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Biochimica et Biophysica Acta 1403 (1998) 179–188

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Iron transport in K562 cells: a kinetic study using native gel electrophoresis and ^{59}Fe autoradiography

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Received 16 December 1997; revised 25 March 1998; accepted 14 April 1998

Abstract

The exact mechanisms of iron transport from endosomes to the target iron containing cellular proteins are currently unknown. To investigate this problem, we used the gradient gel electrophoresis and the sensitive detection of ^{59}Fe by autoradiography to detect separate cellular iron compounds and their iron kinetics. Cells of human leukemic line K562 were labeled with [^{59}Fe]transferrin for 30–600 s and cellular iron compounds in cell lysates were analyzed by native electrophoretic separation followed by ^{59}Fe autoradiography. Starting with the first 30 s of iron uptake, iron was detectable in a large membrane bound protein complex (Band I) and in ferritin. Significant amounts of iron were also found in labile iron compound(s) with the molecular weight larger than 5000 as judged by ultrafiltration. Iron kinetics in these compartments was studied. Band I was the only compound with the kinetic properties of an intermediate. Transferrin, transferrin receptor and additional proteins of the approximate molecular weights of 130 000, 66 000 and 49 000 were found to be present in Band I. The labile iron compounds and ferritin behaved kinetically as end products. No evidence for low molecular weight transport intermediates was found. These results suggest that intracellular iron transport is highly compartmentalized, that iron released from endosomal transferrin passes to its cellular targets in a direct contact with the endosomal membrane complex assigned as Band I. The nature of the labile iron pool and its susceptibility to iron chelation is discussed. © 1998 Elsevier Science B.V.

Keywords: Iron; Transferrin; Transferrin receptor; Ferritin; Labile iron pool; K562 cell; Native gradient gel electrophoresis

1. Introduction

Iron is indispensable for life, yet present in loosely bound forms, it can catalyze the formation of oxidative radicals and the resulting tissue damage is thought to be causally related to plethora of diseases including arthritis, atherosclerosis and cancer [1,2]. Despite the progress in delineating the iron-dependent regulation of iron proteins in the past decade [3], accurate description of intracellular iron transport has been difficult, mainly because of the lack of appropriate techniques for the separation and detection of cellular iron compounds.

Autoradiography with ^{59}Fe of commonly available specific activity allowing the detection of 100 fg of iron per mm^2 after an overnight exposition is a new powerful tool for the study of cellular iron transport [4,5]. In the present work native electrophoresis followed by ^{59}Fe autoradiography was used to dissect

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PII S0167-4889(98)00039-1

early stages of iron uptake from transferrin by cells of the human leukemic cell line K562.

Cells of this line are well characterized with respect to iron metabolism, they were used to study transferrin endocytosis, regulation of ferritin translation and turnover, the function of iron responsive element binding proteins, iron–ascorbate interactions [6,7], inhibition of ribonucleotide reductase [8], and also to investigate the interactions of NO released from tumoricidal macrophages with its protein-bound iron targets [9].

The sensitivity of the techniques employed here enabled us to identify several iron containing compounds in K562 cells and to assess their role in cellular iron transport.

Using the kinetic approach, we detected a large molecular weight compound with the properties of an iron transport intermediate.

2. Materials and methods

2.1. Cells

K562 cells from Dr. P. Goldfarb, Beatson Institute for Cancer Research, Glasgow were grown in Iscove's medium supplemented with 10% fetal bovine serum starting at densities of 10^5 cells/ml. The cells reaching the density of 10^6 cells/ml were collected by centrifugation and washed in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) at 4°C, resuspended in the modified Eagle's minimum essential medium (incubation medium) containing 108 mM NaCl, 10 mM $NaHCO_3$, 25 mM HEPES (pH 7.4, 280 mosM) supplemented with 10 g/l of deionized bovine serum albumin into the final cell concentration of 10^7 cells/ml.

2.2. Transferrin and cell labeling with ^{59}Fe

Human apotransferrin (Fluka) was saturated with radioactive iron ($^{59}FeCl_3$, DuPont-NEN, specific activity 0.74–1.66 GBq/mg) using nitrilotriacetate as the iron chelator [10]. Unbound [^{59}Fe]nitrilotriacetate was removed on a Sephadex G25 column equilibrated with 0.14 M NaCl, 0.01 M HEPES, pH 7.4. Non-radioactive diferric transferrin was prepared by the same method with non-radioactive $FeCl_3$. Diferric [^{59}Fe]transferrin was added to 30 ml of the cell suspension of 10^7 cells/ml in the incubation medium in the final concentration of 2.5 μM and the cells were incubated in a shaking water bath at 37°C. After different times of incubation, samples of 2 ml were taken into 10 ml of PBS at 4°C, centrifuged for 5 min at $1000\times g$ and washed two times in PBS at 4°C. The cell pellets were then frozen and stored at −80°C. In pulse-chase studies, cells prelabeled for 10 and 30 min with [^{59}Fe]transferrin were washed two times with PBS and then reincubated in 4 ml of the incubation medium supplemented with 2.5 μM non-radioactive differic transferrin at the density of 5×10^6 cells/ml.

2.3. Gradient gel preparation

Hoeffer SE 600 vertical electrophoresis system with external circulating water bath was used. Linear 3–20% polyacrylamide gradient was prepared using Hoeffer SG gradient mixer. The ratio of acrylamide and *N*,*N*′-methylene/bis-acrylamide was 29:1, the gel mixture contained 1.5% Triton X-100, 0.375 M Tris, 1.18 mM ammonium persulfate, pH 8.8. The concentrations of TEMED were 5.38 mM for the 3% gel mixture and 2.69 mM for the 20% gel mixture. The gels (1.5 mm spacers) were left overnight at room temperature, 3% stacking gel with 0.25 M Tris, pH 6.8, was then poured and left to polymerize for 2 h.

2.4. Cell lysis and sample preparation for the electrophoretic separation

Cell pellets (10^7 cells) were thawed by the addition of 100 μl the lysing buffer containing 0.14 M NaCl, 0.1 M HEPES, 1.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, pH 7.4 at 4°C. The lysates were vortexed and centrifuged at 4°C for 20 min at $16\,000\times g$. The resulting pellets and supernatants were counted for ^{59}Fe radioactivity on Tesla NA 3601 gamma counter, the protein concentration in the supernatants was 4.5 mg/ml. Supernatants were then made 10% (w/v) in sucrose with trace amount of Bromophenol blue and aliquots corresponding to 0.5–1.2 pg of Fe were applied to the sample wells for the electrophoretic separation.

2.5. Protein determination

To avoid the interference of Triton in the Lowry and dye-binding assays, absorbance at 205 nm was used to measure the total protein in cell lysates [11]. An aliquot of the lysate (5 μl) was added to a quartz cuvette filled with 3 ml of 5 mM phosphate buffer, pH 7.0, containing 50 mM sodium sulfate to prevent protein adsorption.

2.6. Ultrafiltration assay of low molecular weight iron complexes

To detect low molecular weight iron complexes in cell lysates, 30 μl lysate samples (135 μg of protein) were added to 370 μl of 15 mM NaCl, 10 mM HEPES, pH 7.8 (NaCl/HEPES buffer) and centrifuged at $5000 \times g$ for 300 min at 4°C on Millipore Ultrafree-MC membranes with NMW cutoff 5000. The retentate (30 μl) and the ultrafiltrate (370 μl) were then counted for ^{59}Fe radioactivity. When chelatable iron was assayed, 30 μl of the lysate was added to 370 μl of the NaCl/HEPES buffer with 500 μM desferrioxamine (Desferal, Ciba) or 500 μM 1,2-dimethyl-3-hydroxypyrid-4-one (L1, Deferiprone, kindly provided by Dr. G.J. Kontoghiorghes, Department of Haematology, Royal Free Hospital, London) and centrifuged as described above. The adsorption of ^{59}Fe to the ultrafilter was less than 1% for the 5000 NMW cutoff membranes.

2.7. Electrophoresis and autoradiography

Electrophoresis was performed using the electrode buffer containing 0.025 M Tris, 0.192 M glycine, pH 8.3 with external cooling set to 4°C at 110 mA constant current for two gel gradients. The run was terminated after approximately 210 min. The end voltage ranged from 500 to 550 V. The gels were then placed on Whatman No. 3 filter paper impregnated with sodium salicylate and vacuum dried at 80°C for 2.5 h. Dried gels were exposed to the Amersham Hyperfilm-MP radiographic film with one DuPont Cronex Lightening Plus intensifying screen for 24–72 h at −70°C. A portion of the gel was cut off before vacuum drying and proteins were visualized as described previously [12].

2.8. Measurement of ^{59}Fe radioactivity in compounds separated by electrophoresis

Bands detected on a radiographic film were redrawn to a transparent polyethylene foil and the foil was glued to dried gel, using sample wells and stacking gel boundary for alignment. Bands of interest were then cut out with scissors and ^{59}Fe radioactivity in the band and in the remaining portion of the particular lane was counted for 2000 s on the gamma counter.

2.9. Ferritin immunoprecipitation

In preliminary experiments, we observed that the addition of the whole anti-ferritin serum into the lysate caused loss of the LIP pool after electrophoresis due to scavenging of LIP by apotransferrin present in the serum. We prevented this by binding of anti-ferritin immunoglobulins to the immunosorbent beads as follows.

Polyclonal anti-human ferritin serum (50 μl, Sigma F 5012) was added to 50 μl of rehydrated *Staphylococcus aureus*-protein A immunosorbent resuspended in 50 μl of NaCl/HEPES buffer. After shaking for 60 min at 0°C, the immunosorbent was pelleted and washed two times with 500 μl of NaCl/HEPES buffer to remove non-immunoglobulin serum proteins. Control immunosorbent pellets were prepared by the same method except that anti-ferritin serum was substituted with 50 μl of 5% bovine serum albumin. Samples containing 100 μl of cell lysate were added to the anti-ferritin-coated pellet or to the control pellet, shaken for 60 min at 0°C and pelleted. Supernatants of the anti-ferritin-treated and control samples were then analyzed by electrophoresis.

2.10. Immunoprecipitation of transferrin receptor and associated proteins

Transferrin receptor was isolated from 25×10^6 of K562 cells incubated with 2.5 μM Fe transferrin for 30 minutes at 37°C. The cells were lysed as described above. Immunomagnetic beads were prepared using monoclonal anti-human transferrin receptor antibody MEM-75 [13], commercially available from Exbio Prague, catalogue number 11-235. Antibodies were covalently linked to M 450 sheep anti-mouse

IgG1 Dynabeads according to the manufacturer's instruction using dimethyl pimelimidate dihydrochloride.

Lysate (2.25 mg of protein) was incubated at 20°C for 60 min with 5 mg of Dynabeads. The immunomagnetic beads were than washed ten times with 1 ml of the lysing buffer. Control samples were prepared by incubation of the lysate with mock Dynabeads. Immunoadsorbed proteins were stripped from the Dynabeads by heating at 80°C for 10 min in the SDS loading buffer and analyzed by electrophoresis on 8% SDS acrylamide gel [12]. Separated proteins were detected by silver staining [12].

3. Results

3.1. Iron containing compounds in K562 cells

Iron uptake by K562 cells incubated with the 2.5 μM [^{59}Fe]transferrin was linear up to 180 min of incubation and proceeded with the rate of 20 fg of ^{59}Fe/10^6 cells/min. Of the total cellular ^{59}Fe activity, 90% was released into the Triton-soluble supernatant and this proportion did not change with the time of cell labeling with ^{59}Fe-transferrin. No attempts were made to characterize iron compounds in the Triton insoluble residue. To investigate the recovery of radioactivity after the electrophoretic separation the dried gel was cut and lanes were counted for ^{59}Fe activity on the gamma counter. In all samples more then 97% of the activity applied to the gel was detected.

3.2. Distribution of ^{59}Fe-containing compounds in the Triton soluble cellular fraction

In the early intervals of iron uptake, most of the cellular ^{59}Fe activity was detected in a slow-migrating band (Band I, Fig. 1Fig. 3A). It was not possible to detect this compound in the cytosolic fraction from ^{59}Fe-labeled cells obtained by hypotonic lysis [14] and centrifugation at $100\,000 \times g$ for 60 min. However, it was possible to release the Band I from the pellet fraction of hypotonic lysates by the addition of the detergent Triton X-100. Antibodies against transferrin receptor (OKT 9 Ortho Diagnostic, MEM-75 Exbio) shifted the migration of Band I

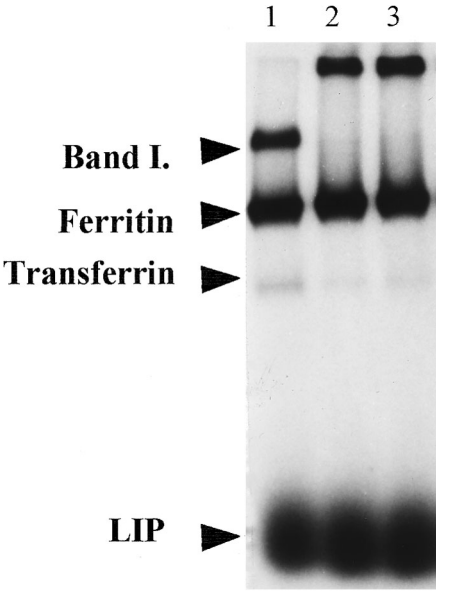

Fig. 1. Shift of Band I by anti-transferrin receptor antibodies. Cells were labeled with [^{59}Fe]transferrin for 30 min at 37°C, washed in PBS at 4°C, lysed and samples containing 90 μg of protein were subjected to native gradient gel electrophoresis and ^{59}Fe autoradiography. Lane 1, control; lane 2, OKT 9 monoclonal antibody was added to the sample before electrophoresis; lane 3, MEM-75 monoclonal antibody was added to the sample before electrophoresis.

when added into the lysate before electrophoresis (Fig. 1). The Western blot of the gel showed signal in the position of Band I when monoclonal antibodies against the transferrin receptor were used (not shown). These findings point out that the Band I contains transferrin receptor. However, the migration of the Band I suggest its molecular weight is bigger than molecular weight of ferritin (400 000). The transferrin–transferrin receptor complex (molecular weight 260 000) thus cannot be the only component of the Band I. We tested the possibility that the Band I is complex of transferrin and transferrin receptor with the additional proteins. When the immunoprecipitate formed by incubation the K562 cell lysate with monoclonal anti-transferrin receptor antibodies covalently linked to the magnetic Dynal beads were analyzed on the SDS polyacrylamide gel by electrophoresis, several bands were detected. Transferrin receptor (molecular weight of 180 000) and transferrin (molecular weight of 80 000) were identified by the silver staining. In addition, bands of the approximate molecular weight of 130 000, 66 000 and

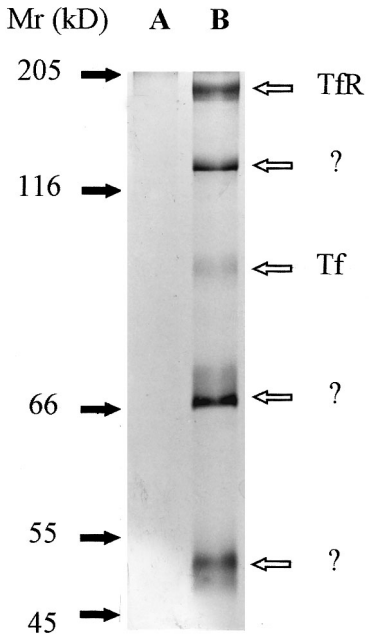

Fig. 2. Eletrophoretic analysis of proteins co-immunoprecipitated with transferrin receptor. The transferrin receptor was immunoprecipitated from K562 cell lysate using MEM-75 monoclonal antibodies covalently linked to the magnetic Dynal beads. The immunoprecipitate was solubilized in SDS-containing loading buffer and analyzed on 8% polyacrylamide gel under non-reducing conditions (B). The control sample was obtained by incubation of the lysate with the mock Dynabeads (A).

49 000 was also found to be present in immunoprecipitates (Fig. 2). There was no non-specific binding to the Dynal beads, as shown by the absence of bands in control sample containing mock Dynal beads. When we analyzed the ^{59}Fe activity in the portions of electrophoretic gel corresponding to Band I by direct counting we found that activity of ^{59}Fe in Band I reached a plateau within several seconds after the addition of [^{59}Fe]transferrin (Fig. 3A). This is consistent with the kinetic properties of an early intermediate. An additional finding pointing to such a role is the rapid chase of ^{59}Fe from this band during the reincubation of prelabeled cells with non-radioactive transferrin (Fig. 4A).

The second band from the top of the gel was identified as ferritin-bound iron. This band was shifted after an anti-ferritin antibody was added into the sample before electrophoresis. Ferritin-bound ^{59}Fe displayed the kinetic properties of an end product, increasing in a linear fashion with time (Fig. 3B).

Transferrin-bound ^{59}Fe not associated with transferrin receptor was identified by comigration with [^{59}Fe]transferrin. This free transferrin associated ^{59}Fe activity increased linearly for 60 min. This is consistent with transferrin taken up into the cells by the fluid phase endocytosis.

A significant proportion of total ^{59}Fe in K562 cells was present in a diffuse rapidly migrating LIP band (Figs. 1 and 3AFig. 4A). A band of similar electro-

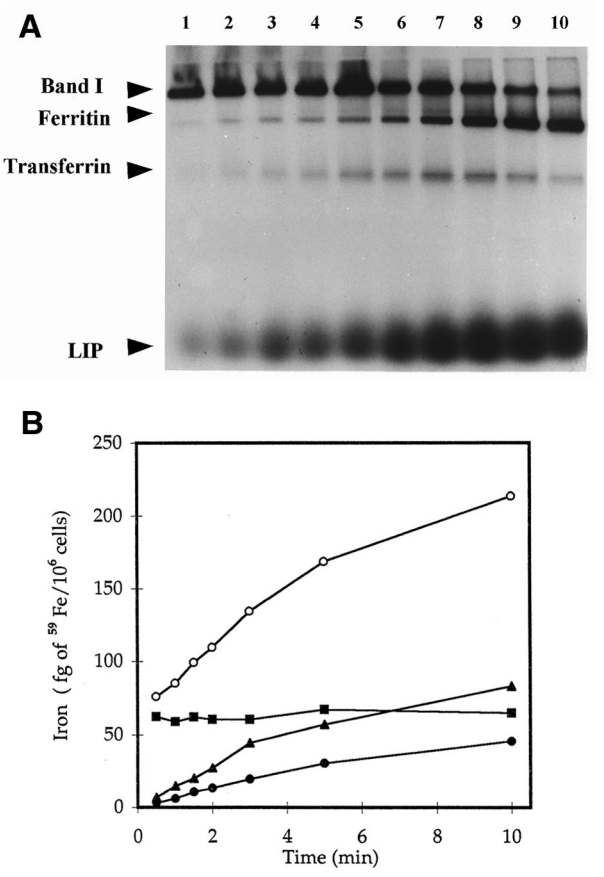

Fig. 3. Kinetics of iron in cells incubated with [^{59}Fe]transferrin. (A) Cells were pulse-labeled with 2.5 μM [^{59}Fe]transferrin for 0.5, 1, 1.5, 2, 3, 5, 10, 30, 60, 120 min (lanes 1–10). After the incubation, transferrin and iron uptake was stopped by the addition of cell suspension aliquots containing 2×10^7 cells to PBS at 4°C. The cells were then washed, lysed and the lysates were subjected to native gradient gel electrophoresis. Samples containing 1 pg of ^{59}Fe were loaded into each well, protein loads ranged from 300 μg for the 30-s incubation interval to 13.5 μg for the 120-min interval. (B) Portions corresponding to Band I, ferritin and LIP were cut out of the gel depicted in (A) and counted for ^{59}Fe radioactivity. The amount of ^{59}Fe in total lysate (○), Band I (■), ferritin (●), and LIP (▲) increased linearly up to 120 min of incubation with ^{59}[Fe]transferrin.

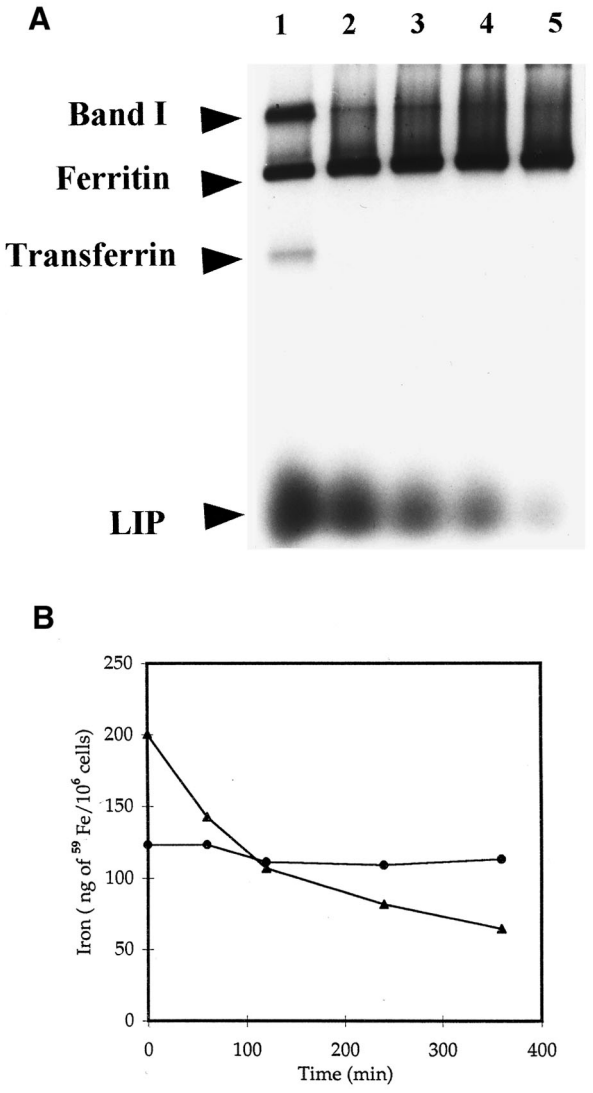

Fig. 4. Kinetics of cellular iron in pulse-chase experiments. (A) Cells labeled with [^{59}Fe]transferrin for 30 min were washed in PBS at 4°C, resuspended in the incubation medium supplemented with 2.5 μM nonradioactive transferrin, reincubated for the specified times at 37°C and harvested by centrifugation. There was no loss of ^{59}Fe from the cells during reincubation. Cell pellets were then lysed, samples containing 110 μg of protein were subjected to electrophoresis and ^{59}Fe autoradiography was performed. Lane 1, cells labeled for 30 min, reincubated for 0 min; lane 2, cells reincubated for 30 min; lane 3, cells reincubated for 60 min; lane 4, cells reincubated for 240 min; lane 5, cells reincubated for 360 min. (B) The LIP band (▲) and ferritin band (●) was cut out from the gel depicted in A) and counted for ^{59}Fe radioactivity.

phoretic mobility and appearance was described previously in erythroid cells, where it was markedly increased under the conditions of heme synthesis inhibition by succinylacetone [4,5]. The presence of analogous compounds was not reported in earlier publications concerning iron transport in K562 cells, probably because it escaped detection by migrating out of the gels [6,7,15]. In preliminary experiments we have ruled out the possibility that LIP is artificially removed from [^{59}Fe]transferrin or hemoglobin in the course of electrophoresis.

Low molecular weight iron complexes were postulated to play a regulatory role in iron transport and to be the common source of iron for cellular iron compounds [16,17]. However, kinetic studies revealed a linear increase of ^{59}Fe in the LIP band, which is in conflict with immediate precursor role of this iron compartment. When we chased the cells prelabeled with ^{59}Fe there was no increase of ^{59}Fe activity in the ferritin band during the chase as judged by measuring the ferritin band cut off from the gel (Fig. 4B).

During the long chase experiments the LIP pool was metabolically active, decreasing with the half-life of about 2.5 h (Fig. 4B). Iron derived from this pool during long chase interval was not incorporated into ferritin. Instead, iron from LIP has becomes incorporated into background bands localized between the

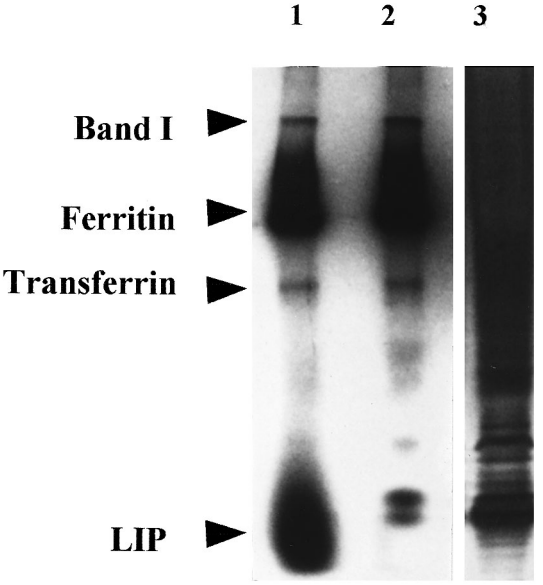

Fig. 5. ^{59}Fe containing bands resistant to DFO chelation. Cells were labeled for 60 min with ^{59}Fe, washed in PBS and lysed. Then control lysate (lane 1) and the lysate supplemented with 500 μM desferrioxamine (lane 2) were subjected to electrophoresis and autoradiography. A portion of the gel was cut off before vacuum drying and the separated compounds were visualized by silver staining (lane 3).

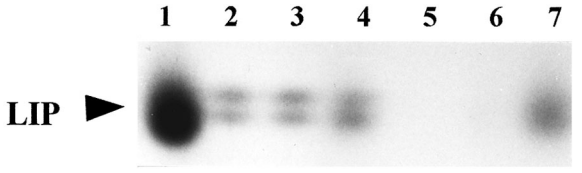

Fig. 6. Differential chelation of LIP ^{59}Fe by DFO and L1. Cells were labeled for 60 min with ^{59}Fe, washed in PBS and lysed. Different concentrations of DFO or L1 were added to the lysate samples before electrophoresis. Lane 1, no chelator added (control); lane 2, 1000 μM DFO; lane 3, 500 μM DFO; lane 4, 100 μM DFO; lane 5, 1000 μM L1; lane 6, 500 μM L1; lane 7, 100 μM L1.

top of the gel and ferritin. This bands are different from Band I and they can be fully resolved on different concentration gradient gel. Molecular weight of iron in the LIP was investigated by ultrafiltration using the membrane with molecular weight cutoff 5000. When lysates from cells labeled with [^{59}Fe]transferrin for times ranging from 30 s to 2 h were subjected to ultrafiltration, only 0.5–1% of the total sample radioactivity appeared in the ultrafiltrate. However, when the ultrafiltration was done in the presence of Deferiprone chelator, the ultrafiltrable activity increased and was found to correspond to the proportion of lysate ^{59}Fe in the LIP band detected by direct counting of the cut gels after electrophoresis. When the ultrafiltration was done in the presence of desferrioxamine, the activity in the ultrafiltrate was about 10% less than in the presence of Deferiprone.

When desferrioxamine was added directly to K562 lysate samples before electrophoresis, most of the diffuse LIP band was removed while satellite bands appeared in the radiogram (Fig. 5Fig. 6). When the same experiments were repeated with deferiprone, all the ^{59}Fe activity in band LIP disappeared (Fig. 6). Complexes of desferrioxamine–^{59}Fe and deferiprone–^{59}Fe were found not to enter the separation gel. Silver staining of portions of the same gels revealed several distinct bands in the region corresponding to the LIP band on the radiograms (Fig. 5). Both chelators were unable to remove any iron from ferritin, at least within the preincubation interval used (10 min at 0°C).

The LIP, like ferritin, is labeled with the kinetics of an end product during the 10 min of incubation with [^{59}Fe]transferrin (Fig. 3B). We explored the possibility that at least a part of iron detected in LIP was originally weakly bound to ferritin, perhaps in a pool of newly incorporated iron not included in the crystalline core. However, immunodepletion of ferritin from the samples before electrophoresis did not affect ^{59}Fe radioactivity in the LIP band (Fig. 7). The iron found in this compartment is thus non-ferritin bound. All the described attributes of the LIP band iron suggest that it is composed of iron weakly bound to compounds of molecular weight higher than 5000, distinct from ferritin or transferrin. In the course of electrophoretic separation in the presence of 0.375 M Tris, this iron is labilized from its carrier(s) and it is visualized as a diffuse band in the radiograms. Iron in this compartment can be differentially chelated, desferrioxamine being a less efficient scavenger for LIP than Deferipron under the conditions used. When the cells were preincubated with an excess of iron or with iron chelators before labeling with [^{59}Fe]transferrin, the proportion of iron incorporated into ferritin and into LIP changed. Cells which were preincubated with various iron complexes and than pulsed with [^{59}Fe]transferrin in-

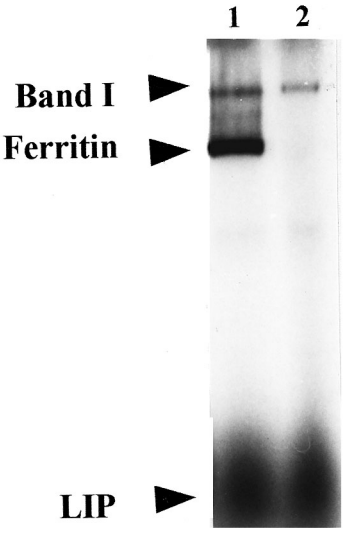

Fig. 7. Removal of ferritin by anti-ferritin antibody coated immunosorbent. Cells were labeled for 60 min with ^{59}Fe, washed in PBS and lysed. Equal portions of the lysate were incubated with anti-ferritin antibody-coated immunosorbent or with control albumin-treated immunosorbent. After pelleting the immunosorbent, supernatants containing 100 μg of protein were subjected to native gradient gel electrophoresis and autoradiography. Lane 1, control; lane 2, anti-ferritin antibody. ^{59}Fe radioactivity of the LIP was not affected by ferritin depletion before the electrophoresis as judged by counting ^{59}Fe in the LIP band cut off from the gel.

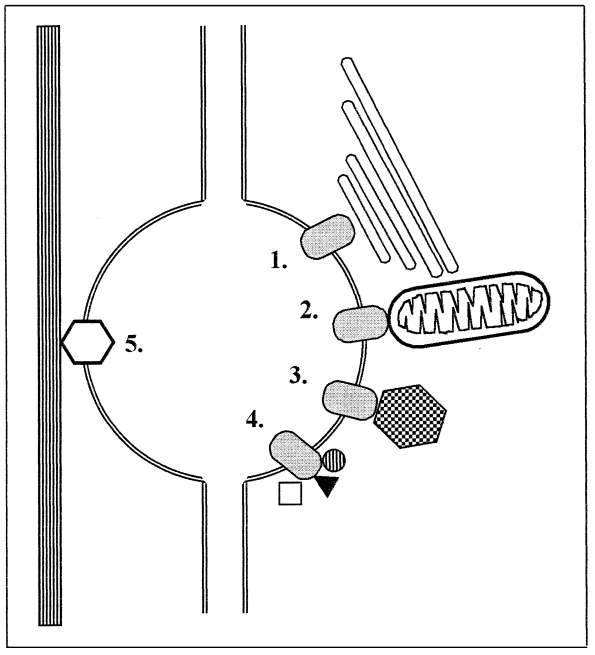

Fig. 8. Putative direct protein–protein contact transport of iron from endosomes to various cellular compartments. Iron is transported from endosomes to cytosolic target proteins or organelles through an Band I iron translocation and processing protein complex (shaded ovals) and presented on the cytosolic side of the endosome membrane by the direct contact to endoplasmic reticulum (1), mitochondria (2), ferritin (3), or to non-ferritin, non-heme proteins (4). The endosome is connected to the cytoskeleton via molecular motor protein (5). Modified from Warren [36].

corporated more iron into LIP and less iron into ferritin than the cells preincubated with iron chelators. This might be explained by a higher rate of iron uptake by ferritin in cells depleted of iron. Conversely, in cells preincubated with high iron concentration, all the ferritin iron binding sites are probably occupied. Ferritin uptake of ^{59}Fe is thus suppressed.

4. Discussion

Ferritin is the major destination of iron entering K562 cells [14,18]. We were able to detect iron in ferritin as early as 30 s after the initiation of [^{59}Fe]transferrin uptake. This is comparable to the incorporation of iron into heme in erythroid cells [5]. Despite extensive studies of iron incorporation into ferritin in vitro using purified ferritin and various iron complexes as donors, detailed pathways of iron uptake by ferritin in vivo remain unknown [19]. Low molecular iron complexes were postulated to play an intermediate role in this process. However, no low molecular weight iron compounds were detected in our current and previous [14] studies on K562 cells.

The only iron component with the kinetic properties of an early intermediate is the slowly migrating Band I. It is not detected in the cytosol of cells subjected to hypotonic lysis unless Triton is added to the lysis buffer. We found that the Band I contains transferrin – transferrin receptor complex in association with proteins of the approximate molecular weight of 130 000, 66 000 and 49 000. The identity of this proteins is unknown at present. However, the protein defective in hereditary hemochromatosis (molecular weight about 48 000) was recently found to be present in non-covalent association with transferrin receptor in human placenta [20] and the NRAMP 2 gene product (molecular weight about 65 000) was implicated in iron uptake and endosomal transport [21,22]. The molecular weights of these two proteins closely corresponds to unknown protein bands we found to be co-immunoprecipitated with transferrin receptor in the Triton lysates of K562 cells. Further study is needed to reveal the identity of this bands. The spectrum of transferrin receptor associated proteins may be cell and tissue specific. Additional proteins weakly bound to the Band I complex may be identified by reversible crosslinking [20].

The other likely constituents are ferrireductase and iron translocating H^+ ATPase [23,24]. Interestingly, an iron binding complex of the molecular weight of 520 000 with ferrireductase activity was recently found in rat intestine [25].

On the basis of present results, we believe the concept of direct iron transfer from endosomes to mitochondria in erythroid cells [5] should be extended to other iron ligands and possibly to other cell types as well. The endosome–mitochondria contact was postulated in reticulocytes, because no low molecular weight iron intermediates were discovered in the reticulocyte cytosol [5], and this idea was later presented as the 'kiss and run' hypothesis [26].

The exact mechanisms of iron incorporation into non-heme proteins such as ferritin, iron sulfur cluster proteins, and other metalloproteins (lipoxygenases, ribonucleotid reductase, protein serine/threonine

phosphatases, proline-, phenylananine- and tyrosine hydroxylases and others) and the cellular compartments involved are currently not known. In our view (Fig. 8), Band I, an endosomal membrane protein complex includes a putative iron translocating channel and presents iron to different proteins or organelles such as mitochondria and endoplasmic reticulum in direct contact with the cytosolic side of the endosomal membrane. The process of iron release from transferrin, iron(III) reduction, its translocation across the endosomal or the mitochondrial membranes and its incorporation into target proteins is probably driven by the consumption of ATP or NAD(P)H. According to this hypothesis, iron transport to the target compounds is tightly compartmentalized and does not involve diffusible low molecular ligands.

The partial loss of the Band I complex after immunodepletion of ferritin (Fig. 7) is in concordance with the scheme represented in Fig. 8, i.e. ferritin can form a transient supercomplex with Band I. Under conditions of immunodepletion this association is probably stable, because of the volume exclusion effects in the interior of agarose bead. However, the Band I and ferritin dissociate when subjected to electrophoresis. This is probably due to smaller pore size of the acrylamide gel compared to agarose.

Iron compounds having the properties of 'labile' (i.e. chelatable) iron were detected as the rapidly migrating LIP band in the present study. A similar band corresponding to 10–50% of cellular iron was also found (D. Vyoral, and J. Petrák, unpublished observation) in the lysates of Friend murine erythroleukemia cells, normal and sideroblastic human bone marrow cells, iron loaded hepatocytes and the neuroblastoma and melanoma cell lines (D.R. Richardson, personal communication).

In K562 cells, iron in this pool is weakly bound to carrier(s) with the molecular weight higher than 5000 and the size of the pool responds to preloading of the cells with iron or to the depletion of cellular iron by chelators.

The concept of 'labile iron' is central to numerous studies concerning free radical generation and free radical induced tissue damage [1,2,27]. However, the term itself might be misleading, unless conditions under which iron becomes labile are rigorously defined. The method for the determination of the cellular labile iron was described which depends on desferioxamine extraction of cell homogenates followed by the quantitation of ferrioxamine complex by high performance liquid chromatography [28]. Desferrioxamine is unable to remove iron from transferrin at the physiological pH 7.4, while microbial exochelins [29] can chelate even 'tightly' bound transferrin iron under these conditions and they can deliver it to bacteria.

Furthermore, proteins binding iron with relatively low affinity may be present in cellular compartments where there are only few or no competing low molecular weight ligands. Only after the disruption and mixing of cell constituents, especially in the presence of Tris-containing buffers, iron released from its physiological carriers rebinds, and becomes 'low molecular weight'. In our experiments, half of the LIP pool was scavenged by 100 mM Tris and passed through the membrane of 5000 NMW cutoff. Labile iron pool detected in our study behaved kinetically as an end product and may be thus composed of numerous proteins binding iron with lower affinity than transferrin and ferritin. It is possible to influence the magnitude of the labile iron pool by preincubation of the cells with high or low iron, the same conditions used to manipulate the activity of IRE-BP. In the K562 cells, iron sulfur cluster protein IRE-BP I is possible candidate to constitute the major part of the LIP pool. Other iron sulfur cluster proteins and numerous housekeeping non-heme, non-sulfur-cluster proteins, such as ribonucleotide reductase [30], can also contribute to this pool.

The second cause of iron lability in cellular lysates may be the sensitivity of proteins containing iron(II) to oxidation. In intact cells, sensitive groups may be shielded by specialized compartments, such as anaerobic hydrophobic lipid domains, by pockets between multiprotein complexes, or by the continuous action of reducing enzymatic systems similar to methemoglobin reductase in erythrocytes [31]. As a result of cell disruption, cell constituents shielded in vivo from oxygen may become aerobic and oxidized. Metallothioneins, proteins known to bind iron(II) in vitro under strictly anaerobic conditions only [32], can contribute to the labile iron pool in this way. Part of the labile iron pool may also come from non-protein macromolecular carriers, such as polysaccharides and nucleic acids, or it could be bound

to polyphosphates, linear polymers of phosphoric acid with the ability to chelate transition metals, which were also recently found in mammalian cells [33].

Fluorescent iron chelators were used for the detection of labile iron pool in various cells, including the cells of K562 line [34,35] and this approach eliminates some of the difficulties caused by artefacts generated after cell disruption. The methods employed here, native electrophoresis coupled with ^{59}Fe autoradiography have the potential of measuring chelatable iron in various separated compounds and can contribute to studies of intestinal iron uptake, hepatocyte iron metabolism, interaction of iron with cytotoxic compounds such as bleomycin or anthracyclines, and to quantitative studies of iron catalyzed free radical formation under various pathological conditions.

Acknowledgements

We thank to Antonín Hradilek and Jaroslav Jelínek for support, helpful suggestions and critical reading of the manuscript and to Eva Mikulová for the cell culture work. This work was supported by grant from IGA MZ CR No. 3565-3.

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