Role of protein synthesis in the accumulation of ferritin mRNA during exposure of cells to iron

Elena MATTIA,* Jan den BLAAUWEN and Jos van RENSWOUDE†

E. C. Slater Institute for Biochemical Research, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands

The iron-induced biosynthesis of ferritin is regulated at the translational level via multiple mechanism. A prolonged exposure of cells to iron leads to a marked increase in ferritin mRNA levels caused by stabilization of the message. Here we show that this stabilization requires the synthesis *de novo* of an iron-inducible protein factor.

INTRODUCTION

Ferritin is an intracellular multimeric iron-storage protein. In all, 24 assembled units of two kinds, heavy (H) and light (L), form a shell in the core of which iron is sequestered [1]. The rate of ferritin biosynthesis is strongly dependent on intracellular iron levels. It is relatively high when cells are exposed to iron and substantially lower when iron availability is reduced by exposure of cells to deferoxamine, a compound which is able to chelate intracellular iron [2]. Upon iron administration, an inactive ribonucleoprotein pool of ferritin mRNA is translationally activated on polysomes [3,4]. A *cis*-acting element, contained within the 5' untranslated region of ferritin mRNA, mediates its translational regulation by forming a complex with a cytoplasmic factor in an iron-dependent fashion [5–7]. The redistribution of cytoplasmic ferritin mRNA occurring upon exposure of the cells for a few hours to iron takes place in the absence of changes in overall ferritin mRNA levels [3]. We have recently shown that the levels of ferritin H- and L-subunit mRNA, in cells exposed for 24 h to iron, are respectively 2–5- and 2–3-fold higher than the control values [8]. This increase is not transcription-dependent and is not prevented when cells are simultaneously exposed to an iron source plus the iron chelator deferoxamine. These findings suggest that: (i) the increment in ferritin mRNA levels might be caused by a decrease in the rate of turnover of ferritin mRNAs, and (ii) the ferritin mRNA accumulation is not mediated by chelatable iron. We hypothesize that some factor induced upon challenging cells with iron protects ferritin mRNAs from degradation. Alternatively, we consider the possibility that the iron-mediated recruitment of ferritin mRNA on to polysomes *per se* results in a lower rate of decay of ferritin mRNA. To gain further insight into the process that in iron-challenged cells leads to ferritin mRNA accumulation, we studied the effect of inhibition of protein synthesis. By using compounds that interfere with different steps of the translation process, we were able to evaluate the effect of ribosome–mRNA interaction on ferritin mRNA accumulation. Our results strongly indicate that the iron-dependent increase of ferritin mRNA levels is regulated by the synthesis of a protein which is involved in the stabilization of ferritin transcripts.

MATERIALS AND METHODS

Cell culture

K562 cells were maintained at $(2\text{--}5) \times 10^5$ cells/ml in RPMI

1640 medium supplemented with 10% (v/v) heat-inactivated newborn-calf serum. Cells were 'iron-loaded' with 30 μM -haemin, prepared as previously described [2].

Inhibitors of protein and RNA synthesis

Cycloheximide and puromycin were obtained from Sigma Chemical Co. Pactamycin was kindly provided by Upjohn Co. Concentrated stock solutions of cycloheximide in water (15 mg/ml), puromycin in water (35 mg/ml) and pactamycin in dimethyl sulphoxide (5 mg/ml) were stored at -20°C . Compounds were used in the experiments in the following final concentrations: cycloheximide, 75 $\mu\text{g/ml}$; puromycin, 100 $\mu\text{g/ml}$; pactamycin, 25 ng/ml. Actinomycin D (Dactinomycin; Merck, Sharp and Dohme) was used at a final concentration of 50 ng/ml.

Analysis of polysome distribution

K562 cells were grown at a density of about 5×10^5 /ml. From the cell culture, aliquots of 50 ml (corresponding to approx. 2.5×10^7 cells) were preincubated for 1 h at 37°C with no addition (control), cycloheximide (75 $\mu\text{g/ml}$), puromycin (100 $\mu\text{g/ml}$) or pactamycin (25 ng/ml). Cells were then centrifuged, resuspended in 5 ml of prewarmed (37°C) medium containing a protein-synthesis inhibitor and further incubated for 30 min at 37°C . After incubation, the cells were rapidly chilled by the addition of 10 vol. of ice-cold phosphate-buffered saline (0.15 M-NaCl/7 mM-sodium phosphate, pH 7.4) solution and centrifuged at 0°C for 5 min at 300 *g*. The cell pellets were lysed by the addition of 0.5 ml of polysome-lysis buffer (0.15 M-KCl/0.5% Nonidet P40/20 mM-dithiothreitol/50 mM-MgCl₂/10 mM-Tris/HCl, pH 7.2). The lysates were then centrifuged at 4°C for 10 min at 10000 *g*. From the supernatants, supplemented with KCl to a final concentration of 0.25 M, approx. 20 A_{260} units were layered on 15 ml 15–40%-(w/v)-sucrose gradients buffered with 0.25 M-KCl/50 mM-MgCl₂/20 mM-dithiothreitol/20 mM-Hepes buffer, pH 7.2. Gradients were centrifuged in a Beckman SW 27 rotor for 4 h at 25000 rev./min. Gradients were collected with a Buchler Auto Densi-flow II C instrument connected to a 2138 LKB Uvicord S monitor to trace the polyribosome profile.

RNA isolation and Northern-blot analysis

K562 cells were exposed for various periods of time up to 12 h to either haemin or haemin plus each of the inhibitors at the concentrations mentioned above. Total RNA was isolated by the

* On leave of absence from the Institute of Microbiology, Faculty of Medicine, University of Rome 'La Sapienza', Rome, Italy.

† To whom all correspondence and reprint requests should be sent.

LiCl/urea procedure described by Auffray & Rougeon [9]. The yield of total RNA from 10^7 cells was about 150–200 μg . RNA samples were electrophoresed on 1%-agarose gels containing 0.66 M-formaldehyde and transferred to Pall Biodyne A membranes (Pall Corporation) as described by Davis *et al.* [10]. RNA fixation was achieved by irradiation of the membranes for 7 min using a 254 nm-wavelength u.v. transilluminator. The 3' restriction-endonuclease-*Pst*I fragment of the ferritin H-chain cDNA and a *Pst*I fragment of the hamster actin gene were ^{32}P -labelled by random priming [11] to a specific radioactivity of 10^8 c.p.m./ μg of DNA. Prehybridization and hybridization were carried out as previously described [8]. The washed filters were exposed to Fuji X-ray film at -70°C for periods of time varying from 12 to 14 h. For quantification, autoradiographs were scanned with a Bio-Rad densitometer.

RESULTS AND DISCUSSION

To study whether ongoing protein synthesis is required for the iron-induced accumulation of ferritin subunit mRNA, we used three different protein-synthesis inhibitors known to affect the translation process via distinct mechanisms. Since the accumulation of ferritin subunit mRNAs becomes manifest during the first 8 h after exposure of cells to an iron source, it was important to choose such a concentration of the inhibitor as to be compatible with cell viability, given the relatively long time frame of the experiment. For this purpose we determined the lowest concentrations of cycloheximide, puromycin and pactamycin that, in K562 cells, were still able to inhibit the incorporation of

$[^3\text{H}]$ leucine into trichloroacetic acid-precipitable material by more than 90%. At 12 h after exposure of the cells to the inhibitors at such concentrations, cells appeared to be viable, as judged by Trypan Blue exclusion and, in the case of cycloheximide, by recovery of cell growth after removal of the inhibitor. Although the mode of action of different inhibitors that interfere with the protein-biosynthetic machinery is well known, the concentration of such inhibitors is often critical, in that different effects are produced at different concentrations [12]. We therefore analysed the polysome distribution obtained upon incubation of K562 cells with cycloheximide, puromycin and pactamycin at the concentrations chosen according to the criteria mentioned above. Fig. 1 illustrates the results of the experiment. The polysome profile of cells treated with cycloheximide shows an enrichment in heavier polysomes. The one from cells exposed to puromycin shows a nearly complete loss of the polysomal fraction, with a concomitant increase in the 40 S and 60 S ribosomal subunits and the 80 S monosomal component. The polysome profile of cells treated with pactamycin shows a total absence of the polysomal fraction, with a large increase in the monosomal component. These data demonstrate that, at the concentrations used in our experiments, the different inhibitors impede protein synthesis at known distinct stages: a block of the elongation step, a premature release of the transcripts and an inhibition of the initiation step respectively.

In order to evaluate the role of protein synthesis in the iron-induced accumulation of ferritin mRNAs, K562 cells were incubated with haemin in combination with either no inhibitor of protein synthesis (control), or with cycloheximide or puromycin or pactamycin at the concentrations mentioned above. Immediately after the additions (0 h) and 4, 8 and 12 h later, total RNA was isolated from samples equivalent to approx. 10^7 cells, electrophoresed, blotted and hybridized with the ^{32}P -labelled H-ferritin cDNA probe. Fig. 2 shows, as previously described [8], that in the presence of the iron source the ferritin H-subunit mRNA concentration gradually increases, reaching an increment of 3–4-fold over the control values after 12 h. By contrast, when any of the protein-synthesis inhibitors is added to the cell culture in combination with haemin, the iron-induced accumulation of ferritin mRNA is completely suppressed. The Northern blots, re-hybridized with an actin probe, revealed no difference in the amount of actin mRNA between the samples derived from the cells treated with haemin plus the antibiotic and those from cells exposed to haemin alone (results not shown). To exclude the

Fig. 1. Polysome distribution of K562 cells after treatment with protein-synthesis inhibitors

Cells were incubated with no addition (control), cycloheximide (75 $\mu\text{g}/\text{ml}$), puromycin (100 $\mu\text{g}/\text{ml}$) or pactamycin (25 ng/ml). The postmitochondrial supernatants were fractionated on 15–40%-sucrose gradients (see the Materials and methods section). Gradients were pumped through an Uvicord monitor, and the absorbance of the effluent was monitored at 254 nm. The 40 S and 60 S ribosomal subunits and the 80 S monosomal fraction are indicated.

Fig. 2. Effect of protein-synthesis inhibitors on iron-induced increase of ferritin mRNA levels

K562 cells were incubated for 4, 8 and 12 h with either 30 μM -haemin or 30 μM -haemin plus each of the inhibitors [cycloheximide (CHX), puromycin (PUR) or pactamycin (PACT)] at the concentrations indicated in the legend to Fig. 1. For each time point, 20 μg of total RNA was size-fractionated, blotted and hybridized with ^{32}P -labelled ferritin cDNA. The autoradiographic signals were quantified by densitometry, and the relative intensities are illustrated in the graph.

Fig. 3. Effect of actinomycin D (ACT. D) on iron-induced ferritin mRNA levels

Cells were incubated with either 30 μ M-haemin or with 30 μ M-haemin plus actinomycin D (50 ng/ml). At the times indicated, total RNA was isolated and analysed as described in Fig. 2.

possibility that protein-synthesis inhibitors would, by themselves, lead to a decrease of ferritin mRNA levels (thereby masking an increment induced by iron), total RNA was also extracted from cells incubated for 12 h with each of the inhibitors. Ferritin mRNA levels determined in cells exposed to the antibiotics appeared to be similar to those observed in control cells (results not shown).

Several studies have indicated that inhibition of protein synthesis differentially influences the rate of eukaryotic mRNA degradation [13]. Because of the different ways in which the tested protein-synthesis inhibitors interfere with ribosome-mRNA interaction, we conclude that the iron-induced increase in ferritin mRNAs is not due to a protection of the messengers from nuclease digestion when they are polysome-associated. On the contrary, data strongly indicate that the rise in ferritin mRNA concentration occurring during exposure of cells to iron depends on active protein synthesis.

A straightforward hypothesis would be that iron in fact *induces* the synthesis of a protein factor that acts as a stabilizer of the message. To test this hypothesis, we studied the effect of actinomycin D, an inhibitor of transcription, on the iron-induced stabilization of ferritin mRNAs. We have previously shown, by nuclear run-off experiments, that the rate of transcription of the ferritin genes is not altered when cells are exposed (for periods of time up to 24 h) to high loads of iron; this indicated that the regulation of ferritin mRNA concentration in response to iron is post-transcriptional [8]. K562 cells were incubated in the presence of 30 mM-haemin plus 50 ng of actinomycin D/ml or, alternatively, in the presence of haemin alone. The use of a relatively low concentration of actinomycin D that can still inhibit by 70 % the incorporation of [3 H]uridine into trichloroacetic acid-precipitable material allowed us to expose the cells to the drug for several hours without loss of viability. The same concentration of actinomycin D, when added to the culture medium for 12 h; reduced the total cellular protein content by 25 % and the synthesis of new proteins by 80 %, as judged by incorporation of

[35 S]methionine into trichloroacetic acid-precipitable material. Total RNA was extracted from control cells as well as from cells treated with haemin plus or minus actinomycin D for 4, 8 and 12 h, and ferritin mRNA levels were determined as described above. The results of this experiment, depicted in Fig. 3, show that the increment in ferritin H subunit mRNA occurring during incubation of cells with iron is prevented entirely when actinomycin D is added to the culture medium together with haemin. In fact, we observed a slight decrease in the concentration of ferritin subunit mRNA. A similar slight decrease was found in cells treated with actinomycin D in the absence of haemin (results not shown). Taken together, these data suggest that actinomycin D prevents the accumulation of ferritin mRNA by impeding the synthesis of an iron-inducible protein involved in the regulation of ferritin mRNA turnover. This protein might act a positive effector and, by forming complexes with *cis*-acting regulatory elements of the ferritin mRNAs, protect them from nuclease digestion. The slight decrease in ferritin mRNA levels observed after a 12 h treatment of the cells with actinomycin D presumably indicates that the ferritin messengers are characterized by a rather long half-life.

It has been recently shown that the binding of a cytoplasmic factor to secondary structures residing within the 3' untranslated region of the transferrin-receptor mRNA regulates its iron-dependent stability [14,15]. It will be of interest to investigate the 3' untranslated region of the H- and L-ferritin mRNA as sites for the formation of protein-mRNA complexes that affect, in an iron-dependent fashion, ferritin mRNA stability. The analysis of this region in eight different species has revealed a high degree of similarity in secondary structure [16], suggesting a functional role of these sequences in ferritin transcripts.

We thank Dr. C. van den Bogert and Dr. H. F. Tabak for helpful comments on the manuscript.

REFERENCES

1. Aisen, P. & Listowsky, I. (1980) *Annu. Rev. Biochem.* **49**, 357–393
2. Mattia, E., Josic, D., Ashwell, G., Klausner, R. D. & van Renswoude, J. (1986) *J. Biol. Chem.* **261**, 4587–4593
3. Rogers, J. & Munro, H. N. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2277–2281
4. Zaehring, J., Baliga, B. S. & Munro, H. N. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 857–861
5. Hentze, M., Rouault, T., Caughman, W., Dancis, A., Harford, J. & Klausner, R. D. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6730–6734
6. Leibold, E. A. & Munro, H. N. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2171–2175
7. Rouault, T. A., Hentze, M., Caughman, S. W., Harford, J. B. & Klausner, R. D. (1988) *Science* **241**, 1207
8. Mattia, E., den Blaauwen, J., Ashwell, G. & van Renswoude, J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1801–1806
9. Auffray, C. & Rougeon, F. (1980) *Eur. J. Biochem.* **107**, 303–314
10. Davis, L. G., Dibner, M. D. & Battey, J. F. (1986) *Methods in Molecular Biology*, p. 150. Elsevier, New York
11. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13
12. Pestka, S. (1971) *Methods Enzymol.* **30**, 261–282
13. Raghoebar, R. (1987) *Trends Biochem. Sci.* **12**, 358–360
14. Koeller, D. M., Casey, J. L., Hentze, M. W., Gerhardt, E. M., Chan, L. L., Klausner, R. D. & Harford, J. B. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3574–3578
15. Mullner, E. W., Neupert, B. & Kuhn, I. C. (1989) *Cell (Cambridge, Mass.)* **58**, 373–382
16. Dickey, L. F., Wang, Y., Shull, G. E., Wortman, I. A., III & Theil, E. C. (1988) *J. Biol. Chem.* **263**, 3071–3074