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BIPHASIC UPTAKE OF IRON-TRANSFERRIN COMPLEX BY L1210 MURINE LEUKEMIA CELLS AND RAT RETICULOCYTES

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The kinetics of the cellular uptake of iron-transferrin complex was studied in L1210 murine leukemia cells and rat reticulocytes using ^{125}I-transferrin. Saturation of transferrin with iron was necessary for optimal uptake. Following the incubation of cells with the radiolabeled complex a biphasic pattern of uptake was observed. The initial phase was rapid and relatively temperature-independent and was not altered by ethylamine, an inhibitor of transglutaminase activity which is necessary for receptor-mediated endocytosis. This phase was considered to result from receptor-ligand interaction which could be reversed to a great degree by replacement with unlabeled transferrin. A plateau was then reached, indicating a saturation of receptors. After 30 min a second phase of uptake was indicated by the second rise in the curve. This phase was slow, relatively temperature-dependent and could be abolished by ethylamine. It was interpreted as evidence of internalization of the ligand. Analysis of the data from competition studies with unlabeled transferrin indicated that the first phase might itself comprise a reversible and an irreversible step with a ratio of 5 to 1.4 for bound transferrin. Thus, the cellular uptake of iron-transferrin complex may consist of a reversible ligand-receptor interaction. Conformational changes may render this interaction irreversible and the internalization of the ligand may then follow.

Introduction

The transport of iron across the cell membrane has been studied in a variety of cell systems [1–7] and in particular in the reticulocyte [8–10]. Iron transport is mediated by the transport glycoprotein, transferrin. Iron-transferrin complex, then, binds to specific cell surface receptor, and iron is taken up by the cell. There has been some debate as to whether the subsequent transmembranous uptake involves iron-transferrin complex or that transferrin releases the iron to the cell and itself dissociates from the receptor. Studies using fractionation [11] and electron microscopy [12–14] with labelled transferrin have suggested that the entire complex is internalized. Double labeling studies using ^{125}I- or ^{131}I-transferrin and ^{59}Fe [15,16] however, suggest that only iron is taken up whereas transferrin dissociates from the receptor.

In the present study the kinetics of the uptake of iron-transferrin complex by L1210 murine leukemia cells and rat reticulocytes were examined using ^{125}I-labeled transferrin. The findings indicate a biphasic uptake. The initial phase is rapid and relatively temperature-independent and remains largely unaltered by ethylamine, a inhibitor of receptor-mediated endocytosis. This phase, itself, appears to consist of a reversible and an irreversible stage. The second phase on the other hand, is slow and relatively temperature-dependent and is abolished by ethylamine. The findings indicate the internalization of the transport protein.

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Materials and Methods

Transferrin and labeling method. Purified rat transferrin used in these experiments was a generous gift from Drs. U. Müller-Eberhard and H.H. Liem (Scripps Clinic and Research Foundation, La Jolla, CA). The purity of the preparation in the forms of apotransferrin and iron-transferrin complex was demonstrated by its migration as a single band on polyacrylamide gel electrophoresis both before and after labeling. The protein was labeled by chloramine T method [17] using 20 μg of chloramine T (Sigma Chemical Co., St. Louis, MO) and 1.0 mCi of ^{125}I (carrier-free Na ^{125}I, New England Nuclear, Boston, MA) per 1 ml of 13.2 μM transferrin. Free ^{125}I was removed by dialysis for 24 h against phosphate-buffered saline (10 mM sodium phosphate/0.15 M NaCl, pH 7.4), or by passing through a Sephadex G-100 column. The fraction containing radioactive transferrin was collected and subsequently concentrated (13.2 to 26.3 μM) by using a Diaflo PM-10 ultrafilter (Amicon Corp., Lexington, MA). The specific activity of the labeled protein was calculated by measuring the radioactivity and the protein concentration [18]). For control yeast hexokinase, mol. wt. 95000, was also labeled with ^{125}I by the same procedure. This protein was selected as control because it has a molecular weight close to that of transferrin but has no known transport function and, hence, it is unlikely to have cell surface receptor.

Saturation of ^{125}I-transferrin with iron. To maximize transferrin binding to specific receptors on the cell surface [1,15], radiolabeled transferrin was then fully saturated with iron [19,20]. Ferric nitrilotriacetate was prepared by adding equal volumes of 1.0 M disodium nitrilotriacetate (Sigma) and 0.33 M ferric nitrate (Sigma) (molar ratio 3 : 1). The mixture was adjusted to pH 6.0 with 1.0 M sodium bicarbonate, and gently stirred for 30 min. Subsequently, 50 μl of ferric nitrilotriacetate so prepared was diluted with an excess sodium bicarbonate (1 M) to 10^{-2} and added dropwise to 1 ml of 13.2 μM iron-poor ^{125}I-transferrin while stirring gently for 30 min. The mixture was dialyzed overnight against phosphate-buffered saline to remove unbound ferric nitrilotriacetate and then measured spectrophotometrically at the wavelength of 470 nm to ensure the binding of iron [21].

Preparation of murine L1210 leukemia cells and reticulocytes. L1210 cells (a gift from Dr. D.W. Jacobsen, Scripps Clinic and Research Foundation) were grown in RPMI 1640 medium supplemented with 5 percent fetal bovine serum (Flow Laboratories, Rockville, MD), 100 U of penicillin per ml and 100 μg of streptomycin per ml (Irvine Scientific, Irvine, CA) at 37°C in 5% humidified CO_2-air mixture. This cell line is a lymphoblastoid cell line derived from DBA/2 mouse lymphoma and is maintained by passages thrice a week in liquid culture. The cells were washed with the growing medium (without fetal bovine serum and antibiotics) by centrifugation at $150 \times g$ for 7 min and then resuspended in the same medium to an approximate volume of $5.5 \cdot 10^6$ cells per ml, as counted by a Coulter Counter (Coulter Diagnostic, Inc., Hialeah, FL). Reticulocytes were obtained from rats with phenylhydrazine-induced hemolysis as described [22].

Uptake of iron bound ^{125}I-transferrin by L1210 cells. The cell suspension was kept at 4°C at least for 10 min before adding ^{125}I-transferrin at a final concentration of 5.3 μM. Incubation was then proceeded for 120 min either at 4 or 37°C in 5% humidified CO_2-air mixture. At various intervals, thereafter, 300 μl of the suspension was removed and placed into 15 ml of ice-cold phosphate-buffered saline as described [23] and centrifuged at $300 \times g$ for 3 min. This procedure was repeated twice. Samples were then placed into separate tubes wherein the radioactivity was measured and the cell count was made. The specific activity of the protein was then used to calculate the data in terms of ng ^{125}I-transferrin taken up per 10^5 cells. Using Avagadro's number and molecular weight of rat transferrin (76000 [24]) the data were then expressed as the number of molecules taken up per cell. The data represent the outcomes of two independent experiments.

Competition and inhibition studies. In competition studies, the cells were pre-incubated with a final concentration of 12.8 μM of non-iodinated transferrin or 14.1 μM of rat serum albumin (Sigma) for 20 min at 37°C. The cells were then washed and resuspended in the growing medium

and then incubated with radiolabeled transferrin. In inhibition studies cells were exposed to 1 mM of ethylamine (Sigma) at 37°C 20 min before and during the incubation with radiolabeled transferrin. Ethylamine, a primary alkylamine is a transglutaminase inhibitor and because glutamination is apparently essential for receptor-mediated endocytosis [25–27], ethylamine has been used to inhibit the internalization phase in receptor-mediated transport [25]. It may also be involved in receptor recycling [28].

Results

Uptake of iron-transferrin complex by L1210 leukemia cells and reticulocytes

The uptake of iron-saturated transferrin by murine L1210 leukemia cells was dependent on the concentration of transferrin in the medium, as demonstrated in Fig. 1. The rate of uptake increased continuously as the concentration increased, and although within the range of concentration studied, the uptake did not reach a plateau (probably because the curve was not corrected for non-specific binding of transferrin), the rate of uptake slowed down considerably at 3 μM concentrations. We, therefore, selected 5.3 μM concentrations as optimum for use in these experiments.

The time course of uptake in L1210 leukemia cells is shown in Fig. 2 demonstrating that the cellular uptake of iron-saturated transferrin is basically biphasic. The initial phase is rapid, reaching a plateau within 5 min. This phase appears to be relatively temperature-independent, as the plateau is almost similar whether the incubation is carried out at 37 or 4°C. The second phase of uptake begins after 30 min incubation. This phase appears to be slower than the initial and is relatively temperature-dependent, continuous and does not reach a plateau for the 2-h period of observation. Fig. 3 shows similar data for rat reticulocytes, showing a comparable biphasic uptake with the second phase being relatively temperature-dependent.

Effect of iron saturation on transferrin uptake

To study the effect of iron saturation on the uptake of transferrin, we compared saturated with iron-poor (less than 10%) ^{125}I-transferrin. The results are shown in Fig. 4 indicating that the uptake of iron-poor transferrin is suboptimal although higher than that of the control hexokinase.

Competition and inhibition studies

When L1210 cells were pre-incubated with albumin, washed and subsequently incubated with

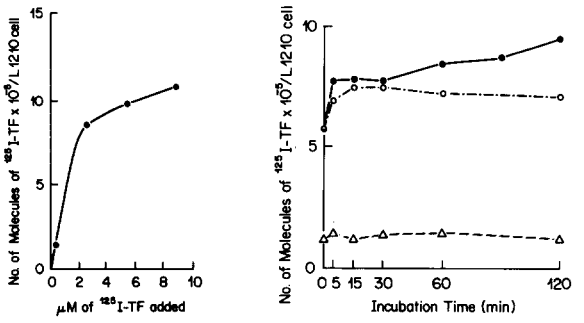

Fig. 1. Binding of the radiolabeled iron-transferrin complex as a function of protein concentration in the incubation medium. the binding increases rapidly but subsequently slows down. No plateau is reached up to 9 μM concentration of added protein. Limited availability of the protein precluded the study of higher concentrations.

Fig. 2. The time course of uptake of iron-transferrin complex by L1210 leukemia cells. At 37°C (●) the uptake is biphasic and consists of an initial rapid phase, reaching a plateau within 5 min. A second, slower, phase begins after 30 min and does not reach a plateau even after 2 h. At 4°C (○) the second, but not the initial, phase is abolished. Hexokinase (△) was used as control. All control experiments were carried out at 37°C.

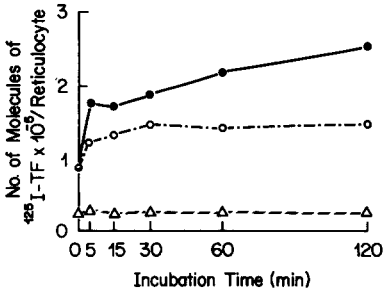

Fig. 3. The time course of uptake of iron-transferrin complex by rat reticulocytes. The biphasic pattern of uptake similar to that for L1210 cells is noted. ●, 37°C; ○, 4°C; △, control (hexokinase) at 37°C.

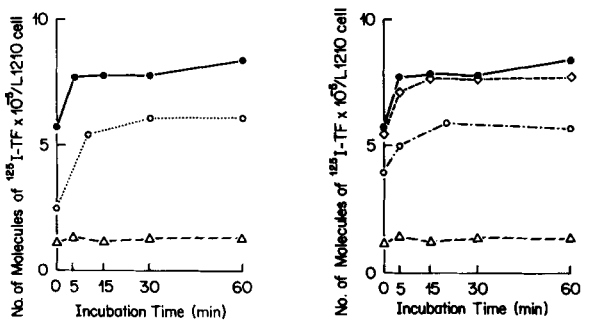

Fig. 4. The effect of saturation of transferrin by iron. Although the pattern of uptake is similar, the uptake of iron-poor transferrin (less than 10%) (○) is suboptimal compared to the iron-saturated protein (●). △, control (hexokinase) at 37°C.

Fig. 5. The effect of pre-incubation with unlabeled transferrin. The uptake of radiolabeled protein is reduced as a result of competition with unlabeled protein (○). Pre-incubation with albumin does not reduce the uptake (◇). ●, uptake without pre-incubation. △, control (hexokinase) at 37°C.

radiolabeled transferrin, the plateau remained unchanged suggesting that the cellular uptake of transferrin was not altered (Fig. 5). However, when preincubation was carried out with unlabeled transferrin, and cells were subsequently washed and incubated with radiolabeled transferrin, the plateau was considerably lower indicating competition of unlabeled and radiolabeled transferrin.

Addition of 1 mM ethylamine, an inhibitor of receptor-mediated endocytosis [25] and possibly receptor recycling [28] to the incubation mixture did not significantly alter the primary phase of uptake but completely abolished the second phase (Fig. 6). In fact there was a slight drop in the cellular uptake of the protein at 2 h compared to 1 h suggesting dissociation of receptor-ligand from the surface. To determine the reversibility of the binding, the medium containing 5.3 μM ^{125}I-transferrin was replaced by a medium containing 13 μM of either unlabeled transferrin or rat serum albumin after 60 min of incubation. The cells were washed twice in the fresh medium followed by centrifugation at $150 \times g$ for 3 min. The supernatant was then removed and the cells were resuspended in the new medium. The results are shown in Fig. 7. Within 15 min there was a considerable drop in the cellular uptake of ^{125}I-transferrin. This recovered partially within an hour but the recovery could be prevented by changing the medium again with the fresh medium containing unlabeled transferrin. This suggests that the receptor-bound transferrin molecules are in state of equilibrium with unbound molecules. The use of serum albumin, instead of unlabeled transferrin also led to a drop in the cellular uptake of ^{125}I-transferrin but the magnitude of the drop was less than in the experiments where the exchanges were done with a medium containing unlabeled transferrin.

Computation of the number of binding sites

The number of transferrin binding sites were computed by back-calculation using the specific activity of the ^{125}I-labeled protein and Avogadro's number (see Methods). Visually, this can be seen as the difference between the plateau part of the curve and the background hexokinase curve (specific transferrin binding) (Figs. 2 and 3). Alternatively the data were plotted according to the method of Scatchard [29] and the number of binding sites was obtained from the intercept of the curve with abscissa. By either method, the number of binding sites per cell were estimated to be $6.4 \cdot 10^5$ for L1210 leukemia cells but varying between 1.5 and $2.5 \cdot 10^5$ for reticulocytes.

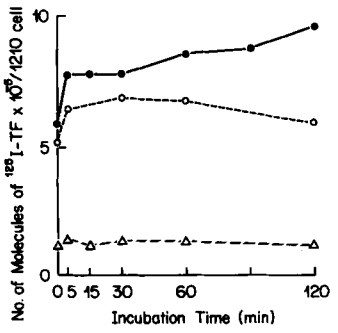

Fig. 6. The effect of ethylamine on the uptake of transferrin by L1210 cells. Ethylamine is a transglutaminase inhibitor which interfers with receptor-mediated endocytosis. Although it does not appreciably alter the first phase of uptake, it causes complete abolition of the second phase suggesting that the second phase is the result of internalization of the ligand. Uptake in absence (●) and presence (○) of ethylamine. △, control (hexokinase) at 37°C.

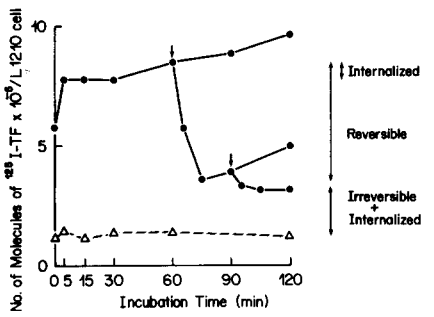

Fig. 7. Partial reversibility of transferrin binding to L1210 leukemia cells. At 60 min the incubation medium is changed with a medium containing a higher concentration of unlabeled transferring (arrow). Molecules of radiolabeled transferrin bound to the cell are displaced by unlabeled transferrin suggesting the binding is partially reversible. The subsequent rise in the uptake of radiolabeled transferrin also suggests equilibrium of unlabeled and labeled protein. This rise can again be aborted by a second change of the medium (arrow). The magnitude of fall in the uptake of labeled transferrin indicates the magnitude of reversible binding and amounts to $5 \cdot 10^5$ molecules per L1210 cell. The number of molecules not displaced by unlabeled transferrin is $2.4 \cdot 10^5$. This is derived at, by subtraction of background from the total number of labeled molecules. At this point in time, the number of internalized molecules (calculated from the magnitude of rise in the second phase of curve) is $1 \cdot 10^5$. Thus, $1.4 \cdot 10^5$ molecules appear to be bound irreversibly to the cell without being internalized. △, control (hexokinase) at 37°C.

Discussion

These results indicate that the uptake of iron-transferrin complex displays a biphasic pattern. The initial phase is rapid, reaching a saturation plateau within 5 min. This phase appears to be relatively temperature-independent giving comparable curves at 4 and 37°C. The second phase is slow, relatively small in magnitude and begins 30 min after incubation but does not reach a plateau even after 2 h. This phase is relatively temperature-dependent. These patterns are similar to the curves obtained by Beamish et al. [1] in rat liver cells. Somewhat similar curves have also been obtained by Octave et al. [7] in rat fibroblasts.

The initial phase of the curve with a steep slope may result from receptor-ligand interaction by an electrostatic binding force [30,31]. The plateau is reached when all receptors are saturated. This interaction may in part be in state of reversible equilibrium because when the ^{125}I-transferrin in the medium is removed or replaced by unlabeled transferrin, there is a sharp fall in the curve followed by slow rise again. Moreover, pre-incubation with unlabeled iron-transferrin complex (but not with albumin) diminishes the magnitude of uptake but does not abolish it.

The second phase of uptake may be explained by internalization of iron-transferrin complex. Alternatively, it is possible that there is recycling or de novo synthesis of receptors which will bind more ^{125}I-transferrin to the cell membrane, leading to the second phase of the uptake. Be that as it may, both the recycling or de novo synthesis of receptors may require internalization of receptor-ligand complexes, because ethylamine, an inhibitor of receptor-mediated endocytosis [25] abolishes the second phase of the uptake. In this regard the work of Basu et al. [28] is of interest. These authors used monensin, an inhibitor of receptor recycling. They found accumulation of ^{125}I-low density lipoproteins in the human fibroblasts with a subsequent fall in the specific binding to the cells.

In support of our interpretation is the relative temperature-dependence of the second phase which suggests a metabolic process and is consistent with receptor-mediated endocytosis [32]. In the same vein, it has been reported that the uptake of iron-transferrin complex by reticulocytes is calcium-dependent at 37°C but not at 4°C [33]. This uptake is also dependent on the integrity of microtubular system [33,34] which may not be maintained in 4°C. Our interpretation is in agreement with several studies using fractionation technique [11], autoradiographic electron microscopy [12,14] and electron microscopy with ferritin or peroxidase conjugated transferrin [13,14]. Using cellular fractionation technique, Zaman et al. [35] found about a fourth of the radioactivity associated with the cytoplasmic fraction. When they immobilized ^{125}I- or ^{59}Fe-transferrin, they again found a considerable proportion of the radioactivity associated with the cytoplasmic fraction. From these experiments, they concluded that internalization of transferrin occurred but this might be the consequence of transferrin-independent internalization and reorganization of cell membrane. Analysis of the data on the uptake of double labeled iron-

transferrin complex (^{59}Fe and ^{3}H) by cultured rat fibroblast, also suggested internalization of the complex with subsequent recycling of transferrin [7]. In this respect transferrin appears to behave similar to such other transport protein as transcobalamin II-cobalamin complex which is also internalized [36]. Our data, therefore, do not support the concept that subsequent to ligand-receptor interaction, iron is released to the cell and transferrin is dissociated from the receptor [15,16]. It is also possible that molecules of transferrin altered by chemical modification or total or partial denaturation are preferentially internalized [35]. Milsom and Batey [37] have found that only denatured transferrin is taken up by the rat liver.

Transferrin fully saturated with iron clearly demonstrated more avidity for the binding sites than iron-poor transferrin. This is consistent with the results reported by other [1,15] indicating that iron uptake increases as a function of the percentage of transferrin saturation with iron.

The number of transferrin binding sites per cell is considerably higher in L1210 cells than in reticulocytes. Moreover, the number of binding sites appear to decrease with maturation of erythroid cells. In fact the number of transferrin binding sites on the marrow normoblasts exceeds several millions and with maturation to reticulocyte, this is reduced to less than one million per cell [10]. The number of binding sites computed here for reticulocytes is in line with those reported by others [38,39], although the values reported by these authors show a wider variation (between 1.9 and $5.6 \cdot 10^5$ per reticulocyte) than those computed here (1.5 to $2.5 \cdot 10^5$ per cell). Nonetheless a variation appears to be present and may be attributed to the age and percentage of reticulocytes in preparations studied. As in the case with erythroid cells, the number of binding sites per reticulocytes decreases with age so that mature red cells have no transferrin binding sites. In this regard L1210 cells or other cultures cells [1–7] may offer an alternate cell system than the reticulocyte to study iron transport since they show little variation in the number of transferrin binding sites.

The computation of binding sites suggests that the initial phase may itself comprise two steps. Fig. 7 shows that the addition of unlabeled transferrin during the second phase of uptake, results in displacement of $5.0 \cdot 10^5$ molecules of ^{125}I-transferrin bound to the cell. Thus $5.0 \cdot 10^5$ molecules are reversibly bound to the cell. This does not however reach the background level of hexokinase, thus leaving the cell with about $2.4 \cdot 10^5$ molecules. At this point the number of internalized molecules (indicated by the magnitude of rise for the second phase over the plateau level) is $1 \cdot 10^5$ and amounts to 16% of the molecules taken up by cell. There remain at this time another $1.4 \cdot 10^5$ molecules which are neither internalized, nor reversibly bound. This may constitute those transferrin molecules irreversibly bound to the receptors.

Thus we believe that uptake of iron-transferrin complex occurs in 3 steps: (a) A reversible interaction occurs between the receptor and the ligand. (b) This may result in conformational changes, rendering the receptor-ligand interaction irreversible. (c) Internalization of iron-transferrin complex, then follows.

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