Insulin-like Growth Factor I and Epidermal Growth Factor Regulate the Expression of Transferrin Receptors at the Cell Surface by Distinct Mechanisms*

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(Received for publication, March 9, 1987)

The transferrin receptor cycles rapidly between cell surface and endosomal membrane compartments. Treatment of cultured cells with epidermal growth factor (EGF) or insulin-like growth factor I (IGF-I) at 37 °C causes a rapid redistribution of transferrin receptors from an intracellular compartment to the cell surface. The effects of EGF and IGF-I on the kinetics of the cycling of the transferrin receptor in A431 human epidermoid carcinoma cells were compared. The primary site of EGF action was found to be an increase in the rate of transferrin receptor exocytosis. The exocytotic rate constant was measured to be 0.11 min⁻¹ in control cells and 0.33 min⁻¹ in EGF-treated cells. In contrast, IGF-I was found to increase the cell surface expression of transferrin receptors by causing a small increase in the rate of exocytosis (from 0.11 to 0.17 min⁻¹) and a decrease in the rate of endocytosis (from 0.33 to 0.24 min⁻¹). It is concluded that the mechanisms for EGF and IGF-I action to increase the cell surface expression of the transferrin receptor are distinct.

A kinetic model of the cycling of the transferrin receptor based on experimentally determined rate constants is presented. The model predicts that the apparent Km for the uptake of dierffer transferrin by cells. This prediction is confirmed by direct measurement of the accumulation of 59Fe-labeled dierffer transferrin by A431 cells. These data demonstrate that the accumulation of iron by cultured cells is a complex function of the rate of cycling of the transferrin receptor and that this process is under acute regulation by growth factors.

Transferrin is a serum protein that binds iron and is an essential requirement for the growth of cultured cells (1). The uptake of iron into cells is mediated by specific cell surface receptors for dierffer transferrin that cycle between the plasma membrane and endosomal membranes (2). This process is acutely regulated by peptide growth factors (3-5). Treatment of fibroblasts with epidermal growth factor (EGF)1 or insulin-like growth factor I (IGF-I) causes a redistribution of transferrin receptors from an intracellular compartment to the cell surface (3, 4). The purpose of the experiments reported here was to investigate the mechanisms of action of EGF and IGF-I by examining the sites of action of these growth factors on the cycling pathway followed by the transferrin receptor. As the receptors for both EGF and IGF-I have been shown to possess intrinsic tyrosine-protein kinase activity, the mechanism of action of these growth factors on the transferrin receptor might be expected to be similar. However, a detailed examination of the kinetics of transferrin receptor cycling revealed that the primary steps in the cycling pathway regulated by EGF are different from those regulated by IGF-I. The contrasting effects of EGF and IGF-I suggest that the mechanisms of action of these growth factors are different.

EXPERIMENTAL PROCEDURES

Materials—EGF was purified as described (6, 7). Porcine insulin was from Dr. Ronald Chance, Lilly. IGF-I produced by recombinant DNA technology (rIGF-I) was obtained from Dr. M. Peters (Amgen Corp.). rIGF-I differs from IGF-I in that it contains an additional eight amino acids at the amino terminus (MKKYWIPM) and a threonine substitution for methionine at position 59. Recent studies (8) have demonstrated that rIGF-I and IGF-I bind to the type I IGF receptor with similar affinity. Human transferrin was from Behring Diagnostics. Diferff transferrin, 125I-labeled transferrin, 125I-OKT9, and 59Fe-labeled dierffer transferrin were prepared as described previously (4).

Cell Culture—A431 epidermoid carcinoma cells were obtained from Dr. G. Todaro (Oncogen) and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum (GIBCO). Hybridoma cells producing monoclonal antibody OKT9 were obtained from the American Type Culture Collection.

Binding of Diferff 125I-Transferrin to Cell Surface Receptors—Binding assays were performed on cells grown in 16-mm wells. The cells were washed with serum-free medium and incubated for 30 min at 37 °C in 125 mM NaCl, 6 mM KCl, 1.2 mM CaCl2, 1 mM MgSO4, 25 mM HEPES (pH 7.4), and 0.2% (w/v) bovine serum albumin. After treatment with and without growth factors, the medium was removed and rapidly replaced with medium at 0 °C. Diferff 125I-transferrin was then added to the cells which were incubated at 0 °C for 180 min. The monolayers were then washed three times with cold medium and solubilized with 900 μl of 1 M NaOH. Radioactivity was quantitated with a Beckman γ-counter. Nonspecific binding was estimated in incubations with a 100-fold excess of dierffer transferrin.

Binding of Monoclonal Anti-transferrin Receptor Antibody (OKT9) to Cell Surface Receptors—The binding of the antibody OKT9 to the cell surface receptors was measured using cells grown in 16-mm wells. The cells were washed and incubated for 30 min at 37 °C in medium containing 120 mM NaCl, 6 mM KCl, 1.2 mM CaCl2, 1 mM MgSO4, 25 mM HEPES (pH 7.4), and 0.2% (w/v) bovine serum albumin. After treatment with and without growth factors, the medium was removed and replaced with medium at 0 °C. 125I-OKT9 (2 μg/ml) was then added to the cells for 180 min at 0 °C. The monolayers were then washed three times and solubilized with 900 μl of 1 M NaOH. Radioactivity was quantitated with a Beckman γ-counter. Nonspecific binding of 125I-OKT9 was estimated in incubations containing 0.2 mg/ml OKT9 antibody.

Steady-state Analysis of Transferrin Receptor Distribution—The distribution of receptor-bound dierffer 125I-transferrin between cell
Regulation of Transferrin Receptor Cycling

Results

In initial experiments to compare the effects of rIGF-I and EGF on the expression of transferrin receptors at the cell surface, the time courses of the effects of these growth factors were examined. Fig. 1 shows that rIGF-I and EGF cause a similar extent of increase in the cell surface expression of the transferrin receptor. However, marked differences in the regulation of the transferrin receptor by EGF and rIGF-I are apparent. First, the effect of EGF is extremely transient, whereas the increased cell surface expression of transferrin receptors caused by rIGF-I is sustained. Second, the effect of EGF is extremely rapid (maximal effect observed at 2 min), whereas the effect of rIGF-I is slower (maximal effect observed at 5 min). These differences between the effects of EGF and rIGF-I on the expression of the transferrin receptor at the cell surface (Fig. 1) suggest the possibility that the mechanisms of action of EGF and rIGF-I may be distinct. Further experiments to characterize the effects of EGF and rIGF-I were therefore performed.

Steady-state Analysis of Transferrin Receptor Expression—The effects of EGF and rIGF-I on the subcellular distribution of transferrin receptors during steady-state diferric transferrin uptake were examined. Receptors cycling between cell surface and endosomal membrane compartments were labeled with \( \text{\textsuperscript{125}I}-\text{transferrin} \) by incubation of cell monolayers with \( \text{300 nM diferric \text{\textsuperscript{125}I}-transferrin} \) for 2 h at 37 °C. The cells were then treated with and without EGF or rIGF-I, and the amount of transferrin bound to receptors localized in the cell surface and endosomal membrane compartments was assessed by the acid washing procedure. Fig. 2 shows that treatment of A431 cells with EGF or rIGF-I causes no change in the steady-state binding of \( \text{\textsuperscript{125}I}-\text{transferrin} \). However, a marked increase in the binding of transferrin to cell surface receptors and a corresponding decrease in the binding of transferrin to receptors located in an intracellular membrane compartment were observed. These results indicate that EGF and rIGF-I increase the expression of the transferrin receptor at the cell surface by causing a redistribution of cycling transferrin receptors from an intracellular compartment to a cell surface compartment. The results are not consistent with the hypothesis that EGF and rIGF-I cause the recruitment of a previously unla-

FIG. 1. Time course of growth factor action to regulate the expression of the transferrin receptor at the cell surface. A431 cells were incubated for different times with \( \text{10 nM EGF or rIGF-I} \) at 37 °C. The cells were then washed and subsequently incubated with \( \text{65Fe}-\text{transferrin} \) for 30 min. Diferric \( \text{65Fe}-\text{transferrin} \) was added and after incubation, the cell surface and endosomal membrane compartments were labeled with \( \text{\textsuperscript{125}I}-\text{transferrin} \) and solubilized with \( \text{0.1% sodium deoxycholate, 20 mM nitroblue tetrazolium (pH 6.4). The solubilized cells were mixed with 4 ml of Optifluor (Packard Instrument Co.), and the associated radioactivity was measured with a y-counter.} \)

Measurement of Transferrin Receptor Occupancy—The occupancy of the transferrin receptor by ligand during steady-state uptake was determined by comparison of the specific \( \text{\textsuperscript{125}I}-\text{transferrin} \) binding to cell surface receptors with \( 0 °C \) with the binding of saturating concentrations of \( \text{\textsuperscript{125}I}-\text{transferrin} \) under equilibrium conditions measured at \( 0 °C \). The binding of diferric transferrin to cell surface receptors at \( 37 °C \) was measured by incubation of cell monolayers with \( \text{300 nM diferric \text{\textsuperscript{125}I}-transferrin} \) for 80 min at \( 37 °C \) and subsequently washing the cells rapidly at \( 0 °C \). The binding of cell surface and intracellular receptors was determined by the acid washing procedure (9, 10). Cell-associated radioactivity was measured with a y-counter.

Steady-state Analysis of Transferrin Uptake—Steady-state analysis of diferric \( \text{\textsuperscript{125}I}-\text{transferrin} \) uptake was performed by the method of Wiley and Cunningham (11). A431 cells in 16-mm wells were incubated in serum-free medium with \( \text{300 nM diferric \text{\textsuperscript{125}I}-transferrin} \). At defined times, the cells were washed rapidly three times with cold medium. The cells were then solubilized with \( \text{1 M NaOH} \), and the associated radioactivity was measured with a y-counter.

Diferric Transferrin Binding to Cell Surface Receptors at \( 37 °C \)—In order to measure the binding of diferric \( \text{\textsuperscript{125}I}-\text{transferrin} \) to cell surface receptors, it is necessary to inhibit internalization of the receptors. To achieve this, A431 cells were transferred rapidly from medium at \( \text{20 mM NaNs} \) to \( \text{37 °C} \). After 15 min, the cells were placed in a 37 °C incubator for 30 min. Diferric \( \text{\textsuperscript{125}I}-\text{transferrin} \) was added and after defined times of incubation at \( 37 °C \), the binding of the ligand to cell surface receptors was determined by washing the cell monolayers three times with cold medium and measuring the amount of radioactivity associated with the cells. Nonspecific binding was estimated in incubations containing a 200-fold excess of diferric transferrin.

Control experiments showed that briefly washing the cell monolayers with buffer (pH 3) caused the dissociation of >90% of the specifically bound radioactivity. This result demonstrates that the internalization of the transferrin receptor is inhibited under these conditions.

Uptake of \( \text{\textsuperscript{55}Fe}-\text{Labeled Diferric Transferrin} \)—The uptake of \( \text{\textsuperscript{55}Fe}-\text{labeled diferric transferrin} \) was measured at \( 37 °C \). A431 cells were incubated in serum-free medium with \( \text{\textsuperscript{55}Fe}-\text{labeled diferric transferrin} \) for 10 and 20 min and subsequently washed rapidly three times at \( 0 °C \). The cells were solubilized with \( 0.5 \text{ mL of 0.5% sodium deoxycholate, 20 mM nitroblue tetrazolium (pH 6.4). The solubilized cells were mixed with 4 ml of Optifluor (Packard Instrument Co.), and the associated radioactivity was measured with a scintillation counter. The rate of accumulation of \( \text{\textsuperscript{55}Fe} \) was calculated from the difference between the radioactivity measured after incubation of the cells for 10 and 20 min with \( \text{\textsuperscript{55}Fe}-\text{labeled diferric transferrin} \).
Regulation of Transferrin Receptor Cycling

The time courses of growth factor actions were investigated by pretreating cells with EGF or rIGF-I for different times prior to the measurement of the rate of release of apotransferrin. It was observed that the effect of EGF to cause stimulated release of 125I-apotransferrin was transient and was complete within 2 min of treatment with EGF (Fig. 4). In contrast, rIGF-I caused a sustained stimulation of 125I-apotransferrin release by A431 cells that only diminished gradually with the time of treatment with rIGF-I (Fig. 4). The transient effect of EGF and the sustained effect of rIGF-I on exocytosis are consistent with the effects of these growth factors on the time courses of transferrin receptor expression at the cell surface (Fig. 1).

Regulation of Transferrin Receptor Endocytosis—The effect of growth factors on the rate of endocytosis of the transferrin receptor was investigated. The endocytic rate constant was measured under steady-state conditions by the ln/Sur method described by Wiley and Cunningham (11). The method involves the measurement of the rate of intracellular accumulation of ligand under conditions where the number of occupied cell surface receptors is constant and no release of accumulated ligand occurs. In control cells incubated at 37 °C with 300 nM diﬀerent 125I-transferrin, a linear rate of uptake of transferrin with time was observed up to approximately 8 min. After this time, a sharp decrease in the rate of accumulation of 125I-transferrin occurred because of the release of accumulated transferrin as apotransferrin (Fig. 5). The first-order endocytic rate constant was calculated to be 0.33 ± 0.03 min⁻¹ (mean ± S.D.). Treatment of A431 cells with rIGF-
I caused a decrease in the transferrin receptor endocytic rate constant. A similar measurement of the endocytic rate constant in EGF-treated cells was not possible using this technique because of the requirement for steady-state conditions during the assay. Fig. 1 shows that the transient nature of the effect of EGF does not allow a steady-state distribution of transferrin receptors to be achieved for long enough to allow the measurement of the endocytic rate constant by the method of Wiley and Cunningham (11).

Diferric Transferrin Binding to Cell Surface Receptors---In addition to regulating the cycling of the transferrin receptor, it is possible that growth factors also directly alter the kinetics of the interaction of diferric transferrin with its receptor. Previously, we have reported that EGF and rIGF-I do not alter the affinity of the transferrin receptor in BALB/c 3T3 cells (4). This result was confirmed using A431 cells (data not shown). To substantiate further the conclusion that EGF and rIGF-I do not directly regulate the interaction of diferric transferrin with its receptor, the effect of growth factors on the rate of diferric transferrin binding to cell surface receptors was investigated. No effect of pretreatment at 37 °C with EGF or rIGF-I was observed on the subsequent rate of binding of diferric 

\[ ^{125}I \text{transferrin} \] to cell surface receptors assayed at 0 °C (data not shown). In this assay, the cells were cooled to 0 °C in order to inhibit the internalization of ligand that occurs at 37 °C. In further experiments, the rate of binding of diferric 

\[ ^{125}I \text{transferrin} \] to cell surface receptors was measured at 37 °C in cells that were metabolically depleted (to inhibit receptor-mediated endocytosis) by incubation with 20 mM 2-deoxyglucose and 10 mM NaCl for 15 min (Fig. 6). The second-order rate constant for diferric 

\[ ^{125}I \text{transferrin} \] binding to cell surface receptors was determined to be 2.7 × 10^7 M^-1 min^-1 (Fig. 6). Pretreatment of the A431 cells with EGF or rIGF-I was observed to have no significant effect on the rate of binding of diferric 

\[ ^{125}I \text{transferrin} \] to cell surface receptors. We conclude that EGF and rIGF-I do not directly regulate the interaction of diferric transferrin with its receptor at the cell surface.

Dissociation of Apotransferrin from Cell Surface Receptors---The rate of dissociation of apotransferrin from cell surface receptors was measured using cells that were metabolically depleted in order to inhibit receptor cycling. A431 cells in 16-mm wells were incubated with 20 mM 2-deoxyglucose and 10 mM NaN3 for 15 min at 37 °C. The medium was then aspirated and replaced with 120 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 25 mM MES (pH 6.0) containing 20 mM 

\[ ^{125}I \text{apoprot} \] transferrin. After 60 min of incubation, the cell monolayers were washed rapidly with cold medium to remove unbound 

\[ ^{125}I \text{apoprot} \] transferrin. Dissociation of 

\[ ^{125}I \text{apoprot} \] transferrin bound to cell surface receptors was then initiated by the addition of 1 ml of HEPES-buffered medium (pH 7.4). At defined times, the monolayers were rapidly washed, and the radioactivity remaining associated with the cells was measured with a γ-counter (Fig. 7). The first-order rate constant for apotransferrin.
The effect of diferric transferrin concentration on the occupancy of cell surface receptors was estimated by this method to be 2.6 min⁻¹ (mean of three separate determinations). No significant effect of treatment of the cells with EGF or rIGF-I was observed.

**Occupancy of Cell Surface Transferrin Receptors at 37 °C—**The steady-state binding of diferric 125I-transferrin to cell surface receptors at 37 °C was compared with equilibrium binding of diferric 125I-transferrin observed after rapidly shifting the cells to 0 °C in order to inhibit receptor cycling. Fig. 8 shows that the apparent $K_a$ for diferric transferrin binding to cell surface receptors is 54 ± 7 nM (mean ± S.D.; $n = 3$). This apparent $K_a$ is significantly greater than the $K_a$ (2.1 nM) measured under equilibrium conditions at 0 °C in which receptor cycling is inhibited. We conclude that the binding of diferric transferrin to cell surface receptors at 37 °C is a process that is maintained far from thermodynamic equilibrium. The apparent $K_a$ is therefore a parameter that could potentially be regulated by growth factors. The effects of rIGF-I and EGF on the steady-state binding of diferric 125I-transferrin to cell surface receptors at 37 °C was determined. Table I shows that treatment of A431 cells for 5 min at 37 °C with 10 nM rIGF-I results in an increase in the occupancy of the cell surface receptors. This result contrasts with the lack of effects of rIGF-I on the affinity of the transferrin receptor measured under equilibrium conditions at 0 °C (4).

In further experiments, the accumulation of iron by cells incubated with 59Fe-labeled diferric transferrin was examined. A431 cells were incubated with different concentrations of 59Fe-labeled diferric transferrin, and the rate of accumulation of radioactivity by the cells was measured. Fig. 9 shows that the apparent $K_m$ for iron accumulation was 57 ± 8 nM (mean ± S.D.; $n = 3$). The apparent $K_m$ for iron uptake is signif-

![Fig. 8](image)

**Fig. 8. Occupancy of cell surface transferrin receptors.** The effect of diferric transferrin concentration on the occupancy of cell surface transferrin receptors at 37 and 0 °C was measured as described under "Experimental Procedures." The results presented are means of four separate determinations. The $K_a$ for diferric transferrin binding observed under equilibrium conditions (0 °C) was calculated to be 2.1 ± 0.6 nM (mean ± S.D.; $n = 3$). The apparent $K_a$ for diferric transferrin binding observed at 37 °C was calculated to be 54 ± 7 nM (mean ± S.D.; $n = 3$).

**TABLE I**

| TABLE I |

| Effect of growth factors on the occupancy of transferrin receptors at the cell surface at 37 °C |

A431 cells in 15-mm wells were treated with 10 nM EGF or rIGF-I for 5 min at 37 °C. The specific binding of 10 nM diferric 125I-transferrin to cell surface receptors was measured as described under "Experimental Procedures." The results presented were obtained in a single experiment and are the means of triplicate observations. In three additional experiments, the occupancy of cell surface transferrin receptors was determined to be: 17, 23, and 21% (control cells); 23, 27, and 26% (EGF-treated cells), and 29, 31, and 38% (rIGF-I-treated cells). On the basis of the results obtained in these four experiments, we calculate that the transferrin receptor occupancy is 21 ± 3% in control cells, 26 ± 2% in EGF-treated cells, and 33 ± 4% in rIGF-I-treated cells (mean ± S.D.).

| Cell surface binding | Occupancy of receptors at 37 °C |
|----------------------|-------------------------------|
| Control              | 946                           | 4302 |
| EGF                  | 2666                          | 9875 |
| rIGF-I               | 2633                          | 7744 |

![Fig. 9](image)

**Fig. 9. Effect of diferric transferrin concentration on the accumulation of iron by cells.** The rate of uptake of 59Fe-labeled diferric transferrin by A431 cells treated at zero time with and without 10 nM rIGF-I was measured as described under "Experimental Procedures." A presents the effect of 59Fe-labeled diferric transferrin concentration on the accumulation of radioactivity by A431 cells. B presents Edie-Schachar analysis of this data ($V = \text{(counts/min accumulated in 10 min)/1284 cpm}$). A similar analysis was performed on the data obtained in three separate experiments. The apparent $K_m$ was calculated to be 57 ± 8 and 31 ± 7 nM (mean ± S.D.; $n = 3$) for control and rIGF-I-treated cells, respectively. The $V_{max}^{-1}$ was calculated to be 17 ± 2.5 and 20.4 ± 1.9 fmol/10⁶ cells/min (mean ± S.D.; $n = 3$) for control and rIGF-I-treated cells, respectively.

**TABLE II**

| Summary of kinetic rate constants |

The rate constants are defined in the schematic diagram presented in Fig. 10. The rate constants $k_1$, $k_2$, and $k_3$ are taken from Figs. 5, 7, and 6, respectively. The rate constant $k_1$ is assumed to be equal to the experimentally determined rate constant $k_1$ (Fig. 3). The rate constants $k_1$ and $k_3$ describing the cycling of the unoccupied receptor were not measured.

| Rate constant | Control | EGF | rIGF-I |
|---------------|---------|-----|--------|
| $k_1$         | 0.33 min⁻¹ | 0.24 min⁻¹ |
| $k_2$         | 0.11 min⁻¹ | 0.28 min⁻¹ |
| $k_3$         | 2.6 min⁻¹  | 2.6 min⁻¹  |
| $k_4$         | 2.7 x 10⁻¹ M⁻¹ | 2.8 x 10⁻¹ M⁻¹ |
| $k_5$         | 2.7 x 10⁻¹ M⁻¹ | 2.7 x 10⁻¹ M⁻¹ |
cantly larger than the \( K_d \) for diferric transferrin binding to receptors (2.1 nM), but is consistent with the measured apparent \( K_d \) for diferric transferrin binding to cell surface receptors (54 nM) at 37 °C.

**Effect of Growth Factors on Iron Accumulation**—We have previously reported that growth factors cause an increase in the rate of iron accumulation by cells by modulating receptor-mediated endocytosis of diferric transferrin (4). This effect was further examined by investigating the dependence of growth factor-stimulated iron uptake on the concentration of diferric transferrin in the medium. rIGF-I was not observed to cause a significant alteration in the \( V_{\text{max}} \) of uptake (16.7 ± 2.5 fmol/10^6 cells/min for control cells versus 20.4 ± 1.9 for IGF-I-treated cells). However, a large decrease in the apparent \( K_a \) of ^59Fe-labeled diferric transferrin uptake was found when A431 cells were treated with rIGF-I. The \( K_a \) values for diferric transferrin uptake were calculated to be 57 ± 8 and 31 ± 7 nM (mean ± S.D.) for control and rIGF-I-treated cells, respectively (Fig. 9). Uptake data for the effect of EGF could not be obtained because of the extremely transient nature of the stimulation of iron uptake caused by EGF (3, 4).

**Analysis of a Model Receptor Cycling System**—Fig. 10 presents a model of the cycling of the transferrin receptor that is consistent with a large body of published data (see Ref. 2 for review). A kinetic description of the cycling has been derived by Ciechanover et al. (12) and Klausner et al. (13) using the assumption that the transferrin receptor is always saturated by its ligand. This assumption is not valid for steady-state analysis (Fig. 8). The steady-state solution for the model presented in Fig. 10 was solved (Table III) using the King-Altman procedure simplified by the addition of multiple lines connecting two corners (14). The solution for \( k_{\text{on}} \) and \( k_{\text{off}} \) for transferrin binding to cells is identical. The solutions for \( k_{\text{on}} \) and \( k_{\text{off}} \) for the fractional expression of the transferrin receptor at the cell surface \((\frac{[R_s]}{[R_T]}\)) were also determined (Table III).

The model for the cycling of the transferrin receptor was used to predict the cell surface expression of transferrin receptors. Substitution of the experimentally determined values for \( k_5 \) (Fig. 8), \( k_{\text{on}} \) (Fig. 9), \( k_2 \), and \( k_5 \) (Table II) into Equation 1 (see Table III) permits the calculation of \( k_{\text{on}}/k_s \). The calculated value for \( k_{\text{on}}/k_s \) was then used to calculate \( \frac{[R_s]}{[R_T]} \) (Equation 3). \( V_{\text{max}} \) was calculated from Equation 2. The results of these calculations are presented in Table IV. Differences between the predicted and experimentally determined values for \( V_{\text{max}} \) and \( \frac{[R_s]}{[R_T]} \) are observed, indicating that the model does not describe the cycling of the transferrin receptor in a completely accurate manner. However, taking into account the simplicity of the kinetic model used for the calculations and the possible systematic errors in the values of experimentally determined parameters, it is apparent that a marked similarity exists between the predicted and experimentally determined values for \( k_{\text{on}} \) and \( \frac{[R_s]}{[R_T]} \). In particular, the model predicts that IGF-I causes a 2.9-fold increase in the expression of the transferrin receptor at the cell surface. This prediction is similar to the observed effects of IGF-I observed on A431 cells (Fig. 1).

Further analysis of the predicted effects of growth factors on the kinetics of transferrin receptor cycling requires knowledge of the ratio \( k_2/k_s \). These rate constants describe the cycling of the transferrin receptor without bound transferrin (15). In the absence of the experimentally determined values for \( k_2 \) and \( k_s \), we have assumed that the reliable ratio \( k_2/k_s \) is altered to the same extent as \( k_2/k_s \). This assumption is a reasonable approximation because the effects of growth factors on the expression of the transferrin receptor at the cell surface are similar whether A431 cells are saturated with or depleted of transferrin (Table V). Using this assumption, the values of \( k_{\text{on}} \), \( V_{\text{max}} \), and \( \frac{[R_s]}{[R_T]} \) can be calculated for conditions in which the regulation of endocytosis or exocytosis occurs (Table IV). The model predicts that the stimulation of exocytosis caused by EGF results in a marked increase in the cell surface expression of transferrin receptors (Table IV). However, the increase in exocytosis caused by IGF-I would result in only a modest increase in transferrin receptor expression at the cell surface. Similarly, the inhibition of endocytosis caused by IGF-I would result in only a small increase in cell surface transferrin receptor expression. In contrast, the simultaneous regulation of exocytosis and endocytosis by IGF-I is predicted to cause a 2.9-fold increase in the expression of the transferrin receptor.
Regulation of Transferrin Receptor Cycling

The experimentally determined values for $K_{\text{app}}$ (Fig. 9), $K_e$ (Fig. 8), and the rate constants $k_2$, $k_3$, and $k_{-2}$ are presented in Table II. The medium used to determine the values of $k_3/k_4$ using Equation 1 (Table III). $k_3/k_4$ values were estimated to be 108 and 36 for control and rIGF-I-treated cells, respectively. For other conditions, it is assumed that $k_2/k_4$ is affected to the same extent as the experimentally determined $k_3/k_4$ (Table II). For EGF-treated cells and for cells in which the effect of alterations of either $k_3$ or $k_2$ was examined, $k_3/k_4$ values were assumed to be 42, 70, and 48, respectively. Using these values of $k_3/k_4$, the predicted values of $[R_0]/[R_0]$ were calculated using Equation 3 (Table III). The measured value of $[R_0]/[R_0]$ was taken from Fig. 1. The predicted $K_{\text{app}}$ values were calculated using Equation 1 (Table III), and the experimentally determined $K_{\text{app}}$ values were taken from Fig. 9. The predicted $V_{\text{app}}$ values were calculated using Equation 3 (Table III). For this calculation, $[R_0]$ was assumed to be $2 \times 10^8$ cells (Fig. 2). The measured $V_{\text{app}}$ for diferric transferrin uptake was calculated as half of the $V_{\text{app}}$ of $^{59}$Fe uptake (Fig. 9).

### Table IV

| Condition | $K_{\text{app}}$ | $V_{\text{app}}$ | $[R_0]/[R_0]$ |
|-----------|-----------------|-----------------|---------------|
| Control   | Measured        | Predicted       | Measured      | Predicted     |
|           | $nM$            | $[^{59}Fe]/10^8$ cells/min | %             |               |
| Control   | 57              | 57              | 8.5           | 2.9           | 0.92          |
| EGF (k_3  | 0.28 min$^{-1}$ | 35              | 3.6           | 8.7           | 2.3           |
| IGF-I endocytic step (k_4  | 0.17 min$^{-1}$ | 45              | 6.4           | 8.5           | 2.7           |
| IGF-I endocytic step (k_5  | 0.24 min$^{-1}$ | 44              | 4.8           | 1.4           |               |

### Table V

**Effect of diferric transferrin on the transferrin receptor expression at the cell surface**

A431 cells in 16-mm wells were incubated in medium supplemented with and without 1 $\mu M$ diferric transferrin or 1 $\mu M$ apotransferrin for 30 min at 37°C. The cells were then treated for 5 min with and without 10 nM EGF or HGF-I. The medium was aspirated and then rapidly replaced with medium (0°C) supplemented with 1 $\mu M$ diferric transferrin. The binding of the monoclonal antibody OKT9 to cell surface receptors was then determined as described under "Experimental Procedures." The results presented were obtained from a single experiment and are the means of triplicate determinations. Similar results were obtained in two separate experiments.

| Condition            | Control | EGF | HGF-I |
|----------------------|---------|-----|-------|
| Cell surface anti-transferrin receptor antibody bound | 2986 | 6589 | 5873 |
| Diferric transferrin (1 $\mu M$) | 2634 | 6114 | 5589 |
| Apotransferrin (1 $\mu M$) | 3091 | 6483 | 5710 |

the transferrin receptor at the cell surface (Table IV). This increase is similar to that observed with A431 cells (Fig. 1).

**DISCUSSION**

The transferrin receptor cycles rapidly between cell surface and endosomal membrane compartments (2). One consequence of this rapid cycling is that the receptors are exposed at the cell surface for only a brief period of time between exocytosis and endocytosis. The endocytotic rate constant, $k_2$ (Fig. 10), in control cells is estimated to be 0.33 min$^{-1}$. The half-life of the receptor in the plasma membrane can be calculated as $\ln 2/k_2 = 2.1$ min. This time is significant because the mean time required for low nanomolar concentrations of diferric transferrin to bind to the receptor is much greater than 2 min (Fig. 6B). As a result, the internalization of unoccupied receptors effectively acts as a competitive inhibitor of the internalization of receptors occupied by ligand. Consequently, the binding of diferric transferrin to cell surface receptors is not an equilibrium process at low concentrations of the ligand.

In order to investigate the consequences of nonequilibrium binding of transferrin to cell surface receptors, we have developed a simplified kinetic model for the cycling of the transferrin receptor (Fig. 10). As a kinetic model, this scheme is limited in that it does not take into account the molecular mechanisms involved in the cycling process. However, as a first approximation, this model is useful for investigating the complex properties of a nonequilibrium process. One success of the kinetic model is that it is able to account for the observation that the apparent $K_a$ for diferric transferrin binding to its receptor (Fig. 8). This is because the kinetic model predicts that the apparent $K_a$ is a function of the exocytotic and endocytotic rate constants (Table III). However, it is clear that this simplified kinetic model cannot account for all of the complex properties of transferrin receptor cycling, and further refinement of the model will be necessary to achieve this.

The purpose of the experiments presented here was to define the kinetic steps in the cycling of the transferrin receptor that are regulated by growth factors. The steady-state distribution of transferrin receptors between cellular membrane compartments is a function of the rate of entry into and exit from each compartment. Thus, the expression of transferrin receptors at the cell surface is a function of both the rate of exocytosis and the rate of endocytosis. The effect of growth factors to increase the cell surface expression of transferrin receptors could therefore be mediated by either a stimulation of exocytosis or an inhibition of endocytosis. The experiments reported here demonstrate that both of these processes are regulated by growth factors.

The primary site of action of EGF to regulate the cell surface expression of transferrin receptors is the exocytosis of the receptor from the endosomal compartment to the plasma membrane (3). The exocytotic rate constant is rapidly increased from 0.11 to 0.28 min$^{-1}$ after EGF treatment (Fig. 3). The location of exocytosis in A431 cells has been demonstrated to be the spreading margin of the cell (16). It is therefore interesting that this region of the cell exhibits membrane ruffling following EGF addition (17, 18). It is likely that this membrane ruffling is a result of the stimulated exocytotic activity. Fig. 3 shows that control and EGF-treated cells exocytose 18 and 46% of intracellular $^{131}$I-labeled ferrin in 2 min, respectively. As the cell surface population of transferrin receptors is less than 10% of the total number of transferrin receptors (Fig. 2), it can be calculated that the effect of EGF to stimulate exocytosis is sufficient to increase the cell surface expression of transferrin receptors by at least...
transferrin receptor endocytosis is independent of the cell surface receptor expression. A more detailed analysis of the simplified kinetic model of the cycling of the transferrin receptor (Fig. 10). This model predicts that EGF causes a rapid increase in the cell surface expression of the transferrin receptor (Table IV). We conclude that the stimulation of endocytosis caused by EGF is sufficient to account for the action of this growth factor to regulate the transferrin receptor (Fig. 1).

It has been reported that rIGF-I causes ruffling of the spreading margin of cultured cells in a similar manner to that caused by EGF (18). However, in contrast to the marked effect of EGF to stimulate the rate of transferrin receptor endocytosis, rIGF-I was observed to cause only a small increase in the rate of exocytosis (Fig. 3). The exocytotic rate constant was increased from 0.11 to 0.17 min⁻¹ after rIGF-I treatment. Fig. 3 shows that control and rIGF-I-treated cells exocytose 47 and 60% of intracellular ¹²⁵I-labeled transferrin in 5 min, respectively. If it is assumed that the velocity of receptor endocytosis is independent of the number of cell surface receptors, it can be calculated that the increased exocytosis is sufficient to increase the cell surface expression of transferrin receptors by at least 2.3-fold over 5 min. This increase is sufficient to account for the effect of rIGF-I on cell surface receptor expression (Fig. 1), but this conclusion is dependent on the assumption that the velocity of transferrin receptor endocytosis is constant under these conditions. Analysis of the predicted effect of rIGF-I to stimulate exocytosis can be made without this assumption using a simplified kinetic model of the cycling of the transferrin receptor (Fig. 10). The model predicts that the action of rIGF-I to cause an increase in the rate of exocytosis would result in only a small increase in the cell surface expression of the transferrin receptor (Table IV). We conclude that the stimulation of exocytosis caused by rIGF-I is not sufficient to account for the action of this growth factor to regulate the transferrin receptor (Fig. 1).

Investigation of the rate of transferrin receptor endocytosis revealed that rIGF-I regulated this step in the cycling pathway (Fig. 5). The endocytotic rate constants were estimated to be 0.33 and 0.24 min⁻¹ in control and rIGF-I-treated cells, respectively. A decrease in the endocytotic rate constant would be expected to cause an increase in the cell surface expression of the transferrin receptor. The inhibited endocytosis of receptor may therefore play a role in the regulation of cell surface expression of the transferrin receptor caused by rIGF-I. Analysis of the effect of the reduced endocytosis on cell surface expression of the transferrin receptor using the simplified kinetic model (Fig. 10) indicates that only a small increase in receptor expression is predicted (Table IV). However, the simultaneous regulation of both the endocytotic and exocytotic rate constants can account for the effect of rIGF-I to regulate the cell surface expression of the transferrin receptor (Table IV).

Treatment of cultured cells with rIGF-I causes an increase in the rate of uptake of ⁵⁹Fe-labeled diferric transferrin (4). rIGF-I stimulation of iron accumulation by cultured cells does not result from an increase in V₅₀₉ for diferric transferrin uptake, but rather from a decrease in apparent Kₘ for uptake (Fig. 9). Kₘ values for diferric transferrin uptake are estimated to be 57 and 31 nm in control and rIGF-I-treated cells, respectively. This decrease in apparent Kₘ for diferric transferrin uptake can be accounted for, in part, by the changes in the endocytotic and exocytotic rate constants caused by rIGF-I (Table IV). The observation that rIGF-I causes an increase in the rate of iron uptake because of a large decrease in the Kₘ for diferric transferrin raises a question about the physiological significance of this phenomenon. Transferrin is a major serum protein present at a concentration of about 10 μM in blood and is approximately 30% saturated with iron. The physiological concentration of diferric transferrin can therefore be estimated to be about 3 μM in serum. This concentration is sufficient to saturate cellular transferrin receptors. Thus, little effect of rIGF-I on iron uptake by cells suspended in serum would be expected. However, it is possible that the concentration of transferrin in the blood is significantly greater than that present in the interstitial space between cells. We are not aware of any direct measurements of the concentration of transferrin in the interstitial space, but there is reason to suspect that the interstitial concentration is less than that in serum. First, transferrin is a large molecule that would be expected to be excluded by the endothelial cells lining blood capillaries. Furthermore, it has been shown that endothelial cells express very few transferrin receptors, except those lining the blood-brain barrier (19). Second, transferrin provides a significant component of the colloidal osmotic pressure of blood and would therefore be expected to be retained within capillaries in order to maintain the venous blood volume. If the concentration of transferrin in the interstitial space is lower than that in serum, it is possible that IGF-I is a physiologically important regulator of the accumulation of iron by cells.

This work has helped to define exocytosis as the primary site of action of EGF to regulate the transferrin receptor and has shown that IGF-I is more complex in its action regulating both exocytosis and endocytosis. Further progress in this field will depend on the identification of the biochemical steps that are regulated by growth factors at these stages of the cycling process. Two hypotheses can be suggested to account for the biochemical mechanism of growth factor action on the transferrin receptor. First, the growth factors may regulate the machinery of the endocytotic and exocytotic steps. Evidence for this is provided by the observation that growth factors cause an increase in fluid-phase endocytosis (20, 21) and cause membrane ruffling (17, 18). Alternatively, the recent demonstration (22) that the transferrin receptor can cycle by two pathways suggests that growth factors could control the distribution of receptors between these pathways. A second hypothesis that can be proposed is that the growth factors regulate the transferrin receptor directly, for example by post-translational modification such as phosphorylation. Evidence for this has been provided by the observation that the phosphorylation of the transferrin receptor at serine 24 by protein kinase C (23) is associated with the regulation of transferrin receptor cycling (24–27).

In addition to the transferrin receptor (5), two other proteins have been shown to redistribute from an intracellular compartment to the plasma membrane after treatment of cells with insulin: the glucose transporter (28, 29) and the type II IGF receptor (30–32). The conclusions we have reached that define the sites of action of rIGF-I on the cycling of the transferrin receptor may be relevant to the regulation of the glucose transporter and the type II IGF receptor by insulin. Further work will be required to investigate whether the regulation of these proteins is mediated by inhibited endocytosis or stimulated exocytosis.

Acknowledgments—The excellent secretarial assistance of Karen Donahue and Mary Halley is greatly appreciated.
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