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Antipeptide antibodies reveal structural and functional characteristics of rat placental lactogen-II

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Summary

The purpose of this investigation was to develop specific immunologic probes to rat placental lactogen-II (PL-II) and to use the immunologic probes to further characterize rat PL-II. Five oligopeptides corresponding to different regions of rat PL-II (amino acids 1–13, 56–70, 89–103, 107–118, 150–164) were chemically synthesized by solid phase methods and purified to homogeneity by reverse phase high performance liquid chromatography. The synthetic peptides were coupled to keyhole limpet hemocyanin (KLH) and the peptide-KLH conjugates were used to immunize rabbits. Antibody production was monitored by enzyme-linked immunoassay (EIA), electrophoresis and immunoblotting analyses.

Each of the antipeptide antisera showed reactivity with the entire rat PL-II protein; however, the extent of the reactivities of each antiserum with rat PL-II was dependent on the conformational state of rat PL-II. Antisera directed to amino acids 56–70 showed the best reactivity toward each of the conformational states of rat PL-II tested. Antibodies generated to the entire rat PL-II protein specifically recognized the 56–70 amino acid sequence but showed limited reactivity with synthetic peptides corresponding to amino acids 1–13, 89–103, 107–118, and 150–164 of rat PL-II. Antisera to amino acids 56–70 of rat PL-II were specific for PLs as demonstrated by their recognition of rat PL-II, mouse PL-II and human PL and by their lack of reactivity with rat pituitary prolactin and growth hormone and with a series of other synthetic peptides to rat PL-II and rat prolactin-like protein-A. The immunorecognition of human PL was restricted to antipeptide antibodies directed to amino acids 56–70 of rat PL-II.

The chemically synthesized peptides representing various regions of rat PL-II did not show significant interactions with prolactin receptors, and antisera directed to the peptides failed to interfere with the binding of either rat PL-II or human PL to prolactin receptors.

In summary, we have generated a series of immunologic probes for studying the structure of rat PL-II. The sequence comprising amino acids 56–70 of rat PL-II was shown to make up at least part of an epitope for rat PL-II and to be a region of significant structural homology with mouse PL-II and human PL.

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Introduction

The rodent placenta is a source of a number of different proteins belonging to the prolactin family (Duckworth et al., 1986c; Ogren and Talamantes, 1988). These proteins include placental lactogen-I (PL-I; Kelly et al., 1975; Robertson and Friesen, 1981; Robertson et al., 1982; Soares et al., 1983, 1985; Colosi et al., 1987a), PL-II (Robertson and Friesen, 1975; Colosi et al., 1982), prolactin-related proteins (Duckworth et al., 1986b, c), proliferin (Linzer et al., 1985; Lee and Nathans, 1987; Lee et al., 1988; Nilsen-Hamilton et al., 1988) and at least one proliferin-related protein (Linzer and Nathans, 1985; Colosi et al., 1988). Complementary DNAs have been isolated and the predicted amino acid sequences determined for all of these proteins (Linzer et al., 1985; Duckworth et al., 1986a, b; Jackson et al., 1986; Colosi et al., 1987b). The temporal pattern of expression of the mRNAs in the placenta during gestation has been determined (Linzer et al., 1985; Duckworth et al., 1986a, b; Jackson et al., 1986; Colosi et al., 1987b) and information exists on the gestational serum concentrations of some of the hormones (Robertson and Friesen, 1981; Soares et al., 1982; Colosi et al., 1988; Lee et al., 1988), but far less is known about the processing and expression of the mature proteins by the placenta.

Availability of amino acid sequence information for the prolactin-like proteins permits the development of immunological probes to specific regions of each protein (see Walter and Doolittle, 1983; Walter, 1986 for reviews of the technique). In this report we describe the development of antisera to specific regions of one of the proteins, rat PL-II. PL-II is the principal prolactin-like hormone present in the circulation during the latter half of gestation (Robertson and Friesen, 1981; Soares et al., 1982) and is a potent stimulator of mammary gland development (Thordarson et al., 1986). Considerable evidence has accumulated suggesting that there are a number of closely related isoforms of rat PL-II (Soares et al., 1988). Some of the isoforms may represent separate gene products (Duckworth et al., 1986b), whereas others may represent post-transcriptional or post-translational modifications. Specific immunologic probes to various regions of the PL-II

sequence will help resolve some of these uncertainties.

The purpose of this investigation was to develop specific immunologic probes to rat PL-II and to use the immunologic probes to further characterize rat PL-II.

Materials and methods

Hormones and reagents

Hormones used for the characterization of the antipeptide antisera included rat prolactin (PRL; NIADDK-rat PRL-B-4), rat growth hormone (GH; NIADDK-rGH-RP-1), ovine PRL (NIADDK-oPRL-1), and human PL. These hormones were obtained from the National Hormone and Pituitary Program (Baltimore, MD, U.S.A.). Human GH was a generous gift from Dr. Wayne Moore of the Department of Pediatrics, University of Kansas Medical Center (Kansas City, KS, U.S.A.). Mouse PL-II was purified according to previously published procedures (Colosi et al., 1982). A semi-purified preparation of rat PL-II was used in the characterization of the antipeptide antisera. It was obtained by a modification of the procedure described for the isolation of mouse PL-II (Colosi et al., 1982). Rat chorioallantoic placentas (obtained from day 17–19 of gestation) were extracted under alkaline conditions. Rat PL-II was then precipitated from the clarified extract with 50% ammonium sulfate, and the pellet was solubilized and chromatographed on Sephacryl S-300 (Sigma Chemical Co., St. Louis, MO, U.S.A.). Fractions were monitored with a PRL radioreceptor assay (Shiu et al., 1973; Soares, 1987). Active fractions were pooled, dialyzed against 50 mM NH_4HCO_3 buffer, pH 9.0, and lyophilized. The activity of the preparation was 0.5 μg of oPRL-like activity/mg of protein. Antiserum to the entire purified rat PL-II protein (Southard and Talamantes, 1987) was generated as previously described (Southard and Talamantes, 1987), and antiserum to human PL was obtained from ICN Immunobiologicals (Lisle, IL, U.S.A.).

Selection of oligopeptides

Our rationale for selecting peptide sequences from rat PL-II (Fig. 1) was based on three criteria: (1) relative hydrophilicity/hydrophobicity of the

RAT PLACENTAL LACTOGEN-II

10	20	30
<u>APNYRMSTGSLYQ</u> RVVGLSHYTHDLASKVF		
40	50	60
IEFDMKFGRTVWTHNLMLS <u>PCHTAAIPTPE</u>		
70	80	90
<u>NSEQVHOAKSE</u> DLLKVSITILQAWQEPLKH		
100	110	120
<u>IYAAVATLPDGS</u> DTLLSRTKELEERIOGLL		
130	140	150
EGLETILSRVQPGAVGSDYTFWSEWSDLQS		
160	170	180
<u>SDKSTKNGVLS</u> YLYRCMRDTHKVDNFLKV		
190		
LKCRDIYNNNC		

Fig. 1. Amino acid sequence of rat PL-II (Duckworth et al., 1986a). Underlined sequences were selected for synthesis. Single letter abbreviations for amino acids are used (aspartic acid = D, threonine = T, serine = S, glutamic acid = E, proline = P, glycine = G, alanine = A, cysteine = C, valine = V, methionine = M, isoleucine = I, leucine = L, tyrosine = Y, phenylalanine = F, lysine = K, histidine = H, arginine = R, tryptophan = W, glutamine = Q, asparagine = N).

region as determined by the computer program of Hopp and Woods (1981, see Fig. 2); (2) presence of *B*-turns (frequently indicated by prolines in sequences, Walter and Doolittle, 1983); and (3) sequence comparisons with other proteins, especially those of the PRL-GH family. Hydrophilic regions and those containing *B*-turns would have

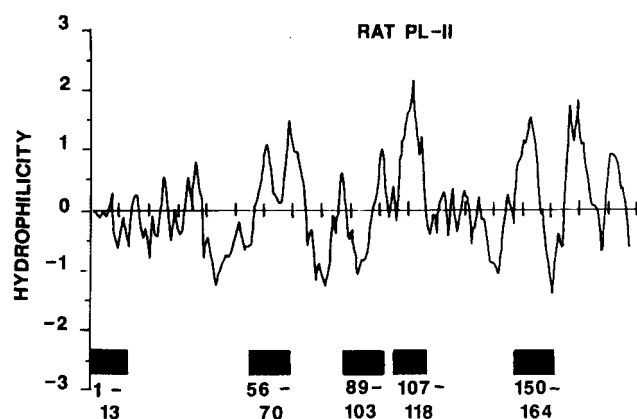


Fig. 2. The hydrophilic character of rat PL-II was determined by the computer program of Hopp and Woods (1981). Hydrophilic regions are shown as an upward excursion from the x-axis, whereas hydrophobic regions are shown as a downward excursion from the x-axis. Positions of peptides along the amino acid sequence are indicated by the black bars at the bottom of the figure.

a presumed superficial location and thus be more likely to be part of an antigenic site. The five peptide sequences selected for this study are shown in Fig. 1.

Synthesis and characterization of the oligopeptides

The oligopeptides were synthesized as carboxy-terminal amides using a Biosearch SAM TWO solid phase peptide synthesizer (Biosearch, San Rafael, CA, U.S.A.) and standard *tert*-butoxy-carbonyl (BOC) methodology (Marglin and Merri-field, 1970). A cysteine residue was added to the amino terminus of each of the peptides to facilitate coupling to the carrier protein. Following cleavage of the peptides from the resin with hydrogen fluoride, the crude peptides were recovered by lyophilization and purified by reverse phase high performance liquid chromatography (HPLC). All of the peptides were chromatographed under similar mobile (0.1% trifluoroacetic acid in water with gradients utilizing various acetonitrile concentrations) and stationary (octadecyl silica, C-18, ES Industries, Marlton, NJ, U.S.A.) phases using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan). Peptides were monitored by absorbance at 230 nm. Major peptides eluting from the column were lyophilized and their amino acid compositions determined as follows. Samples were hydrolyzed in 6 N HCl for 20 h at 105°C. The hydrolyzed samples were dried in an evacuated desiccator over NaOH pellets while low heat was applied. The dried hydrolyzed samples were reconstituted in 0.2 N sodium citrate buffer pH 2.2 and injected onto a Beckman 121 MB amino acid analyzer (Beckman Instruments, Fullerton, CA, U.S.A.) for amino acid composition analysis and total peptide content.

Coupling of synthetic oligopeptides to carrier protein

The oligopeptides were coupled to the carrier protein keyhole limpet hemocyanin (KLH; Calbiochem, LaJolla, CA, U.S.A.) through the amino terminal cysteine residue of each peptide by using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) as described previously (Lerner et al., 1981). KLH (Calbiochem, San Diego, CA, U.S.A.) was solubilized in 0.05 M sodium phosphate buffer, pH 6.0, at a concentration of 5 mg/0.25 ml, and MBS (Calbiochem) was solubi-

lized in *N,N*-dimethyl formamide (DMF, Sigma) at a concentration of 50 mg/ml. 10 μ l of the MBS solution (0.5 mg) were added to 0.2 ml of KLH solution (4 mg). The mixture was incubated for 30 min at room temperature with gentle vortexing. A slight precipitate was normally formed during the incubation and it was removed by centrifugation. The MBS-KLH mixture (supernatant) was then loaded onto a Sephadex G-25 column, pre-equilibrated with 0.05 M sodium phosphate buffer, pH 7.0, to separate free MBS from MBS coupled to KLH. Fractions absorbing at 280 nm were pooled and the protein concentration of the MBS-KLH preparation determined (Bradford, 1976). Purified oligopeptides were initially solubilized in a minimal volume of distilled water and then added to the MBS-KLH preparation in 0.05 M phosphate buffer, pH 7.0, at a 1:1 ratio (5 mg of peptide to 5 mg of MBS-KLH in a final volume of 4 ml). The mixture was allowed to react for 3 h at room temperature with constant shaking. At the termination of the reaction, the peptide-KLH mixtures were aliquoted and stored frozen until used for immunization of rabbits.

Immunization procedure

Adult New Zealand white rabbits obtained from White Hare Rabbitry (Stark City, MO, U.S.A.) were immunized with oligopeptide-KLH preparations ($n = 2-3$ rabbits/peptide). Prior to the immunization all rabbits were bled to obtain pre-immune serum. The rabbits were initially injected with 400 μ g of the oligopeptide-KLH preparation in Freund's complete adjuvant (1:1 ratio of peptide in phosphate buffer to adjuvant, v/v, Organon Teknika Corp., West Chester, PA, U.S.A.) in 30-40 intradermal sites on the back of each rabbit. Two weeks after the initial immunization, the animals were injected subcutaneously with 200 μ g of the oligopeptide-KLH preparation prepared with Freund's incomplete adjuvant (Organon Teknika Corp.) in the upper back and neck region. Animals were injected with the same regimen and in the same location 2 weeks later and then bled within 7-10 days. An injection and bleeding protocol, identical to the previous, was performed at monthly intervals for each of the immunized rabbits.

Characterization of the anti-peptide antisera

Anti-peptide antisera were characterized with two techniques: (1) enzyme-linked immunoassay; and (2) immunoblotting of proteins separated by gel electrophoresis and transferred to nitrocellulose.

Enzyme-linked immunoassay (EIA). A standard EIA was used to screen reactivities of antisera with oligopeptides and various hormones (Engvall, 1980). Polystyrene 96-well microtiter plates (Costar Corp., Cambridge, MA, U.S.A.) were coated with the synthetic peptides representing various regions of rat PL-II (100 ng/well), partially purified rat PL-II (10 ng of prolactin-like activity/well), or with other proteins of the prolactin/growth hormone family (25 ng/well). The peptides and proteins were diluted in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, 3 mM sodium azide, pH 9.6), added to the wells of the microtiter plates (100 μ l/well) and incubated at 4°C for a minimum of 16 h. The plates were washed twice (200 μ l/well) with wash buffer (1.5 mM potassium phosphate, 20 mM sodium phosphate, 2.7 mM potassium chloride, 0.14 M sodium chloride, pH 7.4 containing 0.05% Tween 20, v/v), incubated with 5% Carnation milk in coating buffer (200 μ l/well) for 30 min at room temperature, and the wells then washed twice with wash buffer (200 μ l/well). Primary antisera or pre-immune sera, diluted to various concentrations in coating buffer, were added to the wells (100 μ l/well) and incubated for 3 h at 37°C. Wells were washed 3 times with wash buffer (200 μ l/well) and then incubated with goat anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase conjugate (Sigma, 100 μ l/well) prepared in 10 mM Tris-HCl, 150 mM sodium chloride, pH 8.0, containing 0.05% Tween 20, v/v at a final dilution of 1:1000. The incubation duration was 90 min at room temperature followed by two washes with wash buffer. Assessment of alkaline phosphatase activity was determined by incubation of the wells for 15-30 min at room temperature with 8 mM disodium *p*-nitrophenyl phosphate (200 μ l/well, Sigma) dissolved in a buffer containing 100 mM sodium bicarbonate, 10 mM magnesium chloride, pH 9.5. The reaction was terminated by the addition of 2 N sodium hydroxide (50 μ l/well). Microtiter plates were then read

at 405 nm with a Titertek Multiskan Plate Reader (Flow Laboratories, McLean, VA, U.S.A.).

Antibody titers were estimated by measuring the endpoint dilution, which was calculated as the dilution of antiserum generating absorbance readings equivalent to background (pre-immune serum at a 1:100 dilution). The amounts of peptide/protein used for coating varied in different experiments and the efficiency of peptide/protein coating to the wells was not evaluated. Thus, antibody titers are relative for the peptide/protein coated on the well and comparison of titers between various peptides and proteins is not valid.

Cross-reactivities of rat PL-II and rat PLP-A peptides with antiserum to amino acids 56–70 of rat PL-II were evaluated as follows. Microtiter plates were coated with 5 ng of the peptide representing amino acids 56–70 of rat PL-II per well. The wells were blocked with Carnation milk, incubated with pre-immune serum (1:20 000, final dilution) or antiserum to amino acids 56–70 of rat PL-II (1:20 000, final dilution) and various concentrations of the different peptide preparations. The remainder of the procedure was as described above.

Immunoblotting. Various hormone preparations were separated by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis in 12.5% gels under reducing or nonreducing conditions (Laemmli, 1970) and electrophoretically transferred to nitrocellulose (Towbin et al., 1979). Processing and development of the nitrocellulose membranes for reactivity with various antisera have been described in detail elsewhere (Soares et al., 1988). Antigen-antibody reactivities were visualized by the histochemical development of alkaline phosphatase conjugated to an antibody directed against rabbit IgG (Blake et al., 1984). Specificity of antisera reactivity was determined by substituting each antiserum with pre-immune serum and by incubating the antiserum with an excess amount of peptide.

Interactions of rat PL-II peptides with lactogen receptors

The ability of various concentrations of the five PL-II peptides to compete with 125 I ovine PRL for lactogen receptors (obtained from the lactating rabbit mammary gland) was examined. The ra-

dioreceptor assay was conducted as previously described by this laboratory (Soares, 1987; Soares and Glasser, 1987). Antipeptide antisera were also tested for their ability to interfere with the binding of rat PL-II and human PL to lactogen receptors. Different concentrations of antipeptide antiserum (or pre-immune serum) were incubated with 125 I ovine PRL, lactogen receptors and either rat PL-II or human PL. Specific neutralization of rat PL-II or human PL displacement activity with antipeptide antiserum would provide information on lactogen receptor binding domains within PL-II and human PL.

Results

Reactivities of the antipeptide antisera with rat PL-II and synthetic peptides derived from rat PL-II

Each of the five peptides coupled to KLH successfully generated immune responses in rabbits. The antisera titer to the peptides varied considerably (Table 1). The amino terminal peptide (1–13) provoked the smallest response, whereas

TABLE 1

ANTIPEPTIDE ANTISERUM TITER FOR THE RESPECTIVE RAT PL-II PEPTIDE ANTIGEN ^a

Antigen (peptides)	Rabbit number	Titer ^b	
		Five weeks	Ten weeks
1–13	8	18 000	12 000
	11	nd ^c	nd
	12	8 000	15 000
56–70	26	629 000	600 000
	27	394 000	400 000
89–103	16	225 000	235 000
	17	150 000	150 000
	19	314 000	335 000
107–118	23	271 000	Died
	24	344 000	284 000
	25	236 000	205 000
150–164	2	175 000	202 000
	3	229 000	291 000
	5	231 000	195 000

^a Polystyrene 96-well microtiter plates were coated with the respective peptides at a concentration of 1 µg/ml in a volume of 100 µl.

^b Values are expressed as the reciprocal of the endpoint dilution.

^c Not detectable.

TABLE 2

ANTIPEPTIDE ANTISERUM TITER FOR NATIVE RAT PL-II ^a

Antigen (peptides)	Rabbit number	Titer ^b	
		Five weeks	Ten weeks
1-13	8	600	7192
	11	5757	5875
	12	2457	2575
56-70	26	9317	9640
	27	7417	9646
89-103	16	984	4441
	17	690	1010
	19	775	693
107-118	23	nd ^c	Died
	24	200	350
	25	450	200
150-164	2	nd	nd
	3	758	1200
	5	136	214

^a Polystyrene 96-well microtiter plates were coated with partially purified rat PL-II at a concentration of 100 ng of PRL-like activity/ml in a volume of 100 μ l.

^b Values are expressed as the reciprocal of the endpoint dilution.

^c Not detectable.

the internal sequences generated significant titers. The highest anti-peptide antisera titer was obtained with peptide 56-70. Recognition of native rat PL-II as determined by EIA analysis was achieved with anti-peptide antisera generated to the amino terminal peptide (amino acids 1-13), peptide 56-70 and peptide 89-103 (Table 2). Anti-peptide antisera to peptide 56-70 generated the largest and most consistent reactivity to native rat PL-II. Our inability to detect antibody production to the amino terminal peptide (amino acids, 1-13) in rabbit number 11, while finding that the same immune serum detected native rat PL-II is difficult to explain.

Antisera to the entire rat PL-II protein (Southard and Talamantes, 1987) showed strong reactivity with peptide 56-70, minimal reactivity with the amino terminal peptide (1-13) and very low reactivity with the three other internal rat PL-II sequences. The absorbance reading at 405 nm in a representative EIA using anti-PL-II serum (1:100) for each of the peptides (100 ng of peptide coated

per well) was: 1-13, 0.27; 56-70, 1.43; 89-103, 0.12; 107-118, 0.14; 150-164, 0.16.

Immunoblotting analysis of the reactivities of the anti-peptide antisera indicated that all of the antibodies reacted with reduced, denatured rat PL-II (Fig. 3). Antisera to each of the peptides recognized a prominent protein at 25 000 molecular weight and slightly smaller proteins at molecular weights 23-24 000. This set of immunoreactive bands was also recognized by the antiserum to the entire rat PL-II protein (Fig. 3). Additional low molecular weight bands present in Fig. 3 under reducing conditions were found to be nonspecific (data not shown). Nonreduced rat PL-II was recognized by the antiserum to the entire rat PL-II protein and by antisera to peptides 56-70 and 150-164 but not by antisera to the other three peptides (Fig. 3). Under nonreducing conditions antisera to peptide 56-70 and to the entire rat PL-II protein recognized two major proteins possessing molecular weights of 20 000 and 25 000. Antisera to peptide 150-164 only recognized the 20 000 molecular weight protein. The presence of the 25 000 molecular weight protein in the nonreduced immunoblots suggests that some reduced rat PL-II may exist in the semi-purified rat PL-II preparation. The reactivities of the anti-peptide antisera and anti-rat PL-II antiserum with rat PL-II are summarized in Table 3.

The recognition of rat PL-II on immunoblots by antisera generated to each of the peptides

TABLE 3

SUMMARY OF RELATIVE REACTIVITIES OF ANTI-PEPTIDE ANTISERA WITH RAT PL-II

Antigen (peptides)	Relative reactivity with rat PL-II		
	Native ^a	Denatured ^b	Denatured and reduced ^b
1-13	+	-	+
56-70	++	++	+++
89-103	+	-	++
107-118	-	-	+
150-164	-	+	+
Rat PL-II	+++	+++	+++

^a Derived from EIA data described in the Results section.

^b Derived from electrophoresis and immunoblot data described in the Results section. (+++) strong, (++) moderate, (+) weak, (-) no reactivity.

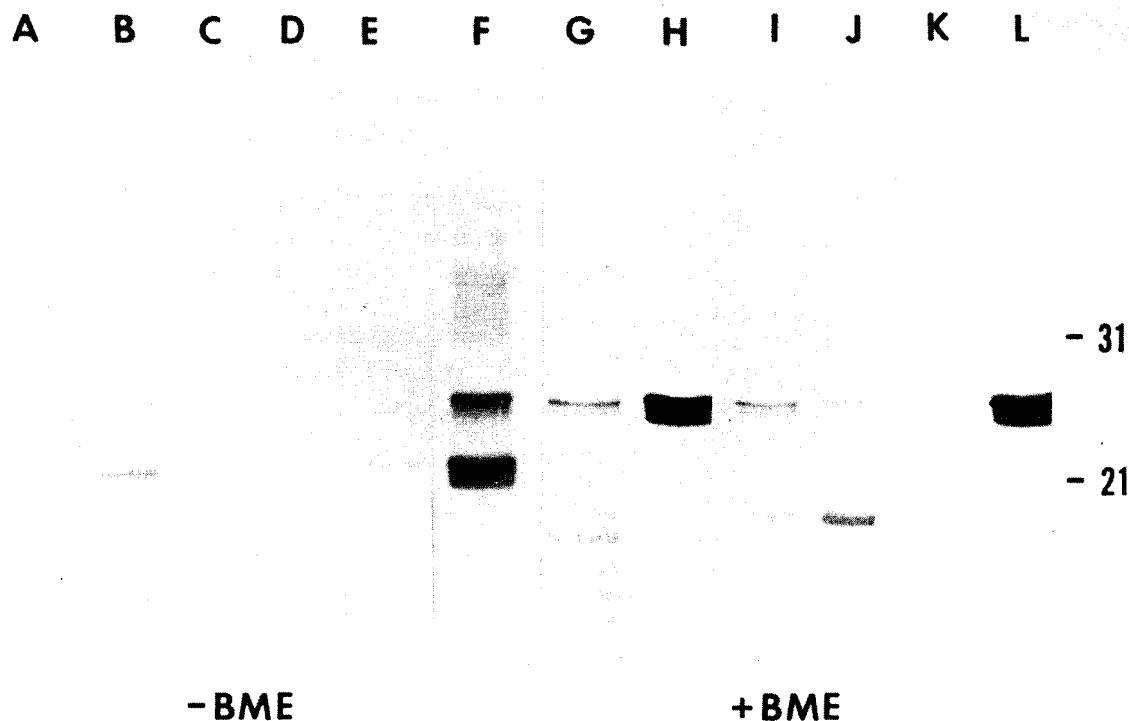


Fig. 3. Immunoreactivities of anti-peptide antisera to rat PL-II. A semi-purified preparation of rat PL-II was separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis in 12.5% gels and electrophoretically transferred to nitrocellulose. Nitrocellulose membranes were probed with anti-peptide antisera directed to different regions of the rat PL-II molecule: amino acids 1-13 (A and G), 56-70 (B and H), 89-103 (C and I), 107-118 (D and J), 150-164 (E and K) and to the entire rat PL-II protein (F and L). Lanes A through F were separated under nonreducing conditions and lanes G through L were separated under reducing conditions. Antisera were used at a final dilution of 1:100.

could be blocked by co-incubation of the anti-peptide antiserum with excess amounts of peptide or by substituting pre-immune serum for the antiserum (Fig. 4). The reactivity of the antiserum to the entire rat PL-II protein with rat PL-II could not be blocked by addition of excess amounts of peptide 56-70 suggesting the existence of antibodies to multiple antigenic sites in the anti-rat PL-II antiserum (Fig. 4).

Cross-reactivity of anti-peptide antiserum to peptide 56-70 with PLs, PRLs and GHs

Comparison of the immunoblotting reactivities of antiserum to peptide 56-70 with various members of the PRL and GH protein family revealed significant reactivity with rat, mouse and human PLs, slight reactivity with human GH, and no reactivity with rat GH or rat PRL (Fig. 5). The reactivity of antiserum generated to amino acids 56-70 of rat PL-II with mouse PL-II and human PL was specific (Fig. 6). Human PL could also be

detected with antiserum to the entire rat PL-II protein but not with antisera to the four other synthetic rat PL-II peptides (Fig. 7).

Examination of the reactivities of PRL- and GH-related proteins with antiserum to peptide 56-70 in the EIA revealed a relationship similar to that observed in the immunoblotting experiments. Only rat PL-II and human PL showed significant reactivity with the antiserum to peptide 56-70 in the EIA.

Examination of the reactivities of a variety of different synthetic peptides with antiserum to peptide 56-70 in the EIA revealed reactivity with peptide 56-70 of rat PL-II, and surprisingly, some limited reactivity with peptide 1-13 of rat PL-II, but no significant reactivities with an assortment of other rat PL-II peptides and rat placental PRL-related protein (PLP-A) peptides (Fig. 8).

A polyclonal antiserum to human PL (ICN Immunobiologicals) specifically recognized human PL but failed to recognize rat PL-II in electro-

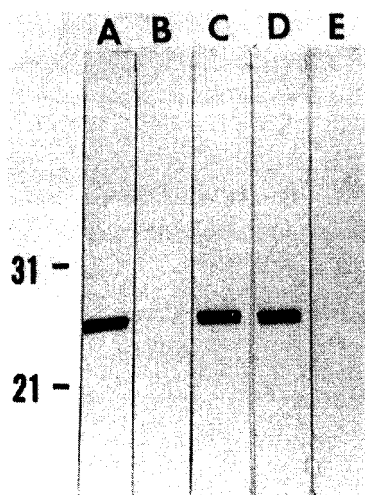


Fig. 4. Examination of the specificity of the reaction of antiserum directed to amino acids 56-70 of rat PL-II with a semi-purified preparation of rat PL-II following electrophoresis under reducing conditions and immunoblotting. Lane A: antiserum to amino acids 56-70, lane B: antiserum to amino acids 56-70+excess peptide representing amino acids 56-70 (16 $\mu\text{g}/\text{ml}$), lane C: antiserum to the entire rat PL-II protein, lane D: antiserum to the entire rat PL-II protein+excess peptide representing amino acids 56-70 (16 $\mu\text{g}/\text{ml}$), lane E: pre-immune serum. Antiserum and pre-immune serum were used at dilutions of 1:2000.

phoretic and immunoblotting experiments. The antiserum to human PL also failed to recognize the peptide representing amino acids 56-70 of rat PL-II in EIA experiments.

Functional characterization of rat PL-II synthetic peptides and anti-peptide antisera

Some functional aspects of various rat PL-II regions were examined by evaluating the effects of adding rat PL-II synthetic peptides on the binding of ^{125}I ovine PRL to lactogen receptors and by examining the ability of anti-peptide antisera to prevent the interaction of rat PL-II or human PL with lactogen receptors. None of the synthetic peptides (used at concentrations $\leq 150 \mu\text{g}/\text{ml}$) successfully competed with radioiodinated ovine PRL for binding to lactating rabbit mammary gland PRL receptors (data not shown). Antiserum to peptide 56-70 also failed to inhibit the binding of rat PL-II and human PL to PRL receptors isolated from the lactating rabbit mammary gland. Since anti-peptide antisera directed to other regions of rat PL-II did not recognize native rat

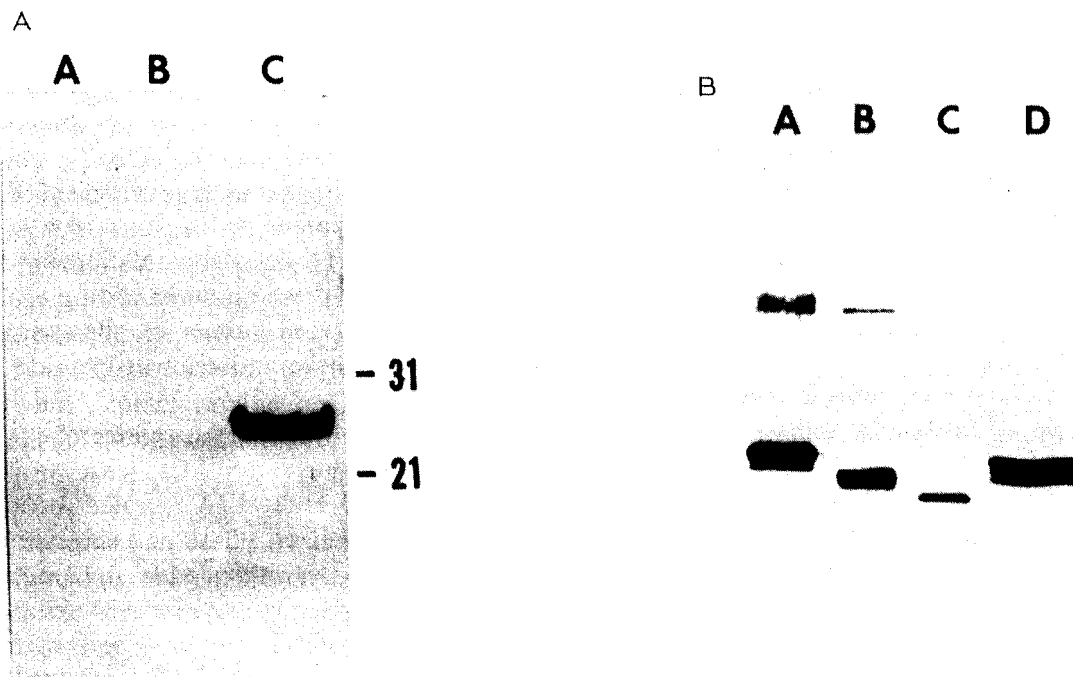


Fig. 5. Cross-reactivity of anti-peptide antiserum to amino acids 56-70 of rat PL-II. Left panel: rat PRL (lane A: 5 μg); rat GH (lane B: 5 μg) and rat PL-II (lane C: 50 ng). Right panel: mouse PL-II (lane A: 1 μg), human PL (lane B: 1 μg), human GH (lane C: 1 μg) and rat PL-II (lane D: 50 ng). Samples were subjected to electrophoresis under reducing conditions and immunoblotting as described above.

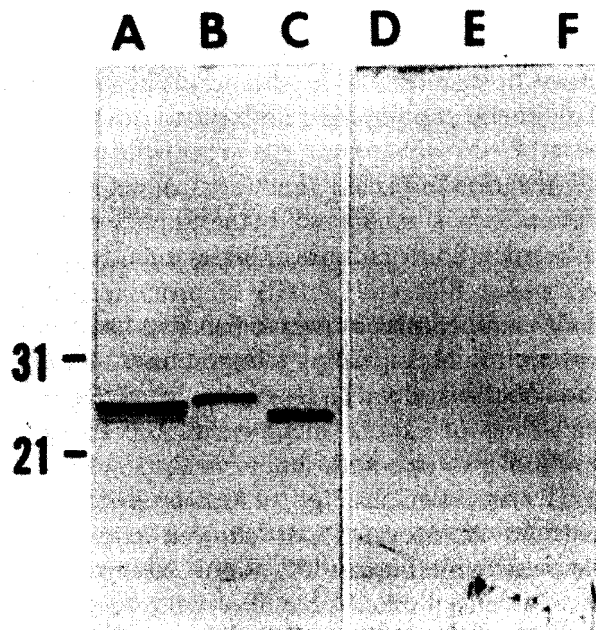


Fig. 6. Specificity of the reactivity of antiserum directed to amino acids 56-70 of rat PL-II with a semi-purified preparation of rat PL-II (lanes A and D, 50 ng/lane), mouse PL-II (lanes B and E, 200 ng/lane) and human PL (lanes C and F, 50 ng/lane). Samples were subjected to electrophoresis and immunoblotting as described above. Lanes A through C were incubated in the presence of antiserum to amino acids 56-70 (1:100) and lanes D and F were incubated with antiserum to amino acids 56-70 (1:100) + excess peptide representing amino acids 56-70 (80 μ g/ml). It was necessary to use 4 times the amount of mouse PL-II as human PL in order to visualize the band generated from the immunoblot.

PL-II or human PL, their effects on the binding of rat PL-II or human PL to lactogen receptors were not evaluated.

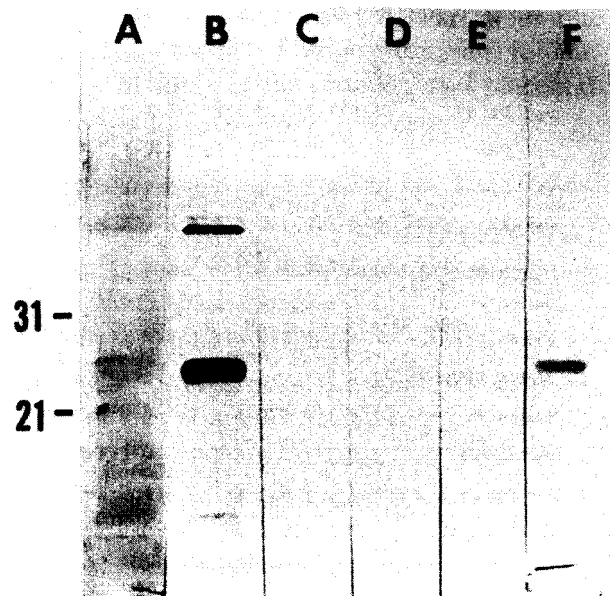
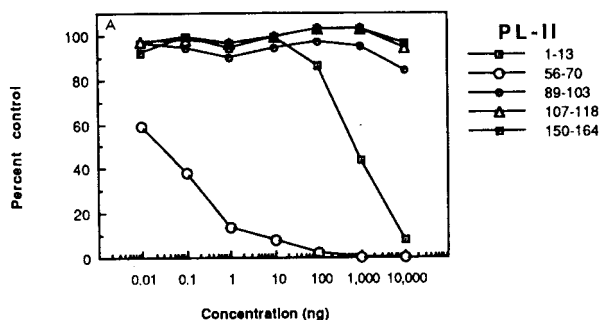


Fig. 7. Cross-reactivity of anti-peptide antiserum to various regions of rat PL-II and antiserum to the entire PL-II protein with human PL (1 μ g/lane). Lane A: antiserum to amino acids 1-13, lane B: antiserum to amino acids 56-70, lane C: antiserum to amino acids 89-103, lane D: antiserum to amino acids 107-118, lane E: antiserum to amino acids 150-164, lane F: antiserum to entire rat PL-II protein. Human PL was subjected to electrophoresis and immunoblotting as described above. All antisera were used at a dilution of 1:100.

Discussion

In this report we have described the generation of antisera to selected regions of the rat PL-II amino acid sequence.

The anti-peptide antibodies showed reactivity with the entire rat PL-II protein; however, the

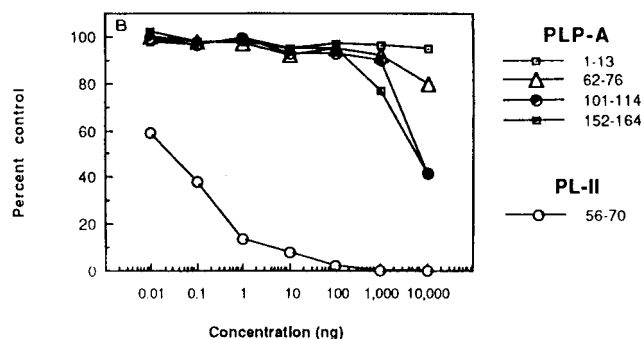


Fig. 8. Examination of the reactivities of various synthetic peptides with antiserum to amino acids 56-70 of rat PL-II determined by EIA. Panel A: rat PL-II peptides. Panel B: rat PLP-A peptides.

SEQUENCE SIMILARITIES

	56	70
rat PL-II	I-P-T-P-E-N-S-E-Q-V-H-Q-A-K-S	
rat PRL	L-A-T-P-E-D-K-E-Q-A-Q-K-V-P-P	
rat PLP-A	L-S-S-P-E-N-K-E-Q-A-Q-Q-F-Q-L	
rat GH	I-P-A-P-T-G-K-E-E-A-Q-Q-R-T-D	
mouse PL-I	I-H-T-P-E-N-R-E-E-V-H-E-T-K-T	
mouse PL-II	I-L-T-P-E-N-S-E-Q-V-H-Q-T-T-S	
mouse PLF	L-P-T-P-E-N-K-E-Q-A-R-L-T-H-Y	
mouse PRP	I-N-V-P-E-T-V-E-D-V-R-K-T-S-F	
human PL	I-P-T-P-S-N-M-E-E-T-Q-Q-K-S-N	
human GH	I-P-T-P-S-N-R-E-E-T-Q-Q-K-S-N	
bovine PL	M-T-T-P-N-N-K-E-A-A-A-N-T-E-D	
bovine PRCI	F-H-A-P-E-E-R-D-I-V-Q-Q-T-N-I	

Fig. 9. Sequence identities of various members of the PRL/GH family of proteins. Single letter abbreviations for amino acids are used. See Fig. 1. References for sequence information: Duckworth et al., 1986a, b; Jackson et al., 1986; Colosi et al., 1987b; Schuler and Hurley, 1987; Schuler et al., 1988.

reactivities of each antiserum with rat PL-II were dependent on the conformational state of rat PL-II. Antisera to each of the selected regions showed positive reactivity following both reduction and denaturation of rat PL-II, whereas only antisera to synthetic peptides corresponding to amino acid regions 56–70 and 150–164 reacted with denatured rat PL-II under nonreducing conditions. Reactivities with native rat PL-II were achieved with antisera to peptides representing amino acids 1–13, 56–70 and 89–103. Antisera directed to amino acids 56–70 showed the best reactivity toward each of the conformational states of rat PL-II tested. The 56–70 amino acid segment was identified as one or part of one of the epitopes of rat PL-II. Polyclonal antibodies generated to the entire rat PL-II protein (Southard and Talamantes, 1987) specifically recognized the 56–70 amino acid sequence but showed limited reactivity with synthetic peptides corresponding to amino acids 1–13, 89–103, 107–118, or 150–164. The results of the antipeptide antiserum reactivities to different conformational states of rat PL-II allow us to make some conservative predictions regarding the rat PL-II structure. We hypothesize that within the native folding pattern of rat PL-II amino acids 56–70 would have a superficial location (accessi-

ble to antibodies) whereas amino acid sequences 1–13, 89–103, 107–118 and 150–164 would be buried (inaccessible to antibodies). The generation of synthetic peptides and antisera to other regions of rat PL-II will permit the identification of additional structural and functional domains of the protein.

Examination of the reactivities of other members of the PRL/GH family of proteins with the PL-II antipeptide antisera provides some important insights regarding the structural characteristics of the antibody recognition sites. Sequence similarities of various members of the PRL/GH family of proteins with amino acids 56–70 of rat PL-II are shown in Fig. 9. Antiserum to amino acids 56–70 of rat PL-II showed considerable reactivity with human PL, some reactivity with mouse PL-II, limited reactivity with human GH and lacked detectable reactivity with rat PRL and rat GH. These observations are illuminating in that they are not entirely consistent with the overall sequence identities of the hormones. Mouse PL-II shows approximately 80% sequence identity with amino acids 56–70 of rat PL-II (Duckworth et al., 1986a; Jackson et al., 1986) yet shows less reactivity with antisera directed towards amino acids 56–70 of rat PL-II than does human PL, which shows approximately 47% sequence identity with amino acids 56–70 of rat PL-II (Duckworth et al., 1986a; Nicoll et al., 1986). Human GH has an amino acid sequence identical to human PL in this region except for a substitution of an arginine for a methionine (Nicoll et al., 1986). This substitution has a marked effect on the reactivity of human GH with antisera directed towards amino acids 56–70 of rat PL-II. Based on these reactivities we can begin to predict which amino acids might be critical for the generation of the epitope represented by amino acids 56–70 of rat PL-II and test these predictions by constructing specific synthetic peptides. Furthermore, we would hypothesize that mouse proliferin shares a similar epitope with rat PL-II. However, the existence of proliferin in the rat has not been determined.

The overall sequence identity of human PL with rat PL-II is only 18%. Antisera generated to other regions of the rat PL-II molecule did not recognize human PL, only antisera to amino acids 56–70 and the antiserum to the entire rat PL-II

protein recognized human PL. The preservation of this structural feature of human PL and rat PL-II is of interest in that human PL is believed to have evolved from human GH (Nicoll et al., 1986), whereas rat PL-II is believed to have evolved from rat PRL (Duckworth et al., 1986c). Retention of the epitope, represented by amino acids 56–70, may suggest a structural requirement for human and rodent placental PRLs; however, it is important to note that bovine placental PRLs show limited sequence homology with the 56–70 amino acid sequence of rat PL-II (Fig. 9; Schuler and Hurley, 1987; Schuler et al., 1988).

Synthetic peptides and antisera to synthetic peptides have proven to be useful tools in elucidating domains involved in hormone action (Socher et al., 1987). Chemically synthesized peptides representing five different regions of rat PL-II did not show significant interactions with lactogen receptors, and antisera to amino acids 56–70 did not prevent rat PL-II or human PL from interacting with lactogen receptors. Other regions of the PL-II protein must be involved in interactions with the lactogen receptors. The receptor binding domain may include other continuous segments of rat PL-II not investigated or discontinuous segments of rat PL-II brought together during protein folding.

In summary we have generated a series of immunologic probes for studying the structure of rat PL-II. The sequence comprising amino acids 56–70 of rat PL-II was shown to make up at least part of an epitope for rat PL-II and to be a region of significant structural homology with mouse PL-II and human PL.

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