**Key Points**

- Hydroxyurea activates nuclear factor-κB to transcriptionally upregulate SAR1.
- SAR1, in turn, activates γ-globin expression through the Gia/JNK/Jun pathway.

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**Introduction**

β-hemoglobinopathies are inherited disorders caused by mutations/deletions in the β-globin chain that lead to structurally defective β-globin chains or reduced (or absent) β-globin chain production. These diseases affect multiple organs and are associated with considerable morbidity and mortality, representing a major public health challenge. During erythropoiesis, SAR1 has been successfully used in the treatment of β-hemoglobinopathies by augmenting the production of fetal hemoglobin (HbF). However, the molecular mechanisms underlying HU-mediated HbF regulation remain unclear. We previously reported that overexpression of the HU-induced SAR1 gene closely mimics the known effects of HU on K562 and CD34+ cells, including γ-globin induction and cell-cycle regulation. Here, we show that HU stimulated nuclear factor-κB interaction with its cognate-binding site on the SAR1 promoter to regulate transcriptional expression of SAR1 in K562 and CD34+ cells. Silencing SAR1 expression not only significantly lowered both basal and HU-elicited HbF production in K562 and CD34+ cells, but also significantly reduced HU-mediated S-phase cell-cycle arrest and apoptosis in K562 cells. Inhibition of c-Jun N-terminal kinase (JNK)/Jun phosphorylation and silencing of Gia expression in SAR1-transfected K562 and CD34+ cells reduced both γ-globin expression and HbF level, indicating that activation of Gia/JNK/Jun proteins is required for SAR1-mediated HbF induction. Furthermore, reciprocal coimmunoprecipitation assays revealed an association between forcibly expressed SAR1 and Gia2 or Gia3 proteins in both K562 and nonerythroid cells. These results indicate that HU induces SAR1, which in turn activates γ-globin expression, predominantly through the Gia/JNK/Jun pathway. Our findings identify SAR1 as an alternative therapeutic target for β-globin disorders. (Blood. 2014;124(7):1146-1156)
the Gis/JNK/Jun pathway, which may provide a novel target for therapeutic intervention aimed at upregulating γ-globin gene expression in hemoglobinopathies.

Materials and methods

Cell culture and transfection

Bone marrow CD34+ cells (LONZA) and K562 (ATCC) cells were cultured as previously described.27 On day 5 of differentiation, SARI–expressing vector or empty vector was electrophoresed into CD34+ cells using the LONZA 4D-Nucleofector system according to the manufacturer’s protocol. SARI-V5 tag– or vector only–stably transducted K562 cells were established according to a previously published protocol.15

Cloning of SARI promoter region and reporter gene assays

SARI promoter fragments were cloned from K562 genomic DNA using the GC-RICH PCR system (Roche) and inserted into the pGL3 basic luciferase vector (Promega). All mutant reporter gene constructs were generated by QuiikChange site-directed mutagenesis (Stratagene). Plasmids were sequenced to verify the integrity of the insert. The level of promoter activity was evaluated by measurement of firefly luciferase activity relative to the internal control Renilla luciferase activity using the Dual Luciferase Assay system (Promega) following the manufacturer’s instructions. K562 cells or CD34+ cells were preincubated with HU for 2 days, then cotransfected with a reporter construct, and a pRL-TK vector that produces Renilla luciferase (Promega). The transfected cells were continually treated with or without HU for another 12 to 48 hours.

EMSAs, antibody-supershift assays, and ChIP assays

Electrophoretic mobility shift assays (EMSAs) and antibody-supershift assays were performed according to a previously described protocol.18 Sequences for each probe were as follows: wild-type Elk-1/κB, 5′-ACGCAGCAGCGGTCGGG-3′; mutant Elk-1/κB, 5′-ACGCAGCAGCGGTCGGTGG-3′. Two micrograms of anti-NF-κB p50, anti-Elk-1, anti-c-Rel, or rabbit immunoglobulin G (IgG) antibody (Santa Cruz Biotechnology) was used in supershift assays. Chromatin immunoprecipitation (ChIP) assays were performed as previously described.27

RNAi assays

A plasmid-based system for production of SARI microRNA (miR) interfering RNA (RNAi) (5′-TGCGTAACCCTGCTCTTGAGCACAGATT TGGCACACTGACTGTGCTTCAAGGAAAGTTGTTTACAGG-3′) or negative control miR RNAi was generated by inserting oligonucleotides into pcDNA3.0 plasmid (Invitrogen). Five micrograms of miR RNAi or shRNA plasmid was transfected into K562 cells using the Nucleofector system (Amaxa Biosystems) according to the manufacturer’s optimized protocol. K562 cells were transfected with control or SARI miR RNAi twice (on day 0 and day 1) followed by 3 days of HU treatment (day 0 to day 2), then subjected to flow cytometry to detect HbF-positive cells. For shRNA-mediated SARI silencing, K562 cells were incubated with or without HU for 2 days after transfection with SARI shRNA or control shRNA, then subjected to 5-bromodeoxyuridine (BrdU) incorporation assay or terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay.

CD34+ bone marrow cells were infected with SARI shRNA or control shRNA lentivirus (Santa Cruz Biotechnology) at day 4 of expansion. On day 6, transduced CD34+ cells were reseeded and grown in differentiation medium with 2 μg/mL puromycin for 14 days. At day 7 of differentiation, cells were stained with phycoerythrin (PE)-conjugated anti-HBF antibody, and subjected to flow cytometry to detect HbF-positive cells. At day 10 of differentiation, transduced CD34+ cells were subjected to high-performance liquid chromatography analysis for measurement of HbF, Giemsa staining for morphologic changes, and flow cytometry for CD71/glycoprotein A (GPA) expression.

For Gis silencing experiments, CD34+ cells were cotransfected with SARI–expressing vector or empty vector and scrambled siRNA or Gis siRNA, and the transfected cells grown in differentiation medium for 2 days before being harvested for real-time polymerase chain reaction (PCR) and western blot analysis.

Coimmunoprecipitation

For coimmunoprecipitation studies, 1 mg of whole-cell lysates from K562 cells stably transected with SARI-V5 tag or control vector was incubated with 2 μg/mL anti-Gis1, -Gis2, -Gis3, -Gro, or -Gb antibody (Santa Cruz Biotechnology) for 3 hours at 4°C. Recombinant Protein G-Sepharose 4B (20 μL; Invitrogen) was then added and gently mixed overnight at 4°C. The beads were washed 3 times with immunoprecipitation buffer and boiled in 35 μL of sample-loading buffer (NuPAGE LDS sample buffer; Invitrogen) for 10 minutes. After a brief spin to remove the protein G-Sepharose beads, the supernatant (25 μL) was run in a 4% to 12% Bis-Tris (tris(hydroxymethyl) aminomethane) gel (Invitrogen) for western blot analysis with anti-V5 antibody (Invitrogen).

Statistical analysis

Results were analyzed using the Student t test for significance of the difference of individual pairs and the Pearson correlation coefficient for the closeness of linear relationship.

Results

Basal and HU-mediated SARI transcriptional regulation requires an intact Elk-1/κB-binding site in the SARI promoter

Our prior work has demonstrated that the SARI gene, which we cloned from primary cultures of human adult erythroid cells, was inducible by HU treatment and upregulated γ-globin expression in K562 and CD34+ cells.15 This finding led us to investigate how SARI is transcriptionally regulated by identifying the promoter elements responsible for SARI induction by HU. When we examined the proximal region of the SARI promoter sequence based on MatInspector software, we found putative-binding sites for several transcription factors, such as EKLF, Elk-1, and NF-κB. The promoter region contains 1 binding consensus site for both Elk-1 and NF-κB (supplemental Figure 1, available at the Blood Web site). A series of recombinant DNA plasmids containing various lengths of the predicted SARI promoter sequence (−977 to +49) in a luciferase reporter vector was prepared and transfected into K562 cells or CD34+ cells at day 6 of differentiation with or without 2 days of HU pretreatment. We found that 24 or 48 hours after transfection, reporter activity was significantly increased in HU-treated K562 cells transfected with the full-length SARI reporter construct compared with non-treated controls (Figure 1A, top panel). HU treatment also increased SARI promoter activity in CD34+ cells in a dose-dependent manner (Figure 1A, bottom panel). Deletion analysis revealed that the reporter construct containing the −41 to +49 region produced a high luciferase activity (Figure 1B) while retaining most of the HU-mediated induction of SARI promoter activity (Figure 1C). In contrast, the luciferase activity generated from the reporter construct containing the +3 to +49 fragment dropped to the level of the vector control (Figure 1B), and its luciferase activity could not be increased by HU (Figure 1C). Moreover, removal of the −30 to +49 fragment in the reporter construct containing the −315 to +49 region not only decreased luciferase activity, but also eliminated the HU-responsive luciferase activity (Figure 1D). These results imply that an element within the region between −41 and +3 is sufficient to...
drive \( SARI \) promoter activity and is responsible for HU-mediated \( SARI \) induction.

To determine which regulatory elements were involved in \( SARI \) gene transcription and HU responsiveness, we introduced mutations in the binding sites for Elk-1, EKLF, or Elk-1/NF-xB. We found that mutations at either the EKLF or Elk-1/NF-xB sites significantly decreased \( SARI \) promoter activity and abolished its responsiveness to HU treatment, and alteration of the Elk-1–binding site increased luciferase activity without compromising HU-induced \( SARI \) promoter activity (Figure 1E). These results suggest that these EKLF and Elk-1/NF-xB–binding sites are important for \( SARI \) transcription and HU-regulated \( SARI \) induction, and the Elk-1–binding site is a functional element that negatively regulates \( SARI \) gene expression.

Transcription factor NF-xB binds to its cognate site in the \( SARI \) 5'-upstream region

To determine whether these promoter sequences were indeed recognized by their putative transcription factors, we performed EMSAs using \( ^{32}P \)-labeled oligonucleotides containing the Elk-1/NF-xB consensus sequence and its corresponding mutant. When nuclear extracts from K562 cells were incubated with the wild-type radiolabeled DNA probe, 2 shift bands of specific protein–DNA complexes were detected (Figure 2A, left panel, complexes A, B). When the
HU activates NF-κB signaling and enhances NF-κB binding to the \( \text{SAR1} \) promoter region

We next set out to explore the effect of HU on NF-κB signaling and binding of NF-κB to the \( \text{SAR1} \) promoter region. Immunofluorescence staining with an Alexa Fluor-conjugated NF-κB p50 antibody showed that HU substantially increased nuclear localization of NF-κB in CD34\(^+\) cells (supplemental Figure 2A). Western blot analysis revealed that HU treatment induced phosphorylation of ataxia-telangiectasia mutated and degradation of the NF-κB inhibitor IκB, and increased levels of nuclear NF-κB p50 protein in a time-dependent manner in K562 cells. The effects peaked at 3 hours post-HU treatment and then gradually declined (supplemental Figure 2B). A similar pattern was observed from EMSA analysis showing that HU enhanced the binding of NF-κB to its cognate site within the \( \text{SAR1} \) promoter region (Figure 2B). To test whether Elk-1 or NF-κB directly binds the regulatory regions of the \( \text{SAR1} \) gene promoter in primary cells, we performed ChIP assays in CD34\(^+\) cells with or without HU treatment. ChIP data indicated that NF-κB antibody immunoprecipitated the \( \text{SAR1} \) promoter region, and that HU treatment enhanced NF-κB binding to the promoter. Surprisingly, although Elk-1 could directly bind the \( \text{SAR1} \) promoter region, HU treatment did not augment the binding (Figure 2C). To provide further evidence that NF-κB is a key transcriptional regulator for \( \text{SAR1} \) promoter activity and that HU influences the activity through recruitment of NF-κB to the gene promoter, CD34\(^+\) cells were preincubated with the NF-κB-specific inhibitor BAY11-7082, transfected with \( \text{SAR1} \) reporter construct, then tested for luciferase activity in the absence or presence of HU treatment. Blocking NF-κB activation by BAY11-7082 in CD34\(^+\) cells produced a dose-dependent downregulation of \( \text{SAR1} \) promoter activity and blocked HU-mediated \( \text{SAR1} \) promoter activity (Figure 2D). Furthermore, BAY11-7082 also significantly reduced basal and HU-induced \( \text{SAR1} \) and \( \gamma \)-globin expression in CD34\(^+\) cells (Figure 2E). The NF-κB inhibitor had a more profound effect on HU-responsive \( \text{SAR1} \) and \( \gamma \)-globin expression than on basal levels. Taken together, our results indicate that HU treatment upregulated \( \text{SAR1} \) transcription through the NF-κB pathway by causing NF-κB to translocate from the cytoplasm to the nucleus and subsequently bind to its recognition site in the \( \text{SAR1} \) promoter.

\( \text{Elk-1/NF-κB mutant probe was used, band A was clearly absent and the density of band B was substantially reduced. The gel-shift signal could be efficiently competed by unlabeled oligonucleotides containing the wild-type sequence (Figure 2A, right panel). These data indicate that the putative Elk-1/NF-κB-binding site is responsible for the formation of the 2 complexes. Antibody-supershift assays were used to further confirm the binding of the transcription factors to their recognition sites on the \( \text{SAR1} \) promoter. Addition of NF-κB p50 antibodies, but not Elk-1 or c-Rel antibodies, eliminated band A and resulted in a new higher molecular weight band termed “ss” (Figure 2A, left panel). These data suggest that the band “A” protein complex is composed of NF-κB p50 protein that may activate \( \text{SAR1} \) transcription through binding to specific sites in the \( \text{SAR1} \) 5’-upstream region. Similar experiments were not able to confirm the specific binding of the transcription factors Elk1F and Elk-1 with their recognition sites (data not shown).}
Overexpression of SAR1 enhances HbF production in K562 and CD34+ cells

We previously demonstrated that HU induced SAR1, and that overexpression of SAR1 increased γ-globin at the transcriptional level in K562 cells and primary cultures of CD34+ cells. Here we furthered those studies by examining HU’s effects at the protein level. HU was found to increase SAR1 protein expression (Figure 3A), and overexpression of SAR1 increased levels of γ-globin chain protein (Figure 3B). SAR1 overexpression also significantly increased the percentage of HbF-positive cells from a mean of 14% to 44% in K562 cells (Figure 3C). We also found that various hemoglobin gene inducers (HU, 5-azacytidine, and Ara-C) could increase γ-globin messenger RNA (mRNA) expression with a concomitant increase of SAR1 mRNA levels in primary CD34+ cells after 72 hours of drug exposure (Figure 3D). These data and our previously published results strongly support the role of SAR1 in the transcriptional regulation of γ-globin expression.

Silencing SAR1 expression in K562 and CD34+ cells reduces basal and HU-induced HbF production

To further confirm that basal and HU-induced HbF expression is mediated through SAR1, we silenced the expression of SAR1 in K562 and CD34+ cells by RNAi targeting SAR1 and then measured HbF production by flow cytometry. Silencing of SAR1 in K562 cells using SAR1 miR RNAi (knockdown efficiency shown in supplemental Figure 4A) reduced basal and HU-mediated HbF expression by 22% and 35%, respectively, compared with mock-transfected cells (Figure 4A). Similar results were obtained when CD34+ cells were transduced with lentiviruses containing SAR1 shRNA to silence SAR1 expression (knockdown efficiency shown in supplemental Figure 3D). In SAR1-knockdown CD34+ cells, basal and HU-induced HbF levels were reduced by 23% and 38%, respectively, compared with control shRNA-transduced cells at day 7 of differentiation (Figure 4B). shRNA-mediated knockdown of SAR1 in CD34+ cells resulted in a 74% decrease in HbF levels (from 5.1% ± 1.4% to 1.3% ± 0.6%, P < .05), with no notable changes in cell morphology and surface expression of GPA/CD71 markers at day 10 of differentiation (supplemental Figure 3A-C). Our findings indicate that SAR1 is an obligatory mediator in HU-induced HbF production, although SAR1 might not be the exclusive mediator of HbF induction by HU.

Silencing SAR1 expression attenuates HU-mediated S-phase cell-cycle arrest and apoptosis

We have previously shown that SAR1 overexpression alone can replicate the known effects of HU in 2 human erythroid differentiation models, including cell-cycle regulation and cytotoxicity. Therefore, if HU inhibits erythroid cell growth through induction of SAR1, one would expect that suppression of SAR1 expression would reverse HU-induced inhibitory effects. Transfection of K562 cells with SAR1 shRNA (knockdown efficiency shown in supplemental Figure 4B) had no notable effect on the cell cycle in untreated cells, but significantly attenuated HU-mediated S-phase cell-cycle arrest in SAR1-knockdown cells. The percentage of S-phase cells was 68%, compared with 83% in control shRNA-knockdown cells (P < .01), as determined by BrdU incorporation assay (Figure 4C). In TUNEL assays, knockdown of SAR1 did not change basal apoptosis levels, but did significantly abolish HU-mediated apoptosis in K562 cells (Figure 4D). These results indicate that HU-induced cell-cycle arrest and apoptosis are mediated, at least in part, through alteration of SAR1 expression.

SAR1 enhances HbF expression through the activation of JNK/Jun signaling

To further investigate the mechanism underlying SAR1-regulated HbF expression, we first knocked down SAR1 expression in CD34+
Figure 4. Silencing SAR1 expression reduces HbF protein expression in K562 and CD34+ cells and attenuates HU-mediated S-phase cell-cycle arrest and apoptosis. (A) K562 cells were transfected with SAR1 miR RNAi or control miR RNAi at day 0 and day 1, and incubated with or without 100 µM HU for 3 days after transfection at day 0. Cells were harvested, stained with PE-conjugated anti-HbF antibody, and subjected to flow cytometry to detect HbF-positive cells. (B) CD34+ bone marrow cells were infected by SAR1 shRNA or control shRNA lentivirus at day 4 of expansion. On day 6, transduced CD34+ cells were reseeded and grown in differentiation medium with 2 µg/mL puromycin for 7 days. Cells were harvested, stained with PE-conjugated anti-HbF antibody, and subjected to flow cytometry to detect HbF-positive cells. Left panels, representative flow cytometry results. Right panels, graphical representation of flow cytometry data for HbF protein expression with or without HU treatment relative to that of mock-transfected (A) or control shRNA lentivirus-transduced (B) cells not treated with HU. (C-D) K562 cells were incubated with or without HU for 2 days after transfection with SAR1 shRNA or control shRNA, then subjected to cell-cycle analysis using a BrdU incorporation assay or TUNEL assays. (C) Panels display representative BrdU incorporation assay results; the mean (±SD) percentage of cells in S-phase is shown below. (D) Apoptotic cells were stained green (GFP) in TUNEL assay; the nuclei of cells were stained blue with DAPI. Left panel, representative TUNEL assay results. Images were acquired with a fluorescence microscope system (Axioplan 2; Carl Zeiss MicroImaging) with a 20×/0.75 NA objective and a Zeiss AxioiMicrICam real-color CCD camera and AxioVision 4 (Zeiss) acquisition software. Right panel, graphical representation of TUNEL assay results showing percent of GFP-positive cells. *P < .05 vs control-transfected cells not treated with HU. **P < .05 vs control-transfected cells with HU treatment. Error bars represent SD of the mean of 3 independent experiments. DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein.
SAR1 is known to function in protein trafficking from the endoplasmic reticulum (ER) to the Golgi via the cytosolic coat protein complex II (COPII) secretory pathway. We examined whether the COPII pathway could account for the HU-induced enhancement of \( \gamma \)-globin production and found that genetic silencing of the COPII component Sec12/Sec23 or chemical inhibition of vesicle protein transport from ER to Golgi with brefeldin A could only partially affect HU-induced \( \gamma \)-globin expression without a change in basal \( \gamma \)-globin level (supplemental Figure 5), suggesting that SAR1 might also use other signal transduction pathways to regulate hemoglobin expression in the context of HU treatment.

To determine the possible signaling cascade involved in SAR1-mediated HbF induction, PowerBlots and micro-western blots were carried out to search for potential candidates. Both approaches indicated that SAR1 increased Jun expression in SAR1-stably transfected K562 cells (data not shown). We confirmed these results by western blot analysis, which revealed that overexpression of SAR1 markedly increased both total and phosphorylated c-Jun, as well as induced phosphorylation of its upstream signaling molecule JNK (Figure 5A). To verify that JNK/Jun signaling accounts for the functionality of SAR1 in upregulating HbF expression, vector control– and SAR1-stably transfected K562 cells were incubated with the JNK phosphorylation inhibitor SP600125 for 5 days, then assessed for Jun activation and HbF expression. SP600125 substantially suppressed basal and SAR1-regulated Jun and JNK phosphorylation (Figure 5B), which was accompanied by a significant reduction in the percentage of HbF-positive cells in vector control– and SAR1-stably transfected K562 cells (Figure 5C).

Given that K562 cells are genetically abnormal, we extended our studies to the more clinically relevant CD34\(^+\) cells to examine the effect of Jun signaling on SAR1-regulated \( \gamma \)-globin expression.

Figure 5. SAR1 enhances HbF expression through the activation of JNK/Jun signaling. (A) Western blot analysis of SAR1- or vector control–stably transfected K562 cells for expression of phospho- and total c-Jun and phospho- and total JNK. (B) Western blot analysis of SAR1- or vector control–stably transfected K562 cells for expression of phospho- and total c-Jun and phospho- and total JNK after incubation in the absence or presence of the JNK phosphorylation inhibitor SP600125 (12.5 \( \mu \)M) for 5 days. \( \beta \)-actin was used as a loading control. (C) SAR1- or vector control–stably transfected K562 cells incubated in the absence or presence of SP600125 (12.5 \( \mu \)M) for 5 days were stained with PE-conjugated anti-HbF antibody and subjected to flow cytometry for measurement of HbF-positive cells. Left panels, representative flow cytometry results. Right panel, Graphical representation of flow cytometry data for HbF protein expression in SAR1- or vector control–stably transfected K562 cells with or without SP600125 treatment. * \( P < .001 \) vs vector control–transfected cells without SP600125 treatment, ** \( P < .001 \) vs SAR1-transfected cells without SP600125 treatment. Error bars represent SD of the mean of >3 independent experiments. (D) Western blot analysis of SAR1- or vector control–transfected CD34\(^+\) cells for expression of phospho- and total c-Jun (left panel) and \( \gamma \)-globin chain (right panel) after incubation in the presence or absence of SP600125 (20 \( \mu \)M) for 3 days. \( \beta \)-actin was used as a loading control. CD34\(^+\) cells were transfected at day 5 of differentiation.
SAR1- and vector control–transfected CD34+ cells were incubated with or without SP600125. SP600125 markedly inhibited Jun phosphorylation and γ-globin expression in both SAR1– and vector control–transfected CD34+ cells (Figure 5D). These results clearly demonstrate that JNK/Jun signaling plays a pivotal role in basal and SAR1–regulated HbF expression.

Exogenously expressed SAR1 binds to Giα2, 3 proteins to activate Jun and increase γ-globin chain in K562 and CD34+ cells

Several lines of evidence have indicated that activation of Jun and heterotrimeric G-proteins, especially Gi2 and Gi3 proteins, are associated with erythroid cell differentiation and γ-globin expression.17,18,33-37 We postulated that SAR1 might activate the JNK/Jun pathway to regulate γ-globin expression through crosstalk between heterotrimeric G-protein and small G-protein.38 To this end, we examined whether SAR1 can regulate Giα activity and what roles Giα played in SAR1-mediated Jun activation and γ-globin induction. Giα activation assays showed that transfection of SAR1 increased the levels of Giα activation (Figure 6A). Knockdown of Giα2 or Giα3, but not of Giα1, in SAR1-stably transfected K562 cells or SAR1-transfected CD34+ cells (knockdown efficiencies shown in supplemental Figures 6-7) substantially reduced SAR1-stimulated c-Jun phosphorylation and was accompanied by a decreased γ-globin expression by approximately one-half compared with silencing control (Figure 6B-C). We also found that silencing of Giα4 or Giα11 had no significant impact on γ-globin expression in K562 cells. These data support our hypothesis that SAR1 promotes γ-globin expression through the Giα/Jun signal pathway.

Next, we performed a communoprecipitation study with vector control—or SAR1-stably transfected K562 cells to test whether SAR1 could directly associate with Giα proteins. Exogenously expressed SAR1-V5 could be communoprecipitated when using a precipitating antibody to endogenous Giα1, 2, 3, or Giq, but not with antibody to Giβ protein (Figure 6D). Because the Giα antibodies were not sensitive enough to detect their relative G-proteins in the lysates precipitated by anti-V5 antibody, we opted to transfet SAR1-V5–stably expressing K562 cells with vectors containing Flag-tagged Giα1, 2, 3, or Giq for reciprocal communoprecipitation. Thus, detection of anti-V5 antibody communoprecipitated proteins could be achieved by the use of anti-Flag antibody in western blot analyses. Using this approach, we found that SAR1-V5 could be communoprecipitated with an antibody recognizing Flag-Giα1, 2, 3, or Giq, and vice versa (Figure 6E). We found similar binding in 293T cells when cells were cotransfected with SAR1-V5 and Giα1, 2, 3 or Giq-Flag (data not shown). Taken together, we concluded that exogenous expression of SAR1 may activate Jun to increase HbF expression in K562 cells via interaction with Giα2 and Giα3 proteins.

Discussion

Our previous studies identified SAR1 as an HU-inducible gene and demonstrated a novel role for this small guanosine triphosphate–binding protein in both cell-cycle control and hemoglobin gene expression regulation in K562 and CD34+ cells.15 Based on these unique functions of SAR1, it is important to identify the regulatory elements in the SAR1 gene promoter region and its cognate DNA–binding proteins that confer HU inducibility, to assess whether SAR1 is an obligate mediator of these functions, and to elucidate the molecular events underlying SAR1-regulated HbF expression. In the present study, we first dissected the SAR1 promoter region and identified the binding site for the transcription factors Elk-1/NF-κB required for HU-mediated SAR1 gene induction. We then demonstrated that SAR1 is necessary and central to the beneficial effects of HU treatment through its induction of HbF. More importantly, we discovered that forcibly expressed SAR1 interacts with Giα proteins to activate the Giα/Jun/JNK pathway and increase HbF expression in the transformed red cell line K562 and ex vivo human hematopoietic progenitor CD34+ cells.

Recently, a new cell-stress signaling model was suggested to be a common pathway for HbF induction.13 Along with other HbF–inducing agents, the replication stress inducer HU has been identified as a potent HbF inducer through its effects in stimulating γ-globin production during adult erythropoiesis by way of activating cell-stress signaling. The function of NF-κB in hemoglobin regulation has been indicated in several previous studies.39-42 Through regulation of its target genes, NF-κB functions as a key modulator of the cellular response to physiological and pathological stress conditions. In this study, the physical and specific interaction between NF-κB and the SAR1 promoter was verified by EMSAs, antibody-supershift assays, and ChiP assays in K562 or CD34+ cells. In addition, mutation of the putative NF-κB–binding site in the SAR1 promoter and inhibition of NF-κB activation by pharmacologic intervention significantly impaired basal and HU-mediated SAR1 and γ-globin transcription. These data, together with our finding that HU induced ataxia-telangiectasia mutated phosphorylation and IκB degradation, imply that HU stimulation may initiate a series of cell-stress signals, including NF-κB, to upregulate SAR1 expression, which subsequently elevates γ-globin transcription. However, we cannot exclude the possibility that other factors to be identified might have distinct roles in regulating HU response and SAR1 transcription.

Overexpression of SAR1 alone can replicate the known effects of HU in K562 and CD34+ cells.15 This led to our interest in identifying whether SAR1 is required for HU-induced γ-globin production. Our results here indicate that blocking SAR1 expression significantly lowered both basal and HU-elicted HbF production in K562 and CD34+ cells and abolished HU-induced apoptosis in K562 cells, revealing a vital role of SAR1 in HbF expression and HU’s effects in these cells. These data suggest that SAR1 could be a potential therapeutic target for β-hemoglobinopathies.

Activation of JNK/Jun signaling could interact with the γ-globin promoter to increase its transcription and regulate erythroid cell differentiation.17,33 Heterotrimeric G proteins (G-proteins) consisting of α, β, and γ subunits are one kind of JNK/Jun upstream signaling protein that can be regulated by G-protein–coupled receptors, regulators of G-protein signaling, and activators of G-protein signaling.13 G-protein signaling regulates a variety of biological processes, including cell growth, proliferation, and differentiation. Their functions are also implicated in erythroid differentiation44,45; in particular, the presence of Giα is required for hemin- and butyrate-mediated erythroid differentiation46,47 and erythropoietin-induced erythropoiesis.34,35 It has been reported that crosstalk occurs between small G-protein and heterotrimeric G proteins38,48,49; we therefore hypothesized that SAR1 might act through G-proteins to activate JNK/Jun and enhance HbF expression. This hypothesis was supported by several lines of evidence from the current studies. Overexpression of SAR1 activated Giα and increased the phosphorylation of JNK/Jun. Inhibition of JNK/Jun signaling by the JNK-specific inhibitor SP600125 markedly reduced HbF expression in SAR1–transfected cells, and knockdown of Giα2 and Giα3 by siRNA substantially attenuated SAR1-stimulated Jun phosphorylation and γ-globin expression. Furthermore, communoprecipitation studies revealed an association between forcibly expressed SAR1 and Giα2 or Giα3.
in both K562 and 293T cells. However, it should be noted that both Giα and Gaq had no effect on SAR1-regulated γ-globin expression, which may further attest to a distinct role of individual Giα proteins in the regulation of γ-globin expression in K562 and CD34⁺ cells.

In summary, to the best of our knowledge, these results demonstrate for the first time that HU activates NF-κB to transcriptionally upregulate SAR1 expression and that SAR1 induces γ-globin expression predominantly through the Giα/JNK/Jun pathway in K562 cells.
and CD34+ cells. The specific function of SAR1 in upregulating γ-globin suggests that it may serve as a useful molecular target for upregulation of γ-globin gene expression and HbF levels in hemoglobinopathies, such as sickle cell disease and β-thalassemia.

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References


Authorship

 Contribution: J.Z. designed and performed the research, analyzed data, and wrote the manuscript; K.C. contributed to immunofluorescence staining, the design of the study, and scientific discussion of the results; W.A. contributed to EMSA studies and analysis; C.K. contributed to the analysis of the flow cytometry data; H.L. contributed to the communoprecipitation studies; and G.P.R. conceived and participated in the design of the study, the evaluation of the results, and the preparation of the manuscript.

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Hydroxyurea-inducible SAR1 gene acts through the Giα/JNK/Jun pathway to regulate γ-globin expression

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