Endocytosis of the Transferrin Receptor Is Altered during Differentiation of Murine Erythroleukemic Cells*

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Carol Anne Mulford† and Harvey F. Lodish‡§

From the †Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, Massachusetts 02142 and the §Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

During terminal differentiation of murine erythroleukemic (MEL) cells, the number of surface transferrin binding sites per cell decreases dramatically, while steady-state ligand uptake and immunoblotting studies demonstrate that the total number of transferrin receptors per cell remains constant. Since the amount of protein per cell decreases 4-fold during this 4-day period, the amount of transferrin receptor protein, relative to total soluble cell protein, increases 4-fold during this time, suggesting continued synthesis of the receptor. Supporting this, we show that the amount of transferrin receptor transcript in equal amounts of total cell RNA also increases as differentiation proceeds. Uninduced cells maintain 52% of the total transferrin binding sites on the cell surface, whereas only 22% of the receptors are on the surface in 4-day induced cells. All ligand endocytosed by either uninduced or induced cells at 37 °C is rapidly and completely exocytosed from the cells, suggesting that all of the cellular receptors are cycling. These studies suggest that, during MEL cell differentiation, an increasing fraction of transferrin receptors are localized to the cell interior, but are nevertheless cycling to the cell surface. This observed redistribution is due to altered kinetic parameters of the receptor. Receptor-bound 125I-labeled transferrin ligand has been followed through a single endocytic cycle. Ligand internalization occurs much more rapidly in induced cells (τ = 2.9 min) than in uninduced cells (τ = 6.9 min). The rates for ligand movement back out to the cell surface and its subsequent release into the medium in both uninduced and induced cells are quite similar.

The delivery of iron into the cell by transferrin constitutes the first step in the incorporation of this essential cofactor into a variety of polypeptides, most notably hemoglobin and several respiratory proteins (Vogt et al., 1969). By receptor-mediated endocytosis, iron-saturated transferrin is internalized into endocytic vesicles (Octave et al., 1983; reviewed in Goldstein et al., 1985). Iron is released from transferrin in these acidic compartments, while apotransferrin remains tightly bound to its receptor. Both the receptor and ligand then recycle together back to the cell surface, where, upon exposure to neutral pH, the apotransferrin dissociates from the receptor (Dautry-Varsat et al., 1983). Both apotransferrin and the receptor are reutilized.

Specific membrane receptors for transferrin were initially demonstrated on immature erythroid cells, which require large amounts of iron for hemoglobin production (Jandl and Katz, 1963). However, during erythroid differentiation, the composition of the plasma membrane undergoes significant alterations (Skutelsky and Danon, 1970; Skutelsky et al., 1974; Ackerman and Clark, 1972); although the transferrin receptor is abundant in erythroid and reticuloctyes, it is completely absent in mature erythrocytes (van Bockxmeer and Morgan, 1979; Frazier et al., 1982). The mechanism by which the transferrin receptor is cleared from the erythroid cell during maturation is unknown.

The transferrin receptor has been characterized in several cell types: it is a glycoprotein covalently associated with fatty acid (Omary and Trowbridge, 1981), with an apparent molecular weight of 95,000. It exists in the membrane as a disulfide-bonded dimer (Seligman et al., 1979; Wada et al., 1979; Schneider et al., 1982; Van Aghoven et al., 1984).

In this paper we examine the transferrin receptor during differentiation of a murine erythroleukemic cell line (MEL) cells. The receptor is evaluated in relation to its effect on the intracellular cycling pathway of its ligand, transferrin. We find that the number of surface binding sites per cell for transferrin decreases dramatically during differentiation. However, the total number of transferrin receptor molecules per cell remains constant and, importantly, accessible to the intracellular receptor-ligand cycling pathway throughout the 4-day program of MEL cell differentiation. In fact, since cell volume and protein content decrease continuously during differentiation, there is continued synthesis of transferrin receptor and, we show, of transferrin receptor mRNA. Finally, we examine the kinetics of ligand internalization and ligand movement back to the cell surface. We find an altered rate of endocytosis of surface transferrin-receptor complexes in induced cells which can account for the observed redistribution of the transferrin receptor.

MATERIALS AND METHODS

Cells—MEL cells were maintained in suspension culture and induced to undergo differentiation, according to published procedures (Volloch and Housman, 1982). Briefly, differentiation was initiated by incubating cells at a density of 5 × 10⁶ cells/ml in Dulbecco's modified Eagle's medium supplemented with 1.8% (v/v) dimethyl sulfoxide, 13% (v/v) heat-inactivated fetal bovine serum, 5% (w/v) bovine serum albumin, 2 mM l-glutamine, and 1.8 mM Imferon. The extent of cell differentiation was assessed by determining the amount of globin present in cells induced for 4 days; sodium dodecyl sulfate gel-resolved protein species were scanned with a microdensitometer. The globin protein comprised at least 60% of the total cellular protein in 4-day induced cells. As expected, no globin was present in uninduced MEL cells.

The abbreviations used are: MEL, murine erythroleukemic; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase.

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Transferrin Receptor Redistribution

duced cells. MEL cells, where indicated, were grown and induced to differentiate attached to fibronectin-coated dishes according to previously published procedures (Patel and Lodish, 1987). In all experiments involving fibronectin-coated dishes, only the cell population attached to the fibronectin was utilized. Cultures were grown at 37°C in a humid 5% CO2 incubator, and cell numbers were determined using a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL).

Transferrin—Human transferrin (Behring Diagnostics) was saturated with iron and radiolabeled with 125I using chloramine T as described previously (Karim and Mintz, 1981). Labeled transferrin was stored at −80°C in the presence of 0.6 mg/ml cytochrome c. Antibodies—Goat monospecific anti-human transferrin receptor antibodies (Enns et al., 1981) were the generous gift of Dr. Caroline Enns (Syracuse University, Syracuse, NY). Rabbit anti-goat IgG fraction was purchased from Cappel Laboratories (Cochrane Hill, PA).

Binding Assays—All assays were carried out at either 4 or 37°C, as indicated, using human 125I-ferrotransferrin. To assess transferrin binding at 4°C, MEL cells, either uninduced or induced for up to 4 days, were washed twice at 4°C with Hank’s saline solution containing 20 mM Hepes, pH 7.4 (binding medium). Cells were resuspended in this buffer to a concentration of 4 x 10⁶ cells/ml and 250-μl aliquots were added to each well of a 24-multiwell tissue culture dish (Falcon Labware, Oxnard, CA). Binding was carried out in duplicate wells at 4°C for 2 h with gentle agitation. Nonspecific binding was determined, also in duplicate, by addition of 50-100-fold excess of unlabeled transferrin just prior to addition of the radiolabeled ligand. Nonspecific binding remained below 10% of the specific binding in these binding experiments. After 2 h, cells were washed with the Hanks’ saline solution and incubated at 37°C for 1 h to dissociate surface-bound transferrin from the cell surface. Cells were then washed several times to remove unbound ligand and incubated at 37°C for 1 h in a 400-μl Eppendorf tube and centrifuged for 10 s at 12,000 x g. The tubes were frozen in liquid nitrogen, the tips containing the cell pellets were cut off, and the radioactivity of both the tip and the remaining tube containing the unbound ligand was determined using a Packard Auto-Gamma 500 γ counter.

Steady state labeling of the transferrin receptor with 125I-ferrotransferrin was assayed at 37°C according to the same procedure with the following modifications: Total RNA was isolated by guanidine thiocyanate, phenol, and chloroform extraction followed by centrifugation through a cesium chloride cushion (Chirgwin et al., 1979). Equal amounts of RNA (20 μg/lane) were electrophoresed on 1.0% agarose, 6% formaldehyde gels, and transferred to nylon membranes (Biotores, ICN). The membrane was hybridized overnight at 42°C in 35% formamide, 5 x SSC to the nick-translated DNA probe, a 4.9-kb BamHI fragment of PCD-TR1 encoding the human transferrin receptor (a generous gift from Dr. F. Rudlde, Yale University, New Haven, CT) (Kuhn et al., 1984). The transfer and hybridization conditions were as recommended in the ICN Bio-res instruction booklet. After hybridization, the membrane was washed in 0.1 x SSC, 0.1% sodium dodecyl sulfate at 50°C, then subjected to autoradiography.

Gel Electrophoresis and Immunoblotting Procedure—Total cell protein was dissolved in sodium dodecyl sulfate, subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate according to the method of Laemmli (1970), using a 10% acrylamide separating gel. The gel was then transferred electrophoretically to sheets of nitrocellulose (Towbin et al., 1979; Israel and Lodish, 1982). The nitrocellulose sheet was reacted sequentially with goat anti-human transferrin receptor antibody, rabbit anti-goat IgG, and 125I-protein A. The nitrocellulose sheet was thoroughly washed after each incubation, dried, and exposed to Kodak XAR film. Quantitation of the transferrin receptor at each stage of cell induction was accomplished by excising each band from the nitrocellulose and determining the associated radiactivity with a γ counter.

Results—Iron-free human transferrin was purchased from Behring Diagnostics. Na125I and affinity-purified 125I-protein A labeled with Bolton and Hunter reagent (40 mCi/mg) were purchased from Amerham Corp. Dithiothreitol-modified Eagle’s medium and fetal bovine serum were obtained from Gibco. All other reagents used in these studies were purchased from standard commercial sources.

Immunological Detection of the Transferrin Receptor—The total number of transferrin receptors present in MEL cells was examined by Western blotting of total cell proteins from equal numbers of cells. Gel-resolved proteins were transferred to nitrocellulose, which was incubated with goat anti-human...
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**Fig. 1.** Immunological detection of the transferrin receptor in equal numbers of cells. The total number of transferrin receptors was examined throughout a 5-day period of differentiation by Western blotting techniques. Equal numbers of cells (5 × 10⁶ cells/lane) from uninduced or 2–5-day induced cells, grown on fibronectin-coated dishes, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, incubated with transferrin receptor antibodies, and ¹²⁵I-protein A, and exposed for autoradiography. Lane a, transferrin receptor from uninduced cells; lane b, 2-day induced cells; lane c, 3-day induced cells; lane d, 4-day induced cells; lane e, 5-day induced cells.

**Fig. 2.** Western blot of the transferrin receptor from equal amounts of cell protein. Equal amounts of total soluble cell protein (200 µg) from uninduced and from cells induced 3–5 days in suspension were electrophoresed, immunoblotted, and exposed for autoradiography as in Fig. 1. The transferrin receptor band is shown in each lane. Lane a, uninduced cells; lane b, 3-day induced cells; lane c, 4-day induced cells; lane d, 5-day induced cells.

transferrin receptor antibodies. This antibody is able to cross-react with the murine transferrin receptor; it specifically immunoprecipitated the transferrin receptor from both ³⁵S-labeled human hepatoma (HepG2) cells and MEL cells yielding a single labeled polypeptide band of the same molecular weight (95,000) (data not shown). Visualization of the receptor band in the Western blot was enhanced by incubating with rabbit anti-goat IgG prior to addition of ¹²⁵I-protein A. Upon autoradiography (Fig. 1), both the uninduced and the induced cells exhibited a single band of M₉ 95,000 present in equal amounts regardless of the extent of cell differentiation, indicating that the total number of transferrin receptors per cell remained constant throughout the differentiation period. This conclusion was confirmed by quantitating the amount of radioactivity in each band excised from the nitrocellulose. The transferrin receptor band from uninduced cells contained 450 cpm of ¹²⁵I; 2-day induced cells, 490 cpm; 3-day, 452 cpm; 4-day, 445 cpm; 5-day, 464 cpm.

However, as differentiation proceeds, the cells divide and the cell volume decreases significantly (Volloch and Houman, 1982). The amount of total soluble protein per cell decreases approximately 3.5-fold from 0- to 4-day induced cells.² Although the amount of transferrin receptor per cell remains constant throughout differentiation (Fig. 1), the amount of receptor protein relative to total cellular protein actually increases during this time (Fig. 2). To show this, equal amounts of total soluble cell protein (200 µg) were examined by immunoblotting techniques and the amount of transferrin receptor in each band increased as differentiation progressed. The radioactivity in each excised band was as follows: uninduced cells, 370 cpm of ¹²⁵I; 3-day induced cells, 674 cpm; 4-day induced cells, 914 cpm; 5-day induced cells, 1337 cpm. There is an overall increase of 3.6-fold in the amount of transferrin receptor from 0–5 days suggesting that the rate of synthesis of the transferrin receptor may increase throughout differentiation.

**Northern Analysis of Transferrin Receptor mRNA**—We carried out Northern analyses on RNA from both uninduced and induced MEL cells to examine the possibility that the transferrin receptor continues to be synthesized during cell differentiation. Probing Northern blots of equal amounts of total cellular RNA with human transferrin receptor cDNA revealed the 28 and 15 S rRNA bands as well as a 4.9-kb putative transferrin receptor transcript. Upon washing these blots under high stringency conditions, the rRNA bands disappeared while only the 4.9-kb transcript was retained (Fig. 3). The amount of transferrin receptor mRNA, isolated from cells differentiated in suspension, increased as differentiation progressed. Attachment of MEL cells to a fibronectin monolayer allows differentiation to proceed longer than in suspension; after 6 days, most of the cells enucleate and form reticulocytes (Patel and Lodish, 1987). Fig. 3 also shows an increase in the transferrin receptor mRNA up to 6 days of differentiation on fibronectin. These observations support the proposal that the transferrin receptor continues to be synthesized during cell development, perhaps at an increased rate.

**Binding of Transferrin to the Surface of MEL Cells**—We next examined receptor function and distribution in MEL cells throughout the differentiation period. The number of functional transferrin binding sites on the surface of MEL cells was determined by binding ¹¹¹I-ferrotransferrin for 2 h at 4°C to prevent endocytosis. Data in Fig. 4 and Table I show that the number of transferrin receptors on the cell surface decreased significantly as cell differentiation pro-

² C. A. Mulford, unpublished results.
binding of human transferrin to mouse teratocarcinoma stem cells (Karin and Mintz, 1981) and to MEL cells (Wikzynska and Schulman, 1980) and have reached the same conclusion.

Cells exhibited decreased numbers of cell surface receptors, but a constant amount of immunoactive transferrin receptors (Fig. 1), suggested that cell differentiation was accompanied by selective internalization of receptor molecules, rather than actual loss of receptors from the cell. In the ligand uptake study described above, it was important to determine whether all cell-associated transferrin was cycling, rather than being sequestered in an intracellular compartment inaccessible to the cycling pool. Thus uninduced cells and cells induced for either 2 or 4 days were incubated with 125I-transferrin for 2 h at 37 °C; unbound ligand was removed and the samples were reincubated with the same concentration of unlabelled transferrin for 30 min. Cell-associated radioactivity was reduced by over 95% of its initial value, demonstrating that all internalized transferrin, presumably together with their bound receptors, were able to return to the cell surface; in the process, all of the 125I-labeled ligand was released to the medium and the transferrin receptors presumably rebound unlabeled transferrin.

Kinetics of Receptor-bound 125I-Transferrin—To elucidate the mechanism by which the observed redistribution of the transferrin receptor during differentiation occurs, the receptor was followed through a single cycle of endocytosis by tracing its labeled ligand, 125I-transferrin. A saturating amount of 125I-transferrin was bound to the surface of uninduced or 4-day induced MEL cells at 4 °C. Unbound ligand was removed, and the cells were incubated for various times at 37 °C in binding medium supplemented with unlabelled transferrin. After quick chilling, the cells were recovered by centrifugation and incubated with Pronase for 1 h at 4 °C. Samples were subsequently centrifuged and the radioactivity associated with the cell pellet after Pronase digestion (internal, C), the Pronase-released supernatant (cell surface, S), and the binding medium (M) was determined. The Pronase-sensitive fraction contained surface ligand, accessible to proteolysis, whereas the intracellular ligand was protected from Pronase digestion and was recovered in the cell pellet. The radioactivity in the medium consisted of ligand (presumably apotransferrin) released from the cell at the completion of the single cycle of endocytosis, as well as surface-bound ligand (holotransferrin) that dissociated directly into the medium without undergoing endocytosis.

In uninduced cells, approximately 20% of the surface-bound 125I-transferrin was internalized within 5 min and was subsequently exocytosed into the medium (Fig. 5). The rate constant, k1, describing the internalization event was calculated from the data to be 0.10 min⁻¹. The rate constant for movement of the internalized ligand back to the cell surface, k2, was found to be 0.31 min⁻¹. Finally, the value of k3, the rate constant for the movement of ligand from the internal pool into the medium, was found to be 0.03 min⁻¹. These assays were also performed using 125I-mouse transferrin as ligand and identical results, with respect to both receptor number and affinity, were obtained. However, the assays using 125I-human transferrin exhibited slightly lower nonspecific binding, so this ligand was used throughout these studies. Other investigators have also examined the specific binding of human transferrin to mouse teratocarcinoma stem cells (Karin and Mintz, 1981) and to MEL cells (Wikzynska and Schulman, 1980) and have reached the same conclusion.

Total Number of Functional Transferrin Receptors in MEL Cells—The decrease in the number of transferrin receptors on the surface of induced cells prompted examination of the total number of functional receptors/cell. Cells were incubated with 125I-ferrotransferrin for 45 min at 37 °C, to allow steady-state accumulation of the receptor-ligand complex. When steady state is reached, the number of cell-associated ligand molecules should equal the total number of cycling transferrin binding sites. The data depicted in Table I demonstrate that, after a 45-min incubation with the ligand, each uninduced cell contained 6.2 × 10⁵ transferrin molecules. Since these cells have 3.2 × 10⁵ surface receptors, 48% of the receptors for transferrin were localized intracellularly. However, in 4-day induced cells, 78% of the receptors were intracellular. Further, the total number of cellular transferrin molecules, and thus functional transferrin receptors, remained constant throughout a 4-day program of cell differentiation (Table I). This result, combined with the finding that induced cells exhibited decreased numbers of cell surface receptors, but a constant amount of immunoactive transferrin receptors (Fig. 1), suggested that cell differentiation was accompanied by selective internalization of receptor molecules, rather than actual loss of receptors from the cell.

Table I

Number of transferrin binding sites

| Cells                  | No. of receptors/cell (×10⁵) | Kd (×10⁻⁹ M) |
|------------------------|-----------------------------|--------------|
| Surface (4 °C)         |                             |              |
| Uninduced              | 3.2                         | 11.6         |
| 3-day induced          | 1.4                         | 6.1          |
| 4-day induced          | 1.1                         | 6.9          |
| Total (37 °C)          |                             |              |
| Uninduced              | 6.2                         |              |
| 2-day induced          | 6.1                         |              |
| 3-day induced          | 6.3                         |              |
| 4-day induced          | 5.0                         |              |

Saturating amount of 125I-human transferrin was bound to the surface of uninduced or 4-day induced MEL cells at 4 °C. Unbound ligand was removed, and the cells were incubated for various times at 37 °C in binding medium supplemented with unlabelled transferrin. After quick chilling, the cells were recovered by centrifugation and incubated with Pronase for 1 h at 4 °C. Samples were subsequently centrifuged and the radioactivity associated with the cell pellet after Pronase digestion (internal, C), the Pronase-released supernatant (cell surface, S), and the binding medium (M) was determined. The Pronase-sensitive fraction contained surface ligand, accessible to proteolysis, whereas the intracellular ligand was protected from Pronase digestion and was recovered in the cell pellet. The radioactivity in the medium consisted of ligand (presumably apotransferrin) released from the cell at the completion of the single cycle of endocytosis, as well as surface-bound ligand (holotransferrin) that dissociated directly into the medium without undergoing endocytosis.

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Total Number of Functional Transferrin Receptors in MEL
of dissociation of ligand from the cell surface into the medium, was 0.050 min⁻¹. These rate constants, together with the integrated form of the differential equation describing our model of endocytosis and recycling (see "Materials and Methods") and were solved for a single endocytic cycle using the normalization so that at \( t = 0 \), surface = 1.0, intracellular = 0, and medium = 0. The rate constants obtained are \( k_0 = 0.056 \text{ min}^{-1} \), \( k_1 = 0.10 \text{ min}^{-1} \), and \( k_2 = 0.31 \text{ min}^{-1} \).

This same procedure was followed for 4-day induced cells, as seen in Fig. 6. Importantly, the rate of internalization of ligand, \( k_1 \), in these cells was 0.24 min⁻¹, a dramatic increase from that observed in uninduced cells. (Graphically, this can be seen in the more rapid drop in the level of surface-bound ligand, and the more rapid and larger increase in the fraction of intracellular ligand.) Further, the rate of exocytosis, \( k_0 \), was 0.27 min⁻¹, just slightly slower than that of uninduced cells (0.31 min⁻¹). Finally, the dissociation rate constant, \( k_2 \), in the 4-day induced cells was not significantly different from uninduced cells, as expected (0.040 min⁻¹). These data suggest an increased rate of receptor-bound ligand internalization in the induced cells and an equivalent or slightly slower rate of movement back to the cell surface and subsequent ligand release into the medium. This would result in a net redistribution of the transferrin receptor to the interior of the cell in 4-day induced cells of the magnitude expected from Table I.

**DISCUSSION**

Upon treatment with dimethyl sulfoxide, murine erythroleukemic cells are induced to differentiate, providing an extremely useful cell system in which to examine the transferrin receptor during erythroid differentiation. MEL cells proliferate in culture as large, nucleated, hemoglobin-poor cells arrested at a developmental stage resembling proerythroblasts (Gordon and Rubin, 1982). Treatment with dimethyl sulfoxide or a variety of other agents (Marks, 1978; Nishioka and Silverstein, 1978) induces MEL cell differentiation which strongly parallels normal erythropoiesis and results in the appearance of globin mRNA (Ross et al., 1972), the accumulation of hemoglobin and other erythrocyte-specific proteins (Eisen et al., 1977, a and b), and eventually the cessation of cellular division and nuclear activity (Gaedicke et al., 1974). In suspension culture, differentiated MEL cells are nucleated and nondividing, contain large amounts of hemoglobin and developmentally resemble small orthochromatic erythroblasts (Eisen et al., 1977a; Gordon and Rubin, 1982) and reticuloctyes (Vcloch and Housman, 1982). The terminally differentiated cells (at 4 days) used in our experiments with suspension cultures resembled late erythroblasts. Cells that were induced to differentiate on fibronectin-coated dishes continue to differentiate to the reticulocyte stage (Patel and Lodish, 1987).

MEL cells grown in culture in the absence of any inducer possess approximately 3.2 \( \times \) 10⁶ transferrin receptor molecules on the surface of each cell (Table I). This is similar to the number of receptors found on the surface of an uninduced human fetal erythroleukemic cell line, K562 cells (Hunt et al., 1984).

Results from 4 °C binding and 37 °C ¹²⁵I-transferrin uptake studies demonstrate that about 52% of the total number of functional cellular receptors in steady state are localized on the surface of uninduced cells; 6.2 \( \times \) 10⁶ total receptors/cell were detected. This result demonstrates the existence of an intracellular pool of receptors; a finding which has been noted in other cell systems as well. A human hepatoma cell line, HepG2, maintains about two-thirds of the total transferrin receptors inside the cell (Ciechanover et al., 1983b), 44% of
the total receptors in human K562 cells are localized intracellularly (Hunt et al., 1984), and the majority of transferrin receptors in cultured HeLa cells are also found inside of the cell (Lamb et al., 1983).

Following induction of MEL cells, the number of surface transferrin receptors decreases by at least 3-fold over a 4-day differentiation period while the total number of receptors per cell remains constant (Fig. 1, Table I). Immunofluorescence microscopy of the receptor in uninduced and induced cells also reveals a dramatic decrease in the number of receptors on the surface of induced cells relative to those on the membrane of uninduced cells (data not shown). A reduction in the number of surface receptors during hemin-induced differentiation of human K562 cells has also been demonstrated by Hunt et al. (1984). These results are in contrast to results of Hu et al. (1977), who found a doubling in the number of receptor molecules on the surface of MEL cells, upon induction, as measured by transferrin binding activity at 37 °C. However, these investigators used 60% iron-saturated transferrin in their assays, rather than 100% iron-saturated transferrin, reporting that the latter ligand gave variable results.

In an attempt to discern a mechanism by which the receptor can undergo this redistribution, we examined the kinetic parameters of the receptor. We found that 4-day induced cells internalized receptor-bound 125I-transferrin 2.4-fold faster than uninduced cells (Figs. 5 and 6). In addition, the rate of ligand movement back to the surface and its release into the medium in induced cells was just slightly slower (0.27 min⁻¹) than the rate in uninduced cells (0.31 min⁻¹). Therefore, this would generate an alteration in the steady-state distribution of transferrin receptors in induced cells; an increased fraction of receptors inside the cell. Further, all transferrin receptors remain functional in the ligand-receptor pathway throughout differentiation. Uninduced and induced cells incorporated equal amounts of 125I-ferrotransferrin at 37 °C and subsequently excystotised the internalized 125I-labeled transferrin completely from the cell.

Importantly, we showed here that the reduction in surface receptors results from an increased rate of internalization of the receptor, rather than a change in the affinity of surface receptor for its ligand (Table I) or actual loss of these molecules from the cell. The total number of receptors per cell remains constant throughout differentiation, as measured by binding assays (Table I) and immunoblotting procedures (Fig. 1).

Selective phosphorylation of the transferrin receptor may be a mechanism by which the receptor could be more rapidly endocytosed by the cell. Hunt et al. (1984) have reported differences in phosphorylation of the human K562 transferrin receptor as hemin-induced differentiation proceeds, noting a shift to the more phosphorylated forms of the receptor. This result is supported by the observation that, using phorbol esters to activate protein kinase C, phosphorylation of the transferrin receptor is accompanied by its internalization and a down-regulation of surface receptors (May et al., 1984; Klaunser et al., 1984). It is probable that phorbol ester-induced receptor phosphorylation is regulating the subsequent receptor internalization and redistribution. In fact, this down-regulation is completely reversible, and is accompanied by dephosphorylation of the receptor (May et al., 1984). Similar results were obtained by Hunt and Marshall-Carlson (1986), treating human erythroblastemic K562 cells with the drug trifluoperazine, which inhibits calmodulin-dependent and calcium-activated phospholipid-dependent kinases. The number of surface transferrin receptors decreased by approximately half, and this was reversible upon removal of the drug.

Perhaps increased phosphorylation of the transferrin receptor during differentiation promotes increased endocytosis of bound transferrin, causing a redistribution of the receptor population. The net result leaves less receptors on the surface at any given time during differentiation, but all receptors are still cycling.

MEL cells divide twice during the early stages of differentiation; they become smaller (Volloch and Housman, 1982) and contain 3-4-fold less protein per cell. The number of transferrin receptors/cell remains constant during this time (Fig. 1). However, the number of transferrin receptors per cell protein mass increases (Fig. 2), as does the abundance of transferrin receptor mRNA per total cellular RNA (Fig. 3). Thus MEL cells are incorporating more transferrin ligand, normalized to cell protein, as differentiation proceeds. The requirement for hemoglobin (and thus, iron) increases during differentiation, which may provide the rationale for the increase in the number of transferrin receptors, relative to the amount of cell protein, and also the increased rate of endocytosis of the ligand-receptor complexes.

During erythropoiesis, some mechanism must exist to clear the cell membrane of transferrin receptors, since they are not present in mature erythrocytes. However, although a number of mechanisms have been proposed, the ultimate fate of the transferrin receptor molecule during cell maturation is controversial (Hunt et al., 1984; Pelicci et al., 1982; Testa et al., 1982, Harding et al., 1985). The experiments presented here describe the recycling events of the transferrin receptor during the first 4 days of differentiation. This may be too early in the differentiating program to provide any clues as to the mechanism by which the receptor is finally lost from the cell. However, the MEL cell system now established in our laboratory (Patel and Lodish, 1987), which allows synchronous differentiation of MEL cells through the enucleation stage, is an ideal system in which to examine the ultimate fate of the transferrin receptor.

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