Co-migration and Internalization of Transferrin and Its Receptor on K562 Cells

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ABSTRACT The incorporation of iron into human cells involves the binding of diferric transferrin to a specific cell surface receptor. We studied the process of endocytosis in K562, a human erythroid cell line, by using tetramethylrhodamine isothiocyanate-labeled transferrin (TRITC-transferrin) and fluorescein isothiocyanate-labeled Fab fragments of goat antireceptor IgG preparation (FITC-Fab-antitransferrin receptor antibody). Because the antireceptor antibody and transferrin bind to different sites on the transferrin receptor molecule it was possible to simultaneously and independently follow ligand and receptor. At 4°C, the binding of TRITC-transferrin or FITC-Fab antitransferrin receptor antibody exhibited diffuse membrane fluorescence. At 20°C, the binding of TRITC-transferrin was followed by the rapid formation of aggregates. However, the FITC-Fab antitransferrin receptor antibody did not show similar aggregation at 20°C unless transferrin was present. In the presence of transferrin, the FITC-Fab antitransferrin receptor antibody formed aggregates at the same sites and within the same time period as TRITC transferrin, indicating co-migration. Although the diffuse surface staining of either label was removed by proteolysis, the larger aggregates were not susceptible to enzyme degradation, indicating that they were intracellular. The internal location of the aggregates was also demonstrated using permeabilized cells that had been preincubated with transferrin and fixed with 4% paraformaldehyde. These cells showed aggregated receptor in the interior of the cell when reacted with fluorescein-labeled antibody to the receptor. This indicated that the transferrin and the transferrin receptor co-internalize and migrate to the same structures within the cell.

Transferrin, an iron-binding serum protein, and its receptor on the plasma membrane are considered to be involved in a major pathway for the transport of iron into cells. Superficially, the receptor appears to act as a cation transporter; however, the detailed mechanism of the cellular internalization of iron is still unclear. Initial studies using 131I-labeled transferrin and cell fractionation found transferrin on the surface of reticulocytes (1). In more recent work, using ascites tumor cells, ferrocyanide staining of transferrin and ferritin-conjugated antibody to transferrin also showed that transferrin is localized on the cell surface (2). In contrast, there is evidence that transferrin is pinocytosed into reticulocytes and normoblasts (3–5). In these studies, ferritin-conjugated antibodies to transferrin (3, 4) and transferrin-colloidal gold complexes (5) were employed to visualize the localization of transferrin.

Experiments demonstrating that transferrin co-purifies with clathrin in human placental tissues (6, 7) imply that the mechanism of transferrin uptake into cells might be similar to that of many peptides, i.e., asialoglycoprotein receptor, yolk proteins, α-2 macroglobulin, and the hormones, insulin and epidermal growth factor (8, 9). Many studies of receptor-mediated endocytosis have been reported (10–15). In most of these cases, receptor internalization has been inferred, and only ligand internalization has been directly observed. Protease digestion of the cell surface or treatment with agents that destroyed ligand binding to its receptor was used in some of these studies to suggest that ligand internalization was receptor mediated.

Our study’s purpose was to directly observe both the receptor and transferrin following the binding of the ligand to the receptor. In these studies, we fluorescently labeled transferrin and an antibody to the transferrin receptor to visually study them independently on the surface of K562 cells, a human erythroid cell line.

MATERIALS AND METHODS

Generation of Fab Fragments from IgG: Fab fragments of an IgG preparation of goat anti-human transferrin receptor in which all cross-reactivity with transferrin was eliminated (16) were prepared by digesting the antibody (6.8 mg/ml) with papain (0.12 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN) in 0.05 M Tris-HCl, 2 mM EDTA, 1 mM...
dithiothreitol, pH 7.4, for 6 h at 37°C. The digested material was loaded onto a Protein A column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with PBS, pH 7.4, and the void volume collected. This material was judged to be free of intact heavy chain by SDS 14% polyacrylamide electrophoresis under reducing conditions (17).

Fluorochrome Labeling of Fab Antitransferrin Receptor and Transferrin: The purified Fab fragment of the antibody to the human transferrin receptor was labeled with fluorescein isothiocyanate (FITC) (Sigma Chemical Co., St. Louis, MO) in 1 M sodium bicarbonate, pH 9.5, for 1 h at room temperature. Unreacted FITC was removed by G-25 gel filtration (Pharmacia Fine Chemicals) in phosphate-buffered saline (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4 [PBS]). Iron-saturated human transferrin (Sigma Chemical Co.) was labeled with 50 μl of tetramethylrhodamine isothiocyanate (2 mg/ml dissolved in acetone) (Nordic Immunology, London) in 1 ml of 1 M sodium bicarbonate at pH 9.5 for 3 h at room temperature. Excess tetramethylrhodamine isothiocyanate (TRITC) was removed by G25 (Pharmacia Fine Chemicals) gel filtration in PBS.

Incubation of Cells with Antibodies and Transferrin: K562 cells were washed twice with PBS and resuspended to 2 x 10^6 cells/ml in PBS with 1 mg/ml ovalbumin. FITC-labeled transferrin and/or TRITC-labeled Fab fragment of antitransferrin receptor were added and incubated at 4°C for 15 min. The cells were then pelleted, washed, and resuspended to 1 x 10^6 cells/ml in PBS with 1 mg/ml ovalbumin. Samples were stored on ice (15 min) until observed or photographed under a fluorescence microscope. Samples (10 μl) were dropped on slides, and a cover slip was placed over the sample. Cells were either taken directly from the ice at 4°C and counted or photographed within 5 min, or else were allowed to warm to room temperature either on the slide (5-10 min) or in the incubation tube (30 min) and then counted or photographed.

Anti-human glycophorin generated in goats was a gift of Dr. Leif C. Anderson (18). FITC-labeled rabbit anti sheep IgG (Antibodies Inc., Davis, CA) was used to identify red blood cells to visualize glycophorin bound to cells. FITC-labeled rabbit anti sheep IgG did not show any fluorescence when incubated by itself with the cells.

Protease Digestion of Cell Surface Proteins: Cells (1 x 10^6 cells/ml) labeled with fluorescent probes were prepared as described in the previous section, except that ovalbumin was omitted from the buffer in the final wash. Samples were warmed to room temperature and either immediately digested with Streptomyces griseus protease (500 μg) (Sigma Chemical Co.) or allowed to incubate for 5 min at 4°C and counted or photographed within 5 min. After incubation for 5 min at room temperature, the cells were washed free of growth media with PBS, resuspended with 0.1 ml of the buffer, and fixed for 5 min at 4°C with 0.1 ml of 1% paraformaldehyde. After washing, the cells were incubated for 4°C with 0.1 ml of 0.01 M Tris-HCl, pH 7.4, for 6 h at 37°C. The digested material was loaded onto a Protein A column (Pharmacia Fine Chemicals) in phosphate-buffered saline (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4 [PBS]). Protein-saturated human transferrin (Sigma Chemical Co.) was labeled with 50 μl of tetramethylrhodamine isothiocyanate (2 mg/ml dissolved in acetone) (Nordic Immunology, London) in 1 ml of 1 M sodium bicarbonate at pH 9.5 for 3 h at room temperature. Excess tetramethylrhodamine isothiocyanate (TRITC) was removed by G25 (Pharmacia Fine Chemicals) gel filtration in PBS.

RESULTS

Time Course and Temperature-dependence of the Internalization of Transferrin and Its Receptor

At 4°C, the binding of either TRITC-labeled transferrin (TRITC-transferrin) or the FITC-labeled Fab fragment of the antitransferrin receptor antibody (FITC-Fab antitransferrin receptor antibody) exhibited diffuse membrane fluorescence. The binding was judged to be specific because unlabeled transferrin added in 100-fold excess simultaneously with TRITC-transferrin could abolish the rhodamine fluorescence. Similarly, excess unlabeled antitransferrin receptor antibody added simultaneously with FITC-Fab antitransferrin receptor antibody could abolish the fluorescein fluorescence. Neither ovalbumin nor nonspecific goat IgG had any effect on the cellular fluorescence of FITC-Fab antitransferrin receptor antibody or TRITC-transferrin. When transferred to room temperature, cells incubated with TRITC-transferrin showed a transition from diffuse membrane fluorescence to brightly fluorescent patches (Fig. 1A). After 30 min at room temperature, the diffuse fluorescence diminished with coalescence of patches into aggregates (Fig. 1C). By contrast, cells incubated with FITC-Fab antitransferrin receptor antibody in the absence of transferrin did not show the same pattern of fluorescence. The fluorescence remained diffuse and showed essentially no patching at room temperature (Fig. 1B). Incubation simultaneously with both fluorescent labels showed co-migration, co-patching, and co-coalescing of TRITC-transferrin and FITC-Fab antitransferrin receptor antibody (Figs. 1, C and D). Table I summarizes the percentage of the cells showing these transitions. It was necessary to use a monovalent Fab fragment of the antitransferrin receptor antibody to show this phenomenon. If purified whole IgG from the antiserum was labeled with FITC and incubated with cells, then the same patterns of aggregation were seen with or without transferrin (Fig. 2, A, B, and C).

Glycophorin Does Not Co-migrate with Transferrin and Its Receptor

We wanted to test whether the aggregation of the receptor/transferrin complex is restricted to these two proteins or whether all the proteins on the surface of the K562 cells co-migrate. Cells incubated at room temperature in the presence of TRITC-transferrin and goat antiglycophorin (an antibody to a protein specific for the erythrocyte membrane) and FITC-labeled rabbit anti-goat IgG or all reagents together showed different membrane fluorescence patterns (Fig. 3, A and B). The TRITC-transferrin exhibited patches identical to those seen in Fig. 1. The fluorescence detected with goat antiglycophorin and FITC anti-goat IgG did not coaggregate with the transferrin and remained as diffuse stippled fluorescence.

Susceptibility of TRITC-Transferrin and FITC-Fab Antitransferrin Receptor Antibody Binding to Protease Treatment

To determine whether the patched fluorescence represented internalized ligand and receptor, we tested the susceptibility of the fluorescence of each to proteolysis. When cells were incubated for 15 min at 4°C with either TRITC-transferrin or FITC-Fab antitransferrin receptor antibody, or both labels, then washed and digested for 5 min with protease, no fluorescence could be detected. However, when cells were incubated at room temperature for 15 min so that both diffuse and patched fluorescence were present (Fig. 4, A and B), and then incubated for 5 min with protease, only the diffuse fluorescence was susceptible to proteolytic attack. Thus, the patched fluorescence was unavailable to proteolysis (Fig. 4, C and D).

Fluorescent Patterns of Fixed Cells

The results of the kinetic observations of the unfixed cells indicated that transferrin and the antibody to the transferrin receptor co-migrated to the cell interior, forming a fluorescent aggregate. Because neither of these probes, antibody or transferrin, is covalently bound to the receptor, we wanted to determine whether the receptor was actually located in the same aggregates in which the transferrin migrates or whether the receptor was located elsewhere in the cell. To examine this possibility, cells were preincubated with TRITC-transfer-
Figure 1 Fluorescence microscope photographs of K562 cells showing specific binding of transferrin and antitransferrin receptor. (A) Cells were incubated with TRITC-transferrin (2.5 × 10⁻⁷ M), washed, and incubated for 15 min at room temperature. (B) Cells were incubated with FITC-Fab fragments of goat anti-human transferrin receptor, washed, and incubated for 15 min at room temperature. (C and D) Cells were incubated with FITC-labeled Fab fragments of goat anti-human transferrin receptor and TRITC-transferrin for 30 min at room temperature. The same field was photographed with a filter combination for rhodamine (C) and fluorescein (D) fluorescence. In all cases the cells were initially washed free of culture media, resuspended at 1 × 10⁶ cells/ml in phosphate buffer saline, incubated with the appropriate fluorescent label or labels (5-10 μl) for 15 min at 5°C, pelleted by centrifugation, and resuspended in PBS (10⁷ cells/ml). Slides were made at room temperature and photographs were taken at the times listed in the figures. × 400.

Table 1
Quantification of Changes in Fluorescent Patterns on Cells with Time at Room Temperature

| min | Sample  | % diffuse | % patched | % diffuse | % patched |
|-----|---------|-----------|-----------|-----------|-----------|
|     | + Fab αTR* | 90 | 0 | 73 | 15 |
| 0-2 | + TF     | 72 | 7 | 62 | 10 |
| 5-10| + Both* | 100 | 0 | 13 | 85 |
| 30  | + TF     | 93 | 2 | 6 | 91 |
| 30  | + Both* | 4 | 83 | 0 | 4 | 83 |

The total number of cells in each field were counted under phase-contrast optics. Each sample counted contained between 100 and 200 cells.

* Fab αTR, FITC-labeled Fab fragment of goat anti-human transferrin receptor antibody.

† TF, TRITC-labeled diferric human transferrin.

* Both, both fluorescent reagents added simultaneously.

Using TRITC-transferrin, we studied the process of transferrin binding and internalization in K562 cells. In addition, the
transferrin receptor has been independently observed using an FITC Fab fragment of an anti-human transferrin receptor antibody generated in a goat. In a previous paper we demonstrated that transferrin does not compete with the binding of this antibody to the receptor (16). This enabled us to observe the receptor and transferrin independently and simultaneously. These experiments provide direct evidence for the internalization of transferrin in association with its receptor. van Renswoude et al. (20) have noted that transferrin is internalized in K562 cells. They used fluorescently labeled transferrin and low pH washes to remove surface-bound transferrin. We have shown that, in addition to the transferrin being internalized, the receptor co-internalizes in the same structure.

A summary schematic of the events associated with transferrin internalization is presented in Fig. 6. When transferrin was initially bound at 4°C it exhibited diffuse fluorescence at the cell surface, and, after the cells were brought to room temperature, there were, first, clustering and, then, aggregation of the fluorescence. There was also a loss of susceptibility of the aggregated fluorescence to proteolytic digestion. This is presumably because the transferrin has accumulated within intracellular aggregates.

An interesting finding was the demonstration that the monovalent Fab fragment of the antibody to human transferrin receptor did not internalize and migrate to the cell interior in the absence of transferrin. In the presence of transferrin it co-migrated to the cell interior forming a fluorescent aggregate. Transferrin may cause clustering and internalization of the
FIGURE 4 Fluorescence of cells digested with pronase. (A and B) Cells were simultaneously incubated with TRITC-transferrin and FITC-Fab fragments of anti-human transferrin receptor as described in Fig. 1. The same field was photographed after a 15-min incubation at room temperature under rhodamine (A) and fluorescein (B) fluorescence. (C and D) Cells were simultaneously labeled with TRITC-labeled transferrin and FITC-labeled Fab fragments of anti-human transferrin receptor. After a 15-min incubation at room temperature, cells were digested with Streptomyces griseus protease for 5 min as described in Materials and Methods. Ovalbumin was added, and the cells were spun down and resuspended in PBS and ovalbumin. The same field of cells is shown under rhodamine (C) and fluorescein (D) fluorescence. × 400.
receptor by inducing a conformational change in the receptor molecule. Alternatively, transferrin binding may act to cross-link receptors close together, as a necessary step for internalization. Perhaps, the bifunctional nature of the two ferric ion-binding sites of the transferrin polypeptide chains is related to this cross-linking function. The experiments with the intact IgG preparation of the antibody to the receptor showed internalization without transferrin being present. However, this is still consistent with a requirement for cross-linking of receptors by a binding agent since the IgG is bivalent.

In addition to the time-course studies showing co-migration, we have shown with fixed cells that the receptor for transferrin can be found in the same intracellular aggregate as previously internalized TRITC-transferrin. The internal location of the aggregate was demonstrated by the need to have Triton X-100 in the incubation buffer for the antireceptor antibody to gain access to this structure. This phenomenon was specific to transferrin and its receptor because control experiments using antibody to a different membrane antigen, glycophorin, did not show co-internalization. This observation that transferrin does not cause the aggregation of another major cell surface protein, glycophorin, is in agreement with the experiments of Ciehanover et al. (21), who demonstrated that the internalization and recycling of the asialoglycoprotein receptor in a human hepatoma cell line is independent of the transferrin receptor. Thus it appears to be a very specific process.

We would like to acknowledge the technical assistance of Agnes Tin in cell culture and Lucille Weiss in the preparation of this manuscript. These studies were supported by National Institutes of Health grants CA13533 and CA33096.

Received for publication 8 November 1982, and in revised form 18 April 1983.
CONCLUSIONS

|       | FITC-FabαTR | TRITC-T | FITC-FabαTR + TRITC-T | FITCαGP + TRITC-T |
|-------|-------------|---------|-----------------------|------------------|
| 5°C   | ![image](5C_5min.jpg) | ![image](5C_5min_TRITC.jpg) | ![image](5C_5min_both.jpg) | ![image](5C_5min_GP.jpg) |
| 23°C  | ![image](23C_30min.jpg) | ![image](23C_30min_TRITC.jpg) | ![image](23C_30min_both.jpg) | ![image](23C_30min_GP.jpg) |
| 23°C  | ![image](23C_15min_protease.jpg) | ![image](23C_15min_protease_TRITC.jpg) | ![image](23C_15min_protease_both.jpg) | ![image](23C_15min_protease_GP.jpg) |

Figure 6: Schematic representation of the results of the time course of the effects of antitransferrin receptor and transferrin binding on K562 cells. Transferrin was labeled with tetramethylrhodamine isothiocyanate (TRITC-T). The Fab fragment of the antibody was labeled with fluorescein isothiocyanate (FITC-Fab TR). In the last column, goat antibody to human glycophorin was visualized by fluorescein isothiocyanate-labeled rabbit anti-goat IgG. TRITC fluorescence is denoted by dashes (--) and FITC fluorescence is denoted by dots (.).

REFERENCES

1. Jandl, J. H., and J. H. Katz. 1963. Plasma-to-cell cycle of transferrin. J. Clin. Invest. 42:314-326.
2. Parmley, T. T., F. Ostroy, R. A. Gains, and L. Deluc. 1979. Ferrocyanide staining of transferrin and ferrin-conjugated antibody to transferrin. J. Histochem. Cytochem. 27:681-685.
3. Baker, E., and E. H. Morgan. Kinetics of the interaction between rabbit transferrin and reticulocytes. Biochemistry. 8:1133-1141.
4. Sullivan, A. L., J. A. Graziano, and L. R. Weinstein. 1976. Micropinoctosis of transferrin by developing red cells: an electron-microscopy study utilizing ferritin-conjugated transferrin and ferritin-conjugated antibodies to transferrin. Blood. 47:133-143.
5. Light, A., and E. H. Morgan. 1982. Transferrin endocytosis in reticulocytes. Scand. J. Haematol. 28:205-214.
6. Pearse, B. M. F. 1982. Coated vesicles from human placenta carry ferritin, transferrin and immunoglobulin G. Proc. Natl. Acad. Sci. USA. 79:451-455.
7. Booth, A. C., and M. J. Wilson. 1981. Human placental coated vesicles contain receptor-bound transferrin. Biochem. J. 196:355-362.
8. Octave, N.-Y., Y. J. Schneider, R. R. Chretien, and A. Trouet. 1981. Transferrin uptake by cultured rat embryo fibroblasts. Eur. J. Biochem. 115:611-618.
9. Karna, M., and B. Miezt. 1981. Receptor mediated endocytosis of transferrin in developmentally transform mouse teratocarcinoma stem cells. J. Biol. Chem. 256:3245-3252.
10. Neufeld, E. F., and G. Ashwell. 1980. Carbohydrate recognition systems for receptor-mediated endocytosis. In The Biochemistry of Glycoproteins and Proteoglycans. Carbohydrate Recognition Systems for Receptor-Mediated Pinocytosis. William J. Lenneate. Plenum Publishing Co., NY. 241-266.
11. Willingham, M. C., and I. Pastan. 1980. The receptosome: an intermediate organelle of receptor-mediated endocytosis in cultured fibroblasts. Cell. 12:67-77.
12. Goldstein, J. L., R. G. W. Anderson, and M. S. Brown. 1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. Nature (Lond.). 279:670-684.
13. Pearse, B. M. F., and M. S. Bretsch. 1981. Membrane recycling by coated vesicles. Annu. Rev. Biochem. 50:83-101.
14. Stahl, P. D., and P. H. Schizen. 1980. Receptor-mediated pinocytosis of mannos/N-acetylglycosamine-terminated glycoproteins and lysosomal enzymes by macrophages. Trends Biochem. Sci. 5:194-196.
15. Kaplan, J. 1981. Polyglycine-binding membrane receptors: analysis and classification. Science (Wash. DC). 212:14-20.
16. Enns, A. J. E. Shindelman, S. E. Tonik, and H. H. Sussman. 1981. Radioimmunochemical measurement of the transferrin receptor in human trophoblast and reticulocyte membranes with a specific antireceptor antibody. Proc. Natl. Acad. Sci. USA. 78:4222-4225.
17. Læmmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680-685.
18. Anderson, L. C., K. Nilson, and C. Gahlenberg. 1979. K562—a human erythroleukemic cell line. Int. J. Cancer 23:143-147.
19. McDowell, E. M., and B. F. Trump. 1976. Histologic fixatives suitable for diagnostic light and electron microscopy. Arch. Pathol. Lab. Med. 100:405-414.
20. van Renswoude, J., K. R. Bridges, J. B. Harford, and R. R. Klausner. 1982. Receptor-mediated endocytosis of transferrin and the uptake of Fe in K562 cells: identification of a nonlysosomal acidic compartment. Proc. Natl. Acad. Sci. USA. 77:6186-6190.
21. Cieshanover, A., A. L. Schwartz, and H. F. Lodish. 1983. The asialoglycoprotein receptor internalizes and recycles independently of the transferrin and insulin receptors. Cell. 32:267-275.