A comparison of lactogenic receptors from rat liver and Nb2 rat lymphoma cells by using cross-linking techniques

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Lactogenic receptors were analysed with the use of the cross-linking agent disuccinimidyl suberate to attach covalently ^{125}I -labelled ovine prolactin or human growth hormone to binding sites from (1) liver from pregnant rats and (2) the rat-derived Nb2 lymphoma cell line. Analysis by SDS/polyacrylamide-gel electrophoresis of the proteins cross-linked to labelled hormone in rat liver indicated a major specifically-labelled complex with an M_r of 68 000–72 000, when run under reducing or non-reducing conditions. With Nb2 cells a major specifically-labelled complex with an M_r of 97 000–110 000 was identified, but only when electrophoresis was run using reducing conditions. Assuming one hormone molecule (M_r 22 000–24 000) per hormone–receptor complex, then the receptor proteins have an M_r of 44 000–50 000 for rat liver and 73 000–88 000 for the Nb2 cells. For both cell types the receptors were of lactogenic specificity; lactogenic hormones competed for binding whereas somatogenic hormones did not. These studies suggest that the lactogenic receptors in rat liver membranes and Nb2 cells differ in two respects. Firstly, the M_r of the labelled receptor protein in Nb2 cells is greater than that of the corresponding receptor protein in rat liver membranes; secondly, the Nb2 cell receptor appears to exist as a disulphide-linked oligomer whereas the receptor in rat liver membranes does not.

INTRODUCTION

Specific binding sites for lactogenic hormones are found in various tissues in the rat (reviewed by Wallis, 1980; Hughes *et al.*, 1985), including liver (Posner *et al.*, 1974; Postel-Vinay, 1976; Borst *et al.*, 1985), mammary gland (Bohnet *et al.*, 1977), ovary (Mitani & Dufau, 1986), lung (Amit *et al.*, 1984), adrenal gland (Calvo *et al.*, 1981) and kidney (Mountjoy *et al.*, 1980). In this study liver from pregnant rat was chosen as a source of normal rat lactogenic receptors, since previous studies have shown that the concentration of hepatic lactogenic receptors is large and that the receptor concentration is much greater in females than in males and increases during pregnancy (Kelly *et al.*, 1974). Lactogenic receptors have also been observed in a malignant rat lymphoma (Nb2) cell line (Shiu *et al.*, 1983), the growth of which is dependent on lactogenic hormones. The receptors from this cell line appear to have a greater affinity for lactogenic hormones than those found in normal rat tissue (Shiu *et al.*, 1983). This could be a factor contributing to the malignancy of the cell line since, in the normal female rat, the prolactin (PRL) concentration rarely falls below the level (1 ng/ml) that maintains maximal growth of Nb2 cells.

The purpose of the work described in this paper was to compare lactogenic receptors on the Nb2 cell line with those from normal rat liver, in order to determine whether the difference in affinity is associated with major structural differences. Cross-linking techniques were used, in combination with SDS/polyacrylamide gel electrophoresis, to determine M_r values for complexes between hormone and receptor (or receptor subunits) for the two tissues. Previous authors have demonstrated that a specific protein–receptor complex can be identified

following cross-linking in rat liver preparations (Hughes *et al.*, 1983; Yamada & Donner, 1984; Borst *et al.*, 1985; Kelly *et al.*, 1985) and recently Ashkenazi *et al.* (1987) have identified such a complex in several lines derived from Nb2 cells using ^{125}I -labelled human growth hormone (hGH). However, a direct comparison of the receptors from normal and lymphoma-derived cells has not been carried out. In this study, receptors were cross-linked to ^{125}I -labelled ovine prolactin (oPRL) or ^{125}I -labelled hGH using the homobifunctional reagent disuccinimidyl suberate (DSS). Both of these hormones are lactogenic in the rat, whereas non-primate growth hormones are not. Emphasis here has been placed on binding specificities and on the use of labelled oPRL, since hGH has both somatogenic (growth-promoting) and lactogenic activity in this species, and may bind to both somatogenic and lactogenic receptors.

MATERIALS AND METHODS

Materials

hGH was a gift from the Wellcome Laboratories and the DHSS/MRC joint advisory committee. Ovine GH (NIH-GH-S10), rat GH (NIAMDD rGH-B-3), ovine PRL (NIAMDD oPRL-14) and rat PRL (NIAMDD rPRL-B-2) were gifts from Dr. A. F. Parlow, Dr. A. E. Wilhelmi and The National Institute of Arthritis, Diabetes and Digestive and Kidney Disorders, NIH, Bethesda, MD, U.S.A. Na^{125}I (NEZ-033A) was from Dupont/NEN, Stevenage, Herts., U.K. Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril) and disuccinimidyl suberate (DSS) were from Pierce and Warriner (U.K.), Chester, Cheshire, U.K. Molecular mass markers were from Sigma Chemical Co., Poole,

Abbreviations used: GH, growth hormone (somatotropin); hGH, human growth hormone; PRL, prolactin; oPRL, ovine prolactin; BSA, bovine serum albumin; DSS, disuccinimidyl suberate.

Dorset, U.K. Fischer's medium, foetal calf serum and horse serum were purchased from GIBCO/BRL, Paisley, Scotland, and tissue culture plasticware was obtained from GIBCO/BRL, Uxbridge, Middx., U.K. Kodak X-ray film DEF-2 was obtained from Kodak, Hemel Hempstead, U.K. Sprague-Dawley rats (14–15 weeks old) were reared in the School of Biological Sciences, University of Sussex. The Nb2 lymphoma cell-line (Gout *et al.*, 1980) was a gift from Dr. R. Aston.

Iodination of hormones

^{125}I -labelled hGH and oPRL were prepared by the iodogen method of Fraker & Speck (1978), as described previously (Cadman & Wallis, 1981), omitting the final purification step using Sephadex G-100. The specific activity of the ^{125}I -labelled hormones was 1.75–3.2 MBq/ μg of hormone.

Preparation of microsomal membranes

Microsomal membranes from several livers (60–100 g) of 18–21 day pregnant rats were isolated by the method of Tsushima & Friesen (1973), as modified by Cadman & Wallis (1981) but using an alternative 'assay buffer' (0.01 M- NaH_2PO_4 , containing 0.15 M- NaCl , 2.5 mM- KCl , 1 mM- EDTA , and 1% BSA adjusted to pH 7.5 with NaOH) in which to suspend the final membrane pellet.

Growth of the Nb2 lymphoma cell-line

Nb2 lymphoma cells were maintained in suspension culture using the method of Gout *et al.* (1980). When cells were grown in Fischer's medium supplemented with 10% (v/v) foetal calf serum, 10% (v/v) horse serum, 30 mg of penicillin/l and 50 mg of streptomycin/l in an atmosphere of CO_2 /air (1:19) at 37 °C the cell population doubled in approx. 20 h. Prior to binding studies, the cells were transferred into the same medium lacking foetal calf serum for 24 h. Under these conditions their growth was retarded ('stationary phase'), and could be stimulated by the addition of lactogenic hormones (Gout *et al.*, 1980).

Binding of labelled hormone to microsomal membranes from rat liver

All dilutions of receptor (microsomal) preparation, labelled hormone and unlabelled hormone were carried out in 'assay buffer' (see above), which was found to give somewhat higher specific binding than the Ca^{2+} -containing buffer used previously in this laboratory (Webb *et al.*, 1986). Where cross-linking was subsequently to be employed, each tube contained 1 ml of microsomal membranes (6–10 mg of protein) and approx. 10^6 c.p.m. of ^{125}I -labelled oPRL or hGH. Binding of ^{125}I -labelled hormone that occurred in the presence of excess (2 μg) unlabelled hormone was considered to be non-specific. After incubation for 16–20 h at room temperature, free and bound labelled hormone were separated by the addition of 2 ml of cold phosphate-buffered saline, (0.1 M- NaCl , 3.7 mM- Na_2HPO_4 , 0.68 mM- KH_2PO_4 , adjusted to pH 7.2 with NaOH), followed by centrifugation (1000 g, 20 min, 4 °C). The pellet was washed twice by resuspension in 2 ml of phosphate-buffered saline and subsequent centrifugation, prior to cross-linking. For binding studies where cross-linking was not to be used subsequently each tube contained a varied (0.2–10 mg) amount of membrane preparation and 30 000–78 000 c.p.m. of labelled hormone, and precipitation of the

hormone-membrane complex was carried out using poly(ethylene glycol) (final concn. 12.5%, w/v).

Binding of labelled hormone to Nb2 cells

Nb2 cells in 'stationary phase' were centrifuged (3 min, 500 g) and the cell pellet was washed twice in Hepes buffer (25 mM-Hepes, 137 mM- NaCl , 5 mM- KCl , 0.7 mM- Na_2HPO_4 , 10 mM-glucose, 1.5 mg of Phenol Red/l, adjusted to pH 7.2 with NaOH). The cell pellet was resuspended in Hepes buffer [(0.5–3) $\times 10^7$ cells/ml]. Each tube contained 1 ml of Nb2 cell suspension, 100 μl of ^{125}I -labelled hormone (approx. 10^6 c.p.m./tube) in the presence or absence of excess (2 μg) unlabelled hormone. After incubation for 1 h at 30 °C, bound and free labelled hormone were separated by addition of 2 ml of phosphate-buffered saline and centrifugation (3 min at 500 g), prior to cross-linking. For binding studies where cross-linking was not used subsequently a similar procedure was used but the cell number was varied and 0.1% BSA was included in the buffer.

Cross-linking procedure

The incubation procedure was identical for rat liver microsomal membranes and Nb2 cells. After binding of ^{125}I -labelled hormone to microsomal membranes or Nb2 cells and washing, the pellets were resuspended in 1 ml of phosphate-buffered saline and freshly prepared 0.1 M-DSS (in dimethyl sulphoxide) was added to give a final concentration of 1 mM. After reaction for 30 min at room temperature, 1 vol. of 0.1 M-Tris/HCl (pH 7.6, adjusted with HCl) was added. The tube contents were centrifuged (20 min at 1000 g for microsomal membranes and 3 min at 500 g for Nb2 cells) and the pellets washed twice with phosphate-buffered saline. The final pellets were resuspended in 100 μl of electrophoresis sample buffer (0.1 M-Tris/HCl, adjusted to pH 6.8 with HCl), containing 2% SDS (w/v), 20% glycerol (v/v), with or without 2% (v/v) β -mercaptoethanol as a reducing agent. After boiling for 5 min the samples were subjected to SDS/polyacrylamide gel electrophoresis, using 5–20% linear gradient gels and a discontinuous Tris/HCl buffer system based on that of Maizel (1971). After electrophoresis the gels were either subjected to autoradiography using Kodak DEF-2 X-ray film or each lane was cut into 2 mm slices and subjected to γ -radiation counting using a LKB 1260 multigamma II gamma counter, counting efficiency 50–55%.

RESULTS

Effect of increasing receptor concentration on binding of ^{125}I -labelled oPRL and ^{125}I -labelled hGH

Lactogenic binding sites were examined from two sources: (1) liver microsomal membranes from pregnant rat and (2) intact Nb2 rat lymphoma cells. For increasing concentration of rat liver membranes, specific binding increased up to a maximum of 50% (labelled oPRL) and 30% (labelled hGH) of the total radioactivity added. For Nb2 cells, specific binding of 5% was attained at 2×10^7 cells/ml (labelled oPRL) and 2.3% at 4×10^6 cells/ml (labelled hGH). There was no suggestion that binding was reaching a plateau at the maximum concentrations of Nb2 cells used, and it is likely that a higher percentage specific binding would have been attained if more cells could have been used.

Fig. 1. Cross-linking of rat liver membranes and Nb2 cells to ^{125}I -labelled oPRL, detected by SDS/polyacrylamide-gel electrophoresis and γ -radiation counting of gel slices

^{125}I -labelled oPRL bound to rat liver membranes (10 mg of protein) or Nb2 cells (1.4×10^7) was treated with 1 mM-DSS and subjected to SDS/polyacrylamide gel electrophoresis in the presence (rat liver membranes, a; Nb2 cells, c) or absence (rat liver membranes, b; Nb2 cells, d) of β -mercaptoethanol. The gel was cut into 2 mm slices and these were subjected to γ -radiation counting. Peak A represents hormone-receptor complexes not entering the gel matrix; peak B represents hormone-receptor complexes and peak C represents labelled hormone that has not linked to receptor protein. Shaded area: labelled oPRL non-specifically bound; unshaded area: total binding of labelled oPRL. Arrows indicate the positions of M_r standards: myosin (206000), β -galactosidase (116000), phosphorylase B (97500), BSA (66000), ovalbumin (45000) and carbonic anhydrase (29000), with the origin (highest M_r) to the left.

Fig. 2. Cross-linking of Nb2 cells and rat liver membranes to ^{125}I -labelled hGH, detected by SDS/polyacrylamide-gel electrophoresis and autoradiography

Nb2 cells and rat liver membranes were cross-linked with ^{125}I -labelled hGH and subjected to SDS/polyacrylamide gel electrophoresis followed by autoradiography. Binding of ^{125}I -labelled hGH in the absence (lanes 1 and 3, Nb2 cells; lanes 5 and 7, rat liver membranes) and presence (lanes 2 and 4, Nb2 cells; lanes 6 and 8, rat liver membranes) of $2 \mu\text{g}$ of unlabelled hGH is shown under reducing (lanes 1 and 2, Nb2 cells; lanes 5 and 6, rat liver membranes) and non-reducing (lanes 3 and 4, Nb2 cells; lanes 7 and 8, rat liver membranes) conditions. $\blacktriangle$ indicate the M_r values of the cross-linked hormone-receptor complexes (estimated from the position of markers run on the same gel), and * indicates the position where uncross-linked labelled hGH ran on the gel. M_r values of the standards (see legend to Fig. 1) are shown to the side.

Cross-linking of rat liver membranes and Nb2 cells with ^{125}I -labelled oPRL and hGH

Following binding of rat liver membranes and Nb2 cells to labelled hormone, cross-linking was carried out by reaction with DSS. Following solubilization, the cross-linked samples were subjected to SDS/polyacrylamide gel electrophoresis in the presence or absence of β -mercaptoethanol, followed by autoradiography or slicing and γ -radiation counting.

Under reducing conditions, analysis of proteins labelled in the liver microsomal membrane preparation revealed one major ^{125}I -labelled oPRL-receptor complex which migrated as a component of M_r 70000–72000 (Fig. 1a, peak B). Under non-reducing conditions the M_r of the main ^{125}I -labelled oPRL-receptor complex in rat liver microsomal membranes remained unchanged (Fig. 1b).

When Nb2 cells were cross-linked with ^{125}I -labelled oPRL and analysed under reducing conditions (Fig. 1c) one major hormone-receptor complex was observed which migrated at an M_r of 100000–112000 (peak B). Under non-reducing conditions (Fig. 1d) this component was virtually eliminated. The major cross-linked proteins are presumably too large to enter the gel matrix ($M_r > 300000$) probably because the receptor in Nb2 cells exists in a disulphide-linked aggregated or polymeric form. The labelling of the receptors observed in rat liver membranes and Nb2 cells was specific, since addition of excess unlabelled oPRL during incubation of the receptor source with ^{125}I -labelled oPRL resulted in almost complete elimination of the cross-linked receptor-hormone complexes.

Similar results were obtained when cross-linking was

Fig. 3. SDS/polyacrylamide gel electrophoresis of rat liver membranes cross-linked to ^{125}I -labelled oPRL in the presence of various hormones

^{125}I -labelled oPRL bound to rat liver membranes (5 mg of protein) was treated with 1 mM-DSS and subjected to SDS/polyacrylamide-gel electrophoresis, followed by autoradiography. Binding of ^{125}I -labelled oPRL to membranes is shown in the absence of unlabelled hormone (lanes 1 and 7), and in the presence of 2 μg of oPRL (lane 2), oGH (lane 3), rat PRL (lane 4), rat GH (lane 5), or hGH (lane 6). M_r values of the standards are shown to the side (see legend to Fig. 1); $\blacktriangle$ indicates the position of the hormone-receptor protein complex.

Fig. 4. SDS/polyacrylamide gel electrophoresis of Nb2 cells cross-linked to ^{125}I -labelled oPRL in the presence of various hormones

^{125}I -labelled oPRL bound to Nb2 cells (2.8×10^7 cells) was treated with 1 mM-DSS and subjected to SDS/polyacrylamide-gel electrophoresis, followed by autoradiography. Binding of ^{125}I -labelled oPRL to Nb2 cells is shown in the absence of unlabelled hormone (lane 1) and in the presence of 2 μg of oPRL (lane 2), rat PRL (lane 3), ovine GH (lane 4), rat GH (lane 5) or hGH (lane 6). M_r values are shown to the side (see legend to Fig. 1); $\blacktriangle$ indicates the position of the hormone-receptor protein complex.

carried out using ^{125}I -labelled hGH, as illustrated by the autoradiograph in Fig. 2. With rat liver microsomal membranes, a major cross-linked hormone-receptor complex was identified, of M_r 68 000–72 000. This was little changed when non-reducing conditions were used for electrophoresis. However, a second component, of M_r 107 000–110 000, was also apparent when ^{125}I -labelled hGH was used, and the amount of this increased when non-reducing conditions were used. When Nb2 cells were cross-linked with ^{125}I -labelled hGH, a single major component of M_r 97 000–110 000 was identified. This

virtually disappeared when non-reducing conditions were used for electrophoresis, as was the case when ^{125}I -labelled oPRL was used for cross-linking.

Specificity of the receptors from rat liver membranes and Nb2 cells

Figs. 3 and 4 show the competition between ^{125}I -labelled oPRL and various unlabelled hormones for binding to rat liver membranes (Fig. 3) and Nb2 cells (Fig. 4). Incubation of membranes or cells with oPRL and unlabelled hormone was followed by cross-linking and SDS/polyacrylamide gel electrophoresis. Results with rat liver membranes and Nb2 cells were very similar; oPRL (2 μg) and hGH (2 μg) displaced ^{125}I -labelled oPRL with almost equal efficacy and rat PRL competed for receptors, but less effectively. Ovine GH and rat GH competed poorly with ^{125}I -labelled oPRL for binding sites.

DISCUSSION

Lactogenic hormones bound specifically to rat liver microsomal membranes and Nb2 cells with a specificity similar to that observed by others (Postel-Vinay, 1976; Tanaka *et al.*, 1980; Shiu *et al.*, 1983). Cross-linking of labelled oPRL or hGH to such receptors, followed by SDS/polyacrylamide-gel electrophoresis, revealed the presence of specific hormone-receptor complexes. In each case the labelled hormone cross-linked to receptor was only a small proportion (10–30%) of the total specifically bound; this presumably reflects the inefficiency of the cross-linking procedure.

The labelled hormone-receptor protein complexes ran as discrete bands on electrophoresis, though in some cases these were rather broad. This may reflect a heterogeneity of receptors, natural or induced by proteolysis. The specificity of formation of hormone-receptor protein complexes was similar to that observed for binding to the membranes and cells from which the receptors were derived; unlabelled lactogenic hormones (PRL, hGH) prevented formation of the complexes whereas unlabelled somatogenic hormones did not.

Cross-linking of rat liver membranes to ^{125}I -labelled oPRL and hGH resulted in the labelling of a complex with an M_r of 68 000–72 000, under both reducing and non-reducing conditions. Allowing for the M_r of oPRL and hGH (22 000–24 000, based on electrophoretic mobility) and assuming binding of only one hormone molecule, this suggests that the lactogenic receptor includes a protein with M_r of 44 000–50 000 which is not covalently bound to other membrane proteins by disulphide bonds. A second cross-linked complex (of M_r 107 000–110 000) was observed with rat liver membranes but only when ^{125}I -labelled hGH was used as the ligand, suggesting the presence of a separate, somatogenic-like receptor protein of M_r 83 000–88 000. The results found here give a slightly higher value for the M_r of the rat liver lactogenic hormone-receptor complex than has been reported previously (Hughes *et al.*, 1983, M_r 66 000; Kelly *et al.*, 1985, M_r 58 000; Borst *et al.*, 1985, M_r 60 000). Similar M_r values have been observed for PRL receptors from other tissues and animal species by using cross-linking techniques (reviewed by Hughes *et al.*, 1985). On the other hand, larger M_r values (100 000–300 000) for lactogenic receptors have been

reported on the basis of gel filtration studies using Triton X-100-solubilized microsomal membranes

When ^{125}I -labelled oPRL or hGH was covalently bound to Nb2 cells a single band of M_r 96000–110000 was identified under reducing conditions. In the absence of reductant this band was much diminished or absent, and the amount of specifically bound hormone–receptor complex unable to enter the gel matrix ($M_r > 300000$ based on behaviour of marker proteins) increased. This suggests that the hormone–receptor complex of M_r 96000–110000 represents a subunit (M_r 72000–88000) of the lactogenic receptor in Nb2 cells and that this is polymerized via disulphide linkages. M_r values similar to that observed here have been obtained from cell lines derived from the original Nb2 strain (Ashkenazi *et al.*, 1987) using ^{125}I -labelled hGH as ligand although a second hormone–receptor complex with M_r 52000 was also reported.

The studies presented here thus indicate that the main lactogenic receptor subunit identified by cross-linking in rat Nb2 cells is different from that in rat liver microsomal membranes, in that it is larger and linked to other subunits or membrane components by disulphide bonds. It cannot be ruled out that the difference reflects a difference between lactogenic receptors in a membrane preparation and intact cells, especially in view of the fact that binding to cells was carried out at a temperature at which internalisation may occur. It is probable, however, that DSS only cross-links cell-surface receptors to labelled hormone since it is unlikely that this compound can cross the plasma membrane. Yamada & Donner (1984) have shown that hormone–lactogenic receptor complexes in intact rat hepatocytes, identified by cross-linking, have similar M_r values (65000 and 50000) to those in microsomal preparations. Attempts to cross-link hormone to receptors using membrane preparations from Nb2 cells have not been successful.

The receptor in Nb2 cells has the specificity of a lactogenic receptor (Fig. 4) but has greater affinity for PRL than 'normal' lactogenic receptors. We have shown that the receptor protein that cross-links to PRL has an abnormally high M_r for a lactogenic receptor. It seems likely that the possession of this abnormal lactogenic receptor underlies the growth-dependence on lactogenic hormones displayed by the Nb2 cell line, and it is possible that the abnormal receptor is involved in the malignancy of this cell line. How this receptor relates to the smaller lactogenic receptor found in other rat tissues remains unclear.

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