Binding and structural characteristics of a soluble lactogen-binding protein from rabbit mammary-gland cytosol

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Specific receptors for prolactin (PRL) are known to be present on plasma membranes and intracellular membranes of mammary gland. We now report, however, the detection and characterization of a soluble lactogen-specific binding protein in high-speed (200 000 g) cytosolic preparations from pregnant- and non-pregnant-rabbit mammary gland. The binding protein was not detectable by poly(ethylene glycol) precipitation; instead, bound and free ^{125}I -labelled human growth hormone (hGH; a potent lactogen) was separated using a mini-gel filtration technique. Specific binding of ^{125}I -hGH reached an apparent equilibrium between 10 and 14 h at 21–23 °C. It was dependent on mammary-gland protein concentration and, partially, on Ca^{2+} or Mg^{2+} concentrations. Scatchard analysis revealed steep curvilinear plots, the high-affinity component having a K_A of $\sim 3 \times 10^{10} \text{ M}^{-1}$. Gel filtration on calibrated Ultrogel AcA34 columns of ^{125}I -hGH–cytosol complexes or of cytosol alone, followed by measurement of ^{125}I -hGH binding in each eluted fraction, indicated that the binding protein had an M_r of 33 000–43 000. A specific binding protein of the same size was observed when ^{125}I -ovine or -human PRL, but not ^{125}I -bovine GH, was used as ligand. The apparent lactogenic specificity was confirmed by a lack of cross-reactivity of the binding protein with an anti-[GH receptor (rabbit liver)] monoclonal antibody. Polyacrylamide-gel electrophoresis of ^{125}I -hGH covalently cross-linked to cytosol with disuccinimidyl suberate revealed binding proteins of M_r 35 000 (non-reduced) and 37 000 (reduced), results comparable with those obtained by gel filtration and indicating an absence of inter-subunit disulphide bonds. These studies have shown the presence of an apparently naturally soluble lactogen-binding protein in the cytosolic fraction of rabbit mammary gland. The relationship between this binding protein and the membrane PRL receptor is not yet known.

INTRODUCTION

PRL receptors have been identified and characterized in a variety of tissues and species. Mammary-gland membranes from rats and rabbits in particular have been well studied, having been detergent-solubilized and purified by PRL- or biotin-affinity chromatography (Shiu & Friesen, 1974b; Haeuptle *et al.*, 1983; Necessary *et al.*, 1984; Katoh *et al.*, 1985a). Both polyclonal (Shiu & Friesen, 1976; Katoh *et al.*, 1984) and monoclonal antibodies (Katoh *et al.*, 1985b) have been raised against these purified preparations and, together with covalent labelling techniques, have been used to probe the PRL-receptor structure (Borst & Sayere, 1982; Haeuptle *et al.*, 1983; Hughes *et al.*, 1983; Necessary *et al.*, 1984; Katoh *et al.*, 1985a). Several of these studies have shown that the primary PRL-binding subunit of the solubilized receptor has an M_r of $\sim 35 000$.

We have recently shown that rabbit liver cytosol contains a soluble, specific, GH-binding protein that was not detected by the widely used poly(ethylene glycol) technique (Ymer *et al.*, 1984). These studies were extended to examine a variety of tissues from the rabbit to determine the ubiquity of this binding protein (Herington *et al.*, 1986). As a consequence, we observed the presence of an additional binding protein with lactogenic specificity in cytosolic preparations from kidney and mammary gland (Ymer & Herington, 1986).

The lactogen-binding factor was also not detectable by poly(ethylene glycol) precipitation.

We now report in detail an examination of the binding and structural characteristics of the soluble lactogen-binding species from rabbit mammary gland using gel filtration and covalent cross-linking techniques. A preliminary account of these studies has been published (Ymer & Herington, 1986).

EXPERIMENTAL

Materials

The following hormones were obtained from the National Hormone and Pituitary Program of the NIADDK, Bethesda, M.D., U.S.A.: hGH for iodination (NIAMDD-hGH-I-1), bGH (NIH-GH-B-18), oGH (NIH-GH-S-11), oPRL (NIAMDD-oPRL-15) and hPL (NIADDK-hPL). bGH used for iodination was generously given by Dr. M. Sonenberg, Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A., hPRL (^{125}I -labelled and unlabelled) was obtained through the WHO Matched Reagent Quality Control Program; hGH used for unlabelled standards was obtained from the Commonwealth Serum Laboratories (CSL), Melbourne, Vic., Australia. All other reagents, including M_r markers for gel chromatography and polyacrylamide-gel electrophoresis, were obtained as

Abbreviations used: GH, growth hormone; PRL, prolactin; PL, placental lactogen (the prefixes b, h, and o refer to the bovine, human and ovine hormones respectively); DSS, disuccinimidyl suberate; PMSF, phenylmethanesulphonyl fluoride; k.i.u., kallikrein-inhibitory unit.

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previously described (Ymer & Herington, 1984, 1985; Ymer *et al.*, 1984; Herington *et al.*, 1986).

Preparation of rabbit mammary-gland cytosol

Mammary glandular tissue was dissected from 28 day-pregnant female New Zealand White rabbits and homogenized in 0.3 M-sucrose (5 ml/g of mammary gland) containing 1 mM-PMSF and 1000 k.i.u. of Trasylol/ml. The homogenate, which was not buffered for pH, was subjected to serial centrifugation at 1500 g (20 min), 15000 g (20 min) and 100000 g (90 min). The 100000 g supernatant was then re-centrifuged at 200000 g (90 min) and the resulting supernatant filtered through a 0.22 μ m-pore-size Millipore filter. This preparation was used as the 'high-speed' cytosol for all binding studies. Protein concentrations (10–13 mg/ml of cytosol and representing a recovery of ~3 mg of protein/g of mammary gland) were estimated by the Coomassie Blue method (Bradford, 1976).

Iodination and binding studies

hGH was iodinated, essentially as previously described (Ymer *et al.*, 1984), by using the Iodogen method (Salacinski *et al.*, 1981), with a hGH/Na¹²⁵I ratio of 2:1. The ¹²⁵I-hGH was separated on a Sephadex G-50 column (0.8 cm $\times$ 19 cm) and subsequently repurified on Ultrogel AcA54 (1 cm $\times$ 55 cm) before use. Specific radioactivities of 30–50 Ci/g were obtained.

Mammary-gland cytosol (50 μ l; 2.4 mg of protein/ml) was incubated with ¹²⁵I-hGH (20000–30000 c.p.m.; 76–114 pM) in 25 mM-Tris/HCl, pH 7.5, containing 10 mM-CaCl₂, 0.1% bovine serum albumin and 0.02% NaN₃ in a final volume of 250 μ l at 21 °C. Separation of bound and free hormone was achieved by gel filtration of the entire reaction mixture on Ultrogel AcA44 mini-columns (0.6 cm $\times$ 22 cm) as described previously (Ymer & Herington, 1985). Specific binding was calculated as the difference in binding in the presence (non-specific binding) and absence (total binding) of an excess (0.1 μ M) of unlabelled hGH.

Gel filtration of mammary-gland cytosol

Characterization of the M_r of the mammary-gland cytosolic binding species was initially carried out by gel filtration at 4 °C on an Ultrogel AcA34 column (1 cm $\times$ 90 cm). Equilibration, elution and calibration of the column were carried out as described previously (Ymer & Herington, 1985). When ¹²⁵I-hGH–cytosol complexes (250 μ l; 20000–30000 c.p.m.), formed in the presence and absence of unlabelled hGH, were gel-filtered, the elution profile of the lactogen-binding species was determined by simply counting the radioactivity of each fraction. When cytosol alone (1–2 ml) was chromatographed, the binding profile was determined by incubation of 50 μ l aliquots of each column fraction (1 ml) with ¹²⁵I-hGH for 16 h, followed by measurement of specific binding by separation of bound and free GH on AcA44 mini-columns.

¹²⁵I-hGH binding in the presence of an anti-(GH receptor) monoclonal antibody

A hybridoma cell line (D6) that produces a monoclonal antibody against the GH-binding region of the purified GH receptor from rabbit liver membranes (Simpson *et al.*, 1983) was a gift from Dr. H. G. Friesen, Department of Physiology, University of Manitoba, Winnipeg,

Canada. Antibody-containing cell culture medium or control medium (150 μ l) was preincubated with mammary-gland or rabbit liver cytosols (50 μ l; 4 and 2 mg of protein/ml respectively) at 23 °C for 17–24 h before the addition of ¹²⁵I-hGH (or ¹²⁵I-oPRL) (20000 c.p.m., 76 pM). Specific binding of ¹²⁵I-hGH or ¹²⁵I-oPRL was determined during the next 2 h (liver) or 4 h (mammary gland).

Affinity chromatography on concanavalin A–Sephacrose

Mammary-gland cytosol (500 μ l; 12 mg of protein/ml) was applied to a column of concanavalin A–Sephacrose (1.7 ml bed volume) equilibrated in 25 mM-Tris/HCl/10 mM-CaCl₂/0.02% NaN₃, pH 7.5. Fractions (15 $\times$ 1 ml) were collected in the same buffer and then elution (15 $\times$ 1 ml fractions) of bound glycoproteins was effected by application of 0.3 M α -methyl mannopyranoside in the absence of Ca²⁺. An aliquot (50 μ l) of each fraction (both 'bound' and 'unbound' peaks) was assayed as above for ¹²⁵I-hGH binding.

Covalent cross-linking of ¹²⁵I-hGH to mammary-gland cytosol

¹²⁵I-hGH was first purified on Ultrogel AcA54 as described above, but in the complete absence of bovine serum albumin, which has been reported in earlier studies (Ymer & Herington, 1984) to become involved in significant non-specific cross-linking reactions. To minimize loss of ¹²⁵I-hGH, the purification and subsequent steps were carried out in silicone-treated glass tubes. The 'peak' tubes were pooled and the buffer changed to 25 mM-sodium phosphate, pH 7.5, by gel filtration on 6 ml-volume Ultrogel AcA202 or Sephadex G-50 mini-columns. ¹²⁵I-hGH [(0.5–1.0) $\times$ 10⁶ c.p.m.] ($\pm$ excess unlabelled hGH or other hormones) was then incubated with mammary-gland cytosol (4–5 mg of protein/ml) in 25 mM-phosphate buffer, pH 7.5, containing 10 mM-MgCl₂ and 0.01% merthiolate, in silicone-treated glass tubes, for 18–24 h at 21 °C in a total volume of 250 μ l. Covalent cross-linking of bound ¹²⁵I-hGH was accomplished, as described previously (Ymer & Herington, 1984), by addition of DSS in dimethyl sulphoxide, to a final concentration of 0.5 mM. The cross-linking reaction was terminated after 15 min at room temperature by removal of 50 μ l aliquots, which were boiled (3 min) in the presence of 2% (w/v) SDS with or without 100 mM-dithiothreitol (final concns.). Each sample was then subjected to SDS/polyacrylamide-gel electrophoresis on vertical slab gels [0.5 mm thick; 7.5% or 10% (w/v) acrylamide] by the general method of Laemmli (1970) as described previously (Ymer & Herington, 1984).

RESULTS

Initial experiments involved the incubation of ¹²⁵I-hGH with rabbit mammary-gland cytosol, followed by an attempt to separate bound and free hormone by precipitation with poly(ethylene glycol) (12.5–16% final concn.). By using this methodology, less than 1% of the ¹²⁵I-hGH appeared to be specifically bound (results not shown). In contrast, however, when equivalent ¹²⁵I-hGH–cytosol complexes were gel-chromatographed on Ultrogel AcA34 (Fig. 1), a distinct peak (36% of total c.p.m.) of specifically bound ¹²⁵I-hGH was observed, eluted at M_r ~ 65000. The specific nature of the binding was demonstrated by the complete absence of this peak when

Fig. 1. A gel filtration profile on Ultrogel AcA34 (1 cm $\times$ 90 cm) of (a) $^{125}\text{I-hGH}$ -binding protein complex formed in the absence (—, total binding) or presence (---, non-specific binding) of excess (1 μM) unlabelled hGH, and (b) mammary-gland cytosol alone, with specific binding of $^{125}\text{I-hGH}$ (●) determined in 100 μl aliquots from individual (1 ml) column fractions

The elution positions of M_r markers and binding protein or hGH-binding protein complex are shown by arrows ($\downarrow$). Abbreviation used: 200K etc., 200000 M_r etc. Similar data were obtained in at least five experiments of each type.

the original mixture included excess unlabelled hGH. Two other peaks of radioactivity were observed: peak 2, eluted in the position of free $^{125}\text{I-hGH}$, and peak 3, eluted at the total volume of the column and probably represents small degradation products or free $^{125}\text{I}^-$. The same specifically bound peak ($M_r \sim 65000$) was observed in other experiments (not shown) when $^{125}\text{I-hPRL}$ or $^{125}\text{I-oPRL}$, but not $^{125}\text{I-bGH}$, were used as ligand. These data strongly suggest that the mammary-gland cytosolic binding species has lactogenic-hormone-specificity. The lactogen-binding activity was also demonstrated in cytosols prepared from non-pregnant-female-rabbit mammary gland, indicating that it is not a pregnancy-specific observation (results not shown). Given that the $^{125}\text{I-lactogen-cytosol}$ complex was eluted with an $M_r \sim 65000$ and assuming a 1:1 binding stoichiometry, one can calculate, on the basis of an M_r of 22000 for GH, that the binding species itself has an $M_r \sim 43000$. A slightly lower M_r (~ 33000) was found, however, when cytosol alone was gel-chromatographed and specific binding detected subsequently by incubation of column fractions with $^{125}\text{I-hGH}$ (Fig. 1b).

This experimental approach clearly demonstrated the presence of a specific-lactogen binding component in mammary-gland cytosol. However, a less time-consuming and more convenient mini-gel-filtration system (Ymer & Herington, 1985) has been used for routine and multiple analysis; all of the following binding data were obtained by using this mini-system.

Specific binding of $^{125}\text{I-hGH}$ was dependent on the cytosolic protein concentration used (Fig. 2a) as well as being partially dependent on the Ca^{2+} or Mg^{2+}

concentrations (Fig. 2b). The time courses for association and dissociation of $^{125}\text{I-hGH}$ are illustrated in Fig. 3. At room temperature (21–23 $^{\circ}\text{C}$), the association reaction was relatively slow, with an apparent steady state being reached between 10 and 14 h. In several experiments this equilibrium was maintained for at least another 24 h, although in the experiment illustrated there was a gradual increase during this time. At 37 $^{\circ}\text{C}$ (Fig. 3b), the binding was more rapid, with a steady state being attained by ~ 8 h. Reversibility of binding was examined after the addition of an excess of unlabelled hGH (0.5 μg) after 3 h of incubation. At 23 $^{\circ}\text{C}$, dissociation of bound $^{125}\text{I-hGH}$ was minimal ($\sim 20\%$) over a period of > 24 h (Fig. 3a). This observation is consistent with either a completely irreversible reaction or with simply a very slow dissociation rate. In effect, the added unlabelled hGH simply prevented any further binding of $^{125}\text{I-hGH}$ without causing any dissociation of previously bound $^{125}\text{I-hGH}$. At 37 $^{\circ}\text{C}$ the same phenomenon was observed, with only 25% reversibility of $^{125}\text{I-hGH}$ binding over 24 h (Fig. 3b). The apparent irreversibility of binding to mammary-gland cytosol was absolute at both 23 $^{\circ}\text{C}$ and 37 $^{\circ}\text{C}$ if the addition of excess unlabelled hGH was delayed until ~ 24 h (i.e. after establishment of the binding steady state) (results not shown).

The hormonal specificity of the cytosolic binding species was tested by incubation with $^{125}\text{I-hGH}$ in the presence and absence of various unlabelled lactogenic and somatotrophic hormones (Fig. 4). Essentially complete displacement was achieved with the lactogenic hormones hGH, oPRL, hPRL and hPL, whereas very little displacement was seen with the non-lactogenic

Fig. 2. Dependence of ^{125}I -hGH-specific binding on (a) mammary-gland cytosol protein concentration and (b) Ca^{2+} and Mg^{2+} ion concentration

Binding was carried out as described in the Experimental section, except for the variable under test. Each data point is derived from Ultrogel AcA44 mini-column separation of bound and free hormone and is the mean of duplicate determinations which differed by less than 6% in all cases. Three experiments of each kind were performed.

Fig. 4. Hormonal specificity of binding of ^{125}I -hGH to mammary-gland cytosol

All unlabeled hormones were added at a final concentration of 100 nM. Binding is expressed as a percentage of total binding in the absence of unlabeled hormone (100%). Similar data were obtained in three experiments.

hormone oGH. The bGH preparation used in this experiment (NIH-GH-B18) did give considerable displacement despite (i) its theoretical non-lactogenic activity, and (ii) a relatively poor cross-reactivity against the lactogen receptor of mammary-gland microsomal membranes (results not shown). Other bGH preparations (USDA-bGH-B-1; NIH-bGH-100 3A) have since been tested and show a much lower degree of cross-reactivity in the cytosol system. Furthermore, as indicated above, no binding of ^{125}I -bGH to mammary-gland cytosol was detectable. Finally, confirmation that the cytosol binding activity did not have specificity for somatotrophic hormones *per se* came from studies utilizing a monoclonal antibody raised against the rabbit liver GH receptor (Simpson *et al.*, 1983) (Fig. 5). Although the antibody was able to block completely ^{125}I -hGH-specific binding to rabbit liver membranes, it was completely ineffective in

Fig. 3. Time course of association and dissociation for the specific binding of ^{125}I -hGH to rabbit mammary-gland cytosol at (a) 23 °C and (b) 37 °C

The binding was determined as for Fig. 2, with each data point being the mean of duplicate measurements. In both (a) and (b), dissociation was initiated in a parallel set of tubes by the addition (indicated by $\uparrow$) of an excess (0.5 μg) of unlabeled hGH in a 10 μl volume to avoid dilution effects. Similar data were obtained in five experiments.

Fig. 5. Effect of anti-(GH receptor) monoclonal antibody on ^{125}I -hGH and ^{125}I -oPRL binding to rabbit mammary-gland and liver cytosols

Data represent the mean of duplicate determinations, which differed by less than 5%, and are expressed as a percentage of control binding (100%; no monoclonal antibody). Two such experiments were performed. The liver cytosol was used as a positive control for a somatotrophic, but not lactogenic-binding, protein preparation (Ymer *et al.*, 1984).

Fig. 6. A representative Scatchard plot for ^{125}I -hGH-specific binding to mammary-gland cytosol

The bound hormone is expressed as fmol of hormone bound per mg of cytosol protein. The calculated mean affinities for the high- and low-affinity sites from six such experiments are given in the text.

blocking the binding of ^{125}I -hGH or ^{125}I -oPRL to mammary-gland cytosol.

Although binding of ^{125}I -hGH to cytosol appears to be largely irreversible, Scatchard analysis has been applied to classical competition curves based on displacement of a fixed concentration of ^{125}I -hGH with unlabelled hGH (Fig. 6). No correction was made for the bindability of the ^{125}I -hGH ($\sim 80\%$; see Fig. 2). This analysis was made, despite its theoretical invalidity, simply to allow some comparison with previous data for lactogen receptors of mammary-gland membranes rather

than as a definitive quantitative measurement of binding affinity. Steep curvilinear Scatchard plots consistent with a poorly reversible reaction were obtained in several experiments. A graphical estimate, based on the analytical method of Weder *et al.* (1974) and assuming two classes of site, gave a high-affinity class (concn. 67 ± 6 fmol/mg of protein) with a K_A of $(2.8 \pm 0.4) \times 10^{10} \text{ M}^{-1}$ (mean $\pm$ S.E.M., $n = 6$). The more-abundant lower-affinity site had a K_A of $\sim 1 \times 10^9 \text{ M}^{-1}$. These data contrast with those for mammary-gland membrane binding of ^{125}I -hGH (not shown), where a single class of site with a K_A of $3 \times 10^9 \text{ M}^{-1}$ was observed. Both this value and that for the high-affinity cytosol site are within the range reported for PRL receptors in a variety of different tissues and species (Shiu & Friesen, 1974a; Bonifacino *et al.*, 1978; Suard *et al.*, 1979; Borst & Sayere, 1982; Liscia & Vonderhaar, 1982; Rae-Venter & Dao, 1982; Gavish *et al.*, 1983; Ferland *et al.*, 1984; Sakai *et al.*, 1985).

The chemical and structural nature of the cytosol binding species has also been studied. Incubation of cytosol (2 mg/ml) for 30 min at 37°C with trypsin (1 mg/ml) in the presence (control) or absence of soybean trypsin inhibitor (10 or 100 mg/ml) led to a substantial reduction in the subsequent ability of the cytosol to bind ^{125}I -hGH (control specific binding, 100%; + trypsin, 21%). This suggests that the binding entity is proteinaceous in nature. Furthermore, application of cytosol alone (Fig. 7) or ^{125}I -hGH-cytosol complex (results not shown) to a concanavalin A-Sepharose affinity column revealed the presence of two types of mammary-gland cytosol binding protein: a glycoprotein, which bound to the column, and a differently- or non-glycosylated protein, which did not bind to the column. The non-bound material did not result from column overloading.

The subunit structural nature of the binding protein was determined by SDS/polyacrylamide-gel electrophoresis and autoradiography after covalent cross-linking of ^{125}I -hGH to the binding protein with DSS. A typical autoradiograph, which also demonstrates the lactogenic

Fig. 7. A representative protein (●) and ^{125}I -hGH binding (▨) profile after affinity chromatography of mammary-gland cytosol on concanavalin A-Sepharose

The experiment was carried out as described in the Experimental section and is one of three such studies performed. Abbreviation: MEM, α -methyl mannopyranoside.

Fig. 8. Autoradiograph of an SDS/polyacrylamide-gel pattern of ^{125}I -hGH covalently cross-linked to rabbit mammary-gland cytosol

The lanes represent total binding (lane A) or non-specific binding (lane B, +excess hGH; lane C, bGH; lane D, oGH; lane E, oPRL). The gel was run under non-reduced conditions. The M_r of protein markers, and specifically labelled bands are shown. Conditions of cross-linking are given in the Experimental section. Similar specifically labelled bands [M_r 55000, non-reduced; M_r 58000, reduced (results not shown)] were obtained in five experiments.

specificity, is shown in Fig. 8. In lane A, one strongly cross-linked band was observed at M_r 55000, which was completely displaced (lane B) when the initial binding incubation was carried out in the presence of excess unlabelled hGH. This band was also displaced by oPRL (lane E), but not by bGH (lane C) or oGH (lane D) and, therefore, represented a specific lactogen-binding protein. A second band ($M_r < 45000$) was deemed non-specific, since it was not displaced by unlabelled hGH in lane B. A second faintly cross-linked, diffuse, but specifically labelled, band was occasionally observed at M_r 70000. In the presence of 100 mM-dithiothreitol (results not shown) the M_r of the major specific band increased slightly to 58000. Assuming a 1:1 binding stoichiometry with ^{125}I -hGH (M_r 20000 non-reduced; M_r 22000 reduced; results not shown, but obtained in separate experiments using 15% polyacrylamide gels), then the M_r of the major mammary-gland cytosol binding protein is 35000 and 36000 under non-reduced and reduced conditions respectively. The mean ($\pm$ S.E.M.) values obtained for a number of gels (7.5 or 10% polyacrylamide) were 35700 ± 200 ($n = 6$, non-reduced) and 36800 ± 730 ($n = 5$, reduced). These changes in M_r with oxidation/reduction state, although minimal, were consistently observed and may reflect the presence of intramolecular disulphide bonds within the lactogen-binding protein. Furthermore, these M_r values agree well with that

estimated by gel filtration (Fig. 1). The lactogenic specificity of the cross-linked ^{125}I -hGH-binding protein complex was demonstrated by the complete disappearance of the bands in the presence of excess hGH and oPRL but their retention in the presence of oGH and bGH. The significance of the faintly cross-linked, higher- M_r (~ 70000), band, which was not observed on all gels, is not yet known.

DISCUSSION

These results have demonstrated that a 200000 g cytosolic preparation from rabbit mammary gland contains a highly specific lactogen-binding glycoprotein. Of note was the observation that this binding protein was not detectable by the traditional poly(ethylene glycol) separation method widely used for soluble hormone-binding systems. This lack of poly(ethylene glycol) detectability is a common feature observed for somatotropin-binding proteins recently identified in similar cytosolic preparations from other tissues and serum of the rabbit (Ymer *et al.*, 1984; Ymer & Herington, 1985; Herington *et al.*, 1986). It is clear, on a number of grounds (size, specificity, antigenicity, binding properties), however, that the mammary-gland cytosol binding protein is distinct from the somatotropin(GH)-binding protein reported in these other tissues. Apparently naturally soluble PRL-binding proteins have been reported previously in lung and liver of female or oestradiol-17 β -treated male rats (Amit *et al.*, 1984) and in the 100000 g supernatant of light-density membrane fractions of pig mammary gland (Sakai *et al.*, 1985). In contrast with our data, however, these other binding proteins were precipitable by poly(ethylene glycol) and were of much higher M_r .

The binding characteristics of the rabbit mammary-gland cytosol binding protein are typical of those for membrane-bound PRL receptors. The binding of ^{125}I -hGH (a potent lactogen) to the soluble binding protein was a time- and temperature-dependent, saturable, process. The hormonal specificity was clearly that of a lactogen-preferring binding protein. Of some interest was the very slow dissociation/apparent irreversibility of the binding. This is a common finding for binding of PRL to the lactogenic receptor of several tissues of rats and rabbits (Shiu & Friesen, 1974a; Rae-Venter & Dao, 1982; Suard *et al.*, 1979). We examined the dissociation reaction at incubation times both before (3 h) and after (24 h) binding had reached an apparent steady state, since previous reports (Van der Gugten *et al.*, 1980; Kelly *et al.*, 1983) had indicated that PRL binding became 'tighter', and therefore less dissociable, with longer incubations. Our data show, however, that the binding was poorly ($\sim 20\%$) reversible, regardless of the duration of incubation or the dissociation temperature (23 or 37 $^{\circ}\text{C}$). Although it has been suggested (Van der Gugten *et al.*, 1980) that the irreversibility of PRL-receptor interactions *in vitro* may not be a true reflection of the situation *in vivo*, no adequate explanation (mechanistic or functional) for this phenomenon is currently available.

The M_r of the mammary-gland binding protein was shown to be 43000 and 33000 on the basis of gel filtration of ^{125}I -hGH-cytosol complex or cytosol alone respectively. The difference may be due to a change in the conformation of the binding protein once complexed to

^{125}I -hGH or to unrecognized protein-protein or protein-gel interactions. The M_r of the binding protein was also shown to be 35000 or 37000 by covalent cross-linking techniques (under either non-reduced or reduced conditions and again assuming a 1:1 hGH/binding protein stoichiometry). These values are much smaller than those (M_r 150000–340000) reported by gel filtration of detergent-solubilized lactogen receptors (Shiu & Friesen, 1974b; Bonifacino *et al.*, 1978; Sasaki *et al.*, 1982; Gavish *et al.*, 1983) or rat liver cytosol PRL-binding protein (Amit *et al.*, 1984). The major exception to this is the work of Liscia & Vonderhaar (1982), who showed that the M_r of 3-[(3-cholamidopropyl)dimethylammonio]propanesulphonic acid ('Chaps')-solubilized rat liver PRL receptor was 37000, a value confirmed more recently for several tissues/species by covalent cross-linking studies (Hughes *et al.*, 1983; Haeuptle *et al.*, 1983; Bonifacino & Dufau, 1984; Necessary *et al.*, 1984; Sakai *et al.*, 1985; Katoh *et al.*, 1985a). Possible explanations for the much larger estimation of M_r by most gel-filtration studies include: (i) overestimation of M_r due to detergent (Triton X-100) complexing [a 30% contribution according to Jaffe (1982)]; (ii) random aggregation of the 35000- M_r binding protein; (iii) the existence of a 35000- M_r primary PRL-binding subunit within a larger non-covalently linked binding-protein complex. The results obtained in the present study, however, indicate (by gel filtration) that the 35000–43000- M_r binding protein exists as a distinct entity in rabbit mammary cytosols, there being no evidence for a higher- M_r form.

The source of the cytosolic binding protein is unknown at present. It is in fact difficult to be sure whether the binding protein resides naturally, in a soluble form, within the cytoplasmic compartment or whether it is normally associated with the plasma membrane or intracellular membranes of the mammary gland. Attempts to differentiate between these possibilities, by very gentle hand homogenization of mammary gland versus mechanical/cutting-blade homogenization, indicated no differences in the ability of each preparation to bind ^{125}I -hGH (S.I. Ymer & A.C. Herington, unpublished work). This suggests that the binding protein may exist as such in the cytosolic environment, although it remains possible that even gentle hand homogenization is sufficient to disrupt the protein from membranes. This may be particularly true of intracellular membranes (Golgi, prelysosomal or lysosomal fractions), which are known (Josefsberg *et al.*, 1979; Khan *et al.*, 1981; Ferland *et al.*, 1984) to contain relatively high concentrations of PRL receptor. Alternatively, the cytosolic binding protein may represent newly synthesized receptor awaiting incorporation into membranes or recently internalized, but not yet degraded, plasma-membrane receptor. Studies with anti-(PRL receptor) antibodies would help resolve some of these possible relationships.

Nevertheless, these data have clearly shown the presence of an apparently naturally soluble lactogen-binding protein in the high-speed cytosolic fraction of rabbit mammary gland. Although the relationship between this binding protein and the membrane PRL receptor is not yet known, the ability to obtain the binding protein in high yield, without the use of detergents, should facilitate the purification and full characterization of this binding protein.

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REFERENCES

- Amit, T., Barkey, R. J., Gavish, M. & Youdim, M. B. H. (1984) *Endocrinology* (Baltimore) **114**, 545–552
- Bonifacino, J. S. & Dufau, M. L. (1984) *J. Biol. Chem.* **259**, 4542–4549
- Bonifacino, J. S., Sanchez, S. H. & Paladini, A. C. (1978) *Biochem. Biophys. Res. Commun.* **85**, 62–69
- Borst, D. W. & Sayere, M. (1982) *Biochem. Biophys. Res. Commun.* **105**, 194–201
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Ferland, L. H., Djiane, J., Houdebine, L.-M. & Kelly, P. A. (1984) *Endocrinology* (Baltimore) **115**, 1842–1849
- Gavish, M., Amit, T., Barkey, R. J. & Youdim, M. B. H. (1983) *FEBS Lett.* **154**, 81–86
- Haeuptle, M.-T., Aubert, M. L., Djiane, J. & Kraehenbuhl, J.-P. (1983) *J. Biol. Chem.* **258**, 305–314
- Herington, A. C., Ymer, S. I., Roupas, P. & Stevenson, J. L. (1986) *Biochim. Biophys. Acta* **881**, 236–240
- Hughes, J. P., Simpson, J. S. A. & Friesen, H. G. (1983) *Endocrinology* (Baltimore) **112**, 1980–1985
- Jaffe, R. C. (1982) *Biochemistry* **21**, 2936–2939
- Josefsberg, Z., Posner, B. I., Patel, B. & Bergeron, J. J. M. (1979) *J. Biol. Chem.* **254**, 209–214
- Katoh, M., Djiane, J., Leblanc, G. & Kelly, P. A. (1984) *Mol. Cell. Endocrinol.* **34**, 191–200
- Katoh, M., Djiane, J. & Kelly, P. A. (1985a) *Endocrinology* (Baltimore) **116**, 2612–2620
- Katoh, M., Djiane, J. & Kelly, P. A. (1985b) *J. Biol. Chem.* **260**, 11422–11429
- Kelly, P. A., Djiane, J. & Leblanc, G. (1983) *Proc. Soc. Exp. Biol. Med.* **172**, 219–224
- Khan, M. N., Posner, B. I., Verma, A. K., Khan, R. J. & Bergeron, J. J. M. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4980–4984
- Laemmli, U. K. (1970) *Nature* (London) **227**, 680–685
- Liscia, D. S. & Vonderhaar, B. K. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5930–5934
- Necessary, P. C., Humphrey, P. A., Mahajan, P. B. & Ebner, K. E. (1984) *J. Biol. Chem.* **259**, 6942–6946
- Rae-Venter, B. & Dao, T. L. (1982) *Biochem. Biophys. Res. Commun.* **107**, 624–632
- Sakai, S., Katoh, M., Berthon, P. & Kelly, P. A. (1985) *Biochem. J.* **224**, 911–922
- Sasaki, N., Tanaka, Y., Imai, Y., Tsushima, T. & Matsuzaki, F. (1982) *Biochem. J.* **203**, 653–662
- Salacinski, P. R. P., McLean, C., Sykes, J. E. C., Clement-Jones, V. V. & Lowry, P. J. (1981) *Anal. Biochem.* **117**, 136–146
- Shiu, R. P. C. & Friesen, H. G. (1974a) *Biochem. J.* **140**, 301–311
- Shiu, R. P. C. & Friesen, H. G. (1974b) *J. Biol. Chem.* **249**, 7902–7911
- Shiu, R. P. C. & Friesen, H. G. (1976) *Biochem. J.* **157**, 619–626
- Simpson, J. S. A., Hughes, J. P. & Friesen, H. G. (1983) *Endocrinology* (Baltimore) **112**, 2137–2141
- Suard, Y. M. L., Kraehenbuhl, J.-P. & Aubert, M. L. (1979) *J. Biol. Chem.* **254**, 10466–10475
- Van der Gugten, A. A., Waters, M. J., Murthy, G. S. & Friesen, H. G. (1980) *Endocrinology* (Baltimore) **106**, 402–411
- Weder, H. G., Schildknecht, J., Lutz, R. A. & Kesselring, P. (1974) *Eur. J. Biochem.* **42**, 475–481

- Ymer, S. I. & Herington, A. C. (1984) *Endocrinology* (Baltimore) **114**, 1732–1739
- Ymer, S. I., & Herington, A. C. (1985) *Mol. Cell. Endocrinol.* **41**, 153–161

- Ymer, S. I. & Herington, A. C. (1986) *Biochem. Soc. Trans.* **14**, 644–645
- Ymer, S. I., Stevenson, J. L. & Herington, A. C. (1984) *Biochem. J.* **221**, 617–622

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