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Insulin-Like Growth Factors I and II and Their Binding Proteins in Rat Milk

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ABSTRACT. IGF-I and -II are peptide growth factors that may be important contributors to the growth-promoting properties associated with milk. IGF in extracellular fluids, including serum and milk, are carried by specific high-affinity binding proteins (IGFBP). In this study, the levels of IGF-I and -II in rat serum and milk were quantified by specific RIA, and the IGFBP were characterized using Western ligand blotting and autoradiography throughout lactation. The levels of IGF-I in both milk and maternal serum decreased during lactation. Serum IGF-I decreased from 743 ± 187 μg/L on d 1 to 391 ± 106 (mean ± SD) on d 21 of lactation, and milk IGF-I levels fell from 30 ± 10 to 13 ± 8 μg/L. Levels of IGF-II in serum and milk were much lower than IGF-I, and were unaffected by lactation. In maternal serum, several IGFBP were identified: IGFBP-3, which migrates as four glycosylated bands with apparent M_r from 38 to 42 kD and one to two nonglycosylated bands with apparent M_r of 28 to 29 kD, and an IGFBP with an apparent M_r of 24 kD. In milk, IGFBP-3, the 24-kD binding protein, and a third IGFBP with an apparent M_r of 29 kD were identified. Treatment of milk and serum with Endoglycosidase F reduced the four glycosylated IGFBP-3 bands (38–42 kD) to two bands with apparent M_r of 35 and 32 kD. In rat milk, but not adult rat serum, the IGFBP with an apparent M_r of 29 kD was immunoprecipitable by an antibody that recognizes IGFBP-2. These results demonstrate that in adult rat serum and milk both IGF-I and IGF-II are present, with IGF-I being predominant. IGFBP are present in rat milk; the presence of IGFBP-2 in rat milk, but not in maternal serum, indicates that IGFBP may be synthesized within the mammary gland. (*Pediatr Res* 29: 50–55, 1991)

Abbreviations

IGFBP, insulin-like growth factor binding proteins
TBS, Tris-buffered saline
au, absorbance unit

Milk contains growth factors that have been shown to be potent stimulators of DNA synthesis and proliferation in a variety of cells in culture (1–3). Among the growth factors present in human (4–6), bovine (7), and porcine (8) milks are the IGF. IGF-I and -II are growth hormone-dependent peptides that share structural and functional homology with insulin and may play an important role in early postnatal growth and development (9, 10). The IGF are present in serum and other extracellular fluids associated with a family of structurally related binding proteins (IGFBP) (11). In the human, three immunologically and genetically distinct binding proteins have been isolated, cloned, and sequenced and designated hIGFBP-3 (12), hIGFBP-2 (13), and hIGFBP-1 (14). Several other distinct binding proteins appear likely (15, 16).

In the rat, three IGFBP that are structurally homologous to the human IGFBP have recently been characterized. Baxter and Martin (17) demonstrated that the predominant IGFBP in adult rat serum is highly homologous to the glycosylated hIGFBP-3. This protein has been recently cloned, sequenced, and designated rIGFBP-3 (18). A second rat IGFBP, first isolated from the conditioned media of the rat hepatoma-derived cell line BRL-3A, has been cloned and identified as a nonglycosylated protein with an apparent M_r of 29.5 kD (rIGFBP-2) (19, 20). IGFBP-2 is the predominant IGFBP in neonatal rat serum, and disappears by the time of weaning (21, 22). A third rat IGFBP, rIGFBP-1, has recently been cloned from a rat deciduoma library using a cDNA for hIGFBP-1 (23). rIGFBP-1 has been shown to be secreted by the rat hepatoma cell line H-35 (24). In addition, both human and rat serum contains an IGFBP with an apparent M_r of 24 kD that has not yet been cloned and sequenced (21, 24).

Milk provides the sole source of nutrition for the neonatal rat for at least the first 2 wk postpartum and may represent a significant source of exogenous growth-promoting factors for the neonate (25, 26). Receptors for the IGF have been identified in the intestines of suckling animals, indicating a possible function for milk-borne growth factors in intestinal development (8, 27). Due to the potential role of the IGF in the growth and development of the neonate, it is of interest to quantify the levels of these growth factors in milk. In addition, the IGFBP increase the stability of the IGF by slowing degradation (28), and modulate the access of IGF to membrane receptors. Their presence in milk may thus be of importance.

MATERIALS AND METHODS

Peptides. Pure biosynthetic human [^{59}Thr]IGF-I was purchased from Amgen Biologicals (Thousand Oaks, CA). Recombinant human IGF-II was generously provided by Dr. Michelle Smith (Lilly, Indianapolis, IN). Iodination was performed by a modification of the chloramine-T technique.

Biologic samples. Timed pregnant Wistar rats (n = 45) were obtained on d 14 of gestation (Simonsen, Gilroy, CA). Dams were housed singly in animal facilities maintained at 25°C with 12-h light-dark cycles. Dams were allowed *ad libitum* access to food (Wayne Rodent Blox, Premier Laboratory Diets, Bartonville, IL) and water. After delivery, which was designated d 1 of lactation, litters were normalized to 10 pups per dam. Serum and milk samples were collected from dams on d 1–4, 7, 10, 14,

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18, and 21 of lactation. Pups were removed from the dams 3 h before milking. Dams were injected intramuscularly with 0.1 mL synthetic oxytocin (200 IU/mL; Sigma Chemical Co., St. Louis, MO) 15 min before milking and were anesthetized during milking. Blood was collected by cardiac puncture under anesthetic. Blood samples were allowed to clot at 4°C for 30 min and were centrifuged at 10 000 × *g* at 4°C for 15 min to separate serum. Milk samples were centrifuged at 180 000 × *g* (Beckman LM-2 ultracentrifuge, SW-60ti rotor, Beckman Instruments, Inc., Palo Alto, CA) at 4°C for 45 min to separate the whey (infranatant) from the fat (supernatant) and casein (pellet) fractions before analysis. Whole milk and milk fractions from d 14 of lactation were assayed for IGF binding activity by ligand blot analysis. All samples were stored at −70°C before analysis. All animal protocols were approved by the Administrative Panel on Laboratory Animal Care at Stanford University.

IGF quantification. To dissociate the IGF from the binding proteins, serum and whey samples were chromatographed in 0.25 M formic acid on a 0.9 × 100-cm column containing Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, NJ). The fractions eluting between 50 and 67 mL, which contain 90% of the IGF activity for both whey and serum and no demonstrable IGFBP, were collected in siliconized glass tubes containing 1.0 mL of 1.0% BSA, frozen, and lyophilized (29). Samples were resolublized in RIA buffer (0.04 M sodium phosphate, 0.15 M sodium chloride, 0.5% BSA, 0.1% sodium azide) to their original volume before assay. The assay was run using 25-, 50-, and 100-μL samples, and parallel dose-response curves were seen for both serum and whey (data not shown). IGF-I content was measured by RIA using [^{125}I]IGF-I as radioligand and a polyclonal antisomatomedin-C/IGF-I antibody. This antiserum (UBK487) was generated by Drs. L. E. Underwood and J. J. Van Wyk, University of North Carolina at Chapel Hill, and is distributed through the Hormone Distribution Program of the National Institute of Diabetes and Digestive and Kidney Diseases to the National Hormone and Pituitary Program. Inter- and intraassay coefficients of variation for the IGF-I assay were 8 and 5%, respectively. IGF-II content was measured by RIA using recombinant [^{125}I]IGF-II and a MAb against rat IGF-II that has less than 10% cross-reactivity with human IGF-I (Amano, Troy, VA). Inter- and intraassay coefficients of variation for the IGF-II assay were 10 and 6%, respectively. Serum samples were run at a 1:100 dilution for IGF-I. Milk IGF-I and serum and milk IGF-II were determined using undiluted samples. The lower limits of sensitivity for both assays is approximately 1.0 μg/L in the original sample.

SDS-PAGE. SDS-PAGE was performed according to the method of Laemmli (30). Twenty μL of a 1:10 dilution of serum or a 1:2 dilution of milk were applied to a 4% stacking gel and electrophoresed through a 10% polyacrylamide gel. Comparable amounts of proteins were loaded for both serum and milk (approximately 0.8 g protein/lane). Prestained molecular weight standards (BRL, Bethesda, MD) were run in parallel lanes. Some IGFBP samples were enzymatically deglycosylated using Endoglycosidase F (Endo F; Calbiochem, La Jolla, CA) as previously described (31). Briefly, samples (20 μL) were adjusted to pH 5.5 and 240 mU of Endo F were added; after incubation at 37°C for 3 h, reactions were terminated by the addition of an equal volume of electrophoresis buffer. Samples were applied to a 10% SDS-PAGE gel and electrophoresed overnight at 50 V.

Electrotransfer and Western ligand blotting. Western ligand blotting was performed following the method of Hossenlopp *et al.* (32). Briefly, after electrophoresis polyacrylamide gels were washed in Towbin buffer (0.025 M Tris-base, 0.192 M glycine, 20% methanol) (33) for 15 min to remove SDS before electrotransfer. Proteins were electroblotted onto nitrocellulose (0.45 μm, Schleicher and Schuell Inc., Keene, NH) using a Gelman Biotrans semidry electrophoresis transfer unit (Gelman Sciences, Ann Arbor, MI) at 180 mA for 90 min, using Towbin buffer. Nitrocellulose membranes were washed with TBS (0.15 M sodium chloride, 0.01 M Tris-HCl) containing 3% Nonidet P-40 (NP-40, Sigma Chemical Co.). Membranes were blocked with TBS containing 1% BSA (Sigma Chemical Co.) for 2 h, followed by TBS containing 0.1% Tween for 10 min. The membranes were then incubated overnight at 4°C with approximately 1.5 × 10^6 cpm of [^{125}I]IGF-I or [^{125}I]IGF-II in 10 mL of TBS containing 1% BSA and 0.1% Tween. Membranes were washed with TBS, air-dried, and visualized by exposure to Kodak X-Omat AR film (Rochester, NY) for 4–14 d at −70°C. Autoradiograms were scanned using laser densitometry and total au·mm were calculated for each lane (UltraScan XL; LKB, Bromma, Sweden).

Immunoprecipitation. A polyclonal antibody to partially purified human IGFBP (hIGFBP-2 and hIGFBP-3) secreted by the human endometrial adenocarcinoma cell line HEC 1A (αHEC 1) was generated in New Zealand White rabbits and protein A purified, as previously described (31). In the rat, this antibody has been shown to be specific for rIGFBP-2, inasmuch as it does not recognize rIGFBP-3 (21). To immunoprecipitate rIGFBP-2 in rat milk and serum, *Staphylococcal aureas* protein A (Pansorbin, Calbiochem) was washed in Tris buffer (50 mM Tris HCl, pH 7.4) and resuspended in Tris buffer at the original volume. Fifty μL of the washed protein A were incubated with 5 μL of αHEC 1 or 5 μL of normal rabbit serum on a rotating shelf at 4°C overnight. Milk (40 μL, 1:2 dilution) and serum (40 μL, 1:10 dilution) samples were then added to the antibody-Staph A complex and incubated at 4°C on a rotating shelf for 3 h. Samples were centrifuged at 10 000 × *g* for 5 min and the pellet washed three times with Tris buffer. Samples were prepared for electrophoresis by the addition of sample buffer (Tris-SDS, glycerol), vortexed, boiled for 5 min, revortexed vigorously, and centrifuged at 10 000 × *g* for 5 min. The resulting supernatants were electrophoresed, electrotransferred, and ligand-blotted with [^{125}I] IGF, as previously described.

Statistical analysis. Differences in milk and serum IGF peptide concentrations were analyzed using a nonpaired analysis of variance and differences were considered significant if $p < 0.05$ by Fischer protected least significant difference test (Statview, Brainpower, Inc., Santa Monica, CA). Correlations between milk and serum IGF and IGFBP were determined by Pearson's method.

RESULTS

Serum and milk IGF-I and IGF-II concentrations. Serum IGF-I and IGF-II concentrations throughout lactation are presented in Table 1. Rat serum IGF-I content decreased significantly ($p = 0.05$) during lactation, from mean ± SD levels of 743 ± 187 on the first postpartum d to 391.7 ± 38.1 μg/L by d 21 postpartum. This compares to a concentration of 577 ± 77 μg/L for IGF-I in virgin females. The major drop in serum IGF-I concentration occurred between d 3 and 4 postpartum, with levels staying relatively constant thereafter. Serum IGF-II levels in the adult

Table 1. *IGF-I and IGF-II in rat serum and milk throughout lactation (μg/L)**

	IGF-I		IGF-II	
Day	Serum	Milk	Serum	Milk
1	743 ± 187	30 ± 10.0	9.3 ± 6.7	1.3 ± 0.5
2	833 ± 201	29 ± 5.3	7.0 ± 2.0	1.0 ± 0.3
3	907 ± 238	26 ± 5.3	6.3 ± 1.2	<1
4	558 ± 62	26 ± 3.6	9.0 ± 1.0	<1
7	461 ± 68	18 ± 4.3	4.0 ± 1.0	<1
11	440 ± 56	18 ± 3.5	6.3 ± 1.5	<1
14	501 ± 71	17 ± 7.8	7.0 ± 0.1	<1
18	449 ± 105	15 ± 6.1	5.3 ± 0.6	<1
21	392 ± 38	13 ± 8.0	4.5 ± 0.6	<1
Control†	577.0 ± 77.0		10.0 ± 3.0	

* Values represent mean ± SD of five animals/d.

† Virgin females of similar age and weight.

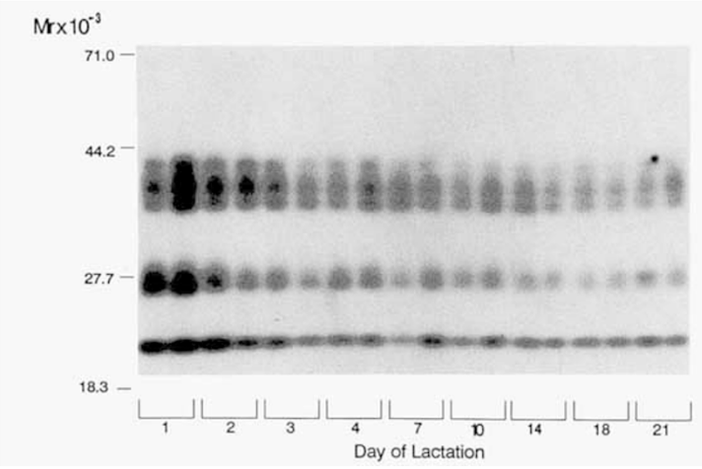

Fig. 1. Rat serum IGFBP throughout lactation. Autoradiograph of a Western ligand blot of rat serum during lactation. Each lane represents samples from a different dam. Samples (20 μL; 1:10 dilution) were separated by SDS-PAGE on a 10% gel, Western ligand blotted using [^{125}I]IGF-I, and exposed to film for 4 d. Bands with apparent M_r of 38–42, 28, and 24 kD were visualized.

Table 2. *IGFBP in rat serum and milk throughout lactation (au·mm)**

Day	Serum†	Milk‡
1	16.9 ± 5.2	5.4 ± 0.7
2	11.2 ± 1.1	6.2 ± 1.2
3	7.4 ± 2.1	5.6 ± 3.9
4	7.3 ± 0.7	6.8 ± 1.7
7	5.3 ± 1.0	3.7 ± 3.1
10	5.6 ± 1.9	6.4 ± 0.1
14	4.6 ± 1.7	5.8 ± 0.1
18	3.1 ± 0.2	3.5 ± 0.1
21	4.4 ± 0.1	3.2 ± 0.1

* Values represent mean ± SD of two animals/d.
† Serum data from densitometric analysis of Figure 1.
‡ Milk data from densitometric analysis of Figure 3.

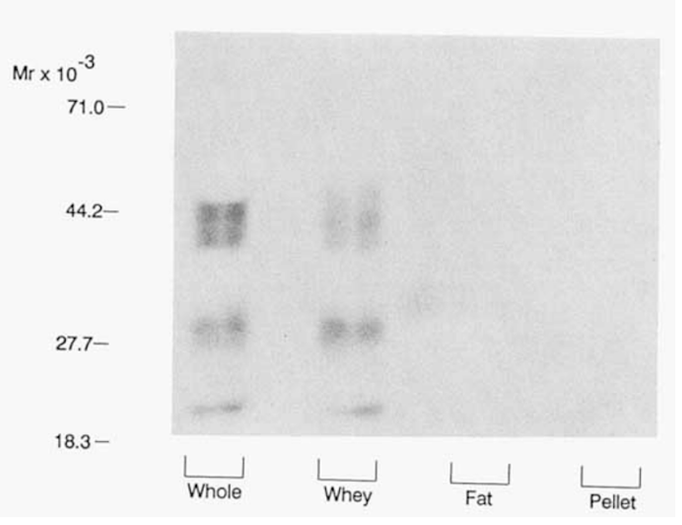

Fig. 2. Comparison of IGFBP in whole rat milk and milk fractions. Autoradiograph of a Western ligand blot of rat milk and milk fractions from d 14 of lactation. Milk fractions were separated by ultracentrifugation at 180 000 × *g* at 4°C into fat, whey, and pellet (casein) fractions. Samples (20 μL; whole milk 1:2 dilution and whey 1:2 dilution, fat undiluted and pellet undiluted) were separated by SDS-PAGE on a 10% gel, Western ligand blotted using [^{125}I]IGF-I, and exposed to film for 14 d. Bands with apparent M_r of 38–42, 29, 28, and 24 kD were visualized.

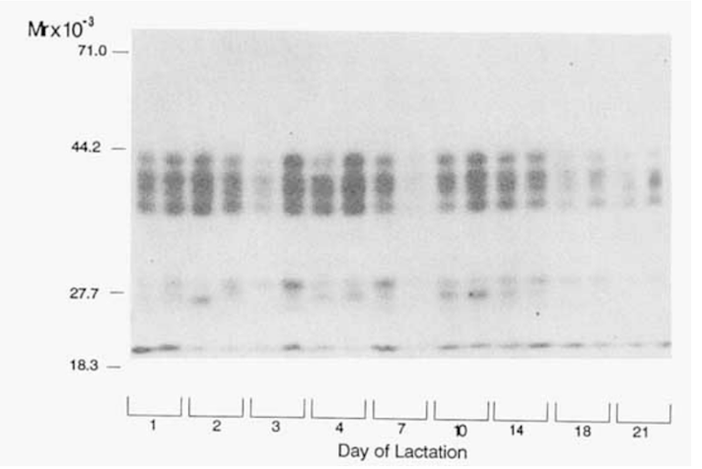

Fig. 3. Rat milk IGFBP throughout lactation. Autoradiograph of a Western ligand blot of rat milk whey during lactation. Each lane represents samples from a different dam. Whey samples (20 μL; 1:2 dilution) were separated by SDS-PAGE on a 10% gel, Western ligand blotted using [^{125}I]IGF-I, and exposed to film for 7 d. Bands with apparent M_r of 38–42, 29, 28, 27, and 24 kD were visualized.

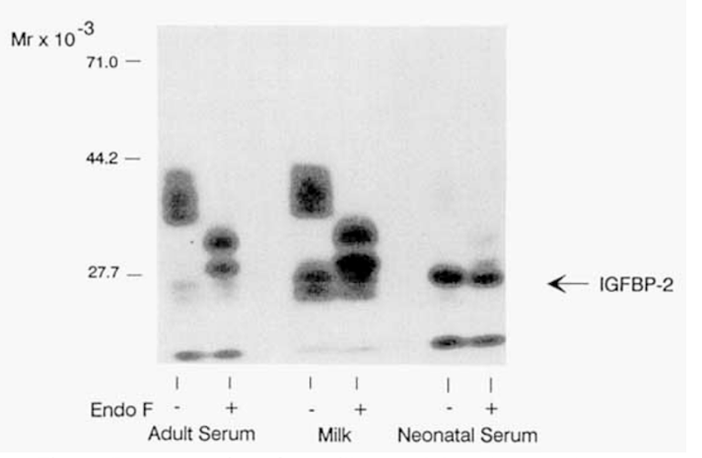

Fig. 4. Deglycosylation of rat milk and adult and neonatal rat serum IGFBP by Endoglycosidase F. Autoradiograph of a Western ligand blot of samples deglycosylated with Endoglycosidase F. Whey samples (20 μL; 1:2 dilution) and serum samples (20 μL; 1:10 dilution) were deglycosylated by Endoglycosidase F for 3 h at 37°C. All samples were from postpartum d 4. Native and deglycosylated samples were separated by SDS-PAGE on a 10% gel, Western ligand blotted using [^{125}I]IGF-I, and exposed to film for 7 d. Bands with apparent M_r of 38–42, 29, 28, and 24 kD were visualized. The *arrow* indicates the location of rIGFBP-2.

rat are extremely low (34), with levels in this study ranging between 4.0 and 9.0 μg/L, or 1–2% of the serum IGF-I levels. Serum IGF-II concentration in the serum of virgin rats was 10 ± 3.0 μg/L. Serum IGF-II concentrations were unaffected by lactation. The levels of IGF-I and -II in rat milk are also shown in Table 1. Milk IGF-I content decreased during lactation from 30 ± 10 μg/L on d 1 of lactation to 13 ± 8.0 μg/L on d 21 of lactation. IGF-II concentration in rat colostrum (d 1–2 postpartum) was 1.0–1.3 μg/L; after d 2 the levels fell below 1.0 μg/L, which is below the lowest level of detection for our assay.

Western ligand blot of rat serum IGFBP. A Western ligand blot of IGFBP in rat serum throughout lactation, visualized with [^{125}I]IGF-I, is shown in Figure 1. Adult rat serum contains six bands with apparent M_r values of 42, 41, 40, 38, 28, and 24 kD. The bands migrating at 38–42 kD and the 28-kD band, a proteolytic fragment of the 38- to 42-kD bands, have previously been identified to be rIGFBP-3 (35). In some serum samples, a doublet can be observed in the 28- to 29-kD region. These bands most likely represent rIGFBP-2 (29 kD) and the proteolytic

inhibit the actions of the IGF on their target tissues (39, 40), or, conversely, potentiate IGF action (41, 42). In addition to being present in the circulation, mRNA for both IGF-I and IGF-II have been identified in multiple organ systems throughout the body, suggesting that the growth factors may be synthesized and act locally via paracrine or autocrine mechanisms (43) and be regulated by locally produced IGFBP (39–41).

Several lines of research have indicated that the IGF are important mediators of mammary growth and development. IGF-I has previously been described in the milk of a variety of species (4–8) and has been shown to be a potent stimulator of mammary cell growth and DNA synthesis in cultured bovine (44) and ovine (45) mammary tissue. In addition, increased milk production observed when growth hormone is administered to the cow (46) or the goat (47) may be mediated via IGF-I. Lastly, both type I and type II IGF receptors have been characterized on bovine mammary plasma (48) and microsomal (47) membranes and rat mammary tissue (49). In both the cow and the rat, the onset of lactation is associated with a dramatic increase in IGF binding to mammary tissue, which is mainly due to an increase in the number of type II rather than type I receptors (49, 50).

In this study, we have characterized the presence of IGF-I and -II in rat serum and milk throughout lactation. IGF-I represents the predominant IGF in adult rat serum and milk. The levels of IGF-I in rat milk are 30 to 50 times higher than the IGF-II levels, but are present at only 3–5% of the levels found in serum from the lactating rat. IGF-I in serum and milk decreased after the first 3 to 4 d of lactation, and then remained stable throughout the remainder of lactation. Concentrations of IGF-I and -II in rat milk are compared with milks of other species in Table 3. IGF-I levels of 25–30 μg/L in rat colostrum are in the range of what has been reported in human milk (7–27 μg/L), but are far below the levels reported in bovine (100–600 μg/L) and porcine (67–357 μg/L) colostrum. Levels of IGF-I in mature rat milk ranges from 13–18 μg/L, which is comparable to the levels reported in the milks of other species (Table 3). The levels of IGF-II in rat milk also decrease after the first few days of lactation, although the levels are extremely low even in early lactation. The concentrations of IGF-II in human and porcine milk are currently unreported.

The source of the IGF in rat milk is not known. Messenger RNA for both IGF-I and -II were very low, or undetectable, in rat mammary glands isolated from dams d 11 postpartum (43), suggesting that maternal serum is the most likely source of IGF. Previous studies with epidermal growth factor in mice (51) and goats (52) demonstrated the transfer of epidermal growth factor from maternal circulation, most likely via epidermal growth factor receptors located in the mammary glands. The presence of IGF receptors in mammary tissue may sequester IGF from the maternal serum in an analogous manner. In the rat, suckling stimulus is necessary for final differentiation of the mammary glands. Therefore, the elevated levels of both IGF-I and -II observed in early lactation may represent leakage from the maternal circulation via paracellular or "leaky" junctions. Alternatively, a major role for IGF in the mammary gland may be to stimulate either the growth or differentiated function of the mammary epithelial cells. Therefore, the levels of IGF-I and -II in milk may simply represent intracellular mammary IGF that is released during milk secretion. It is interesting to note that although serum and milk IGF-I were significantly correlated with one another, the correlation was rather weak ($r^2 = 0.25$), suggesting that serum may not be the sole source of the milk IGF-I.

IGF binding proteins have been demonstrated in human (4–6) and cow (53) milk, and have been shown to be secreted by bovine mammary glands in culture (54). In our study, the presence of rIGFBP-2 in milk, but not in the serum of the lactating rat, and the lower levels of the 24-kD binding protein in milk than in maternal serum suggest that some IGFBP are synthesized in rat mammary epithelium rather than transported from the maternal circulation. The observation that the levels of IGFBP-2 in maternal serum are absent or extremely low (21, 22) and the absence of detectable message for rIGFBP-2 in adult rat liver (55) further support the hypothesis of *de novo* synthesis within the mammary gland. In addition, there is a relatively weak correlation between the levels of IGFBP-3 in maternal serum and milk ($r^2 = 0.34$). Further studies of rat mammary gland using probes for rIGFBP mRNA may elucidate the source of IGFBP in milk.

Growth factors contained in milk, particularly epidermal growth factor, have been postulated to have a role in the developing gastrointestinal tract (25). The gastrointestinal tract undergoes rapid growth and proliferation during the neonatal period. The presence of colostrum or early milk has been shown to stimulate intestinal growth in the suckling rat (26). Although a direct role for the milk-borne IGF remains to be demonstrated, the presence of both type I and type II IGF receptors in the intestinal epithelium of rats suggests that exogenous IGF may be able to interact with the intestine and elicit mitogenic responses (27). A role of IGF in intestinal growth is supported by increased binding of IGF to proliferative intestinal cells, located in the crypts, than to the differentiated enterocytes located on the tips of the villi (27). In addition, a recent study investigating the absorption of [^{125}I]IGF by the suckling rat demonstrated that approximately 78% of administered IGF-I was retained by the animals, predominantly in the stomach and intestinal lining (56). Because it appears that in the suckling animals IGF may partially survive digestion, further studies investigating the *in vivo* effects of IGF-I and -II on the developing rat intestine are warranted.

In summary, the presence of both IGF-I and IGF-II has been demonstrated in rat milk and serum during lactation. IGF-I is predominant in both adult rat serum and milk and decreases significantly after the first 3 to 4 d postpartum. The IGFBP in rat milk and serum have been characterized by Western ligand blotting. Adult rat serum contains predominantly rIGFBP-3 and a low molecular weight 24-kD binding protein. In addition, very small amounts of rIGFBP-2 or rIGFBP-1 may be present. Rat milk contains rIGFBP-2, in addition to rIGFBP-3 and the 24-kD binding protein, and may also contain small amounts of rIGFBP-1. Previous studies have demonstrated that although levels of rIGFBP-2 are high in the neonatal rat, rIGFBP-2 is virtually absent in the serum of the adult rat (21, 22); in this study we have confirmed by immunoprecipitation that no rIGFBP-2 was detectable in adult rat serum. Therefore, it appears that the rIGFBP-2 in milk arises from synthesis within the mammary gland. Further studies are necessary to elucidate the source of both the IGF and the IGFBP in rat milk and to determine the role of milk IGF in neonatal growth and development.

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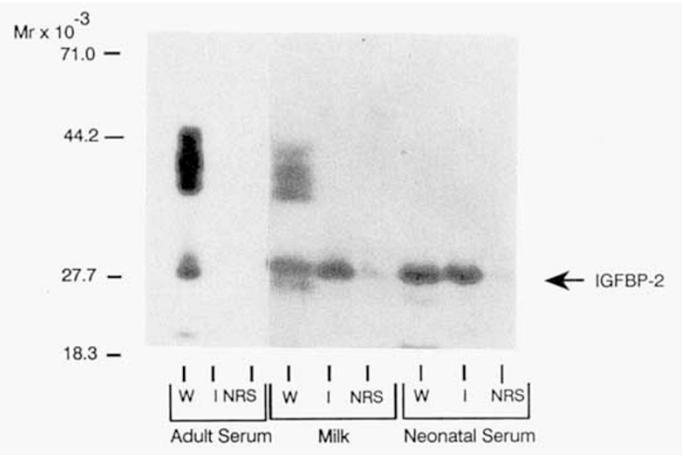

Fig. 5. Immunoprecipitation of rat milk and adult and neonatal serum IGFBP with αHec 1 antibody. Autoradiograph of a Western ligand blot of adult rat serum (*lanes 1–3*), rat milk (*lanes 4–6*), and neonatal rat serum (*lanes 7–9*). All samples were from postpartum d 14. Each sample is represented in three lanes with the first lane being a ligand blot (*W*) (*lanes 1, 4,* and *7*), the second lane an immunoprecipitation with αHec 1 (*I*) (*lanes 2, 5,* and *8*), and the third lane an immunoprecipitation with nonimmune rabbit serum (*NRS*) (*lanes 3, 6,* and *9*). Milk samples (40 μL; 1:2 dilution) and serum sample (40 μL; 1:10 dilution) were immunoprecipitated, then separated by SDS-PAGE on a 10% gel, Western ligand blotted using [^{125}I]IGF-I, and exposed to film for 10 d. Bands with apparent M_r of 38–42, 29, 28, and 24 kD were visualized. The *arrow* indicates the location of rIGFBP-2.

Table 3. *Comparison between milk IGF-I and IGF-II levels in milks of different species (μg/L)*

	IGF-I		IGF-II	
Species	Colostrum	Mature	Colostrum	Mature
Human (4)*	7–27	6–9	NA†	NA
Pig (8)	65–357	4–14	NA	NA
Cow (44)	100–600	22–26	600	107–127
Rat	28–30	13–18	1–2	<1

* Numbers in parentheses denote the reference from which data was compiled. Rat data is from the current study.

† NA = values not available.

fragment of rIGFBP-3 (28 kD) (21, 24). In addition, a 24-kD IGFBP was identified in serum throughout lactation. Results of densitometric analysis of Figure 1 are shown in Table 2. Results for each day represent the mean ± SD of the au·mm of the serum IGFBP for the two dams. Data is presented to show relative changes in IGFBP content throughout lactation. The level of IGFBP in maternal serum was higher during the first 4 d postpartum than in later lactation. The decrease in IGFBP activity during lactation mirrored that of the serum IGF-I content (Table 1).

Western ligand blot of rat milk IGFBP. A Western ligand blot of IGFBP in whole milk and the whey, fat, and casein fractions of rat milk, visualized with [^{125}I]IGF-I, is shown in Figure 2. IGFBP were identified only in whole milk and the whey fraction, validating the use of the whey fraction for further analysis. The presence of large amounts of casein protein in whole rat milk causes a compression of the IGFBP-3 bands due to their similar molecular weights. The removal of casein by centrifugation was verified by staining the SDS-PAGE gel with Coomassie blue and comparing whole milk *versus* whey (data not shown). By removing the casein proteins by centrifugation, the compression of the IGFBP bands can be relieved, allowing better resolution of the autoradiographic bands. IGFBP in rat milk whey throughout lactation are shown in Figure 3. When electroblotted milk proteins were exposed to [^{125}I]IGF-I, seven bands, with apparent M_r values of 42, 41, 40, 38, 29, 28, and 24 kD, were identified. In some milk samples, an 8th band with an apparent M_r of approximately 27 kD was visualized. The triplet of bands migrating in the 29- to 27-kD region have previously been identified as rIGFBP-2 (29 kD), rIGFBP-3 (28 kD), and the proteolytic fragment of rIGFBP-1 (27 kD) (24). In milk, the 24-kD binding protein was less apparent than that observed in maternal serum and was absent in some samples. Results of densitometric analysis of Figure 3 are shown in Table 2. Results for each day represent the mean ± SD of the au·mm for milk IGFBP from the two dams. Unlike the maternal serum, levels of IGFBP in milk did not show a significant decrease during lactation.

Effect of deglycosylation. The effect of deglycosylation of rat milk, adult rat serum, and neonatal rat serum is shown in Figure 4. In milk and adult rat serum, treatment with Endoglycosidase F (Endo F) reduces the four higher molecular weight bands of rIGFBP-3 (42–38 kD) to two bands, with approximate M_r of 35 and 32 kD. The apparent M_r of the 24-kD protein and of the 29-kD protein, which occur in neonatal serum and rat milk, were unaffected by Endo F digestion, confirming the lack of N-linked glucosamine groups associated with these proteins. These results suggest that the predominant 29-kD protein in milk is rIGFBP-2, which is nonglycosylated. In adult serum, the predominant band in the 28- to 29-kD region is most likely the proteolytic fragment of rIGFBP-3 (21, 22, 24), rather than rIGFBP-2. Previous investigators have demonstrated that this rIGFBP-3 band can be reduced to a band with an apparent M_r of 24 kD by deglycosylation with N-glycanase (36).

Immunoprecipitation of IGFBP. Immunoprecipitation studies were performed to test for immunologic similarities between the IGFBP contained in adult and neonatal rat serum and rat milk. Samples were preincubated with either an antibody to rIGFBP-2 (αHEC 1) or nonimmune rabbit serum. Immunoprecipitates were electrophoresed, electroblotted onto nitrocellulose, and exposed to [^{125}I]IGF-II as described above. In Figure 5, each sample is represented in three separate lanes, with the first lane being a Western ligand blot, the second lane an immunoprecipitation with αHEC 1, and the third lane an immunoprecipitation with nonimmune rabbit serum. A band of approximately 29 kD (rIGFBP-2) was visualized in both the Western ligand blots and the immunoprecipitations of neonatal rat serum and rat milk. It is of note that a band of the same approximate size is not immunoprecipitated from adult rat serum, confirming the paucity of rIGFBP-2 in adult rat serum (21, 22, 24). The bands associated with rIGFBP-3 can be observed in the Western ligand blots of both adult rat serum and rat milk, but were not immunoreactive with αHEC 1. In addition, the 24-kD binding protein, which is present in all samples, was not immunoprecipitated by the antibody. No bands were observed in samples incubated with nonimmune rabbit serum.

Regression analyses of rat milk and serum IGF (μg/L) and IGFBP (au·mm) content. The following comparisons were made by regression analysis: serum IGF-I *versus* milk IGF-I, serum IGFBP-3 *versus* milk IGFBP-3, serum IGF-I *versus* serum IGFBP-3, and milk IGF-I *versus* milk IGFBP-3. IGF-I concentrations in serum and milk were correlated ($r^2 = 0.25$; $p = 0.03$) as were serum and milk IGFBP-3 ($r^2 = 0.34$; $p = 0.01$). Serum IGF-I and IGFBP-3 were strongly correlated with an $r^2 = 0.76$ ($p = 0.001$), whereas milk IGF-I and IGFBP-3 were not significantly related ($r^2 = 0.13$, NS).

DISCUSSION

IGF-I and IGF-II are peptide growth factors that exhibit mitogenic effects and can stimulate functional differentiation (37, 38). IGF in serum and extracellular fluids are found to be associated with a family of binding proteins. Although the function of the IGFBP remains unknown, several potential actions have been proposed. IGFBP have been shown to prolong the $t_{1/2}$ of circulating IGF from several minutes to several hours (29),

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