Communication

Biosynthetic Regulation of the Human Transferrin Receptor by Desferrioxamine in K562 Cells*

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Treatment of K562 cells with the iron chelator desferrioxamine results in the gradual increase in total cell receptors for transferrin. Receptor number rises 2.5-4.5-fold over 24 h and remains at the elevated level if the chelator is continuously present. Preincubation of the chelator with ferric chloride abolishes the effect. The drug has no effect on the 7-h half-life of the receptor. The increased number of receptors can be accounted for by a specific increase in the rate of receptor biosynthesis which reaches 3-4 times that seen in untreated cells by 6 h after the addition of the chelator. Isolation of mRNA from treated cells reveals that, after 8 h in the presence of desferrioxamine, there is a 3-fold increase in the specific translation of transferrin receptor over untreated cells. Total protein synthesis is not changed under these conditions.

All living cells require iron for cell growth. The delivery of iron into cells is mediated by transferrin, a serum glycoprotein, via specific receptors that reside on the cell surface (1, 2). Following binding of transferrin to its receptor, the bound transferrin is internalized via coated pits and rapidly enters into a non-lysosomal acidic compartment (3-8). It is within this compartment that iron is released from transferrin and, through some as yet understood mechanism, the majority is deposited within cytosolic ferritin. The amount of iron entering a cell could be regulated by altering the number of transferrin receptors. Furthermore, we present evidence, using an in vitro translation assay, that the increased receptor level results from an increase in the level of mRNA for the receptor.

This increase is specific for the receptor in that no change in either the amount or profile of total protein synthesis is observed.

MATERIALS AND METHODS

Chemicals and Cells—Human transferrin was purchased from Calbiochem. Desferrioxamine was obtained from Ciba Geigy. Protein A-Sepharose CL-4B was purchased from Pharmacia. 5-[35S]methionine (1190 Ci/mmol) was from Amersham. Aprotinin and phenylmethylsulfonyl fluoride were obtained from Sigma. Vanadyl ribonucleoside complex was from Bethesda Research Laboratories and rabbit reticulocyte lysate was from Amersham. Oligo(dT)-cellulose was from Bethesda Research Laboratories. Human erythroleukemia K562 cells were grown in RPMI 1640 with 25 mM Heps supplemented with 10% heat-inactivated fetal bovine serum (Gibco Laboratories) and l-glutamine. Cells were maintained in log phase at 2-5 x 10⁵ cells/ml.

Desferrioxamine Induction and Pulse Labeling of Transferrin Receptor—Log phase growing cells (5 x 10⁵ cells/ml) were treated for different lengths of time with 50 μM desferrioxamine at 37 °C. Following the incubations, 10⁶ cells were washed with 5 ml of RPMI 1640 (containing 1.5 mg/liter of methionine), incubated for 15 min in 2 ml of this low methionine medium and then labeled with 200 μCi of [35S]methionine for 15 min in 1 ml of methionine-free RPMI 1640. The cells were then washed three times with cold phosphate-buffered saline before they were lysed. 30-minute pulse labeling was employed for studies of the receptor half-life. Treatment of the cells with 50 μM desferrioxamine had no effect on total protein synthesis or cell growth rate over 48 h.

Immunoprecipitation of Receptor from K562 Cells—Labeled cell pellets were solubilized in Tris-buffered saline containing 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 trypsin inhibitory units/ml of aprotinin (final volume 0.25 ml) for 30 min at 4 °C. After centrifugation for 2 min at 4 °C in an Eppendorf microcentrifuge, 100 μl of the supernatant was removed and mixed with 15 μl of mouse monoclonal anti-transferrin receptor antibody, (OKT9, Ortho Pharmaceutical Corp.) and incubated overnight at 4 °C. Protein A-Sepharose (Pharmacia) was saturated with rabbit anti-mouse IgG by incubation for 1 h at 4 °C. The protein A-Sepharose was then washed and suspended at 0.1 mg/ml, and 100 μl of this suspension was added to the OKT9-receptor complex and tumbled for 1 h at 4 °C. The complexes were centrifuged and washed three times with 1 ml of Tris-buffered saline containing 0.5% deoxycholate (Na), 1% Triton X-100, 1 mg/ml of bovine serum albumin. Proteins were released from the Sepharose pellet by boiling for 10 min in electrophoresis sample buffer.

SDS Polyacrylamide Gel Electrophoresis and Autoradiography—SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli, using 12.5% polyacrylamide gels (20). The gels were fixed and dried for autoradiography after impregnation with 1 M sodium salicylate. Kodak X-Omat film was exposed to the gels for 12-48 h at -70 °C, and the receptor was quantitated using a Zienhe densitometer.

Isolation of Total Cellular RNA—K562 cells were grown to a density of 5 x 10⁵ cells/ml. To ten 100-ml flasks, desferrioxamine was added at a final concentration of 50 μM and incubated for 8 h. A total of 10⁶ cells (control or drug-treated) were pelleted and washed twice with cold phosphate-buffered saline. The cells were homogenized in a Dounce homogenizer (type B) in 10 mM Tris-HCl, pH 7.4, containing 10 mM vanadyl ribonucleoside. After 15 strokes, the homogenate was made isotonic with 10 mM Tris-HCl, pH 7.4, 1.7% NaCl, 0.5 mM MgCl₂, and 10 mM vanadyl ribonucleoside. The homogenate was centrifuged at 2000 rpm to remove nuclei and cell debris. Proteinase K buffer was added to the supernatant to a final concentration of 0.1 M Tris-HCl, pH 7.5, 12.5 mM EDTA, 0.15 M NaCl, and 1% (w/v) SDS. Proteinase K was added to a final concentration of 200 μg/ml and...
incubated for 30 min at 37 °C. The homogenate was then extracted 3 times with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was recovered and 2.5 volumes of ethanol was added and incubated at −20 °C for 4 h. The nucleic acid precipitate was centrifuged at 5000 × g in an HB-5 rotor at 0 °C for 10 min and the pellet was dissolved in 1 ml of buffer containing 50 mM Tris-HCl, pH 7.5, and 1 mM EDTA. MgCl₂ and 50 mM ethylenediamine tetraacetic acid complex were added to a final concentration of 10 mM and 2 mM, respectively. Pancreatic DNase I was added to a final concentration of 10 µg/ml and incubated at 37 °C for 30 min. After the incubation, EDTA and SDS were added to a final concentration of 10 mM and 0.2%, respectively. The solution was then extracted once with an equal volume of phenol:CHCl₃: isoamyl alcohol. 3 M sodium acetate, pH 5.2, was added to the aqueous phase to give a final concentration of 0.3 M and the RNA was precipitated with 2 volumes of absolute ethanol (12).

Selection of Poly(A⁺) mRNA—The RNA precipitate was dissolved in 1 ml of sterile water. The solution was heated to 65 °C for 5 min and cooled rapidly. The solution was then adjusted to final concentrations of 20 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 1 mM EDTA, and 0.1% SDS, and loaded onto an oligo(dT)-cellulose column previously equilibrated with 20 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 1 mM EDTA, and 0.1% SDS. The column was washed with equilibration buffer until the effluent was zero. The poly(A⁺) RNA was eluted with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.05% SDS. Sodium acetate (pH 5.2) was added to a final concentration of 0.3 M and the RNA was precipitated with 2.2 volumes of ethanol at −20 °C. The yield of total RNA and poly(A⁺) RNA was not effected by prior treatment of cells with desferrioxamine (12).

In Vitro Translation and Immunoprecipitation—5 µg each of poly(A⁺) RNA from both control and treated cells were translated in a rabbit reticulocyte lysate according to the manufacturer's instructions containing 100 mM KCl, 50 µM amino acids minus methionine, 50 µCi of [³⁵S]methionine, and 50 units of RNasin. The mixture was incubated at 30 °C for 90 min and immunoprecipitated with polyclonal goat anti-human transferrin receptor (14) by using the immunoprecipitation procedure as described above except that the final wash buffer contained 0.1% SDS. The amount of product translated was linear as a function of mRNA over the range used in this study.

RESULTS

Effect of Desferrioxamine on Transferrin Receptor Number—The effect of desferrioxamine on the number of transferrin receptors was determined. After the drug was added to exponentially growing cells, there was a gradual increase in total number of surface receptors which reached its maximum by about 24 h (Fig. 1). In multiple experiments, the plateau value was between 2.5 and 4.5 times the control value. The drug had no effect on binding affinity. Total cell receptor number was measured using a soluble receptor assay described previously (5). Surface receptors in control cells represent 25–30% of the total receptors. Desferrioxamine resulted in a parallel increase in total receptors and at no point during drug treatment did the ratio of surface to total receptors change. Prolonged incubation of solubilized cells with the drug had no effect on receptor number, thus mitigating against a direct effect of the desferrioxamine on the receptor. The number of surface receptors/cell in the uninjected population was approximately 170,000. In the experiment shown in Fig. 1, the induced cells attain a total receptor number/cell of about 1.5 × 10⁶.

Degradation of the Transferrin Receptor—An alteration in the number of receptors/cell could arise due to changes in biosynthesis, degradation, or both. We examined the rate of degradation of the transferrin receptor in the absence and presence of desferrioxamine. These cells displayed a relatively rapid rate of turnover of the receptor with a half-life of between 7 and 9 h. Incubation of the cells with 50 µM desferrioxamine had no effect on receptor degradation (Fig. 2). Pretreatment of cells with the drug for 8 h before measuring the half-life did not alter the value observed.

Effect of Desferrioxamine on Transferrin Receptor Synthesis—To study the effect of desferrioxamine treatment on transferrin receptor biosynthesis, cells incubated for different lengths of time with the drug were pulsed with [³⁵S]methionine for 15 min and lysed with Triton X-100. Cell extracts were immunoprecipitated with the OKT9 mouse monoclonal antibody against human transferrin receptor. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (Fig. 3). Cells incubated with 50 µM desferrioxamine show an increase of transferrin receptor synthesis as early as 2 h after adding the chelator. The rate of synthesis increases and reaches a peak level of about 3-fold over control synthesis by 6 h after the addition of the desferrioxamine. This increase was specific for transferrin receptor since total protein synthesis, as estimated by the radioactivity incorporated into trichloroacetic acid-precipitable material, obtained from control and induced cell extracts, was not significantly different in an 8-h incubation following desferrioxamine addition. The effect of desferrioxamine could be completely abolished by saturating the chelator with iron. Furthermore, the membrane-impermeable iron chelator, diethylentriaminopentacetic acid (13), fails to increase either receptor number of receptor biosynthesis (data not shown). This suggests that intracellular iron must be chelated in order to affect receptor biosynthesis.

In Vitro Translation of mRNA for the Transferrin Receptor—5 µg of poly(A⁺) mRNA from K562 cells were translated in a rabbit reticulocyte lysate at 30 °C for 90 min. The translated products were immunoprecipitated with OKT9 as well as with polyclonal antibodies directed against purified transferrin receptor (14). The only protein precipitated from metabolically labeled K562 cells by either of these antibodies is the transferrin receptor (data not shown). As seen in Figure 4A, the polyclonal antibody was able to immunoprecipitate a protein of 80,000 daltons, whereas this (or other) monoclonal antibody was unable to specifically recognize any translated product. This molecular weight agrees with a recent report of the in vitro translation and immunoprecipitation of the transferrin receptor using a different receptor-specific polyclonal
antibody (15). Lane 5 of Fig. 4A depicts the core-glycosylated receptor that has been metabolically labeled in K562 cells.

In an attempt to rule out the possibility that the 80,000-dalton protein was transferrin, the translated product was pretreated with rabbit anti-transferrin antibody followed by the addition of protein A-Sepharose. The supernatant was then immunoprecipitated with the goat anti-receptor antibody. As seen in Fig. 4B, the 80,000-dalton protein was immunoprecipitated with anti-receptor antibodies clearly showing that it was indeed the transferrin receptor. Titration of the antibody demonstrated that the precipitations shown were performed using a large excess of anti-receptor activity.

In order to determine if the desferrioxamine induction of transferrin receptor biosynthesis was expressed at the level of translatable message, we isolated poly(A) mRNA from both uninduced and induced K562 cells. Five μg of RNA of each of the RNA preparations were translated, the synthesized proteins were immunoprecipitated, and the specific receptor bands were scanned in a densitometer. As seen in Fig. 4C, for the same amount of total poly(A) RNA, the induced cells direct three times the amount of receptor synthesis as compared with the RNA from the uninduced cells. This correlates with the observed 3-fold induction in biosynthesis of receptor when cells are metabolically labeled with [35S]methionine after treatment with desferrioxamine for 8 h. Total in vitro protein synthesis directed by mRNA from treated or untreated cells was identical. For both control and induced mRNA, translation yielded approximately 500,000 cpm of trichloroacetic acid-precipitable [35S]methionine/μg of mRNA. Furthermore, densitometer scanning of identical but undefined proteins other than the receptor showed no increased band other than the receptor in translations directed by mRNA from induced cells. Both of these observations point to the specificity of the increment in receptor mRNA levels. The translation of total product and of immunoprecipitable transferrin receptor was linear with concentration of added mRNA in the range used for all of the experiments reported here.

**DISCUSSION**

Under physiological conditions, most of the iron that cells require for growth is delivered by transferrin. Iron-saturated transferrin, bound to specific cell surface receptors, is internalized in endocytic vesicles. Upon acidification of the endosome, Fe3+ is released while receptor and apotransferrin cycle back to the cell surface (3–8). The majority of iron delivered in this way is ultimately stored in intracellular ferritin (6), but the steps involved between the release from transferrin and the deposition into ferritin are not known (1).

Interrupting the normal intracellular iron balance by the iron chelator, desferrioxamine, results in an up-regulation of transferrin receptors. Both surface and total receptor number rises over 24 h and then remain at a level 2.5–4.5 times the control as long as the chelator is present. In this study, we
The inhibition of the desferrioxamine effect by iron is consistent with the hypothesis that it induces receptor synthesis by sequestering cellular iron. Over the time course reported here, we observe no decrease in cellular iron after addition of desferrioxamine (data not shown). Thus, the effect of the chelator is likely to be the result of sequestration of an intracellular iron pool which regulates the expression of the receptor gene.

The size and nature of the regulatory iron pool remain to be determined. The regulation of transferrin receptor expression by iron is analogous to the regulation of low density lipoprotein receptors by cholesterol (10). Elucidating the molecular mechanisms underlying these regulatory influences will be a major goal of future research into the molecular biology of receptors. Other factors regulate the expression of the transferrin receptor, including cell proliferation and mitogens (16–19). It is tempting to speculate that intracellular iron may be involved in mediating regulation of expression of the receptor in these circumstances as well. Answers to these questions will require study of the gene for this receptor and examination of its regulatory elements.

REFERENCES

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