Alterations in the Transferrin Receptor of Human Erythroleukemic Cells after Induction of Hemoglobin Synthesis*

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When K562 human erythroleukemic cells are induced to differentiate by addition of hemin to their medium, the number of binding sites for transferrin on the cell surface is substantially reduced. This reflects an internalization of receptors since no such reduction is observed when the total binding sites in soluble extracts of uninduced and differentiating cells are compared. The internalization of transferrin receptors has also been shown using lactoperoxidase-mediated radiodination of cell surfaces and by immune precipitation of total and surface labeled receptors using an anti-receptor monoclonal antibody. Transferrin receptors from uninduced and differentiating cells were partially purified by affinity chromatography on transferrin-Sepharose and shown to be disulfide-bridged homodimers of a polypeptide with an apparent molecular weight of approximately 80,000. This protein is a phosphorylated polypeptide that can be resolved by isoelectric focusing into three major and two minor forms. By digestion of phosphorylated polypeptides, it has also been shown using lactoperoxidase-mediated radiodination of cell surfaces and by immune precipitation of total and surface labeled receptors using an anti-receptor monoclonal antibody, that at least four of these forms are probably phosphorylation variants of a single polypeptide. As differentiation proceeds, the proportions of the individual forms of the receptor change with a shift to the more phosphorylated polypeptides.

EXPERIMENTAL PROCEDURES

Materials

Cyanogen bromide-activated Sepharose, phosphoamino acids, polyethylene glycol, lactoperoxidase, glucose oxidase, and neuraminidase were obtained from Sigma; human transferrin was obtained from Miles laboratories, and bacterial alkaline phosphatase from Worthington. Polycel 400 cellulose thin-layer plates were obtained from Brinkmann Instruments. All other materials were of reagent grade and the highest quality commercially available.

Methods

Cell Culture—Human erythroleukemic K562 cells were cultured and induced to differentiate as previously described (5).

Radiodination of Transferrin—Transferrin was radiodinated using chloramine T. One mg of transferrin was dissolved in 1 ml of 0.1 M sodium phosphate buffer (pH 7.5) and incubated with 50 μl of a 1 mg of chloramine T/ml solution in water. After 5 min at 4 °C, 50 μl of 2 mg of sodium bisulfite/ml and 50 μl of 0.1 M potassium iodide were added, and the solution was applied to a Sephadex G-50 column equilibrated in 0.1 M sodium phosphate buffer (pH 7.5) containing 1 mg of BSA/ml and 1 mg of sodium azide/ml. The column was eluted using the same buffer, and the eluate was monitored by gel counting.

Estimation of Cell-surface Binding Sites—10^6 washed cells in PBS were incubated at 4 °C to inhibit endocytosis for 90 min with 100 ng to 15 pg of 125I-transferrin and various concentrations (100 ng to 15 μg) of unlabeled transferrin in a total volume of 80 μl. Control experiments showed that saturation of receptors was achieved under these conditions. The cell suspension was then laid over 150 μl of 15% sucrose and centrifuged in a 400-μl tube in a Beckman microfuge.

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The abbreviations used are: NP40, Nonidet P-40; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PBS, Dulbecco's phosphate-buffered saline.
The tube was frozen in liquid nitrogen, and the tip of the tube containing the cell pellet was cut off. The radioactivity in the pellet and the supernatant fraction was determined by γ counting. All incubations were carried out in duplicate and were corrected for nonspecific background binding by subtracting the radioactivity bound in similar incubations by cells preincubated in PBS containing 200 pg of transferrin/ml.

The binding was analyzed using Scatchard plots (6) and the best straight line fitted to the binding data using linear regression analysis and the PROPHET computer.

**Analysis of Total Cell Receptors**—Cells were dissolved in PBS containing 0.1% Triton X-100, 1 mg of BSA/ml, and 1 mM phenylmethylsulfonyl fluoride and centrifuged to remove insoluble material. Extract equivalent to 10⁶ cells in 80 μl was incubated with 100 ng of ¹²⁵I-transferrin and various concentrations of unlabeled transferrin as described above. After 90 min, 800 μl of 12% (w/v) polyethylene glycol (approximate molecular weight of 6000) was added in 0.1 M Tris citrate (pH 5) containing 0.1% Triton X-100 and 1 mg of BSA/ml and incubated at 4 °C for 30 min. The precipitated receptor-transferrin complex was recovered by filtration through Millipore filters (0.45-pm pore size) presaturated with BSA and washed with Tris citrate/Triton X-100/BSA solution. Bound radioactivity was corrected for nonspecific binding in similar incubations in which 100 μg of transferrin were included.

**Affinity Chromatography**—Cyanogen bromide-Sepharose was coupled to human transferrin or BSA as previously described (7). The column (3-ml affinity matrix) was washed with 5 column volumes of extraction buffer containing 0.1 M Tris-HCl (pH 7.4) containing 0.5 mM Tris/200 pg of transferrin/ml and 1 mM phenylmethylsulfonyl fluoride and then in 2 volumes of extraction buffer containing 1 mM ferric ammonium citrate to saturate the transferrin with iron. Cell extracts dissolved in the extraction buffer were passed over the column which was then washed thoroughly (5 column volumes) with extraction buffer followed by wash buffer (50 mM Tris-HCl (pH 7.4) containing 0.5% NP40). The radioactivity was monitored until no more radioactivity was eluted from the column, after which the specifically bound transferrin receptor was eluted from the affinity column using 0.2 M glycine/HC1 (pH 2.3). The pH of the eluted receptor was immediately returned to neutrality using 1 M Tris. The material that did not bind to the affinity matrix was passed again through an affinity column to ensure as complete binding of the transferrin receptor as possible. The neutralized glycine/HC1-eluted fractions were reapplied to a transferrin-Sepharose column. The column was washed and eluted as before.

The contents of the eluted fractions from the second column were monitored by SDS-polyacrylamide gel electrophoresis.

**Preparation of Radioactively Labeled Cell Extracts**—K562 cells (uninduced or induced to differentiate) were washed in methionine-free medium and grown for a further 24 h (greater than one generation time) in the deficient medium containing 12 μCi of [³⁵S]methionine/ml. The cells of 50 ml of cells were harvested five times in PBS, and the specific radioactivity was checked by scintillation counting to ensure that the incorporated radioactivity/cell was equal when control and differentiating cells were to be compared. This was usually the case since the growth rate of noninduced and differentiating cells were similar over the 96-h differentiation period (3). 30 washed cells were dissolved in 1 ml of extraction buffer at 4 °C and the solution was centrifuged at 20,000 rpm in an SW 50.1 rotor for 30 min at 4 °C. Again, the specific radioactivity was monitored and equal amounts of radioactivity were applied to the affinity column or were immunoprecipitated.

**Labeling of the External Surface of Cells**—Equal numbers of noninduced and differentiating cells were labeled at 4 °C using lactoperoxidase-catalyzed iodination as previously described (8).

**Immune Precipitation**—Cell extracts, prepared as described above, were preadsorbed with Panobin (formalin-fixed Staphylocoecus aureus (Cowan strain)) and then mixed with various amounts (see figure legends) of B3/25 monoclonal antibody against the transferrin receptor (diluted 1:250) and incubated for 1 h at 4 °C. Rabbit anti-mouse IgG (10 μl of a 1:50 dilution) was then added, and the incubation was continued for another hour. Thirty μl of a 10% suspension of Panobin were added, and after another hour, the Panobin was removed in an Eppendorf microfuge. The immune complex was washed five times in 50 mM Tris-HCl (pH 7.4) containing 0.5% NP40 before dissolving the material for electrophoresis.

**Enzymic Digestion of the Transferrin Receptor**—[³⁵S]Methionine-labeled affinity-purified preparations of the transferrin receptor in 50 mM Tris-HCl (pH 7.4) containing 10 mM MgCl₂ were incubated for 1 h at 37 °C with 12-25 units of bacterial alkaline phosphatase/ml or 0.25-1 unit of neuraminidase/ml. The digestion was terminated by heating the mixture of 100 °C for 2 min, followed by preparation of the sample for two-dimensional, isoelectric SDS-gel electrophoresis. Complete digestion was assumed to have taken place when no further change in the isoelectric points of the receptor components occurred at a higher concentration of the digesting enzyme. The enzymes were checked for proteolytic activity against the transferrin receptor by analysis of the digests on single-dimension SDS-polyacrylamide gels followed by autoradiography. No change in the apparent radioactivity of the 90-kilodalton receptor was observed. Only minor changes in molecular weight occurred, compatible with the loss of phosphate or neuraminic acid.

**Polyacrylamide Gel Electrophoresis**—For single-dimensional separations, 10% polyacrylamide-SDS gels were employed as previously described (9) using the Laemmli (10) buffer system. Two-dimensional O’Farrell (11) gels, in which the proteins were first separated by isoelectric focusing and then in the second dimension by SDS-polyacrylamide gel electrophoresis, were prepared and used as previously described (9). The pH profile along the isoelectric-focusing gel was determined using a Bio-Rad microelectrode. To determine the molecular weight of the transferrin receptor components under nonreducing and reducing conditions, another two-dimensional system was used. First, the proteins, dissolved in Tris-buffered (pH 8.3) SDS without reducing agent but containing 50 μg of 5,5'-dithiobis-(2-nitrobenzoic acid) per ml, were separated on the 90-kilodalton gel. These gels were then soaked in SDS/Tris sample buffer containing 100 mM dithiothreitol and laid horizontally on the stacking gel of a 10% polyacrylamide slab gel prior to electrophoresis in the second dimension.

Gels were stained in 0.25% Coomassie Blue, destained, and dried. Autoradiographs were prepared by treating the gel with either EN'FANCE or 0.25 mM salicylic acid, a modification of the procedure in Ref. 12, and exposing to Kodak X-AR-5 film. For autoradiographs, intensifying screens were not used.

**Western Blotting**—Cell fractions or purified receptor preparations were analyzed by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting (13). The replicas were incubated with 3% BSA in solution A (50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and 0.1% Triton X-100) to block protein-binding sites and then sequentially in solution A containing B3/25 monoclonal antibody against transferrin receptor (for 2 h), solution A (two washes), rabbit anti-mouse IgG in solution A (for 2 h), solution A (four washes), and ¹²⁵I-Protein A in solution A (for 1 h). After extensive washing in solution A, the nitrocellulose paper was autoradiographed.

**RESULTS**

**Binding of Transferrin to the Surfaces of Uninduced K562 Cells and Those Induced to Synthesize Hemoglobin**—K562 cells were subcultured into RPMI 1640 medium, and at 72, 48, and 24 h prior to harvest, the medium of individual bottles of cells was supplemented with 0.05 mM hemin to induce differentiation. At the end of the incubation period, all cell cultures, whether induced or not, were in log phase of growth. Binding of ¹²⁵I-transferrin to these cells at 4 °C, to inhibit endocytosis, followed by Scatchard plot analysis of the binding data showed that by 24 h after the addition of hemin to the medium, the number of surface binding sites was dramatically reduced to approximately 25% of the binding sites on uninduced cells. This lower level of surface receptors remained relatively constant at least until 72 h after induction of differentiation. The remaining receptors were of higher affinity than those of uninduced cells (Fig. 1 and Table I).

The reduction in surface receptors in the presence of hemin is not the result of a change in cellular proliferation rate, a factor known to alter transferrin receptor levels (14, 15), since all cultures used in these experiments were in log phase of growth. When cells were grown under similar conditions for 120 h in the absence of hemin, at which time they were no
The activity was determined by incubating with 100 pg of transferrin/assay. The data were analyzed in duplicate with various concentrations of transferrin (Tf) and 100 ng of [¹²⁵I]-transferrin free in reaction mixture. The cells were then separated from unbound ligand by centrifugation through 15% sucrose solution, and the bound and unbound radioactivity was determined by γ counting. The data shown are the mean of duplicate determinations corrected for nonspecific binding in parallel duplicate incubations in which whole cells were supplemented with 16 μg of unlabeled transferrin/assay or soluble extracts were supplemented with 100 μg of transferrin/assay. The data were analyzed using Scatchard plots and the best straight line fitted using linear regression analysis. All cells with the exception of those grown for 120 h without hemin were in log phase of growth. Values in parentheses were arbitrarily set at 100%.

**TABLE I**

| Growth conditions | Surface Total | Compared to | Non-differentiating log-phase cells<sup>a</sup> | External | Non-differentiating log-phase cells<sup>a</sup> | External |
|-------------------|---------------|-------------|---------------------------------|----------|---------------------------------|----------|
|                   | Surface Total | %           | %                               | %        | %                               | %        |
| **pote**<sup>b</sup> | 0.34 0.63 (100) | (100) | 55 |
| No hemin, 72 h    | No hemin, 120 h | 0.19 ND<sup>a</sup> | 56 | ND |
| Hemin, 24 h      | 0.09 0.79 | 26 | 126 | 11.4 |
| Hemin, 48 h      | 0.07 0.88 | 21 | 141 | 8.0 |
| Hemin, 72 h      | 0.10 0.8 | 29 | 130 | 12.3 |

<sup>a</sup> No hemin, 72 h growth.

<sup>b</sup> ND, not determined.

longer actively proliferating, the number of receptors was reduced compared to actively proliferating cells, but to a much smaller extent than differentiating cells (Table I).

**Total Receptors in Uninduced and Induced Cells**—The dramatic fall in surface transferrin receptors soon after the onset of K562 cell differentiation could result from either a reduction in the total number of receptors in the cell or from the sequestration of hitherto surface receptors to a location within the cell. In order to distinguish these possibilities, the number of binding sites was also measured in detergent extracts of cells at various stages of differentiation. These studies showed that the total number of transferrin-binding sites is not reduced during differentiation; in fact, a slight increase in receptor number was consistently found (Fig. 2 and Table I), suggesting that the induction of hemoglobin synthesis was accompanied by internalization of transferrin receptors rather than loss from the cell.

**Purification of Transferrin Receptors**—In order to determine whether any change occurs in transferrin receptors as a result of the internalization that accompanies differentiation, it was necessary to purify receptors from K562 cells, and this was carried out using affinity chromatography on transferrin-Sepharose columns.

K562 cells were metabolically labeled for in excess of one generation time using [⁵⁷⁷³]methionine and were dissolved in an NP40-containing buffer supplemented with BSA. Approximately 65% of the trichloroacetic-acid-precipitable [⁵⁷⁷³]methionine was contained in the soluble fraction. This material was applied to a transferrin-Sepharose column, eluted with glycine/HCl buffer at pH 2.3, neutralized, applied to a second transferrin-Sepharose column, and again eluted and neutralized. Of the total soluble cellular proteins, 0.6% was eluted at pH 2.3 from the second column (Table II). The total NP40-soluble extract, the material that did not adhere to the first affinity column, and the material that eluted from the second affinity column were assayed for transferrin binding activity.
the extract was then applied to a Sepharose affinity column, the washed in extraction buffer. When no more protein was washed from nonbound material was allowed to pass through, and the column was washed in extraction buffer. When no more protein was washed from nonbound material was allowed to pass through, and the column was washed and eluted in the same way. To ascertain the specificity of glycine/HCl at pH 2.3. The eluted material was neutralized and reapplied to a second transferrin (Tf-Sepharose column which was washed and eluted in the same way. To ascertain the specificity of binding to the transferrin-Sepharose affinity column, a second aliquot of the detergent extract of K562 cells was applied to a transferrin-Sepharose column twice in the above manner after addition of transferrin to a final concentration of 0.5 mg/ml, and a further fraction was applied twice to a BSA-Sepharose column. Transferrin binding assays were carried out on total cell extracts, the nonbound material from the first column, and the material specifically eluted from the second affinity column. The binding data were analyzed using Scatchard plots. Protein was estimated by Bradford’s method (34) in assays were carried out on total cell extracts, the nonbound material from the first column, and the material specifically eluted from the second affinity column. The binding data were analyzed using Scatchard plots. Protein was estimated by Bradford’s method (34) in fractions where sufficient amounts were available and by recovery of [35S]methionine in the glycine-eluted fractions assuming a constant specific activity of [35S]methionine in all fractions.

| Sample                          | Tf binding | Total yield of binding activity | Yield of protein |
|---------------------------------|------------|---------------------------------|-----------------|
| Total soluble cell extract      | 4.8        | 39.2 (100%)                     | 8.2 (100%)      |
| Material not binding to first Tf-Sepharose column | 0.7        | 4.9 (12.4%)                     | 7.3 (89%)       |
| Material eluted from second Tf-Sepharose column | 453.3      | 27.9 (71.2%)                    | 0.06 (0.7%)     |
| Material eluted from second Tf-Sepharose column; sample preincubated with Tf | 0          | 0                               | 0.05 (0.6%)     |
| Material eluted from second BSA-Sepharose column | 0          | 0                               | 0.05 (0.5%)     |

Purification of transferrin receptors from K562 cells

Transferrin receptors were purified from [35S]methionine-labeled K562 cells by dissolving washed cells in NP40-containing extraction buffer and removing undissolved material by centrifugation. Part of the extract was then applied to a Sepharose affinity column, the nonbound material was allowed to pass through, and the column was washed in extraction buffer. When no more protein was washed from the column, the specifically bound material was eluted using 0.2 M glycine/HCl at pH 2.3. The eluted material was neutralized and reapplied to a second transferrin-Tf-Sepharose column which was washed and eluted in the same way. To ascertain the specificity of binding to the transferrin-Sepharose affinity column, a second aliquot of the detergent extract of K562 cells was applied to a transferrin-Sepharose column twice in the above manner after addition of transferrin to a final concentration of 0.5 mg/ml, and a further fraction was applied twice to a BSA-Sepharose column. Transferrin binding assays were carried out on total cell extracts, the nonbound material from the first column, and the material specifically eluted from the second affinity column. The binding data were analyzed using Scatchard plots. Protein was estimated by Bradford’s method (34) in fractions where sufficient amounts were available and by recovery of [35S]methionine in the glycine-eluted fractions assuming a constant specific activity of [35S]methionine in all fractions.

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| Material eluted from second Tf-Sepharose column; sample preincubated with Tf | 0          | 0                               | 0.05 (0.6%)     |
| Material eluted from second BSA-Sepharose column | 0          | 0                               | 0.05 (0.5%)     |

Using [35S]methionine and Scatchard plot analysis. Of the total binding sites applied to the first column, 12% did not adhere, while 71% eluted from the second column (Table II). Thus, the major part of the recovered binding activity was retained by the first and second column at neutral pH and eluted at pH 2.3. In fact, it is likely that more than 71% of the binding protein is bound and eluted specifically from the column since two cycles of pH 2.3 treatment are likely to inactivate binding sites in this fraction preferentially.

To test the specificity of the transferrin-Sepharose affinity columns, cell extracts were also applied to a BSA-Sepharose column or were presaturated with 50 μg of transferrin/ml prior to application to a transferrin-Sepharose column. In each case, a small amount of protein was eluted from the column, rebound at neutral pH, and again eluted on lowering the pH. Soluble transferrin-binding site assays of the eluted fractions, however, showed no activity in these fractions (Table II). The transferrin-Sepharose affinity column, therefore, retains the binding sites specifically by virtue of their interaction with immobilized transferrin.

Characterization of Transferrin Receptors of K562 Cells—K562 cells were labeled metabolically with [35S]methionine or exclusively on their external surfaces using lactoperoxidase-catalyzed iodination, and transferrin receptors were then isolated by affinity chromatography on transferrin-Sepharose columns; The preparations were then analyzed by SDS-polyacrylamide gel electrophoresis under either nonreducing or reducing conditions, that is, in the absence or presence of dithiothreitol. The iodinated transferrin receptors have an apparent molecular mass of approximately 90 kDa (Fig. 3, lane D), in accord with the receptors of other cell types (16, 17). The [35S]methionine metabolically labeled receptor preparation also contained a major 90-kDa protein plus a protein of 45-kDa and a less intense band at 76 kDa, together with some minor components (Fig. 3, lane B). Under nonreducing conditions, iodinated receptor preparations contain a prominent band with the apparent molecular weight of the receptor dimer (180,000) and a minor band, the proportion of which varied considerably from experiment to experiment, with an apparent size of 230 kDa (Fig. 3, lane C). Similar proteins are seen using [35S]methionine-labeled receptor preparations (Fig. 3, lane A) together with minor contaminants. Much of the 230- and 180-kDa proteins does not enter the stacking gel under nonreducing conditions, accounting for the relative prominence of the minor contaminants in this gel. Although the nonreduced protein of the size of the homodimer was expected, the reason for the presence of the larger protein on nonreducing gels was obscure. That this is also a complex that gives rise to monomers of 90 kDa was shown using a two-dimensional polyacrylamide gel system in which the proteins were first separated under nonreducing conditions and then in the second dimension under reducing conditions. Fig. 4 shows that the 230-, 180-, and 90-kDa proteins and a protein that migrates with an apparent molecular mass of 115 kDa under nonreducing conditions all migrate with an apparent size of 90 kDa under reducing conditions. Using [35S]methionine metabolically labeled receptor preparations, it could be shown that no additional major protein was associated with the 90-kDa protein in the 230-kDa complex (data not shown).
transferrin receptors were isolated from lactoperoxidase-iodinated K562 cells by affinity chromatography on transferrin-Sepharose columns. The labeled receptors were then separated under conditions that did not cleave disulfide bonds using tube 10% polyacrylamide containing solution that partially cleaved disulfide bonds and was laid on the top of a 10% polyacrylamide slab gel. The proteins were then separated in the vertical dimension. The gels. The first-dimension gel was then soaked in a dithiothreitol-

K562 cells by affinity chromatography on transferrin-Sepharose col-

ular mass and isoelectric point on two-dimensional O'Farrell di-

appendages. All of the components migrate in both dimensions with a molecular mass of 90 kDa.

It is, therefore, likely that the 230-kDa protein is also a dimer of the 90-kDa protein, but that it has different hydrodynamic characteristics from the 180-kDa dimer under nonreducing conditions that result from different disulfide-bridging configurations. If this is the case with the transferrin receptor of K562 cells, it reflects a similar situation to that found with the insulin receptor of a different cell type which also exhibits different hydrodynamic forms of the same polypeptide that are dependent on disulfide bridging (18).

The Identity of the 45- and 76-kDa Proteins—In addition to the 90-kDa receptor, after two rounds of binding to the affinity column, the receptor preparation contains two major additional proteins. The 45-kDa protein has the same molecular mass and isoelectric point on two-dimensional O'Farrell (11) polyacrylamide gels as actin.

The possibility that the 76-kDa protein was a precursor or degradation product of the 90-kDa protein was investigated using a monoclonal antibody that specifically interacts with human transferrin receptor (19). Cell extracts and preparations of transferrin receptors were analyzed by SDS-polya-

crylamide gel electrophoresis and transferred to nitrocellulose paper by electroblotting. The nitrocellulose paper was then incubated sequentially with B3/25 anti-transferrin receptor mouse IgG, rabbit anti-mouse IgG, and 125I-protein A. Fig. 5 shows that the 90-kDa, but not the 76- or 45-kDa polypeptide, binds the antibody specifically, further confirming the identification of the 90-kDa protein as the transferrin receptor.

The failure of the 76-kDa protein to bind to the transferrin receptor antibody argues against it being a precursor of the 90-kDa protein and also against it being a degradation product, although it is possible that it could have lost the recognition site for B3/25 antibody. To rule out the possibility of degradation further, the 90, 76, and 45-kDa bands were excised from polyacrylamide gels, radioiodinated, and subjected to complete trypic digestion using the procedure of Elder et al. (20). The peptides were then separated by two-dimensional thin-layer electrophoresis and chromatog-

raphy (20). No similarity was detected between the three proteins (not shown), suggesting that they are indeed not related.
In Fig. 5 (lanes 9 and 10), it can be seen that affinity chromatography on transferrin-Sepharose in the presence of free transferrin or on BSA-Sepharose not only fails to isolate the 90-kDa receptor but also fails to bind actin and the 76-kDa protein. Thus, the latter seem not to be contaminants that nonspecifically bind to and elute from the transferrin-Sepharose column, but appear to interact with the column via the transferrin receptor. It is possible that the receptor interacts with actin of the cytoskeleton in a transmembrane manner.

The observation that no stainable (Fig. 5, lane 9) or $[^{35}S]$ methionine-labeled (not shown) protein of 90 kDa binds to the transferrin-Sepharose affinity column when the cell extract is supplemented with free transferrin suggests that the band of 90 kDa is relatively pure receptor, and although on high-resolution two-dimensional gel electrophoresis (11), this band separates into five forms of similar isoelectric points, four of these are interconvertible and probably phosphorylation variants of one another (see below). No other major spot migrates with a molecular mass of 90 kDa on these gels.

Alterations in the Transferrin Receptor after Induction of Hemoglobin Synthesis—In order to confirm the results obtained using $[^{125}I]$-transferrin binding, K562 cells were grown under conditions that either did or did not promote hemoglobin synthesis, that is in the presence or absence of 0.5 mM hemin, and were either labeled metabolically or labeled exclusively on their external surfaces; transferrin receptors were then detected by affinity chromatography on transferrin-Sepharose columns or by immune precipitation using anti-receptor monoclonal (B3/25) antibody.

Equal numbers of noninduced cells and cells that had been induced to synthesize hemoglobin by growth in hemin-containing medium for 3 days were labeled metabolically to the same specific radioactivity by growth for a further 24 h in $[^{35}S]$methionine-containing medium, washed to remove endogenous transferrin, and then dissolved in extraction buffer. The transferrin receptors were then isolated by affinity chromatography. Fig. 6, lanes A and B, shows that a similar amount of radioactive receptor was isolated from either uninduced or differentiating cells, again suggesting that induction of erythroid differentiation has essentially no effect on the total amount of receptor present in the cells. In contrast, when external receptors were iodinated in equal numbers of cells, considerably more radioactive receptor was isolated from noninduced than from induced cells (Fig. 6, lanes C and D). In both the metabolically labeled and externally labeled cells, the total radioactivity/cell was similar regardless of their differentiation state. These experiments confirm the binding studies in suggesting that the amount of receptor does not change with differentiation, the amount of surface receptor is reduced when globin synthesis is induced.

The affinity chromatographic purification of transferrin receptors and the transferrin binding studies measure transferrin-binding sites, and it is possible that differences in transferrin binding might reflect changes in the receptor other than internalization; for example, there might be a differentiation-dependent conformational change in the surface receptor that reduces binding, or endogenous transferrin might block the binding of exogenous ligand despite the washing of the cells. To confirm the relative amounts of surface and total receptor in uninduced and differentiating cells, immune precipitation of surface or metabolically labeled cells by B3/25 anti-transferrin receptor antibody was used. This antibody has been shown to bind to the receptor at a site other than the transferrin-binding site and, therefore, not to compete with transferrin for binding to the receptor (19).

Induced or uninduced cells were labeled metabolically by growth in $[^{35}S]$methionine-containing medium or externally using Na$^{125}$I and lactoperoxidase-catalyzed iodination and were then dissolved in NP40-containing buffer. Again, $[^{35}S]$ methionine-labeled immune precipitates showed little difference between the amount of radiolabeled receptor before and after induction (Fig. 7B), while the amount of surface iodinated receptor was appreciably reduced after the onset of hemoglobin synthesis (Fig. 7A).

Five Forms of Transferrin Receptor Exist and They Are Altered in Their Relative Amounts on Induction of Globin Synthesis—Transferrin receptors were isolated from uninduced and induced K562 cells that were metabolically labeled with $[^{35}S]$methionine. The receptor preparations were then analyzed using two-dimensional polyacrylamide gel electrophoresis. Nondifferentiating cells contain five transferrin receptor spots (1–5 with increasing isoelectric point in Fig. 8), some with a slightly different mobility in the second dimension; that is, some forms have a slightly different molecular weight from others. The isoelectric points of the five forms are: 1, 5.15; 2, 5.23; 3, 5.30; 4, 5.35; and 5, 5.45. The acidic isoelectric points of these forms of the receptor are very similar to that of the single transferrin receptor species of Molt-4 cells, a line derived from a T-cell acute leukemia (21).
beled cells were then subjected to immune precipitation using an either metabolically with [%]methionine under conditions that did or did not induce differentiation and labeled uninduced and differentiating cells using lactoperoxidase-catalyzed iodination. The dissolved labeled extracts were used, and the proteins were labeled to the same specific radioactivity. Lanes 1 and 6, 100 µl of diluted antibody; lanes 2 and 7, 50 µl of diluted antibody; lanes 3 and 8, 10 µl of diluted antibody; lanes 4 and 9, 5 µl of diluted antibody; lanes 5 and 10, no antibody. B, immune precipitation of receptor from metabolically labeled uninduced (lanes 1–3) and differentiating (lanes 4–6) cells. In each incubation, equal amounts of labeled extract were used. Lanes 1 and 4, 100 µl of diluted antibody; lanes 2 and 5, 50 µl of diluted antibody; lanes 3 and 6, no antibody.

In this case, the pl was approximately 5.2. Three forms (2–4 in Fig. 8A) are present in approximately equal amounts, the other two (1 and 5) are present to a lesser degree. One day after addition of hemin to the cells, form 3 is the most abundant form (Fig. 8B), which is also the case on day 2 (Fig. 8C). After differentiation has proceeded for 4 days (Fig. 8D), two forms of the receptor predominate; forms 2 and 3 are equally intense with form 4 now less intensely labeled and of similar intensity to 1 and 5.

All Major Forms of the Receptor Are Present at the Cell Surface—In order to determine which of the five forms of the receptor were exposed at the cell surface and whether any might be internal and possibly precursors of the external receptor, uninduced and differentiating cells were labeled using lactoperoxidase-catalyzed iodination. In this case, the total cellular proteins were analyzed using two-dimensional electrophoresis without prior affinity chromatography. Prior fractionation was unnecessary because it was possible to recognize the transferrin receptor proteins on two-dimensional gels in which only labeled surface proteins were analyzed. In Fig. 9, it can be seen that all forms of the receptor in uninduced cells become radioactively labeled; again forms 2–4 are the predominant species and are labeled to approximately equal intensity. Fig. 9 shows that less radioactivity is incorporated into the receptors of induced cells when an equal number of cells is labeled, confirming the previous observation that fewer receptor molecules are accessible to the labeling reagents at the surface after the onset of differentiation. As with the results after metabolic labeling, two forms (2 and 3) again predominate in the differentiating cells. In each part of Fig. 9, an inset in the bottom left corner shows the results of a different experiment in which the autoradiograms have been exposed so that the major spots were of approximately equal intensities. Again, the change from three major forms to two is evident.

The Transferrin Receptor Contains Both Neuraminic Acid and Phosphate—The five forms of the transferrin receptor are separable on the basis of their different isoelectric points. It is possible that these are the products of different genes, are post-translational modifications of the product of one gene, or possibly both explanations may be applicable. To determine whether the different forms might be phosphorylated variants of the same protein, [35S]methionine-labeled transferrin receptor preparations were incubated with bacterial alkaline phosphatase. This resulted in the conver-
FIG. 9. Two-dimensional gel electrophoresis of lactoperoxidase-iodinated K562 cells. Uninduced (A) and induced (B) K562 cells were labeled using lactoperoxidase-catalyzed iodination. Equal amounts of radioactivity were separated on two-dimensional gels. In each panel, the series of spots that represent the transferrin receptor is indicated between the open arrows. In the bottom left-hand corner of each panel is an inset that shows the result of a different experiment in which uninduced and differentiating cells were similarly iodinated and in which the autoradiograms were exposed so that the major spots had similar intensities. The components of the receptor are labeled in order of increasing isoelectric point.

FIG. 10. Digestion of the transferrin receptor with bacterial alkaline phosphatase and neuraminidase. [35S]Methionine-labeled differentiating K562 cells were dissolved, and transferrin receptors were isolated by affinity chromatography. The receptors were analyzed on two-dimensional polyacrylamide gels without further treatment (A) or after digestion with 25 units of bacterial alkaline phosphatase/ml (B) or 1 unit of neuraminidase/ml (C). The spots are numbered according to increasing isoelectric point. The gel in A is the same as in Fig. 9D to facilitate comparison, but all preparations of receptor from 96-h differentiating cells gave fairly similar ratios of the components of the receptor preparation. The alignment of the gels was facilitated by the position of a non-phosphorylated, non-glycosylated protein in the [35S]methionine-labeled receptor preparations that had a similar isoelectric point to form 2 of the receptor preparation but a lower molecular weight. This protein is not shown.

DISCUSSION

Human erythroleukemic cells grown in the absence of an inducer of erythroid differentiation bear approximately 200,000 surface transferrin receptors/cell (a figure very similar to that found by others (22)); in addition to these receptors, a further 175,000 are found intracellularly. Many of these internal receptors may be involved in the transferrin cycle in which surface-bound transferrin is internalized via coated pits and travels through low pH endosomes and multivesicular bodies before returning to the cell surface (22–24). It is in the low pH endosomes that transferrin releases its iron atoms which are then transported to the cytoplasm.

Addition of hemin to the medium reduces the number of surface transferrin receptors, but our results, in contrast to the results of Pelicci and colleagues (25), suggest that this diminution is due to greater internalization of the receptors rather than loss from the cell, either because demand for iron increases and therefore more receptor is employed in delivering iron to the interior of the cell or because elevated intracellular heme causes down-regulation of the transferrin receptor, as is the case in some other cell lines.3

Using immunoelectron microscopy to visualize the distribution of most of the radioactive proteins to one isoelectric form that co-migrated with form 4 (Fig. 10). It is, therefore, likely that receptor form 4 represents the dephosphorylated form of the receptor. Receptor form 5 remained at the same intensity after bacterial alkaline phosphatase treatment.

The transferrin receptor is also post-translationally modified by glycosylation. To investigate the possibility that multiple forms may differ in the number of neuraminic acid residues, purified, [35S]methionine-labeled receptor was digested with neuraminidase. After extensive neuraminidase digestion, five classes of receptor remained, but their isoelectric points were changed to higher values (Fig. 10C). This indicates that all forms of the receptor do contain neuraminic acid, but the presence of this sugar is not the basis of the multiple forms of the receptor.

3 Kaplan, J., Ward, J. H., and Kushner, J. (1983) Proceedings of the Sixth International Conference, Proteins of Iron Storage and Transport, Sapporo, Japan, p. 99.
bution of transferrin receptors over the surface of uninduced and differentiating K562 cells, we have noted a change in the distribution of receptors as differentiation proceeds. In non-differentiating cells, receptors are found in coated pits on the cell surface, but are also found randomly distributed over the noncoated parts of the plasma membrane. As differentiation proceeds, more of the surface receptor is clustered into coated areas of the membrane, suggesting the possibility that more receptor is recruited into the endocytotic cycle of the cell. High degree of clustering of receptors on the surfaces of differentiating cells could also explain the increased affinity of these receptors for transferrin. Such a recruitment of the transferrin receptor would be a logical consequence of increased demand for iron during elevated hemoglobin synthesis; that is, it would be expected that if, before the onset of hemoglobin synthesis, much of the receptor were not participating in internalization and were, therefore, not moved into clathrin-coated areas, the level of receptor detected on the surface would be high. However, after the onset of hemoglobin synthesis when demand for iron is raised, it would be expected that available receptor would be recruited into the endocytotic cycle and would rapidly become associated with coated pits and internalized. In this case, much less receptor should be detected at the cell surface. There is a precedent for the existence of two classes of receptor, those participating in endocytosis and those not doing so, for such has been observed in the endocytosis of low-density lipoprotein by fibroblasts (26).

The recruitment of receptors for hormones or growth factors into the endocytotic cycle of the cell has important implications in the control of cell proliferation. This is especially the case for the transferrin receptor which has been shown to be one of the most prominent transmembrane-specific surface antigens in a variety of cell lines and tissues (19, 27–29); however, the mechanism by which various membrane glycoproteins with receptor activity become selectively internalized while others are maintained on the cell surface is unknown. Recently, it has been shown (30, 31) that the insulin receptor contains a tyrosine kinase activity that is stimulated on binding of the hormone to the receptor. This activity phosphorylates the receptor which is subsequently internalized through clathrin-coated pits (32). Analogous results have also been found when epidermal growth factor binds to its receptor on the cell surface; again the receptor becomes phosphorylated on a tyrosine residue (33). From our investigations, it appears that as transferrin receptors are sequestered to an intracellular location, a change in the proportions of the various phosphorylation forms occurs with the more phosphorylated forms becoming the most prominent. How phosphorylation of the receptor may be involved in the endocytosis of the receptor (if indeed it is) is unknown, but it may result in a conformational change in the cytoplasmic region of the receptor that allows interaction with a component of the coated pit. Selective phosphorylation of the receptor would thus be a mechanism by which different receptors could be internalized under different circumstances through the common mechanism of the coated pit.

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REFERENCES
1. Tong, B. D., and Goldwasser, E. (1981) J. Biol. Chem. 256, 12656-12672
2. Geidusche, J. B., and Singer, S. J. (1979) Cell 16, 149–163
3. Skutelsky, E., and Farquhar, M. G. (1976) J. Cell Biol. 71, 218–231
4. Frazier, J. L., Caskey, J. Y., Yaffe, M., and Seigman, P. A. (1982) J. Clin. Invest. 69, 853–865
5. Hunt, R. C., and Marshall, L. M. (1981) Mol. Cell. Biol. 1, 1150–1162
6. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660–672
7. Pharmacia Fine Chemicals (1979) Affinity Chromatography, p. 6, Pharmacia Fine Chemicals, Uppsala, Sweden
8. Hubbard, A. L., and Cohn, Z. A. (1972) J. Cell Biol. 55, 390–406
9. Marshall, L. M., and Hunt, R. C. (1982) J. Cell Sci. 49, 97–113
10. Lemmil, U. K. (1971) Nature (Lond.) 227, 680–685
11. O’Farrell, P. H. (1975) J. Biol. Chem. 250, 4007–4021
12. Chamberlain, J. P. (1979) Anal. Biochem. 98, 132–135
13. Towbin, H., Stachel, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
14. Galbraith, G. M. P., Galbraith, R. M., and Faulk, W. P. (1980) Cell Immunol. 49, 215–222
15. Larrick, J. W., and Cresswell, P. (1979) J. Supramol. Struct. 11, 579–586
16. Godling, J. W., and Burns, G. F. (1981) J. Immunol. 127, 1256–1258
17. Wada, H. G., Hass, P. E., and Sussman, H. H. (1979) J. Biol. Chem. 254, 12629–12635
18. Maturo, J. M., Hollenberg, M. D., and Aglio, I. S. (1983) Biochemistry 22, 2571–2586
19. Trowbridge, I. S., and Orany, M. B. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3039–3043
20. Elder, J. H., Pickett, R. A., III, Hampton, J., and Lerner, R. A. (1977) J. Biol. Chem. 252, 6610–6615
21. Schneider, C., Sutherland, R., Newman, R., and Greaves, M. (1982) J. Biol. Chem. 257, 8516–8522
22. Klausner, R. D., Van Renswoude, J., Ashwell, G., Kempf, C., Schechter, A. N., Dean, A., and Bridges, K. R. (1983) J. Biol. Chem. 258, 4715–4724
23. Van Renswoude, J., Bridges, K. R., Harford, J. B., and Klausner, R. D. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6196–6190
24. Harding, C., Heuser, J., and Stahl, P. (1983) J. Cell Biol. 97, 329–339
25. Pellici, P. G., Tabilio, A., Theophopoulos, P., Titeux, M., Vainchenker, W., Rochant, H., and Testa, U. (1982) FEBS Lett. 145, 380–384
26. Basu, S. K., Goldstein, J. L., Anderson, R. G. W., and Brown, M. S. (1981) Cell 24, 492–502
27. Larrick, J. W., and Logue, G. (1980) Lancet ii, 862–863
28. Sutherland, R., Delia, D., Schneider, C., Newman, R., Kemshaw, J., and Greaves, M. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4515–4519
29. Orany, M. B., Trowbridge, I. S., and Minowada, J. (1980) Nature (Lond.) 286, 888–891
30. Avruch, J., Nemenoff, R. A., Blackshear, P. J., Pierce, M. W., and O’Rahony, R. (1982) J. Biol. Chem. 257, 15162–15166
31. Kasuga, M., Zick, Y., Yeffer, M., and Witz, A. H., and Kain, C. R. (1982) J. Biol. Chem. 257, 9891–9894
32. Pi³ch, P. F., Shia, M. A., Benson, R. J. J., and Fine, R. E. (1983) J. Cell Biol. 96, 133–138
33. Ushiro, H., and Cohen, S. (1980) J. Biol. Chem. 255, 8363–8365
34. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
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