

MULTIPLE MOLECULAR FORMS OF HUMAN LACTOFERRIN

Identification of a Class of Lactoferrins that Possess Ribonuclease Activity and Lack Iron-binding Capacity

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Lactoferrin (Lf)¹ is an 80-kD iron-binding glycoprotein found in high concentrations in human milk and at much lower but detectable levels in a number of other secretions of glandular epithelium (1). The principal functions of Lf are thought to be iron transport and storage (2), and bacteriostasis through strong chelation of iron required for microbial growth (3). However, Lf is also a major constituent of the secondary or specific granules of neutrophils (4), and has been implicated in several operational and regulatory functions in the immune and hematopoietic systems (5). To date, only a single form of Lf has been described, and is presumed to account for all of the diverse functions of the molecule.

One of us (M. R. Das) previously reported (6) the presence in human milk of a unique and potent RNase activity, termed human milk RNase (hmRNase). This enzyme was initially identified based on its interference with the detection of retroviral-like RNAs in human milk, and was subsequently shown to be present in low concentrations in a consanguineous community with a high incidence of breast cancer, the Parsi women of Bombay. The hmRNase was detected in high concentrations only in human milk and was thus considered to be potentially a marker for breast epithelium as well as for risk to development of breast cancer.

The hmRNase has recently been purified to homogeneity and revealed to be a high molecular mass (80 kD) glycoprotein with a preference for mRNAs, viral RNAs, and purine homopolymers (7). Activity of the enzyme is influenced by various cations and is optimal at pHs of 7.5–8.0.

We report here that hmRNase is an isoform of Lf, sharing physical, chemical, and antigenic properties with the major species of Lf, but differing from it in the possession of potent nuclease activity and in the lack of significant iron-binding capacity. These findings establish the presence of multiple forms of Lf, with very distinctive properties, that may be related to the highly diverse physiological functions of the molecule.

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¹ *Abbreviations used in this paper:* hmRNase, human milk RNase; Lf, lactoferrin.

Materials and Methods

Materials. Human milk Lfs were obtained from Sigma Chemical Co. (St. Louis, MO). Rabbit antiserum against human Lf was obtained from Accurate Chemical & Scientific Corp. (Westbury, NY). All reagents used were the highest grade commercially available. Human milk was kindly provided by Drs. R. Ceriani and J. Peterson (John Muir Cancer and Aging Research Institute, Walnut Creek, CA).

Purification of Human Milk RNase. The high molecular weight hmRNase was purified from human milk as previously described (7). Briefly, dialyzed milk plasma was chromatographed on phosphocellulose with a linear gradient of 0.2–1.5 M NaCl. Fractions eluting between 0.7 and 0.9 M NaCl, containing the ribonuclease activity, were combined and further purified by gel filtration chromatography on Sephadex G-200 in 10 mM Tris HCl, pH 7.5. The peak of ribonuclease activity eluting at ~80 kD was concentrated and dialyzed against 10 mM Tris-HCl, pH 7.5. This material was homogeneous by SDS-PAGE and devoid of contaminating DNase, alkaline phosphatase, phosphodiesterase, or esterase activity.

SDS-PAGE. Gels (15% acrylamide, 0.75-mm thick) were prepared according to the method of Laemmli (8), and run at 7 mA in a Mini-Vertical Slab Apparatus (Hoefer Scientific Instruments, San Francisco, CA). Molecular mass standards (Bio-Rad Laboratories, Richmond, CA) were included on each gel. Gels were stained with Coomassie Blue for protein or periodic acid-Schiff for carbohydrate.

Western Blots. SDS-PAGE gels were blotted onto nitrocellulose sheets (0.45 μ m; Schleicher & Schuell, Inc., Keene, NH) as previously described (9). After transfer, the sheets were blocked by soaking in 3% BSA in PBS at room temperature for 30 min. Nitrocellulose strips were then incubated with hybridoma culture supernatant (mAb) for 1 h at room temperature, washed with PBS three times for 15 min each with constant rocking, incubated with horseradish peroxidase-conjugated goat anti-mouse Ig (diluted 1:1,000 in PBS; Sigma Chemical Co.), washed, and developed with 0.5% 3,3' diaminobenzidine tetrahydrochloride (Polysciences, Inc., Warrington, PA), 0.1 M Tris-HCl, pH 7.2, 0.01% H₂O₂, 0.04% NiSO₄.

Production of mAbs. 8-wk-old BALB/c female mice were immunized with an intraperitoneal injection of 10 μ g purified hmRNase emulsified in Freund's complete adjuvant. One week later, the mice were reinjected ip with 10 μ g hmRNase in IFA. 2 wk later, the animals were boosted with an intravenous injection of 10 μ g hmRNase in saline, and 2 d later, the mice were test bled to determine antibody response by ELISA (see below). 3 d after the last antigen injection, the spleens of mice with the highest anti-hmRNase titers were removed and their cells fused with the myeloma line P3/NS1/1-Ag4-1, using polyethylene glycol, as described (10). Fused cells were selected in HAT medium. Culture supernatants were tested by ELISA and selected hybridomas were cloned by limiting dilution. The studies reported herein were carried out with mAbs 1A-G4 and 2A-B7, both IgG1s with κ L chains.

ELISA. Antigen preparations (100 μ l of 10 μ g/ml solutions in PBS) were added to the wells of microtiter plates and allowed to adsorb overnight at 4°C. The antigen solution was then discarded and replaced with blocking solution (1% BSA in PBS). After incubation for 1 h at room temperature with continuous shaking, the blocking solution was replaced with 50 μ l hybridoma supernatant (diluted with an equal volume of PBS) or controls and the plates were incubated for 1 h at room temperature with continuous shaking. The wells were washed three times with PBS and secondary antibody added (100 μ l of alkaline phosphatase-conjugated goat anti-mouse Igs [Bio-Rad Laboratories] diluted 1:1,000 in PBS). The plates were incubated for 1 h at room temperature and washed three times with PBS. Substrate solution was added (100 μ l of 1 mg/ml disodium *p*-nitrophenyl phosphate in 0.75 M 2-amino-2-methyl-1,3 propanediol; both from Sigma Chemical Co.). The plates were read at 405 nm in an EL309 reader (Bio-Tek Instruments, Burlington, VT).

For inhibition assays, the same method was used except that the primary antibody was diluted with PBS containing 1% BSA to a concentration giving ~50% maximum reactivity. Aliquots (50 μ l) of the diluted antibody were mixed with the indicated amounts of competing antigen in 50 μ l PBS, incubated overnight at 4°C, and then tested for residual reactivity by ELISA.

Partial Peptide Mapping. Proteins were compared structurally using a modification of the partial proteolytic cleavage method (11). Test proteins (20 μ g in 20 μ l of 1 mM Tris-HCl,

pH 7.4) were mixed with 2 μ l 1% SDS and 2 μ l glycerol, and heated at 100°C for 2 min. After cooling on ice, 5 μ l of enzyme was added (0.05 mg/ml trypsin [type III; Sigma Chemical Co.] or 0.1 mg/ml papain [type IV; Sigma Chemical Co.]) and the mixture incubated at 37°C for 30 min with continuous shaking. The reactions were terminated by addition of 5 μ l 10% SDS, 2 μ l 2-ME, and heating at 100°C for 2 min. The peptides were analyzed by SDS-PAGE.

RNase Assay. Samples were diluted into 40 μ l buffer (20 mM Tris-HCl, pH 7.4, autoclaved and filtered through a 0.45- μ m membrane filter). Substrate ($[^3\text{H}]$ poly-uridylic acid, 30 μ l containing 10⁴ cpm, 3.7 Ci/mmol uridine monophosphate; New England Nuclear, Boston, MA; or 30,000 cpm ^{32}P -poly-uridylic acid, 2.23 Ci/mmol uridine monophosphate, kindly provided by Dr. Opendra Sharma, AMC Cancer Research Center) at 37°C for 30 min with continuous shaking. Reactions were terminated by addition of 10 μ l of 10 mg/ml tRNA (Sigma Chemical Co.) and 0.5 ml ice-cold 5% TCA. The samples were held on ice for 30 min and filtered through glass fiber filters (GF/C; Whatman Inc., Clifton, NJ). The filters were washed with 5 ml ice-cold 5% TCA, dried, and counted in a scintillation spectrometer.

Iron-binding Assays. Test protein samples (5 μ g in 50 μ l PBS) were added to 150 μ l of 0.1 M NaHCO_3 followed by 10⁵ cpm ^{59}Fe citrate (12.7 $\mu\text{Ci}/\mu\text{g}$ Fe, New England Nuclear), and the mixture was incubated for 1 h at 37°C with continuous shaking. Bound and unbound ^{59}Fe were separated by chromatography on a 1 \times 2-cm column of AG1-X8 resin (Bio-Rad Laboratories) eluted with 5 ml of 5 mM veronal buffer (5 mM Na barbital-HCl, 50 mM NaCl, pH 7.4). Fractions of 0.5 ml were collected and counted to determine protein-bound (eluted) Fe.

Purification of Human Milk Lactoferrin on Cibacron Blue-Sepharose. The method used was that previously described (3). Briefly, 5 ml of pooled human milk was skimmed and decaseinated by centrifugation (10,000 g, 40 min), acidification (to pH 4.7 with HCl), heating (40°C, 30 min), and recentrifugation (10,000 g, 40 min). The milk whey was dialyzed overnight against 500 ml veronal buffer. The whey was diluted to 25 ml with veronal buffer. An aliquot (50 μ l) was removed, labeled with ^{59}Fe -citrate (see above), and added back to the remaining whey. The entire sample was then applied to a 1 \times 10-cm column of Cibacron Blue Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ). The column was washed with 25-ml veronal buffer and eluted with a 30-ml linear gradient of 0.5–1 M NaCl in veronal buffer. 1-ml fractions were collected for analysis. The entire procedure, with the exception of the heating step, was carried out at 4°C.

Amino Acid Sequencing. The NH_2 -terminal amino acid sequence of hmRNase was determined by the phenyl isothiocyanate procedure (12) using a 1090 liquid chromatography system (Hewlett-Packard Co., Palo Alto, CA).

Results

Characterization of the RNase Purified from Human Milk. RNase isolated from human milk migrated as a single band on SDS-PAGE with a calculated M_r of 80 kD (Fig. 1). The band stained with periodic acid-Schiff reagent, indicative of the presence of carbohydrate. Gel fractions eluted and assayed for RNase gave a single prominent peak of activity corresponding to the 80-kD glycoprotein band. A minor band of RNase activity with an M_r of 14 kD was sometimes detected in the gels, which may be a breakdown product of the 80 kD RNase (7). The hmRNase also gave a single prominent peak of protein and RNase activity in IEF-PAGE, with pI of 8.7.

The mAbs developed against hmRNase reacted only with the 80-kD RNase band, as determined by direct ELISA of eluted SDS-PAGE gel fractions (Fig. 1), IEF-PAGE fractions, or Western blotting (Fig. 2). Blots of human milk whey also gave a single band of reactivity with the mAbs, which comigrated with the reactive band obtained using purified hmRNase. Under these conditions, no reactivity of the mAb was seen on blots of human spleen and liver (Fig. 2) or pancreas, kidney, and serum (not shown).

Thus, the hmRNase was a homogenous 80-kD glycoprotein, expressed primarily

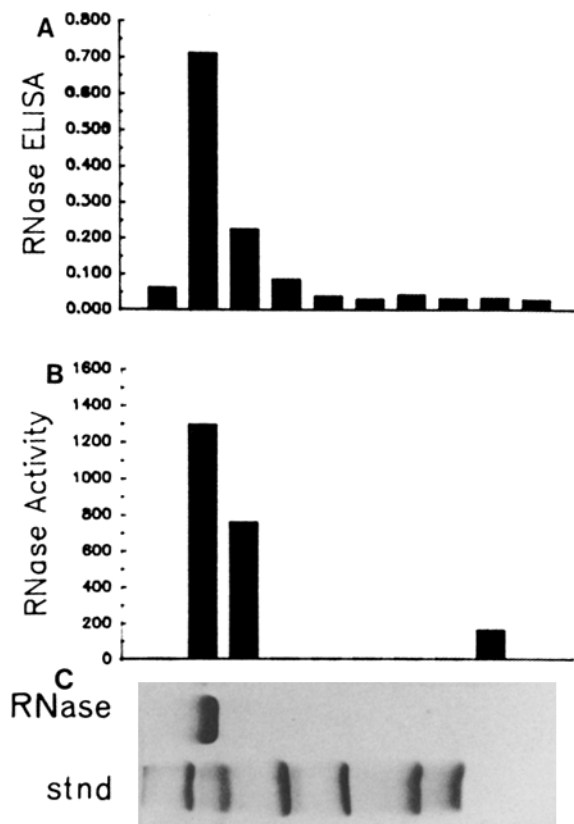


FIGURE 1. Characterization of purified hmRNase. (C) Coomassie Blue-stained SDS-polyacrylamide gel of hmRNase and molecular mass standards (phosphorylase B, 97.4 kD; BSA, 66.2 kD; OVA, 42.7 kD; bovine carbonic anhydrase, 31.0 kD; soybean trypsin inhibitor, 21.5 kD; hen eggwhite lysozyme, 14.4 kD). (B) RNase activity of eluted fractions of hmRNase gel (cpm [^3H]poly-uridylic acid solubilized minus cpm solubilized by control [lowest gel fraction, 672 cpm]). (A) ELISA of eluted fractions (OD₄₀₅ minus OD₄₀₅ of no antigen well).

in human milk. mAbs prepared against the hmRNase appeared to be specifically reactive with the glycoprotein.

Identity of hmRNase with Human Lf. In the course of these studies, a striking similarity was noted between the properties of hmRNase and one of the major bands of human milk on SDS-PAGE (Fig. 3). Based on molecular weight and relative concentration, the corresponding milk component was tentatively identified as Lf, an 80-kD iron-binding glycoprotein present in high amounts in milk (1).

To examine the relationship between hmRNase and Lf, we compared hmRNase with several commercial, homogeneous preparations of authentic human Lf. The molecules comigrated on SDS-PAGE, with calculated M_r s of 80 kD (Fig. 3), and stained similarly with periodic acid-Schiff reagent. Subtle differences in migration of hmRNase and different Lfs were sometimes noted on the gels, but overall the characteristics of the preparations as determined by SDS-PAGE were the same. The pIs of hmRNase and Lf, determined by IEF in PAGE, were both 8.7.

hmRNase and Lf reacted identically with mAbs against hmRNase, as determined by inhibition of ELISA (Fig. 4), direct ELISA, and Western blotting. Monospecific rabbit anti-human Lf also reacted identically with Lf and hmRNase in these assays (not shown).

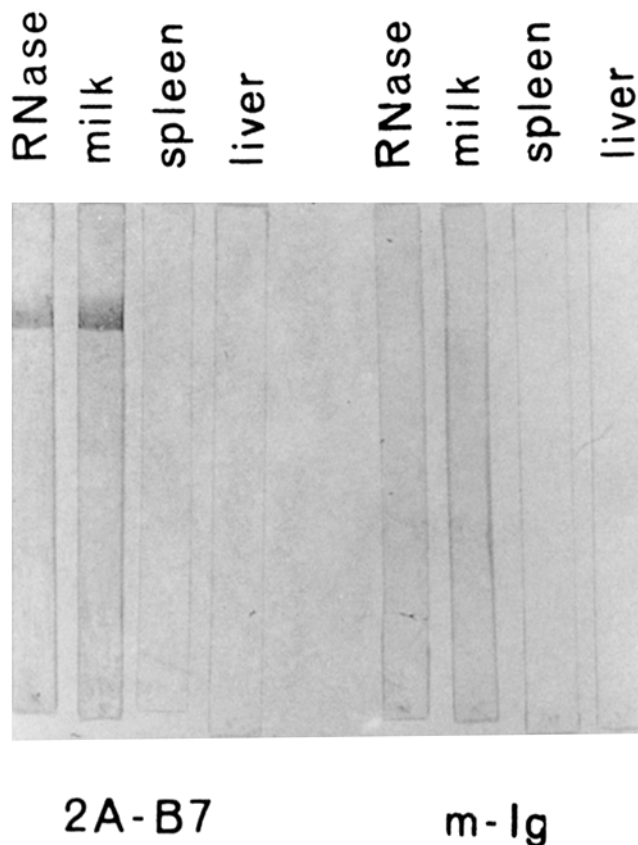


FIGURE 2. Western blot detection of hmRNase in purified hmRNase preparation (RNase), human milk whey, human spleen extract, and human liver extract using anti-hmRNase mAb 2A-B7 or normal mouse IgG (M-Ig).

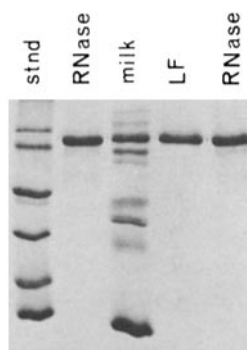


FIGURE 3. SDS-PAGE of purified hmRNase, human milk whey, and human Lf. Standards (stnd) are the same as those in Fig. 1.

Further comparison of the composition of hmRNase and Lf was made by partial proteolytic digestion and peptide analysis by SDS-PAGE. As shown in Fig. 5, the two glycoproteins yielded identical peptide patterns after treatment with trypsin or papain. The NH₂-terminal amino acid sequence of hmRNase was determined (gly-arg-arg-arg-ser-val-glu) and found to be the same as that reported for Lf (13).

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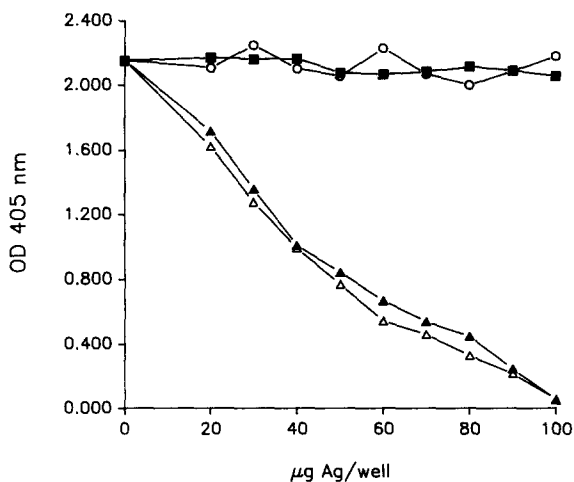


FIGURE 4. Inhibition ELISA of the relationship between hmRNase and Lf using anti-hmRNase mAb 1A-G4. (▲) purified hmRNase; (Δ) human Lf; (■) purified human lactalbumin; (○) purified bovine lactoperoxidase.

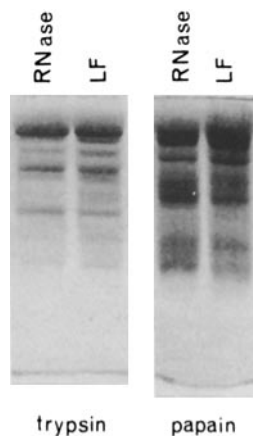


FIGURE 5. Comparison of hmRNase and human Lf by partial proteolytic cleavage with trypsin and papain.

By these criteria, hmRNase and Lf share physical, chemical, and antigenic properties and were, therefore, considered to be very closely related or the same.

Enzymatic and Iron-binding Properties of hmRNase and Lf. Despite the extensive homology between the two glycoproteins, no RNase activity was detected in several different commercial preparations of human Lf (Table I). Removal of iron from RNase or Lf, or saturation with iron under several different conditions, did not alter the results.

In addition, hmRNase did not exhibit the major functional characteristic of Lf, iron binding (Table II). No iron binding by hmRNase was detected in the presence or absence of HCO_3^- , using different anionic forms of Fe^{3+} (ferric chloride, ferric citrate, ferric ammonium sulfate), and in the presence or absence of reducing agent (ascorbic acid).

Thus, although the two molecules appeared to be very similar, if not identical, on a structural basis, they failed to coordinately express the respective primary functional characteristics: hmRNase did not bind iron, and Lf had no RNase activity.

TABLE I
Ribonuclease Activity of Human Milk Proteins

Protein	RNase activity*
Buffer only	11,800
hmRNase (1 μ g)	155
human Lf (1 μ g)	10,895

* TCA precipitable cpm 32 P-RNA remaining after 30-min incubation at 37°C.

TABLE II
Iron-binding Activity of Human Milk Proteins

Protein	59 Fe bound*
hmRNase (5 μ g)	1,970
Human Lf (5 μ g)	22,391
BSA (5 μ g)	0

* cpm 59 Fe bound minus cpm 59 Fe eluting in the absence of protein (452 cpm).

Isolation of Multiple, Functionally Distinct Forms of Human Lf. The finding of functional differences between hmRNase and Lf in the face of their strong structural similarities could be due to the existence of multiple forms of Lf with distinct properties, or to selective inactivation of function (i.e., RNase or iron binding) in the course of purification of the respective molecules. To examine these possibilities, Lf was purified from human milk using the procedure of Bezwoda and Mansoor (14). The salient feature of this method is affinity chromatography of milk whey on Cibacron Blue F3G-A-Sepharose. Lf is identified in the chromatographic fractions based on the presence of bound 59 Fe, added at the initial stages of purification.

The chromatographic pattern obtained with human milk whey is shown in Fig. 6, and is identical to that previously reported (14). A single 59 Fe-containing protein peak was obtained (peak 3), which eluted at ~ 0.75 M NaCl. Analysis of this peak by SDS-PAGE revealed a single band that comigrated with authentic Lf. No significant RNase activity was detected in this peak.

However, when all the column fractions were assayed, RNase activity was detected in two non- 59 Fe-containing protein peaks eluting at ~ 0.20 and 0.40 M NaCl, peaks 1 and 2, respectively (and the unbound fractions, Fig. 6). Analysis of these components by SDS-PAGE revealed essentially a homogeneous material comigrating with authentic Lf and hmRNase (Fig. 7). In some preparations, peak 1 contained minor components migrating with *M*_rs of 115 and 64 kD. Subtle differences in the migration of peaks 1, 2, and 3 were observed, similar to those noted above for authentic hmRNase and Lf. The RNase activity corresponded to the major protein bands in SDS-PAGE fractions of peaks 1 and 2.

The relationship between peaks 1, 2, and 3, hmRNase, and Lf was further examined by partial proteolytic cleavage. Peptide patterns obtained after digestion of the five preparations with papain (Fig. 8) and trypsin (not shown) were identical. SDS-PAGE bands of all three peaks, hmRNase, and Lf stained with periodic acid-Schiff reagent (Fig. 9). The intensity of staining with periodic acid-Schiff relative to pro-

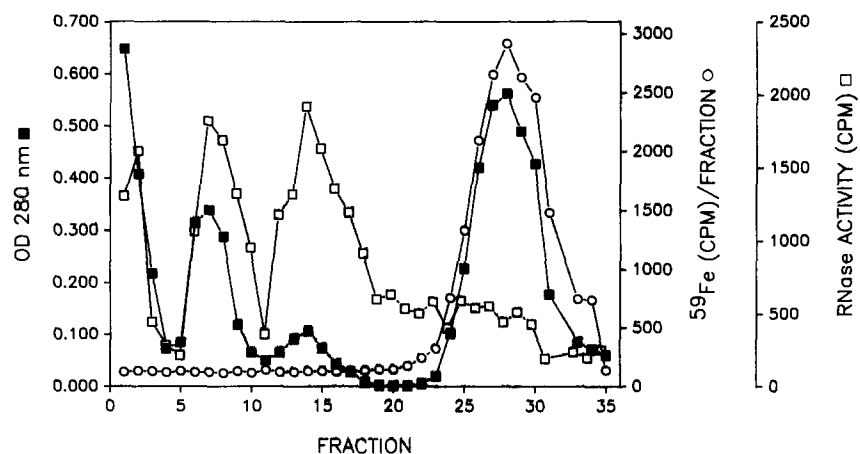


FIGURE 6. Purification of Lf from human milk by affinity chromatography on Cibacron Blue F3G-A-Sepharose and analysis of fractions for protein, iron binding, and RNase activity.

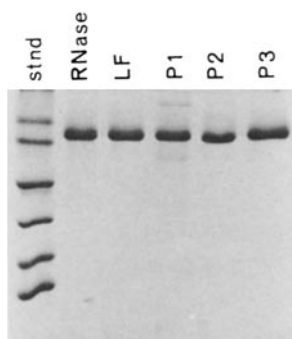


FIGURE 7. SDS-PAGE of hmRNase, Lf, and peaks 1, 2, and 3 of affinity-purified human Lf (Fig. 6). Standards (stnd) are the same as those in Fig. 1.

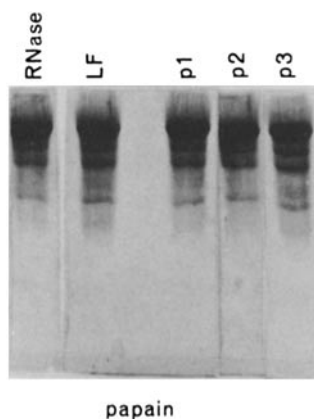


FIGURE 8. Comparison of hmRNase, commercial Lf, and peaks 1, 2, and 3 of affinity-purified human milk (Fig. 6) by partial proteolytic cleavage with papain.

tein staining of the same amount of sample on replicate gels was slightly but reproducibly less for Lf and peak 3 than for RNase, peak 1, and peak 2.

The antigenic relationship between the different Lf-like components was examined by inhibition ELISA using anti-hmRNase mAb 2A-B7. All three peaks, as well as hmRNase and authentic human Lf, were equally reactive (Fig. 10). The same results were obtained using other anti-hmRNase mAbs and monospecific rabbit anti-human Lf.

Thus, we concluded that peaks 1, 2, and 3 represent functionally distinct isoforms of human Lf.

Interconversion of Lf Isoforms. When rechromatographed on Cibacron Blue F3G-A-Sepharose, peaks 1, 2, and 3 eluted at the same NaCl concentrations as they did initially. Addition or removal of Fe had no effect on the chromatographic patterns (not shown).

Affinity chromatography of the original hmRNase preparation, which had been isolated by phosphocellulose and gel filtration chromatography (see Materials and Methods), revealed the presence of all three peaks corresponding to those obtained from human milk whey, with a predominance of peak 2. Affinity chromatography

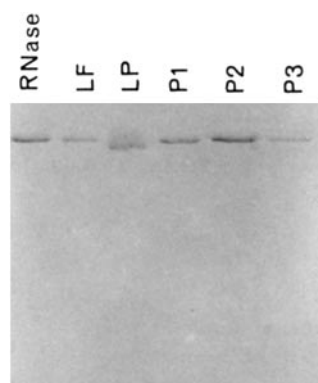


FIGURE 9. SDS-PAGE analysis of hmRNase, Lf, and peaks 1, 2, and 3 of affinity-purified human milk (Fig. 6), and bovine lactoperoxidase (LP). Gel was stained with periodic acid-Schiff reagent.

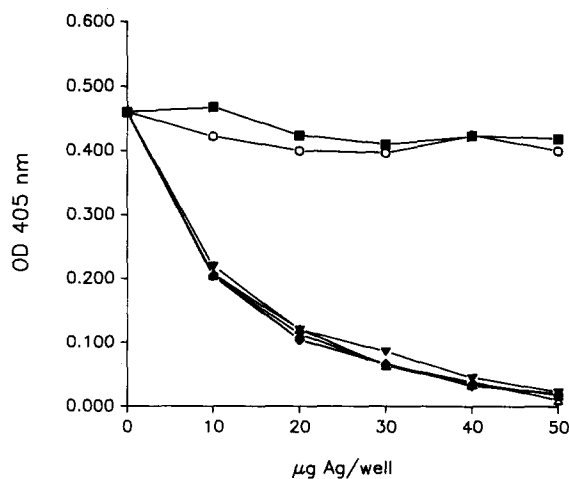


FIGURE 10. Inhibition ELISA of the relationship between hmRNase (▲), Lf (Δ), peaks 1 (▼), 2 (▽), and 3 (◆), human lactalbumin (■), and bovine lactoperoxidase (○), using anti-hmRNase mAb 1A-G4.

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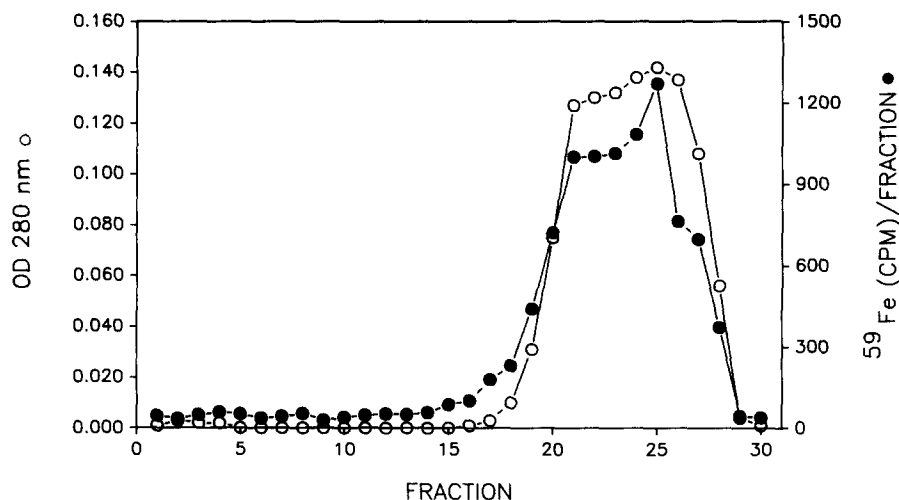


FIGURE 11. Affinity chromatography of commercial human Lf (Sigma Chemical Co.) on Cibacron Blue F3G-A-Sepharose.

of commercial human Lf (No. L3639; Sigma Chemical Co.), which lacks RNase activity, yielded a single ^{59}Fe -binding protein peak, eluting at the same NaCl concentration as peak 3 from human milk, and no detectable amounts of peaks 1 and 2 (Fig. 11).

Discussion

These studies were originally undertaken with the purpose of characterizing an unusual RNase activity in human milks. The enzyme had previously been reported to be expressed in low concentrations in the milks of Parsi women (6), a consanguineous population in Bombay with a particularly high incidence of breast cancer relative to other communities in India. Thus, it was considered that hmRNase might serve as a marker for breast cancer risk, or because of its apparent highly restricted tissue distribution, as a marker for human mammary epithelium. In the course of these studies, we found that hmRNase is a functionally distinct isoform of Lf.

Lf and hmRNase exhibited very similar, if not identical, migrations in SDS-PAGE, IEF-PAGE, partial peptide maps, NH_2 -terminal amino acid sequences, and reactivities with a panel of anti-hmRNase mAbs and monospecific anti-Lf antiserum. The preparations could be resolved into three distinct Lf isoforms by affinity chromatography, with one form having the characteristics of conventional Lf and the other two exhibiting RNase activity and no iron binding.

Lf, identified originally as the major iron-binding constituent of milk (1), and serving a major physiological role in iron storage and transport in lactation, has also been implicated in a wide variety of other functions involving a number of different organ systems (5). Lf is a significant component of the secondary granules of neutrophils (4) and is thought to play a major role in antimicrobial activity of granulocytic cells (3) and milk (15) by chelating iron required for microbial growth. The affinity of

Lf for iron is greater than that of transferrin (1), and thus, Lf removes both available, free iron as well as bound iron from plasma or milk. After release from granulocytes and saturation with iron, Lf is removed from the circulation by the reticuloendothelial system through specific receptors for iron-saturated Lf (16). This is thought to be a mechanism of the hyposiderinemia accompanying an acute inflammatory response. Since neutrophil degranulation is considered to be the only source of circulating Lf (17), serum Lf levels have been proposed as a measure of inflammation or the acute phase response (18, 19), and the size of the total granulocyte pool (20).

Hematopoietic regulatory functions have also been ascribed to Lf. Broxmeyer et al. (21) have shown that Fe-saturated Lf binds to receptors on macrophages and inhibits their release of GM-CSF. In vivo, Lf decreases myelopoiesis in normal mice and dampens rebound myelopoiesis in animals treated with cyclophosphamide (22). Lf inhibits replication of the SFFV component of the Friend erythroleukemia virus complex and prolongs survival of mice infected with the virus (23). Abnormalities in Lf production and in responsiveness to Lf have been detected in leukemia patients (24, 25).

In addition, Lf has been shown to promote neutrophil adhesiveness, reduce lysozyme regeneration, stimulate NK activity, suppress in vitro primary immune responses, enhance growth of certain human lymphoid cells in culture, and decrease numbers and replication of early erythroid colony-forming cells and multipotential hematopoietic progenitors, among other activities (reviewed in references 5, 26, 27). Some of these effects may be mediated through Lf suppression of IL-1 production (5).

Ambruso and Johnston (28) reported that Lf catalyzes hydroxyl radical production from superoxide anion and hydrogen peroxide. This activity was detected in both milk Lf and neutrophil Lf, and required Fe saturation. Production of hydroxyl radical may be involved in antimicrobial activity as well as tissue injury occurring at sites of inflammation.

Until now, all of these varied functions of Lf have been attributed to a single molecule. Lfs isolated from human milk and human neutrophils were reported to have the same M_r , pI, reactivity with monospecific antibody, and clearance from the circulation (29). However, Broxmeyer et al. (27) found a small, but consistent difference in migration of milk and neutrophil Lf on SDS-PAGE, and Ambruso and Johnston (28) found that neutrophil Lf catalysis of hydroxyl radical production was optimal at 10-fold lower concentrations than milk Lf. We have detected small differences in the migration of Lf peaks 1, 2, and 3 on SDS-PAGE that may be due to the differences in iron content (although this would represent an M_r difference of only ~0.15%) or changes in carbohydrate composition.

The amino acid sequence of Lf has been determined (13). The protein consists of 703 amino acid residues, which comprise two domains, each with a single Fe-binding site and an *N*-glycosylation site. A partial cDNA clone for Lf has been isolated from human myeloid leukemia cells (30). Numbers of Lf genes or their organization have not yet been reported. There are no apparent relationships between the amino acid sequence of Lf and known RNases.

The nature of the structural differences that give rise to the profound functional changes in the isoforms of Lf are not known. It does not appear that they are due to Fe, since neither removal nor addition of Fe had any effect on RNase activity. The fact that peaks 1, 2, and 3 gave the same partial proteolytic digestion patterns

and NH₂-terminal sequence suggests that the primary amino acid structures of the isoforms are very similar, although small differences obviously cannot be detected in this way. All three forms stain with periodic acid-Schiff reagent, demonstrating that all are glycoproteins, but neither quantitative nor qualitative differences in carbohydrate content can be ruled out at this time; a small but consistent difference in the ratio of periodic acid Schiff (PAS) stain intensity to the amount of protein on the gel has been observed. Other post-translational modifications, including phosphorylation, could also be involved in the functional differences among the isoforms.

The finding of a single polypeptide, or closely related congeners, with multiple discrete functions is not itself novel. Many examples exist of genes that become duplicated, diverge, and evolve into homologous but functionally distinct entities. There are also several examples of a single gene/polypeptide with both structural or regulatory and enzymatic functions in different tissues, including lens crystallins shown to be identical with certain metabolic enzymes (31-33), and the identity between neuroleukin and glucosephosphate isomerase (34, 35).

The precise relationship of the RNase activity of two of the forms of Lf to the various proposed functions of the glycoprotein also remains to be determined. Nuclease activity could obviously play a role in microbial degradation or inflammation. Lf binds to DNA (36) and to cell membrane DNA on B cells (37) and neutrophils (38), as does hmRNase (7), which implies a nucleic acid recognition capacity for the primary molecule that could be related to its enzymatic and regulatory activities. Furthermore, while some investigators have shown localization of Lf in the specific granules of neutrophils (4), others have reported its presence in neutrophil nuclei (39), also suggestive of a regulatory role related to nucleic acid recognition.

hmRNase was initially detected primarily in human milk. The lack of detection of material reactive with the hmRNase mAb in human spleen extracts (Fig. 1) or other tissues was due to the fact that the concentration of Lf (hmRNase) in milk is ~1,000-fold higher than in granulocytes (1). Studies with granulocytes purified from human peripheral blood have revealed the presence of all three isoforms of Lf determined by Cibacron Blue-Sepharose chromatography (unpublished results). In addition, Western blots of extracts of purified human granulocytes and immunofluorescent staining of human peripheral blood smears with mAbs directed against hmRNase demonstrated specific immunoreactive material in granulocytes.

Thus, Lf, a molecule with a particular tissue distribution and numerous proposed metabolic and regulatory functions, exists in multiple forms with distinctive properties. We propose the following nomenclature for these isoforms of Lf: lactoferrin- α , the traditional iron-binding form (peak 3); lactoferrin- β , the RNase, non-iron-binding form of Lf eluting from Cibacron Blue Sepharose at 0.40 M NaCl (peak 2); lactoferrin- γ , the RNase, non-iron-binding form of Lf eluting at 0.20 M NaCl (peak 1). Studies are presently in progress to further define the comparative structures of the three Lfs and to relate their properties to the varied functions of the molecule.

Summary

Lactoferrin (Lf), the major iron-binding component of milk, also a major constituent of the specific granules of neutrophils involved in antimicrobial activity and a glycoprotein thought to play a role in regulatory functions in the hematopoietic

system as well as other physiologic activities, is shown to occur in three isoforms. One, Lf- α , binds iron; the other two, Lf- β and Lf- γ , express potent RNase activity, but do not bind iron. The three isoforms are very similar or identical in M_r , pI, partial proteolytic peptide patterns, NH₂-terminal amino acid sequence, and reactivity with mAbs and polyclonal antisera against the RNase and Lf, respectively. The finding of structurally similar but enzymatically distinct forms of Lf may be related to the diverse functions of the molecule.

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