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A GLYCOSYLATED PROLACTIN SPECIES IS COVALENTLY BOUND TO IMMUNOGLOBULIN IN HUMAN AMNIOTIC FLUID

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SUMMARY: Western immunoblots performed during separation of glycosylated prolactin from amniotic fluid revealed that some of the glycosylated prolactin is covalently bound to another protein. Using high performance liquid chromatography and other protein isolation techniques we have demonstrated that a glycosylated prolactin species is linked to immunoglobulin by disulfide bonds in amniotic fluid.

The protein hormone prolactin is now known to be secreted and to circulate both as a glycosylated (G-PRL) and as a nonglycosylated form (PRL) (1,2). The *in vivo* function of G-PRL is unknown, but G-PRL has altered receptor binding and biologic activity when compared to PRL (3,4).

Recently it has been shown that the different molecular weight species of prolactin reported in human serum are composed of different ratios of G-PRL and PRL (5). The higher molecular weight species are made up of only G-PRL, whereas lower molecular weight species are comprised of mixtures of both G-PRL and PRL. These molecular weight species of prolactin may represent a way of segregating prolactins with different biologic activities.

During the course of purifying G-PRL from amniotic fluid, we observed that the G-PRL associated with the >100 kDa molecular weight fractions was tightly bound to another amniotic fluid protein that is unrelated to prolactin. We report here the evidence for the covalent disulfide binding of G-PRL to immunoglobulin in human amniotic fluid.

MATERIALS AND METHODS

Human amniotic fluid was obtained following a protocol approved by the Committee for the Protection of Human Subjects from Risk of the Brigham and

ABBREVIATIONS: G-PRL, glycosylated prolactin; PRL, prolactin; kDa, kiloDaltons; HPLC, high performance liquid chromatography; TFA, trifluoracetic acid; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; NTC, nitrocellulose; TBS, Tris buffered saline; BSA, bovine serum albumin; TBST, TBS + 0.1% Tween-20.

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Women's Hospital. The fluid was centrifuged at 3290 x g, filtered to remove fetal cellular debris and stored at 4°C. Freezing and thawing, lyophilization, and concentration of material to more than twice starting concentrations were avoided because of problems with aggregation and precipitation of the G-PRL complex. Sample buffers were changed by dialysis against 100x starting volumes and volume reductions were performed using Centricon filters (Amicon).

Separation Procedures

All HPLC was performed on a dual pump system with an automated interface to gradient control software (Biorad). Buffer gradients were monitored using a conductivity meter and proteins, by measuring absorbance at 280 nm. Both detectors were interfaced with a data acquisition system.

The following separation techniques were used either alone or in combination.

Anion exchange HPLC: A TOSOHAAS TSK DEAE 5PW, 10 μm,1000 A, 21.5 x 150 mm column, equilibrated with 50 mM Tris, 0.01% NaN_3, 6 M urea, pH = 8.5, was eluted stepwise with equilibration buffer containing 250 mM NaCl.

Hydrophobic interaction HPLC: A NEST PROPYL ASPARTAMIDE, 5 μm, 300 Å, 4.6 x 200 mm column, equilibrated with 1.6 M $(NH_4)_2SO_4$, pH=7.0, was eluted stepwise with 10 mM phosphate buffer, pH=7.0.

Cation exchange HPLC: A BAKERBOND ABx, 5 μm, 300 Å, 7.75 x 100 mm column, equilibrated with 20 mM 2-(N-morpholino)ethane sulfonic acid, pH=5.5, was eluted stepwise with 0.1 M $(NH_4)_2SO_4$, pH=7.0.

Reverse phase HPLC: A VYDAC PROTEIN C4, 10 μm, 300 Å, 4.6 x 250 mm column protected by a BIORAD MICROGUARD C4, 5 μm, 330 Å, 4.6 x 40 mm column, equilibrated with 0.05% trifluoroacetic acid, pH=2.0, was eluted stepwise with equilibration buffer plus 80% acetonitrile.

Gel filtration HPLC: A BECKMAN SW 125, 10 μm, 125 Å, 7.5 x 75 mm column in series with a BIORAD BIOSIL SW 250, 10 μm, 250 Å, 7.5 x 300 mm and a TOSOH TSK SW 400, 13 μm, 400 Å, 7.5 x 300 mm column, equilibrated with PBS, pH=7.0, was eluted with isocratic gradients of either PBS, PBS + 6M urea or 50 mM Tris, pH=7.0.

Fractionation of amniotic fluid precipitated G-PRL at 43% $(NH_4)_2SO_4$ and 75% ethanol. Protein A Sepharose-4B (Pharmacia) precipitation was performed using 20 μl of a 50% suspension per ml of amniotic fluid.

Electrophoresis and Immunoblotting

Detection of G-PRL was by immunoblotting electrophoretically separated proteins with anti-hPRL serum (anti-hPRL-IC-4); G-PRL has an apparent molecular weight increase of 2,000-4,000 Daltons above PRL.

Samples were solubilized in Tris-glycine buffer, pH 8.9, containing 10% 2-mercaptoethanol and electrophoresed in the discontinuous system of Laemmli on 12% SDS polyacrylamide gels (6). Proteins were fixed for silver staining or transferred to a 0.2 μm NTC membrane. Membranes were blocked with 50 mM TBS; pH 7.4, containing 3% Carnation powdered nonfat dry milk and incubated at room temperature overnight in a 1:1000 solution of anti-hPRL serum in TBS + 1%BSA (TBS-BSA). The membrane was washed with TBST, rinsed in TBS and exposed to 500,000 cpm/ml of $[^{125}I]$protein A in TBS-BSA for at least two hours. The membrane was then washed in TBST, dried and autoradiographed.

Standards used for PRL and G-PRL, respectively, were h-PRL-I-7, and ovine G-PRL. The specificity of the immunobinding was confirmed by incubating duplicate blots in the presence of nonspecific rabbit immune serum and by performing several immunoblots using another PRL antiserum, VLS-2.

Molecular weights in Daltons of markers used for electrophoresis were lactoglobulin = 14,200; carbonic anhydrase = 29,000; ovalbumin = 45,000; BSA = 66,000; phosphorylase B = 92,500.

Identification of Immunoglobulin Classes

Identification of the immunoglobulin classes present in the fractions containing G-PRL was by immunoblotting using a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories). Fifty or 100 μl samples were applied for 60 min to a NTC membrane using the BioDot apparatus and blocked for 30 min using TBS-BSA. Wells were washed with TBST and 100 μl of biotinylated goat

anti-human immunoglobulin chain specific antiserum was added to each well and incubation continued for 30 minutes. The NTC paper was removed from the apparatus, washed five times with TBST and incubated 30 min with an avidin-biotin-conjugated-horseradish peroxidase H reagent (Vectastain; Vector). The paper was washed again and the color developed using 0.5 mg/ml 4-chloro-1-napthol plus 0.18% H_2O_2 in 16% methanol in water. Immunospecific binding was identified by visual inspection of the dried NTC membrane. Unfractionated amniotic fluid was used as a positive control for all antibodies; BSA was used as the negative control. Purified G-PRL gave no reaction.

RESULTS

Typical immunoblots obtained after the high molecular weight fraction of amniotic fluid G-PRL had been partially purified using a sequence of standard biochemical techniques are shown in Figure 1. The protein mixture had been reduced to relatively few proteins as shown in the silver stained gel in Figure 1A.

Comparison of the two immunoblots (Figure 1B and 1C) shows specific binding of anti-hPRL at 25 kDa and nonspecific binding of ^{125}I protein A at 92, 51, 43, 40 and 35 kDa. The densely stained 51 kDa band is thought to be immunoglobulin heavy chain containing the Fc region that binds to Protein A. The minor bands staining nonspecifically with the protein A aggregates and may be breakdown products of the heavy chains present in both amniotic fluid and the column fractions. The electrophoretic migration and immunostaining of a commercial preparation of human IgG (Sigma) prepared by DEAE fractionation of serum is included on the blots for comparison. The profile of the specific and nonspecific staining of the IgG is similar to the partially purified amniotic fluid containing G-PRL, indicating that some G-PRL is bound to immunoglobulin in serum also.

Altogether, nine different biochemical techniques were used in attempts to separate the 25 kDa G-PRL from the contaminating immunoglobulin; these are listed in Table 1. Many of these techniques were used in series and although >100 fold purification of G-PRL was achieved using several sequences, all failed to separate the 25 kDa and 51 kDa proteins apparent on each immunoblot. Identical blots were obtained when VLS-2 was used as the PRL antiserum.

The resistance of the protein complex to separation except during reducing SDS-PAGE suggested that disufide bonds might be involved in the interaction of the 25 and 51 kDa species. This was confirmed by repeating several of the separation techniques in the presence of 10% 2-mercapto-ethanol. When reverse phase HPLC of amniotic fluid, previously subjected to weak cation exchange HPLC under conditions that maximized immunoglobulin binding, was performed in the presence of 2-mercaptoethanol, some of the protein moved to a less hydrophobic region of the chromatogram (Figure 2).

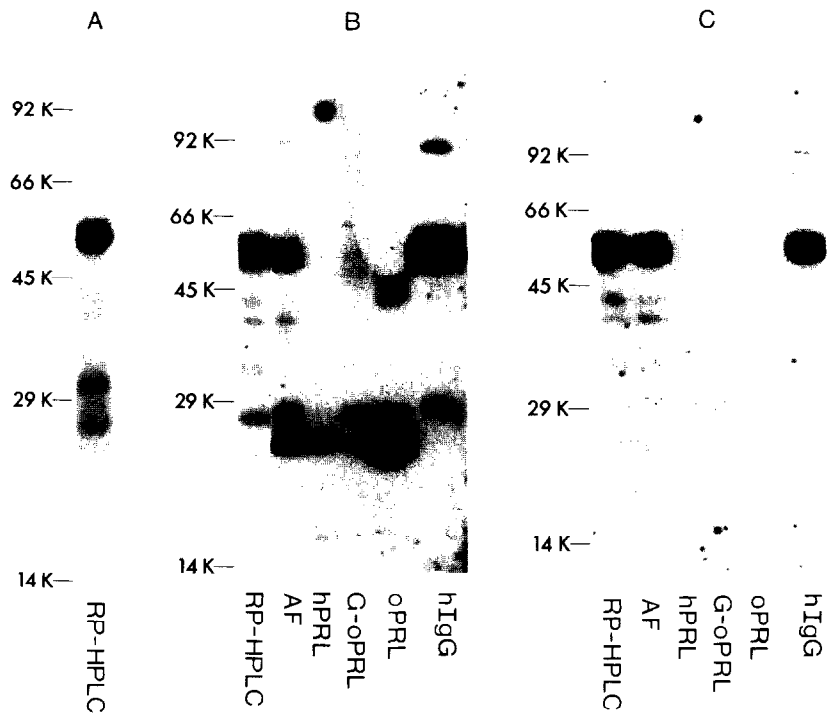

FIGURE 1
Silver stained gel and two Western blots demonstrating the association of immunospecific anti-hPRL binding to the 25 kDa protein thought to be G-PRL and the nonspecific binding of protein A to the 51 kDa band thought to be immunoglobulin heavy chains.
Amniotic fluid was subjected to ultrafiltration against a 50 kDa exclusion filter, chromatographed using DEAE-HPLC in the presence of 6M urea and the unbound fractions subjected to reverse phase - HPLC. Each of the six peaks eluted from the RP-HPLC column were then concentrated and electrophoresed on a 12% polyacrylamide gel. Only peak VI, which eluted at 45-55% acetonitrile, contained immunoreactive PRL.
A. Silver stain of RP-HPLC peak VI. B. Western blot of RP-HPLC peak VI and standards using rabbit anti-PRL serum and radioactive detection with $[^{125}I]$protein A. C. Western blot of same, using nonimmune rabbit serum and radioactive detection with $[^{125}I]$protein A. hIgG = 6 μg human IgG (Sigma); oPRL = 3 μg ovine PRL (Sigma); G-PRL = 500 ng ovine G-PRL; hPRL = 60 ng NIADDK hPRL I-7; AF = 50 μl amniotic fluid; RP-HPLC = aliquot of eluant in peak VI from reverse phase HPLC, equivalent to 500 μl starting volume of amniotic fluid.

Immunoblotting showed that G-PRL moved into this region of the chromatogram, which corresponds to the position of purified G-PRL. No such movement was observed in the absence of 2-mercaptoethanol. Similar results were obtained with SDS-PAGE and gel filtration HPLC of amniotic fluid. In the absence of 2-mercaptoethanol, the G-PRL was associated with a large complex that contained immunoglobulin; in the presence of a reducing agent, the G-PRL and immunoglobulin separated. Substantially fewer immunoglobulin breakdown products were found in these blots, presumably because of the absence of prior urea treatment.

TABLE 1. Techniques which did not separate the immunoglobulin and G-PRL complex in amniotic fluid

HPLC	Precipitation
DEAE +/- 6M urea	Ammonium sulfate
Hydrophobic interaction	Ethanol
Reverse phase	Protein A Sepharose
Gel filtration	
ABx (weak cation)	

Each of the above techniques was applied at least twice to amniotic fluid in attempts to separate the 25 kDa G-PRL from the contaminating immunoglobulin. Several series of separations were also attempted: these included ethanol precipitation followed by DEAE HPLC and then reverse phase HPLC or hydrophobic interactive HPLC; DEAE with 6M urea followed by reverse phase HPLC or protein A precipitation and ABx HPLC followed by reverse phase HPLC. Detection of G-PRL was by immunoblotting. None of these techniques singly or in series were successful in separating the G-PRL from the immunoglobulin complex.

Demonstration of intact immunoglobulin in the fractions containing G-PRL was by molecular weight determinations using SDS-PAGE and gel filtration HPLC. The molecular weights of the G-PRL plus immunoglobulin complex by both methods was approximately 160 kDa consistent with a ratio of G-PRL to immunoglobulin of 1:1. Additionally, the dot blots performed to identify

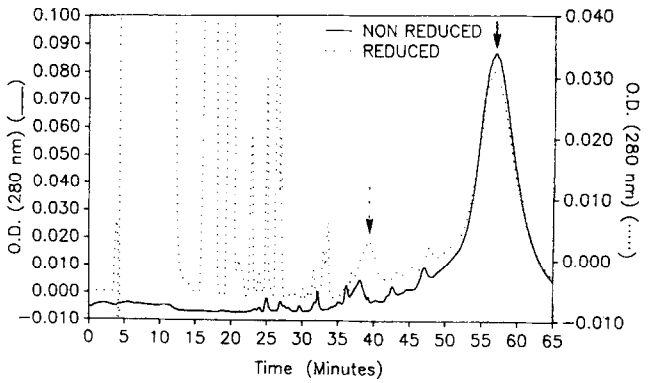

FIGURE 2
Reverse phase HPLC chromatograms of amniotic fluid under reducing and nonreducing conditions which demonstrate movement of G-PRL following reduction. Amniotic fluid was first chromatographed on a weak cation exchange column (ABx) under conditions which maximized immunoglobulin recovery. The samples were then rechromatagraphed on reverse phase HPLC in a TFA-acetonitrile system using a continuous elution gradient. Immunoblots were then performed to determine the fractions containing G-PRL.
The solid line indicates the chromatogram obtained when the ABx eluant was chromatographed without prior reduction; the dotted line shows the chromatogram when the ABx eluant was reduced with 10% 2-mercaptoethanol prior to reverse phase HPLC. The arrows indicate the position of G-PRL in the unreduced (⟶) and reduced (⇢) states. Purified G-PRL elutes at 40 minutes in this system.

TABLE 2. Dot-blot results demonstrating the immunoglobulin classes present in fractions containing G-PRL

	HPLC Fraction				
Antiserum Specificity	AF 1X	DEAE+urea 5X[a]	RP+DEAE 5X	ABx 4X	ABx+RP (unreduced) 5X
γchain	++[b]	++++	++++	++	++
αchain	++	+	0	+	++
μchain	+	+	0	0	0
κchain	+	++	+++	+	+++
λchain	++	++++	++++	++	++++

[a] Concentration of HPLC fractions relative to starting volume of 50 μl of amniotic fluid (AF).

[b] Indicates density of color development at each spot; ++++ was maximum and 0 corresponded to no color present above background.

Immunoglobulin classes present in select HPLC fractions known to contain G-PRL were determined using a dot-blot technique with chain specific anti-human immunoglobin antibodies. Detection of immunospecific binding was by an avidin biotin-horseradish-peroxidase-H system with color development of 4-chloro-1-napthol.

the class(es) of immunoglobulin binding to G-PRL all included antisera to both kappa and lambda light chains. All chromatography fractions which were obtained without reducing agents and which were positive for G-PRL were positive for both heavy and light chains (Table 2).

The dot blots indicated that G-PRL was consistently associated with the γ heavy chain of IgG; many relatively pure fractions also contained small amounts of α chain, suggesting that G-PRL may also bind to IgA.

DISCUSSION

Our results show that a human G-PRL species can be found tightly bound to immunoglobulins in amniotic fluid and suggest that they are also bound together in human serum. It is clear from the data that at least one or more subclasses of IgG bind G-PRL; it is quite possible that G-PRL can bind to IgA as well. Circulating G-PRL from some patients with hyperprolactinemia has recently been reported to associate with very large molecular weight complexes (>600 kDa) (7). These very large complexes may be aggregates of IgA which in humans can be found as trimers or higher molecular weight polymers (8).

Immunoglobulin fractions of pregnancy sera have previously been reported to contain prolactin as well as several gonadotropins measurable by radioimmunoassay (9). Serum fractionation by cold ethanol precipitation reduced, but did not totally remove, these contaminating proteins. Our data

indicate that the prolactin remaining bound to immunoglobulin was the glycosylated form. It is interesting to note that all the hormones which bind tightly to immunoglobulin are cysteine containing glycoproteins.

The potential functions of the G-PRL plus immunoglobulin complex are numerous. The immunoglobulin may protect G-PRL from removal from the circulation, thus prolonging the half-life of G-PRL. The immunoglobulin may deliver G-PRL to otherwise inaccessible sites such as the fetal circulation via placental transfer of immunoglobulins (10). It is also possible that G-PRL may block a recognition or binding site on immunoglobulins and modify their actions. G-PRL secreted by the decidua could effectively alter local immune function within the uterus during pregnancy.

Finally, it has recently been shown that there are a minimum of two isoforms of pituitary G-PRL that can be distinguished by lectin binding (11). Now that G-PRL can be separated from immunoglobulin in amniotic fluid, it will be possible to determine whether there are differences in isoform binding to the classes of immunoglobulins to which amniotic fluid G-PRL binds.

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