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Expression of Sara2 Human Gene in Erythroid Progenitors

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A human homologue of Sar1, named Sara2, was shown to be preferentially expressed during erythropoiesis in a culture stimulated by EPO. Previous studies, in yeast, have shown that secretion-associated and Ras-related protein (Sar1p) plays an essential role in protein transport from the endoplasmic reticulum to the Golgi apparatus. Here, we report the molecular analysis of Sara2 in erythroid cell culture. A 1250 bp long cDNA, encoding a 198 amino-acid protein very similar to Sar1 proteins from other organisms, was obtained. Furthermore, we also report a functional study of Sara2 with Real-time quantitative PCR analysis, demonstrating that expression of Sara2 mRNA increases during the initial stages of erythroid differentiation with EPO and that a two-fold increase in expression occurs following the addition of hydroxyurea (HU). In K562 cells, Sara2 mRNA was observed to have a constant expression and the addition of HU also up-regulated the expression in these cells. Our results suggest that Sara2 is an important gene in processes involving proliferation and differentiation and could be valuable for understanding the vesicular transport system during erythropoiesis.

Keywords: Erythropoiesis, Hydroxyurea, K562 cells, Sar1, Vesicular traffic

Introduction

Erythroid differentiation, one of the few human processes of continuous non-malignant cellular proliferation and differentiation, is stimulated by erythropoetin (EPO), which possibly regulates the expression of many genes interacting with specific high affinity receptors on erythroid progenitors cells (Spivak *et al.,* 1991). The identification of genes preferentially expressed during human erythroid proliferation and differentiation, particularly in the presence of EPO stimulus, could provide further insight into the molecular mechanisms that underlie hematopoietic differentiation and the appearance of hematopoietic malignancies.

A transcriptional profile of genes from peripheral blood mononuclear cells, arising only in response to EPO, has been published (Gubin *et al.,* 1999). Among the 719 expressed sequence tags (ESTs) found in this study, available at the Hembase database, we focused our attention on three related ESTs named Ax35f04, Ax35g04 and Ax40e08. These ESTs are related to the human gene, Sara2, homologous to the *S. cerevisiae* Secretion-associate and Ras-related gene (Sar1). The Sar1 gene, originally isolated as a multiple copy suppressor of a temperature sensitive mutant of the Sec12 gene (Nakano and Muramatsu, 1989), is required for transport from the endoplasmatic reticulum to the Golgi apparatus in the yeast, *S.cerevisiae* (Nakano *et al.,* 1988; Takai *et al.,* 2001). Sar1 protein is a member of the small GTPases family, which are very important in mediating multiple signaling transduction pathways involved in the regulation of cellular proliferation and differentiation (Barbacid 1987).

The function of Sar1 in vesicle budding has been extensively characterized in the yeast, *S. cerevisiae,* but is less studied in mammals (Schekman and Orci 1996). Two Sar1 related genes (Sara1 and Sara2) have been found in mammals and in humans (Shen *et al.,* 1993; He *et al.,* 2002; Jones *et al.,* 2003). Sara2 was first sequenced in humans in pituitary tumor (GenBank accession no. AF092130). This gene was further described as highly expressed in human liver cancer (He *et al.,* 2002) and, recently, mutations in Sara2 gene have been associated with some lipid absorption disorders (Jones *et al.,* 2003).

To the best of our knowledge, there is no description available of Sara2 gene expression in hematopoietic tissue. Thus, we carried out cDNA cloning and the first functional study of the Sar1 human homologue Sara2 during erythropoiesis "in vitro". The expression pattern of this gene was analyzed during erythroid differentiation with EPO stimulus and with the addition of hydroxyurea (HU), a drug used to treat sickle cell disease. The precise mechanism by

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which this drug exerts its effects is not yet completely understood, but probably involves the expression of several genes (Idriss *et al.,* 1999). Our results indicate that Sara2 gene presents an increased expression during the initial stages of erythroid differentiation and a two-fold increase in expression following the addition of HU.

Materials and Methods

Erythroid cell cultures Blood from normal volunteers was cultured using a two-phase liquid culture procedure, as previously described (Pope *et al.,* 2000).

Mononuclear cells were isolated from the peripheral blood samples by centrifugation in a Ficoll-Hypaque gradient and cultured for 7 days (Phase I) in IMDM medium (Invitrogen, San Diego, USA), supplemented with 20% fetal calf serum (Invitrogen), 1ug/ml cyclosporin A (Sandoz), and 10% conditioned medium collected from the culture of human bladder carcinoma 5637 cell line. Cells were incubated at 37°C, in an atmosphere of 5% CO_2 and 92% extra humidity. After 7 days, the non-adherent cells were harvested and re-cultured in phase II medium, IMDM supplemented with 30% fetal calf serum (Invitrogen), 1% deionized bovine serum albumin (BSA-Sigma), 10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, USA), 1.5 nmol/l glutamine (Invitrogen), 300 mg iron saturated transferrin (Sigma) 10^{-6} M dexamethasone, 5 ng/ml human stem cell factor (SCF-Calbiochem), 1 U/ml human recombinant erythropoetin (Cilag), 2.5 ug/ml funzigone (Invitrogen), 50 ug/ml streptomycin (Invitrogen) and 25 ug/ml glutamicin (Invitrogen). For the cultures with hydroxyurea (HU), 80 mMol/L of HU (Sigma) was added after 72 h of phase II. Cell samples were collected from phase II cultures at 0, 48, 72, 96, 120, 144, 168 and 240 h. Cell numbers and viability were determined by Trypan Blue exclusion. Samples of 5×10^6 cells were pelleted and ressuspended in Trizol (Invitrogen) and stored at −80°C for total RNA extraction and cDNA synthesis. For morphological cell analyses, cytospin slides were prepared and were stained with Leishman before examining with an Eclipse E-600 microscope (Nikon) using the Image Pro-Express 4.0 software (Media Cybernetic, LP) to morphologically analyze the cell differentiation stage.

K562 cell culture The human leukemia cell line, K562, was obtained from ATCC, Philadelphia, PA, USA. Cells were cultured in RPMI containing 10% fetal calf serum and glutamine with the addition of penicillin/streptomycin and amphotericin B, and maintained at 37°C, 5% CO_2. For experiments, cells were seeded at a density of 3×10^5 cells/ml and cultured for 4 days. K562 cells were incubated, or not, with 80 mMol/L of HU (Sigma). Cell samples were collected at 24, 48, 72 and 96 h after the beginning of the culture.

Flow cytometry analysis of the cell surface markers, transferrin receptor and glycophorin A Expression of cell surface markers using flow cytometry was evaluated by dual staining using FITC-conjugated anti-transferrin receptor and PE-conjugated anti-glycophorin A (Dako, Glostrub, Denmark) for 30 min, at 4°C, in the dark. The cells were washed with PBS and suspended in 1% paraformaldehyde prior to analysis. Data from 10,000 events were acquired for analysis using Cell Quest Software (Becton Dickinson, Mountain View, USA). Non-specific staining was established from the corresponding isotype control and subtracted from the corresponding positive population percentage.

RNA extraction and cDNA synthesis Cells were collected at specific time points during phase II and total RNAs were isolated using the Trizol Reagent Protocol (Invitrogen). Two μg RNA samples were incubated with 1 U DNaseI (Invitrogen) for 15 min at room temperature and EDTA was added to a final concentration of 2 mM to stop the reaction. The enzyme was subsequently inactivated for 10 min at 65°C. The DNaseI-treated RNA samples were reverse transcribed with 200 U SuperScript II (Invitrogen) for 50 min at 42°C. Two U of RNaseH (Invitrogen) were subsequently added and the samples were incubated at 37°C for 20 min. The cDNA samples were quantified using the UV spectrophotometer, GeneQuant (Pharmacia, Uppsala, USA).

PCR, cDNA cloning and sequencing Specific primers for Sara2 were designed based on the sequence deposited in BLAST (GenBank Accession No. AF092130) using the GeneRunner software (available at http://www.generunner.com). Their sequences are: Sara Forward (5'-TTCGGTCTCCTGGTGTACGG-3'); Sara Reverse (5'-ACCAAACATCTCTCGCAACCTC-3'); Sara reverse 1261 (5'-CATTAGAGTTTGTTTTATTGC-3'). The locations of the primers in relation to the transcript sequence are depicted in Fig. 1. PCR amplification was performed on the cDNA from the erythroid cells cultured with EPO, as follows: 1 μl of template was amplified in a 20-μl volume mixture containing 2 μl of 10x PCR buffer, 5 μl of 1.25 mM dNTPs, 1 μl of 50 μM $MgCl_2$, 1 unit of Taq DNA polymerase (Invitrogen) and 1.2 μl of 15 pMol of each specific primer. PCR conditions were an initial denaturation step at 95°C for 2 min, followed by 39 cycles at 95°C for 30 s, annealing (depending on primer Tm) for 45 s, and 1 min. at 72°C. Finally an additional elongation step was carried out at 72°C for 7 min. The PCR products were resolved in 1% agarose gel stained with ethidium bromide. PCR products were then purified from agarose gel with specific kits (Concert-Invitrogen), consequently the amplified fragments were recombined into the pGEM-T easy vector system (Promega, Madison, USA) and transformed into *E. coli* DH5αf'. Five positive clones were selected, the plasmids were extracted and the fragment was sequenced using T7 and SP6 primers in an ABI377 automated sequencer.

Northern blotting A 532 bp probe for Sara2 was amplified using Sara Forward and Sara Reverse primers, labeled with (α-^{32}P)dATP according to the Megaprime Labeling Kit (Amersham, Richmond, USA) protocol and used for hybridization to poly(A)$^+$ mRNA isolated from different normal human tissues (Human 12-lane Multiple Tissue Northern Blot and Human Multiple Tissue Northern Blot III, Clontech, Palo Alto, USA), according to the manufacturer's manual. The membranes were exposed to a phosphor screen at room temperature for 24 h and scanned with STORM 840 (Molecular Dynamics). The cDNA expression levels were determined using the Image Quant 5.2 software (Molecular Dynamics) and Microsoft Excel 9.0. β-Actin was used as an internal control and to normalize the cDNA expression levels in all hybridization experiments.

Fig. 1. Nucleotide sequence of human Sara2 gene. Nucleotide sequences and deduced amino acid sequences of the open reading frame are shown. This sequence was confirmed in erythroid cells in differentiation by PCR and sequencing. Arrows indicate the PCR primers used to amplify Sara2.

Real-time PCR analysis Gene expression analysis was performed using a relative analysis, in which the gene expression is measured by the quantification of cDNA corresponding to the target gene relative to a calibrator sample serving as a physiological reference. An expression control was also used. In the case of cultured samples, the reference was 0 (zero) h. The relative quantification value was expressed as $2^{-\Delta\Delta Ct}$, as recommended by the manufacturer (Applied Biosystems, Foster City, USA).

Synthetic oligonucleotide primers were designed using PrimerExpress™ software (Applied Biosystems) and synthesized by Invitrogen. Real-time detection of amplification was performed in an ABI 5700 Sequence Detector System (Applied Biosystems) using SybrGreen PCR Master Mix (Applied Biosystems). Twenty ng of each cDNA sample and 400 nM of b-actin control forward and reverse primers (FW: 5'-TCACCGAGCGCGGCT-3'; RW: 5'-TAATGTCACGCACGATTTCCC-3') or 900 nM of Sara2 specific forward and reverse primers (FW: 3-TCTTGGATTGGAT AATGCAGGAA-5'; RW: 3'-AACGTCATGCCAGCAATGG-5') were used. No template controls (NTCs) were also included for each primer pair. The dissociation protocol was performed at the end of each run to check for non-specific amplification, as well as analysis in 2.0% agarose gel with ethidium bromide (β-actin primers amplified a 60 pb fragment and Sara2 primers amplified a 122 pb fragment). Three replicas were run on the same plate for each sample and each sample was run twice independently. Previous experiments were performed to guarantee the maximum efficiency of amplification of the primers. The relative expression levels were determined using the software Gene Amp 5700 SDS 1.3 (ABI Prism; Applied Biosystems) and Microsoft Excel 9.0.

Results

Sequence of erythroid Sara2 In an attempt to confirm the full-length sequence of this gene in erythroid cells, we designed primers according to the sequence covered by Sara2, as described in pituitary tumor. PCR and sequencing of erythroid culture cDNA were used to determine this sequence (Fig. 1). The expression of Sara2 was confirmed during two-phase cell culture of erythroid cells, obtaining a cDNA that was 1250 bp long with an open reading frame of 596 bp, resulting in an 198-aminoacid-containing protein. Our sequence was completely identical to the sequence previously obtained in human pituitary tumor, except that it was 30 bp shorter in the non-codifying region, confirming the identity of this sequence among human tissues. A search, using the ClustaW algorithm, revealed that the amino acid sequence and the size of the Sara2 gene product (Sar1b) were highly conserved compared with Sar1 proteins from other organisms,

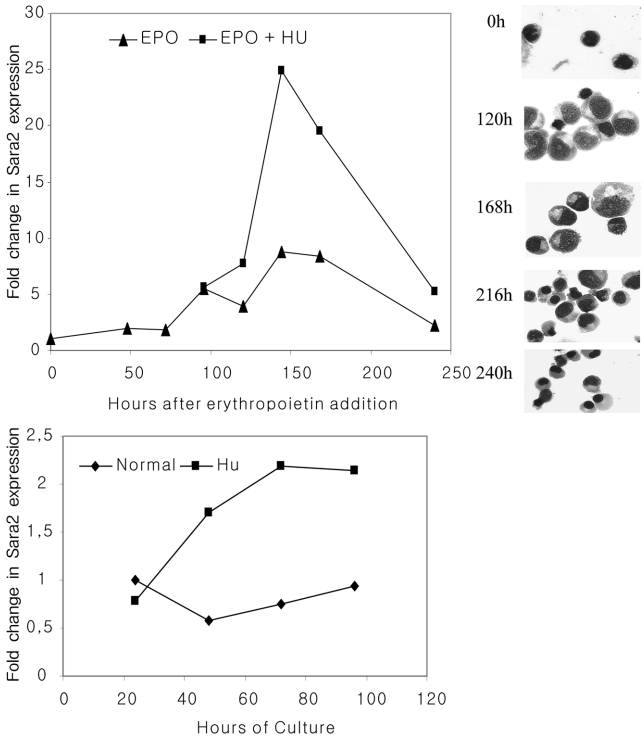

Fig. 2. Real-time detection of amplification of Sara2 gene during erythroid differentiation in two-phase cell culture (A). During the cultures with only EPO and with EPO + HU, the expression pattern of Sara2 was similar, although HU produced a significant increase in Sara2 expression levels. Here, we also present morphological analysis of two-phase cell culture demonstrating erythroid differentiation. (B) Real-time detection of amplification of Sara2 gene during K562 cells culture without stimulus (normal) and with HU.

as previously described (Shen *et al.,* 1993, He *et al.,* 2002 and Jones *et al.,* 2003).

Northern blot analysis of poly(A)$^+$ RNA isolated from a panel of different human tissues was performed to determine Sara2 expression in human tissues and results obtained were very similar to those reported in previous studies (He *et al.,* 2002 and Jones *et al.,* 2003). These data are available upon request. Abundant expressions were detected in liver and skeletal muscle; moderate expressions were detected in heart, kidney, placenta and bone marrow; Sara2 had a very low expression in the other tissues tested.

Two-phase liquid culture of erythroid cells Cytospin preparations of cells at various stages of phase II are depicted in Fig. 2A. Proerythroblasts begin to be discernible on days 3-4 as large, round, smooth cells which, after staining with Leishman, reveal a deep blue cytoplasm and a large central nucleus. As these cells multiply they form clusters. During the intermediate phases, cells gradually give way to an increasing proportion of basophilic erythroblasts (days 5-7) and polychromatic and orthocromatic cells (days 8-10).

Differentiation in phase II was determined by flow cytometry using a FITC-conjugated anti-transferrin antibody and a PE-conjugated anti-glycophorin A antibody. Results demonstrated that cell differentiation towards the erythroid lineage occurred during culture, as reflected by the increase in the percentage of transferrin-positive cells and percentage of glycophorin A-positive cells (0.03% and 0.11% on day 0, respectively, increasing to 32.79% and 8.15% on day 9, respectively), as previously described (Pope *et al.,* 2000).

Sara2 expression during erythroid differentiation in two-phase cell culture To verify whether Sara2 plays a role in erythroid cell differentiation, Sara2 expression was analyzed with real-time PCR during erythropoiesis in two-phase cell culture with EPO. Differentiation was counted as h after EPO addition and followed by morphology and flow cytometry studies (Fig. 2A). Our results demonstrated that Sara2 had an increasing expression pattern culminating in a nine-fold increase in expression 6 days (144 h) after addition of EPO, followed by a highly decreasing expression level until the end of the culture at 240 h (Fig. 2A).

Sara2 expression following addition of hydroxyurea In order to characterize the influence of hydroxyurea (HU) upon Sara2 expression, real-time PCR analysis was performed during erythroid differentiation with addition of 80 mMol/L HU on the third day of phase II (72 h). Cell morphology studies, during this culture, demonstrated the cells to be identical to those of two-phase culture without HU. We observed that the expression of Sara2 mRNA significantly increased after HU addition (Fig. 2A).

The Sara2 mRNA expression pattern was also analyzed using the real-time PCR approach in a culture of K562 cells. The effect of HU treatment (80 mmol/L) upon levels of Sara2 was also studied. Sara2 mRNA was observed to have a constant expression in K562 cells without any stimulus during the 96 h of culture. The addition of HU up-regulated the expression of Sara2 during culture (Fig. 2B).

Discussion

We have identified a human homologue of *S.cerevisiae* Sar1 gene, denominated as Sara2, during erythroid culture *in vitro*. A previous study, realized during erythroid differentiation in two-phase cell culture (Gubin *et al*., 1999), showed that an EST, probably related to this gene, was differentially expressed during erythropoiesis with EPO, suggesting a possible specific role for Sara2 in this process. The human Sar1b protein, related to the Sara2 gene presented in this study, reveals several structural features which are characteristics of the small GTPase superfamily. The GTP-binding motifs (GX_4GK, DXXG and NKID), which are conserved in the small GTPase superfamily (Bourne *et al.,* 1990), were present in Sar1b. In addition, this protein contained a C-terminal Sar1-specific motif, RPXEVFM(C/

V)S(V/I)(V/L), thought to be essential for targeting Sar1 to the ER membrane (D'enfert *et al*., 1992).

The Sara2 gene expression demonstrated, herein, during erythroid cell culture suggests that this gene may be important in erythroid differentiation. Our results show an increase in Sara2 expression culminating with a peak 144 h after the addition of EPO, followed by a reduction in expression until 240 h. These findings are consistent with the morphological observations of erythroid cells and the dynamics of erythropoiesis. At 0 h, CFU-Es were observed, whilst at 120 h pro-erythroblasts, cells with high levels of erythropoietin receptors (Sawada *et al*., 1990) dominated. In these cells, erythropoetin appears to stimulate all the biochemical processes characterizing erythropoiesis (i.e., heme synthesis, globin synthesis, and synthesis of cytoskeletal proteins) (Papayannopoulou and Abkowitz 1991) and, immediately after this period, we observe a peak in Sara2 expression (144 h after EPO). The hypothesis that EPO directly, or indirectly, influences the transcription of Sara2, among other genes, is consistent with the fact that during the pro-erythroblast and basophilic erythroblast phases (120 and 168 h in our culture) the highest rates of RNA synthesis are observed (Papayannopoulou and Abkowitz 1991) and that Sara2 was expressed with EPO stimulus (Gubin *et al*., 1999). The findings reported herein support this hypothesis, since Sara2 expression was observed to occur secondary to EPO stimulation similar to the rate of RNA synthesis in erythropoiesis. During the orthochromatic erythroblast stage (240 h after EPO addition), a decrease in RNA synthesis was observed, reflecting lower levels of Sara2 expression, due to the reduction of protein and hemoglobin synthesis.

Following the addition of hydroxyurea on the third day of phase II during erythroid culture and during K562 cell culture, the expression of Sara2 mRNA approximately doubled. During erythroid culture the kinetics of cell differentiation were not modified by HU, since the EPO and EPO+HU cultures showed similar patterns, although Sara2 expression levels were significantly higher in the presence of HU. The detailed mechanism of action of HU remains uncertain, however, despite the numerous studies to investigate its action. It seems that HU exerts bi-modal dose-dependent effects on erythropoiesis in human erythroid cells, up-regulating some genes and down-regulating others (Wang *et al.,* 2002) through changes in transcriptional regions influencing the assembly of the transcription complex (Ji *et al.,* 2003). Our results indicate that Sara2 is another gene that is up-regulated by HU.

A recent study has demonstrated an association between mutations in Sara2 with some lipid absorption disorders (Jones *et al.,* 2003), such as Anderson's disease. It is known that some patients with this syndrome present, in addition to other clinical and biochemical features, hematological symptoms including anemia (Dannoura *et al*., 1999), which additionally indicate that Sara2 could be important in erythropoiesis.

In conclusion, we have characterized a human homologue of *S.cerevisiae* Sar1, Sara2, in human erythroid progenitors cells in liquid culture, and demonstrated that its mRNA expression is up-regulated during the early phases of erythroid differentiation "in vitro", and following the addition of HU. Our results demonstrate that Sara2 plays a specific role in some tissues, under certain conditions, and in the presence of certain stimuli, such as EPO and HU. This study extends our understanding of the secretion pathway in erythroid cells, and contributes to our knowledge of hematological disorders.

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