Extra-mitochondrial localisation of frataxin and its association with IscU1 during enterocyte-like differentiation of the human colon adenocarcinoma cell line Caco-2

Fabio Acquaviva[1], Irene De Biase[1], Luigi Nezi[1], Giuseppina Ruggiero[1], Fabiana Tatangelo[2], Carmela Pisano[3], Antonella Monticelli[1], Corrado Garbi[1], Angela Maria Acquaviva[1,*] and Sergio Cocozza[1]

[1]Dipartimento di Biologia e Patologia Cellulare e Molecolare, Via S. Pansini 5, Istituto di Endocrinologia ed Oncologia Sperimentale Centro Nazionale delle Ricerche, Università 'Federico II', Napoli, Italy
[2]Unità Operativa Complessa di Anatomia Patologica, Istituto Nazionale Tumori, Via M. Semmola, 80131 Napoli, Italy
[3]Unità Operativa Complessa di Oncologia Medica B, Istituto Nazionale Tumori, Via M. Semmola, 80131 Napoli, Italy
*Author for correspondence (e-mail: angacqua@unina.it)

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Summary

Friedreich's ataxia is a recessive neurodegenerative disease due to insufficient expression of the mitochondrial protein frataxin. Although it has been shown that frataxin is involved in the control of intracellular iron metabolism, by interfering with the mitochondrial biosynthesis of proteins with iron/sulphur (Fe/S) clusters its role has not been well established. We studied frataxin protein and mRNA expression and localisation during cellular differentiation. We used the human colon adenocarcinoma cell line Caco-2, as it is considered a good model for intestinal epithelial differentiation and the study of intestinal iron metabolism. Here we report that the protein, but not the mRNA frataxin levels, increase during the enterocyte-like differentiation of Caco-2 cells, as well as in in-vivo-differentiated enterocytes at the upper half of the crypt-villus axis. Furthermore, subcellular fractionation and double immunostaining, followed by confocal analysis, reveal that frataxin localisation changes during Caco-2 cell differentiation. In particular, we found an extramitochondrial localisation of frataxin in differentiated cells. Finally, we demonstrate a physical interaction between extramitochondrial frataxin and IscU1, a cytoplasmic isoform of the human Fe/S cluster assembly machinery. Based on our data, we postulate that frataxin could be involved in the biosynthesis of iron-sulphur proteins not only within the mitochondria, but also in the extramitochondrial compartment. These findings might be of relevance for the understanding of both the pathogenesis of Friedreich's ataxia and the basic mechanism of Fe/S cluster biosynthesis.

Key words: Frataxin, Caco-2 cells, Differentiation, Mitochondrial mass, Subcellular localisation

Introduction

Friedreich's ataxia (FRDA) is the most common inherited ataxia. It is an autosomal recessive degenerative disorder that involves mainly the nervous system, but is also frequently associated with cardiac hypertrophy and diabetes (Harding, 1981). The most common mutation causing the disease is a large trinucleotide expansion in the *X*25 gene encoding frataxin (Campuzano et al., 1996). This mutation causes a severe reduction in frataxin transcript and steady-state protein levels (Campuzano et al., 1997; Bidichandani et al., 1998). Frataxin has been localised by indirect immunofluorescence and immunoelectron microscopy to the mitochondria of transfected and non-transfected cells (Campuzano et al., 1997). Yeast cells lacking the yeast frataxin homologue yfh1 exhibit mitochondrial iron accumulation and constitutive activity of the high-affinity iron uptake system (Babcock et al., 1997; Cavadini et al., 2000; Foury and Talibi, 2001). Due to the fact that yfh1 has been shown to form a high molecular mass complex with iron, it has been proposed to act as an iron chaperone (Adamec et al., 2000; Cavadini et al., 2002; Park et al., 2002). Moreover, impaired biosynthesis of iron-sulphur (Fe/S) clusters and defective activities of Fe/S-containing enzymes have been demonstrated in a Δ-Yfh1 yeast strain (Babcock et al., 1997).

Iron/sulphur proteins (ISPs) represent a heterogeneous group of proteins with different functional features and different subcellular localisation. ISPs are more frequently found in mitochondria, although they have also been located in the cytosol and the nucleus (Muhlenhoff and Lill, 2000). ISPs play an essential role in a variety of processes including redox reactions, DNA repair and the sensing of iron and oxygen levels (Cammack, 1992; Bainert and Kiley, 1999). Impaired activity of proteins with Fe/S clusters has been demonstrated both in frataxin KO mice and in tissues from FRDA patients (Rotig et al., 1997; Puccio et al., 2001). Finally, it has been demonstrated that frataxin is involved in the biosynthesis of Fe/S proteins physically interacting with Isu1/Nfs1, a component of the Fe/S cluster assembly machinery (Huynen et al., 2001; Muhlenhoff et al., 2002, Duby et al., 2002; Yoon and Cowan, 2003; Gerber et al., 2003). The

present study investigates frataxin expression during intestinal epithelial cell differentiation. The human colon carcinoma cell line Caco-2 is considered a good model for studying iron metabolism (Pinto et al., 1983; Jumarie and Malo, 1991) and cell differentiation in enterocytes (Halleux and Schneider, 1991; Alvarez-Hernandez et al., 1991). Indeed, Caco-2 cells display many features of the intestinal epithelial cells that differentiate into mature enterocytes in vivo. During their functional maturation, enterocytes modify the expression patterns and activities of many proteins that are implicated in iron metabolism in response to body iron stores and act as modulators of iron absorption (Babyatsky and Podolsky, 1999; Ekmekcioglu et al., 1998; Griffiths et al., 2000).

In the present study, we report an increase in frataxin protein levels during enterocyte differentiation both in vitro and in vivo. Furthermore, we demonstrate that frataxin is unexpectedly present in an extramitochondrial location during Caco-2 cell differentiation. We also show a physical interaction between frataxin and IscU1, the cytosolic isoform of the human Fe/S assembly machinery, suggesting that frataxin is involved in the biosynthesis of ISPs not only within mitochondria, but also in the extramitochondrial compartment.

Materials and Methods

Cell culture

Caco-2 cells were seeded at 4×10^3 cells cm^{-2} in 100 mm plastic dishes, and grown as previously described (Pinto et al., 1983; Pignata et al., 1994). After a lag period of about 48 hours from the seeding, the cells begin an intense proliferating phase. At day 4, cell growth is typically in logarithmic phase. Cells reach confluence at about 6-7 days and are in the stationary phase after 9 days of culture. Morphological differentiation begin in the early proliferative phase, while brush-border-associated enzymatic activities are first detected at day 9 and increase progressively until functional differentiation is complete (Pinto et al., 1983).

Western blot analysis

Protein extracts obtained from proliferating (day 4 of culture) and differentiated (day 13 of culture) cells were denatured and separated on a 15% SDS polyacrylamide gel, transferred to Hybond ECL nitrocellulose membrane (Amersham) by electroblotting and blocked in 10% milk overnight. The mouse monoclonal anti-frataxin antibody 1G2 (Chemicon International) was used at 1:10,000 dilution. The goat polyclonal anti-Voltage Dependent Anion Channel (VDAC-1, Santa Cruz Biotechnology), the mouse monoclonal anti-Citrate Synthase antibody (Chemicon International) and the goat polyclonal anti-IscU1 antibody (Santa Cruz Biotechnology) were all used at 1:1000 dilution. The goat anti-mouse and the monkey anti-goat IgG peroxidase conjugates (Sigma) were used to visualise the specific immunoreactive band (ECL, Amersham). The relative intensities were quantified using NIH Image software. Values were normalised according to the intensity of tubulin (anti-tubulin antibody, Santa Cruz Biotechnology).

Immunoprecipitation

Protein extracts (300 μg) obtained from proliferating (day 4 of culture) and differentiated (day 13 of culture) cells were incubated overnight with 10 μl of anti-frataxin antibody or with anti-IscU1 antibody. Following the incubation, protein A-G Sepharose was added for 1 hour. Pellets were washed in cold lysis buffer several times and then resuspended in 2× loading buffer for western blot analysis.

Tissue samples and immunohistochemistry

Formalin-fixed, paraffin-embedded 4 μm tissue sections of normal colorectal mucosa, were immunoassayed using the biotin-streptavidin-peroxidase method (YLEM). Sections were subjected to routine deparaffinization and rehydratation before being immersed in 10 mM sodium citrate buffer (pH 6.0), boiled for 10 minutes on a hot plate, and allowed to cool for 20 minutes. Sections were then incubated for 10 minutes in 3% hydrogen peroxide in distilled water, washed in PBS three times for 5 minutes each and treated with 10% normal horse serum in PBS for 30 minutes. After three washes in PBS buffer, the sections were treated overnight at 4°C with 2 μg m^{-1} of anti-frataxin antibody. The sections were then incubated with biotin-labelled secondary antibody (1:30) and streptavidin-peroxidase (1:30) for 20 minutes each. Slides were stained for 5 minutes with 0.05% 3,3′-diaminobenzidine tetrahydrochloride freshly prepared in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.024% hydrogen peroxidase and then counterstained with haematoxylin, then dehydrated and mounted in Diatex. After three washes in PBS buffer (5 minutes each), the sections were incubated for 10 minutes with biotinylated secondary antibody, washed in PBS three times for 5 minutes, and incubated for 10 minutes with the avidin-biotin complex according to the manufacturer's instructions (YLEM). Finally, the sections were washed and incubated with diaminobenzidine in 0.1 M Tris buffer (pH 7.6) with 0.03% hydrogen peroxide, rinsed in tap water, counterstained and mounted. Negative controls were obtained by omitting the primary antibody.

Indirect immunofluorescence staining

Caco-2 cells were seeded on glass coverlips. At day 4 and 13 of culture, the standard growth medium was removed. Cells were incubated for 1 hour in the presence of 100 nM MitoTracker and then extensively washed in PBS/CaMg. Cells were then, fixed with 3% Paraformaldehyde and 60 mM Sucrose, in PBS for 20 minutes at room temperature and consecutively washed in NH_4Cl and PBS/CaMg. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes and blocked by the addition of 1% BSA in PBS/CaMg for 30 minutes. After a 1-hour-incubation at 37°C with the anti-frataxin antibody or with a non-specific antibody as negative control, cells were extensively washed and further incubated with FITC-tagged goat anti-mouse secondary antibody. Cells were washed three times with PBS/CaMg and the coverlips were mounted on a microscope slide using a 50% glycerol solution in PBS/CaMg. Microscopic analysis was performed using a Zeiss LSM510 confocal microscope. Every double-immunostaining was also performed with a control antibody to ensure the specificity of the frataxin signal.

Subcellular fractionation

Proliferating and differentiated cells collected at day 4 and 13 respectively, were washed in PBS and scraped into a buffer containing 250 mM Mannitol, 0.5 mM EGTA, 5 mM HEPES and 0.1% BSA. Cells were then centrifuged at 1000 ***g*** for 5 minutes at 4°C to pellet nuclei, membranes and unbroken cells. The supernatant was centrifuged at 10,500 ***g*** for 15 minutes at 4°C to separate a 'soluble cytosolic fraction' from a pellet containing membranes and mitochondria. The pellet was then resuspended in lysis buffer. Western blot analysis was performed according to the previously described method. The enrichment for mitochondria in the 'membranes fraction' and the absence of them in the soluble cytosolic fraction was assessed using the mitochondrial markers VDAC-1 and Citrate Synthase.

Fluorescence activated cell-sorting (FACS) analysis

Cells at day 4 and 13 of culture were trypsinized, pelleted at 600 ***g*** for 5 minutes and then resuspended in PBS/2% FCS/0.1% EDTA

buffer at 3 to 5×10^6 cells m^{-1}. For surface staining, cells were incubated for 40 minutes with the anti-frataxin antibody or with an antibody against mitochondrial manganese superoxide dismutase (Mn-SOD) (Bender MedSystems). After extensive washing, the binding was revealed using the FITC-conjugated goat anti-mouse IgG (SIGMA). Cells treated only with FITC conjugated goat anti-mouse IgG (SIGMA) were used as isotypic controls. To obtain intracellular staining, cells were permeabilized using Cytofix/Cytoperm kit (Becton Dickinson). Permeabilized cells were incubated as described above for the non-permeabilized condition. Flow cytometry and data analysis were carried out using a FACScalibur Becton Dickinson cytometer and the CellQuest analysis software, as described (Lanier and Recktenwald, 1991). For each analysis, 10,000 events were recorded. The result of one of three independent experiments is reported as the proportion (%) of positive cells.

Quantitative analysis of mtDNA and frataxin mRNA

A quantitative assay for mtDNA and frataxin mRNA expression was established using the GeneAmp 7000 Sequence Detection System (Applied Biosystem). Quantitative real time amplification of D-loop and Cytochrome-b was used to estimate mtDNA quantity relative to the single copy β-actin gene. Primers used are as follows. D-Loop (66 bp fragment): 5′-TGA CCC CCC TCA GAT AGG-3′ (forward), 5′-ACT CTT GTG CGG GAT ATT GAT TT-3′ (reverse). Cytochrome-b (70 bp fragment): 5′-CCT AGG CGA CCC AGA CAA TTA T-3′ (forward), 5′-TCA TTC GGG CTT GAT GTG G-3′ (reverse). In the experiments evaluating frataxin expression, total RNA from Caco-2 cells was extracted using guanidine thiocyanate. First strand cDNA was prepared from total RNA (1 μg) using Superscript II RNaseH reverse transcriptase (Invitrogen) according to the manufacturer's method. Frataxin mRNA expression levels were measured by the quantification of cDNA, converted from frataxin mRNA, relative to the basal level of frataxin mRNA at day 4 of culture. Quantifications were normalised to an endogenous control, using a 101 bp fragment spanning the 3′ region of the human beta-glucuronidase. *X25* (132 bp fragment): 5′-TGG GAG TGG TGT CTT AAC TGT C-3′ (forward), 5′-CCC AGT CCA GCA TAA CGC TTA-3′ (reverse). Human β-glucuronidase (101 bp fragment): 5′-GAA AAT ATG TGG TTG GAG AGC TCA TT-3′ (forward), 5′-CCG AGT GAA GAT CCC CTT TTT A-3′ (reverse).

PCR was performed using the SYBR Green PCR Master Mix 2× (Applied Biosystem) and 50 ng of total DNA or cDNA in a total reaction volume of 15 μl. The PCR cycling program started with AmpErase UNG incubation for 2 minutes at 50°C and AmpliTaq Gold activation for 10 minutes at 95°C as pre-denaturation steps, and was followed by 50 two-step cycles at 95°C for 15 seconds and at 60°C for 60 seconds. The temperature was then gradually raised from 60°C to 95°C with continuous data acquisition to prepare melting curves for the produced fragments. Quantitative real-time analysis was made using the 2[–Delta Delta C(T)] method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001).

Results

Frataxin expression during enterocyte differentiation

We evaluated frataxin expression during enterocyte-like differentiation of Caco-2 cells. In particular, we used actively proliferating undifferentiated cells at day 4 of culture and either pre-confluent or post-confluent cells at different stages of differentiation (day 7, 10 and 13 of culture). The relative levels of frataxin protein were determined by western blot analysis using a specific anti-frataxin antibody. As shown in Fig. 1A, the mature form of frataxin was expressed in both the proliferative (day 4) as well as the differentiating phases (day 7, 10, 13). Densitometric analysis of the experiments demonstrated that frataxin protein levels increased during cell differentiation by 8-fold at day 7 of culture and by 4 to 5-fold during all following phases of culture compared with the level in undifferentiated cells at day 4. It has previously been demonstrated that frataxin localises into the mitochondrial structure (Campuzano et al., 1997). To evaluate whether the increase of frataxin expression during cell differentiation reflected an increase of cell mitochondrial mass, we firstly determined the amount of the mitochondrial protein VDAC-1 before performing copy number analysis of mtDNA. The data obtained by western blot (Fig. 1A) showed an increase of VDAC-1 expression at the beginning of the differentiation process (day 7). In the following phases, a marked reduction

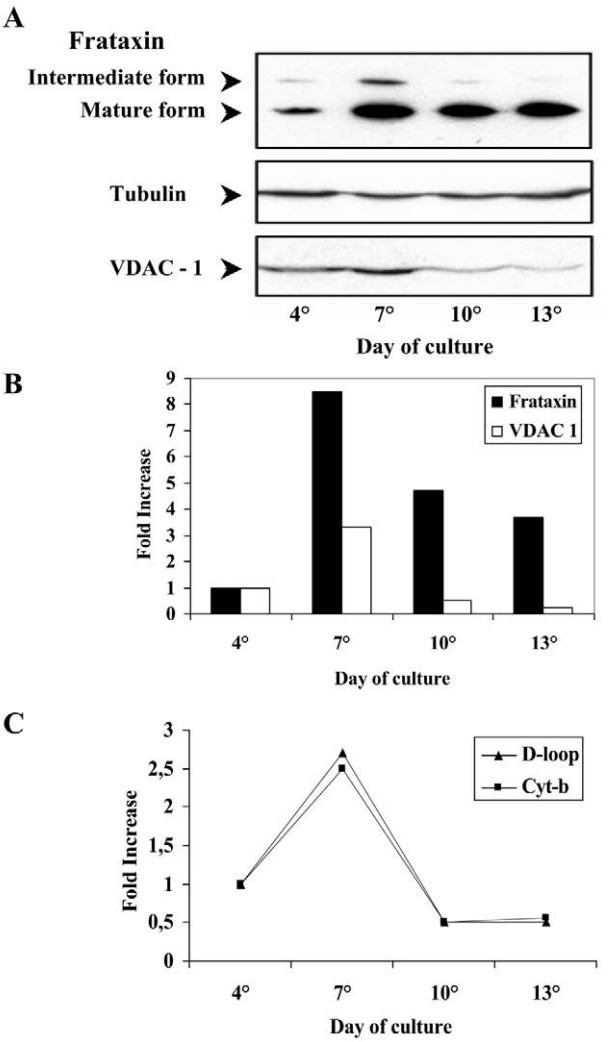

Fig. 1. (A) Western blot analysis of frataxin and VDAC-1 expression in Caco-2 cells extracts at different stages of culture. (B) Relative densitometric analysis of mature frataxin and VDAC-1 expression was normalised to tubulin using NIH Image Software. Data is expressed as fold change over control levels at day 4. (C) D-loop and Cytochrome-b mtDNA expression analysis in Caco-2 cell after the indicated days of culture. Results are expressed as fold change over control levels at day 4. Experiments were carried out three times; results presented are taken from one representative experiment.

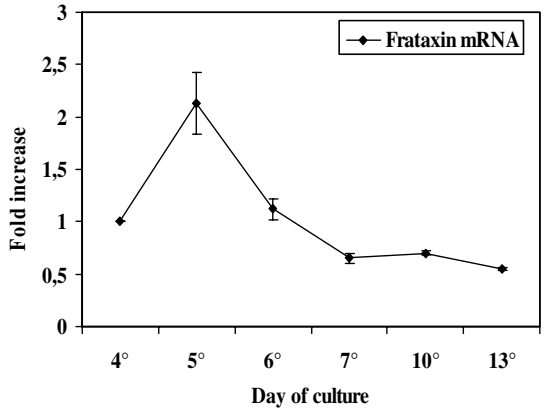

Fig. 2. Quantitative gene expression of frataxin using real-time PCR in Caco-2 cells at the indicated days of culture. Fold changes (y-axis) represent the relative expression arbitrarily setting the mRNA frataxin level at day 4 to 1. Indicated values, normalised to β-glucuronidase mRNA level, are means ±s.d. of three independent experiments.

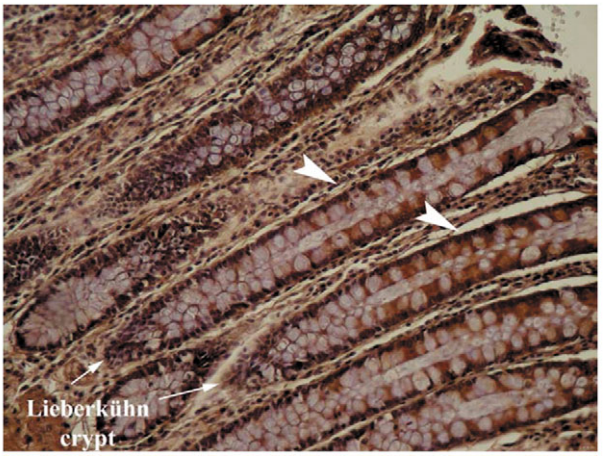

Fig. 3. Immunohistochemical staining of human intestinal mucosa. The staining for frataxin was performed using anti-frataxin antibody. Along the crypt-villus axis the stem cells committed to become mature enterocytes migrate and differentiate. Proliferating undifferentiated cells are located towards the base of the crypt compartment, while differentiated cells are located at the tips of the villus. Frataxin signal corresponding to the brown staining become clearly evident in the upper half of the villus (arrowheads indicate). Magnification used is 40×.

of VDAC-1 levels was observed when compared with proliferating cells (Fig. 1A,B). In addition, the amounts of two distinct regions of mtDNA, namely D-Loop and Cytochrome-b, were analysed by real-time PCR. We observed a 2.5 to 3-fold increase in mtDNA at day 7 of culture, followed by a strong decrease during the following differentiation phases (Fig. 1C). Staining for mitochondria at different days of culture, obtained with MitoTracker Green, demonstrated the presence of integral mitochondrial structure at each analysed time point of culture (data not shown). To evaluate if the increase in frataxin protein level was related to an improved transcriptional rate, we analysed the amount of frataxin mRNA by real-time PCR. As shown in Fig. 2, frataxin mRNA levels, after an initial increase at the beginning of the differentiation process, strongly decreased. In particular, an approximately 2.5-fold increase was observed at day 5 followed by a constant reduction down to approximately 0.5 fold at day 13 compared with day 4. Thus, the observed frataxin protein increase was not merely related either to mitochondrial mass trend or to an increase in transcriptional rate and was probably due to a strong protein stabilisation event.

Frataxin expression was also evaluated in vivo by immunohistochemical staining of human intestinal mucosa. The stem cells committed to become mature enterocytes migrate and differentiate along the crypt-villus axis. Proliferating undifferentiated cells are located towards the base of the crypt compartment, while the differentiated cells are located at the tip of the villus. As shown in Fig. 3, a clear frataxin signal was detected in the upper half of the villus where differentiated cells were located, while weaker, almost non-detectable staining was observed at the base of the crypt. We conclude that frataxin protein, but not mRNA, levels increase during enterocyte differentiation either in vitro or in vivo probably as result of a protein stabilisation event.

Frataxin subcellular localisation

To investigate the subcellular localisation of frataxin during enterocyte differentiation, we performed double-immunostaining and confocal analysis of Caco-2 cells with anti-frataxin antibody. In proliferating Caco-2 cells, frataxin signal was located in cytoplasmic organelles identified as mitochondria by MitoTracker Red staining (Fig. 4A). Differentiated cells were smaller in size and contained a small amount of cytoplasm when compared with proliferating cells. Frataxin staining was still observed within the mitochondria of these cells; however a diffused cytoplasmic staining was also clearly evident (Fig. 4B). Additionally, immunostaining of frataxin was observed at the cell periphery in regions of cell-cell contact, as indicated by the arrows in Fig. 4B.

This unexpected extramitochondrial localisation of frataxin was further investigated by two additional independent approaches. Firstly Caco-2 cell extracts were fractionated into a membrane/organelle-enriched fraction and a cytoplasmic soluble fraction. Enrichment of mitochondria in the membrane/organelle fraction was checked and confirmed two markers: voltage-dependent anion channel (VDAC-1), a mitochondrial membrane marker and citrate synthase, a soluble mitochondrial marker. In proliferating cells (day 4) frataxin is present in the membrane/organelle fraction, and is virtually undetectable in the cytosolic fraction (Fig. 5). In differentiated Caco-2 cells (day 13), a frataxin signal is still present in the membrane/organelle-enriched fraction; however frataxin is predominantly seen in the cytosolic soluble fraction that lacks mitochondria (Fig. 5).

To further investigate the extramitochondrial localisation of frataxin, FACS analysis was performed on undifferentiated and differentiated Caco-2 cells in permeabilizing and non-permeabilizing conditions. Permeabilization efficiency was checked using the mitochondrial manganese superoxide dismutase (Mn-SOD) antibody. In permeabilizing conditions, a specific frataxin fluorescence is present in 100% of Caco-2 cells at both time points (Fig. 6B). In non-permeabilized proliferating

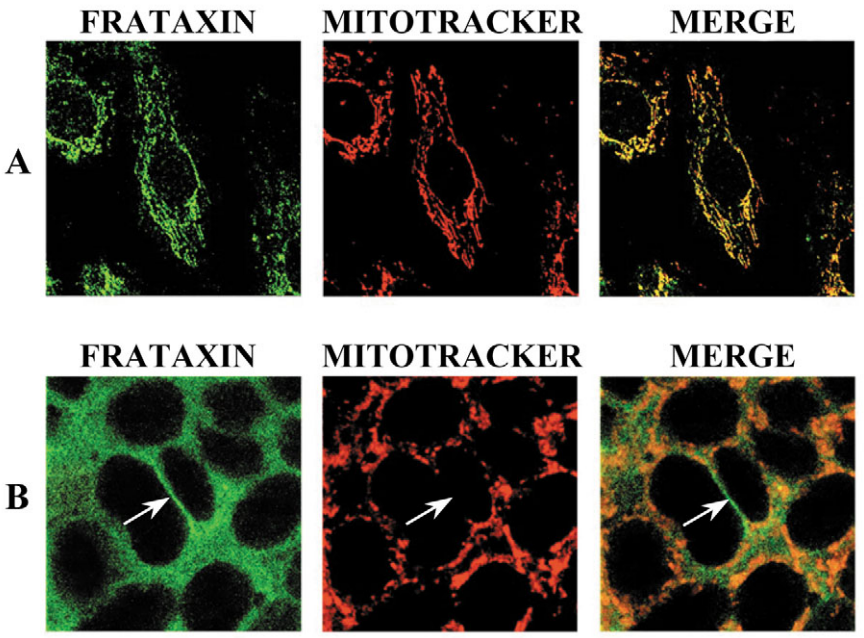

Fig. 4. Double-immunostaining and confocal analysis of permeabilized Caco-2 cells with anti-frataxin antibody. (A) In proliferating cells at day 4 of culture, frataxin localises in elongated cytoplasmic organelles identified as mitochondria by MitoTracker Red staining. (B) Differentiated cells (day 13) were smaller in size and contained a small amount of cytoplasm. Frataxin staining appears diffuse in the cytosol and co-localises only in part with MitoTracker Red signal. The arrows indicate a site of cell-cell contact clearly positive for frataxin. Magnification used is 100×.

undifferentiated cells, the FACS analysis did not show any surface fluorescence with the anti-frataxin antibody (Fig. 6A). By contrast, specific fluorescence for frataxin was seen on the cell surface in differentiated cells (Fig. 6A). Quantitative analysis of the experiments showed that the fraction of cells expressing membrane bound fluorescence for frataxin increased from 3% at day 4 to 28% at day 13 of culture (Fig. 6C).

Cytosolic frataxin associates with the IscU1 cytosolic isoform of the human Fe/S cluster assembly enzyme

To investigate the role of extramitochondrial frataxin during differentiation of Caco-2 cells we looked for an interaction between frataxin and the IscU1 cytosolic isoform of the human Fe/S cluster assembly enzyme. Three previously identified IscU1 isoforms were present in undifferentiated actively proliferating and differentiated Caco-2 cells (Fig. 7). As expected IscU1 was only present in the cytosolic fractions. To determine whether cytosolic frataxin interacts with IscU1, we immunoprecipitated the proteins of the cytosolic fraction with anti-frataxin and anti-IscU1 antibodies and analysed the immunoprecipitate by western blot. As shown in Fig. 8 (upper panel), the anti-frataxin antibody was able to co-immunoprecipitate the IscU1 15 kDa isoform in differentiated cells (day 13 of culture), but not in undifferentiated proliferating cells (day 4 of culture). Similarly the anti-IscU1 antibody was able to co-immunoprecipitate frataxin in differentiated, but not in undifferentiated cells (lower panel Fig. 8).

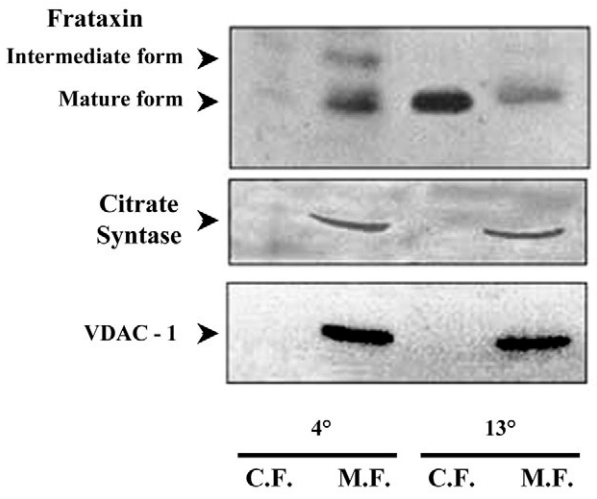

Fig. 5. Subcellular localisation of frataxin, VDAC-1 and citrate synthase in proliferating and differentiated Caco-2 cells. The enrichment of mitochondria in membrane/organelle fractions was checked by the use of VDAC-1 and citrate synthase mitochondrial markers. Membrane/organelle-enriched fractions contain mitochondria (M.F.), while the cytoplasmic fraction (C.F.) does not.

Discussion

We have evaluated frataxin expression and cellular localisation during the differentiation of intestinal cells. Firstly we investigated the expression level of frataxin in vitro using the Caco-2 cell line that shows a proliferative undifferentiated phase followed by a spontaneous enterocyte-like differentiation. Frataxin was expressed in both proliferative and differentiative phases with a protein level increasing during differentiation, as demonstrated by western blot analysis of Caco-2 cells protein extracts. In addition, we have shown that frataxin protein expression increases in differentiating enterocytes along the crypt-villus axis.

Determination of mitochondrial mass and analysis of frataxin mRNA levels by real-time PCR suggested that the observed increase in frataxin protein level during enterocyte-like differentiation of Caco-2 cells was not merely attributable to either mitochondrial mass trend or an increased transcriptional rate. In differentiated cells frataxin mRNA levels and mitochondrial mass were found to be very low compared with the basal level, while the protein levels

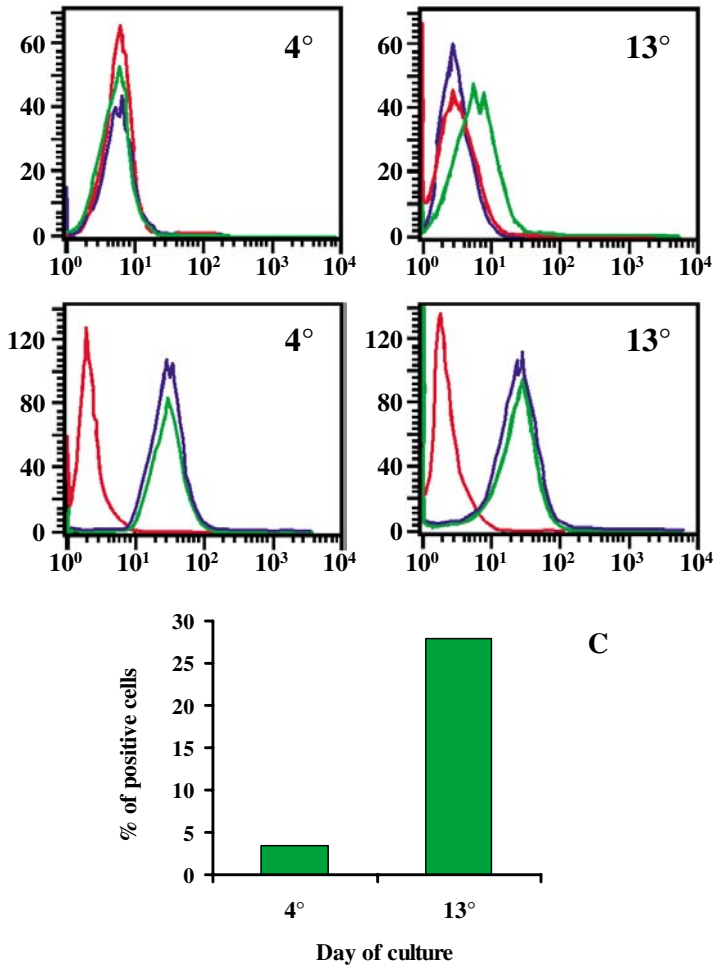

Fig. 6. Fluorescence activated cell-sorting analysis of Mn-SOD and frataxin expression in permeabilized and non-permeabilized Caco-2 cells. The red lines indicate the non-specific anti-mouse antibody fluorescence, while the blue and green lines indicate Mn-SOD and frataxin specific fluorescence respectively in proliferating and differentiated Caco-2 cells. (A) Non-permeabilized Caco-2 cells. Frataxin-specific cell-surface fluorescence is evident at day 13. (B) Permeabilized Caco-2 cells. (C) Percentage of surface-frataxin positive cells; analysis of one of three independent experiments.

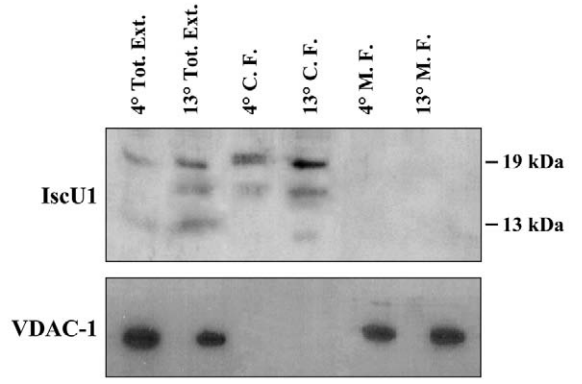

Fig. 7. Western blot analysis of IscU1 and mitochondrial marker VDAC-1 expression during Caco-2 cell differentiation. IscU1 and VDAC-1 protein levels from total, cytosolic (C.F.) and mitochondrial (M.F.) fractions of Caco-2 cells at day 4 and 13 of culture are shown.

remained elevated. These observations support the existence of a mechanism of protein stabilisation. It is worth noting that at this stage of Caco-2 cell differentiation we also observed extramitochondrial frataxin staining and the presence of frataxin in the soluble, without mitochondria, fraction.

Regulation of frataxin expression during differentiation has not been studied extensively. It has however been shown that frataxin expression increases during neuronal differentiation of P19 embryonic carcinoma cells. A possible role for frataxin in neuronal differentiation has been suggested by the finding that knocking down frataxin by anti-sense technology increases apoptosis (Santos et al., 2001). However, the levels of both the frataxin protein and mRNA are down regulated in Friend cells undergoing erythroid differentiation (Becker et al., 2002). The authors postulate that this down-regulation induces the mitochondrial iron accumulation required for the induction of heme and haemoglobin synthesis (Becker et al., 2002).

Based on both these observations and our data, we can speculate that the variability in frataxin protein expression during differentiation may reflect the specific role of frataxin in the intracellular iron metabolism of different cell compartments. Therefore, the increase in frataxin amounts that we described in differentiated enterocytes could be related to their role in iron intestinal uptake. The mature enterocytes are specialised for absorption and transport of iron and differ from the undifferentiated precursors in their expression of iron uptake and iron transport proteins (Ekmekcioglu et al., 1998; Griffiths et al., 2000). Undifferentiated cells can be considered as sensors of body iron requirement responding to serum iron levels (Schumann et al., 1999; Morgan and Oates, 2002). Upon differentiation, enterocytes become able to transport iron, and Fe/S-iron regulatory proteins (IRPs) play a central role in the regulation of the enterocytes' iron uptake machinery. In this context, frataxin could be involved in intestinal iron absorption because of its role in the biogenesis of the Fe/S proteins (Adamec et al., 2000; Park et al., 2002).

Here we identify an extramitochondrial localisation of frataxin for the first time. This finding – obtained by different experimental approaches – was unexpected, since the mitochondrial localisation of frataxin is well established in the literature. Based on co-immunofluorescence, we demonstrate the re-localisation of mature frataxin protein in the cytosol. The above observation is confirmed by subcellular fractionation showing the mature form of frataxin in the mitochondria lacking soluble cytoplasmatic fraction of differentiated cells.

Several other mitochondrial proteins appear to re-localise in alternative sites (Soltys and Gupta, 1999). For example, the mitochondrial HSP70 has also been observed at the plasma membrane, in cytoplasmic vesicles and in cytoplasmic granules (Ran et al., 2000). It is intriguing to note that the mitochondrial HSP70 yeast homologue (Ssq1) is involved in frataxin processing (Knight et al., 1998; Voisine et al., 2000) as well as in the biogenesis of the Fe/S proteins (Dutkiewicz et al., 2003; Lutz et al., 2001).

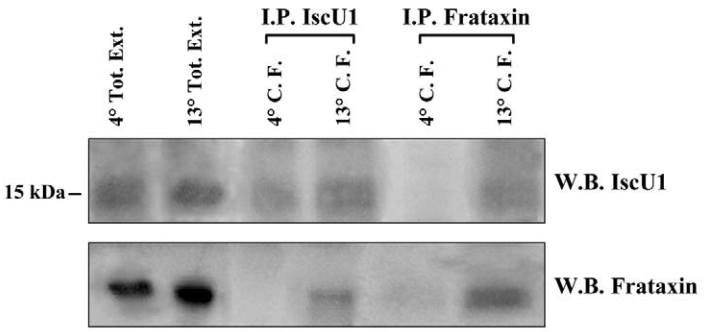

Fig. 8. Cytosolic association of IscU1 with frataxin during Caco-2 cell differentiation. Western blot analysis of IscU1 and frataxin protein levels on total cell extract and on cytosolic fraction (C.F.) at day 4 and 13 of culture following immunoprecipitation with anti IscU1 or anti-frataxin antibody.

The question of how mitochondrial proteins end up in other cellular compartments is so far unresolved but several mechanisms have been proposed. For example, in the case of the mitochondrial enzyme fumarase, it has been hypothesised that molecules destined for the cytosol re-enter this compartment by retrograde movement through the mitochondrial translocation pore (Knox et al., 1998). A different mechanism has been suggested for IscU, a protein involved in Fe/S cluster biosynthesis and reduced in frataxin-deficient cells. Here alternative splicing of a common pre-mRNA results in synthesis of two isoforms that differ at the N-terminus and localise either to the cytosol (IscU1) or to the mitochondria (IscU2) (Tong and Rouault, 2000). It has been well established that frataxin is targeted to mitochondria where it gets processed by a mitochondrial peptidase (Koutnikova et al., 1998; Branda et al., 1999). However, no evidence exists for alternative splicing or extramitochondrial processing of frataxin mRNA. Based on the above data, and on our finding of the presence of the mature form of frataxin in the cytosol, we support the hypothesis that frataxin enters the mitochondria, where it is processed before exiting the organelle during the enterocyte-like differentiation of Caco-2 cells. The mechanism of frataxin exit from the mitochondria remains to be investigated; however, the presence of the mature form of frataxin in the cytosol suggests that a cytoplasmic redistribution of frataxin follows its mitochondrial processing.

Another interesting question addresses the role of frataxin outside the mitochondria. Evidence from different groups demonstrates a direct role for frataxin in the formation of mitochondrial Fe/S clusters (Huynen et al., 2001; Muhlenhoff et al., 2002; Yoon and Cowan, 2003). Moreover, it has been shown that the yeast frataxin homologue yfh1 is involved in the biosynthesis of Fe/S proteins that physically interact with Isu1/Nfs1 (Gerber et al., 2003). The synthesis of Fe/S clusters in all eukaryotes takes place mainly in the mitochondria (Muhlenhoff and Lill, 2000). The observation that proteins included in the Fe/S cluster machinery are also found in the cytosol and the nucleus suggests that their biosynthesis takes place in multiple subcellular compartments. It has also been shown that the expression levels of all known members of the 'iron regulon' are increased in the Δ-Yfh1 yeast strain (Foury and Talibi, 2001). This suggests that frataxin-deficient cells are starved of cytosolic iron. The data presented herein show that frataxin localises in an extramitochondrial compartment in differentiated Caco-2 cells and is physically associated with the cytosolic Fe/S cluster assembly enzyme IscU1. In support of this hypothesis, it has recently been demonstrated that frataxin is a component of the human Fe/S cluster assembly machinery and plays a role in the maturation of both mitochondrial and cytosolic Fe/S proteins in human cells (Stehling et al., 2004). In this context, the extramitochondrial localisation of frataxin is compatible with the hypothesis that frataxin is directly involved in iron homeostasis – possibly by mediating iron efflux. It has been hypothesised that the relative expression of both the mitochondrial and cytosolic isoform of IscU could be affected by iron availability (Tong and Rouault, 2000). It will be interesting to investigate if the intracellular iron status may also modify the cellular localisation of frataxin in Caco-2 cells.

Despite the great advances in the knowledge of frataxin function, the pathogenesis of Friedreich's ataxia remains largely unknown. The unexpected observation of the extramitochondrial localisation of frataxin and of its physical interaction with the Fe/S cluster assembly protein IscU1 reported here may help to elucidate the mechanism by which loss-of-frataxin function causes Friedreich's ataxia.

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