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# A partially purified putative iron P type-ATPase mediates $Fe^{3+}$-transport into proteoliposome

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**Abstract**

We report that two fractions containing proteins from rat hepatocyte nuclei, obtained by nondenaturing gel electrophoresis, were able to bind iron and ATP, and to hydrolyze ATP. Electroelution of these two active fractions followed by SDS–PAGE analysis showed an identical protein pattern, each one containing four proteins in a range of 62–80 kDa. Phosphorylated protein bands were also detected in acid gel and disappeared after treatment with hydroxylamine/acetate or KOH, and upon chasing with cold ATP. A proteoliposome system, made by the incorporation of these partially purified protein fractions into phosphatidylcholine vesicles, carried out $Fe^{3+}$–citrate uptake in a $Mg^{2+}$-ATP-dependent way; $Fe^{3+}$ accumulation increased with time reaching a plateau in 30 min. Iron uptake was not supported by AMP-PNP, was partially inhibited by orthovanadate and was not affected by a mix of specific inhibitors of known ATPases. These results support our previous hypothesis that a putative nuclear membrane $Fe^{3+}$-ATPase is involved in nuclear iron homeostasis.


*Keywords:* Iron uptake; P-type ATPase; Acid gel; Nuclear membrane; AMP-PNP; Vanadate; $Fe^{3+}$–citrate; Proteoliposome system; Vesicles

Iron is a ubiquitous heavy metal present in the structure of many enzymes and proteins in the cells. Its ionic form is prone to participate in one-electron transfer reactions, an essential characteristic as prosthetic group in enzymes that catalyze redox reactions. However, this property also makes iron a potentially hazardous metal, because it can generate reactive oxygen species (ROS) [1]. It has been shown that iron uptake and storage are tightly controlled by a sophisticated system that maintains a pool of "free" iron low enough not to cause cell damage [2]. Alterations in cellular iron pool are related to neurodegenerative diseases and cancer [3]. Thus, many efforts have been made to uncover new mechanisms of iron transport in the cells and across biological membranes.

Iron transporters have been recently identified in mice as well as in rats [4–7]. Divalent metal transporter 1 (DMT1) has been shown to be responsible for ferrous iron uptake by the enterocytes and the basolateral transporter Ireg1/ferroportin/MTP-1/SLC40A1 for its release into the plasma [7]. Interestingly, the expression of DMT1 and Ireg1 in the intestine has been shown to be regulated by Hepcidin, an iron sensor peptide produced by hepatocytes [8,9]. In addition, a recent report has identified a putative mitochondrial iron transporter (MTABC3) ubiquitously expressed in rats [10]. A putative $Fe^{2+}$-ATPase in mice macrophage was also described and suggested to promote ferrous uptake in microsome membranes [11].

Iron has been also detected in the nucleus of cells in different forms [12–15] and a controlled nuclear iron transport has been described [15]. Isolated rat liver nuclei transported iron in an Mg·ATP-dependent manner, possibly promoted by a P-type $Fe^{3+}$-ATPase. The ferric transport

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occurred when $Fe^{3+}$–citrate or $Fe^{3+}$-ATP was used as an iron source in the presence of magnesium. Iron uptake was not stimulated by AMP-PNP, a nonhydrolysable ATP analogue and was partially inhibited by vanadate, a phosphate analogue and P-ATPase inhibitor. Other known ATPase inhibitors had no effect on iron uptake. These data strongly suggested that an $Fe^{3+}$-ATPase, distinct from other known ATPases, might be responsible for iron transport in the nuclei [15].

Given the importance of an ATPase pump as a feasible controller of ferric ions transport into the nucleus, proteins from rat liver nuclei were partially purified, using nondenaturing-PAGE system and incorporated into phosphatidylcholine vesicles, in order to originate a proteoliposome system to evaluate iron transport. We found that this system supported ferric iron uptake in a similar way to that of purified nuclei. $P^{32}$-phosphorylated protein bands were also detected in acid gel and disappeared upon cold ATP-chasing or after treatments with hydroxilamine/acetate or KOH, as has been shown before for known P-ATPases [16,17].

## Methods

### Materials

All biochemical reagents were from Sigma. $^{55}FeCl_3$ (2.6 μCi/μL) and [$\gamma$-$^{32}$P] ATP (2 μCi/μL) were from Amersham (Buckinghamshire, UK). BioBeads were from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA) and microfibre glass membranes (GF/C) were from Whatman (Whatman, Inc).

### *Isolation of nuclei and partial purification of the putative $Fe^{3+}$-ATPase by nondenaturing-polyacrylamide gel electrophoresis*

Rat hepatocytes nuclei were isolated and incubated in standard reaction (SR) medium containing 25 mM Hepes, pH 7.0, 125 mM KCl, 4 mM $MgCl_2$ as previously described [15]. Nondenaturing polyacrylamide gel electrophoresis (nondenaturing-PAGE) was performed as described by Laemmli [18] using a 6% polyacrylamide gel and 1 mg/mL $C_{12}E_8$[1] (polyoxyethilene-8-lauryl ether) instead of sodium dodecyl sulfate in the gel and running buffer. Nuclear proteins were solubilized in loading buffer (62.5 mM Tris–Cl, pH 6.8, 10% glycerol plus 1 mg/mL $C_{12}E_8$) and centrifuged at 8000*g* for 5 min. For each gel lane, an aliquot of the supernatant containing 300 μg of nuclear extract was subjected to electrophoresis for approximately 4 h, at 75 V in a cold room. After electrophoresis, the gel was washed for 40 min in SR medium, supplemented with 1 mg/mL $C_{12}E_8$. One gel lane was cut out and incubated at 37 °C for approximately 2 h in fresh SR medium, supplemented with 10 mM $CaCl_2$ and 5 mM. ATP, to detect ATPase activity in the form of white bands of calcium phosphate precipitate. Another gel lane was incubated in the same conditions with the addition of a inhibitors mix containing classical ATPase inhibitors at known inhibitory concentration: 1 μM thapsigargin [19,20], 100 μM DCCD [21–23], 100 μM ouabain [24,25], 1 μM FCCP [23,26], 0.5 μM antimycin A [23,26]. Corresponding bands of other lanes were cut off and electroeluted under nondenaturing conditions in a Gel Eluter (Hoefer Scientific Instruments, San Francisco, CA) for 6–8 h at approximately 50 V in 300 μL of 4× modified Laemmli buffer (0.1 M Tris–HCl and 0.768 M glycine, pH 8.3, plus 1 mg/mL $C_{12}E_8$) [18]. Protein concentration was determined according to Lowry [27]. Eluted samples were used for incorporation of proteins into phosphatidylcholine vesicles or submitted to SDS–PAGE (8%). Gels were stained with Coomassie Blue.

### *ATPase activity, ATP or iron binding under nondenaturing conditions*

Isolated rat liver nuclei (3 mg/mL) were incubated with SR medium supplemented with 1 mM [$\gamma$-$^{32}$P]ATP, in the presence or absence of 315 μM $Fe^{3+}$–citrate, at the indicated times and temperatures to detect ATP binding. Nuclei were also incubated with SR medium at 37 °C for 40 min, supplemented with 315 μM $^{55}Fe^{3+}$–citrate in the presence of 1 mM ATP to detect iron binding. In some experiments one of the following reagents was added to observe its effect on iron binding: 500 μM vanadate, 2 mM DFO, or a mix of ATPase inhibitors mentioned above. After incubation, the nuclear protein was centrifuged at 8000*g* for 5 min. The pellet was solubilized in loading buffer (62.5 mM Tris–Cl, pH 6.8, 10% glycerol plus 1 mg/mL $C_{12}E_8$) and centrifuged at 8000*g* for 5 min. For each gel, aliquots of the supernatant containing 300 μg of nuclear proteins were subjected to nondenaturing-PAGE for approximately 4 h, at 75 V. After electrophoresis, the gel was washed for 40 min in SR medium, supplemented with 1 mg/mL $C_{12}E_8$. A gel portion was incubated to observe ATPase activity, as described above, and another portion was quickly washed in SR medium, dried and used to verify iron or ATP binding by autoradiography. Autoradiographies were quantified using Scion Image Program (Scion Corporation).

### *Phosphorylation reaction and acid gel analysis*

The analysis of protein phosphorylation was performed essentially as described by Tsai and Linet [17] with some modifications. Proteins from LM (low mobility) and HM (high mobility) bands were electroeluted under nondenaturing conditions as described above and diluted to 400 μg/mL in the SR medium containing 30 μM of pre-formed $Fe^{3+}$–citrate complex (except in the cases where DFO was present). The reactions were started by addition of 2 μCi [$\gamma$-$^{32}$P]ATP (1 μM). After 10 s, the reactions were stopped by the addition of ice-cold TCA to a final concentration of 10% and placed in ice bath for 10 min. The precipitated reaction mixtures were washed with water and dissolved in acid gel loading buffer. Samples treated with DFO were pre-incubated with 2 mM DFO for 10 min at 37 °C before ATP addition.

In assays to determine the effects of alkali and hydroxylamine, the TCA-precipitated pellets were incubated with either 0.5 M KOH for 5 min in an ice bath or dissolved in a solution of 250 mM hydroxylamine and 50 mM sodium acetate for 10 min at room temperature. After incubation, samples were precipitated by the addition of 10% TCA and washed as described [16,17].

For pulse-chase experiments, the phosphorylation reactions were carried out as described and after incubation with [$\gamma$-$^{32}$P]ATP, an additional 1 mM of unlabeled ATP was added to the reaction mixture followed by incubation for 30 s at room temperature. Reaction was then stopped by addition of with TCA (10% final concentration). Samples were prepared for electrophoresis as described above.

The reactions were subjected to SDS–PAGE 8% at pH 6.4. After electrophoresis, gels were dried under vacuum at 80 °C and isotope incorporation was analyzed by autoradiography. Autoradiographies were quantified using Scion Image Program (Scion Corporation).

### *Inclusion of electroeluted nuclear proteins into liposomes and measurement of ferric ion uptake*

Hen egg yolk phosphatidylcholine (PC) was solubilized to 10 mg/mL by mixing the phospholipids with SR medium, 1.5 mg $C_{12}E_8$/mg PC and 7.5 mg of electroeluted protein/mg PC. Stepwise removal of detergent was

[1] *Abbreviations used:* $C_{12}E_8$, polyoxyethilene-8-lauryl ether; NEM, *N*-ethylmaleimide; DFO, desferrioxamine; PC, phosphatidylcholine; DCCD, dicyclohexylcarbodiimide; FCCP, carbonyl cyanide-*p*-trifluorometoxyphenylhydrazone; AMP-PNP; adenosine-5′-(β-γ-iminodiphosphate); SR, standard reaction medium; HM, high mobility; LM, low mobility.