Separation of Fe^{+3} from transferrin in endocytosis

Role of the acidic endosome

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Received 27 April 1983; revised version received 6 July 1983

NH_4Cl and monensin, two agents which neutralize intracellular acidic compartments, block the segregation of iron from transferrin after endocytosis, while neither of these reagents affects internalization of diferric transferrin into the cell. In conclusion the molecular separation of iron from transferrin inside the cell requires a non-lysosomal acidic compartment

Transferrin *Endocytosis* *K562 cell* *Acidic compartment* *Monensin* *NH_4Cl*

1. INTRODUCTION

The general intracellular pathway of receptor-mediated endocytosis is shared by many types of molecules including hormones, viruses, toxins, and carriers of essential nutritional components such as LDL and transferrin [1]. As the details of each individual system are elucidated, specific variations unique to different receptor–ligand systems become clear. The finding that ligand rapidly enters an acidic non-lysosomal organelle has become the focus for early molecular events in this pathway [2]. Several events may take place in this compartment, because of its acidic pH: (i) ligand-receptor separation [3,4]; (ii) toxin penetration [5]; (iii) virus envelope–membrane fusion [5]; and perhaps, (iv) receptor recycling [7,8]. Transferrin delivers iron to cells via receptor-mediated endocytosis [9–11]. It has been shown that transferrin rapidly enters an acidic non-lysosomal vesicle upon internalization [12]. At some point before the internalized transferrin is recycled out of the cell, the iron is removed and transferred to cytosolic ferritin [12]. It is reasonable to assume that the acidic environment is necessary for dissociating the iron from the transferrin, since it is known that the affinity of iron for the protein is dramatically reduced at low pH [13]. In this report we present evidence to show that agents that neutralize intracellular acidic compartments inhibit the dissociation of iron from transferrin.

It is clear that iron is released from transferrin at some point during the transferrin cycle. We designed a series of experiments to demonstrate the intracellular separation of iron from transferrin and to ask whether an intracellular acidic environment was necessary for this separation.

2. MATERIALS AND METHODS

[^{125}I]Transferrin and [^{59}Fe]transferrin were prepared as in [10,12]. Anti-transferrin antibody was obtained from Boehringer (Mannheim). All other reagents used were of the highest quality commercially available.

Specific activities were determined by counting each labelled protein in a Beckman 5500 gamma counter with windows set for each nuclide to count 95% of cpm. Protein concentrations were accurately determined by using the method of Lowry. Full saturation of the transferrin was confirmed by finding a ratio of absorbance of 0.046 (465/280). Protein concentrations were confirmed by the VV absorbance at 280 nm.

Published by Elsevier Science Publishers B.V.
00145793/83/$ 3.00

Transferrin binding to and uptake into K562 cells was determined as in [9,10,14]. For the purpose of immunoprecipitation, 200 μl cell suspension (10^7 cells/ml) was solubilized in 1% Triton X-100, 10 μl anti-transferrin antibody was added and incubated at 4°C for 2 h; 50 μl fixed *Staphylococcus aureus* cells were then added and incubated at 4°C for 1 h. The fixed *S. aureus* cells were washed and the radioactivity was measured. Non-specific precipitation was determined by using the same procedure without the specific antibody.

3. RESULTS AND DISCUSSION

K562 human leukemia cells were incubated at either 0°C or 37°C, with either [^{59}Fe]transferrin or [^{125}I]transferrin. At the end of this period the cells were cooled to 0°C and washed free of unbound ligand. Aliquots of the cells were exposed to an acid (pH 2.5) wash as in [9,10,14] to determine cell surface (acid-releasable) *vs* internal (acid-resistant) counts. We found that >95% of the ^{125}I-counts were on the cell surface after a 0°C incubation while 80–85% of the ^{125}I-counts were internalized at the end of a 37°C incubation. Upon Triton X-100 solubilization of the cells after a 0°C incubation, 95–100% of both ^{125}I and ^{59}Fe could be precipitated with anti-transferrin antibody. The ratio of the precipitated ^{59}Fe/^{125}I very closely reflected the ratio of the specific activities of the two labelled ligands (^{59}Fe/^{125}I = 0.055). This demonstrates that, at 0°C, ^{59}Fe and transferrin have not separated from each other. The ratio of counts precipitated from cells solubilized after a 37°C incubation was quite different. In this case, 97–100% of the ^{125}I was specifically precipitated, while a markedly lower ^{59}Fe/^{125}I ratio was found: about 0.03. This represents a loss of about 45% of the iron from the cell-associated transferrin. When we correct for the 15–20% of the cell-associated transferrin still on the cell surface with a presumed ratio of 0.055, we calculate that the internal transferrin is only about 45% saturated. Application of this technique allows us to assess the separation of iron from transferrin during the endocytic transferrin cycle. It should be noted that the 45% value represents an average of all degrees of iron-saturation found with the transferrin that resides inside the cell at the time of solubilization and immunoprecipitation. In previous experiments we found that transferrin that leaves the cell after a normal cycle, carries no iron [9].

Next, we examined the effect of two drugs, NH_4Cl and monensin, on this intracellular separation. NH_4Cl is a weak 'lipophilic' base that will neutralize acidic compartments [15]. Monensin is a carboxylic proton ionophore that will dissipate proton gradients across membranes [16]. These two drugs, with different mechanisms of action, will both neutralize acidic cellular compartments. It has previously been demonstrated that agents such as NH_4Cl interfere with the transferrin cycle and inhibit the continued uptake of iron from transferrin by cells [9,10]. These studies, however, fail to distinguish between whether these agents act by inhibiting some aspect of the endocytic cycle, such as receptor recycling, or whether they inhibit the molecular dissociation of iron from transferrin. Neither NH_4Cl nor monensin inhibit the binding of transferrin to cells at 0°C or the uptake and internalization of transferrin at 37°C. However, they both completely inhibit the intracellular dissociation of iron from transferrin at 37°C (table 1). From the ratio of immunoprecipitated ^{59}Fe to ^{125}I

Table 1

Immunoprecipitation of ^{125}I and ^{59}Fe with anti-transferrin

Temp. (°C)	Drug	^{59}Fe/^{125}I (ppt)	% Transferrin saturation	Corrected % saturation
0	–	0.056	102	–
0	NH_4Cl	0.057	103	–
37	–	0.033	60	49
37	NH_4Cl	0.052	95	94
0	–	0.053	96	–
0	Monensin	0.054	98	–
37	–	0.028	50	38
37	Monensin	0.054	98	97

K562 cells were allowed to take up 25 μg of [^{125}I]transferrin or [^{59}Fe]transferrin at 10^7 cells/ml at 37°C for 60 min in RPMI 1640 + 0.1% bovine serum albumin both with and without 20 mM NH_4Cl or 50 μM monensin with RPMI + 0.1% bovine serum and resuspended in TBS (10 mM Tris–HCl, pH 7.4, 0.85% NaCl containing 1 μM phenyl methyl sulfonyl fluoride and 0.1 mg/ml leupeptin). Immunoprecipitation of the transferrin was done as in section 2.

we calculate that the total cellular transferrin is 95 and 98% iron-saturated for NH_4Cl and monensin-treated cells, respectively. These numbers do not significantly change when we correct the $^{59}Fe/^{125}I$ ratio for the 15–20% of the ligand present on the cell surface.

The immunoprecipitation demonstrates that the cell-associated transferrin never loses its iron in the presence of NH_4Cl and monensin. However, we do not know at which point in the cellular transferrin cycle iron is normally removed from its carrier. It is possible that these drugs prevent the internalized transferrin from reaching the cellular compartment where iron is removed. It is clear that drugs such as these can inhibit the normal movement of receptors through the endocytic pathway [7,8]. To test where in the endocytic cycle agents like NH_4Cl and monensin would act, we examined whether internalized transferrin could be released from these cells in the presence of an alkalinizing drug and whether this transferrin, having completed the endocytic cycle, would still contain its iron. K562 cells were allowed to internalize ^{125}I- or ^{59}Fe-labelled transferrin at 37°C. At the end of a 15 min incubation the cells were cooled to 0°C and washed free of unbound ligand. In either the absence or presence of 20 mM NH_4Cl, >85% of the counts had been internalized. The cells were then warmed again to 37°C and the release of ^{125}I or ^{59}Fe from the cells was followed. We have shown that the complete cycling of transferrin was slowed markedly, but not completely inhibited by NH_4Cl [10]. Here we found (fig.1) that in control cells, all of the [^{125}I]transferrin could be released while all of the ^{59}Fe remained with the cells. There is no degradation (as measured by trichloroacetic acid precipitation) and all of the released ^{125}I is immunoprecipitable with anti-transferrin antibodies. In cells treated with NH_4Cl, the rate of release of transferrin was markedly reduced [10]. However, almost 50% of cell-associated ^{125}I was released, an amount significantly greater than the 15% remaining on the cell surface. Thus, in the presence of NH_4Cl some of the internalized ligand can be released from the cell. In contrast to the cells that had not been treated with NH_4Cl, these cells release exactly commensurate amounts of ^{125}I and ^{59}Fe. All of this ^{59}Fe is immunoprecipitable with anti-transferrin antibodies. Therefore, the transferrin released in the presence of NH_4Cl is still diferric. In the presence of NH_4Cl, diferric transferrin that has reached the cell surface undergoes dissociation. In the presence of excess unlabelled transferrin, the molecular reassociation of labelled diferrin transferrin is inhibited and hence the inclusion of excess unlabelled transferrin allows us to observe the release of diferric transferrin from cells treated with NH_4Cl. During the process of internalization, intracellular residency and release from the cell NH_4Cl has prevented it from releasing any of its iron. Although NH_4Cl has a wide variety of cellular effects, it appears to directly inhibit the intracellular dissociation of iron from transferrin. It is not surprising that low pH is required for the release of iron from transferrin. As already mentioned above, it is well known that low pH dramatically reduces the affinity of iron for this protein [13]. It is not known whether the primary effect of acid is on the iron–protein interaction directly, or via protonic attack on the anion whose binding to transferrin is an obligate step in the binding of iron [17].

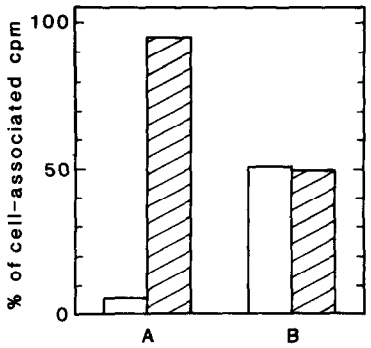

Fig.1. K562 cells at 10^7/ml in RPMI 1640 + 0.1% bovine serum albumin were incubated at 37°C for 10 min with and without 20 mM NH_4Cl; 25 μg of [^{125}I]transferrin, or [^{59}Fe]transferrin was added and the incubation carried out for an additional 15 min. The cells were washed with RPMI 1640 + 0.1% bovine serum albumin at 0°C and resuspended to 10^7 cells/ml. The cells were then incubated at 37°C for 40 min with 100 μg/ml of unlabelled transferrin. 100 μl of cell suspension was centrifuged through 150 μl of dibutylpthalate in a 400 μl Eppendorf tube to measure the loss of cell-associated [^{125}I]transferrin or [^{59}Fe] iron. Shown is the cell-associated transferrin (unhatched) or ^{59}Fe (hatched) after the final 40-min incubation in absence (A) or presence of NH_4Cl (B), respectively. All of the released transferrin is undegraded [9].

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