The hydroxyurea-induced small GTP-binding protein SAR modulates \(\gamma\)-globin gene expression in human erythroid cells

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Abstract

Hydroxyurea (HU), a drug effective in the treatment of sickle cell disease, is thought to indirectly promote fetal hemoglobin (Hb F) production by perturbing the maturation of erythroid precursors. The molecular mechanisms involved in HU-mediated regulation of \( \gamma \)-globin expression are currently unclear. We identified an HU-induced small GTP-binding protein, secretion-associated and ras-related (SAR), in adult erythroid cells using differential display. Stable SAR expression in K562 cells increased \( \gamma \)-globin mRNA expression and resulted in macrocytosis and cells that appeared immature. SAR-mediated induction of \( \gamma \)-globin also inhibited K562 cell growth by causing arrest in G1/S, apoptosis, and delay of maturation, cellular changes consistent with the previously known effects of HU on erythroid cells. SAR also enhanced both \( \gamma \)- and \( \beta \)-globin transcription in primary bone marrow CD34+ cells, with a greater effect on \( \gamma \)-globin than on \( \beta \)-globin. Though upregulation of GATA-2 and p21 were observed both in SAR-expressing cells and HU-treated K562 cells, PI3 kinase and phosphorylated ERK were inhibited specifically in SAR-expressing cells. These data reveal a novel role of SAR distinct from its previously known protein trafficking function. We suggest that SAR may participate in both erythroid cell growth and \( \gamma \)-globin production by regulating PI3 kinase/ERK and GATA-2/p21-dependent signal transduction pathways.
Introduction

Hydroxyurea (HU), a ribonucleotide reductase inhibitor and an S-phase–specific cytotoxic agent, has been successfully used in the clinic to treat sickle cell disease because it augments the production of fetal hemoglobin (Hb F). In a multicenter study (MSH), HU treatment dramatically reduced the frequency of hospitalization due to the incidence of pain, acute chest syndrome, and the blood transfusion requirements in patients with sickle cell disease. After two years of HU treatment, Hb F levels increased from 5% of total Hb to 9% in the majority of patients. Similarly, as Hb F levels became elevated after HU treatment, the rate and extent of sickle hemoglobin (Hb S) polymerization appeared to be reduced. A nine-year follow-up of 233 patients in the original MSH cohort indicated, among other findings, that taking HU was associated with a 40 percent reduction in mortality.

Several mechanisms have been proposed to account for the induction of Hb F by HU. Because HU directly kills late erythroid progenitor cells and triggers rapid erythroid regeneration, HU has been assumed to promote Hb F production by perturbing the maturation of erythroid precursors. Accelerated erythropoiesis increases the chance of premature lineage commitment and the induction of F-cell formation. HU also increases erythropoietin (EPO) production and enhances the proliferation of erythroid precursors and their ability to synthesize Hb F. HU could effectively stimulate erythropoiesis and production of Hb F through multiple mechanisms. It has been reported that induction of Hb F by HU is mediated by upregulation of GATA-2 and downregulation of GATA-1. Recent studies from our laboratory have also demonstrated that, in addition to regulating
GATA expression, HU also induces expression of genes related to the cell cycle and apoptosis; the actions of HU depend upon its concentration.\(^7\) Induction of peroxidation by HU can generate nitric oxide (NO),\(^9,10\) a known inducer of soluble guanylate cyclase (sGC), which, in turn, can modulate the expression of \(\gamma\)-globin.\(^11\) However, the molecular mechanisms by which HU regulates \(\gamma\)-globin gene expression need to be further elucidated. To elucidate the molecular mechanisms underlying the HU-induced elevation of \(Hb\) F and to explore new signal transduction pathways related to HU-mediated hemoglobin gene expression, we used mRNA differential display to identify HU inducible gene(s) in human adult erythroid cells. Here, we identify a new small GTP-binding protein, secretion-associated and ras-related (SAR), as a specific HU-inducible gene.

The small GTP-binding protein superfamily comprises more than 100 members in eukaryocytes.\(^12,13\) Members of the SAR/ADP ribosylation factor (Arf) family are GTPases\(^14-16\) active in protein trafficking and maturation.\(^17\) SAR itself functions in cargo selection and export of proteins from the endoplasmic reticulum to the Golgi via the cytosolic coat protein complex II (COPII) secretory pathway. It has been reported that several Ras superfamily members participate in hematopoietic cell proliferation and differentiation processes involving endo- and exocytosis or cell adhesion.\(^18-21\) However, it is unknown whether SAR participates in hemoglobin gene regulation, and which signal transduction pathway SAR uses. Here, we demonstrate that HU-induced SAR plays a pivotal role in \(\gamma\)-globin gene induction by causing cell apoptosis and G1/S-phase arrest through the reduction of PI3 kinase and ERK phosphorylation and increased p21 and
GATA2 expression. Localization of SAR in the endoplasmic reticulum was also confirmed.

Methods

Cell cultures

Two-phase liquid cultures of human adult erythroid cells (HAECs) were prepared from the peripheral blood of healthy donors. Mononuclear cells were isolated by centrifugation over Ficoll–Hypaque (1.077 g/mL, Organon Teknika Co, Durham, North Carolina), washed twice with Dulbecco's phosphate-buffered saline (PBS), and cultured according to a previously published two-phase liquid culture protocol. Cells were treated with 100 µM HU on days 7 and 8, and RNA was collected on day 10 of phase II culture for differential display. K562 cells grew in RPMI 1640 medium supplemented with 10% FBS plus 4 mM glutamine, 10 U/ml penicillin, and 10 µg/ml streptomycin. Growth curves were performed by seeding cells at a density of 2.5 x 10^4 cells/ml, then counting them every 24 h for four days. The morphology of K562 cells was observed after hematoxylin & eosin staining. Enriched primary bone marrow AC133+ cells and CD34+ cells were purchased from Poietic Technologies (Gaithersburg, MD) and cultured according to a previously described protocol.

Molecular cloning of the SAR gene using differential display

Differential display was performed using an RNA Image Kit (GeneHunter, Nashville, TN). We used a MessageClean DNase I kit (GeneHunter) to eliminate chromosomal
DNA from total RNA before differential display experiments. Reverse transcription–polymerase chain reaction (RT-PCR) followed the manufacturer’s protocol. Reproducible differential bands were excised and eluted by boiling the gel slices for 15 min. Eluted DNA was re-amplified using the same primer set, purified, and cloned into the PCR II TOPO vector (Invitrogen, Carlsbad, CA) for sequencing. The human SAR gene was cloned by 5’- and 3’-RACE with the Marathon™ cDNA Amplification Kit using bone marrow Ready Marathon™ cDNA (Clontech, Palo Alto, CA). Homology searches of GenBank and EST databases were performed using GCG software (Genetics Computer Group, Madison, WI).

**SAR protein expression in vitro**

A full-length SAR cDNA was cloned into the pGEM-T Easy vector (Promega, Madison, WI), then transcribed and translated into an ^35^S-labeled peptide using a TNT Quick T7-coupled transcription/translation system (Promega Madison, WI) with ^[^35]^S-methionine (15 mCi/ml, Amersham Pharmacia Biotech, Piscataway, NJ). The translation product was subjected to electrophoresis in 4%–12% Bis–Tris gels. The gels were exposed to film to determine the size of the translated product by comparison to known protein standards.

**Northern and Western blotting**

A SAR cDNA probe was hybridized to Multiple Tissue Northern (MTN™) Blots (Clontech, CA) according to the manufacturer’s protocol. Membranes were stripped between probes by incubating the blots in sterile 0.5% SDS solution at 95°C for 10 min. The Human Cell Cycle filter membrane containing various human cDNAs sequences was
purchased (Superarray, Frederick, MD). For hybridization probes, 5 µg of total RNA from K562 cells transfected with SAR or with vector alone was converted to cDNA by random hexamer labeling with Moloney MuLV (MMLV) reverse transcriptase and [\(^{33}\)P]-dCTP (370 MBq/ml, NEN Life Sciences, Boston, MA, USA), according to the protocol provided by the supplier. The filter arrays were scanned in a Fuji BAS-1500 phosphoimager (Fuji, Stamford, CT). Before quantitative analysis, hybridization signals for each probe were normalized using GAPDH cDNAs as reference controls. For Western blots, cell lysates containing 50 µg of protein were loaded on Tris-glycine gels (Invitrogen), transferred to nitrocellulose membranes (Invitrogen, CA), and then immunoblotted with antibodies to PI3 kinase (BD Bioscience, CA), phospho- and total ERK, GATA-1, and GATA-2 (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Washing and detection using an ECL (Enhanced Chemiluminescence) Western Blotting Analysis System (Amersham Pharmacia Biotech, Piscataway, NJ) was performed according to the manufacturer’s protocol.

**Powerblot screen**

Proteins were separated on 4-15% gradient SDS-polyacrylamide gel and transferred onto Immobilon-P membrane (Millipore, Bedford, MA). After transfer, the membrane was dried and re-wet in methanol, then blocked for one hour with blocking buffer (LI-COR). Next, the membrane was clamped with a Western blotting manifold that isolated 41 channels across the membrane. In each channel, a complex antibody cocktail was added and allowed to hybridize for one hour at 37°C. The blot was washed and hybridized for 30 minutes at 37°C with secondary goat anti-mouse conjugated to Alexa680 fluorescent...
dye (Molecular Probes, Eugene, OR). The membrane was washed, dried and scanned at 700nm using the Odyssey Infrared Imaging System (LI-COR). The bands were identified, and molecular masses were assessed using specialized software. Each observed band, where possible, was manually matched against the expected molecular mass of a protein recognized by an individual antibody in the mixture.

**Transfection and gene expression of SAR in K562 cells**

We cloned the SAR cDNA into pEF6/V5-His-TOPO (Invitrogen, CA) and stably transfected it into K562 cells by electroporation (Amaxa Biosystems, Cologne, Germany). Stable SAR-expressing clones were selected by culturing the cells in 6 µg/ml blasticidin for two weeks. An antibody directed against the His-tag was used to identify high and low levels of SAR by Western blotting; expression was confirmed by Northern blotting and RT-PCR.

**Retrovirus generation and gene transduction in CD34+ hematopoietic stem cells**

The SAR coding region with a green fluorescent protein (GFP) tag at the 3’ end or GFP alone was subcloned into the pMSCV retroviral expression vector (Clontech, CA). The retroviruses were packaged into PT67 packaging cells. The viral titers were determined and high-titer viral clones were selected. For SAR transduction in CD34+ hematopoietic stem cells, 2 ×10⁶ viral particles were preloaded onto a RetroNectin-coated plate (Takara, JP) and incubated at 37°C for 5 h. Just prior to infection, the viral supernatant was discarded and the plate was washed with PBS; 1x10⁵ CD34+ cells were then added to the preloaded viral plate with growth medium, and the plate was incubated at 37°C. This
procedure was repeated twice at 24-h intervals. On day 7, the infected CD34+ cells were subjected to GFP sorting, and GFP-positive CD34+ cells were selected and cultured. The sorted cells were harvested on day 10, day14, and day18 for further analysis.

RNA isolation, RT-PCR, and real-time PCR,

RNA was isolated with Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's recommended protocol. RT-PCR was carried out using SuperScript™ reverse transcriptase and Taq DNA polymerase (Invitrogen, CA) according to the manufacturer's instructions. PCR primer pairs were as follows: γ-globin sense (SN): 5'-AAGATGCTGGAGGAGAAAC-3', antisense (ASN): 5' -TGCTTGCAGAATAAAG CC-3'; SAR SN 5’-CAATATCCAATGTGCAATCCTTA-3’, ASN 5’-GAAAACCCTCGCC GTACCTT3’; GAPDH SN 5’-SN TGAAGGTCCGAGTCAACGGATTTGGA-3’, ASN 5’- CATGTGGGCCATGAGGTCCAC-3’; Glycophorin C (GlyC) SN: 5’-TGGGG AAGCAAGGAAATAAG-3’; ASN 5’-AGCTGGATCAATGGCAGAC-3’. Real time PCR was accomplished with Platinum QPCR SuperMix-UDG (Invitrogen, CA) according to the manufacturer’s instructions. The γ-globin and β-globin gene primers and probe sequences were published previously; 24 control β-actin primers and probe were ordered from Applied Biosystems (Foster City, CA).

Cellular localization of SAR protein

GFP was tagged onto the 3’ end of the SAR coding region by cloning it into the pGFP-N1 vector (Clontech, CA); the resulting construct was transfected into K562 cells by
electroporation. After 8 h or 18 h of culture in fresh medium, transfectants were collected and fixed with 2% paraformaldehyde in PBS. Cells were then washed three times before being cytospinned onto glass slides. The cells were then incubated with an anti-calreticulin antibody (Affinity Bioreagent, Golden, CO) for 4 h and anti-rabbit antibody for 1 h before being visualized under a Zeiss TV200 confocal fluorescence microscope with a 100X objective. Emission wavelengths were 488 nm and 567 nm for FITC and rhodamine, respectively. The images were collected on an ORCA ER CCD (Hamamatsu Corporation, Bridgewater, NJ).

**Cell cycle and apoptosis analysis**

Cells were counted and centrifuged at 1000 rpm for 5 minutes. The cell pellets were resuspended at a density of 0.5–1.0 x 10^6 cells/ml; 400 µl Quickstain solution (FAST Systems, Inc., Gaithersburg, MD) was then added to each sample, and the samples were incubated in darkness for 30 min at 22–28°C. We used flow cytometry (EPICS-XL Model, Beckman Coulter) with signal acquisition at 675 nm to determine cell cycle distributions. Laser excitation was at 488 nm; emission filters for FITC and propidium iodide were 525 and 630 nm, respectively. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay for cell apoptosis was performed according to the manufacturer's protocol (Promega).
Statistical analysis

The statistical analysis of the results were obtained using the Student's \( t \) test for significance of the difference of individual pairs and the Pearson of correlation coefficient for the closeness of linear relationship.\(^{25}\)

Results

*Molecular cloning, SAR protein production, and localization in erythroid cells*

In vivo studies have shown that continuous infusion of 20 mg/kg HU into patients maintains a serum concentration of HU at 100 \( \mu \)M.\(^{26}\) In order to obtain an exposure regimen for the differential display study that closely resembled the in vivo pharmacokinetics of HU treatment in patients, HAECs were treated with 100 \( \mu \)M HU on day 7 and day 8, and RNA was collected on day 10. A reverse dot blot was used to confirm differential expression, and one particular transcript was identified that was significantly induced in response to HU treatment. This gene had 92\% similarity to the mouse small GTP-binding protein SAR1 by sequence analysis. We then obtained the full-length human SAR cDNA sequence (2878 bps) by screening a bone marrow library with 5’- and 3’-RACE. The human SAR gene was mapped to chromosome 10q22 using an NCBI genomic data search. Alignment of the cDNA with the chromosome 10 genomic sequence (Celera Discovery System, Rockville, MD) revealed that the SAR gene contains 8 exons and 7 introns. Sequence analysis showed a 599-bp open reading frame that includes three GTP binding motifs.
Human multiple-tissue RNA blots probed with the SAR cDNA revealed transcripts of 3.0 and 0.8 kb in most tissues. SAR mRNA was abundant in glandular tissues, such as prostate, thyroid, and adrenal gland. In contrast, relatively low levels were found in spleen, small intestine, spinal cord, lymph node, and trachea, and very low levels were detected in leukocytes, bone marrow, and ovary (Figure 1a). SAR mRNA was barely detectable in primary cultures of HAECs, but increased by at least 4-fold after 48 h of 100 µM HU treatment (Figure 1b). *In vitro* transcription and translation of full-length SAR cDNA produced a single 22-kDa peptide (Figure 1c).

To localize SAR expression within cells, we transiently transfected GFP-tagged SAR (SAR-GFP) cDNA into the K562 cells. Cells were fixed after transfection, and then incubated with an anti-calreticulin antibody for comparison to the SAR-GFP localization; calreticulin is expressed exclusively in the endoplasmic reticulum. Under a confocal fluorescence microscope, the SAR-GFP signal appeared predominantly in the cytoplasmic and perinuclear areas (Figure 1d). K562 cells transfected with GFP alone showed primarily nuclear fluorescence (data not shown). The SAR-GFP colocalized with calreticulin, suggesting that SAR was present in the endoplasmic reticulum, as expected from SAR’s known protein trafficking function.

*SAR-induced γ-globin gene expression in K562 cells*

To determine whether and how SAR regulates γ-globin gene expression in human erythroid cells, we stably transfected SAR into K562 cells and then analyzed multiple SAR-expressing and vector-transfected clones. Northern blotting revealed significantly
higher \( \gamma \)-globin mRNA levels in SAR-transfected cells than in vector-transfected cells (Table 1). To better clarify the correlation between SAR and \( \gamma \)-globin expression, we divided the SAR-expressing cells into two groups: clones with high \( \gamma \)-globin gene expression (>2 fold induction) and clones with lower \( \gamma \)-globin gene expression (<2 fold induction). As shown in Table 1, both groups had significant induction of \( \gamma \)-globin mRNA as compared with the vector control (\( p<0.001 \)), ranging from a 1.5-fold to a 4.3-fold increase in \( \gamma \)-globin mRNA levels. On average, SAR-expressing clones had 3.5-fold higher levels of \( \gamma \)-globin mRNA than vector controls. Coexistence of SAR mRNA and high levels of \( \gamma \)-globin mRNA in K562 cells indicate that HU-induced SAR may be involved in \( \gamma \)-globin mRNA induction in erythroid cell development.

To investigate whether SAR is a common or specific inducer for the \( \gamma \)-globin gene, we designed three sets of experiments. First we used semi-quantitative RT-PCR to analyze and correlate SAR and \( \gamma \)-globin mRNA levels in SAR-expressing K562 cells; we found a correlation coefficient of 0.76 between SAR and \( \gamma \)-globin mRNA levels (Figure 2a). Second, when parental K562 cells were treated with various hemoglobin gene inducers (hydroxyurea, butyrate, and 5-azacytidine) for 48 h, a Northern blot indicated that all three inducers upregulated \( \gamma \)-globin mRNA, from 2.5-fold for butyrate to 3.0-fold for hydroxyurea. In the same experiment, SAR mRNA increased in parallel with the \( \gamma \)-globin mRNA, from 1.5-fold after butyrate treatment to 2.1-fold after hydroxyurea (Figure 2b and 2c). Third, the expression of SAR and \( \gamma \)-globin mRNA in cultured primary bone marrow AC133\(^+\) cells was determined by RT-PCR at different time points. In the presence of EPO, both SAR and \( \gamma \)-globin mRNA began to be expressed at day 10,
reached a peak at day 21, and declined to baseline at day 28, reflecting a similar time course and pattern of gene expression (Figure 2d). These data further indicate that there is a close temporal relation between SAR and γ-globin mRNA expression in primary bone marrow cells, and strongly suggest that the SAR and γ-globin genes coexist in cells of this erythroid lineage.

**SAR-induced hemoglobin gene expression in CD34+ hematopoietic stem cells**

We next investigated the effects of SAR using primary bone marrow stem cells. A SAR-GFP fusion construct was produced using the pMSCV retroviral expression system. A pure population of SAR-infected CD34+ stem cells was obtained after cell sorting. Both γ- and β-globin mRNA transcripts were measured dynamically using real-time PCR (Figure 3a, b). SAR enhanced both γ-globin and β-globin mRNA expression in the CD34+ stem cells, but the effects on γ-globin were larger and more sustained than the effects on β-globin (Figure 3c). SAR enhanced γ-globin gene expression 3-fold at day 10, 5.4-fold at day 14, and 15.8-fold at day 18 in SAR-infected CD34+ cells, compared to 5.7-fold at day 10, 1.6-fold at day 14 and 2.9-fold at day 18 for β-globin mRNA induction.

**G1/S phase accumulation and apoptosis in SAR-expressing cells**

We also noted that SAR-transfected K562 cells grew more slowly than vector-transfected controls. Parental and vector-transfected cells doubled in approximately 18 h, as compared to 30 h in SAR transfectants (Figure 4a). Hematoxylin & eosin staining of these cells showed the existence of many enlarged cells. The nuclear-to-cytoplasmic ratio
was increased in some of these cells, which also exhibited macrocytic morphology, implying that SAR delayed erythroid maturation. HU-treated K562 cells had similar morphological features, particularly after 4 days of HU treatment (Figure 4b).

HU not only causes apoptosis in K562 cells, it also upregulates apoptosis-related molecules. As expected, the incidence of apoptosis was notably higher in SAR-expressing K562 clones than in vector controls. A TUNEL assay showed that a sizable fraction of SAR-expressing cells underwent apoptosis (Figure 4c). This fact and the prolonged doubling time in SAR-expressing cells indicate that the cell cycle might be impacted due to the forced SAR expression. As shown in Figure 4d, the percentage of cells in G2/M decreased from 19.3 ± 0.2% in vector-transfected cells to 5.3 ± 3.0% in SAR transfectants, while the G1/S phase fraction increased to 21% in SAR-expressing cells, suggesting that SAR-expressing cells accumulate in G1 and S phase. By measuring the forward angle light scatter (FALS), an indicator of cell volume, we observed a significant increase in SAR-expressing cells (49.1 ± 1.4%) compared with vector-transfected cells (33.7±0.9%) (P < 0.05). The effects on cell volume and G1/S accumulation by SAR were again consistent with the known effects of HU on erythroid cells.

**Increased protein levels of GATA-2 and p21 in SAR-expressing K562 cells**

The erythroid transcription factors GATA-1 and GATA-2 are considered to be active modulators of γ-globin gene expression. We therefore investigated the GATA-1 and GATA-2 proteins in SAR-expressing K562 cells with Western blots. GATA-2 protein
levels, but not GATA-1, were significantly elevated in these cells compared to vector controls (p < 0.05; Figure 5a). In the same experiment, similar effects on GATA-1 and GATA-2 protein levels were observed in K562 cells after 3 day of HU treatment. Interestingly, p21 mRNA was also elevated 2.4-fold in SAR-expressing cells compared to the vector control (Figure 5b).

**Decreased ERK phosphorylation and PI3 kinase accompanied SAR over-expression**

Because there was observable growth inhibition in SAR-expressing cells, overexpressing SAR may impact growth-related signals. It has been reported that the inhibition of Ras/ERK enhances erythroid cell differentiation. Therefore, we examined mitogen- and stress-activated signaling molecules in the SAR-expressing cells and vector controls. Interestingly, phospho-ERK (pERK) levels, but not total ERK protein, decreased in all three SAR-transfected clones compared with vector-transfected cells (Figure 5c). In contrast, neither the phosphorylation state nor the total level of p38 protein was altered in response to SAR expression (data not shown). ERK phosphorylation transiently decreased after 3 h exposure to 100 µM HU in K562 cells, but returned to baseline by 24h. By using a PowerBlot screen, we also found that the most significant protein change was an 11-fold reduction of PI3 kinase in SAR-expressing clones as compared to vector controls. This result was further confirmed by Western blot (Figure 5d).
Discussion

Using differential display, we identified the small GTP-binding protein SAR as an HU-inducible gene. The induction of γ-globin mRNA by HU was correlated with an increase of SAR mRNA in human erythroid cells, leading us to hypothesize that SAR is a specific factor involved in γ-globin production after HU treatment. This hypothesis is supported by 1) the induction of SAR mRNA by HU in both HAECs and K562 cells, 2) the induction of γ-globin in both K562 cells and CD34+ cells expressing SAR, 3) the correlation of SAR and γ-globin mRNA expression in SAR-transfected K562 cells, 4) parallel increases in mRNA levels for both genes in response to three γ-globin gene inducers in K562 cells, and 5) parallel induction of both γ-globin and SAR mRNA in AC133+ cells cultured with EPO. These observations indicate that the effects of SAR expression in hematopoietic cells not only closely resemble the effects of HU in these cells, but also that the induction of SAR is a specific event in the HU-induced enhancement of γ-globin production.

SAR has long been known to participate in protein trafficking from the ER to the Golgi complex.16,17 The localization of SAR in the endoplasmic reticulum and its association with γ-globin gene expression demonstrated in this study suggest that SAR may also play a special role in hemoglobin regulation. Although the precise pathway(s) and molecules through which SAR regulates the γ-globin gene remain unknown, SAR may increase the transport of membrane-bound transcription factor precursors from the endoplasmic reticulum to the Golgi. In this model, the proteolytic cleavage of the precursor proteins in
the Golgi would activate cytosolic fragments that enter the nucleus and regulate erythroid-specific transcription factors, such as GATA, eventually modulating γ-globin gene expression. Alternatively, increased SAR levels in erythroid cells may influence local concentrations and intracellular locations of GTP and cGMP. Increased availability of intracellular GTP could affect γ-globin expression either directly or indirectly. It has been reported that guanine, guanosine, and guanine ribonucleotides, but not ATP, CTP, and UTP, induce K562 differentiation, which is associated with the accumulation of γ-globin mRNA. GTP also dramatically increases the half-life of γ-globin mRNA. Activators of the sGC and protein kinase G (PKG) pathways have been implicated in the regulation of γ-globin gene expression in both erythroleukemic cells and in primary erythroblasts. Expression of γ-globin and the sGC alpha subunit are correlated, indicating that GTP-binding proteins may participate in γ-globin induction. The finding of a direct interaction between SAR and erythroid-specific factors will strengthen these speculative models.

Stable transfection of SAR into K562 cells arrested the cells at a premature stage, which is associated with increased synthesis of γ-globin. K562 cells express γ-, ε-, and δ-globin but not β-globin, and HU is reported to promote both γ- and β-globin production. We therefore transduced the SAR gene into CD34+ cells using a retroviral system to investigate its effects on β- and γ-globin production. We found that SAR and HU were similarly able to regulate hemoglobin production in the CD34+ stem cells, and SAR induced both γ- and β-globin production. SAR-mediated γ-globin induction was more
sustained and profound than the induction of the β-globin gene. This indicates that γ-globin is an important target of SAR and HU during erythroid maturation.

Alterations in the p21, GATA-2, and ERK signal transduction pathways were seen in SAR-expressing cells. The transcription factors GATA-1 and GATA-2 are required for normal hematopoiesis. GATA-1 is essential for survival during primitive and definitive erythropoiesis and terminal maturation. GATA-2 is required for the expansion of multipotential hematopoietic progenitors, but is dispensable for terminal differentiation of erythroid cells and plays a critical role in both the proliferation and survival of early hematopoietic cells. GATA-1 and GATA-2 are equally important in GTP-induced γ-globin gene regulation. In the regulatory loop between SAR and the γ-globin gene, GATA-1 and GATA-2 can interact to enhance γ-globin promoter activity. It has been shown that a decline in GATA-1 levels alone, without a substantial change in GATA-2, does not affect γ-globin gene expression. Increasing GATA-2 levels increase ε-globin and γ-globin transcripts in K562 cells. In the present study, GATA-2 protein levels increased significantly in SAR expressing cells without a significant change in GATA-1, consistent with the effect of HU on parental K562 cells. We posit that SAR may disturb erythroid maturation by upregulating GATA-2, resulting in an increase in γ-globin production.

p21 plays a regulatory role within the G1/S cell cycle checkpoint. Forced GATA-2 expression inhibits cytokine-dependent growth of normal hematopoietic stem/progenitor
cells by modulating p21 protein levels. In p21 transgenic mice, p21 was found to regulate proliferation and differentiation of early hematopoietic progenitors and, to a lesser extent, of erythroid cells. In this study, SAR-expressing cells not only upregulated GATA-2, but also had a 2- to 3-fold increase in p21 levels. It is likely that p21 induction in SAR-transfected cells exceeded a growth-inhibiting threshold; this would have prevented cells from differentiating into terminal erythroid cells, thereby keeping them at a stage conducive to Hb F production. Our data are consistent with a recent report that GATA-2 inhibited the growth of HSCs as well as other hematopoietic cell lines. The authors suggested that overexpression of GATA-2 may alter cytokine signals and shift the binding partners of GATA-2.

Small GTP-binding proteins interact with PI3 kinase binding proteins to facilitate vesicle-mediated vacuolar protein sorting. It has been reported that PI3 kinase regulates erythroid cell differentiation and hemoglobin production, and inhibition of PI3 kinase activity could dephosphorylate ERK. Enforced SAR expression in K562 cells downregulated PI3 kinase and dephosphorylated ERK, which may imply that SAR blocks ERK phosphorylation through downregulation of PI3 kinase. Inhibition of ERK has been shown to inhibit cell growth and gene transcription. ERK activity can be elevated in response to proliferative factors, as well as in response to differentiating agents; related signal molecules can show differences depending on the genetic makeup of cells used in each experiment. ERK dephosphorylation might induce hemoglobin synthesis and inhibit cell proliferation. Therefore, our data suggest that HU-induced increases in γ-globin gene mRNA levels may involve the PI3 kinase-ERK signal transduction pathway.
Taken together, our findings suggest that SAR induces γ-globin gene expression in human erythroid cells. HU upregulates SAR gene expression in erythroid cells, implicating SAR in γ-globin gene induction and erythroid cell maturation. We have shown that enforced SAR expression alone can replicate the known effects of HU on erythroid cells, including cytotoxicity, G1/S-phase accumulation, macrocytosis, and γ-globin induction. The induction of SAR mRNA expression by HU also suggests that selective enhancement of membrane trafficking may play a role in erythrocyte maturation and proliferation. SAR is likely to be involved in a variety of normal erythrocyte functions.

This study represents the first evidence that the small GTP-binding protein SAR participates in cell cycle control through upregulation of GATA-2 and p21 in erythroid maturation, as depicted in Figure 6. Elucidation of the relationship among SAR’s vesicular trafficking functions and γ-globin induction may allow new therapies for sickle-cell disease and β-thalassemia. Further studies will determine whether SAR is an obligatory mediator of these effects and will explore the specific molecular pathways involved. Direct interactions between SAR and associated binding partner(s) should be established to provide the molecular basis of SAR function in hemoglobin protein production.
Acknowledgments

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</tr>
<tr>
<td></td>
<td>3.50</td>
<td>(1.53)</td>
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<tr>
<td><strong>Vector only</strong></td>
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<tr>
<td>clones</td>
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<tr>
<td></td>
<td>1.20</td>
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</table>

* Fold increase compare to k562 cells

** P value compared with vector clones
Figure Legends

Figure 1. SAR mRNA expression in different tissues and HU induction of SAR. (a) mRNA blots of multiple human tissues were hybridized with a $^{32}$P-labeled SAR-specific probe. (b) Human adult erythroid cells were treated with (+) or without (-) 100 µM HU, as detailed in the Methods. Total RNA was isolated on day 10 and probed for SAR mRNA by Northern blotting. (c) In vitro transcription/translation of the SAR protein produced a 22-kDa peptide. (d) Cellular localization of SAR tagged with GFP at the 3’ end in SAR-transfected K562 cells. Green indicates GFP fluorescence; red indicates staining for calreticulin, which indicates the endoplasmic reticulum; yellow (in the merged image) represents overlapping green and red fluorescence. Cells were incubated for 8 or 18 h.

Figure 2. SAR and \(\gamma\)-globin gene transcription in SAR transfectants and K562 and AC133\(^+\) cells treated with globin inducers. (a) Correlation of \(\gamma\)-globin and SAR mRNA. \(\gamma\)-globin (Gamma) and SAR mRNA levels were normalized to glycophorin C determined by quantitative RT–PCR in SAR-transfected K562 cells. (b) Northern blot analysis of \(\gamma\)-globin and SAR mRNA expression in K562 cells 48 h after treatment with HU, butyrate, or 5-azacytidine, as detailed in the Methods. GAPDH was used as an internal control. (c) Quantitative analysis of \(\gamma\)-globin and SAR mRNA levels normalized to GAPDH from Northern blots. (d) RT-PCR determination of SAR and \(\gamma\)-globin mRNA expression (normalized to GAPDH) in AC133\(^+\) cells (with EPO) cultured for the indicated amounts of time.
**Figure 3. γ-globin and β-globin gene transcription in CD34+ cells determined by real time PCR.** (a) γ-globin genes expression in CD34+ cells infected with an SAR retrovirus and vector control cells, normalized with beta-actin gene. (b) β-globin genes expression in CD34+ cells infected with an SAR retrovirus and vector control cells, normalized with beta-actin gene. (c) Comparison of SAR effect on γ-globin and β-globin genes in SAR-expressing CD34+ cells, shown fold increase comparing to vector control cells.

**Figure 4. Effects of HU and SAR expression on K562 cell growth.** (a) Growth curve generated as described in the Methods. Open diamonds, parental K562 cells; closed circles, HU-treated parental K562 cells; open circles, vector-transfected cells; closed squares, SAR-expressing cells. Cell doubling times were calculated from the graph. (b) Enforced expression of SAR (2 clones are shown) led to dramatic macrocytosis and a relatively immature appearance in some K562 cells, as seen with hematoxylin–eosin staining. HU-treated parental cells are also shown (HU). (c) A sizable fraction of SAR transfectants underwent apoptosis, as shown by TUNEL assays. Apoptotic cells are stained green; the nuclei of cells stained with DAPI are blue. Similarly, K562 cells treated with HU for 24 h showed some apoptotic cells. (d) Cell cycle and FACS analysis. SAR-expressing cells were arrested in G1/S-phase and showed a decreased G2M fraction. Cell cycle analysis was performed after cells were plated for 48h.
Figure 5. Expression of GATA-1, GATA-2, ERK and PI3 kinase proteins, and p21 mRNA in parental and SAR-transfected K562 cells. (a) Comparison of GATA-1 and GATA-2 protein levels in parental K562 cells (grown in the presence or absence of HU for three days), vector-transfected cells, and SAR-expressing cells. Left panel, Western blot; right panel, fold increase over untransfected K562 cells. (b) p21 mRNA levels in SAR-expressing cells and vector controls detected by hybridization. (c) Total and phosphorylated ERK in SAR transfectants and K562 cells treated with HU for three days. (d) PI3 kinase protein in SAR transfected and vector control K562 cells. Samples containing 50 µg of protein were subjected to electrophoresis on Tris–glycine gels and to Western blotting with antibodies directed against either phosphorylated ERK, total ERK, or PI3 kinase.

Figure 6. Schematic pathways of SAR-mediated globin gene induction. Enforced SAR expression alone can replicate the known effects of HU on K562 cells, including cytotoxicity (apoptosis), G1/S-phase arrest, and γ-globin induction. SAR-induced GATA-2 expression not only increases γ-globin gene induction, it also induces p21 protein. Induction of p21 causes inhibition of cell proliferation and G1/S arrest. Reduction of PI3 kinase (PI3K) and phosphorylated ERK (pERK) can be triggered by SAR.
Figure 1

a. 

Spleen  Thymus  Prostate  Testis  Ovary  Small Intestine  Colon  Leukocyte  Stomach  Thyroid  Spinal Cord  Lymph Node  Trachea  Adrenal Gland  Bone Marrow

b. 

HU

-  +  -  +

3.0kb

3.0kb

0.8kb

3.0kb

22kDa

d. 

SAR-GFP  Calreticulin  Merge

8h

18h
Figure 2

a. 

\[
\begin{align*}
\text{R} &= 0.76 \\
\gamma\text{-globin} &\quad \text{SAR} \\
0.0 &\quad 0.2 \\
0.4 &\quad 0.6 \\
0.8 &\quad 1.0 \\
2.0 &\quad 3.0
\end{align*}
\]

b. 

- K562
- HU
- Butyrate
- 5-Aza

\[
\begin{align*}
\gamma\text{-Globin} \\
\text{SAR} \\
\text{GAPDH}
\end{align*}
\]
c.  

Gene Expression (fold)  

K562  K562/HU  K562/Butyrate  K562/5AZA  

Gamma globin  

SAR  

Days in Culture  

D10  D14  D21  D28  

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Figure 3

a.

![Bar graph showing β-Globin gene expression over days in culture for Vector and SAR groups.](image)

b.

![Bar graph showing β-Globin gene expression over days in culture for Vector and SAR groups.](image)
Gene Expression Induction (Fold)

- **Gamma globin**
- **Beta globin**

Days in Culture:
- **day10**
- **day14**
- **day18**

Gene Expression Induction (Fold)
Figure 4

a.

![Graph showing cell number over time for K562, HU, Vector, and SAR treatments.]

b.

<table>
<thead>
<tr>
<th>K562</th>
<th>V</th>
<th>SAR</th>
<th>HU (100µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Day 2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Day 4
c. K562 V | HU (µM) | SAR
| 100 | 200 | 400 |
TUNEL
DAPI
d. K562 V SAR
G2/M phase 19.0% 19.3 ± 0.2% 5.3 ± 3.0% *
FALS Mean 34.4% 33.7 ± 0.9% 49.1 ± 1.4% *
Figure 5

a. K562  HU  SAR  Vector

GATA-2
Actin
GATA-1
Actin

b. V  SAR

p21 expression (fold)

Gene expression (fold)
Figure 6

HU

↑SAR

↑Apoptosis  ↑GATA-2  ↑G1/S arrest

↑γ-globin  ↑p21

↓Cell proliferation

↓PI3 Kinase

↓pERK
The hydroxyurea-induced small GTP-binding protein SAR modulates \( \gamma \)-globin gene expression in human erythroid cells

Delia C Tang, Jianqiong Zhu, Wenli Liu, Kyung Chin, Jun Sun, Ling Chen, John A Hanover and Griffin P Rodgers