Characterization of transferrin receptors on plasma membranes of lactating rat mammary tissue

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Little is known about the transport of iron into the mammary secretory cell and the process of milk iron secretion. The concentration of iron in milk is remarkably unaffected by maternal iron status, suggesting that the uptake of iron into the mammary gland is regulated. It is known that iron enters other cells via transferrin receptor-mediated endocytosis. This study was designed to isolate and characterize the mammary gland transferrin receptor in lactating rat mammary tissue using immunochemical techniques. The existence of functional mammary gland transferrin receptors in lactating rodents was demonstrated using radiolabel-binding techniques. Isolation of mammary transferrin receptors by affinity chromatography was confirmed using immunoelectrophoresis and slot blot analysis. The intact transferrin receptor was found to have a molecular weight of 176 kd as determined by Western blotting followed by scanning densitometry. Reduction of the receptor with β-mercaptoethanol gave a molecular weight of 98 kd. An additional immunoreactive band of 135 kd was observed. The presence of transferrin receptors in normal lactating rat mammary tissue is likely to explain iron transport into mammary tissue for both cellular metabolism and milk iron secretion.

Keywords: Transferrin receptors; lactation; milk; iron; mammary gland

Introduction

Little is known about the transport of iron into the mammary secretory cell and the subsequent process of milk iron secretion. During pregnancy and lactogenesis, normal mammary tissue is rapidly proliferating. Once lactation is established, there is continual maintenance of the enlarged glands. The mammary gland must accumulate its iron needs from the plasma iron pool, in which iron predominantly is carried by transferrin. Therefore, mammary cells should have a mechanism to accrue iron from plasma transferrin, not only for normal cellular metabolism but also for secretion into milk.

It is well recognized that all cells have a mandatory requirement for iron[1] and that iron can enter cells via transferrin receptor-mediated endocytosis.[2,3] Recent studies done in our laboratory,[4] as well as others,[5,6] suggest the presence of transferrin receptors on the membrane of lactating rodent mammary tissue using radiolabel-binding techniques. While demonstrating specificity, these techniques are limited in providing further characterization of the receptors. Immunochemical techniques, using a monoclonal antibody against the receptor, enable further investigation.[3] As a first step in understanding the process of iron accrual by the mammary gland and its regulation, this study was designed to use immunochemical techniques in identifying and characterizing lactating rat transferrin receptors.

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Materials and methods

Plasma membrane isolation

On day 14 of lactation, Sprague-Dawley rats (Charles River, Wilmington, MA, USA) were anesthetized with CO_2. Inguinal mammary tissues were removed and frozen at −70°C until further use. Preparations of enriched plasma membranes were obtained using a modification of the procedure of Maeda et al.[7] Following rapid thawing, the tissue was finely minced. A three-fold excess (wt/vol) of cold homogenization buffer (10 mM sodium phosphate buffer, pH 7.4, containing 1 mM $MgCl_2$, 30 mM NaCl, 1 mM dithiothreitol, 0.005 mM phenylmethylsulfonyl fluoride, and 0.02% NaN_3) was added. Homogenization was achieved using a Janke & Kunkel Ultra-Turrax Tissumizer at 70 rpm (three to five 10-second bursts separated by 30 seconds of cooling). The mixture was filtered through nylon mesh. The filtrate was layered (3:1) over a 41% sucrose gradient and centrifuged at 95,000 × *g* for 1 hour in a Sorvall TH 641 swinging bucket rotor. The white interfacial band of membranes was collected, resuspended in two- to three-fold excess of homogenization buffer, and pelleted at 95,000 × *g* for 20 minutes. The pelleted membranes were washed twice. The final pellet was resuspended through a 22-gauge needle in the homogenizing buffer. Protein content was determined by a modified Lowry procedure.[8] 5′-Nucleotidase activity was used as a membrane marker, and was assayed to assess relative purity of the preparation.[9]

These preparations were used immediately in binding studies. Preparations processed without dithiothreitol were frozen at −70°C for use in immunologic studies.

Tissue homogenate preparation

Mammary tissue homogenates were prepared using the modified procedures of Seligman and Allen.[10] For comparison, liver homogenates were also prepared. Finely minced tissue, in a buffer (1:2) of 10 mM K_2HPO_4 containing 150 mM NaCl, 0.005 mM phenylmethylsulfonyl fluoride, and 0.02% NaN_3, was homogenized as described above. The mixture was centrifuged (Sorvall RC-5B) at 2,000 × *g* for 15 minutes. The supernatant was respun at 30,000 × *g* for 90 minutes, and the pellet resuspended through a 20-gauge needle in homogenization buffer. These preparations were frozen at −70°C for use in the immunologic studies.

Solubilization of plasma membranes and tissue homogenates

For solubilization, Triton X-100 (reduced) at a final concentration of 1% (Aldrich Chemical Co., Milwaukee, WI, USA) was added to membrane and homogenate preparations. The mixtures were placed overnight in a cold room (4°C) under constant stirring and were then centrifuged at 30,000 × *g* for 1 hour. The supernatant was respun for an additional 30 minutes. The solubilized preparations were stored at −70°C. Protein content and 5′-nucleotidase activity were determined as described above.

Binding studies

Qualitative binding studies were performed using ^{59}Fe(III) chloride alone (as $^{59}FeCl_3$ in 1 N HCl, specific activity 74 μCi/g; ICN Biomedicals, Irvine, CA, USA), ^{59}Fe-transferrin (unconjugated rat transferrin, Rockland, Gilbertsville, PA, USA), or ^{59}Fe-bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA). Each of the labeled compounds was incubated at 37°C with plasma membrane preparations in an appropriate volume of phosphate-buffered saline (PBS) for varying times ranging from 1 to 30 minutes. Aliquots were removed, applied to a 0.45-μm Millipore Type HA filter, vacuum filtered, and washed three times with PBS. The filters were placed in scintillation vials and counted in a Beckman Gamma 8500 well-type counter (Fullerton, CA, USA). Specific binding was demonstrated using a 20-fold excess cold transferrin. All values were corrected for nonspecific binding by using the same filtration system without plasma membranes.

Transferrin receptor isolation

Transferrin receptor isolation was achieved following procedures described by van Driel et al.,[11] with modifications suggested by Rudolph and Regoeczi.[12] Briefly, an affinity chromatography column was prepared by coupling rat transferrin with CNBr-activated Sepharose 4B gel as described by Pharmacia (Piscataway, NJ). The transferrin-coupled gel was then loaded with iron and equilibrated with PBS containing 0.5% Triton X-100 reduced. Solubilized plasma membranes were applied to the column, followed by washes of the equilibration buffer and of 0.1 M citrate buffer, pH 5.0, containing 0.2% Triton X-100 reduced and 50 μg/ml sodium nitrilotriacetic acid (NTA). Transferrin receptors were eluted with 1 mM Tris (Trizma Base and Trizma HCl, Sigma), pH 8.0, containing 1 M sodium iodide, 0.2% Triton X-100 (reduced), and 50 μg/ml NTA.

Immunologic detection studies

Immunoelectrophoresis. The purified transferrin receptors and solubilized mammary and liver plasma membranes were assayed with a mouse monoclonal rat transferrin receptor antibody (MRC-OX26) (USA Bioproducts for Science, Indianapolis, IN, USA) using agarose gels (Bio Rad Laboratories, Richmond, CA, USA) in 0.025 M Tris/Tricine buffer, pH 8.6. The agarose was treated to eliminate all charged groups.[13] Electrophoresis was carried out for 3 hours at 300 V and maximum current. The plate was washed and stained as described by Weeke.[14]

Slot blotting. Purified receptor, solubilized liver and mammary plasma membranes, and liver and mammary homogenates were applied directly onto 0.45 μM ni-

trocellulose (Schleicher and Schuell, Keene, NH, USA) as described by Hawkes et al.[15] using the Bio-Dot SF Blotting Apparatus (Bio Rad Laboratories). Solutions containing approximately 0.25 mg/ml, 0.5 mg/ml, and 1.0 mg/ml protein were used. The nitrocellulose membrane was placed in a blocking solution of 5% nonfat dry milk solids in Tris-buffered saline (NFDMS-TBS), pH 7.5, containing 0.02% NaN_3. After blocking overnight at room temperature, the nitrocellulose was gently shaken in a 1:83 dilution of transferrin receptor antibody in 1% NFDMS-TBS for 2 hours, followed by four 5-minute washes with TBS containing 0.05% Tween (Sigma) and one 5-minute wash with TBS. Incubation of the nitrocellulose with alkaline phosphatase-conjugated anti-mouse IgG antibody (Promega, Madison, WI, USA) in TBS (1:7,500 dilution) was followed by three 5-minute washes with TBS-Tween and one 5-minute wash with TBS. Detection of the receptor was performed using the Proto-Blot detection system (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate) (Promega).

Western blotting. One-dimensional electrophoresis was conducted on 7.5% polyacrylamide gels (Integrated Separation Systems, Hyde Park, MA, USA) (10 cm × 8 cm × 3 mm) containing 0.1% (wt/vol) sodium dodecyl sulfate (SDS) according to the directions of Laemmli.[16] After removal of Triton X-100 reduced using Bio-Beads SM-2 Adsorbent (Bio Rad Laboratories) and resolubilization in 0.3% SDS, liver and mammary plasma membrane preparations were diluted in an equal volume of 1.25 M Tris-HCl buffer, pH 6.8, containing 10% SDS, 0.1% bromophenol blue, and 40% sucrose. When samples were to be reduced, 2% β-mercaptoethanol was included. All samples were held at 100°C for 3 minutes before loading onto the gel. The gel was calibrated with 10 μl high molecular weight standard (Pharmacia). Protein transfer was monitored with individual prestained standards (Integrated Separation Systems). The gel was run for 1 hour at 50 mA and maximum voltage. Proteins were transferred onto a 0.45-μm nitrocellulose membrane using the ABN PolyBlot Electroblotting System (American Bionetics, Hayward, CA, USA) according to the procedures outlined by Kyhse-Andersen.[17] The molecular weight standards were cut from the membrane, stained with Amido Black for 3 minutes, and destained with deionized water. The remaining nitrocellulose was processed as described above. Molecular weights were determined using the GS-370 data system attached to a GS-300 Scanning Densitometer (Hoeffer Scientific Industries, San Francisco, CA, USA).

Results

Binding studies

Binding of ^{59}Fe-labeled transferrin to the isolated plasma membranes was shown to reach equilibrium between 15 and 30 minutes (*Figure 1*) and the binding

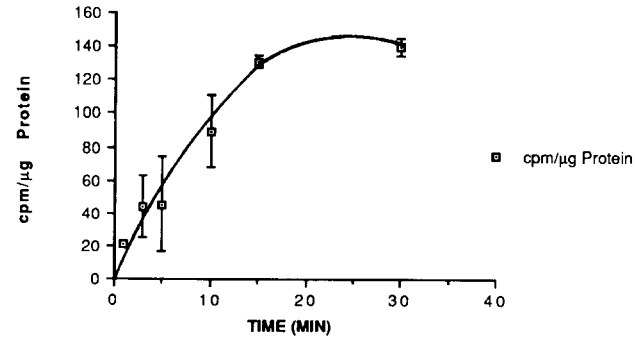

Figure 1 Time course of ^{59}Fe-transferrin binding (transferrin concentration, 14.3 μM) to rat mammary plasma membranes (day 14 of lactation). Results of three experiments.

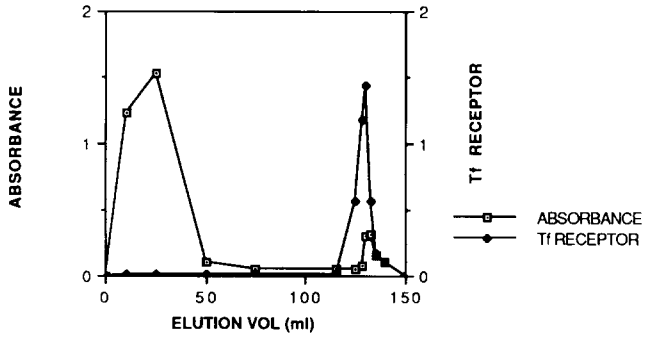

Figure 2 Elution of the transferrin receptor from the transferrin-affinity chromatography column. At 100 ml of buffer, 1 M NaI was added to elute the receptor.

was specific, as shown by inhibition with excess cold iron-transferrin complex.

Receptor isolation

Monitoring of the eluted fractions from the affinity column was performed using immunoelectrophoresis. Protein concentration was assessed spectrophotometrically at 280 nm. After washing the column, the receptor was eluted with the Tris-HCl: sodium iodide buffer (*Figure 2*).

Immunologic studies

Immunoelectrophoresis/slot blotting. Application of purified receptors, purified membrane preparations from liver and mammary tissue, and liver and mammary homogenates onto agarose gels containing the monoclonal antibody demonstrated a positive, but weak, reaction. The appearance of faint rockets formed during immunoelectrophoresis is common when using a monoclonal antibody (data not shown). Slot blotting, followed by detection with an alkaline-phosphatase–conjugated system, provided a better method for identification and quantification (*Figure 3*).

Western blotting. As shown in *Figure 4*, a band of approximately 176,000 d was observed for plasma membranes and homogenates from rat mammary tis-

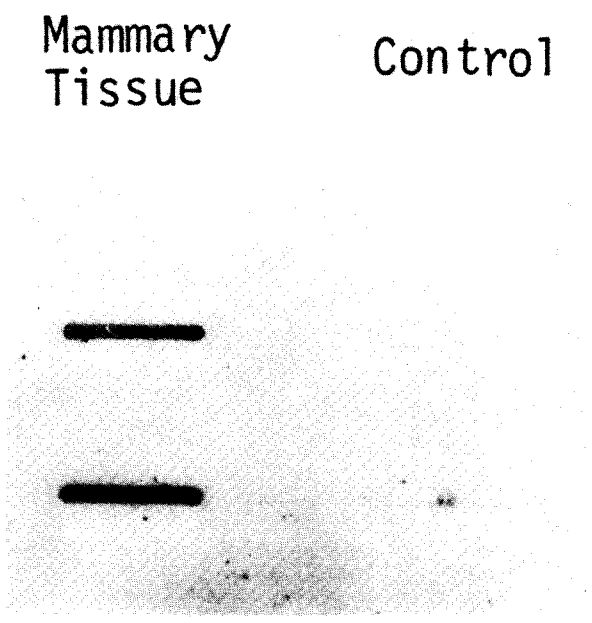

Figure 3 Representative slot blot analyses following application of solubilized lactating rat mammary tissue (day 14 of lactation) or TBS (control) onto a nitrocellulose membrane (see Materials and Methods).

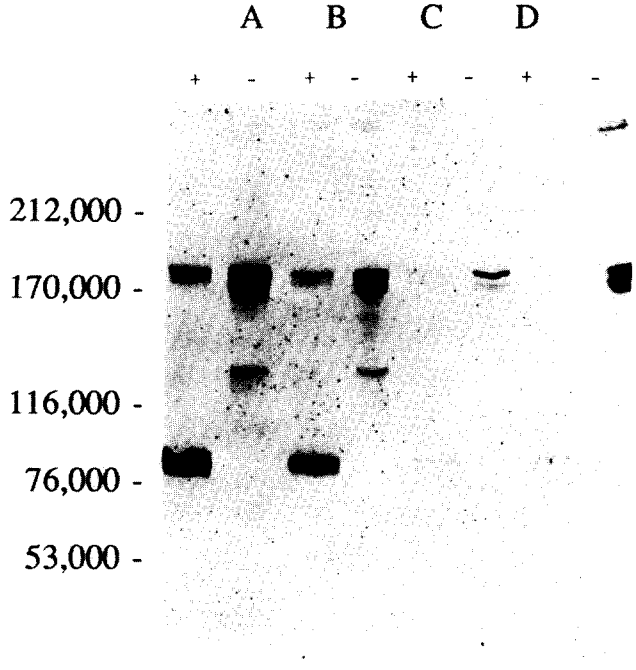

Figure 4 Identification of transferrin receptors in lactating rat mammary gland compared with liver preparations using Western blot analysis. Plasma membranes and homogenates were transferred onto nitrocellulose via electroblotting. Rat transferrin receptor antibody and rabbit anti-rat alkaline phosphatase-conjugated IgG were used for detection of the receptor. +, Reduced with β-mercaptoethanol; −, unreduced. Lane A, mammary plasma membrane; lane B, mammary homogenate; lane C, Liver plasma membrane; lane D, Liver homogenate.

sue (lanes A and B) and rat liver (lanes C and D). When samples were reduced with β-mercaptoethanol, a band of 98,000 d appeared (mammary, lanes A and B; liver, lanes C and D). In addition, a band with an approximate molecular weight of 135,000 d was observed in mammary plasma membranes and homogenates without treatment with β-mercaptoethanol (lanes A and B).

Discussion

The use of traditional radiolabel binding studies, followed by Scatchard analysis, for the quantitation of transferrin receptor in various cell types results in a wide range of values which are magnified by coefficients of variation of one third to one half of the means.[18] Additionally, there are particular technical difficulties when working with lactating mammary tissue, as evidenced by plasma membrane enrichment of no greater than 10- to 15-fold (T. Keenan, personal communication). Given these two restrictions, the binding experiments in this study were of a qualitative, rather than quantitative, nature. Once we had demonstrated specific binding of iron-transferrin complex to plasma membranes from lactating rats,[4] which was recently confirmed by others,[5,6] the immunochemical studies were undertaken.

The molecular weight of 176,000 d observed for the intact mammary transferrin receptor is similar to the 176,000 to 190,000 d reported for human transferrin receptor[19] and the value of approximately 176,000 d for rabbit transferrin receptor.[20] Treatment with a reducing agent yielded a wide band reading at 98,000 d, similar to the value of approximately 95,000 d obtained for the human placental trophoblast transferrin receptor after reduction.[21] This value is slightly higher than would be expected from cleavage of the dimer, but may be a result of the width of the band formed. The unknown band of about 135,000 d found only in the mammary plasma membrane and mammary homogenate may be a product of degradation due to the processing of mammary tissue. After treatment with β-mercaptoethanol, this band disappeared, suggesting the existence of a disulfide bridge. Mammary tissue contains large quantities of connective tissue and fat. Therefore, it is considerably more difficult to homogenize efficiently than other soft tissues. It is possible that this band corresponds to the portion of the transferrin receptor which extends into the extracellular space. This fragment appears to have a molecular weight of about 140,000 d.[3] The functional activity of this unknown band remains to be determined.

Consistent with the estimation of 180,000 transferrin receptors per mammary cell calculated by Grigor et al.,[5] a remarkably low number when compared with over 10^6 for other cell types, we found it logistically impractical to isolate enough receptor material to adequately identify the transferrin receptor by standard gel electrophoresis using either Coomassie or silver staining techniques. This is a further indication of the need for the use of immunochemical procedures in any

future studies involving mammary transferrin receptors.

The mammary gland is unique in that it undergoes most of its morphogenesis in the adult animal. For most other organs, this process is completed by birth, with subsequent development being mainly enlargement or replication of preexisting structures. Iron is mandatory for normal cellular growth and function. During times of rapid proliferation, the need for iron and transferrin increases. It can be assumed that during mammogenesis and lactogenesis, both stromal and parenchymal cells would require significant amounts of iron and transferrin. Consistent with this would be the existence of transferrin receptor on cells within the mammary gland to allow for iron transfer. These receptors should be similar to those found in other cell types. Our finding of such a transferrin receptor on the plasma membrane from mammary glands of lactating rats therefore further provides support for such a mechanism.

Once iron has entered the mammary cell, it becomes available for cellular metabolism as well as for the subsequent export into milk. It can therefore be hypothesized that mammary transferrin receptor endocytosis could be the initial step in making maternal iron available for transport into milk. Further studies are needed to follow the regulation of this transferrin receptor during the course of lactation, in response to varying iron status, and in relation to the concentration of iron in milk.

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