by silver staining (lane 1) or by immune staining using MAb (F10) (lane 2). The position of M_r marker standards are shown in the left.

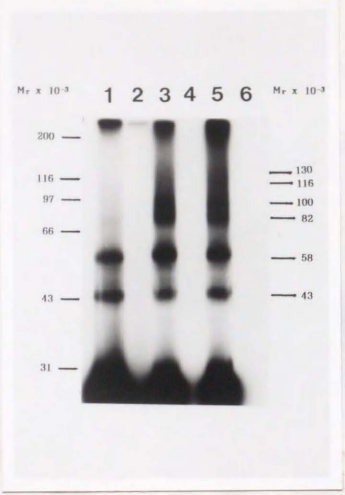

Fig. 3. Cross-linking of ^{125}I-PRL to its receptor at various purification stage

PRL receptors (100 fmol) in Triton extract (560 μg protein), first MAb purified fraction (about 1 μg) and second MAb purified fraction (about 30 ng) were incubated with ^{125}I-PRL (3×10^5 cpm) in the presence (lane 2, 4, 6) or absence (lane 1, 3, 5) of unlabeled PRL (10 μg) overnight at room temperature. After removing of unbound PRL, PRL-receptor complex was cross-linked with 0.75 mM DSS. The cross-linked sample was analyzed by SDS-PAGE (8.5 % acrylamide gel) under reducing conditions and by autoradiography; lane 1, 2, Triton extract; lane 3, 4, first MAb purified fraction; lane 5, 6, second MAb purified fraction.

Fig. 4. Autoradiogram of limited peptide-digested PRL receptors

An aliquot (2,000 cpm) of electroeluted and radioiodinated PRL receptors were digested with 1 μg of chymotrypsin (lane 3, 4), V8 protease (lane 5, 6) or lysyl endopeptidase (lane 7, 8). Intact sample (lane 1, 2) or digested samples were analyzed by SDS-PAGE (13 % acrylamide gel) under reducing conditions. Lane 1, 3, 5, 7, $\underline{M}_r$=77,000 receptor; lane 2, 4, 6, 8, $\underline{M}_r$=41,000 receptor.

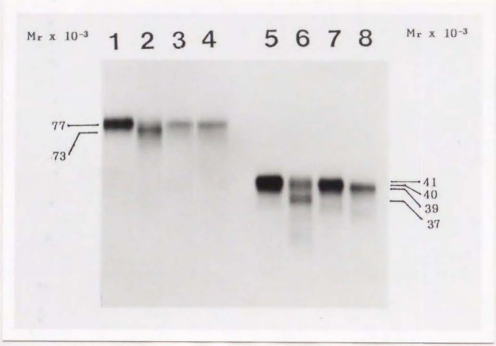

Fig. 5. Autoradiogram of deglycosylated PRL receptors

An aliquot (1,000 cpm) of electroeluted and radioiodinated PRL receptors (M_r=77,000 and 41,000) was reacted with 1.0 unit of N-glycosidase F (lane 2, 6), 0.02 unit of neuraminidase (lane 3, 7), or 0.02 unit of neuraminidase plus 1.0 m unit of O-glycan-peptide hydrolase (lane 4, 8). Intact sample or deglycosylated sample were analyzed by SDS-PAGE (10 % acrylamide gel) under reducing conditions. Lane 1, 2, 3, 4, M_r=77,000 receptor; lane 5, 6, 7, 8, M_r=41,000 receptor.

Table 1. Summary of PRL receptor purification

Sample	Dissociation constant(Kd) ($\times 10^{-11}$M)	Binding capacity (pmol/mg)	Recovery (%)
Microsome	9.1-14.7	0.13-0.19	100
Triton-solubilized	3.3-4.9	0.17-0.19	80-86
MAb-Sepharose(1)	1.5-2.5	98-108	38-50
MAb-Sepharose(2)[a)]	1.3-1.8	2,000-4,000	28-36

Dissociation constant and binding capacity were calculated by Scatchard analysis of ^{125}I-PRL displacement experiment.

Values were pooled from three different experiments.

a) : Values were pooled from two different experiments.

CHAPTER 6

Effect of Deglycosylation of Prolactin Receptors on the Hormone Binding to its Receptors

SUMMARY

Effect of deglycosylation of the prolactin (PRL) receptor on the hormone binding was examined. Treatment of the purified receptor for 6 h with N-glycosidase F, neuraminidase or O-glycan-peptide hydrolase affected little on the affinity and specificity for hormones, and the binding site for PRL. Immunoblot analysis showed that no-enzyme-treated receptor reacted with monoclonal antibody F10, its M_r value were 77,000, 63,000, 56,000, 44,000 and 41,000. Treatment of the receptor with either neuraminidase or O-glycan-peptide hydrolase for 6 h resulted in a decrease in the M_r value of all binding species by about 1,000. By 6 h N-glycosidase F-treatment, all the PRL binding species did not react with F10. These findings suggest that although PRL receptor contained N- and O-linked carbohydrate chains in addition to sialic acid, these carbohydrates did not participate in the binding of PRL.

INTRODUCTION

It has been implicated that prolactin (PRL) receptor contains carbohydrate moieties with evidence that PRL receptor is retained by and eluted from a lectin-column (Mitani and Dufau, 1986) and that the binding of PRL to its membrane receptor is inhibited by the presence of lectins (Costlow and Gallagher, 1979; Bhattacharya and Vonderhaar, 1982). Furthermore, the deduced amino acid sequence from cloned cDNA analysis predicted the existence of three asparagine-linked carbohydrate chains in the extracellular domain (Boutin et al., 1988; Edery et al., 1989). Savoie et al. (1986) showed that injection of tunicamycin, an inhibitor of N-linked glycosylation, resulted in a decrease in the PRL binding activity of the receptor on the rat liver membrane, suggesting that N-linked carbohydrate chains are important in the insertion into the membrane or the acquisition of binding activity for the hormone. However, no information is available about the role of carbohydrate chains on PRL binding to the receptor at the molecular level. The utilization of carbohydrate-chain-cleaving enzymes will allow to characterize the carbohydrate chains linked to the PRL receptor (Haldosen et al., 1989; Chapter 5).

Therefore, the effect of deglycosylation of the receptor on PRL binding activity was examined with carbohydrate-chain-cleaving enzymes. Furthermore, to assess the effectiveness of deglycosylation, I performed immunoblot analysis of the deglycosylated receptor probed with monoclonal antibody (MAb) specific to the rabbit mammary PRL receptor.

MATERIALS AND METHODS

Materials

Ovine PRL (NIADDK-oPRL 17, 30 I.U./mg) was a gift from NIADDK, Bethesda. N-glycosidase F, neuraminidase and O-glycan-peptide hydrolase were purchased from Boehringer Mannheim (Penzberg, Germany). All other chemicals were of analytical grade. Characteristics of MAbs C3 and F10 have been described in Chapter 2,3,4, and 5.

Preparation and affinity purification of PRL receptors

Mammary glands from mid-lactating New Zealand White rabbits were used. Solubilization and purification were described in Chapter 5. In brief, crude membrane preparations were collected by differential centrifugation and solubilized 1 % Triton X-100. Triton X-100-solubilized receptors were purified twice by C3-coupled-affinity chromatography.

Deglycosylation of PRL receptors

Deglycosylation of PRL receptors was performed as described in Chapter 5 with a slight modification. Total volume was adjusted to 30 $\mu\ell$ and reaction temperature was 37 ℃ . Purified receptors (500 fmol) were reacted with 1.0 unit of N-glycosidase F in 10 mM phosphate buffer (pH 7.4) containing 5 mM 3-[(3-cholamidopropyl)-dimethylammonio]-propanesulfonic acid (Chaps), 0.01 % sodium dodecyl sulfate (SDS), 0.1 % Triton X-100 and 1 mM ethylenediaminetetraacetic acid (EDTA) (N-glycosidase F treatment). Purified receptors were reacted with 0.02 U of neuraminidase or 1 mU of O-glycan-peptide hydrolase in 10 mM phosphate buffer (pH 7.4) containing 5 mM Chaps (neuraminidase or O-glycan-peptide hydrolase treatment).

Binding studies

PRL was iodinated by the lactoperoxidase method as described in Chapter 2. Specific radioactivity of iodinated PRL was 2.6-2.9 MBq/μg.

Binding of ^{125}I-PRL to purified receptors was described in Chapter 5. In brief, purified receptors (deglycosylated or intact) were incubated with 20,000 cpm of ^{125}I-PRL in 25 mM Tris-HCl (pH 7.4) containing 10 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.2 % bovine serum albumin (BSA) and 5 mM Chaps for 15 h at room temperature (final volume 0.5 ml). After incubation, PRL-receptor complexes were precipitated with γ -globulin and poly(ethylene glycol) by centrifugation as described in Chapter 3. Nonspecific binding was determined in the presence of 1,000-fold excess of unlabeled PRL. Specific binding was the difference between total binding and nonspecific binding.

Scatchard analysis

Purified receptors were incubated with ^{125}I-PRL (20,000 cpm) in the presence of 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6 and 2.4 ng of unlabeled PRL for 16 h at room temperature. The dissociation constant and the concentration of binding sites were calculated according to Scatchard (1949). Statistical significance was determined using Student's t-test.

Immunoblotting

Immunoblotting was performed as described in Chapter 5. In brief, purified receptors were separated by SDS-PAGE (Laemmli, 1970) and transferred electrophoretically onto a membrane. The blotted membrane was blocked with 3 % gelatin, followed by incubation with F10 or normal IgG. After incubation, anti-(mouse IgG)-alkaline phosphatase conjugate was added and visualized by

adding the substrate.

RESULTS

Purified PRL receptors were treated for 6, 24, or 48 h with N-glycosidase F, neuraminidase or O-glycan-peptide hydrolase, and effect of deglycosylation on the PRL-binding activity of the receptor was examined (Fig. 1). In the enzyme-free control, the PRL-binding activity of the receptor was decreased as incubation of the receptor was prolonged. By 6 or 24 h-incubation, the receptor lost the PRL-binding activity by greater than 40 or 80 % of the 0-h control, respectively. The receptor, incubated for 48 h, had almost no PRL-binding activity. The receptor was treated with N-glycosidase F, neuraminidase or O-glycan-peptide hydrolase. Using the enzyme-treated receptor, the PRL-binding activity was determined by incubation with ^{125}I-PRL in the presence or absence of unlabeled PRL for 16 h. Patterns of the time-dependent change in the PRL-binding activity, however, were essentially similar to that of the enzyme-free control. Competitive binding assay showed that regardless of any kind of enzyme used, the binding of ^{125}I-PRL to its receptor was inhibited by PRL or human growth hormone (GH), but not by ovine GH, luteinizing hormone, follicle stimulating hormone and insulin.

Effects of deglycosylation of the PRL receptor on its molecular weight was examined using a combination of SDS-PAGE and immunoblotting analysis (Fig. 2). After 6 h of incubation, the enzyme-free control had seven protein species capable to react with MAb F10, and their M_r values were estimated to be of 77,000, 63,000, 56,000, 44,000, and 41,000 as compared to the mobility of M_r marker standards. By digestion of the receptor with N-glycosidase F, no bands were appeared on the immunoblot. Digestion with neuraminidase or O-

glycan-peptide hydrolase caused a decrease in the M_r value by about 1,000. The M_r values of digested protein species were decreased to be 76,000, 62,000, 55,000 43,000 and 40,000. The band with M_r of 70,000 was non-specific. Results obtained by 24- or 48-h digestion were essentially similar to above.

Scatchard analysis of the binding of ^{125}I-PRL to the enzyme-treated receptors was performed in order to examine changes in the dissociation constant for PRL binding and the concentration of the binding site. The data are summarized in Table 1. Both the dissociation constant and binding sites between enzyme-treated and no-treated sample were not different.

Effects of deglycosylation of the receptor on immunologic property were examined (Fig. 4). Specific binding of ^{125}I-PRL was inhibited by the presence of C3 or F10 dose dependently. Treatment of the receptor with N-glycosidase F or O-glycan-peptide hydrolase did not affect the inhibition potency of C3 or F10 on PRL binding. However, treatment with neuraminidase decreased the inhibition potency of C3 or F10 slightly.

DISCUSSION

Treatment of purified PRL receptors with N-glycosidase F, neuraminidase, or O-glycan-peptide hydrolase for 6 h affected little on the concentration of the binding site, and the affinity and specificity for hormone binding to the receptor. However, immunoblot analysis of 6 h enzyme-treated receptors showed that all PRL binding species were susceptible to N-glycosidase F, neuraminidase and O-glycan-peptide hydrolase.

Although glycosylated PRL had less bioactivity and receptor binding activity than unglycosylated PRL (Markoff et al., 1988; Atkinson et al., 1988), this study indicated that carbohydrate chains of the receptor were not

greatly involved in PRL binding. Supporting this notion, the earlier observations that the PRL-receptor complexes were able to bind to the Concanavalin A (Con-A)-Sepharose and that the hormone binding to the solubilized receptor was not inhibited by the presence of Con-A, suggested that Con-A binding site and the hormone binding site were not overlapped (Costlow and Gallagher, 1979; Bhattacharya and Vonderhaar, 1982). Neuraminidase treatment of rabbit mammary receptors did not affect the binding of PRL (Shiu and Friesen, 1974), but the pI value was shifted to an alkaline side (Waters et al., 1984). Physiological significance of the carbohydrate chains of the receptor is unclear at present.

In Chapter 5, both electroeluted M_r=77,000 and 41,000 species contained N-linked, O-linked carbohydrate chains and sialic acid, of which contributing M_r values were 4,000, 1,000 and 1,000, respectively. In this study, M_r of N-linked carbohydrate chains could not be determined since treatment of N-glycosidase F resulted in disappearance of immunoreactive bands. However, the results showed that all PRL binding species contained N-linked carbohydrate chains. Treatment of neuraminidase or O-glycan-peptide hydrolase resulted in the shift of immunoreactive band to be smaller. The results suggested that each PRL binding species contained both sialic acid and O-linked carbohydrate chains, of which the M_r were equally 1,000. In the rat liver PRL receptor, the existence of N-linked oligosaccharides and sialic acid but not O-linked oligosaccharides have been demonstrated (Haldosen et al., 1989).

Although F10 could not react with the deglycosylated receptor fixed on a membrane, F10 was able to inhibit the binding of PRL to the deglycosylated receptor in a dose-dependent manner. The reason is unclear at present. It

is probable that the structure of the binding site for F10 may be altered by N-glycosidase F digestion and following denaturing.

In conclusion, PRL receptor in the mammary gland contains N-linked and O-linked carbohydrate chains and sialic acid. However, the carbohydrate chains did not participate in PRL binding.

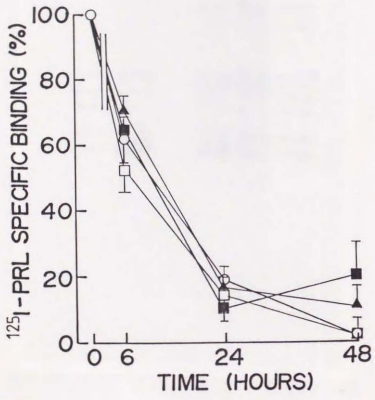

Fig. 1. Time course of ^{125}I-PRL specific binding to intact or deglycosylated receptors

Purified receptors were incubated with buffer only (○), 1.0 U of N-glycosidase F (■), 0.02 U of neuraminidase (□) or 1 mU of O-glycan-peptide hydrolase (▲) at 37 ℃ for the indicated time. After incubation, the sample was subjected to binding assay as described in Materials and Methods section. Nonspecific binding was 6.5-7.5 % of total radioactivity added. Results were compared to the amount of ^{125}I-PRL specific binding obtained at 0 h incubation. Values are the mean± S.D. of three different experiments.

Fig. 2. Immunoblot analysis of deglycosylated receptors

Purified receptors (500 fmol) were incubated for 6 h as the same conditions shown in Fig. 1. After incubation, the samples were separated by SDS-PAGE under reducing conditions and transferred electrophoretically onto a membrane. The membrane was incubated with F10 (10 μg/ml) overnight at room temperature. Immunoreactive bands were visualized as described in Materials and Methods section. The arrow indicates non-specific bands, which can be detected in the absence of the receptor. Lane 1, incubation with buffer only; lane 2, N-glycosidase F treated; lane 3, neuraminidase treated; lane 4, O-glycan-peptide hydrolase treated.

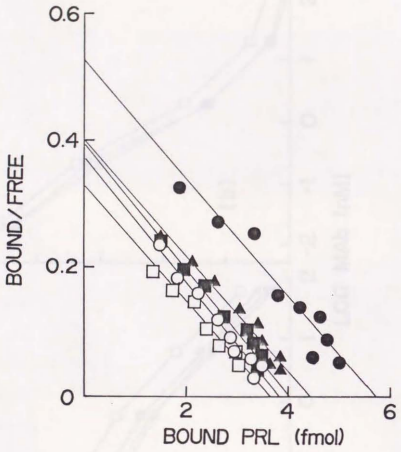

Fig. 3. Scatchard analysis of the binding of PRL to deglycosylated receptor

The same amount of purified receptors were treated with buffer (○), N-glycosidase F (■), neuraminidase (□), or O-glycan-peptide hydrolase (▲) for 6 h at 37℃ . Deglycosylated or intact (●) receptors were incubated with ^{125}I-PRL in the presence of various amount of unlabeled PRL for 15 h at room temperature. Results of two other experiments were exactly the same.

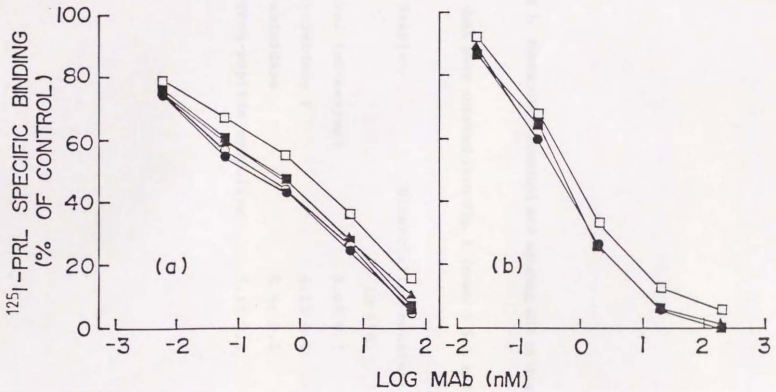

Fig. 4. Inhibition of PRL binding to deglycosylated receptor by MAbs

Deglycosylated receptors [buffer (○), N-glycosidase F (■), neuraminidase (□), or O-glycan-peptide hydrolase (▲) treated] or intact receptors (●) were incubated with ^{125}I-PRL in the presence of various amounts of C3 (a) or F10 (b). Results were compared to ^{125}I-PRL specific binding of the intact control (●). Values are the mean of three different experiments.

Table 1. Dissociation constant and binding site of deglycosylated receptors.

The data were obtained from Fig. 3. (mean ± S.D. n=3).

Sample	Dissociation constant ($\times 10^{-11}$M)	Binding sites (fmol/tube)
Control (no-enzyme)	2.0± 0.1	2.9± 0.8
N-glycosidase F	2.1± 0.1	3.0± 0.2
Neuraminidase	2.3± 0.5	2.5± 0.2
O-glycan-peptide hydrolase	2.1± 0.2	3.3± 0.5

CHAPTER 7

General Discussion

1. Production and characterization of monoclonal antibodies (MAbs)

Antireceptor antibodies, especially MAbs, have been effective tools in analyzing structure and function of prolactin (PRL) receptor (review, Kelly et al., 1988). MAbs directed against the PRL receptor in the rabbit mammary gland and rat liver have been produced (Katoh et al., 1985b, 1987; Okamura et al., 1989b). I also produced MAbs using a partially purified mammary PRL receptor as an antigen. The binding capacity of the antigen used was 44.4 fmol/mg protein, which represents 0.2-1.5 % of purity. Using a partially purified receptor as an antigen, four receptor-specific MAbs were obtained. Two MAbs (C3 and F10) were characterized and used in this study.

The properties of two MAbs are as follows: 1) Typing analysis showed that subclass of C3 or F10 was IgG_{2b} or IgG_1, respectively. 2) The M_r of IgG form (C3 and F10) was 150,000, estimated by SDS-PAGE. 3) The binding site for C3 is distinct from that for PRL or F10. 4) The binding site for F10 is very close to that for PRL but not the same as that for C3. 5) The denatured receptor could be detected with F10, suggesting that F10 recognizes primary structure. The denatured receptor could not be detected with C3, suggesting that C3 recognizes tertiary structure. 6) Both C3 and F10 discriminates PRL receptor from GH receptor. 7) Both C3 and F10 recognize the receptors in all the rabbit tissues tested.

Using two receptor-specific MAbs, I examined immunological relations of the receptor among rabbit mammary glands, livers, kidneys, ovaries and adrenal glands. By direct comparison of inhibition curves among the tissues, tissue specific receptor was observed when microsomes were used as a receptor source. After solubilization, however, the heterogeneity was not observed. These findings suggested that the heterogeneity is due to the

conformational difference in the microsomal PRL receptor among tissues.

Scatchard analysis of ^{125}I-MAb (C3 or F10) binding to the mammary microsome showed a curvilinear line, suggesting that the mammary gland contains a high and low affinity receptor for MAb binding. In contrast, Scatchard analysis of ^{125}I-PRL binding to the same microsome showed a linear regression line. Two major receptors (M_r of 77,000 and 41,000) have a close value of dissociation constant of PRL binding as demonstrated by Sakai et al. (1986, 1987).

2. Identification of PRL binding proteins by MAbs

To identify the species recognized by C3 or F10, immunoprecipitation and immunoblot experiments were performed (Chapter 4). The sample used was PRL-affinity-purified receptors in the rabbit mammary gland. Three bands (M_r of over 200,000, 77,000 and 41,000) were identified with F10 in the immunoblots. Four specific bands (M_r of over 200,000, 77,000, 41,000 and 25,000) were observed in the immunoprecipitation experiments by using C3 or F10 as a detection probe. The species of M_r=25,000 may be a component or subunit of the holo PRL receptor or PRL receptor itself. This species, however, did not react with MAbs under denatured conditions. Cross-linking studies showed that this preparation contained four PRL binding species (M_r of over 200,000, 96,000, 60,000 and 43,000). The differences in M_r before and after PRL binding were close to the M_r of monomeric PRL, suggesting that each PRL binding species reacts with one PRL molecule. These results were comparable with the findings of Sakai and Ike (1987) showing that two separate types of PRL receptors (M_r of 83,200 and 36,800) exist in the microsome and Chaps-solubilized fraction.

The existence of M_r=about 40,000 species in the mammary gland have been reported by several investigators. This was demonstrated by cross-linking of PRL-receptor complex (Haeuptle et al., 1983; Hughes et al., 1983; Katoh et al., 1985a; Ashkenazi et al., 1987) or by the purification of the receptor and SDS-PAGE analysis (Haeuptle et al., 1983; Necessary et al., 1984). In addition to the mammary gland, other tissues contain this PRL binding species (Katoh et al., 1985a). This species appears to be the major PRL-binding species in most tissues. PRL receptor cDNA were cloned in the liver from rats (Boutin et al., 1988) and from mice (Davis and Linzer, 1989). The predicted M_r value of the receptor is about 33,000 and is very close to that estimated by SDS-PAGE. As shown in Chapter 5, the M_r of 41,000 species has the molecular mass of carbohydrate with about 6,000. The existence of M_r=about 80,000 species in the mammary gland have been demonstrated in this study and others (Bonifacino and Dufau, 1985; Sakai and Ike, 1987). The M_r of about 80,000 species existed in the rat ovary (Mitani and Dufau, 1986; Buczko et al., 1989), testis (Bonifacino and Dufau, 1985) and Nb_2 cell line (Webb and Wallis, 1988). The existence of this binding species in the mammary gland have been controversial. In the earlier cross-linking studies and a combination of purification and SDS-PAGE analysis, the existence of the high M_r receptor was not reported. Recently, the other PRL receptor cDNA was cloned in the rabbit mammary gland (Edery et al., 1989), human hepatoma (Boutin et al., 1989), rat ovary (Zhang et al., 1990; Shirota et al., 1990) or rat liver (Shirota et al., 1990). Its size was longer than that isolated earlier and the predicted M_r was about 66,000.

The identification and M_r estimation of the receptor have been performed mainly by using the cross-linking technique. The M_r of the

receptor has routinely been estimated by subtracting the M_r of monomeric PRL on the assumption that the binding of PRL to the receptor is a reaction of 1 : 1. There is no evidence that according to this experimental procedure, the M_r of the receptor is evaluated precisely. To overcome this problem, immunoblot analysis and immunoprecipitation analysis were carried out in order to identify the receptor and to estimate the M_r of the receptor alone.

3. Multiple forms of PRL binding proteins

Purification of PRL receptor with high purity and recovery has been difficult due to very low concentrations of the receptor. The receptor is a membrane-integrated protein. The receptor must be solubilized prior to purification. Therefore, the solubilized fraction potentially contains proteolytic enzymes which will modify the native structure of the receptor. During purification, we should keep the activity of proteolytic enzymes low. Inclusion of detergent is also essential to keep the receptor soluble. These make it difficult to purify the receptor by the common protein purification technique. To purify the receptor successfully, highly specific method is necessary. Shiu and Friesen (1974b) initially attempted to purify the PRL receptor from rabbit mammary glands by hGH-coupled affinity chromatography. The subsequent purification studies utilizing PRL- or hGH-coupled affinity chromatography revealed that both the recovery and purity were low (Liscia and Vonderhaar, 1982; Haeuptle et al., 1983; Katoh et al., 1985; Sakai et al., 1985; Ashkenazi et al., 1987; Chapter 2). It is apparent: 1) high concentrations of magnesium chloride, a dissociating reagent, reduces the PRL binding activity and 2) proteolytic damage of the receptor cannot be by-passed. Therefore, I purified the receptor in the presence of a number of protease

inhibitors. The Triton X-100-solubilized receptors were passed through a normal IgG-coupled Sepharose column and then applied to a C3-coupled Sepharose column. The receptors were eluted with 1 M NaI instead of 4 or 5 M $MgCl_2$. About 20,000-fold purification over microsomes were achieved and the recovery was about 30 %. SDS-PAGE and silver staining revealed that the purified fraction contained ten species of protein. Among them, seven species were reacted with F10 on immunoblots. Cross-linking studies also showed that at least seven bands were observed. Enzymatic digestion of the M_r of 77,000 and 41,000 species indicated that both receptors had many identical peptide fragments, suggesting that both species were derived from the same gene. Glycanase digestion studies showed that size-heterogeneity is not due to the difference in carbohydrate chains. It is more likely that the size-heterogeneity is caused by the difference of polypeptide length. This is also supported by Northern blot analysis using the receptor-specific cDNA probe.

Northern blot analysis using the rabbit cDNA probe showed that several species of mRNA were present in the rabbit mammary gland (Edery et al., 1989). Furthermore, size-heterogeneous mRNAs were found in the rat ovary (Zhang et al., 1990), liver (Shirota et al., 1990) and human breast cancer (Boutin et al., 1989). These findings suggest that size-heterogeneous receptors were produced by alternative splicing.

5. Significance of multiple forms of PRL receptor and its distinct role on signal transduction

The probable explanation of significance of multi-forms of PRL receptors in the mammary gland is that one PRL binding protein exists in one

type of a cell and that each PRL binding species transduces its specific signal through the membrane. In the mammary gland, PRL is involved in development of mammary cells and lactogenesis (Kelly et al., 1984; Meites, 1988). The concentration of the receptor varies depending on the physiological conditions (Kelly et al., 1974; Djiane et al., 1977; Guillaumot et al., 1984). In the mammary gland, the number of the receptor increases gradually during pregnancy and remains high during lactation (Bohnet et al., 1974; Holcomb et al., 1976; Djiane et al., 1977; Hayden et al., 1979; Sakai et al., 1981; Grisson and Littleton, 1988). Furthermore, an increase in the affinity in late lactating rabbits was reported (Perry and Jacobs, 1978). In addition, the affinity of the receptor in the liver fluctuates during estrous cycle (Guillaumot et al., 1988). Dominant species of receptor in the liver and Nb_2 cell have M_r of about 40,000, and 80,000, respectively (Webb and Wallis, 1988). PRL induces proliferation of Nb_2 cells (Shiu et al., 1983). Probably, the M_r of about 80,000 species are involved in mediating proliferative action of PRL. The cDNA analysis of short liver PRL receptor (protein M_r=about 40,000) revealed that it has a short cytoplasmic domain (Boutin et al., 1988) like transferrin (Schneider et al., 1984), low density lipoprotein (Yamamoto et al., 1984) and insulin-like growth factor II /mannose-6-phosphate (Morgan et al., 1987) receptors. Boutin et al. (1988) suggested that this species is involved in transporting PRL from one compartment to another. PRL can be detected in milk, cerebral spinal fluid and semen.

Like other peptide hormones, PRL initially binds to its cell surface receptor in the mammary gland and ultimately regulates lactogenic activity. Even though a hormone has different biological functions depending on a kind of target organs, one type of the receptor has been identified regardless of

difference in the target organs. As shown here, however, the lactating mammary gland has many size-heterogeneous prolactin receptors. It is unclear about the biological significance of these receptors at present. It is well-known that PRL regulates the mammary growth, differentiation and milk synthesis. After parturition, the rate of the cell proliferation becomes to be a very low level and PRL regulates mainly the synthesis of the milk proteins. Mammary glands contain heterogeneous cell population; one synthesizes milk proteins; another proliferates. It is conceivable that one type of PRL receptor is present in one cell and that each receptor has its distinct role; growth, differentiation, and milk synthesis. I present here the hypothesis showing that the mammary gland switches the PRL action by changing the dominant species of the PRL receptors depending on the physiological conditions and that the binding of PRL to the target organ can generate the different PRL signals.

6. Conclusions

1) Rabbit mammary gland contains seven prolactin (PRL) binding species of protein, of which M_r is over 200,000, 100,000, 77,000, 63,000, 56,000, 44,000 and 41,000. Primary structure of PRL binding proteins are highly homologous each other. Size-heterogeneity is caused by the difference of polypeptide length.

2) All the PRL binding species of protein contain N- and O-linked carbohydrate chains in addition to sialic acids. The M_r of N-, O-linked chains or sialic acids is 4,000, 1,000 or 1,000, respectively. Carbohydrate chains linked to PRL receptor do not affect the affinity and specificity for hormones.

3) Two types (C3 and F10) of monoclonal antibodies (MAbs) directed to PRL receptor in the rabbit mammary gland were produced. Both MAbs recognize PRL receptor in the rabbit mammary glands, livers, adrenal glands, kidneys and ovaries. Tissue specific difference was observed when C3 and microsomes were used. After solubilization, tissue specificity was not observed. These findings suggest that similar but distinct receptor exist in the rabbit.

4) PRL binds to its receptor in the molar ratio of one to one.

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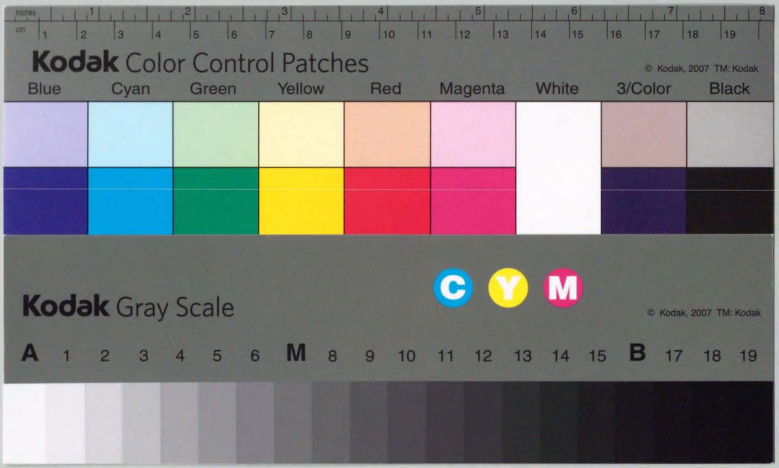
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