Biochem. J. (1989) 264, 35–46 (Printed in Great Britain)

Comparison of the kinetics of cycling of the transferrin receptor in the presence or absence of bound diferric transferrin

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The kinetics of cycling of the transferrin receptor in A431 human epidermoid-carcinoma cells was examined in the presence or absence of bound diferric transferrin. In order to investigate the properties of the receptor in the absence of transferrin, the cells were maintained in defined medium without transferrin. It was demonstrated that Fab fragments of a monoclonal anti-(transferrin receptor) antibody (OKT9) did not alter the binding of diferric ^{125}I-transferrin to the receptor or change the accumulation of [^{59}Fe]diferric transferrin by cells. OKT9 ^{125}I-Fab fragments were prepared and used as a probe for the function of the receptor. The first-order rate constants for endocytosis ($0.16 \pm 0.02\,min^{-1}$) and exocytosis ($0.056 \pm 0.003\,min^{-1}$) were found to be significantly lower for control cells than the corresponding rate constants for endocytosis ($0.22 \pm 0.02\,min^{-1}$) and exocytosis ($0.065 \pm 0.004\,min^{-1}$) measured for cells incubated with 1 μM-diferric transferrin (mean ± S.D., $n = 3$). The cycling of the transferrin receptor is therefore regulated by diferric transferrin via an increase in both the rate of endocytosis and exocytosis. Examination of the accumulation of OKT9 ^{125}I-Fab fragments indicated that diferric transferrin caused a marked decrease in the amount of internalized ^{125}I-Fab fragments associated with the cells after 60 min of incubation at 37 °C. Diferric transferrin therefore increases the efficiency of the release of internalized ^{125}I-Fab fragments compared with cells incubated without diferric transferrin. These data indicate that transferrin regulates the sorting of the transferrin receptor at the cell surface and within endosomal membrane compartments.

INTRODUCTION

Iron is essential for normal cellular proliferation [1]. The accumulation of iron via the receptor-mediated endocytosis of diferric transferrin is regulated by several independent processes that control the expression of transferrin receptors at the cell surface. Long-term regulation of transferrin-receptor expression is mediated, in part, by the action of iron to regulate the rate of transcription of the receptor gene [2] and to regulate the stability of the receptor mRNA [3–5]. In addition to the alterations in the rate of biosynthesis of the transferrin receptor, the expression of the receptor at the cell surface is acutely regulated. This short-term regulation of the expression of the transferrin receptor is mediated by the actions of growth factors, which cause a redistribution of intracellular receptors to the plasma membrane [6–12].

The mechanisms by which growth factors regulate the cell-surface expression of the transferrin receptor are not understood. The initial approach that has been taken to tackle this problem is to identify the sites of action of growth factors on the cycling pathway followed by the transferrin receptor. In these experiments diferric ^{125}I-transferrin has been used as a ligand to follow the kinetics of the cycling of the transferring receptor. By using these methods it has been observed that the primary site of action of epidermal growth factor is the stimulation of the rate of exocytosis of the transferrin receptor from an intracellular membrane compartment to the cell surface [6,11]. By contrast, insulin-like growth factor 1 (IGF-1) and insulin were observed to cause both a stimulation of exocytosis [11,12] and an inhibition of endocytosis [11]. A significant problem with these studies [6,11,12] is that, because diferric ^{125}I-transferrin has been used to investigate receptor cycling, the results obtained are only relevant to the cycling of receptors bound to the ligand, namely diferric transferrin. Until recently it was thought that only the occupied form of the transferrin receptor was competent for endocytosis [13–16]. However, it has recently been elegantly demonstrated that the transferrin receptor does internalize (with undefined kinetics) in the absence of bound diferric transferrin [17–19]. This information suggests that the regulation of the expression of cell-surface transferrin receptors by growth factors may be mediated by changes in the kinetics of the cycling of the unoccupied as well as the occupied receptor.

The purpose of the experiments described here was to develop a method to investigate the kinetics of the cycling of the transferrin receptor in the absence of bound diferric transferrin. To achieve this we have cultured A431 human epidermoid-carcinoma cells in defined medium lacking transferrin. The transferrin-depleted cells were used for the investigation of the kinetics of cycling of the transferrin receptor in the absence or presence of bound diferric transferrin using ^{125}I-Fab fragments of the monoclonal anti-(transferrin receptor) antibody OKT9 [20] as a probe for receptor

Abbreviation used: IGF-1, insulin-like growth factor 1.

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function. These studies extend previous reports describing the kinetics and morphology of the cycling of the transferrin receptor bound to diferric transferrin in A431 cells [11,21–24]. We present evidence that the kinetics of the cycling of the unoccupied transferrin receptor is distinct from that observed for the receptor bound to diferric transferrin.

EXPERIMENTAL

Materials

$Na^{125}I$ was obtained from Amersham. IGF-1 was from Amgen Corp. Human transferrin was from Behring Diagnostics. The monoclonal antibody OKT9 was purified by protein A–Sepharose chromatography. Fab fragments of OKT9 were prepared by papain digestion using reagents purchased from Pierce Chemical Co. and used according to the manufacturer's directions. The papain digestion mixture was applied to a protein A–Sepharose column and the flow-through fractions were collected. The Fab fragments in the flow-through fractions were further purified by Sephacryl S-200 chromatography. The Fab fragments were iodinated by the Iodogen method (Pierce) to a specific radioactivity of 5–12 Ci/g according to the manufacturer's directions. The ^{125}I-Fab fragments were isolated by Sephacryl S-200 chromatography. Diferric ^{125}I-transferrin (5–7 Ci/g) and [^{59}Fe]diferric transferrin (30–40 mCi/g) were prepared as described previously [8].

Cell culture

A431 human 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). Serum-free culture of A431 cells was performed using Ham's F12 medium (Gibco) supplemented with 100 pM-IGF-1. Hybridoma cells secreting the monoclonal antibody OKT9 were obtained from the American Type Culture Collection.

Binding of diferric ^{125}I-transferrin and OKT9 ^{125}I-Fab fragments 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 120 mM-NaCl/6 mM-KCl/1.2 mM-$MgCl_2$/1 mM-$CaCl_2$/25 mM-Hepes, pH 7.4, and 30 μM-bovine serum albumin. After experimental manipulation of the cells, the medium was removed and rapidly replaced with medium at 4 °C. Diferric ^{125}I-transferrin or ^{125}I-Fab fragments of the antibody OKT9 were then added to the cells, which were incubated for 180 min at 4 °C in a final volume of 0.5 ml. The monolayers were then washed three times with cold medium and solubilized with 900 μl of 1 M-NaOH. Radioactivity was quantified with a Beckman γ-radiation counter. Non-specific binding was estimated in incubations with a 200-fold excess of unlabelled ligand.

Analysis of the kinetics of OKT9 Fab fragment binding to cell-surface receptors at 37 °C

In order to measure the binding of ^{125}I-Fab fragments to cell-surface receptors, it is necessary to inhibit the rapid internalization of receptors that occurs at 37 °C. Previously we have reported that the metabolic depletion of A431 cells causes an inhibition of the endocytosis of transferrin receptors [11]. The metabolic depletion was performed by the addition of 0.45 ml of medium (4 °C) supplemented with 20 mM-2-deoxyglucose and 10 mM-NaN_3 to A431 cells in 16 mm-diam. wells. After 15 min the cells were placed in a 37 °C incubator for 30 min. ^{125}I-Fab fragments (50 μl) were added to the incubations (0.5 ml final volume) and, after defined times, 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. Non-specific binding was estimated in incubations containing a 200-fold excess of unlabelled ligand. Control experiments demonstrated that washing the cell monolayers with 150 mM-NaCl/50 mM-glycine, pH 3, for 3 min at 4 °C caused the dissociation of more than 90% of the specifically bound radioactivity. This result indicates that the endocytosis of the transferrin receptor was inhibited in metabolically depleted A431 cells. In order to establish further that the endocytosis of transferrin receptors was inhibited, the accumulation of iron by cells was examined. Rapid accumulation of radioactivity was observed by A431 cells incubated with 300 nM-[^{59}Fe]diferric transferrin. However, no significant accumulation of radioactivity was observed in experiments using metabolically depleted A431 cells incubated with [^{59}Fe]diferric transferrin. Together these data indicate that the internalization of the transferrin receptor was inhibited in metabolically depleted A431 cells.

The rate of dissociation of the ^{125}I-Fab fragments from cell-surface receptors at 37 °C was measured by using metabolically depleted cells. A431 cells were transferred from medium at 37 °C to medium (0.5 ml) at 4 °C supplemented with 20 mM-2-deoxyglucose and 10 mM-NaN_3. After 15 min the cells were placed in a 37 °C incubator for 30 min. The cells were then incubated (0.5 ml final vol.) with 10 nM-^{125}I-Fab fragments at 37 °C for 60 min in order to achieve equilibrium binding to cell-surface receptors. The cells were rapidly washed and subsequently incubated at 37 °C or 4 °C. At defined times the cell monolayers were washed rapidly at 4 °C, and the radioactivity remaining associated with the cells was measured by using a γ-radiation counter. Non-specific binding was estimated in incubations containing a 200-fold excess of unlabelled ligand.

Measurement of transferrin-receptor endocytosis

The endocytotic rate constant for transferrin-receptor internalization was measured by the In/Sur method described by Wiley & Cunningham [25], ^{125}I-Fab fragments of an anti-receptor antibody (OKT9) being used as a ligand. 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. A431 cells in 16 mm wells were incubated at 37 °C with 10 nM-OKT9 ^{125}I-Fab fragments (0.5 ml). At defined times the binding of the ^{125}I-Fab fragments to the cells was measured by rapidly washing the cell monolayers at 4 °C with medium and determining the associated radioactivity with a γ-radiation counter. Cell-surface and intracellularly bound ^{125}I-Fab fragments were determined by incubation of the cells for 3 min at 4 °C with 1 ml of 150 mM-NaCl/50 mM-glycine, pH 3.0. Intracellular ^{125}I-Fab fragments were estimated by measurement of the cell-associated radioactivity after

acid washing. Cell-surface-bound ^{125}I-Fab fragments were estimated by subtraction of the intracellularly bound ^{125}I-Fab fragments from the total specific binding of ^{125}I-Fab fragments to cell monolayers observed. Non-specific binding of OKT9 ^{125}I-Fab fragments was estimated in incubations containing a 200-fold excess of unlabelled ligand. The rate of endocytosis of diferric ^{125}I-transferrin (10 nM) by cells was measured by using the same procedure as that described for the internalization of ^{125}I-Fab fragments and has been previously described [11].

Measurement of transferrin-receptor exocytosis

The rate constant for the exocytosis of transferrin receptors was estimated by measuring the rate of exocytosis of ligands for the transferrin receptor (^{125}I-apo-transferrin and OKT9 ^{125}I-Fab fragments) using a pulse–chase technique. A431 cells were seeded in 16 mm wells and grown to a density of 5×10^4 cells per well. The cells were then incubated in 0.5 ml of medium supplemented with 300 nM-diferric ^{125}I-transferrin or 10 nM-^{125}I-Fab fragments for 5 min at 37 °C. The medium was then removed and the cells were washed at 0 °C and subsequently incubated for 3 min at 0 °C with 150 mM-NaCl/50 mM-glycine, pH 3.0, to remove ligand bound to the cell surface. The medium was then removed and the cells were incubated at 37 °C for defined times, washed rapidly with cold medium and subsequently washed with 150 mM-NaCl/50 mM-glycine, pH 3.0. The radioactivity remaining associated with the cells was measured with a γ-radiation counter.

The data obtained for the release of ligands was mathematically fitted as both a single- and a double-exponential decay process. To test which model provides a better description of the data, a simple statistical analysis (*F* test) was performed. The null hypothesis is that the single- and dual-exponential decay models provide equally reliable fits of the experimental data. We square, then sum, the deviation of the experimental data from the predicted curves as $\Sigma_I(\text{err})^2$ (abbreviated to S_I) and $\Sigma_{II}(\text{err})^2$ (abbreviated to S_{II}) for the single- and double-exponential models respectively. The explained deviation, E, is given as:

$$S_I = S_{II} + (S_I - S_{II}) = S_{II} + E$$

S_{II} representing the unexplained deviation. The F value is given by the equation:

$$F = \frac{E/y}{S_{II}/N - y - 1}$$

where y represents the number of parameters in the double-exponential decay model and N the number of experimental points in the data set.

Analysis of OKT9 ^{125}I-Fab fragment processing by A431 cells

The degradation of Fab fragments of OKT9 by A431 cells during incubation at 37 °C was investigated by incubation of the cells with ^{125}I-Fab fragments. The medium was removed, the cells were rapidly washed at 4 °C and cell-surface-bound ^{125}I-Fab fragments were removed by acid washing. Intracellular ^{125}I-Fab fragments were solubilized using 1% Triton X-100/25 mM-Hepes/1 mM-phenylmethanesulphonyl fluoride/leupeptin (10 μg/ml), pH 7.5. The three compartments of ^{125}I-Fab fragments (medium, bound to the cell surface, and intracellular) were then analysed by gel-filtration chromatography using Sephadex G-50 and by polyacrylamide-gel electrophoresis followed by autoradiography.

Analysis of the expression of the transferrin receptor

The effect of the antibody OKT9 and Fab fragments of this antibody on the expression of transferrin receptors in A431 cells was investigated. A431 cells were seeded in 22 mm wells and grown to a density of 1×10^5 cells per well. The cells were then transferred to medium containing 10 μM-[^{35}S]methionine (20 μCi/ml) for 24 h. The cells were treated with antibodies or Fab fragments during the last 12 h of this incubation. The transferrin receptors were then immunoprecipitated from the solubilized cells and analysed by polyacrylamide-gel electrophoresis and fluorography as described in [26].

RESULTS

Characterization of the binding of OKT9 Fab fragments to A431 cells

In order to use OKT9 Fab fragments as a probe for the function of transferrin receptors in intact A431 cells, it was necessary to characterize the kinetic and equilibrium-binding properties in detail. Therefore experiments were designed to investigate the kinetics of the interaction of the Fab fragments with the surface of A431 cells. The binding of ^{125}I-Fab fragments to the surface of A431 cells was examined at 37 °C (Fig. 1). In order to prevent

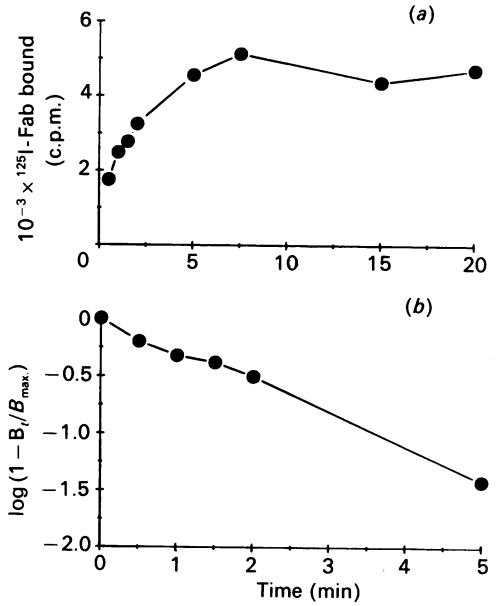

Fig. 1. Time course of ^{125}I-Fab fragment binding to cell-surface receptors

A431 cells were incubated with 20 mM-2-deoxyglucose and 10 mM-NaN_3 in order to inhibit receptor internalization. The specific binding of 10 nM-^{125}I-Fab fragments at 37 °C to the A431 cell monolayers was then measured after different times of incubation. The results presented are the means of the data obtained in three experiments. The second-order rate constant for ^{125}I-Fab fragment binding to the surface of A431 cells was estimated to be 7×10^7 M$^{-1} \cdot$ min^{-1}. B_t and $B_{max.}$ refer to the binding observed at time t and the maximum binding respectively.

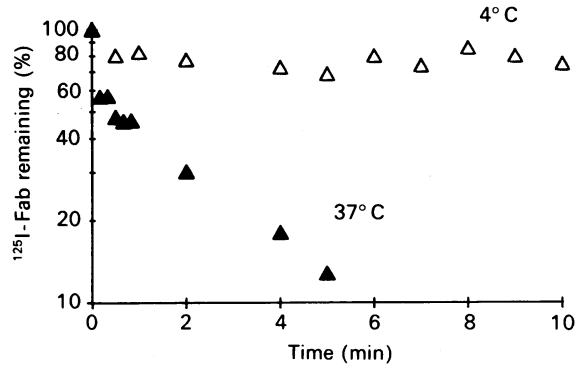

Fig. 2. Time course of dissociation of ^{125}I-Fab fragments

A431 cells were incubated with 20 mM-2-deoxyglucose and 10 mM-NaN_3 in order to inhibit receptor internalization. The cells were then incubated with 10 nM-^{125}I-Fab fragments for 3 h at 4 °C in order to obtain equilibrium binding to the cell-surface receptors. The cells were washed rapidly at 4 °C and then incubated at 37 (▲) or 4 °C (△) for defined times. The medium was aspirated, and the radioactivity that remained associated with the cell monolayers was measured. The results are expressed as the amount bound at different times of dissociation relative to the equilibrium level of binding observed. The first-order rate constants for the dissociation were estimated to be 0.03 and 0.6 min^{-1} at 4 °C and at 37 °C respectively.

receptor internalization, the cells were metabolically depleted [11] by incubation with 2-deoxyglucose and azide before the addition of ^{125}I-Fab fragments. Incubation of cell monolayers with 10 nM-^{125}I-Fab fragments resulted in rapid and specific binding to the cells. Half-maximal binding was observed after 1 min, and maximal binding was observed within 5 min of incubation at 37 °C (Fig. 1). The second-order rate constant for ^{125}I-Fab fragment binding to the cell surface was calculated to be 7×10^7 $M^{-1} \cdot min^{-1}$ (Fig. 1). This rate is similar to that described for the kinetics of binding of diferric ^{125}I-transferrin to A431 cells, namely 2.7×10^7 $M^{-1} \cdot min^{-1}$ [11].

The dissociation of ^{125}I-Fab fragments from the surface of A431 cells was measured at 4 °C and at 37 °C (Fig. 2). The cells were metabolically depleted to inhibit receptor internalization and then incubated at 4 °C for 3 h with 10 nM-^{125}I-Fab fragments. [Control experiments demonstrated that at 4 °C steady-state binding of ^{125}I-Fab fragments was observed after 3 h of incubation (results not shown).] The cells were washed rapidly at 4 °C and then incubated at 4 or at 37 °C to initiate dissociation. Rapid dissociation of the ^{125}I-Fab fragments was observed at 37 °C (Fig. 2). By contrast, the dissociation at 4 °C was slow (Fig. 2). The first-order rate constants for the dissociation of ^{125}I-Fab fragments from the surface of A431 cells was estimated to be 0.03 min^{-1} and 0.6 min^{-1} at 4 and 37 °C respectively. These rates of dissociation are lower than the rate of dissociation of ^{125}I-apotransferrin from A431-cell receptors, 2.6 min^{-1} [11].

Fig. 3 shows the investigation of the ^{125}I-Fab fragment binding isotherm presented by the method of Scatchard [27]. The linear plot obtained is consistent with the presence of a single class of high-affinity (K_d = 1.8 nM) binding sites (26000/cell) on the surface of A431 cells. In

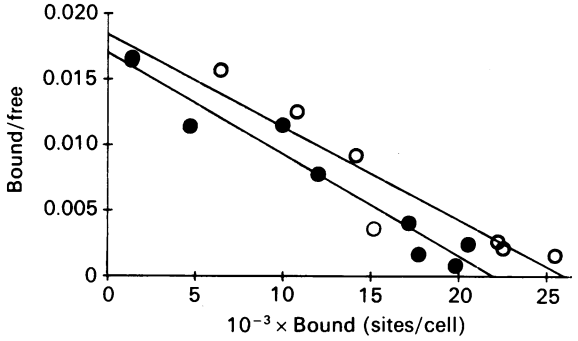

Fig. 3. Analysis of the OKT9 ^{125}I-Fab fragment binding isotherm

The ^{125}I-Fab fragment binding isotherm was determined at 4 °C in the presence (●) or absence (○) of 1 μM-diferric transferrin. Cells were incubated with different concentrations of ^{125}I-Fab fragments for 3 h in order to achieve equilibrium binding. Non-specific binding was determined in incubations within a 200-fold excess of unlabelled ligand. Non-specific binding was less than 7% of the total binding observed at all of the concentrations of ^{125}I-Fab fragments examined. The specific binding observed was analysed using the computer program EBDA (Elsevier Biosoft). The data were plotted as described by Scatchard [27] and represent those obtained in five separate experiments. A linear plot was obtained for control cells and for cells incubated with 1 μM-diferric transferrin. The data are consistent with a single class of high-affinity binding sites expressed at the cell surface (control: K_d 1.8 nM, 2.6×10^4 sites per cell; diferric transferrin: K_d 1.4 nM, 2.3×10^4 sites per cell).

the presence of 1 μM-diferric transferrin, a small decrease in the binding of ^{125}I-Fab fragments to high-affinity (K_d = 1.4 nM) sites (23000/cell) was observed. No significant change in the affinity of binding of the ^{125}I-Fab fragments was observed (Fig. 3). The mechanism by which diferric transferrin causes a small decrease in the apparent number of Fab-fragment-binding sites at the surface of A431 cells is not understood. It is possible that diferric transferrin causes a small fraction of the cell-surface transferrin receptors to be sequestered in a cryptic state.

The equilibrium and kinetic rate constants for the binding of transferrin and OKT9 Fab fragments to the surface of A431 cells are summarized in Table 1. The

Table 1. Summary of the equilibrium binding and kinetic rate constants for ligand binding to the transferrin receptor at pH 7.4 and 37 °C

The constants for the binding of OKT9 Fab fragments were taken from Figs. 1–3. The equilibrium binding constant for diferric transferrin and the rate constants for the association of diferric transferrin and the dissociation of apotransferrin were taken from [11].

Parameter (units)	^{125}I-transferrin	OKT9 ^{125}I-Fab fragments
K_{on} ($M^{-1} \cdot min^{-1}$)	2.7×10^7	7×10^7
K_{off} (min^{-1})	2.6	0.6
K_d (nM)	1.5	1.8

binding properties of these two ligands for the receptor are similar at pH 7.4, with the exception that the rate of dissociation of ^{125}I-apotransferrin (2.6 min^{-1}) is markedly higher than the rate of dissociation of OKT9 ^{125}I-Fab fragments (0.6 min^{-1}). The high rate of dissociation of apotransferrin at pH 7.4 ensures that this ligand is released from the receptor after exocytosis to complete the transferrin-receptor cycle. The lower rate of Fab-fragment release may result in the incomplete dissociation of Fab fragments from the receptor at pH 7.4 after exocytosis during receptor cycling. As some endosomes are acidified, the rate of dissociation of ^{125}I-apotransferrin and OKT9 ^{125}I-Fab fragments was also measured at pH 4.8 (4 °C). The first-order rate constants for dissociation were measured and found to be 0.036 and 0.044 min^{-1} for OKT9 ^{125}I-Fab fragments and ^{125}I-apotransferrin respectively (results not shown). The low rate of dissociation from the receptor at pH 4.8 of both Fab fragments and apotransferrin indicates that little release of these ligands occurs in acidified endosomes.

Effect of incubation of cells with OKT9 Fab fragments

The binding properties of OKT9 Fab fragments are similar to those of transferrin (Table 1). These data indicate that Fab fragments could be used to investigate transferrin-receptor cycling using protocols similar to those described, employing ^{125}I-transferrin as a ligand for the receptor. However, before OKT9 Fab fragments can be used as a probe for the function of transferrin receptors in A431 cells, it is important to demonstrate that the binding of the Fab fragments does not alter the properties of the receptor. We therefore investigated the effect of incubation of cells with OKT9 Fab fragments on the expression and cycling of the transferrin receptor.

It has been reported that the OKT9 antibody causes a redistribution of transferrin receptors in K562 erythroleukaemia cells and results in the lysosomal degradation of the receptors and the antibody [28]. The effect of this enhanced degradation is that the

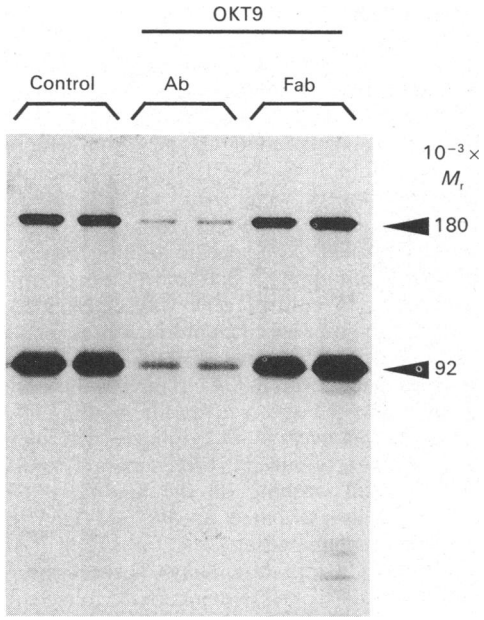

Fig. 4. Effect of OKT9 Fab fragments on the expression of transferrin receptors by A431 cells

A431 cells were incubated for 24 h with 10 μM-[^{35}S]methionine (20 μCi/ml). During the last 12 h of this incubation the cells were incubated without (Control) or with 5 nM-OKT9 antibody (Ab) or 10 nM-OKT9 Fab fragments (Fab). The transferrin receptors were then isolated by immunoprecipitation and reduced with 50 mM-dithiothreitol. The immunoprecipitates were electrophoresed on a 7%-(w/v)-polyacrylamide gel, and the transferrin receptors were revealed by fluorography using En^{3}Hance (du Pont–New England Nuclear) and Kodak X-Omat AR film. Under these conditions the dimeric (M_r 180000) receptor is incompletely reduced to the monomeric (M_r 92000) form. The difficulty in reducing the disulphide-linked dimeric receptor to the fully reduced monomeric form has been observed in previous studies [26].

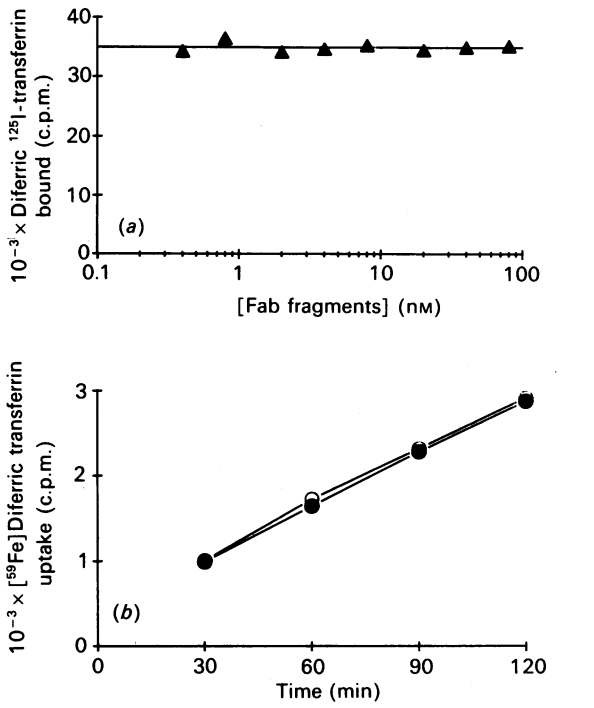

Fig. 5. Effect of OKT9 Fab fragments on the binding of diferric transferrin

(*a*) Effect of Fab fragments on diferric ^{125}I-transferrin binding. A431 cells were incubated at 4 °C for 3 h with 10 nM-diferric ^{125}I-transferrin in the presence or absence of different concentrations of OKT9 Fab fragments. The cells were rapidly washed at 4 °C, and the specific binding of diferric ^{125}I-transferrin to cell-surface receptors was measured. The total binding of diferric ^{125}I-transferrin to control cells was 26532 c.p.m. and non-specific binding was estimated to be 365 c.p.m. The specific binding to control cells was calculated to be 26167 c.p.m. The results presented are averages for triplicate determinations. Similar results were obtained in three separate experiments. (*b*) Effect of OKT9 Fab fragments on [^{59}Fe]diferric transferrin uptake. A431 cells were incubated at 37 °C with 300 nM-[^{59}Fe]diferric transferrin in the presence (●) or absence (○) of 20 nM-OKT9 Fab fragments. At defined times the cells were rapidly washed at 4 °C and the radioactivity associated with the cells was determined using a β-radiation counter. The results presented are averages for triplicate determinations. Similar results were obtained in three separate experiments.

steady-state number of transferrin receptors is markedly decreased when cells are incubated with the OKT9 antibody [28]. The action of the OKT9 antibody to decrease transferrin-receptor expression was also observed when A431 cells were incubated with the antibody (Fig. 4). However, no effect of the Fab fragments was observed on the level of transferrin-receptor expression (Fig. 4). These data demonstrate that, although the bivalent OKT9 antibody causes the degradation of transferrin receptors, the univalent Fab fragments prepared from this antibody do not significantly regulate the expression of transferrin receptors in A431 cells.

The rate of degradation of transferrin by cultured cells is extremely low because the internalized diferric transferrin is exocytosed from the cell as apotransferrin (reviewed by May & Cuatrecasas [29]). By contrast, the OKT9 antibody after binding to the transferrin receptor is rapidly degraded in lysosomes [28]. This observation indicates that the OKT9 antibody is processed as a transferrin-receptor ligand by cells in a manner different from that described for transferrin. It was therefore important to investigate the processing of OKT9 Fab fragments by A431 cells. To do this, cells were incubated with 10 nM-^{125}I-Fab fragments for 0.5, 1, 2, 3, 4 and 5 h at 37 °C. The state of degradation of the Fab fragments was examined by gel-filtration chromatography using Sephadex G-50 and by polyacrylamide-gel electrophoresis. The radioactivity extracted from the incubations co-migrated with a reference ^{125}I-Fab fragment sample (results not shown). We conclude that OKT9 Fab fragments are not degraded during the incubation of the cells at 37 °C.

To examine further the possible effect of OKT9 Fab fragments to perturb transferrin-receptor function, experiments were designed to investigate the effect of Fab fragments on the binding and endocytosis of diferric transferrin. Fig. 5(*a*) shows that Fab fragments were observed to have no significant effect on the binding of diferric ^{125}I-transferrin to A431 cells. Furthermore, the Fab fragments did not significantly change the rate of accumulation of radioactivity by cells incubated with [^{59}Fe]diferric transferrin. Together these data indicate that OKT9 Fab fragments are suitable as a probe for transferrin-receptor function that does not alter the properties of the interaction of the receptor with diferric transferrin.

Accumulation of OKT9 Fab fragments by A431 cells

In order to use OKT9 ^{125}I-Fab fragments as a probe for the cycling of the transferrin receptor, it was necessary to be able to distinguish between the ligand bound to the cell surface and intracellular ^{125}I-Fab fragments. Previous studies of transferrin-receptor cycling have employed acid washing of cells at 4 °C to distinguish between acid-labile (cell-surface) and acid-resistant (internalized) ^{125}I-transferrin [30,31]. The use of this approach to examine the internalization of OKT9 ^{125}I-Fab fragments was investigated. Fig. 6(*a*) shows that the binding of ^{125}I-Fab fragments to the surface of A431 cells is markedly inhibited by incubation of the monolayers for 3 min at low pH. The highest pH at which maximal displacement of ^{125}I-Fab fragment binding occurred was pH 3. Acid washing at pH 3 was therefore used in further experiments to investigate the subcellular distribution of bound ^{125}I-Fab fragments.

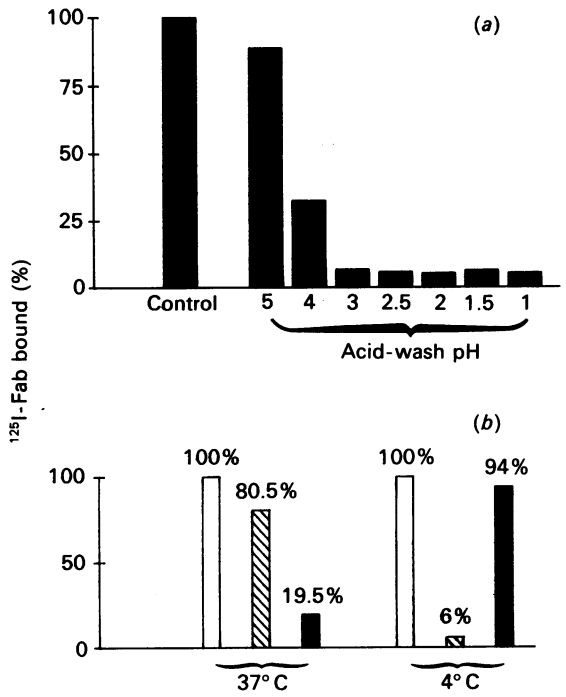

Fig. 6. Effect of pH on the binding of OKT9 ^{125}I-Fab fragments to A431 cells

(*a*) Effect of the pH of acid washing on the binding of OKT9 ^{125}I-Fab fragments. A431 cells were incubated with ^{125}I-Fab fragments at 4 °C for 3 h. The cells were washed rapidly and then incubated at 4 °C for 3 min with medium (control) or with 150 mM-NaCl/50 mM-glycine (pH 1–5). The cell monolayers were then washed rapidly with medium (4 °C) and the radioactivity associated with the cells was determined. Non-specific binding was examined in incubations containing a 200-fold excess of unlabelled ligand. Binding to control cells was determined to be 16632 c.p.m., and non-specific binding was estimated to be 458 c.p.m. The specific binding of ^{125}I-Fab fragments to the cells was calculated to be 16174 c.p.m. The data presented are normalized to the observed specific binding to control cells and are the average of triplicate determinations. Similar results were obtained in three separate experiments. (*b*) Effect of acid washing on the binding of ^{125}I-Fab fragments to cells incubated at different temperatures. A431 cells were incubated at 37 °C for 2 h or at 4 °C for 3 h with 10 nM-^{125}I-Fab fragments. The cells were then washed rapidly at 4 °C and then incubated for 3 min (4 °C) with or without 150 mM-NaCl/50 mM-glycine, pH 3. The cell monolayers were rapidly washed at 4 °C and the radioactivity associated with the cells was measured. At 37 °C the total binding observed was 20972 c.p.m. and the non-specific binding was estimated to be 1251 c.p.m. The total specific binding observed at 37 °C was calculated to be 19721 c.p.m. At 4 °C the total binding and non-specific binding were measured and found to be 8313 c.p.m. and 472 c.p.m. respectively. The total specific binding (□) observed at 4 °C was calculated to be 7841 c.p.m. The amount of ^{125}I-Fab fragments internalized (▨) was estimated from the radioactivity associated with the cells after acid washing, and the amount of cell-surface bound ^{125}I-Fab fragments (■) was calculated as the difference between the total binding and the binding that was resistant to the acid washing. The data presented are the averages of triplicate determinations and are normalized to the total specific binding observed (100%). Similar results were obtained in three separate experiments.

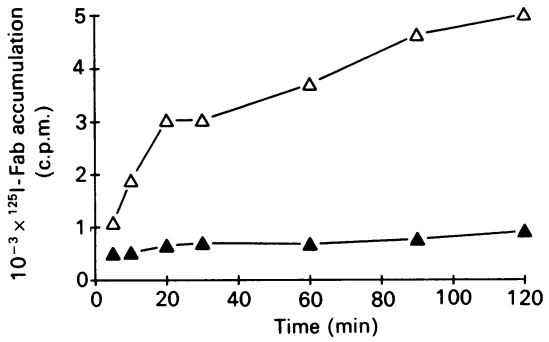

Fig. 7. Accumulation of OKT9 ^{125}I-Fab fragments by cells at 37 °C

A431 cells were incubated in the presence (▲) or absence (△) of 1 μM-diferric transferrin at 37 °C with 10 nM-^{125}I-Fab fragments. At defined times the cells were rapidly washed at 4 °C and the radioactivity associated with the cells was measured. The results presented are the average of triplicate determinations. Similar results were obtained in five separate experiments.

To test the acid-washing procedure, cells were incubated at 4 and at 37 °C with ^{125}I-Fab fragments (Fig. 6*b*). At 4 °C, receptor internalization is inhibited and the bound ^{125}I-Fab fragments are expected to be at the cell surface. Acid washing resulted in the displacement of 94% of the bound radioactivity, consistent with the cell-surface location of the ^{125}I-Fab fragments (Fig. 6*b*). At 37 °C, rapid internalization of the transferrin receptor is observed, resulting in substantial intracellular accumulation of ligand bound to the receptor. Under these conditions, acid washing at 4 °C caused the displacement of only 19.5% of the bound radioactivity. These data indicate that there is a marked decrease in the effectiveness of the acid-washing procedure to displace ^{125}I-Fab fragments bound to cells at 37 °C compared with that found at 4 °C. This result is consistent with the expected intracellular accumulation of ^{125}I-Fab fragments at 37 °C. We conclude that acid washing at pH 3 for 3 min at 4 °C can be used to distinguish between cell-surface- and intracellularly bound ^{125}I-Fab fragments. This procedure was used in further experiments to investigate the cycling of the transferrin receptor.

In initial experiments to investigate the cycling of the transferrin receptor using ^{125}I-Fab fragments as a ligand for the receptor, we examined the time course of accumulation of the ^{125}I-Fab fragments by A431 cells at 37 °C (Fig. 7). The ^{125}I-Fab fragments were initially rapidly taken up by the cells, and steady-state accumulation was achieved after 2 h of incubation at 37 °C (Fig. 7). A markedly different result was obtained when the cells were incubated with 1 μM-diferric transferrin, which caused a large decrease in the extent of accumulation of ^{125}I-Fab fragments (Fig. 7). Although diferric transferrin does cause a small decrease in the number of ^{125}I-Fab-fragment-binding sites expressed at the surface of A431 cells (Fig. 3), the inhibition of ^{125}I-Fab-fragment accumulation caused by diferric transferrin is much greater than that which can be accounted for by the inhibition of binding (Fig. 7). An alternative explanation of the observations is that the accumulation of ^{125}I-Fab fragments is decreased because diferric transferrin changes the cycling kinetics or the morphological pathway followed by the receptor. Therefore, in order to examine the cause of the decreased accumulation of ^{125}I-Fab fragments by A431 cells, experiments were designed to test the hypotheses that diferric transferrin (1) inhibits the rate of transferrin-receptor endocytosis and (2) stimulates the rate of transferrin-receptor exocytosis.

Endocytosis of OKT9 Fab fragments by A431 cells

The effect of diferric transferrin on the rate of receptor endocytosis was measured under steady-state conditions by the In/Sur method described by Wiley & Cunningham [25]. 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. Linear intracellular accumulation of ^{125}I-Fab fragments was observed for up to 6 min of incubation at 37 °C (results not shown). After this time a decrease in the rate of accumulation was observed because of the exocytosis of accumulated ^{125}I-Fab fragments. The first-order endocytotic rate constant for control cells was estimated to be $0.16 \pm 0.02\ min^{-1}$ (mean ± S.D., $n = 3$). In the presence of 1 μM-diferric transferrin the rate constant was calculated to be $0.22 \pm 0.02\ min^{-1}$ (mean ± S.D., $n = 3$). These data demonstrate that the decreased accumulation of ^{125}I-Fab fragments caused by diferric transferrin was not due to an inhibition of receptor endocytosis. By contrast, the data indicate that diferric transferrin causes an increase in the rate of endocytosis of receptors bound to ^{125}I-Fab fragments. The measured endocytotic rate constant in the presence of apotransferrin was $0.17 \pm 0.02\ min^{-1}$ (mean ± S.D., $n = 3$). This rate is not significantly different from the rate measured using control cells, but is significantly lower than the rate of internalization of ^{125}I-Fab fragments measured in the presence of diferric transferrin. The measured endocytotic rate constants are summarized in Table 2.

The rate of endocytosis of ^{125}I-Fab fragments measured in the presence of 1 μM-diferric transferrin (Table 2) is significantly lower than the reported rate of endocytosis of diferric ^{125}I-transferrin ($0.33\ min^{-1}$) by A431 cells [11]. This result suggests that the endocytosis of ^{125}I-Fab fragments may not be a reliable measure of the rate of

Table 2. Measurement of the kinetic rate constant of transferrin-receptor endocytosis

The rate of transferrin-receptor endocytosis was estimated by measuring the rate of internalization of OKT9 ^{125}I-Fab fragments as described in the Experimental section. The rate of ^{125}I-Fab-fragment endocytosis was determined for cells incubated in the absence of transferrin (control) or in the presence of 1 mM-diferric transferrin or 1 μM-apotransferrin. The rate of endocytosis of diferric ^{125}I-transferrin was also measured. The data presented are the means ± S.D. of the results obtained in three separate experiments.

	Rate constant (min^{-1})	
	OKT9 ^{125}I-Fab	Diferric ^{125}I-transferrin
Control	0.16 ± 0.02	–
Diferric transferrin	0.22 ± 0.02	0.24 ± 0.03
Apo-transferrin	0.17 ± 0.02	–

internalization of transferrin receptors, and this possibility was further examined in control experiments. The A431 cells used in the present study were maintained in defined (serum-free) medium in the absence of diferric transferrin and may have properties different from those of A431 cells reported in previous studies in which the cells were maintained in medium containing 5% (v/v) calf serum. The rate of endocytosis of diferric ^{125}I-transferrin was therefore examined in transferrin-depleted cells, so that the measured rate could be directly compared with that found for the internalization of ^{125}I-Fab fragments. Table 2 shows that the measured endocytotic rate constant for diferric ^{125}I-transferrin was $0.24 \pm 0.03\ min^{-1}$ (mean ± S.D., $n = 3$). This rate is similar to that found for the endocytosis of ^{125}I-Fab fragments ($0.22 \pm 0.02\ min^{-1}$). We conclude that the internalization of ^{125}I-Fab fragments provides a reliable estimate of transferrin-receptor endocytosis and that the rate of endocytosis observed is higher in cells grown in serum compared with serum-free culture medium.

Exocytosis of OKT9 Fab fragments by A431 cells

Diferric transferrin increased the endocytotic rate constant for the internalization of ^{125}I-Fab fragments (Table 2). By contrast, it was observed that diferric transferrin markedly reduced the accumulation of ^{125}I-Fab fragments by cells (Fig. 7). To account for the decreased accumulation of ^{125}I-Fab fragments concomitant with an increased endocytotic rate constant it is necessary to propose that diferric transferrin causes an increase in the rate of ^{125}I-Fab-fragment exocytosis. This hypothesis was directly tested by measuring the rate of exocytosis of ^{125}I-Fab fragments from cells incubated with and without diferric transferrin using a pulse–chase procedure (Fig. 8). In control cells, a rapid release of intracellular ^{125}I-Fab fragments was observed (Table 3). The first order rate constant for the exocytosis of ^{125}I-Fab fragments was calculated to be $0.056 \pm 0.004\ min^{-1}$ (Table 3). The measured first-order rate constant for the exocytosis of ^{125}I-apotransferrin ($0.072 \pm 0.005\ min^{-1}$; results not shown) is significantly higher than the rate of release of OKT9 ^{125}I-Fab fragments ($0.056 \pm 0.004\ min^{-1}$). These data suggest that the rate of exocytosis of the transferrin receptor is higher in the presence than in the absence of diferric transferrin. To test this hypothesis we measured the rate of release of ^{125}I-Fab fragments from cells in the presence or absence of diferric transferrin. It was observed that diferric transferrin caused

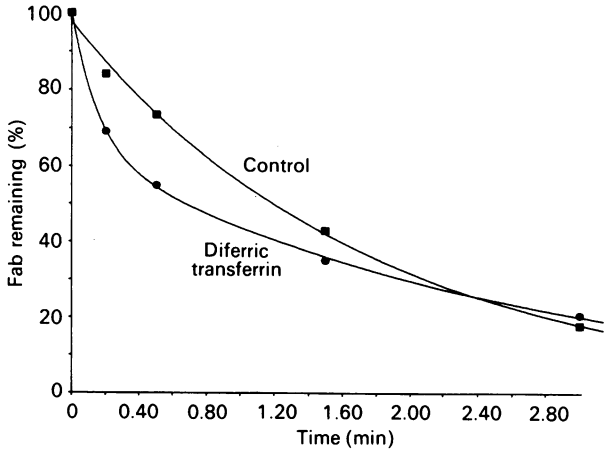

Fig. 8. Exocytosis of ^{125}I-Fab fragments from A431 cells

A431 cells were incubated for 5 min at 37 °C with 10 nM-^{125}I-Fab fragments in the presence (●) or absence (■) of 1 μM-diferric transferrin. The cells were rapidly washed at 4 °C and then incubated for 30 min with 120 mM-NaCl/50 mM-glycine, pH 3, at 4 °C in order to remove cell-surface-bound ^{125}I-Fab fragments. Exocytosis of ^{125}I-Fab fragments was then initiated by incubation of the cells in medium at 37 °C. At defined times the cells were rapidly washed at 4 °C and acid-washed for 3 min. The radioactivity remaining associated with the cells was measured. The data presented are the means of triplicate determinations obtained in four separate experiments. The data were examined using the computer program ENZFITTER (Elsevier Biosoft), and the results of this analysis are presented in Table 3. The curves drawn correspond to the fit of the data to a single-exponential-decay model (control) and to a double-exponential-decay model (diferric transferrin) as described in Table 3.

Table 3. Measurement of the kinetic rate constant of transferrin-receptor exocytosis

The rate of transferrin-receptor exocytosis was estimated by measuring the rate of release of OKT9 ^{125}I-Fab fragments and ^{125}I-transferrin as described in the Experimental section. The rate of ^{125}I-Fab fragment release was determined for cells incubated in the absence (control), or in the presence, of 1 μM-diferric transferrin. The data obtained were analysed by using the computer program ENZFITTER (Elsevier Biosoft) and mathematically fitted to either a single- or a double-exponential-decay model. The first-order rate constants for exocytosis (mean ± S.D.) were calculated on the basis of data obtained in three separate experiments. The total data set for each determination comprises 90 data points. A statistical analysis (F test) of the quality of the fit of the two models using the null hypothesis that the two models provide an equally accurate description of the experimental data was performed. In the absence of diferric transferrin, no statistical significance between the quality of fit of the data by the two models was found ($P > 0.05$). In the presence of diferric transferrin, the double-exponential-decay model is a significantly better description ($P < 0.001$) of the experimental data than the single-exponential-decay model.

	Single exponential decay	Double exponential decay			
	First-order rate constant (min^{-1})	Initial value (%)	First-order rate constant (min^{-1})	F	P
Control	0.056 ± 0.004	77 ± 52.0 23 ± 52.0	0.064 ± 0.03 0.036 ± 0.05	2.12	> 0.05
Diferric transferrin	0.065 ± 0.003	38 ± 3.0 62 ± 3.0	0.64 ± 0.11 0.038 ± 0.003	12.6	< 0.001

an increase in the rate of ^{125}I-Fab fragment release compared with that observed from control cells (Table 3). The measured first-order rate constants for ^{125}I-Fab fragment release in the absence or presence of diferric transferrin were calculated to be 0.056 ± 0.004 min^{-1} and 0.065 ± 0.002 min^{-1} (mean $\pm$ S.D., $n = 3$) respectively. We conclude that diferric transferrin stimulates the rate of exocytosis of the transferrin receptor (Table 3).

The experimental data for the exocytosis of ^{125}I-Fab fragments were mathematically fitted to both a single- and a double-exponential-decay model (Table 3). In the absence of transferrin there was no statistically significant difference between the quality of fit of the data by the two models. Therefore we take the simplest model, the single exponential-decay model, to be an adequate description of the experimental data. However, in the presence of transferrin the double-exponential-decay model was found to be a significantly better description of the experimental data obtained. The double-exponential-decay model for the exocytosis of ^{125}I-Fab fragments in the presence of transferrin comprises two kinetically distinct processes. The first is rapid exocytosis (0.64 min^{-1}) and accounts for the release of 38% of the internalized ^{125}I-Fab fragments. The second is slower (0.038 min^{-1}) and accounts for the release of 62% of the intracellular ^{125}I-Fab fragments. The rate of the slow component of exocytosis in the presence of diferric transferrin is similar to the rate of exocytosis measured in control cells. By contrast, the fast component of exocytosis is not observed in control cells and represents an 11-fold increase in the exocytotic rate constant compared with the rate measured in control cells (Table 3).

Effect of diferric transferrin on the cell-surface expression of transferrin receptors

Diferric transferrin increased the endocytotic rate constant for the internalization of the transferrin receptor (Table 2). An increase in the rate of exocytosis was also observed (Table 3). As the cell-surface expression of the transferrin receptor is a function of both the endocytotic and the exocytotic rates, we examined whether diferric transferrin regulated the cell-surface expression of receptors. A431 cells were incubated without and with 1 mM-diferric transferrin for different times at 37 °C. The medium was aspirated and then replaced with medium (4 °C) supplemented with 10 nM-^{125}I-Fab fragments and 1 μM-diferric transferrin. No marked change in the binding of ^{125}I-Fab fragments was observed when cells were incubated at 37 °C for different times with diferric transferrin (results not shown). We conclude that, at steady state, the cell-surface expression of transferrin receptors is similar in the presence or in the absence of diferric transferrin. This conclusion contrasts with previous reports describing marked changes in transferrin-receptor distribution after diferric transferrin addition to HL60 cells and rabbit reticulocytes [32,33]. The results obtained for these cells may reflect differences between the cycling of the transferrin receptor in different cell types.

DISCUSSION

Use of OKT9 Fab fragments as a probe for transferrin-receptor function

OKT9 is a murine monoclonal antibody that binds to the human transferrin receptor [20]. The epitope that is recognized by the antibody is located in the extracellular domain of the receptor. The binding of the antibody OKT9 has been reported to result in no significant change in the binding of diferric transferrin to the receptor [28]. It can therefore be proposed that the antibody OKT9 can be used as a probe for the receptor that is independent of the binding of diferric transferrin. However, two properties of the bivalent antibody limit its use as a probe for transferrin receptor function. First, it has been reported that equilibrium binding is an inappropriate description of the interaction of bivalent antibodies with cell-surface antigens [34]. Thus the data obtained from the analysis of the binding of OKT9 to cell-surface transferrin receptors are difficult to interpret. Secondly, it has been shown that the OKT9 antibody causes a redistribution of transferrin receptors in K562 erythroleukaemia cells and results in the degradation of both the antibody and the transferrin receptors in lysosomes [28]. Together these considerations indicate that the antibody OKT9 is not suitable for the investigation of transferrin-receptor function. As an alternative approach to the use of the bivalent antibody, we have examined the properties of OKT9 Fab fragments.

In order to investigate whether OKT9 Fab fragments cause changes in the function of the transferrin receptor, several control experiments were performed; their results were as follows: (i) OKT9 Fab fragments did not regulate the binding of diferric ^{125}I-transferrin to cells (Fig. 4*a*); (ii) the accumulation of [^{59}Fe]diferric transferrin by cells was not altered by incubation of cells with OKT9 Fab fragments (Fig. 4*b*); (iii) in contrast with the marked receptor degradation caused by the OKT9 antibody, incubation of cells with Fab fragments did not alter the expression of transferrin receptors (Fig. 5); (iv) whereas the OKT9 antibody is degraded by cells in lysosomes [28], Fab fragments were not observed to be significantly degraded when incubated with cells; (v) the rate of endocytosis of ^{125}I-Fab fragments by cells incubated with diferric transferrin was similar to that observed for diferric ^{125}I-transferrin (Table 2); (vi) the rate of exocytosis of ^{125}I-Fab fragments from cells incubated with diferric transferrin was similar to the rate of release of ^{125}I-apotransferrin (Table 3). These data indicate that, for the properties of the transferrin receptor investigated, the binding of Fab fragments does not perturb the function of the receptor. We conclude that OKT9 Fab fragments are suitable for use as a probe for transferrin cycling.

Comparison of the kinetics of binding of transferrin and OKT9 Fab fragments at 37 °C to the surface of A431 cells indicated that the binding properties of the two ligands are similar (Table 1). However, two distinct differences between transferrin and Fab-fragment binding were noted and are described below.

***(a)* The rate of dissociation of Fab fragments from the transferrin receptor is lower than that measured for the rate of dissociation of apotransferrin (Table 1).** The high rate of dissociation of apotransferrin ensures that this ligand is released from the receptor after exocytosis to complete the transferrin-receptor cycle. The lower rate of Fab-fragment release may result in the incomplete dissociation of Fab fragments from the receptor after exocytosis during receptor cycling. However, the half-time for receptor internalization in control cells, namely

4.1 min (Table 2), is long compared with the measured half-time for Fab-fragment dissociation, namely 1.2 min (Table 1). These data indicate that the Fab fragments as well as the apotransferrin dissociate from the receptor after exocytosis before the initiation of a new cycle of endocytosis.

(*b*) The effect of low pH on binding to the transferrin receptor. Fig. 6 shows that incubation of A431 cells at pH 5 causes a 12% reduction in the level of ^{125}I-Fab fragment binding compared with that observed at neutral pH. In contrast, ^{125}I-transferrin at pH 5 releases iron to yield apotransferrin which maintains a high affinity for the receptor [35,36]. The effect of pH on the binding of Fab fragments and transferrin to the receptor is significant because the acidification of endosomes (pH 5) occurs after endocytosis [37,38]. Consequently, in the acidified endosomes, some dissociation of ligands from the receptor may take place. In order to compare the expected extent of dissociation of ^{125}I-Fab fragments with that of ^{125}I-apotransferrin, the dissociation rate constants for these two ligands were compared. Klausner *et al.* [36] have reported that, at pH 4.8, the dissociation rate constant for ^{125}I-apotransferrin is 0.041 min^{-1} (4 °C). In experiments using A431 cells we have investigated the dissociation of ^{125}I-apotransferrin at pH 4.8 and measured the rate to be 0.044 min^{-1}. Under the same conditions the rate of Fab fragment dissociation was measured to be 0.036 min^{-1}, which is similar to that observed at pH 7, namely 0.030 min^{-1} (Fig. 2). Thus the extent of dissociation of ^{125}I-Fab fragments and ^{125}I-apotransferrin in acidified endosomes is expected to be similar. Klausner *et al.* [36] have argued that ^{125}I-apotransferrin does not significantly dissociate from the receptor within endosomes, because the dissociation of even one molecule within a small endocytic vesicle (with a volume of 10^{-17} litre) would result in a theoretical ligand concentration of approx. 170 nM. This concentration is very large relative to the measured K_d for the binding of both transferrin and Fab fragments (Table 1). Thus both receptor ligands can be expected to remain associated with the receptor within endocytic vesicles. Evidence that the Fab fragments do remain associated with the receptor is provided by the observation of similar rate constants for the exocytosis of ^{125}I-apotransferrin and ^{125}I-Fab fragments from cells at 37 °C (Table 3). Furthermore, if the Fab fragments did dissociate from the receptor in endosomes, it is possible that the Fab fragments would be degraded in lysosomes. This degradation was not observed. Instead, rapid and efficient exocytosis of the internalized Fab fragments was found (Table 3). However, if Fab fragments do dissociate from the receptor within endocytic vesicles, then the measured rates of exocytosis of Fab fragments will correspond to the sum of the exocytosis of Fab fragments bound to receptors and the exocytosis of the luminal contents of the endosome.

The considerations discussed above must be applied as a caveat to conclusions drawn concerning the relationship between the kinetics of exocytosis of Fab fragments and the rate of transferrin-receptor exocytosis.

Effect of diferric transferrin on receptor endocytosis

In contrast with previous studies in which it has been reported that only the receptor bound to diferric transferrin was competent for internalization [13–16], the endocytosis of OKT9 ^{125}I-Fab fragments by cells demonstrates that the transferrin receptor does internalize in the absence of diferric transferrin [Table 2]. Similar conclusions have recently been reported based on experiments employing different techniques [17–19]. The first-order rate constant for the internalization of OKT9 ^{125}I-Fab fragments (0.16 min^{-1}) was found to be significantly lower than the rate of internalization of diferric ^{125}I-transferrin (0.24 min^{-1}). This result could be caused by either the inefficient internalization of ^{125}I-Fab fragments or an effect of diferric transferrin to stimulate receptor endocytosis. To distinguish between these hypotheses, the effect of transferrin on the rate of endocytosis of ^{125}I-Fab fragments was examined. It was observed that diferric transferrin, but not apotransferrin, caused a significant stimulation of the rate of internalization of ^{125}I-Fab fragments (Table 2). The stimulated rate of endocytosis of ^{125}I-Fab fragments was similar to the measured rate of internalization of diferric ^{125}I-transferrin (Table 2). Together these data demonstrate that diferric transferrin binding to the transferrin receptor stimulates the endocytosis of the receptor.

Watts [17] has iodinated the surface of K562 erythroleukaemia cells at 4 °C and subsequently initiated internalization by warming the cells rapidly to 37 °C. Internalized receptors were identified on the basis of proteinase resistance. Using this experimental approach it was observed that the rate of endocytosis in the presence and absence of diferric transferrin was not significantly different. This is in contrast with the results obtained using ^{125}I-Fab fragments and A431 cells, where an effect of diferric transferrin to stimulate receptor internalization was observed (Table 2). The reason for the difference in the data obtained may be due to several factors. First, it is possible that the data reflect a difference between the kinetics of transferrin-receptor cycling in K562 erythroleukaemia cells and A431 epidermoid-carcinoma cells. Secondly, the cell-surface iodination and warming technique described by Watts [17] may not provide sufficient temporal resolution to allow a stimulation of endocytosis to be observed, because of the rapid cycling of the receptor. Thirdly, it may be significant that, in the procedure described by Watts [17], the internalization process was initiated by warming the cells from 4 to 37 °C. At 4 °C the receptors may be preclustered in coated pits and rapidly endocytosed during warming. In the steady state at 37 °C, only a fraction of the transferrin receptors are clustered in coated pits on the surface of A431 cells [21–23]. The technique used to investigate the internalization of ^{125}I-Fab fragments employs steady-state conditions at 37 °C and does not utilize the warming procedure.

Effect of diferric transferrin on receptor exocytosis

Exocytosis of OKT9 ^{125}I-Fab fragments from control cells was rapid and could be mathematically described as a single-exponential-decay process (Table 3). The calculated first-order exocytotic rate constant was calculated as 0.056 min^{-1}. This model of the data assumes that the exocytosis occurs from a single uniform pool of internalized transferrin receptors. Ajoika & Kaplan [18] have demonstrated, using HeLa cells, that transferrin receptors, internalized at times separated by as little as 1 min, are not physically associated within the cell. This datum indicates that there is not a simple pool of intracellular transferrin receptors available for exocytosis,

but that the receptors are segregated in the cycling pathway. However, Salzman & Maxfield [47] have presented evidence that the physical segregation of internalized transferrin receptors may be relevant to only specific cell types. This is because mixing of internalized receptors was observed in Chinese-hamster ovary cells, but not in HeLa cells [47]. Whether significant mixing of internalized receptors occurs in A431 cells is not known. Consequently, although the exocytosis of ^{125}I-Fab fragments can be modelled as a single-exponential-decay process, this model does not represent the morphology of the cycling pathway.

In the presence of diferric transferrin, an increased rate of exocytosis of ^{125}I-Fab fragments was observed (Table 3). The first-order rate constant for release was calculated to be 0.065 ± 0.003 min^{-1} and is significantly greater than the rate constant for the exocytosis of ^{125}I-Fab fragments from control cells (Table 3). Comparison of the calculated first-order rate constants for the release of ^{125}I-apotransferrin (0.072 ± 0.005 min^{-1}) from cells with the rate of release of OKT9 ^{125}I-Fab fragments in the presence of diferric transferrin (0.065 ± 0.003 min^{-1}) indicates that these processes are closely related. However, further examination of the experimental data for ^{125}I-Fab-fragment exocytosis in the presence of diferric transferrin indicated that it was mathematically modelled significantly better as a double-exponential-decay than as a single-exponential-decay process (Table 3). The two kinetic components of the exocytosis resolved by this analysis were characterized with first-order rate constants of 0.64 and 0.038 min^{-1}. The lower rate, accounting for 62% of the total exocytosis, was similar to the rate of exocytosis observed for control cells. The higher rate of exocytosis accounted for 38% of the total exocytosis of ^{125}I-Fab fragments and was not observed in control cells. The relationship between the two resolved kinetic components of exocytosis of ^{125}I-Fab fragments and the physical process of exocytosis is not known.

The stimulation of ^{125}I-Fab fragment exocytosis by diferric transferrin suggests that the rate of exocytosis of the transferrin receptor is increased in the presence of diferric transferrin. The mechanism by which diferric transferrin stimulates receptor exocytosis could be due to either an increase in the rate of the exocytotic pathway or to the sorting of the receptors to a pathway with more rapid kinetics of exocytosis. Previously it has been reported that, in A431 cells, transferrin-receptor cycling occurs via a pathway involving peripheral endosomal membrane compartments and by a pathway that involves the juxtanuclear region (Golgi) of the cell [21]. A Golgi-associated pathway of cycling has been identified based on morphological [39,40] and biochemical [19,41] evidence. Kinetic data have indicated that the peripheral cycling system is rapid [21], and it has been proposed that the juxtanuclear cycling system is significantly slower [18,21]. A possible mechanism of action of diferric transferrin to stimulate receptor exocytosis is that the binding of the ligand to the receptor controls the sorting of the receptor between these cycling pathways. Evidence supporting this hypothesis is provided by the effect of diferric transferrin to inhibit the accumulation of OKT9 ^{125}I-Fab fragments by A431 cells at 37 °C (Fig. 7). Whereas control cells accumulate significant quantities of ^{125}I-Fab fragments, poor accumulation of the ^{125}I-Fab fragments was observed in the presence of diferric transferrin (Fig. 7). As diferric transferrin stimulates endocytosis of the transferrin receptor, the slow accumulation of ^{125}I-Fab fragments observed in the presence of diferric transferrin is anomalous. A possible explanation for this phenomenon is that the receptor bound to diferric transferrin efficiently cycles between the plasma-membrane and endosomal-membrane compartments, whereas, in the absence of ligand, a significant fraction of the receptors cycle through the juxtanuclear region and this results in the accumulation of ^{125}I-Fab fragments.

Regulation of receptor cycling by diferric transferrin

The mechanism by which diferric transferrin regulates the endocytosis and exocytosis of the transferrin receptor is not understood. One possibility is that the binding of diferric transferrin to the receptor induces a conformational change in the receptor that results in an alteration in the kinetics of cycling. A second possibility is that the effects are mediated by the covalent modification of the receptor. The receptor is a phosphoprotein [20]. However, it has been demonstrated that the major site of phosphorylation, serine-24 [26], is not required for the normal cycling of the receptor [9,42–44]. Covalent modification of the receptor by phosphorylation is therefore unlikely to be the mechanism by which diferric transferrin regulates the cycling of the receptor. Acylation with palmitic acid is another covalent modification of the receptor that has been reported [45,46], but the role of this modification has not been critically tested. An important goal for future research is the identification of the molecular mechanism by which ligand regulates the cycling of the transferrin receptor.

This work was supported in part by a grant (GM37845) from the National Institutes of Health. Dr. A. Carruthers is thanked for assistance with the statistical treatment of the data. We thank Karen Donahue for excellent secretarial assistance.

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Received 6 January 1989/13 June 1989; accepted 27 June 1989