Vol. 171, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1990 Pages 26-32

THE CHICKEN OVIDUCT AND EMBRYONIC RED BLOOD CELL TRANSFERRIN RECEPTORS ARE DISTINCT MOLECULES

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Received July 2, 1990

We recently described an estrogen-inducible transferrin receptor from the chicken oviduct. We now report on the comparison of the oviduct transferrin receptor with the transferrin receptor obtained from chick embryo red blood cells. Western blot analysis reveals that rabbit polyclonal antibodies raised against one receptor do not cross react with the heterologous receptor. Furthermore, peptide map analyses of either affinity purified, native [^{125}I]-labelled transferrin receptors (dimers) or dissociated, and repurified monomers obtained from oviducts and embryonic red blood cells yield distinct patterns. Therefore, the estrogen-modulated oviduct transferrin receptor appears to be structurally distinct from the iron-modulated red cell transferrin receptor.

Iron is an essential element for normal cellular function and proliferating cells have an increased iron requirement. Complex systems for acquiring and storing iron have evolved and vertebrate cells acquire iron via receptor-mediated endocytosis of serum diferric transferrin. The structure, function and regulation of mammalian transferrin receptors have been extensively reviewed (1-3). Generally, the transferrin receptor is a disulfide linked homodimeric glycoprotein with an M_r 180 KDa. However, there is species variability in receptor fine structure as judged by sequence comparisons and immunological cross reactivity (4-6). The transferrin receptor is strongly regulated by iron availability, with iron deficiency eliciting increased receptor expression (7). This regulation is mediated by iron regulatory elements (IREs) in the 3'-non-translated region of the receptor mRNA which affect mRNA half-life (8,9). Receptor expression is also modulated by several growth factors (10,11).

We have recently purified and characterized an estrogen-inducible glycoprotein (M_r 91 KDa) from hen oviduct membranes that exhibits properties of a transferrin receptor. Thus, it is a subunit of a disulfide linked homodimer (M_r 180 KDa), which can be purified by affinity chromatography on an immobilized ovotransferrin column (12). To further characterize the oviduct transferrin receptor we have compared it with the chick embryo red blood cell transferrin receptor employing immunological cross reactivity experiments and peptide mapping analyses. Chick embryo red blood cells have a high number of transferrin receptors as they have a high iron

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0006-291X/90 $1.50

requirement for hemoglobin synthesis. The avian red blood cell receptor has been partially characterized (13,14). Our results indicate that the estrogen-inducible chicken oviduct transferrin receptor is distinct from the embryonic red blood cell receptor. This result is unique; in other organisms the transferrin receptors from different tissues appear to be identical (15).

MATERIALS AND METHODS

Na[^{125}I](100 mCi/ml) was from Amersham Corp. Affi-Gel-10 and SDS-PAGE protein molecular weight standards were from Bio-Rad. Ovotransferrin and heparin were from Sigma Chemical Co. Ovotransferrin was coupled to Affi-Gel at 15 mg/ml gel and equilibrated with 2.5M excess of ferrous ammonium sulfate as described previously (16). [^{125}I]protein G (2-15 μCi/μg) and Staphylococcus aureus V8 protease were from ICN Immunobiologicals. Desferrioxamine was from CIBA-Geigy. Fertilized White Leghorn eggs were obtained either from the Poultry Science Dept., Cornell University, Ithaca, N.Y. or Oliver Merrill and Sons, Londonderry, New Hampshire.

Isolation of Chick Embryo Red Blood Cells: Chick embryo red blood cells were prepared essentially as described by Mason and Brown (17). Fertilized eggs were incubated at 37°C, 80% humidity for 14 days. The shells were cracked open at the top and the Y shaped vein was ruptured with a curved forceps. A small portion of PBS (0.1 M, pH 7.4) containing 200 μg/mL of heparin was added to aid the blood flow. The embryo was tipped onto a nylon screen which was rubber banded to a beaker to collect the blood. Typically, one dozen embryos yield about 3 mL of blood. The blood was centrifuged at 2000 rpm in a Beckman J-21 rotor at 4°C, the supernatant was decanted, and the red blood cells were suspended in 5 volumes of Earle's salt solution containing 2.5 mg/mL BSA. The cells were incubated at 37°C under 5% CO_2 -95% air for 10 min with shaking. The cells were centrifuged and the incubations were repeated three times.

Purification of Oviduct Transferrin Receptor: Hen oviduct membranes were prepared as previously described (18). The oviduct transferrin receptor was purified as described previously with minor modifications (12). Oviduct membranes (approximately 15 mg protein) were solubilized in 2 ml of TBS (0.05 M Tris-HCl, pH 7.4), 1 mM PMSF, and 1% NP-40 and loaded on the ovotransferrin Affi-Gel column (2 mL gel). The column was washed with 200 mL of TBS, 1 mM PMSF, and 0.1% NP-40 followed by 50 mL sodium acetate (0.15 M, pH 5.0), 1 mM PMSF, and 0.1% NP-40. Iron was removed by washing the column with 50 mL of citrate buffer (0.05 M, pH 5.0), 1 mM PMSF, 0.1% NP-40 and 50 μg/ml desferrioxamine. The transferrin receptor was eluted with 10 mL of either glycine-NaOH (0.1 M, pH 10.0), or NH_4HCO_3 (0.1 M, pH 8.0) containing 1 mM PMSF, 0.1% NP-40, and 50 μg/mL desferrioxamine. No difference was found in the eluted product using either eluant. The eluate was concentrated to 200 μL in a Centricon microconcentrator.

Purification of Red Blood Cell Transferrin Receptor: Chick embryo red blood cells (8-10 mL) were lysed in PBS (pH 7.4), 1 mM PMSF and 0.5% NP-40 (70-90 mL) (19). The lysate was held at 4°C for 30 min, and then centrifuged at 800 XG for 10 min at 4°C. The supernatant was centrifuged at 100,000 XG for 60 min in a Beckman Type-30 rotor at 4°C. The resultant supernatant was loaded on an ovotransferrin Affi-Gel column (2 mL) and the column was washed with 100 mL of lysis buffer. The column was sequentially washed with 25 mL of sodium acetate (0.15 M, pH 5.0), 1 mM PMSF, 0.1% NP-40, and 50 mL sodium citrate (0.1 M, pH 5.0), 1 mM PMSF, 0.1% NP-40 and 50 μg/mL desferrioxamine. The transferrin receptor was eluted with 15 mL of NH_4HCO_3 (0.1 M, pH 8.0), 1 mM PMSF, 0.1% NP-40 and 50 μg/mL desferrioxamine. The eluate was concentrated to 500 μL in a Centricon microconcentrator.

Purification of the red blood cell receptor yielded a mixture of dimeric and monomeric species whereas purification of the oviduct receptor primarily yielded dimers.

Electrophoresis and Western Blotting: Polyacrylamide gel electrophoresis (SDS-PAGE) was conducted in a slab gel apparatus as described by Laemmli (20). The proteins were transblotted onto nitrocellulose (Whatman) as described by Towbin et al. (21). Blocking and antibody treatments were performed as described (22). The antigen-antibody complexes were detected using [^{125}I]protein G and autoradiography employing Kodak AR X-Omat film and Dupont

Cronex intensifying screens at -70°C (23). Molecular weight standards included rabbit muscle myosin (200 KDa), E. coli β-galactosidase (116 KDa), muscle phosphorylase b (97 KDa), bovine serum albumin (66 KDa), ovalbumin (44 KDa), carbonic anhydrase (31 KDa), soybean trypsin inhibitor (24.4 KDa) and lysozyme (14.4 KDa).

Immunological Cross Reactivity Experiments: The rabbit polyclonal antiserum against the oviduct transferin receptor has been described in an earlier report (12). Antiserum against the chick embryo red blood cells was produced in rabbits by subcutaneous injection of 15 μg of receptor in Freund's complete adjuvant. Serum was obtained after 5 booster injections of 15 μg of antigen in incomplete adjuvant at ~ 10 day intervals. The sera from multiple bleedings was pooled and precipitated with 45% ammonium sulfate. The pellet was dialyzed against PBS. The antibody preparation was treated by multiple passes over an ovotransferrin Affi-Gel column until an ELISA assay revealed the complete absence of ovotransferrin antibodies. The antisera were used to probe Western blots of the oviduct and red blood cell receptors.

Peptide Map Analysis: The purified transferrin receptors from embryo red blood cells and oviduct were radiolabelled with ^{125}I using Iodobeads (Pierce) according to the manufacturer's instructions. The radiolabelled receptors were separated from free $^{125}I^-$ by chromatography through a column of Sephadex G-50 (12 cm X 1 cm), equilibrated with Tris-HCl buffer (0.06 M, pH 6.8), 1 mM PMSF, 0.1% NP-40. The radiolabelled receptors were further purified by SDS-PAGE on 9% gels in the presence of 5% β–mercaptoethanol. Portions of the gels corresponding to dimeric or monomeric transferrin receptors were excised with a razor blade and electrophoretically eluted into a dialysis bag. The radiolabelled transferrin receptors were digested with varying amounts of S. aureus V-8 protease at 37°C in 0.125 M Tris-HCl (pH 6.8), and 1% SDS. The partially hydrolyzed receptors were subjected to SDS-PAGE on gradient gels. The gels were stained briefly with Coomassie brilliant blue to visualize the protein standards, destained, dried and exposed to Kodak X-Omat AR film using Dupont Cronex intensifying screens at -70°C.

RESULTS AND DISCUSSION

Previous results from this laboratory demonstrated that an estrogen-inducible 91 KDa chicken oviduct glycoprotein exhibited properties expected of a transferrin receptor (12). The overall properties of the 91 KDa protein are similar to those of transferrin receptors identified in chicken erythroblasts transformed with avian erythroblastosis virus and on chick embryo red blood cells (13,14). In order to determine the relatedness of the red blood cell and oviduct transferrin receptors, the following immunologic and peptide mapping experiments were undertaken.

Purified receptors from hen oviduct membranes and embryonic red blood cells were probed by Western blotting using specific polyclonal antisera. As shown in figure 1A, an antiserum directed against the oviduct transferrin receptor does not cross react with the red blood cell transferrin receptor. Similarly, the anti-embryonic red blood cell transferrin receptor antiserum does not show any cross reactivity with the oviduct transferrin receptor (Fig. 1B). The conditions employed for probing the blots were chosen to enhance the possibility of detecting cross reactivity. For example, the antiserum directed against the oviduct receptor was used at a dilution of 1:750, although the antiserum will detect the receptor at ng levels at a 1:2000 dilution on Western blots of oviduct membranes. In additional experiments (not shown) the antiserum directed against the oviduct receptor did not cross-react in an ELISA assay with the red blood cell receptor nor did the antiserum directed against the red blood cell receptor cross-react with oviduct membranes. The complete lack of cross-reactivity by the antibodies against the heterologous receptors demonstrates that the two receptors have distinct epitopes. These results suggested to us that the two receptors might be distinct molecules.

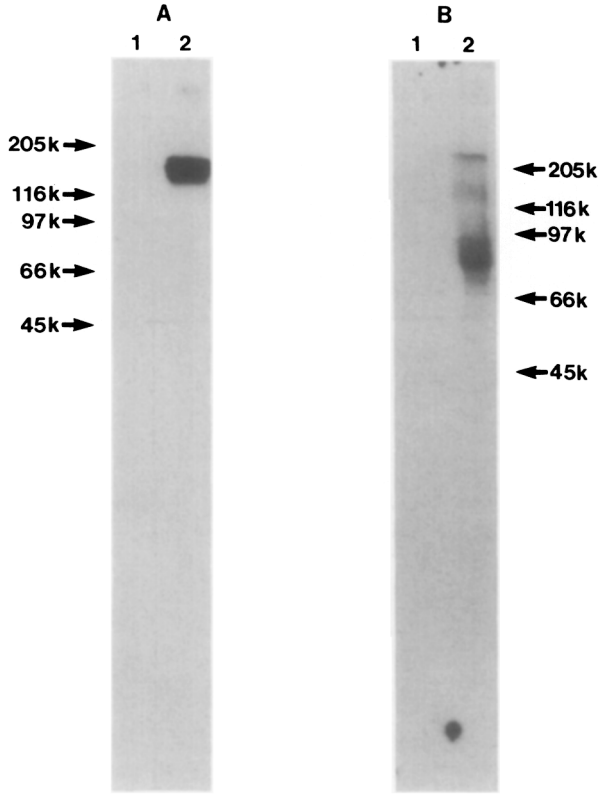

Fig. 1. Lack of Immunological reactivity between oviduct and embryonic red blood cell transferrin receptors. The intact, unlabelled receptors (10 μg) were subjected to 12% SDS-PAGE under nonreducing conditions, transblotted and probed with antisera and ^{125}I-protein G as described in Materials and Methods. Panel A: The receptors were probed with a 1:750 dilution of anti-oviduct transferrin receptor (anti-91 KDa) antibodies. Lane 1, embryonic red blood cell receptor; Lane 2, oviduct receptor. Panel B: The receptors were probed with a 1:200 dilution of anti-embryonic red cell transferrin receptor antibodies. Lane 1, oviduct receptor; Lane 2, embryonic red cell receptor (note the prevalence of monomers).

Therefore, the relationship of the oviduct and red blood cell transferrin receptors was further studied using peptide maps. Purified, radiolabelled transferrin receptors from hen oviducts and embryonic red blood cells were subjected to partial proteolytic digestions with varying amounts of S. aureus V8 protease and the resultant peptides were analyzed by SDS-PAGE. Peptide maps generated from dimeric receptors yielded two distinct patterns (Fig. 2). In each case the released peptides were predominantly under 40 KDa. Although the peptide patterns obtained from the dimeric receptors are clearly different from each other the differences were confirmed by peptide map analysis of the respective monomers. Because neither of the dimeric receptors could be completely reduced to monomers by treatment with disulfide bond reducing agents, the peptide analyses were performed on radiolabelled monomers isolated by SDS-PAGE and electroelution. The results in figure 3 demonstrate that the monomers also yield distinct peptide maps. It is interesting to note that although the patterns are distinct there are at least seven peptides which have

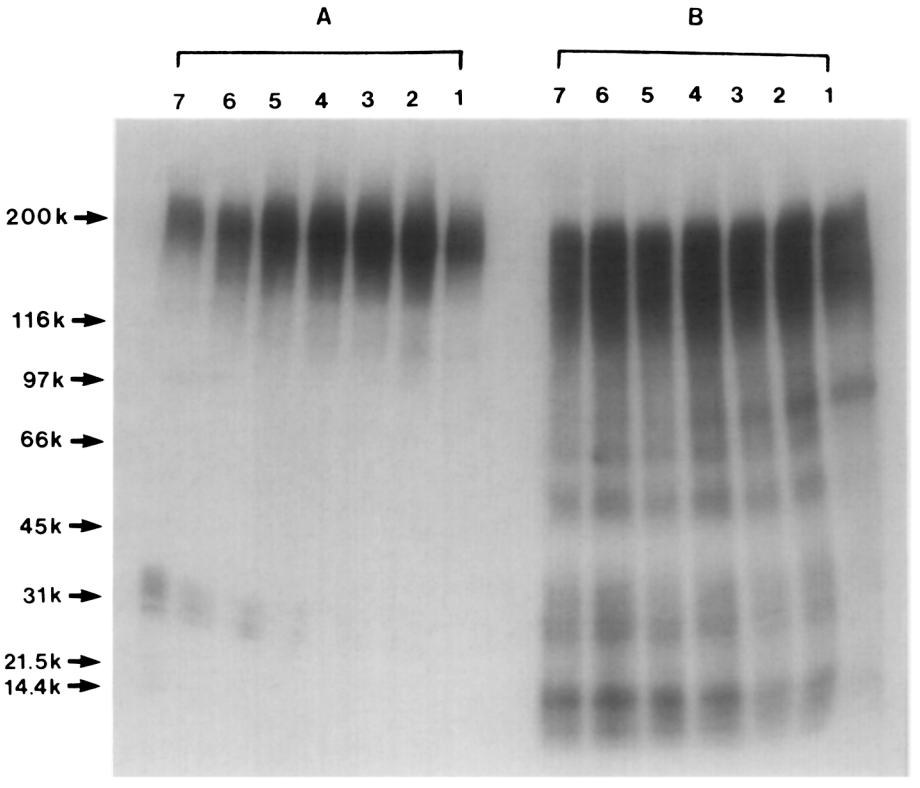

Fig. 2. Peptide maps of transferrin receptors under non-reducing conditions. ^{125}I-labelled dimeric receptors were digested with S. aureus V8 protease for 2 min. as described in Materials and Methods. The digestions were terminated by adding an equal volume of 10% SDS and 2% glycerol. Samples were electrophoresed on 6-18% gradient SDS-PAGE. In each panel, lanes 1-7 were digested with 0, 0.2, 0.4, 0.6, 0.8, 1 and 1.2 μg of protease respectively. Panel A, oviduct receptor; Panel B, embryonic red blood cell receptor.

similar mobilities. These results suggest that although the two transferrin receptors are different, they may share some limited primary peptide structure, perhaps at the ligand binding site.

Although it is clear that transferrin receptors from different species differ from each other (4-6) the presence of distinct transferrin receptors from two different tissues of the same species has not previously been reported. Thus, the human placental and red blood cell receptors were shown to be identical by peptide map analysis (15). In addition, human transferrin receptors from various tissue culture cell lines seem to be identical despite some microheterogeneity, which is probably due to differences in post-translational modifications (24,25).

The existence of two distinct transferrin receptors seems to suggest that the receptors may play different roles in the tissues in which they are located. In this regard it should be noted that evidence has accumulated for a unique role of transferrin and the transferrin receptor in the central nervous system (recently reviewed by Espinosa de los Monteros et al., (26)). The suggestion that the oviduct and red blood cell receptors may have unique functions is further strengthened by our earlier observation that the oviduct receptor is regulated by estrogen (12), whereas the red blood

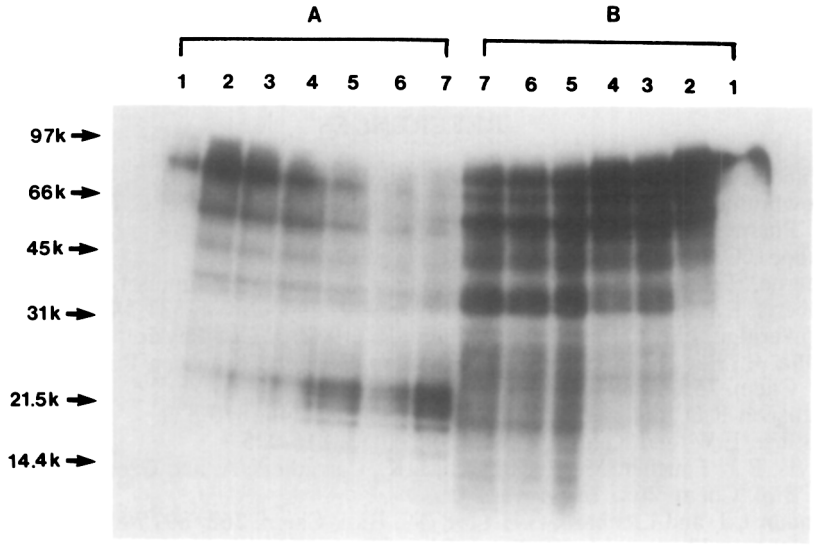

Fig. 3. Peptide maps of transferrin receptor monomers. ^{125}I-labelled monomeric receptors were digested with varying amounts of S. aureus protease in 0.125 M, Tris-HCl (pH 6.8), 1% SDS and 2% β-mercaptoethanol. The digestions were terminated by adding an equal volume of 10% SDS, 2% glycerol and 2% β-mercaptoethanol. The products were electrophoresed on 9-18% gradient SDS-PAGE. In each panel, Lanes 1-7, were digested with 0, 0.2, 0.4, 0.6, 0.8, 1 and 1.2 μg of protease respectively. Panel A, embryonic red blood cell receptor monomer; Panel B, oviduct receptor monomer.

cell receptor is regulated by iron (7). In either case, the uptake of iron-laden transferrin from the serum is presumed to be the major function, although this point remains to be firmly established for oviduct cells. The well-known increased requirement for iron by proliferating cells probably accounts for the apparently greater incorporation of radioactive precursors into the 91 KDa protein observed by DeRosa and Lucas at earlier stages of estrogen treatment of chicks (18). At later stages of estrogenization the initial burst of cell proliferation has passed and the receptor presumably functions only to maintain iron homeostasis.

Although evidence from a limited number of mammalian organisms suggests that the transferrin receptor from different tissues is identical there are several other receptors that exhibit structurally distinct molecular forms. Thus, the atrial natriuretic factor receptor shows distinct forms in rat smooth muscle, kidney and Leydig cells (27). Similarly, two unique forms of the tumor necrosis factor receptor have been observed in human cell lines (28). Presumably these distinct receptor molecules play individualized roles tailored to the particular cell's needs.

Additional studies are underway to further define the unique structural and functional properties of the oviduct and red blood cell transferrin receptors and to elucidate any cell-specific roles for these vital molecules.

ACKNOWLEDGMENTS

This work was supported by grants HD09046 (JJL) and DK/ML31729 from the National Institutes of Health to Dr. R.C. Woodworth who supported ABM. We thank Dr.

Caroline Enns, Syracuse University for numerous helpful discussions and Debra Knapp for her excellent typing of the manuscript.

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