RESEARCH COMMUNICATION

Ferritin expression modulates cell cycle dynamics and cell responsiveness to H-ras-induced growth via expansion of the labile iron pool

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Repression or overexpression of ferritin accelerated or retarded cell cycling respectively, via changes in the cellular labile iron pool (LIP). A rise in LIP is caused by ferritin repression enhanced growth, induced by H-ras, and reverted growth arrest is induced by dominant negative H-ras. The studies indicate that repression

of ferritin expression provides a mechanism by which certain oncogenes lead to cell growth stimulation.

Key words: antisense, metals, oncogenes.

INTRODUCTION

The expression of cell ferritin (FT) has been implicated in the regulation of cell growth based on its potential for modulating the cellular labile iron pool (LIP) [1–3]. The major role of iron in cell growth and proliferation has been associated with the activation of ribonucleotide reductase (RR), which is the rate-limiting enzyme for *de novo* DNA synthesis [4]. However, iron is also an important cofactor of the G1 phase cyclins E/cdk2, D/cdk4 [5], D1 [6] and the S-phase cyclin A/cdk2 [7]. Accordingly, pronounced overexpression of the heavy FT chain (H-FT) in HeLa cells attenuated normal cell growth in a manner that was dependent on H-FT's capacity to incorporate iron [1]. On the other hand, moderate overexpression of H-FT [2] as well as partial repression of both FT subunits produced no significant effect on normal cell growth [3].

As opposed to the lack of effect of FT depletion on cells growing in normal culture conditions, depletion of FT did affect chemically induced growth. Thus repression of FT synthesis seemingly facilitates cell cycle progression by increasing iron availability [3]. Since FT repression was also observed in cells stimulated by some oncogenes [8–10], it was surmised that this repression served as a means for rendering iron available for the oncogene-stimulated growth. The purpose of the current work was to assess whether the involvement of FT expression in growth modulation depends on conditions of oncogene-induced or otherwise stimulated growth, in which demand for accessible iron is imminent. As iron-requiring growth conditions, we focused on the kinetics of progression through a single cell cycle and on responsiveness to H-ras activation. H-ras was chosen as a growth-inducing system because, as opposed to other oncogenes [9,10], it apparently does not perturb FT expression. Therefore, FT expression and its effects on the LIP could be manipulated directly and independently of any cross-interaction with the oncogene. This enabled us to assess how the control of iron availability in the cell can modify, and even rate limit, the

proliferative response of cells to a growth-stimulating factor such as an oncogene.

EXPERIMENTAL

Materials

Oligodeoxynucleotides (ODNs) were procured from Eisenberg Brothers (Givat Shmuel, Israel) and muristerone A from Alexis (San Diego, CA, U.S.A.). Unless specified otherwise, all other chemicals were from Sigma (St. Louis, MO, U.S.A.) or the best available grade. Recombinant H-FT and L-FT, and antibodies against them were kindly provided by Dr P. Santambroggio (Department of Biological and Technological Research, Milan, Italy). Monoclonal mouse anti AU5 antibodies were kindly provided by Dr D. Engelberg (Hebrew University, Jerusalem, Israel). Polyclonal antibodies against cyclin A were a kind gift from Dr M. Brandeis (Hebrew University, Jerusalem, Israel).

Cells

All cells were grown in Dulbecco's minimal essential medium supplemented with 10% (v/v) foetal-calf serum. Viable cells were counted in a Coulter Cell Counter (Coulter Electronics, Harpenden, Herts, U.K.). Mouse erythroleukaemia (MEL) cells overexpressing H-FT were stably transfected with an iron responsive element (IRE) mutant of H-FT, which precluded it from being feedback modulated by iron and enabled direct control of its expression. Parental (pVgRXR transfected) HEK 293 cells (a kind gift from Dr D. Engelberg, Department of Biological Chemistry, Hebrew University of Jerusalem, Israel) were kept at constant selection pressure with 200 µg/ml Zeocin (Cayla, Toulouse, France), against which resistance was provided by the *Sh ble* gene in the pVgRXR plasmid. After transfection with the response plasmid, based on the pIND vector (Invitrogen, Carlsbad, CA, U.S.A.; kindly provided by Dr D. Engelberg), geneticin (G418; 400 µg/ml) was also added to the growth

Abbreviations used: FT, ferritin; H-FT, heavy ferritin chain; L-FT, light ferritin chain; LIP, labile iron pool; AS, antisense; IN, reversed-antisense oligodeoxynucleotide; DFO, desferrioxamine; FAC, ferric ammonium citrate; ODN, oligodeoxynucleotide; MEL, mouse erythroleukaemia; VgEcR, ecdysone receptor; RXR, retinoid X receptor; IRE, iron responsive element; RR, ribonucleotide reductase.

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Figure 1 Effect of H-FT overexpression on the percentage of MEL cells in S-phase following release from growth arrest by mevalonate

The relatively moderate (○) and relatively high (▲) H-FT-overexpressing clones (Cl.) 16 and 6 respectively and wild-type (WT) cells (■) were treated with 10 μ M lovastatin for 24 h, washed and resuspended in 2 mM mevalonate. Clone 6 cells were also resuspended in a medium containing 2 mM mevalonate and 50 μ M ferric ammonium citrate (FAC) (x). (A) Percentage of cells in the S phase was analysed by flow cytometry 3, 6 and 9 h after the resuspension. The cell cycle distributions are shown. (B) The change in the percentage of cells in S-phase over time. Data were pooled from three experiments.

medium, providing a selection based on the neomycin resistance gene in the different pIND constructs.

Depletion of FT subunits

H- and L-FT subunits were repressed in K562 cells using antisense oligodeoxynucleotides (AS-ODNs), as described in [3]. AS-ODNs

were introduced into HEK 293 cells using liposomes, as described in [11].

FT quantification and LIP determination

FT was determined using an ELISA [12] and the LIP was determined using the calcein method, as described elsewhere [13].

Stable transfection of HEK 293 cells with inducible H-ras mutants

HEK 293 cells stably transfected with the pVgRXR vector were used as parental cells. The pVgRXR vector encodes for the ecdysone receptor (VgEcR) under the control of the cytomegalovirus promoter and the retinoid X receptor (RXR) under the control of the Rous sarcoma virus promoter. In the presence of an ecdysone analogue (such as muristerone A) RXR and VgEcR associate and bind a hybrid ecdysone response element which has been incorporated upstream of the gene of interest in the pIND constructs. Binding of the muristerone-A-activated VgEcR-RXR hybrid drives transcription of the gene of interest via a minimal heat-shock promoter. The parental HEK 293 cells were transfected with 3 different pIND constructs: pIND (an empty vector); pIND-AU5-H-ras[Val12], encoding for H-ras[Val12] 5' fused to an AU5 tag; pIND-AU5-H-ras[Asn17], encoding for H-ras[Asn17] 5' fused to AU5. Transfection of stable clones was performed by calcium phosphate-DNA co-precipitation, as described in [1]. Twenty-four hours after the dilution of the transfected cells, selection medium was added and clones were screened for inducible expression of H-ras in the following way. When cells became nearly confluent they were induced with either 0.67 μ g/ml muristerone A or DMSO (negative control) for 24 h. Cells were then dissolved in sample buffer [10% (v/v) glycerol, 30 mg/ml SDS, 0.05 M Tris/HCl, 5% (v/v) 2-mercaptoethanol, pH 6.8] and analysed for protein content using a modified Bradford assay [14]. Aliquots (30 μ g per lane) of each induced/non-induced lysate were analysed by SDS/PAGE (12% gel). The AU5 tag of the H-ras mutants was detected on immunoblots using mouse anti-AU5 monoclonal antibodies and mouse monoclonal anti-actin IgM (Amersham International, Little Chalfont, Bucks., U.K.). As secondary antibodies goat anti-mouse antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, U.S.A.) were used in combination with ECL* (Amersham) detection. The clones in which the muristerone A-induced to non-induced AU5 expression ratio was maximal (see Figures 3A and 3C) were used for further experiments.

Cell cycle analysis

For assessing the percentage of K562 cells in S-phase following growth arrest, cells (initially at 200 000/ml) were treated with none, FT AS, reversed-antisense oligodeoxynucleotide (IN), or FT AS + 20 μ M desferrioxamine (DFO) for 24 h, supplemented with 10 μ M lovastatin and harvested after 10, 20 and 24 h. The cells were subsequently fixed in 75% methanol (1 h at -20°C), washed and treated with 5 μ g/ml propidium iodide. The cell cycle distribution of at least 10 000 stained cells was obtained by flow cytometry (FacsCalibur; BDIS, San Jose, CA, U.S.A.). In order to provide the percentage of cells in S-phase, the cell cycle data were analysed quantitatively by ModFit LT Cell Cycle Analysis software (Verity, Topsham, ME, U.S.A.).

The percentage of MEL cells in S-phase, following the release from lovastatin arrest, was determined by treating cells with 10 μ M lovastatin for 24 h, washing and transferring to media containing the lovastatin inhibitor mevalonate (2 mM). At 3, 6

and 9 h the cells were analysed for cell-cycle distribution as above.

Cell-cycle distribution in HEK 293 H-ras mutants was obtained as follows. After 5 h incubation with the ODN probes (either IN or FT AS) cells were washed, incubated for 24 h and then induced for another 24 h with either 0.67 $\mu\text{g}/\text{ml}$ muristerone A or its solvent DMSO alone, as a negative control. The cells were then harvested and analysed for either cell-cycle distribution by flow cytometry (see above) or cyclin A expression by Western blot. For the latter, 30 μg of protein were run on SDS/polyacrylamide gels (10%) and immunoblotted using rabbit anti-(cyclin A) polyclonal antibodies as primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit antibodies as secondary antibodies. Actin served as a normalizing control and was detected as described above.

RESULTS AND DISCUSSION

We examined whether modulation of LIP by either repression or overexpression of FT affects cell-growth properties, as reflected by the rate of their progression through a single cell-cycle (short-term growth effects). Cells overexpressing H-FT were blocked at G1 with lovastatin, which mediates G1 arrest by stabilizing the

cyclin-dependent kinase inhibitors p21 and p27 [15]. The percentage of cells in the S-phase was monitored after lifting the lovastatin block by mevalonate. As shown in Figure 1, the renewal of DNA synthesis was significantly delayed in H-FT-overexpressing cells, compared with cells that had normal expression of H-FT. The fact that the FT-overexpressing cells showed a significantly lower LIP than the control [2] suggests a possible link between FT effects on the LIP and on cell growth. Support for that putative link was obtained in FT-overexpressing cells which were relieved from growth retardation by iron supplementation (Figure 1).

As a complementary approach to FT overexpression, we assessed the effects of a reduction in FT expression, attained by AS-ODN treatment. The AS-ODN treatment, which resulted in an approx. 70% reduction in FT expression, increased the cellular LIP and the renewed proliferation of cells pre-arrested at the G1 phase [3]. We assumed that since the FT-repressed cells progressed faster through the cell cycle following their lifting from a G1 block, adding lovastatin to the culture should result in faster arrest at G1. Addition of lovastatin caused faster cell cycle arrest in cells treated with the H- and L-FT AS-ODNs, as apparent from the lower percentage of cells in the S-phase (Figure 2A) and the higher percentage of cells in G1 with time (results not shown). When cells were released from a lovastatin-

Figure 2 Effect of H- and L-FT repression on the percentage of cells in S-phase following initiation of growth arrest, and cyclin A expression following release from growth arrest

(A) Cell-cycle distributions and (B) pooled data from three experiments, after human K562 cells were treated with: nothing (C) (●), 1 nM reversed H-FT AS-ODN (IN) (▲), 1 nM H- and L-FT AS-ODN (AS) (x), or 1 nM H- and L-FT AS-ODN with 20 μM DFO (AS + DFO) (○). Twenty-four hours later, 10 μM lovastatin was added and cells were harvested and analysed by flow cytometry 10, 20 and 24 h following the addition of lovastatin. (C) Cells were incubated for 24 h alone (Untr.), or with one of the following: 1 nM reversed H-FT AS-ODN (IN), 1 nM FT AS-ODN (AS), 20 μM FAC (FAC), 1 nM FT AS-ODN with 20 μM DFO (AS + DFO), or 50 μM DFO (DFO). The cells were subsequently growth-arrested by incubation with 10 μM lovastatin, washed and resuspended in media containing 2 mM mevalonate. DFO and FAC were added together with the lovastatin. The cells were harvested after 9 h and analysed for cyclin A expression by immunoblotting.

Figure 3 H- and L-FT repression and its effects on LIP in H-ras mutants

HEK 293 cells stably transfected with H-ras[Val12] (A), or with H-ras[Asn17] (C) were not induced (DMSO), induced for H-ras expression by muristerone A (mur.) and treated with 5 μM of AS-ODN against H- and L-FT (FT AS, 2.5 μM of each), reversed H-FT AS-ODN (IN), or 50 μM DFO. Forty-eight hours later FT content was analysed by ELISA. Values in H- and L-FT AS and DFO treated cells were different to the values from not induced, or induced cells treated with a reversed H-FT AS-ODN. This difference was with levels of significance $**P = 0.01$, or $*P = 0.05$ as determined by independent *t* tests. **Inserts** immunoblots showing muristerone A-inducible expression of AU5 tagged H-ras mutants. In parallel, LIP levels in H-ras[Val12] (B) and H-ras[Asn17] mutants (D) were determined as described in [13]. In brief, the cells were loaded with the permeant acetomethoxy calcein. Extracellular fluorescence was quenched by calcein antibodies (CA-Abs) and the amount of LIP was estimated by the level of fluorescence dequenching following the addition of the strong chelator salicylaldehyde isonicotinoyl hydrazone (SIH). Bars represent the amount of LIP in units of intracellular calcein concentration ($\mu\text{M CA}_{in}$).

mediated G1 arrest, they progressed much faster through the cell cycle, as manifested by the elevated expression of the S-phase cyclin, cyclin A (Figure 2B). These results corroborate our previous findings of accelerated entry into the S-phase by reducing FT levels [3]. In accordance with those previous results, the effect of FT repression was mediated by an increase in LIP, while the iron chelator DFO reversed this effect (Figure 2B, compare lanes 'FT AS', 'FT AS + DFO' and 'DFO'). Taken together, our results suggest that FT repression can expedite cell cycling by expanding the LIP. We hypothesize that the elevated iron availability, caused by FT repression might subserve the acute demand for iron, typical of short-term growth, and facilitate growth. The iron-dependent RR [4] and possibly some iron-dependent cyclins [16] are potential candidates for this iron utilization.

As FT depletion facilitated pharmacologically induced cell growth, we tested the hypothesis that FT repression might condition oncogene stimulation of cell growth by rendering cell iron available for metabolism. This possibility is of particular interest since some oncogenes were observed to transcriptionally repress FT expression [8–10] and iron deprivation or over-supplementation demonstrably retarded or accelerated cell growth respectively (Figure 2). The experimental system chosen

for testing the above hypothesis, relied on HEK 293 cells engineered to express H-ras when exposed to muristerone A. The choice was based on two facts: (a) induction of H-ras itself did not affect FT and the LIP levels (Figure 3), thus allowing FT and the LIP levels to be manipulated independently of oncogene stimulation, and (b) HEK 293 cells are less prone to undergo differentiation steps which might affect FT expression, as found in haemopoietic cell lines [17,18].

The H-ras mutants were selected based on muristerone A inducibility of H-ras expression (Figures 3A and 3C). As a means for manipulating the LIP levels via the repression of FT expression we used AS-ODN against both H- and L-FT subunits. The FT AS-ODNs were able to reduce both H- and L-FT expression in H-ras transformed HEK 293 cells by up to 3- and 6-fold, respectively (Figures 3A and 3C). This reduction in FT expression caused 2.22-fold and 3.58-fold increases in the LIP of H-ras induced H-ras[Val12] and H-ras[Asn17] mutants respectively (Figures 3B and 3D). The iron chelator DFO, which can repress FT post-transcriptionally [19], was also able to repress FT expression in HEK 293 cells. In addition, the FT subunit specificity of the AS-ODNs was shown elsewhere [3] and administration of an AS-ODN in which the base order was reversed did not affect the FT levels (Figures 3A and 3C).

Figure 4 Effect of H- and L-FT repression on cell-cycle profiles and cyclin A expression in H-ras[Val12] and H-ras[Asn17] transfected HEK 293 cells

HEK 293 parental cells transfected with the pVgRXR plasmid were stably transfected with the pIND vector into which either the H-ras[Val12] constitutive mutant (**A** and **B**) or the H-ras[Asn17] dominant negative mutant (**C** and **D**) were inserted. The cells were pretreated (when applicable) with ODN probes for 24 h and then induced (when applicable) for another 24 h with muristerone A. The cells were harvested and analysed for either cell-cycle distribution by flow cytometry (**A** and **C**), or cyclin A and actin expression by immunoblotting (**B** and **D**). The actin normalized cyclin A band intensities pooled from three independent experiments are portrayed below the blots in (**B** and **D**). (**A**) Cell-cycle profiles of (from left to right) cells transfected with the empty pIND vector, parental cells, non-induced H-ras[Val12] cells, muristerone-induced H-ras[Val12] cells pretreated with a reversed H-FT AS-ODN (IN), and muristerone-induced H-ras[Val12] cells pretreated with H and L FT AS-ODN's. (**B**) Cyclin A and actin expression of the respective treatments as in (**A**). (**C**) Cell-cycle profiles of (from left to right) non-induced H-ras[Asn17] cells, muristerone-induced H-ras[Asn17] cells pretreated with a reversed H-FT AS-ODN (IN), and muristerone-induced H-ras[Asn17] cells pretreated with FTAS-ODNs. (**D**) Cyclin A and actin expression of the respective treatments as in **C**. A representative experiment repeated three times.

Table 1 Cell-cycle distributions of HEK 293 cells

The Table compiles data given in Figures 4 (A and C). Cell-cycle distribution of (from left to right) cells transfected with the empty pIND vector, parental cells, non-induced H-ras[Val12] cells, muristerone-induced H-ras[Val12] cells pretreated with a reversed H-FT AS-ODN (IN), muristerone-induced H-ras[Val12] cells pretreated with H and L FT AS-ODN's, non-induced H-ras[Asn17] cells, muristerone-induced H-ras[Asn17] cells pretreated with a reversed H-FT AS-ODN (IN), and muristerone-induced H-ras[Asn17] cells pretreated with FT AS-ODNs.

Cell-cycle stage	Treatment (% cells at each cell-cycle stage)							
	pIND	pVgRXR	H-rasV12 + DMSO	H-rasV12 + mur. + IN	H-rasV12 + mur. + FT AS	H-rasN17 + DMSO	H-rasN17 + mur. + IN	H-rasN17 + mur. + FT AS
G1/G0	58.35	55.34	57.43	43.44	34.99	49.77	85.24	54.69
S	34.26	36.32	34.65	40.10	47.16	38.62	13.36	33.91
G2/M	7.39	8.35	7.92	16.46	17.85	11.61	1.40	11.40

In several independent experiments, a 24 h induction of the constitutively active H-ras[Val12] [20] in HEK 293 cells caused between 1.5- and 2-fold increases in cell count, compared with the non-induced cells (results not shown). We therefore surmised that induction of expression of H-ras[Val12] affects cell proliferation by expediting the transition from G1 to S-phase [21]. These effects should be manifested as an increase in the proportion of cells in S-phase at the expense of cells in G1 phase in non-synchronized cells. A flow cytometry analysis of the cell-

cycle profiles (Figure 4A and Table 1) combined with a protein blot analysis of cyclin A (Figure 4B) revealed an increase in the percentage of cells in S-phase following H-ras[Val12] induction. Depletion of FT and augmentation of the LIP prior to H-ras[Val12] induction caused a further increase in the proportion of cells in S-phase (Figure 4A) and concomitantly in cyclin A expression (Figure 4B).

Competition of the dominant negative H-ras[Asn17] mutant with endogenous oncoproteins on binding of guanine-nucleotide-

exchange factor reduces their activation and delays proliferation [22]. A 24 h induction of H-ras[Asn17] led to a 3-fold reduction in cell counts (results not shown). Consequently, we checked whether cells were arrested at G1 by H-ras[Asn17] induction and whether pretreatment with a FT-repressing agent can rescue them from the arrest. The results shown in Figure 4(C) and Table 1 attest that H-ras[Asn17]-mediated cell-cycle arrest can be, at least partially, reversed by FT repression and expansion of the LIP (Figures 3C and 3D). Following H-ras[Asn17] induction, changes in the proportion of cells in S-phase were commensurate with changes in cyclin A expression similar to the H-ras[Val12] induction (Figure 4D). Pretreatment of H-ras[Asn17] cells with a reversed FT AS-ODN (IN) did not modulate the H-ras-mediated effects.

We have shown here that by modulating the levels of the LIP, FT expression can affect cell growth in the short term. Expansion of the LIP facilitated the recovery from cell-cycle arrest and the arrest process itself, while contracting the LIP by FT overexpression slowed down cell cycling. Our results also provide strong support for the suggestion that oncogenes repress FT expression as a preparatory step for their growth stimulation. The FT depletion-mediated expansion of the LIP supports growth stimulation by constitutively active H-ras and circumvents cell-cycle arrest by a dominant negative H-ras.

FT repression is apparently not involved in H-ras growth stimulation but is associated with growth stimulation by other oncogenes. This difference might result from variability in cell type, signal transduction pathways and in initial levels of the LIP, which may alter growth dependence on iron supplementation. In spite of the lack of H-ras effect on FT expression and the LIP, iron was rate limiting to H-ras-induced growth (Figure 4). This observation demonstrates that iron may be rate limiting to growth even if it is not a genuine component of a growth stimulation mechanism. Iron can enhance H-ras growth stimulation either by activating RR and thus DNA synthesis [23], or by up modulating superoxide production and contributing to a novel H-ras pathway involving Rac, NADPH oxidase and nuclear factor κ B [24].

The main finding of the current work is that FT repression can support oncogene-induced growth by acting as an active LIP-expanding agent. Previous studies did not attempt to support experimentally a functional link between oncogene stimulation of cell growth and transcriptional modulation of FT expression affecting the cellular LIP [8–10]. Since in most studies, FT expression was measured only at the mRNA level, the possibility that the changes in FT expression are merely passive responses to other cellular processes cannot be dismissed. Put together, our results show that FT repression can expedite both pharmacologically and oncogene-stimulated growth. These observations provide experimental support for the assumption that transcriptional FT repression is a cellular strategy for enhancing growth stimulation by oncogenes.

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