Rabbit Reticulocyte Coated Vesicles Carrying the Transferrin–Transferrin Receptor Complex: I. Purification and Partial Characterization

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Coated vesicles bearing the transferrin–transferrin receptor complex were isolated from rabbit reticulocytes by freeze–thaw cell lysis, followed by differential centrifugation with pelleting of vesicles at 100,000 g. Electron microscopy demonstrated the vesicles to have the characteristic morphology of coated vesicles, including the appearance of triskelions. The protein composition of the vesicles as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis included transferrin, transferrin receptor, and proteins of apparent mol wt of ~180,000, 140,000, 100,000, and 47,000 daltons. The 180,000 and 100,000 mol wt proteins were identified as clathrin and coated vesicle assembly factor proteins, respectively, by Western blot analyses. The vesicles had a Mg2+-dependent ATPase with a specific activity of ~8.5 nmoles ATP converted/min/mg vesicle protein. The vesicles could acidify the intravesicular space, as evidenced by the stimulation of the Mg2+-ATPase by the protonophore FCCP. Reticulocytes appear to be an excellent source of coated vesicles and as such should provide a model for studying the endocytosis of transferrin and the steps of iron uptake that precede in these vesicles.

A M P L E EVIDENCE from studies both with reticulocytes and cultured cells shows that transferrin, after binding to its specific receptor on the cell surface, is internalized into a compartment in which release of iron from transferrin occurs. Fluorescent or electron-dense markers and ultrastructural studies have been used to demonstrate that transferrin enters cells through coated vesicles. In reticulocytes, part of the initial coated vesicle population may form multivesicular bodies that then undergo endocytosis. In this process, transferrin appears to be located within a vesicular compartment, since bound transferrin is resistant to exchange from the cell and to digestion with pronase. Many of these same studies, as well as others, demonstrate that the compartment into which transferrin is internalized is acidified. The acidic milieu is essential both for the release of iron from transferrin and for the continued binding of apo-transferrin to the transferrin receptor, allowing the apo-transferrin to cycle out of the cell undegraded.

Many of these studies suggest that the internalized compartment must have both a proton pump driven by an ATPase capable of acidifying the vesicle and a reductase capable of reducing iron. In the following studies, we isolated from rabbit reticulocytes clathrin-coated vesicles bearing the transferrin–transferrin receptor complex, characterized the unique protein composition of these vesicles, and defined a Mg2+-dependent ATPase that can acidify the intravesicular space.

MATERIALS AND METHODS

**Vesicle preparation.** Rabbit reticulocytes, derived at least 4 days after a course of phenylhydrazine, were depleted of bound transferrin by two 10-minute incubations at 37°C in phosphate-buffered saline (PBS) supplemented with 5 mmol/L glucose and 0.2% bovine serum albumin (BSA). The cells were then incubated for 8 minutes at 37°C with rabbit deferoxate Fe, prepared as previously described, and the labeled cells were then incubated at 4°C in PBS-5% rabbit serum to exchange labeled transferrin that was bound but not internalized. The cells were suspended in 1 vol lysis buffer consisting of 100 mmol/L 2-[N-Morpholino] ethanesulfonic acid (MES) (pH 6.5), 1 mmol/L EGTA, 0.5 mmol/L MgCl2, 0.2 mmol/L phenylmethyl sulfonyl fluoride (PMSF) and lysed by two cycles of rapid freezing in dry ice-acetone followed by thawing by continuous swirling in an ice bath. The lysed cells were pelleted at 9,500 rpm for 10 minutes in a Sorvall SS-34 rotor, and the supernatant (S1) was centrifuged at 45,000 rpm for 45 minutes, with the gradients subsequently harvested from the bottom of the tube. The vesicles had a Mg2+-dependent ATPase with a specific activity of ~8.5 nmoles ATP converted/min/mg vesicle protein.
proteins separated by PAGE were transferred to nitrocellulose paper for Western blotting. The presence of clathrin and the ~100,000 dalton clathrin assembly factor protein was assayed with rabbit anti-clathrin or 100,000-dalton protein antibodies using a horseradish peroxidase-coupled goat anti-rabbit IgG as the second antibody. Characterization of these antibodies against coated vesicle proteins will be described in detail elsewhere (S.T. Mosley, and D. Branton, manuscript in preparation).

Measurement of ATPase activity. The ATPase activity was assayed by monitoring NADH oxidation in a modification of the coupled assay system described by Cantley and Josephson. Vesicle preparations were resuspended in 100 mmol/L MES (pH 6.5), 1 mmol/L EGTA to a protein concentration of 0.5 to 1.0 mg/mL, and the reaction was started by the addition of aliquots of 30 to 100 μL to the same MES-EGTA buffer containing 1.5 mmol/L phosphoenolpyruvate (Sigma, St Louis), 7 to 10 U/mL of a mixture of pyruvate kinase and lactic dehydrogenase (Sigma), 3 mmol/L ATP, 8 mmol/L MgCl₂, and 0.1 mg/mL NADH. The change in absorbance at 338 nm was monitored in a dual-beam spectrophotometer using the reaction mixture without vesicles in the reference cuvette. The effect of the protonophore FCCP were studied at 1 mmol/L, 0.5 mmol/L, 2 mmol/L, 10 μmol/L, and 100 μmol/L, respectively, after preincubation with vesicles for 1 to 2 hours at room temperature. The effect of the protonophore FCCP was assayed by addition to a final concentration of 5 μg/mL to the cuvette during the reaction.

RESULTS

Electronmicroscope characterization of vesicles isolated from reticulocytes. As shown in Fig 1, vesicles were visualized both by negative staining with uranyl acetate and rotary-shadowing electronmicroscopy. The predominant material (Fig 1A) was clathrin-coated vesicles, which constituted almost all of the material in P₄ and appeared similar to brain-coated vesicles (not shown). By rotary-shadowing (Fig 1B), the characteristic triskelions, released from coated vesicles during spraying in glycerol, were found. This confirmed the identity of the isolated material in P₄ as clathrin-coated vesicles.

Protein composition and characterization of the vesicles. The yield of vesicles obtained after freeze-thaw lysis was ~1 mg of vesicle protein from 10 mL packed RBCs with a reticulocyte count of ~50%. This yield compares favorably with the yields obtained both with bovine brains and hepatocytes. The density of the vesicles as measured by Percoll density gradient centrifugation was 1.043 g/cm³, similar to the density of coated vesicles isolated from other sources. The uniqueness of the protein composition of the vesicles was demonstrated by examining the vesicles by SDS-PAGE. As shown in Fig 2A, lane 3, the protein composition of the reticulocyte-coated vesicles fraction is clearly distinct and markedly different from reticulocyte plasma membrane. Six proteins, either not discernible, or barely so, in the plasma membrane but quite prominent in the vesicles are transferrin, transferrin receptor, and proteins of apparent mol wt 180,000, 140,000, 100,000, and 47,000. In contrast, erythrocyte plasma membrane bands 1, 2, 2.1, and 3 are depleted from the vesicle preparations. Further purification of vesicles by gel filtration chromatography through Biogel A1.5 did not change the protein pattern on subsequent analysis by SDS-PAGE or change the relative amounts of the major proteins, suggesting that contamination by other cellular structures, if any, is negligible. The yield and enrichment of transferrin (and hence the transferrin receptor) during purification of vesicles was assessed by determining the recovery of ¹²⁵I-transferrin bound to reticulocytes. As shown in Table 1, an enrichment of transferrin of ~1,000-fold was achieved between the freeze-thaw lysate, SI, and the coated vesicles in P₄.

In addition to the data from electronmicroscopy, three lines of evidence suggest that these are clathrin-coated vesicles: (a) The 180,000-dalton polypeptide was released from the vesicles on treatment with 10 mmol/L Tris, pH 8.5 (Fig 2A, lane 4), as has been described for the release of clathrin from coated vesicles derived from brain. In addition, the proteins of mol wt of ~140,000 and 100,000 daltons were also released under these conditions. (b) Western blot analysis with anti-clathrin polyclonal antibody showed binding to a single polypeptide of 180,000 daltons (Fig 3). (c) Antibody against the ~100,000-dalton polypeptide associated with coated vesicle bound to a polypeptide of similar mol wt in the reticulocyte vesicle preparation (Fig 3).

Sidedness of the vesicles. Evidence that transferrin was contained within the vesicles was obtained from the ability to
Fig 2. SDS-PAGE of vesicle preparations and identification of transferrin (Tf) and transferrin receptor (TfR) by Western blot analyses. Vesicles were prepared and electrophoresed as described in the Materials and Methods Section, and the gel was stained with Coomassie blue (A) or transferred onto a nitrocellulose paper to identify Tf and TfR, using anti-Tf and TfR antibodies, respectively (B). (A) Lane 1 represents mol wt markers; lane 2, reticulocyte plasma membrane; lane 3, coated vesicle preparation (P4); lane 4, clathrin-depleted vesicles in 10 mmol/L Tris, pH 8.0; and lane 5 represents purified rabbit Tf. (B) Lanes 1 and 2 are Coomassie blue staining of mol wt markers and Tf (lane 1) and clathrin-depleted vesicles (lane 2); lanes 3 and 4 are Western Blot analyses with goat anti-rabbit Tf antibody (lane 3) and goat anti-rabbit Tf receptor antibody (lane 4) using horseradish peroxidase-conjugated rabbit anti-goat IgG as a second antibody. Cross-reaction of Tf receptor antibody with Tf in lane 4 is due to the Tf contamination in Tf receptor preparation used for immunization. Molecular masses of the marker polypeptides given figure are in kilodaltons.

exchange at 0°C <5% of bound 125I-transferrin with unlabeled excess transferrin. The asymmetrical distribution of the proteins could also be demonstrated by 125I-labeling of the proteins of vesicles prepared with unlabeled transferrin. Iodination with 125I by lactoperoxidase to label the externally facing vesicle proteins, followed by SDS-PAGE and radioautography demonstrated that the clathrin as well as the transferrin receptor, a known transmembrane protein, were labeled while transferrin itself was not (data not shown).

ATPase activity and acidification of the vesicles. The ATPase activity of the vesicles was assayed by monitoring NADH oxidation at 338 nm. The ATPase activity of the vesicles was Mg2+ dependent and had an average specific activity of 8.5 nmoles ATP converted/min/mg vesicle protein at room temperature. As shown in Table 2, the ATPase activity of the vesicles could be distinguished from the ATPase activity of plasma membrane and mitochondria by the pattern of inhibition with ouabain, oligomycin, NBD-Cl, sodium azide, and vanadate. The vesicle ATPase activity was not inhibited by ouabain, minimally inhibited by oligomycin, vanadate and sodium azide, and inhibited ~40% by NBD-Cl. FCCP, an electrogenic protonophore, stimulated the ATPase activity, suggesting that the ATPase was acidifying the interior of the vesicle. This hypothesis has been tentatively confirmed by determining the ATP-dependent alkalinization of the extravascular medium using a pH-sensitive electrode and quenching of acridine orange fluorescence. In contrast to the above, there was a lack of effect of FCCP on ATPase activities of plasma membrane and mitochondria.

DISCUSSION

The reticulocyte is uniquely suited for isolation of the internalized compartment containing the transferrin–transferrin receptor complex. Transferrin receptors are the predominant cell surface receptor; internal membrane structures are reduced in reticulocytes as compared with other cells; and, as shown in the present studies, a high yield of clathrin-coated vesicles may be obtained. The reticulocytes were disrupted under mild hypotonic conditions by freeze-thaw lysis to preserve the integrity of the vesicles. Low-speed centrifugation was sufficient to remove unbroken cells and
mitochondria, whereas the subsequent ultracentrifugation removed plasma membrane fragments. The resulting vesicle preparations had a characteristic protein composition. The major vesicle protein had an apparent mol wt by SDS-PAGE of 180,000 daltons, and our studies with anti-clathrin antiserum. Antisera were diluted 1:1,000 in PBS/5% newborn calf serum/O.1% Tween 20 for incubation with nitrocellulose strips. Antibodies were diluted 1:1,000 in PBS/5% newborn calf serum/0.1% Tween 20 for incubation with nitrocellulose strips. Antisera were diluted 1:1,000 in PBS/5% newborn calf serum/O.1% Tween 20 for incubation with nitrocellulose strips. Antisera were diluted 1:1,000 in PBS/5% newborn calf serum/O.1% Tween 20 for incubation with nitrocellulose strips.

Fig 3. Western blot of SDS-PAGE-fractionated bovine brain coated vesicles (lanes 1, 3, and 5) and reticulocyte coated vesicles (lanes 2, 4, and 6). Lanes 1 and 2 show total protein by Ponceau S stain; lanes 3 and 4 are blotted with anti-clathrin antiserum; lanes 5 and 6 are blotted with anti-100,000 dalton assembly factor antiserum. Antisera were diluted 1:1,000 in PBS/5% newborn calf serum/0.1% Tween 20 for incubation with nitrocellulose strips.

The uniqueness of the protein composition of the reticulocyte, with the hypothesis that erythrocyte clathrin reflects residual clathrin from endocytic vesicles in immature cells, and a report on the presence of coated pits in reticulocytes. The uniqueness of the protein composition of the reticulocyte-coated vesicles was further substantiated by finding that cytoskeletal proteins were minimally present, consistent with the observations that the invading domains of membrane in the process of endocytosis are devoid of spectrin. The major transmembrane protein, band 3, was not detected in the vesicles, whereas transferrin and the transferrin receptor, which are barely detectable on electrophoresis of reticulocyte plasma membranes, were prominent constituents of the vesicles. Furthermore, the purification of transferrin (and hence the transferrin receptor) was considerable, suggesting that a unique array of proteins was isolated in the vesicles. Dickson et al have also demonstrated enrichment of transferrin receptors in endosomes from a human cell line.

Acidification of the compartment into which transferrin is internalized is essential for iron uptake in reticulocytes. Presumably, reticulocytes lower the pH of the endocytic vesicles by an ATP-dependent proton transport similar to that described for coated vesicles derived either from brain or liver. The ATPase activity of the vesicles could be demonstrated by response to cations and various inhibitors to be similar to that of clathrin-coated vesicles derived from calf brain and distinct from those of reticulocyte plasma membrane or mitochondria. The lack of inhibition of vesicle ATPase activity by ouabain and sodium azide distinguishes this ATPase from ATPases in plasma membrane or mitochondria. In contrast to the findings with brain coated vesicles, inhibition of erythrocyte ATPase activity by NBD-CI was not nearly as potent or as specific for coated vesicles. The ability to stimulate vesicle ATPase activity with the protonophore FCCP provided indirect evidence that the ATPase from ATPases in plasma membrane or mitochondria was similar to that previously observed with brain coated vesicles.

These vesicle preparations provide a relatively rapid method for isolating large quantities of coated vesicles bearing transferrin bound to the transferrin receptor. As such, these vesicles provide a model for studying several of the components essential for iron uptake, including an ATP-driven proton pump, a membrane iron carrier, and a reductase to reduce Fe (III) to Fe(II) for transport into the cytosol. Because of the relative simplicity of the reticulocyte system, the ease of obtaining reticulocytes, and the extent of knowledge of the RBC membrane proteins, the reticulocyte vesicles may also provide a model for studying the early events of receptor-mediated endocytosis, including the segregation of unique proteins into the coated pit, the process of vesiculation of the coated pit, and the passage of receptor–ligand complexes from coated vesicles into endosomes.

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REFERENCES


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