Differential tissue localization of oviduct and erythroid transferrin receptors

(immunocytochemistry/estrogen induction/oogenesis/blood–brain barrier)

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ABSTRACT The tissue distributions of the estrogen-inducible hen oviduct transferrin receptor and the chicken embryonic erythrocyte transferrin receptor were studied. Tissue sections were investigated by immunofluorescence microscopy using specific polyclonal antisera against each receptor. The receptor originally identified and characterized in the oviduct strongly stained liver and ovary; localized staining was observed in the brain (especially endothelial cells). Staining of breast muscle and heart tissue occurred only in occasional interstitial cells. Antiserum against oviduct transferrin receptor did not stain erythrocytes, either from embryos or from mature animals. On the other hand, the antiserum directed against the embryonic erythrocyte receptor stained embryonic erythrocytes; it did not stain any other tissues. The fluorescence microscopy observations were confirmed by Western blot analysis. The strong staining of oviduct, liver, and ovary suggests a major role for the oviduct transferrin receptor in oogenesis.

The acquisition of iron is vital for all cells. Vertebrate organisms have evolved complex mechanisms for the absorption of iron and its distribution to peripheral tissues. Iron is transported in the serum by transferrin and individual cells acquire iron by receptor-mediated endocytosis of diferric transferrin. The properties of the specific transferrin receptor and the mechanism of transferrin uptake and recycling have been extensively reviewed (1–4). Generally, mammalian transferrin receptors are disulfide-linked homodimeric glycoproteins with molecular masses of ≈ 180 kDa. Although much of the information regarding the structure and function of the transferrin receptor has been obtained from experiments with human-derived tissue, other species have proteins with similar properties. However, there is considerable species variability in receptor structure, as judged by sequence comparisons and the general lack of immunological cross-reactivity (5–9).

A protein exhibiting properties of a transferrin receptor has been identified in chicken embryo erythroid cells and avian erythroblastosis virus-transformed erythroblasts (10–12). An estrogen-inducible membrane glycoprotein from chicken oviduct that binds to transferrin affinity columns has also been characterized (13). The oviduct protein shares a number of properties with the chicken erythrocyte transferrin receptor. Thus, it is a disulfide-linked homodimer with a monomeric molecular mass of ≈ 95 kDa. We have shown, however, that the oviduct transferrin receptor is distinct from the erythroid receptor (14). This conclusion is based on a lack of immunological cross-reactivity and peptide map analysis. To date, conventional thought has been that each organism possesses but a single transferrin receptor (15, 16). In the present paper we demonstrate that the chicken erythroid receptor and

oviduct receptor are localized to different sets of tissues, further supporting the distinction between the oviduct and embryonic erythrocyte transferrin receptors.

MATERIALS AND METHODS

Antibodies and Tissues. The polyclonal antiserum directed against the oviduct transferrin receptor (α OV-TfR) has been described (13). Purified chicken embryo erythrocyte transferrin receptor and antiserum against the embryonic erythrocyte transferrin receptor (α RBC-TfR) were kindly provided by Anne B. Mason, Department of Biochemistry, University of Vermont; their preparation has been described (14). Biotinylated goat anti-rabbit IgG and fluorescein-conjugated avidin were obtained from Vector Laboratories.

White Leghorn laying hens and fertilized chicken eggs were purchased from the Department of Poultry Science, Cornell University, Ithaca, NY. Hens were sacrificed by decapitation and the following tissues were excised: brain, heart, kidney, liver, breast muscle, ovarian follicles, and oviduct. Vitelline membranes were prepared by puncturing ovarian follicles with a scalpel, removing the developing oocyte, and rinsing the remaining tissue in phosphate-buffered saline (PBS). Tissue specimens (10 mm $\times$ 10 mm) were embedded in OCT compound (Miles) and immediately frozen in liquid N₂. Samples were stored at -70°C prior to cryosectioning. Mature hen blood and chicken embryonic blood smears were prepared on microscope slides and air-dried at room temperature. Chicken embryo blood was obtained from 10-day embryos by puncturing vitelline arteries with a hypodermic needle, and mature hen blood was obtained from a wing vein.

Immunofluorescence Microscopy. Tissue specimens were sectioned on a Reichert Frigocut model 2000 cryostat. Sections (6 μm) were mounted on microscope slides and two fixatives were applied to determine optimum tissue preservation. Sections and smears were fixed in acetone/methanol, 1:1 (vol/vol), for 90 sec or in formalin for 5 min before being washed in PBS. The slides were treated with blocking solution [PBS/4% (wt/vol) bovine serum albumin/5% (wt/vol) nonfat dry milk/10% (vol/vol) normal goat serum] and rinsed in PBS. Antisera were applied at a 1:300 dilution in blocking solution; undiluted normal serum was used as a control. After a 1-hr incubation, the sections were washed in PBS containing 0.5% bovine serum albumin and subsequently incubated with biotinylated goat anti-rabbit IgG at a 1:150 dilution for 45 min. The slides were washed as described above and stained for 30 min with fluorescein-conjugated avidin at a 1:1000 dilution. Stained slides were photographed using a Nikon Microphot microscope fitted with plan apo lenses and a B-2E fluorescein filter cube.

Electrophoresis and Western Blot Analysis. Membrane fractions sedimenting between $6500 \times g$ and $48,000 \times g$ were obtained from all tissues, except erythroid cells, as described for oviduct membranes (17). Samples of membrane fractions

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Table 1. Immunodetection with specific antisera

Tissue	Transferrin receptor immunodetection	
	α OV-TfR	α RBC-TfR
Brain	++	—
Embryonic blood	—	+++
Mature blood	—	—
Heart	++	—
Kidney	±	±
Liver	+++	—
Muscle	+	—
Oviduct	+++	—
Ovary	+++	—

+++ , Strong specific response; ++ , clearly visible specific response; + , some response, perhaps nonspecific; — , no response or nonspecific staining.

were separated by SDS/PAGE on 9% slab gels according to Laemmli (18) and transferred to nitrocellulose using a semi-dry blot apparatus (Integrated Separation Systems, Cambridge, MA). Transferrin receptors were immunodetected using the relevant primary antiserum and horseradish peroxidase-conjugated goat anti-rabbit IgG (ICN) as described (13). Antisera were employed at dilutions of 1:1,000. Molecular mass standards were obtained from Bio-Rad.

RESULTS AND DISCUSSION

In earlier studies an estrogen-induced oviduct membrane glycoprotein that exhibits the properties of a transferrin receptor was identified (13). This protein is distinct from the chicken erythroid transferrin receptor (14). The present study was undertaken to determine whether these two transferrin binding proteins also exhibit distinct tissue distributions.

Immunofluorescence microscopy was employed to survey the distribution of specific transferrin receptors in brain, heart, kidney, liver, oviduct, ovary, and blood cells. Our results demonstrate strongly contrasting patterns of immunostaining between the α OV-TfR and α RBC-TfR polyclonal antisera (Table 1).

In the oviduct, tubular gland cells are heavily labeled by α OV-TfR (Fig. 1A). Ovarian tissue is also prominently labeled (Fig. 1D). Similarly, hepatocytes are intensely stained with α OV-TfR and the labeled cells are uniformly distributed throughout the liver (Fig. 2G). Oviduct, ovary, and liver yielded identical patterns when stained with affinity-purified antibodies purified by elution from nitrocellulose (data not shown). In brain, α OV-TfR stains scattered cell bodies evenly dispersed throughout the parenchyma of the cerebral cortex (Fig. 2D). At present, these transferrin receptor-containing cortical cells cannot be definitely classified as either neurons or glia. The α OV-TfR also intensely stains endothelial cells in brain blood vessel walls (Fig. 2D, arrow). Several other tissues were also probed with the α OV-TfR (data not shown). In heart muscle only scattered interstitial cells react with α OV-TfR. Breast muscle exhibits a similar pattern but staining is even weaker than with heart muscle. In kidney, the staining is weak and not much greater than the background labeling obtained with normal rabbit serum. The α OV-TfR does not, however, label immature erythrocytes to any extent (Fig. 2A).

On the other hand, the α RBC-TfR stains only immature erythrocytes (Fig. 2B).

A pronounced difference in the staining of mature blood cells with α RBC-TfR as a direct result of the fixation method is observed. Formalin fixation results in a high level of nonspecific staining of mature blood cells. As a consequence, mature blood cells retained in vessels of oviduct, liver, and

FIG. 1. Indirect immunofluorescence detection of transferrin receptors in oviduct and ovary. (A–C) Oviduct. (D–F) Ovarian tissue. Tissue was stained with α OV-TfR (A and D), α RBC-TfR (B and E), and normal rabbit serum (C and F). ($\times 220$.)

FIG. 2. Indirect immunofluorescence of transferrin receptors in embryonic blood, brain, and liver. (A–C) Smears of embryonic chicken blood. (D–F) Brain sections. (G–I) Liver sections. Sections were stained with α OV-TfR (A, D, and G), α RBC-TfR (B, E, and H), and normal rabbit serum (C, F, and I). (A–C, $\times 340$; D–I, $\times 220$.)

brain sections and mature blood smears appear labeled after incubation with α RBC-TfR. However, this staining is non-specific, as the staining pattern of mature blood cells in normal-serum-treated sections is identical with the patterns observed in α RBC-TfR-treated sections. Compare, for example, normal serum staining in oviduct blood vessels (Fig. 1C, arrows) with blood vessels in brain (Fig. 2E, arrows) and liver (Fig. 2H, arrow) that react with the α RBC-TfR. Conversely, the failure of α RBC-TfR to stain acetone/methanol-fixed mature blood cells is consistent with the observations of Schmidt *et al.* (11) and binding studies (ref. 19 and Anne B. Mason, personal communication), which indicate the lack of receptors in mature erythrocytes.

The dissimilarities observed using fluorescence immunomicroscopy were verified by Western blot analysis. α OV-TfR and α RBC-TfR do not cross-react with the heterologous receptors (14). The results shown in Fig. 3 confirm the immunofluorescence microscopy studies. The α OV-TfR reacts strongly with oviduct, ovary, and liver membranes, exhibiting dominant bands at ≈ 97 kDa (Fig. 3). The same staining pattern was observed using affinity-purified α OV-TfR prepared as described (ref. 20 and data not shown). No staining of muscle, heart, brain, or kidney membranes with α OV-TfR is seen on Western blots, despite reactivity of those tissues upon immunofluorescence microscopy. Apparently, the concentration of receptors in these tissues is below the limits of detection, a conclusion consistent with the weakly scattered immunofluorescent staining.

In contrast, the α RBC-TfR does not stain any of the membrane preparations under investigation (data not shown).

FIG. 3. Western blot of membrane preparations probed with α OV-TfR. Lanes: 1, brain; 2, heart; 3, liver; 4, purified embryonic erythrocyte transferrin receptor (10 μ g); 5, oviduct; 6, ovary; 7, muscle; 8, kidney; 9, mature blood. Each well contained 300 μ g of total membrane protein. Molecular mass markers: myosin, 200 kDa; *Escherichia coli* β -galactosidase, 116.3 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa.

The results presented in this report clearly demonstrate that the oviduct and embryonic erythrocyte transferrin receptors are localized to different tissues. It is widely accepted that the uptake of iron is regulated by the iron status of cells. During periods of increased iron demand expression of the transferrin receptor is increased (21). This is true for developing erythrocytes and other rapidly proliferating cells (3, 22). Increased receptor expression is primarily due to increased stability of the transferrin receptor mRNA, which is mediated by specific iron regulatory elements located in the 3' noncoding region of the mRNA (23, 24). However, the regulation of the oviduct transferrin receptor by estrogen (13) adds another dimension to regulation of transferrin receptor expression. The transferrin receptor originally identified in oviduct is present in the highest amounts in estrogen-sensitive tissues—oviduct, ovary, and liver. Each of these tissues plays a vital role in oogenesis and each can be expected to have an increased iron demand during oogenesis. Thus, egg yolk has a high iron content, which is obtained by uptake of serum transferrin, which is synthesized by the liver. Transferrin synthesis in the liver is known to be stimulated by estrogen (25) and laying hens have a higher serum iron–transferrin content (26). It appears, therefore, that the oviduct transferrin receptor plays an important role in the mobilization of iron during the reproductive process.

It should be noted that some brain cells also express the receptor and that evidence has accumulated that transferrin and the transferrin receptor play a role in the central nervous system (for review, see ref. 27). The intense staining of the endothelial cells lining blood vessels suggests a role for the oviduct transferrin receptor in the blood–brain barrier (cf. ref. 28) and is consistent with earlier suggestions regarding other organisms (29–31).

Our observations also demonstrate the absence of the embryonic erythrocyte transferrin receptor in mature chicken tissues. This is an unusual observation. In other species in which the question has been examined, evidence suggests that the erythroid transferrin receptor is identical to the receptor found in other tissues (15, 16). The absence of the embryonic erythrocyte receptor in other chicken tissues suggests that the erythroid receptor may play a role only in early erythrocyte development. The more widely distributed estrogen-inducible oviduct receptor may, therefore, play a more prominent role in iron metabolism in mature animals.

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