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Reconstitution of the Transferrin Receptor in Lipid Vesicles. Effect of Cholesterol on the Binding of Transferrin†

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ABSTRACT: Purified rabbit reticulocyte transferrin receptors were incorporated into phosphatidylcholine vesicles containing varying amounts of cholesterol. The binding of transferrin to the receptor in the reconstituted vesicles had three distinct characteristics: (1) The binding of transferrin exhibited the two components characteristic of transferrin binding to erythroid cells, a saturable, specific component and a nonsaturable, nonspecific component. (2) Transferrin binding exhibited positive cooperativity at low cholesterol/phospholipid (C/P) molar ratios. However, the cooperativity diminished and then disappeared as the C/P molar ratios were increased to the levels found in circulating red blood cells. (3) The amount of specific transferrin binding to the reconstituted vesicles also decreased as the C/P molar ratio was increased. These results indicate that in the reconstituted system the lipid environment plays a significant role in the expression of transferrin receptors.

After transferrin binds to its receptor on erythroid cells, a series of still poorly understood events take place which result in the transfer of iron from transferrin to the plasma membrane for transport into the cell and the subsequent release of transferrin to the circulation to undergo another cycle of iron delivery (Jandl & Katz, 1963). The process or transferrin binding and iron release is carried out from the proerythroblast stage of differentiation through that of circulating reticulocytes (Nunez et al., 1977). The reticulocyte undergoes rapid maturation into an erythrocyte upon entering the circulation; simultaneously, the cell loses its ability to bind transferrin. Several hypotheses have been advanced to explain the absence of transferrin binding in mature erythrocytes: (a) the receptor is lost from the cell (VanBockxmeer & Morgan, 1979); (b) the receptor undergoes intrinsic chemical modification that

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renders it inactive (Liebman & Aisen, 1977); and (c) increased levels of cholesterol in the plasma membrane cause the receptor to "sink into" the membrane and become nonreactive (Muller & Shinitzky, 1979).

We have recently described a method for isolation and purification of the transferrin receptor from reticulocytes (Glass et al., 1980; Nunez et al., 1981). Evidence suggests that the receptor is a glycoprotein of about M_r 80000–90000. A next step in the study of the transferrin receptor would be to reconstitute the receptor into liposomes. A reconstituted system would allow the analysis of two major aspects of the reaction of transferrin with its receptor on the reticulocyte plasma membrane: (a) the loss of transferrin binding activity as reticulocytes mature to erythrocytes and (b) the mechanism of detachment of iron from transferrin after the latter is bound to its receptor.

In the studies to be presented, the purified transferrin receptor is inserted into lipid vesicles, with reconstitution of transferrin binding activity. Factors affecting transferrin binding to the reconstituted system are investigated, including the role of cholesterol in modification of binding activity.

Materials and Methods

Materials. Egg phosphatidylcholine, brain phosphatidylserine, cholesterol acetate, and cholesterol oleate were obtained from Sigma. DL-Cholesterol and sulfobetaine 3-14 (Swittergent 3-14) were purchased from Calbiochem. Iodine-125 and [^{14}C]dipalmitoylphosphatidylcholine were from New England Nuclear.

Purification of Transferrin Receptor by Absorption to Anti-transferrin Antibodies. Transferrin–transferrin receptor complexes from rabbit reticulocyte plasma membranes were solubilized with 0.4% sodium dodecyl sulfate, isolated by gel filtration, and precipitated with goat anti-rabbit transferrin as described (Glass et al., 1980; Nunez et al., 1981). For dissociation of the receptor from the precipitate, the complex was suspended in a 2.5 mg/mL solution of sulfobetaine 3-14 (Gonenne & Ernst, 1978) in 100–200 μL of 20 imosM Tris-HCl[1] (pH 7.6). After incubation for 5 min on ice, the immunoprecipitate was pelleted and the clear supernatent containing the receptor collected. By repetition of this process 1–2 times, most of the receptor dissociates from the anti-transferrin–transferrin complex. After dialysis for 48 h with several changes against H_2O, the receptor was lyophilized and stored at −20 °C.

Liposome Preparation. A number of methods of liposome preparation were evaluated. The method of MacDonald & MacDonald (1975), a variation of the reverse-phase evaporation method of liposome formation, was finally adopted because of the high efficiency of incorporation of the receptor and the expression of transferrin binding activity. A typical liposomal preparation was made as follows: 4.5 mL of chloroform–methanol (2:1) and 1 mg either of phosphatidylcholine or of phosphatidylcholine plus cholesterol (in chloroform–methanol) were added to a 10-mL round-bottom flask. Lyophilized receptor was suspended as a 1–2.5 mg/mL solution in 20 mM Hepes–0.15 M NaCl (pH 7.5), and 20 μL of this solution was added with a 50-μL Hamilton syringe to the solution of organic solvents. The organic solvents were then removed in the rotary evaporator under a water pump vacuum. The samples were subsequently hydrated in the rotary evaporator with 2.5 mL of 20 mM Hepes–0.15 M NaCl (pH 7.5). In both steps, the temperature was kept at 37–40 °C, and the time involved was 10 min. The liposomes were pelleted at 25000*g* for 20 min and resuspended to a concentration of 10 mg of lipid/mL with 20 mM Hepes–0.15 M NaCl (pH 7.5). Control liposomes were prepared similarly but without addition of the receptor protein.

Transferrin Binding Assay. Reactions were carried out in 3-mL Pyrex or Corex tubes that had been pretreated with 20 mM Hepes (pH 7.5)–0.15 M NaCl–1% bovine serum albumin (pH 7.5) and then washed extensively. Ten microliters of liposome suspension and varying amounts of ^{125}I-labeled transferrin in 20 mM Hepes–0.15 M NaCl (pH 7.5) were added to make up a final volume of 50 μL. The reaction was incubated for 20 min at 37 °C unless otherwise stated. The reaction was stopped with 2.5 mL of cold 20 mM Hepes–0.15 M NaCl (pH 6.5), and the liposomes were centrifuged at 25000*g* for 20 min in a Beckman J2-21 centrifuge. The supernatant was aspirated, and the walls of the tubes were carefully wiped dry. The liposome pellets were then transferred into fresh tubes, and ^{125}I radioactivity was measured. This method makes use of the low pH (pH 6.5) of the reaction termination buffer to stabilize the transferrin–receptor complex which otherwise dissociates rapidly. Drying of the tubes eliminates most of the free transferrin without necessitating a second centrifugation. Nonspecific radioactivity associated with the liposomes was determined by using similar aliquots of control liposomes at each transferrin concentration. The radioactivity associated with the control liposomes was subtracted from the radioactivity associated with the receptor-containing liposomes, the resulting difference representing the specific binding of transferrin.

Other Methods. Preparation of reticulocytes and iodination of transferrin and of plasma membrane proteins were as previously described (Glass et al., 1980; Nunez et al., 1981). Negative staining electron microscopy was performed as described by Enoch & Strittmatter (1979).

Results

Incorporation of the Transferrin Receptor into Liposomes. The reverse-phase method for forming liposomes had two major advantages. The liposomes were easily pelleted by centrifugation, and the transferrin receptor was incorporated into the liposomes with high efficiency as determined by using ^{125}I-labeled receptor. The percent of receptor incorporated changed very little when the molar ratio of cholesterol to phosphatidylcholine was altered. As the molar ratio increased from 0 to 1.0, the percent incorporation of receptor was 57.2 ± 12.3% and 60.5 ± 11.2%, respectively (mean ± SD of three independent experiments). Between 85 and 90% of the lipids were recovered in the pelleted vesicles as determined by tracer labeling of lipids with [^{14}C]dipalmitoylphosphatidylcholine.

The size of the vesicles obtained by this method depended on the cholesterol/phosphatidylcholine molar ratio. By negative staining electron microscopy, the mean diameters ± the standard error for 100 liposomes were 0.023 ± 0.009, 0.046 ± 0.004, and 0.064 ± 0.003 μm, respectively, at cholesterol/phosphatidylcholine molar ratios of 0, 0.5, and 1.0. Dependence of liposome size on the lipid composition seems to be a characteristic of vesicles prepared by the reverse-phase evaporation technique (Szoka & Papahadjopoulous, 1980). By negative staining electron microscopy, vesicles of all three lipid compositions appeared as monolamellar structures.

Transferrin Binding to the Transferrin Receptor in Lipid Vesicles. An increase in the ability to bind transferrin was observed when increasing amounts of receptor were incorporated into lipid vesicles (Table I). The dose–response effect

[1] Abbreviations: C/P, cholesterol/phospholipid ratio; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

Table I: Effect of Varying Receptor Levels on the Binding of ^{125}I-Labeled Transferrin to Receptor-Lipid Vesicles[a]

receptor/ phosphatidylcholine ratio (w/w)	specific transferrin binding (cpm)
0.05	8200
0.0125	2900
0.005	1200
0.0005	150

[a] Liposomes containing a constant amount of lipid and increasing amount of receptor, or containing no receptor (control), were incubated for 20 min at 37 °C with 5 μM ^{125}I-labeled transferrin. The reaction mixture was diluted 50-fold and centrifuged. Specific binding to liposome-containing receptor was determined after subtraction of radioactivity associated with control liposomes.

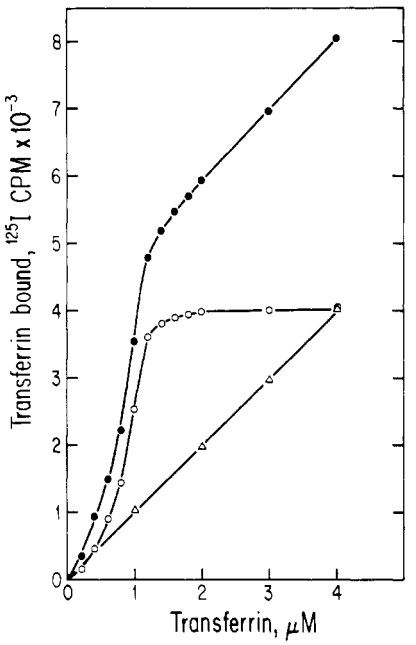

FIGURE 1: Binding of transferrin to receptor incorporated into phosphatidylcholine vesicles. Vesicles containing 2.31 μg of receptor/aliquot were incubated with varying amounts of ^{125}I-labeled transferrin (1.86 × 10^6 cpm/nmol) as described under Materials and Methods. The reaction was stopped with cold 20 mM Hepes–0.15 M NaCl (pH 6.5), and vesicles were collected by centrifugation. (●) Total transferrin binding to receptor containing liposomes. (△) Nonspecific binding obtained by the binding of transferrin to receptor-free vesicles. (○) Specific transferrin binding calculated by subtracting the nonspecific binding from the total binding. The specific binding represented approximately 2.15 × 10^{-12} mol of transferrin bound per aliquot of vesicles.

demonstrated that the transferrin binding capacity of the receptor appears to be conserved during the process of purification and reconstitution. In subsequent experiments, a receptor/total lipid ratio of 0.02 to 0.05 (w/w) was used. This corresponds to approximately 2000–5000 molecules of phosphatidylcholine for each receptor molecule, which has an approximate M_r of 80 000.

Binding of Transferrin to the Transferrin Receptor in Lipid Vesicles. When receptor-containing vesicles were incubated with increasing concentrations of ^{125}I-labeled transferrin, two components of transferrin binding could be detected (Figure 1). One binding component was saturable at about (1–2) × 10^{-6} M transferrin while the other component did not saturate up to at least 10 × 10^{-6} M. The saturable component appears akin to the so-called specific component, and the nonsaturable component to the nonspecific component, of the binding of transferrin to reticulocytes (Baker & Morgan, 1969). The

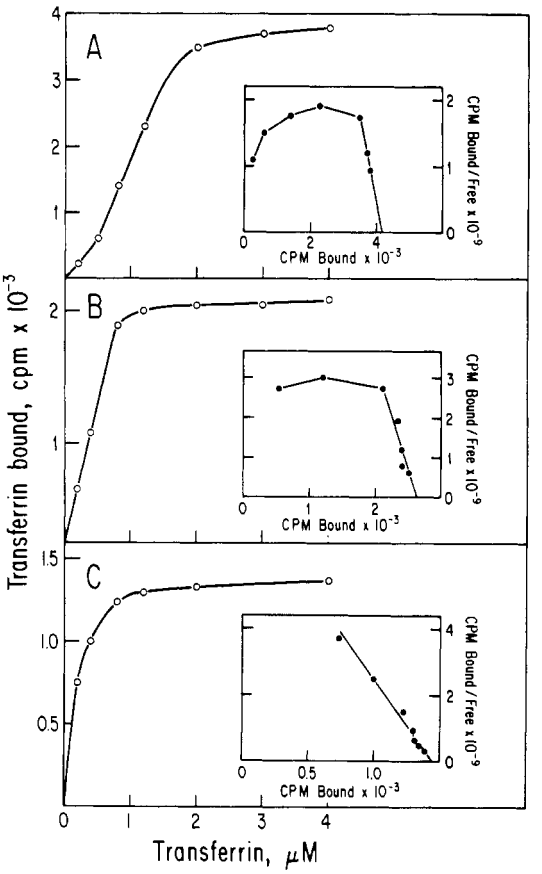

FIGURE 2: Specific binding of transferrin to vesicles with different C/P molar ratios. The transferrin receptor was reconstituted in vesicles with C/P = 0.25 (A), C/P = 0.5 (B), and C/P = 1.0 (C). Panels A–C represent the specific transferrin binding under conditions of Figure 1. Inserts: Scatchard plots of the binding data in (A), (B), and (C), respectively. Specific activity of transferrin was 1.44 × 10^6 ^{125}I cpm/nmol.

specific component of binding was fully reversed by nonlabeled transferrin. The maximum specific binding observed corresponds to about 10% of the receptor binding capacity, assuming the binding unit to have an M_r of about 80 000. The slopes of the nonsaturable component in liposomes containing the receptor and in control liposomes not containing the receptor protein were the same, perhaps representing interaction of transferrin directly with the lipid vesicles.

Positive cooperativity was observed at low transferrin concentrations as demonstrated by the sigmoid-shaped binding curve (Figure 2A,B). Scatchard plot analysis of the binding data produced biphasic, bell-shaped curves as described for hormone receptor systems with positive cooperativity (DeLean & Rodbard, 1969). Hill plots of the binding data yielded a Hill coefficient of 3–4, also indicative of positive cooperativity. This phenomenon has not been observed in the interaction of transferrin with reticulocytes.

Effect of Cholesterol on Transferrin Binding. The suggestion has been made that the cholesterol content of the cell membrane may modify the activity of the transferrin receptor in erythroid cells (Muller & Shinitzky, 1979). Since the molar ratio of cholesterol to phospholipid in lipid vesicles can be altered without affecting the incorporation of the transferrin receptor, the effect of cholesterol on the binding of transferrin can easily be studied in this experimental system. Figure 2 illustrates two marked effects of cholesterol on transferrin binding to receptors incorporated into liposomes: (a) At a C/P[1] molar ratio of 0.25, marked cooperativity is observed. At a C/P ratio of 0.5, positive cooperativity is diminished, and

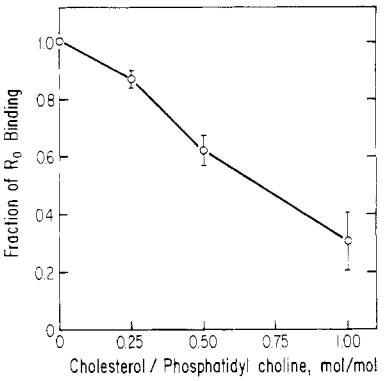

FIGURE 3: Relation of the amount of specific transferrin binding as a function of the C/P ratio. Maximum transferrin binding to receptor-containing vesicles using conditions similar to those in Figure 2 was calculated from Scatchard plots. The results, expressed as the fraction of binding at C/P = 0, are the means ± SE of three experiments.

at a ratio of 1.0, cooperativity is no longer seen. This phenomenon is emphasized by the change of the shape of the Scatchard plots from bell-shaped at a C/P ratio of 0.25 to linear at a C/P ratio of 1.0 (Figure 2, inserts). The affinity constants for transferrin binding to receptor–liposomes with low C/P ratios will vary as a function of the fractional occupancy of the receptor (DeMeyts & Roth, 1975). From the data presented in Figure 2, the average affinity at high fractional occupancy was 2.6×10^6, 5.2×10^6, and 5.6×10^6 M^{-1} for C/P ratios of 0.25, 0.5, and 1.0, respectively. (b) The specific binding of transferrin to the receptor is greatest in liposomes of low C/P ratio (Figure 2A) and decreases with increasing cholesterol content (Figure 2B,C). This inverse relationship is further illustrated in Figure 3. Only 30% of the maximum binding was found at a C/P molar ratio of 1.0 while the specific binding of transferrin is practically abolished at a C/P ratio of 1.4 (not shown). The same correlation was observed by using liposomes composed of phosphatidylserine instead of phosphatidylcholine as well as cholesterol acetate or cholesterol oleate instead of cholesterol.

Discussion

In this paper, we have described the insertion of the transferrin receptor purified from rabbit reticulocytes into lipid vesicles and the properties of binding of transferrin to its receptor in this reconstituted system. The system offers a unique opportunity to examine aspects of the physiology of the transferrin binding system and was used to study the role of cholesterol in this process.

The transferrin receptor was incorporated very efficiently into the liposomes. The high degree of incorporation presumably reflects the affinity of the receptor for lipophilic areas and contrasts with the low efficiency of incorporation of transferrin and serum albumin into liposomes of the same type (data not shown).

Although the efficiency of receptor incorporation was high, only about 10% of the incorporated protein bound transferrin. The apparent efficiency of binding may reflect the orientation of the receptor in the bilayer with a fraction of the receptors being oriented intravesicularly (MacDonald & MacDonald, 1975). Other factors which may influence the efficiency of binding include inactivation of binding activity by the reconstitution process and the lipid composition of the liposome.

While transferrin binding to reconstituted vesicles exhibited the two binding components characteristic of transferrin binding to erythroid cells (Baker & Morgan, 1969), the binding to vesicles with low C/P molar ratios had a characteristic previously undescribed for erythroid cells. In these vesicles, positive cooperativity was always observed at low transferrin concentrations. The cooperativity was demonstrable both as a sigmoid curve in the transferrin concentration curves (Figures 1 and 2A) and as a bell-shaped curve in the Scatchard analysis (inserts of Figure 2A,B). The cooperativity decreased as the molar ratio of cholesterol to phospholipid increased to levels found in circulating red blood cells (Nelson, 1976). The effect was similar in liposomes composed of brain phosphatidylserine instead of egg phosphatidylcholine and therefore is apparently unrelated to the nature of the polar head of the phospholipid. The same changes could be achieved if cholesterol was replaced by cholesterol acetate or cholesterol oleate, and these are therefore not a function of the hydrophilic 3-hydroxyl group of cholesterol. The basis of this cooperativity is not yet clear although it may reflect an effect of the C/P molar ratio on the interaction of putative subunits of the receptor molecule.

The maximum apparent association constant of transferrin to its receptor was 5.6×10^6 M^{-1}. Although this value is similar to that observed with reticulocytes, it must be considered an approximation of the reticulocyte value since the association can be influenced by the cooperativity described above (DeMeyts & Roth, 1975) as well as factors not yet studied such as fatty acid chain length and degrees of saturation.

A strong correlation also existed between an increase in the molar ratio of cholesterol to phosphatidylcholine and a decrease in the specific binding of transferrin. It is tempting, therefore, to conclude that an increase in the membrane cholesterol level is the mechanism by which transferrin binding to reticulocytes and consequently iron uptake are turned off as the reticulocyte matures. This hypothesis implies that the C/P ratio increases after reticulocytes are released into the circulation. The cholesterol/phospholipid ratio in plasma membranes of red blood cells is regulated by exchange of cholesterol between the plasma and the cell membrane (Cooper et al., 1972; Cooper, 1978). This exchange process occurs over a time course of 6–12 h in vivo (Cooper & Jandl, 1969) and could therefore account for loss of transferrin binding activity in circulating reticulocytes. However, older studies have reported no variation in the cholesterol/phospholipid ratio of "young" and "old" cells (Prankerd, 1958; Westerman et al., 1963). These studies are faulted by the use of blood with low reticulocyte counts and poor separation of the different aged cell populations. For substantiation of our hypothesis, a method of cell separation giving close to 100% reticulocytes is required to determine if the cholesterol/phospholipid ratio is lower in new reticulocytes than in mature circulating cells.

The effect of cholesterol on lipid bilayers has been intensively studied. Among its myriad effects, cholesterol modulates motion of phospholipid molecules toward an intermediate fluid state (Chapman & Plenkett, 1969; Rand & Luzatti, 1968), enhances the movement through the membrane of hydrophobic ionophores such as A23187, nigresin, and valinomycim (LaBelle & Racker, 1977), reduces the permeability of liposomes for hydrophilic substances such as glucose and water (DeGier et al., 1968; Demel et al., 1972), and reduces the mean molecular area of phospholipids (Lecuyer & Dervichian, 1969). Inclusion of cholesterol with saturated phosphatidylcholine up to an acyl chain length of 16 increases the width of the lipid bilayer while distearoylphosphatidylcholine with an acyl chain length of 18 reduces the width of the bilayer (McIntosh, 1978). Additionally, a correlation has been reported between decreased amounts of cholesterol and lower microviscosities in

membranes of lymphoma cells (Shinitzky & Inbar, 1974).

Any of the above properties of cholesterol could contribute to the observed decrease in transferrin binding in the reconstituted system. Muller & Shinitszky (1979) have previously suggested that the expression of transferrin receptors in erythroid cells is a function of the cholesterol content of the cell membrane, hypothesizing that the increase in microviscosity produced by cholesterol decreases the exposure of the transferrin receptor. Our data strongly support an effect of cholesterol on modulation of transferrin binding activity. Nevertheless, several reservations exist regarding the model proposed by Muller and Shinitzky: (1) As mentioned earlier, an increase in the C/P molar ratio has yet to be demonstrated in the membranes of developing erythroid cells which undergo an 8-fold decrease in the number of transferrin receptors during their maturation in the bone marrow and peripheral blood (Nunez et al., 1977). (2) The timing of the loss of transferrin binding and iron uptake coincides with completion of hemoglobinization in the red blood cell (Leventhal & Stohlman, 1966; Stohlman, 1970). This apparently finely tuned coordination seems unlikely to be the result entirely of a passive mechanism of cholesterol acquisition. (3) We were able to demonstrate residual transferrin binding at a C/P molar ratio of 1.0, the ratio found in mature circulating red cells which have no transferrin binding activity; clearly, however, binding must be different in several respects in these two systems.

It is possible, therefore, that the membrane C/P molar ratio may be an important factor in the regulation of the expression of transferrin receptors in the later stages of red blood cell differentiation. It will be of great interest if the membrane C/P ratio is also a factor in the expression of transferrin receptors in earlier erythroid precursors where membrane protein biosynthesis occurs.

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