Vol. 105, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1982 Pages 194-201

PHOTOACTIVATED CROSS-LINKING OF PROLACTIN TO HEPATIC MEMBRANE BINDING SITES

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Received January 8, 1982

SUMMARY: [^{125}I]prolactin was found to show high affinity (K_d =1.8x10^{-10} M), saturable, and specific binding to microsomal membranes from rat liver. The specifically bound hormone was then cross-linked to membrane proteins using N-hydroxysuccinimidyl azidobenzoate and these proteins were separated by SDS gel electrophoresis. This treatment resulted in a band of radioactivity (60,000 daltons) that was not seen in controls lacking reagent or light activation. The cross-linked product was eliminated by competition during binding with unlabeled lactogenic hormones (such as ovine prolactin, rat prolactin, or human growth hormone). These data indicate that prolactin (24,000 daltons) was cross-linked to a peptide of 36,000 daltons. This peptide may be the binding site of the prolactin receptor.

INTRODUCTION

Like many other protein hormones, prolactin interacts with target cells by binding to receptor proteins on plasma membranes from a variety of tissues (1,2,3). In addition, the receptor has been partially purified from rabbit mammary tissue using affinity chromatography (4); however, the subunit composition of this 220,000 dalton complex has not yet been investigated.

One approach for determining the molecular characteristics of hormone receptors is to covalently link radioactively-labeled hormones to the receptor protein. The cross-linked products can then be analyzed by techniques such as SDS gel electrophoresis which would otherwise dissociate the complex. This approach has been used successfully in studies of receptors for several other hormones, including epidermal growth factor (5), insulin (6,7), and multiplication stimulating activity (8). A particularly straightforward procedure for cross-linking peptide hormones to their receptors was recently reported (9). In that study, N-hyroxysuccinimidyl azidobenzoate, a light activated hetero-bifunctional reag-

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ent, was used to cross-link membrane bound radiolabeled glucagon to its receptor. Here, we have used a similar procedure to analyze the prolactin receptor in rat liver membranes. An abstract reporting this work has appeared earlier (10).

MATERIALS AND METHODS

Rat prolactin (NIH B-2, 20 IU/mg), ovine prolactin (NIH oPRL-15, 31 IU/mg), human growth hormone (NIAMDD hGH I-1), follicle stimulating hormone (NIH S-12), luteinizing hormone (NIH S-20), and bovine growth hormone (NIH GH-B18) were kindly provided by the National Pituitary Agency, NIAMDD. Porcine insulin (lot 615-D63-10) was the gift of Eli Lilly and Co. Bovine serum albumin (BSA) was purchased from Calbiochem, and N-hyroxysuccinimidyl azidobenzoate (NHSAB) was purchased from Pierce Chemical Co. Ovine prolactin for iodination was the gift of C.H. Li (University of California, San Francisco). Lactoperoxidase was purchased from Sigma and Na[^{125}I] was purchased from Amersham. For gel electrophoresis, [^{14}C]-labeled protein molecular weight standards (BSA, ovalbumin, and carbonic anhydrase) were purchased from New England Nuclear.

Iodination of prolactin: [^{125}I]oPRL was produced using a modified lactoperoxidase procedure (11). 10 µg of ovine prolactin was suspended in 0.3M sodium phosphate buffer (pH 7.5) and 0.5 to 1 µg of lactoperoxidase was added in the same buffer. After the addition of 1 mCi Na[^{125}I], 30 ng of hydrogen peroxide (in distilled water) was added. The total reaction volume was 50 µl. Seven minutes after the addition of hydrogen peroxide, the reaction products were placed on a Sephadex G25 column (5 ml) and eluted with PBS (0.03M phosphate, 0.9% NaCl, pH 7.5) containing 0.1% BSA. Transfer of radioactivity to the hormone (the radioactivity in the excluded volume divided by the total Na[^{125}I] used) was from 40 to 60% resulting in specific activity of 40-60 µCi/µg protein.) Prolactin iodinated by this method had full biological activity when tested with an in vitro mammary gland bioassay (12).

Before its use in binding studies, the iodinated protein was further purified on a Sephadex G100 column (10 ml) using PBSM-BSA (PBS containing 10 mM $MgCl_2$ and 0.1% BSA.) Fractions eluting with the peak or immediately thereafter had the highest specific binding; when incubated with excess rat liver membranes, 50-70% of this material was specifically bound. These fractions were pooled and used for binding studies. Greater than 95% of this radioactive material was precipitable with 6% TCA and co-migrated with prolactin as a single band on SDS-gel electrophoresis followed by autoradiography.

Preparation of hepatic membranes: Liver tissue from virgin or estradiol-treated (1 µg/day for 10 days) Sprague-Dawley rats was homogenized in buffered sucrose (0.25 M sucrose, 0.01M $NaHCO_3$) using a Polytron PT 20 homogenizer (Brinkmann) for 60 seconds at full speed. Two low speed pellets (12,000 x g, for 15 minutes) were discarded, and the microsomal pellet (90,000 x g, for 2 hours) obtained from the supernatant fraction. Microsomal membranes were resuspended in PBSM-BSA and aliquots were frozen. The concentration of membrane proteins in microsomal preparations was determined by the method of Lowry (13).

Prolactin binding studies: Frozen microsomal membranes from the livers of estrogen-treated rats were thawed, gently homogenized (Dounce), pelleted (1,000 x g, for 5 min), and resuspended in PBSM-BSA buffer. Aliquots of membrane containing 100-200 µg protein and [^{125}I]oPRL (40,000 DPM) were co-incubated in 0.2 ml PBSM-BSA at 4 C for 48 hours. The specificity of binding was determined by adding unlabeled protein hormones to the [^{125}I]oPRL before the membranes were then added. At the end of the binding period, one ml of cold PBSM-BSA was added and the membrane components pelleted (1,000 x g, for 5 min). The pellet was washed with a another aliquot of buffer, and the radioactivity retained in the washed pellet was counted in an Amersham-Searle gamma counter (efficiency, 43%).

Cross-linking studies: Microsomal membranes from liver tissue of estrogen-treated rats were incubated with [^{125}I]oPRL (200,000 DPM) and then washed as described above. The membrane pellets were resuspended in one ml cold PBSM (containing no BSA.) NHSAB was dissolved in dimethyl sulfoxide to give a concentrated stock solution (50 mM). The reagent was diluted 200-fold with the resuspended pellet (final concentration 250 μM) and incubated in dim light for six minutes at room temperature. At the end of this incubation, the reagent was photoactivated with a flash of light from an electronic flash unit (Alfon Model #635). After cross-linking, the membranes were centrifuged and the pellet was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% acrylamide. Samples were run both in the presence and absence of 2-mercaptoethanol. Gels were stained with Coomassie blue, and the migration of radioactivity determined by autoradiography (5-10 day exposure) using an intensifier screen (Dupont Cronex). [^{14}C]protein molecular weight standards were added to one track of the gel.

RESULTS

Kinetic Studies. In preliminary studies, we found that the binding of prolactin to hepatic membrane preparations reached a maximum after 48 hours at 4 C (data not shown). Using these conditions, approximately 30% of the [^{125}I]oPRL was specifically bound to microsomal membranes from the liver of estrogen-treated rats. As would be expected for a hormone receptor, these membrane sites were saturated with increasing doses of prolactin (see Fig. 1). As shown in the Scatchard analysis (Fig. 1), this binding had high affinity (K_d = 1.8×10^{-10} M), and there were 6.6×10^{-13} moles of binding sites per mg membrane. Similar binding characteristics were observed with microsomal membranes from virgin animals, except that the amount of specific binding was reduced (data not shown).

The specificity of [^{125}I]oPRL binding to the membrane receptor was determined by competition studies with other hormones. Lactogenic hormones, such as rat prolactin, human growth hormone, and ovine prolactin competed effectively with [^{125}I]oPRL for the membrane binding sites. Non-lactogenic hormones, such as insulin, follicle stimulating hormone and luteinizing hormone did not compete. Bovine growth hormone, though non-lactogenic, showed some competition at the highest dose tested (1 μg) (Fig. 2); this may reflect minor contamination of the preparation with bovine prolactin.

Cross-Linking Studies. [^{125}I]oPRL, specifically bound to hepatic membranes from estrogen-treated rats, was cross-linked to other membrane proteins with NHSAB (250 μM). The cross-linked product was then separated by SDS-PAGE and the

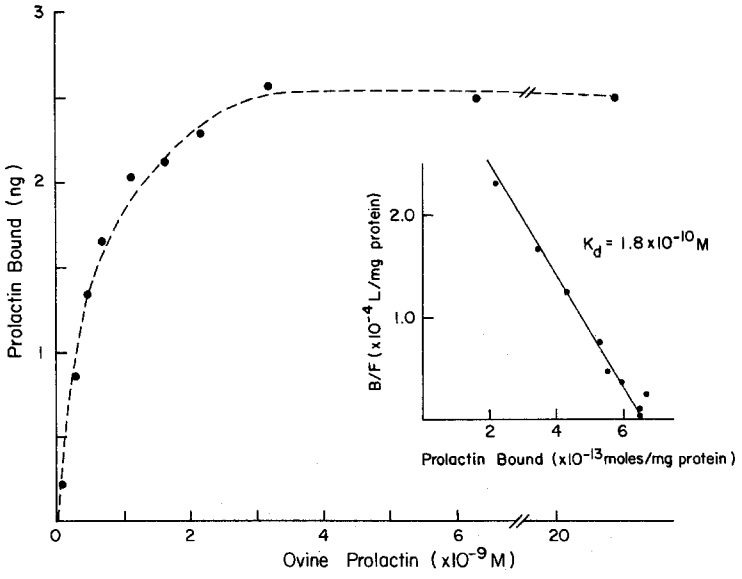

Figure 1: Prolactin binding to rat hepatic membranes. [^{125}I]oPRL (6×10^{-10} M) was incubated for 48 hours at 4 C with microsomal membranes (160 μg of protein) from estrogen-treated rats in the absence and presence of various concentrations of unlabeled ovine prolactin. Each point represents the mean of duplicate determinations in a representative experiment; "non-specific" binding (6% of the total binding), was subtracted from each value. Inset: Scatchard plot of prolactin binding.

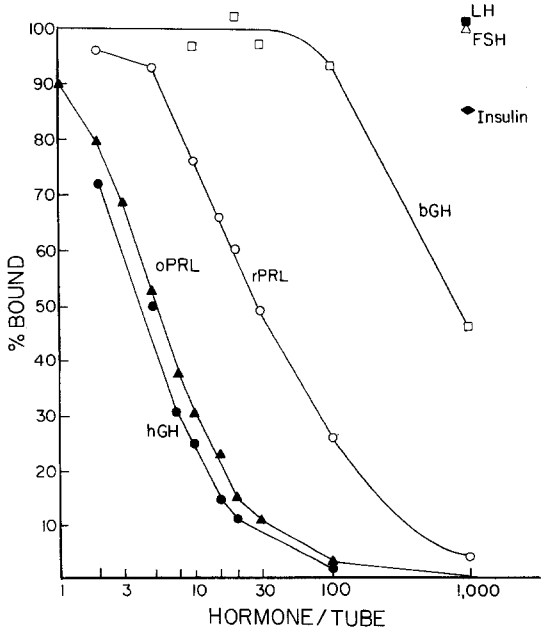

Figure 2: Specificity of prolactin binding. Membranes were incubated (see Fig. 1) in the absence or presence of lactogenic or non-lactogenic hormones: (oPRL = ovine prolactin; hGH = human growth hormone; rPRL = rat prolactin; bGH = bovine growth hormone; FSH = follicle stimulating hormone; LH = luteinizing hormone.) The radioactivity specifically bound at each dose of competitor is expressed as the percentage of [^{125}I]oPRL specifically bound in the absence of hormone.

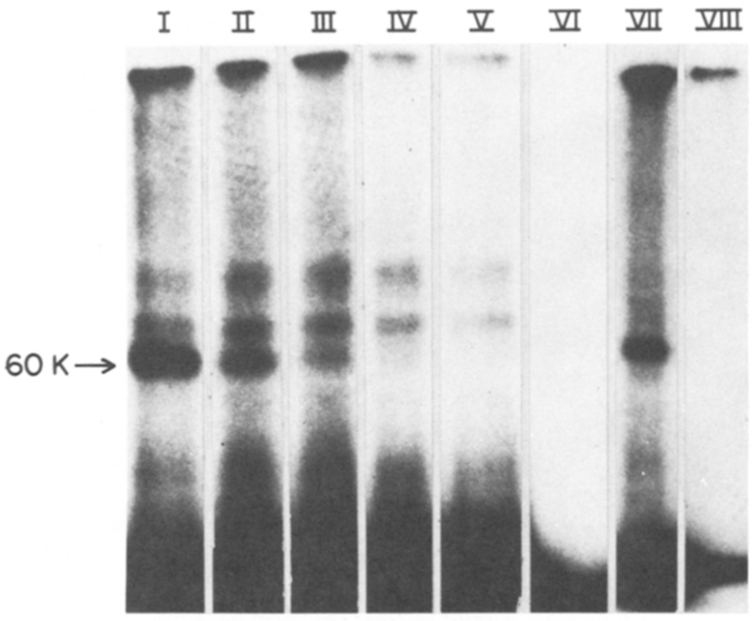

Figure 3: Cross-linking of prolactin to hepatic binding sites. Autoradiogram of SDS-PAGE (7.5% acrylamide) separation of [^{125}I]oPRL covalently-linked to proteins from hepatic microsomal membranes. Track: 1 = 250 μM NHSAB; 2 = 50 μM NHSAB; 3 = 10 μM NHSAB; 4 = 250 μM NHSAB, no photoactivation; 5 = no reagent, no photoactivation; 6) same as track 1, but binding in presence of 1 μg unlabeled ovine prolactin; 7) = 250 μM NHSAB, sample run without 2 mercaptoethanol; 8) same as track 7, but binding in presence of 1 μg unlabeled ovine prolactin.

migration of radioactive prolactin was detected by autoradiography. As is seen in Figure 3 (track 1), a portion of the radioactivity migrated with a molecular weight of 60,000 (60K) after treatment with NHSAB, while the rest of the radioactive material co-migrated with prolactin. In experiments where these two regions were cut from the gel and counted, the efficiency of cross-linking was found to be approximately 5%. Longer incubation times (up to 30 min), multiple light exposures, and a higher concentration of reagent (750 μM) did not increase the amount of radioactive material in this band (data not shown). However, the intensity of this band decreased when less reagent (50 and 10 μM) was used (Fig. 3, tracks 2 and 3, respectively). In addition, the 60K band was not seen when samples were not photoactivated in the presence or absence of NHSAB (Fig. 3, tracks 4 and 5).

Incubation of membrane samples with [^{125}I]oPRL and an excess (1 μg) of unlabeled ovine prolactin (Fig. 3, track 6) yielded no radioactive cross-linked

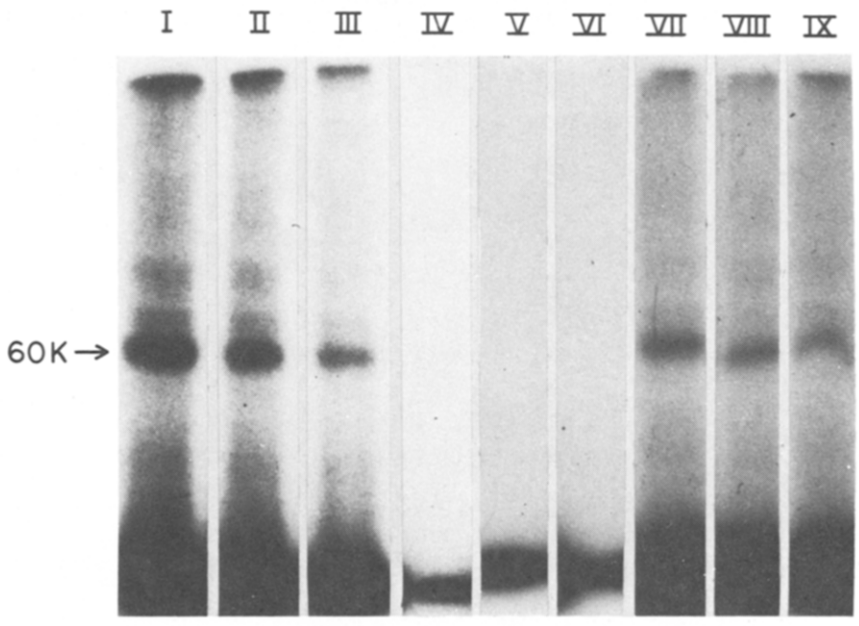

Figure 4: Prolactin cross-linking to hepatic binding sites after competition with other protein hormones. Autoradiogram of SDS-PAGE as in Figure 3. The indicated protein hormones were added to [^{125}I]oPRL during the initial binding reaction. All samples were cross-linked with 250 μM NHSAB. Track: 1 = no added hormone; 2 = 3 ng ovine prolactin; 3 = 10 ng ovine prolactin; 4 = 1 μg ovine prolactin; 5 = 1 μg human growth hormone; 6 = 1 μg rat prolactin; 7 = 1 μg bovine growth hormone; 8 = 1 μg follicle stimulating hormone; 9 = 1 μg insulin.

product. This strongly suggests that the 60K band is composed of [^{125}I]oPRL and its specific binding site. The cross-linked preparations separated by SDS-PAGE without 2-mercaptoethanol also yielded a 60K band (Fig. 3, track 7) that was eliminated by the incubation of the membrane samples with [^{125}I]oPRL and an excess (1 μg) of unlabeled ovine prolactin before cross-linking (Fig. 3, track 8). This indicates that the membrane protein cross-linked to the [^{125}I]oPRL is not covalently attached to other membrane proteins by disulfide bonds. Microsomal membranes from virgin rats were also observed to yield a 60K band upon cross-linking with [^{125}I]oPRL as described above (data not shown).

Further evidence that the cross-linked product reflects the association of [^{125}I]oPRL with a high affinity, lactogenic receptor site comes from the following evidence. Incubation of membranes with [^{125}I]oPRL and low doses of unlabeled ovine prolactin (3 and 10 ng) before cross-linking decreased the intensity of the 60K band (Fig. 4; tracks 2 and 3); and as described above, treatment with

an excess of unlabeled ovine prolactin (1 μg) eliminated the band entirely (Fig. 4, track 4). Furthermore, incubation of membranes with other lactogenic hormones (rat prolactin and human growth hormone) also eliminated the 60K band (Fig. 4, tracks 5 and 6). Non-lactogenic hormones (bovine growth hormone, follicle stimulating hormone, insulin) had no effect (Fig. 4, tracks 7 - 9).

DISCUSSION

The binding of [^{125}I]oPRL to membranes from rat liver had kinetic properties typical of hormone-receptor interactions: high affinity, saturability and specificity. We have covalently linked this membrane-bound prolactin to its putative binding protein using NHSAB. Cross-linking was dependent upon the amount of reagent present, and only occurred upon activation with light. Furthermore, cross-linking was inhibited by unlabeled lactogenic hormones but not by other protein hormones.

When [^{125}I]oPRL was covalently coupled to membrane binding sites, the product migrated with a molecular weight of 60,000. This indicates that prolactin (24,000 daltons) is being linked to a membrane protein of approximately 36,000 daltons. The mobility of this protein in non-reducing SDS-PAGE suggests that it is not covalently bound to other membrane proteins. The 36K protein is either the prolactin binding site or a protein that is close enough to the binding site to be readily cross-linked to the hormone. Using a different approach, Haeuptle, et al. recently reported (14) that a protein associated with the lactogenic binding site in rabbit mammary gland and liver has a similar molecular weight to the protein we have found in rat hepatic tissue. Neither finding excludes the possibility that the prolactin receptor complex is composed of additional subunits, nor that the 36K peptide is the product of a partially degraded larger protein.

ACKNOWLEDGMENTS

We wish to thank Ms. Marina Fikiet for technical assistance. Supported by grant BC-344 from the American Cancer Society.

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