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PURIFICATION OF DECIDUAL PROLACTIN-RELEASING FACTOR, A PLACENTAL PROTEIN THAT STIMULATES PROLACTIN RELEASE FROM HUMAN DECIDUAL TISSUE

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Decidual prolactin-releasing factor (PRL-RF), a placental protein that stimulates the release of prolactin from human decidual tissue, has been purified from conditioned medium of human placental explants. The purification scheme consisted of ethanol extraction, anion exchange chromatography on DEAE-cellulose, size exclusion chromatography on Spherogel TSK-3000, and either a) immunoaffinity chromatography using an antiserum to a partially purified PRL-RF preparation or b) acetic acid-urea/SDS 2-dimensional PAGE. The apparent molecular weight of the purified releasing factor, estimated by SDS-PAGE, was 23,500 Da; and the half-maximal dose for the acute stimulation of prolactin release from human decidual cells was 0.05-0.1 ug/ml (2.2-4.4 nM).

Human decidual tissue synthesizes and releases a prolactin which is identical to pituitary prolactin in its chemical and biological properties (1-3). However, thyrotropin releasing hormone, dopamine, and other factors that affect the release of pituitary prolactin do not affect the release of decidual prolactin (4,5). These results indicate that the regulation of decidual prolactin release is different than that of pituitary prolactin.

Several years ago, we observed that decidual explants co-cultured with human placental explants (which do not synthesize prolactin) or incubated in medium conditioned by placental explants release significantly more prolactin than control decidual explants (6). The stimulatory activity of placental conditioned medium (PCM) was 1) heat (56°C for 30 minutes) and pH (pH 3.0-10.0) stable, 2) non-dialyzable (MW >10,000), 3) unaffected by lipid extraction, and 4) sensitive to proteolytic digestion with trypsin and pronase. The stimulation was not due to a non-specific effect of conditioned medium since conditioned medium from other human tissues did not stimulate prolactin release and PCM did not stimulate the release of other decidual proteins. In addition, PCM had no effect on the release of prolactin and other pituitary hormones from cultured rat pituitary cells. Taken together, these studies strongly suggest that the human placenta releases a protein(s) which selectively stimulates the release of decidual prolactin. We report here the

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purification of a placental protein which stimulates decidual prolactin release. We have designated this novel regulatory protein decidual prolactin-releasing factor (PRL-RF).

MATERIALS AND METHODS

Preparation of placental extracts and placental conditioned medium

Human term placentas were obtained from women delivering at Duke University Medical Center and Durham County General Hospital. Permission to obtain the placentas was approved by the Human Investigation Committee from Duke University Medical Center, and informed consent was obtained in each instance.

After the fetal membranes were removed and placentas were washed extensively with saline to remove as much blood as possible, a crude placental extract was prepared by homogenization of the placentas in a blender with ice-cold absolute ethanol (2 ml ethanol/gm placenta). The extract was filtered through Whatman No. 1 filter paper on a Buchner funnel. The retained material was then rinsed with cold ethanol and dried at 4°C for 16 hours. The dried material was resuspended in 50 mM sodium phosphate, pH 7.2 containing 1 mM phenylmethylsulfonylfloride (15 ml buffer/gm homogenate) and stirred for 16 hours at 4°C to solubilize proteins. The insoluble material was pelleted by centrifugation for 15 minutes at 2400 x g, followed by centrifugation of the resulting supernatant for another 30 minutes at 19,000 x g. The final supernatant was then dialyzed exhaustively (8000 MW cut-off) against deionized water at 4°C, lyophilized, and frozen at -70°C until processed further.

Placental conditioned medium (PCM) was prepared by a 16 hour incubation of term placental explants in RPMI-1640 containing 50 ug/ml gentamicin and 50 ug/ml Fungazone (approximately 100 gm explants/500 ml medium). The explants were maintained at 37°C in an atmosphere of 95% air - 5% CO_2 on a shaking platform in a humidified incubator. The medium was decanted through two layers of cheesecloth, centrifuged at 2,600 x g for 15 minutes, and clarified further by filtration through a 0.45 micron Millipore filter. The medium was then dialyzed exhaustively against 10 mM Tris-HCl, pH 10.0 and subjected to anion exchange chromatography.

Chromatographic and electrophoretic methods

Anion exchange chromatography was performed using diethylaminoethyl (DEAE)-cellulose (Whatman DE-52). The placental extract or PCM was applied to a column, 1.9 cm i.d.x 35 cm (bed volume 80 ml), of DEAE-cellulose equilibrated with 10 mM Tris-HCl, pH 10. The column was washed with 5 bed volumes (400 ml) of equilibration buffer until the OD 280 nm of the eluant was less than 0.005 and then eluted with either 5 bed volumes of equilibration buffer containing 100 mM sodium chloride or a linear gradient of 5 bed volumes of equilibration buffer containing 200 mM sodium chloride as the limit buffer. The active material from the DEAE-cellulose column was dialyzed exhaustively against 50 mM sodium phosphate, pH 7.2 and concentrated by vacuum dialysis (8000 MW cutoff).

Size exclusion chromatography was performed with either Sephadex G-75 or Spherogel TSK-3000. Sephadex G-75 chromatography was performed at 4°C using a column (2.5 cm i.d. x 90 cm) of gel equilibrated and eluted with 50 mM sodium phosphate, pH 7.2. Spherogel TSK-3000 chromatography was performed at 25°C on a Beckman High Performance Liquid Chromatography System using a column (21.5 mm i.d. x 60 cm, bed volume 218 ml) equilibrated and eluted with 50 mM sodium phosphate, pH 7.2 containing 150 mM sodium chloride, 1 mM EDTA and aprotinin

(7.5 trypsin units/L). The active fractions from the Sephadex G-75 or Spherogel TSK-3000 column were pooled and concentrated by vacuum dialysis.

Preparative native polyacrylamide gel electrophoresis (PAGE) was performed with the discontinuous buffer system of Laemmli (7) without sodium dodecyl sulfate (SDS) using a linear acrylamide gradient (7.5-17.5%; acrylamide:bis, 30:0.8). The bioactivity of the various fractions from the native gel was determined following electrophoretic elution of the proteins (8). Analytical SDS-PAGE using 12% acrylamide separating gels was performed by the method of Laemmli (7). The gels were stained with either Coomassie Brilliant Blue R (9) or silver (10). For determination of the apparent molecular weight of PRL-RF, the mobility of purified PRL-RF on SDS-PAGE was compared to that of 8 molecular weight standards (14-96 kDa). Acetic acid-urea gel electrophoresis was performed by the method of Panyim and Chalkley (11). Immunoaffinity column chromatography was performed by the method of Mayes (12) using an antiserum (see below) to partially purified PRL-RF isolated by preparative native gel electrophoresis. The IgG fraction of the antiserum, prepared by ammonium sulfate precipitation and chromatography on DEAE-cellulose(13), was coupled to Affi-Gel 10 (Bio-Rad), an N-hydroxysuccinimide ester of derivatized crosslinked agarose gel beads.

Antibody studies

An antisera to PRL-RF was produced in a New Zealand male rabbit using material prepared by preparative native gel electrophoresis. A single protein band containing the active material was homogenized in 2 ml phosphate buffered saline (PBS) and mixed with an equal volume of complete Freund's adjuvant. The animal initially received approximately 100 ug protein intradermally into the paraspinal region, followed by two intradermal injections of approximately 25 ug protein each at two week intervals for a total of three injections.

The effect of the antiserum on the stimulatory activity of crude placental extract and PCM was determined by incubating solutions of crude placental extracts for 16 hours at 37°C with 10 ul/ml of undiluted antiserum or pre-immunized serum from the same rabbit collected several weeks before immunization was begun. The extract and PCM were then reacted with protein A (50 ul/ml) and centrifuged at 2600 x g for 30 minutes. The biological activities of the placental extracts and PCM that had been reacted with antiserum or normal rabbit serum were compared to those of untreated placental extract and PCM.

Western blot analyses of PRL-RF at various stages of purification were performed by the method of Burnette (14).

PRL-RF bioassay

PRL-RF was detected by measuring the amount of prolactin released from human decidual explants over a 30-60 minute period as previously described (6). Each placental extract or PCM fraction was tested in triplicate cultures with a minimum of 2 concentrations per assay. The specific activity of each fraction was determined by calculating the amount of protein required to stimulate a 2-fold increase in prolactin release. Statistical differences between sample means were determined by analysis of variance and treatment contrasts were performed by Dunnett's test or the Newman-Keul's test. P values equal to or less than 0.05 were considered statistically significant.

Other assays

Prolactin was determined by a specific homologous radioimmunoassay (15), and protein concentrations were determined by the method of Bradford (16).

RESULTS

In initial experiments, PRL-RF was partially purified from crude placental extracts because of the large amounts of prolactin stimulatory activity detected in the placenta. The purification scheme consisted of chromatography on DEAE-cellulose with a stepwise elution with 100 mM sodium chloride, followed by chromatography on DEAE-cellulose with a 0-200 mM linear sodium chloride gradient, and size exclusion chromatography on Sephadex G-75 (Table 1). These successive steps resulted in increases in specific activity of prolactin stimulatory activity of 18, 165, and 20,000, respectively. Greater than 90% of the active material was adsorbed by DEAE-cellulose at pH 10.0, and greater than 95% of the activity eluted from the DEAE-cellulose between 0-20 mM sodium chloride. Greater than 95% of the active material eluted from Sephadex G-75 with an apparent molecular weight of 23,500. Preparative native PAGE (7.5-17.5% gradient) of the pooled active fractions from the Sephadex column yielded 4 protein (Coomassie) bands. After electrophoretic elution of the individual protein bands, greater than 80% of the prolactin releasing activity was detected in the second band from the cathode (Figure 1). Analysis of the protein in this band by SDS-PAGE revealed 2 proteins with the major protein migrating at an apparent molecular weight of 23,500.

The area of the unstained portion of the native gel corresponding to the second band from the cathode was excised, emulsified in adjuvant, and administered to a rabbit at bi-weekly intervals for 6 weeks for the production of antisera. Incubation of crude placental extract (20, 200 and 2000 ug/ml) with the antiserum resulted in an almost complete loss of stimulatory activity.

Table 1. Partial purification of PRL-RF from placental extracts

Purification Step	Weight	Estimated recovery %	Specific activity
1. crude placental extract	286.0 gm	-	-
2. ethanol extraction	40.2 gm	90	-
3. dialysis against water	2.0 gm	90	1
4. DEAE-cellulose chromatography, stepwise elution with 100 mM sodium chloride	120.0 mg	70	18
5. DEAE-cellulose chromatography, gradient elution with 0-200 mM sodium chloride	12.2 mg	90	165
6. Sephadex G-75 chromatography	101.1 ug	90	20,000
7. preparative native PAGE	42.1 ug	82	46,200

PRL-RF was partially purified from placental extracts as described in Methods. As shown in Figure 1, the band containing the active material from the preparative native PAGE revealed two proteins by SDS-PAGE. This material was used for the production of PRL-RF antiserum and the subsequent purification of PRL-RF by immunoaffinity chromatography.

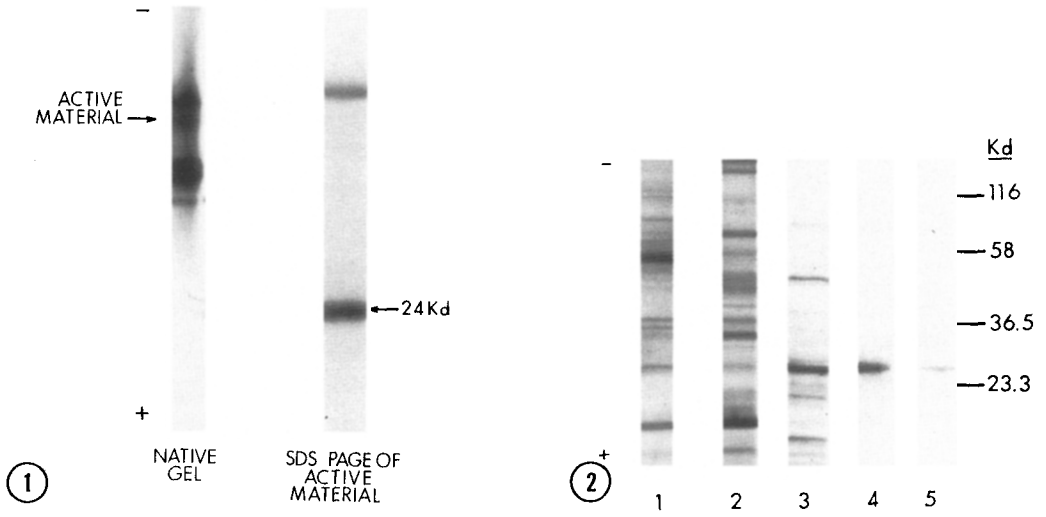
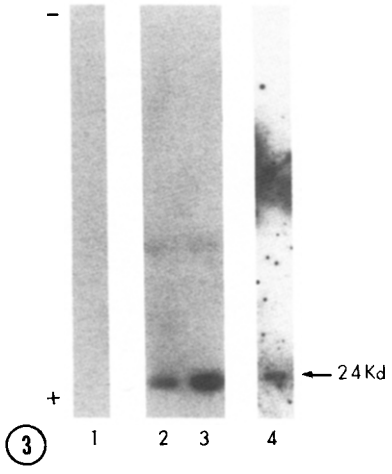


Figure 1. Preparative native polyacrylamide gel electrophoresis of PRL-RF. Approximately 900 ug of the active material prepared by successive chromatographies of a placental extract on DEAE-cellulose and Sephadex G-75 was subjected to preparative native PAGE as described in Methods. The gel on the left shows the protein pattern (Coomassie Blue) following native PAGE. The arrow indicates the band containing the active material. SDS-PAGE of the active material from the native gel yielded the protein pattern shown on the right.

Figure 2. Polyacrylamide gel electrophoresis of PRL-RF at various stages of the purification from PCM. PRL-RF was purified from PCM as indicated in Methods. Approximately 5 ug protein from various stages of the purification was subjected to SDS-PAGE. The proteins were detected by silver staining. Stages of purification: 1) starting material (crude PCM), 2) following DEAE-cellulose chromatography, 3) following DEAE-cellulose and Spherogel TSK-3000 chromatography, 4) following DEAE-cellulose, Spherogel TSK-3000, and immunoaffinity chromatography, and 5) following DEAE-cellulose and Spherogel TSK-3000 chromatography and acetic acid-urea/SDS 2-dimensional PAGE.

Decidual explants exposed for 0.5 hours to 20, 200, or 2000 ug of a placental extract partially purified by DEAE-cellulose chromatography released 2.1 $\pm$ 0.2 (mean $\pm$ SEM, n = 3), 2.8 $\pm$ 0.3, or 3.6 $\pm$ 0.4 times as much prolactin as control explants. Decidual explants exposed to the same concentrations of the placental extract that had been preincubated for 16 hours with PRL-RF antiserum released only 1.1 - 1.3 times as much prolactin as control explants. In contrast, explants exposed to placental extract that had been preincubated with pre-immunized serum in place of antiserum released the same amounts of prolactin as those exposed to placental extract alone.

Subsequent purification of PRL-RF was performed with placental conditioned medium as starting material since SDS-PAGE indicated that PCM contained fewer proteins than crude placental extract. Following chromatography on DEAE-cellulose and Spherogel TSK-3000, the active material was purified to homogeneity by either immunoaffinity column chromatography (using the antiserum to the partially purified PRL-RF from the preparative native PAGE) or by



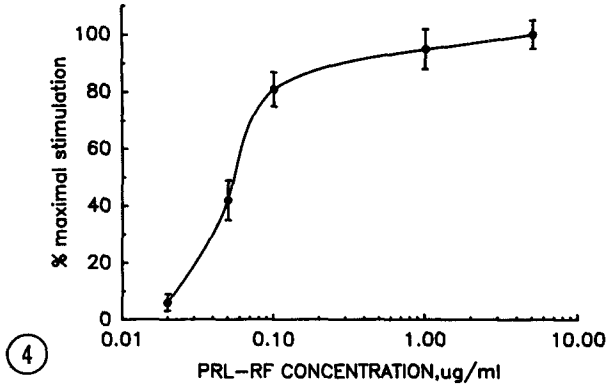


Figure 3. Immunoblot of PRL-RF preparations with PRL-RF antiserum. Partially purified and purified preparations of PRL-RF were subjected to western blot analysis as described in Methods. Following incubation with PRL-RF antiserum or normal rabbit serum (NRS), the gels were incubated with ^{125}I-Protein A and then subjected to autoradiography. Approximately 10-12 ug protein was applied to each lane. 2) placental extract which had been subjected to DEAE-cellulose chromatography, 3) placental extract which had been subjected to successive chromatographies on DEAE-cellulose and Sephadex G-75, 1) same as 3 but reacted with NRS in place of PRL-RF antiserum, and 4) PCM which had been subjected to DEAE-cellulose chromatography, Sephadex G-75 chromatography, and acetic acid-urea/SDS 2-dimensional PAGE.

Figure 4. The effects of purified PRL-RF on prolactin release from human decidual explants. PRL-RF that had been purified from PCM by DEAE-cellulose, Spherogel TSK-3000, and immunoaffinity chromatographies was assayed for prolactin releasing activity over a 0.5 hour interval as described in Methods. Each point represents the mean of triplicate cultures, and the brackets enclose ± SEM.

acetic acid-urea/SDS 2-dimensional PAGE. SDS-PAGE analysis of the active fractions at various stages of the purification procedure is shown in Figure 2. The active material prepared by both immunoaffinity chromatography and 2-dimensional PAGE contained a single protein band by silver staining. Western blot analyses of PRL-RF prepared by either method detected a single band which migrated in an SDS gel at a position identical to the silver-stained band. (Figure 3). Crude placental extract also contained a single band of immunoreactivity by Western blot analysis. The apparent molecular weight of purified PRL-RF by SDS-PAGE was 23,500. Immunoaffinity-purified PRL-RF stimulated a dose-dependent increase in the acute release of prolactin from decidual explants with a half-maximal dose in the range of 0.05 to 0.1 ug/ml (2.2-4.4 nM)(Figure 4).

DISCUSSION

The results of these studies indicate that the placenta releases a protein of 23,500 MW which stimulates the acute release of prolactin from human deci-

dual tissue. The protein was purified to homogeneity from placental conditioned medium by two different purification procedures. In the first, PCM was subjected sequentially to DEAE-cellulose chromatography, size exclusion chromatography and immunoaffinity chromatography using an antibody produced against a partially purified PRL-RF preparation from a placental extract. In the other purification scheme, the active material from size exclusion chromatography was subjected to acetic acid-urea PAGE followed by SDS-PAGE. Homogeneity of the purified PRL-RF was established by the demonstration that the protein purified by both purification schemes revealed single protein bands on SDS-PAGE by silver staining and Western blot analysis.

Recent studies from our laboratory indicate that PRL-RF has biological actions on decidual cells besides the acute stimulation of prolactin release (17). PRL-RF also stimulates the synthesis of decidual prolactin and causes a secondary and prolonged increase in prolactin release beginning about 6 hours after exposure. In addition, decidual cells exposed to PRL-RF synthesize significantly more DNA after 48-72 hours of culture than control cells. The demonstration that PRL-RF is mitogenic for decidual cells suggests that PRL-RF may also be a decidual growth factor. The availability of purified PRL-RF and an antiserum to PRL-RF will greatly facilitate additional studies of the chemistry and physiology of this newly described releasing factor.

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