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HS1 Interacts with Lyn and Is Critical for Erythropoietin-induced Differentiation of Erythroid Cells*

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Erythroid cells terminally differentiate in response to erythropoietin binding its cognate receptor. Previously we have shown that the tyrosine kinase Lyn associates with the erythropoietin receptor and is essential for hemoglobin synthesis in three erythroleukemic cell lines. To understand Lyn signaling events in erythroid cells, the yeast two-hybrid system was used to analyze interactions with other proteins. Here we show that the hemopoietic-specific protein HS1 interacted directly with the SH3 domain of Lyn, via its proline-rich region. A truncated HS1, bearing the Lyn-binding domain, was introduced into J2E erythroleukemic cells to determine the impact upon responsiveness to erythropoietin. Truncated HS1 had a striking effect on the phenotype of the J2E line—the cells were smaller, more basophilic than the parental proerythoblastoid cells and had fewer surface erythropoietin receptors. Moreover, basal and erythropoietin-induced proliferation and differentiation were markedly suppressed. The inability of cells containing the truncated HS1 to differentiate may be a consequence of markedly reduced levels of Lyn and GATA-1. In addition, erythropoietin stimulation of these cells resulted in rapid, endosome-mediated degradation of endogenous HS1. The truncated HS1 also suppressed the development of erythroid colonies from fetal liver cells. These data show that disrupting HS1 has profoundly influenced the ability of erythroid cells to terminally differentiate.

Erythropoiesis, the process of red blood cell development, is primarily controlled by erythropoietin (epo).[1] Several model systems have been used to study erythropoiesis *in vitro*. Although primary erythroid cells provide the ideal cell type for analysis, heterogeneity in preparations and insufficient numbers can preclude biochemical analysis of epo signaling. A number of erythroid cell lines have been derived which provide useful models for analyzing epo-induced signaling cascades, including the SKT6 and J2E lines (1, 2). The J2E cell line was used in this study because it proliferates, remains viable, produces hemoglobin and undergoes morphological maturation in response to epo (2, 3). Following epo stimulation of J2E cells, phosphorylation changes to the epo receptor, janus kinase-2 (JAK2), signal transducer and activator of transcription-5 (STAT5), *ras*-GTPase activating protein, phosphatidylinositol 3-kinase, phospholipase Cγ, and MAP-kinase are identical to the kinetics reported in other cell systems (4, 5).

Previously, we reported that epo-initiated signaling was disrupted in a J2E subclone (J2E-NR), which remained viable in the presence of epo but did not differentiate or undergo enhanced proliferation following hormonal stimulation (4). The tyrosine kinase Lyn was shown to be severely reduced in the J2E-NR cells, and reintroduction of Lyn restored the ability of the cells to terminally differentiate (5). Lyn pre-associated with the epo receptor in parental J2E cells, and inhibition of its activity suppressed differentiation (5). Chin *et al.* (6) confirmed the binding of Lyn to the epo receptor and demonstrated that it may play a role in regulating the JAK/STAT pathway. Lyn is a member of the Src family of membrane-associated tyrosine kinases, which is present mainly in lympho/hemopoietic cells and is involved in signal transduction from numerous receptors (7–15).

Lyn is most closely related to the tyrosine kinase Lck which plays an essential role in T cell activation and development (16). The SH2 domain of Lck binds to tyrosine-phosphorylated CD45 and ZAP-70, whereas its SH3 domain associates with phosphatidylinositol 3-kinase, p120, and HS1 (17–21). HS1, or LckBP1, is a 75-kDa intracellular protein expressed mainly in hemopoietic and lymphoid cells (22) and is a major substrate for several Src family kinases (21, 23, 34). It contains a proline-rich region, an SH3 domain, an acidic α-helix, and a basic segment resembling the DNA-binding motif of the helix-turn-helix family, suggesting it could play a role in both signal transduction and transcriptional regulation (21). HS1 is phosphorylated following activation of B cell and T cell receptors (21, 23–25) but not after stimulation of IL-3, GM-CSF, or SCF receptors (26). Significantly, Lyn has been shown to associate with HS1 in B and T cells (23, 24). Like Lyn knockout mice, studies on HS1-deficient mice have revealed a central role for HS1 in B cell responsiveness (27, 28). While this manuscript was in preparation, HS1 was shown to bind the novel hematopoietic progenitor kinase (HPK1) in erythroid cells (29).

In this study we attempted to identify downstream effectors of Lyn in erythroid cells using a yeast two-hybrid screen of wild type and a kinase inactive mutant (Y397F) of Lyn. Of the seven Lyn-interacting proteins identified, we report here on the in-

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[1] The abbreviations used are: epo, erythropoietin; epo-R, epo-receptor; HS1, hematopoietic lineage cell-specific protein; tHS1, truncated HS1; SH, Src homology; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; β-gal, β-galactosidase; FCS, fetal calf serum; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate; MEL, murine erythroleukemia; MAP-kinase, mitogen-activated protein kinase.

teraction between Lyn and HS1, and the crucial role of HS1 for epo-induced differentiation of erythroid cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Cells were grown in Dulbecco's modified Eagle's medium, 5% fetal calf serum (FCS). Differentiation of J2E (2), clone 11, and clone 24 (30) as well as ME17 (31) cell lines was initiated with epo (5 units/ml) or sodium butyrate (0.5 mM), whereas murine erythroleukemia (MEL) cells were stimulated with Me_2SO (1.5%). Viability was determined by eosin dye exclusion (4) and hemoglobin synthesis by benzidine staining (32). Note that benzidine staining levels reported here were higher than in previous reports (3) because of batch variations in FCS. Cell morphology was examined by cytocentrifugation onto glass slides and Wright-Geimsa staining (3). Proliferation was assayed by [^{3}H]thymidine incorporation as described previously (3).

Molecular Biology Techniques—Total RNA was extracted by the method of Chomczynski and Sacchi (33), from which poly(A)$^+$ RNA was isolated using the Poly(A)-Tract mRNA isolation system (Promega). Northern blots were performed essentially as described by Sambrook *et al.* (34). Restriction enzyme digestions (Promega) and ligation reactions (Life Technologies, Inc., Gaithersburg, MD) were performed as recommended by the manufacturers. PCR reactions were performed using either *Pfu* (Stratagene) or *Taq* (Promega) polymerases on a PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA). DNA was sequenced using the ABI-Prism method (PE Applied Biosystems, Branchburg, NJ).

Yeast Two-hybrid Analysis—The yeast two-hybrid procedures used were essentially as described by Vojtek *et al.* (35), using the *Saccharomyces cerevisiae* L40 strain (*MATa*, *his3Δ200*, *trp1-901*, *leu2-3*, *112*, *ade2*, *LYS2*::(*lexAop*)$_4$-*HIS3*, *URA3*::(*lexAop*)$_8$-*lacZ*, *GAL4*). The 1.5-kilobase wild-type Lyn and a dominant negative Lyn (Y397F) were subcloned into pBTM116 (35) to generate LexA DNA binding domain Lyn and LynY397F fusions. The L40 strain was transformed separately with pBTM116-Lyn and pBTM116-LynY397F. Both yeast strains were then used as the "bait" in two yeast two-hybrid screens of a cDNA library in the VP16 transcriptional activation domain fusion plasmid (pVP16) made from mRNA derived from the lymphohemopoietic progenitor cell line EML C.1 (36). The pVP16 plasmids from the Lyn and LynY397F-specific His$^+$/Lac$^+$ colonies were then rescued into *Escherichia coli* and sequenced. These plasmids were subsequently co-transformed with pBTM116-Lyn, pBTM116-Lyn Y397F, or pBTM116-HLS7 into the yeast L40 strain before performing *HIS3* and β-gal assays.

Plasmids expressing LexA fusions of the unique (pBTM116-Un), SH2 (pBTM116-SH2), and SH3 (pBTM116-SH3) domains of Lyn for the yeast two-hybrid system were generated by ligating PCR fragments into pBTM116. The generation of plasmid pBTM116-Δ243Lyn expressing a LexA fusion of the first 243 amino acids of Lyn has been described previously (5). Plasmids were also generated for pGAD-HS1 and pGAD-P1-P2 expressing transcription activation domain fusions of full-length HS1 and the P1-P2 region of HS1 (amino acids 324–350), respectively.

In Vitro Binding Assay—Plasmids expressing glutathione *S*-transferase (GST) fusion proteins of Lyn (pGEX-Lyn), Lip-1 (pGEX-Lip-1), and Lip-2 (pGEX-Lip-2) were generated. GST fusion proteins were expressed and purified, with minor modifications of the method described by Smith and Johnson (37). Cells were lysed in PBS containing 1% Triton X-100, 10 mg/ml lysozyme, 10 units/ml DNase I, 1 mM benzamidine for 2 h at 37 °C, centrifuged, and processed as described by Smith and Johnston (37). Soluble Lyn was produced by thrombin (Sigma) cleavage of the GST fusion. Binding experiments were performed by the addition of purified soluble Lyn (~100 ng) to GST, GST-Lip-1, or GST-Lip-2 (~500 ng) attached to glutathione-agarose beads in PBS containing 1% Triton X-100, 5 mM dithiothreitol, 5 mM EDTA and incubated at 4 °C for 2 h. Bound Lyn was detected by SDS-polyacrylamide gel electrophoresis and Western blotting.

Immunoprecipitation and Western Blot Analysis—Cells were lysed in 20 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1.0% Nonidet P-40, 10 mM β-glycerophosphate, 10 mM NaF, 1 mM Na_3VO_4, 2 mM EDTA, 10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin. For co-immunoprecipitations, clarified cell lysates were incubated with antibodies to Lyn, Lck, Src, Hck, Fyn (SC-15G, SC-15, SC-13, SC-19, SC-72, SC-16, Santa Cruz Biotechnology Inc., Santa Cruz, CA), HS1 (21), epo-R (#187; Ref. 38) or STAT5a and STAT5b (Santa Cruz Biotechnology Inc., SC-1081, SC-835) for 2 h at 4 °C, then collected with protein A-Sepharose beads for 16 h before washing and analyzing by Western blotting. Additional antibodies used in Western blotting directed against MAP-kinase, v-Raf, phosphotyrosine, and GATA-1 were from Santa Cruz Biotechnology Inc. (SC-154, SC-133, SC-7020, SC-265). Antibodies to EKLF (39), NF-E2 (40), and globin (#55447, Cappel Research, Organon Technika, Belguim) were also used for Western blotting. Secondary antibodies were coupled to horseradish peroxidase (Amersham Pharmacia Biotech, Uppsala, Sweden) and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Indirect Immunofluorescence Microscopy—Cells were cytocentrifuged onto slides, fixed in 50% methanol, 50% acetone, and then processed essentially as described by Harlow *et al.* (41) for indirect immunofluorescence using anti-Lyn (SC-15, Santa Cruz Biotechnology Inc.) or anti-HS1 (21) antibodies and a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody (Silenus, Melbourne, Australia). DNA was counterstained with Hoechst 33258. Slides were mounted in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 2.5% 1,4-diazabicyclo-{2.2.2} octane and visualized using a Bio-Rad MRC-1000/1024 UV laser scanning confocal microscope (Bio-Rad, Hercules, CA).

Retroviral Infection of Cells—Sense and antisense truncated HS1 cDNAs, encoding amino acids 271–409, were generated by PCR. The fragments were subcloned into the pMSCV2.2 neo vector (42) creating pMSCV-Ds and pMSCV-Da for the sense and antisense orientation, respectively. The packaging cell lines PA317 and ψ2 were transfected, and supernatants containing amphotropic and ecotropic retroviruses, respectively, were collected. Amphotropic retroviruses were used to infect J2E and MEL cells, clones were isolated, and viral integration was confirmed by Southern analysis while expression levels were determined by Northern analysis (5). Ecotropic viruses infect at least 50% of fetal liver erythroid progenitors (43) that emerge in colony assays (44).

Flow Cytometry—Cells (10^6) were incubated with antibodies to the epo-R (189; Ref. 38), transferrin receptor (R17 208; Ref. 46), c-kit (47), or Ter119 (48) for 30 min on ice, washed three times in PBS, 2% FCS followed by incubation with a secondary antibody conjugated to FITC (Silenus) for 30 min on ice, then washed three times in PBS-2% FCS before analysis on an Epics XL/MCL flow cytometer (Beckman-Coulter, Palo Alto, CA). Cells incubated in the absence of primary antibody were analyzed as controls.

RESULTS

Lyn Interacts with HS1—The yeast two-hybrid system was used to identify molecules that could associate with wild type or a dominant negative Lyn (Y397F). Two screens, using Lyn and LynY397F, of 10^7 clones each yielded 67 and 82 *HIS3* positive clones, respectively, of which 34 and 68 were also β-gal positive. After curing and rescuing, several clones emerged, which were termed Lyn interacting proteins (Lip). Here we report on Lip-1 and Lip-2 (Fig. 1*A*), which were identical to HS1 between amino acids 268–486 (Lip-1) and 346–486 (Lip-2) (28). To determine whether the association between Lyn and Lip-1/Lip-2 was via a direct interaction, *in vitro* binding was performed with purified immobilized GST, GST-Lip-1, or GST-Lip-2 and with purified, soluble Lyn. A clear association was seen between Lip-1 or Lip-2 and Lyn, with no appreciable binding of Lyn to GST alone (Fig. 1, *B* and *C*). These data show that Lyn and HS1 can interact directly *in vitro*.

The SH3 Domain of Lyn Binds the Proline-rich Region of HS1—The ability of HS1 to interact with the various domains of Lyn was assessed. Deletion of the kinase domain of Lyn (LynΔ243) did not prevent the interaction (Fig. 1*D*). This observation is compatible with the yeast two-hybrid data, where Lip-1 and Lip-2 bound both Lyn and LynY397F (Fig. 1*A*), indicating a functional kinase domain was not required for the association. However, the interaction was eliminated when only the unique domain of Lyn was retained. This result suggested that the SH2 and/or SH3 domains of Lyn were required for the association. However, Fig. 1*D* shows that the SH3, but not the SH2, domain was responsible for the interaction between Lyn and HS1.

The sections of HS1 encoded by Lip-1 and Lip-2 encompass the SH3 domain and part, or all, of the proline-rich region. Fig. 1*E* shows that a section of the proline-rich region containing two consensus SH3-binding sites, P1 (amino acids 324–329) and P2 (amino acids 345–350), was able to interact with Lyn.

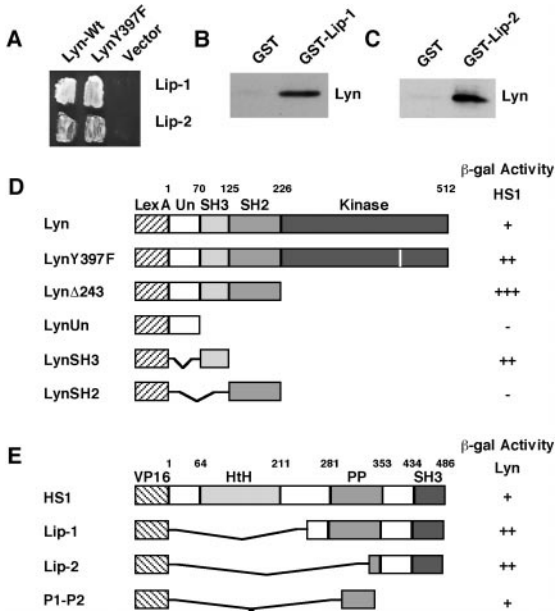

FIG. 1. **Lyn interacts with HS1 in yeast and *in vitro*.** *A*, yeast strain L40 was co-transfected with the plasmids pBTM116 (*Vector*), pBTM1116Lyn (*Lyn-wt*), or pBTM116-LynY397F (*LynY397F*) and pVP16-Lip1 (*Lip-1*) or pVP16-Lip2 (*Lip-2*). The resultant colonies were replated onto Leu^-/Trp^-/His^- plates and assayed for *HIS3* activity. *B*, *in vitro* binding assay of wild-type Lyn and GST-Lip-1. *C*, *in vitro* binding assay of wild-type Lyn and GST-Lip-2. *D*, yeast strain L40 was co-transfected with the plasmids pBTM1116Lyn (*Lyn*), pBTM1116LynY397F (*LynY397F*), pBTM1116LynΔ243 (*LynΔ243*), pBTM1116LynUn (*LynUn*), pBTM1116LynSH3 (*LynSH3*), or pBTM1116LynSH2 (*LynSH2*), and pGAD-HS1 (*HS1*). *LexA* represents the vector, whereas *Un* represents the unique region of Lyn. The domain boundaries are indicated graphically and by residue number. The resultant colonies were replated onto Leu^-/Trp^- plates, and β-gal activity was determined by liquid assay. *E*, yeast strain L40 was co-transfected with the plasmids pGAD-HS1 (*HS1*), pVP16-Lip-1 (*Lip-1*), pVP16-Lip2 (*Lip-2*), or pGAD-P1-P2 (*P1-P2*), and pBTM116-Lyn (*Lyn*). VP16 is the vector, while *HtH* represents the helix-turn-helix motif of HS1, and *PP* represents the proline-rich domain. The domain boundaries are indicated graphically and by residue number. The resultant colonies were replated onto Leu^-/Trp^- plates, and β-gal activity was determined by liquid assay.

Taken together, these data suggest that the SH3 domain of Lyn associates with the consensus SH3-binding sites (P1 and P2) of HS1.

Lyn and HS1 Interact in Erythroid Cells—To demonstrate an *in vivo* association between Lyn and HS1, Lyn was immunoprecipitated from several erythroid cell lines. Fig. 2*A* shows that HS1 co-immunoprecipitated with Lyn in each of these lines. Although HS1 associated with Lyn in erythroid cells, it also interacted with Lck and Fyn, but not with Hck or Src (Fig. 2*B*). To determine whether the association between Lyn and HS1 was altered after epo stimulation, J2E cells were exposed to the hormone and HS1 was immunoprecipitated. Interestingly, maximal association with Lyn occurred between 15 and 30 min of epo stimulation (Fig. 2*C*). However, no phosphorylation of HS1 was detected after epo stimulation.

Indirect immunofluorescence microscopy showed that Lyn and HS1 have distinct, but overlapping, subcellular localizations in J2E cells (Fig. 2*D*). Lyn had fairly uniform cytoplasmic staining, with some concentration around the cell membrane and a small amount in the nucleus. While HS1 also localized primarily in the cytoplasm, the staining was more punctate than Lyn; some HS1 staining was present in the nucleus. Thus, the intracellular co-localization of Lyn and HS1 support the *in vitro* binding data (Fig. 1) and the co-immunoprecipitation results (Fig. 2, *A–C*).

Truncated HS1 Alters the Phenotype of Erythroid Cells—In

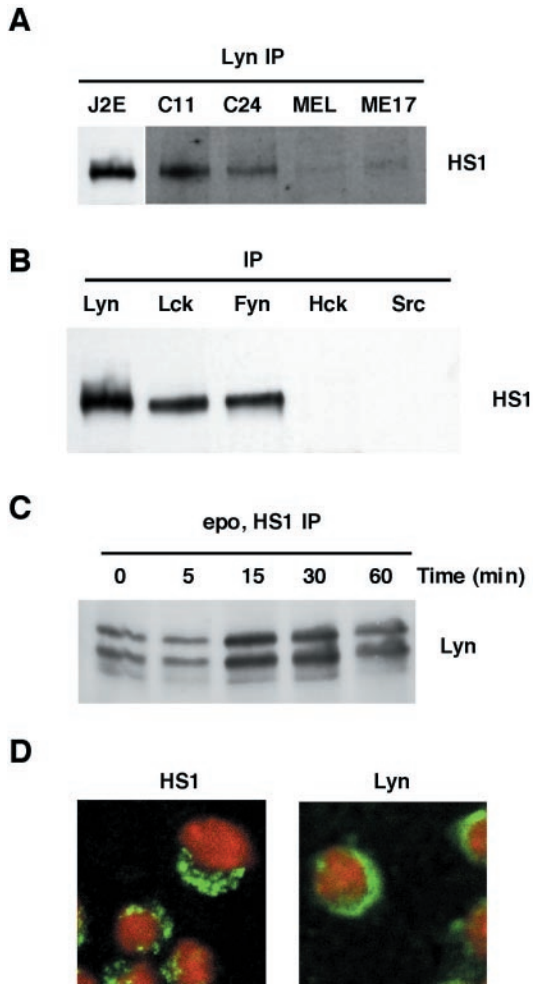

FIG. 2. ***In vivo* association of Lyn and HS1.** *A*, co-immunoprecipitation of Lyn and HS1 from erythroid cell lines J2E, C11, C24, MEL, and ME17 (in logarithmic growth phase) following immunoprecipitation of Lyn and immunoblotting with antibodies to HS1. *B*, co-immunoprecipitation of HS1 with Lyn, Lck, and Fyn but not with Hck or Src, in J2E cells. Src family members were immunoprecipitated with the antibodies shown and then immunoblotted with antibodies to HS1. *C*, co-immunoprecipitation of Lyn and HS1 is maximal 15–30 min after epo stimulation of J2E cells. Lysates from epo-treated J2E cells were immunoprecipitated with anti-HS1 antibodies and probed with antibodies to Lyn. *D*, overlapping subcellular localization of Lyn and HS1 in uninduced J2E cells. The cell nuclei were stained with Hoechst 33258 (*red*), while Lyn and HS1 were identified with FITC-conjugated antibodies (*green*).

an attempt to determine the biological role of HS1 in erythroid cells, a truncated form of HS1 (tHS1) encompassing the proline-rich Lyn-binding domain (amino acids 271–409) was introduced into J2E cells, which were then called JDs cells. It was anticipated that tHS1 would bind Lyn and disrupt signaling via endogenous HS1. The corresponding reverse orientation construct was also expressed in these cells (termed JDa) and used as a control. Numerous clones were isolated, expressing comparable amounts of sense and antisense tHS1 RNA. Each of the clones had the same phenotype, and a representative example is shown in Fig. 3. J2E cells and vector-alone control (JM5) cells grew in large clusters in culture and had a distinctive proerythroblast morphology (Fig. 3, *A* and *B*). However, when cells expressed tHS1 a significant change in morphology was observed. Not only did the cells grow in isolation, but they also developed the appearance of basophilic erythroblasts (Fig. 3, *A* and *B*). The JDs cells were noticeably smaller, with a more condensed cytoplasm than the proerythroblastoid JM5 and JDa lines (Fig. 3, *B* and *C*). Unlike the JM5 and JDa lines, cells

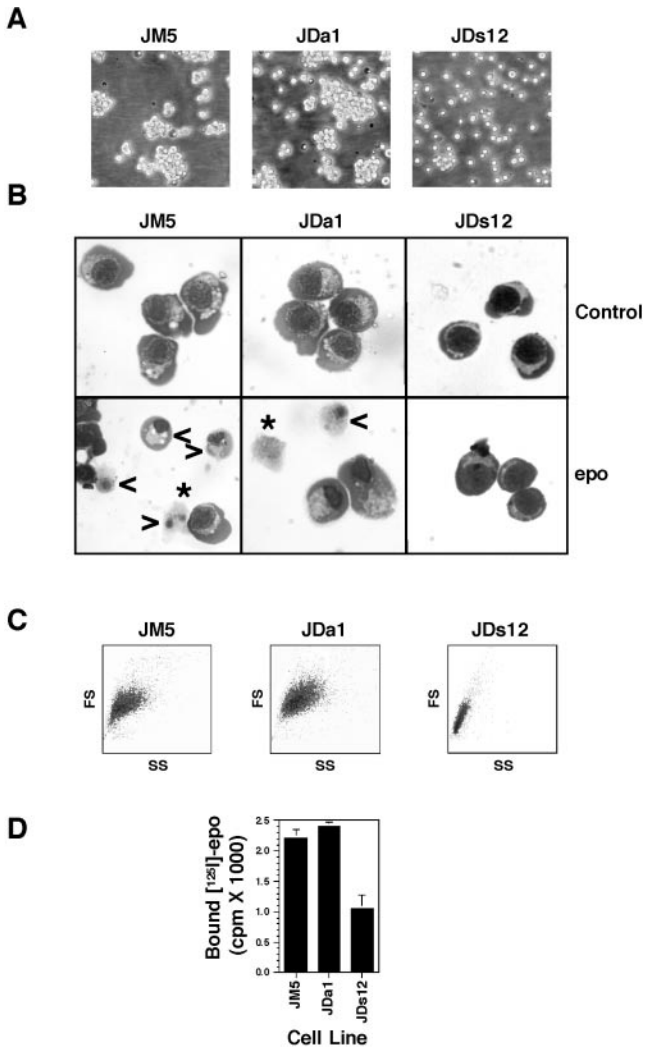

FIG. 3. **Truncated HS1 alters the phenotype of J2E cells.** *A*, suspension cultures showing that JM5 and JDa1 cells formed large clumps, while JDs12 cells grew in isolation. *B*, Wright-Geimsa-stained cells showing the proerythroblastoid morphology of JM5 and JDa1 cells, while the JDs12 cells were more basophilic with darker condensed cytoplasms. After 48 h in the presence of epo, JM5 and JDa1 cells underwent morphological maturation (*arrows*) which included the appearance of reticulocytes (*asterisk*), while JDs12 cells showed no noticeable changes. *C*, flow cytometric analysis of forward scatter (*FS*) and side scatter (*SS*) shows that JDs12 cells were smaller and less heterogeneous than JM5 and JDa1 cells. *D*, saturation epo-receptor binding assay of JM5, JDa1, and JDs12 cells using ^{125}I-epo. Each *bar* represents the mean ± S.D. (n = 3).

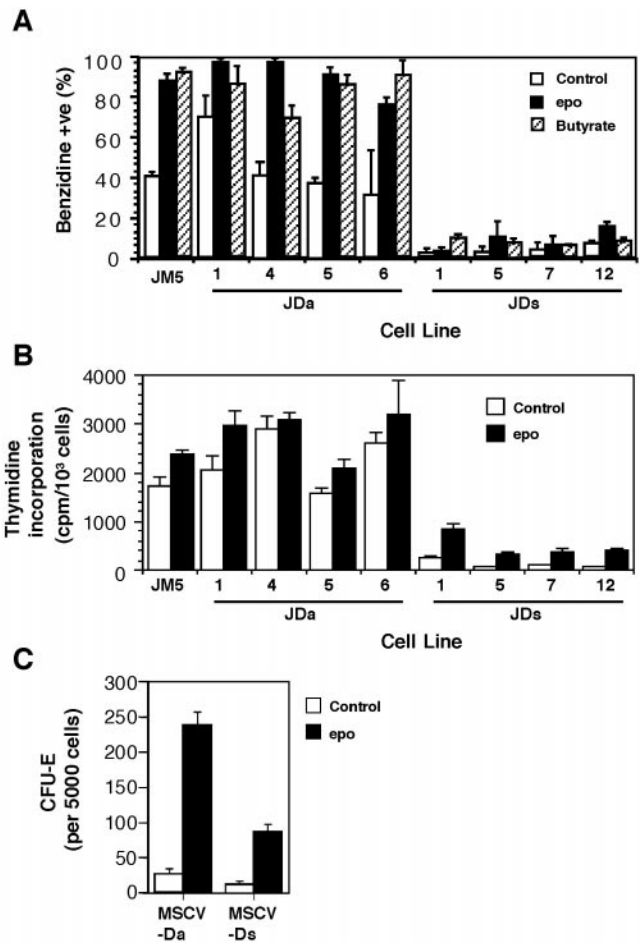

FIG. 4. **Truncated HS1 inhibits differentiation and alters proliferation of erythroid cells.** *A*, epo and sodium-butyrate-induced hemoglobin production by JM5, and four independent JDa and JDs clones measured by benzidine staining after 48 h. *B*, proliferation rate with, and without, epo of JM5, JDa, and JDs clones as measured by [^{3}H]thymidine incorporation. *C*, epo-induced erythroid colony formation (*CFU-E*) in methylcellulose after infection of fetal liver cells with MSCV-Da or MSCV-Ds. Each point is the mean ± S.D. (n = 3).

expressing tHS1 displayed no signs of morphological maturation when epo was added to the cultures (Fig. 3*B*). Moreover, the JDs cells grew much slower than the JM5 or JDa lines, with almost twice the doubling time (data not shown). While the cell surface expression of Ter119, c-kit, and TfR were unaltered (data not shown), the level of surface epo receptors was approximately half in the JDs cells (Fig. 3*D*). These alterations were observed in each of the JDs clones analyzed, indicating that the tHS1 construct had a significant impact upon the cells.

Truncated HS1 Inhibits Erythroid Differentiation and Proliferation—Because tHS1 had a major effect on the phenotype of J2E cells, the effect of this mutant on erythroid differentiation was examined. Strikingly, hemoglobin synthesis was severely repressed in each of the JDs clones studied (Fig. 4*A*). Production of the oxygen carrier was inhibited in both uninduced and epo-treated JDs cells. Similarly, the capacity to manufacture hemoglobin in response to sodium butyrate was almost extinguished (Fig. 4*A*). Thus, introduction of tHS1 had a significant inhibitory effect on the biochemical differentiation of J2E cells. These observations are commensurate with the inability of JDs to undergo morphological maturation (Fig. 3*B*). In contrast, tHS1 had no effect on the Me_2SO-induced differentiation of MEL cells (data not shown).

The impact of tHS1 on proliferation and viability of J2E cells was investigated next. As JDs cells grew much slower than JM5 and JDa controls, it was not surprising to observe reduced thymidine incorporation by these cells (Fig. 4*B*). While the JM5 and the JDa cells displayed the characteristic modest increase in DNA synthesis after epo stimulation (3), the increased proliferation rate of JDs cells remained well below that of the controls. However, tHS1 had no effect on the viability of J2E cells (data not shown). Together these data demonstrate that tHS1 had a dramatic impact on the proliferation and differentiation of J2E cells.

To determine whether the effects of tHS1 could be extended to non-transformed cells, fetal liver cells were exposed to the retrovirus containing this construct, and CFU-E development was enumerated. Fig. 4*C* shows that colony numbers for these erythroid progenitors were also reduced by tHS1. It was concluded that HS1 plays an important role in erythroid differentiation, and the truncated molecule had a dominant negative effect on maturation.

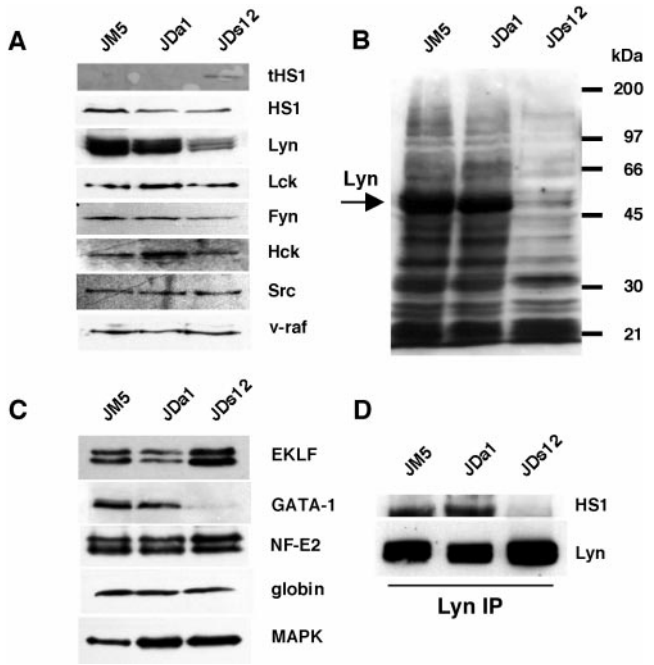

FIG. 5. **Intracellular alterations in JDs cells.** *A*, immunoblot analysis of tHS1, HS1, Lyn, Lck, Fyn, Hck, and Src expression in JM5, JDa1, and JDs12 cells. v-raf was used as a loading control. *B*, anti-phosphotyrosine Western blot of total cell extracts from JM5, JDa1, and JDs12 cells. *C*, immunoblot analysis of EKLF, GATA-1, NF-E2, and globin expression in JM5, JDa1, and JDs12 cells. MAP-kinase (*MAPK*) was used as a loading control because of the separation required to detect globin proteins. *D*, immunoblot analysis of HS1 and Lyn from Lyn immunoprecipitates of JM5, JDa1, and JDs12 cells. All protein lysates were from unstimulated cells. Note that 10-fold more protein was used in the immunoprecipitates from JDs12 cells to compensate for the lower Lyn expression.

Intracellular Alterations in JDs Cells—Having observed phenotypic differences in JDs cells, and an inhibition of proliferation and differentiation, biochemical alterations were then investigated. Initially, the expression of several Src family kinases was examined in these cells. Fig. 5*A* shows the presence of tHS1 in the JDs cells but not the control JM5 or JDa lines. Interestingly, the level of endogenous HS1 was unaltered in cells bearing either the truncated or antisense constructs. In contrast, the amount of Lyn was reduced by approximately 80% in JDs cells, although other Src kinases were not appreciably affected by the presence of the truncated HS1 (Fig. 5*A*). The reduction in Lyn levels may account in part for the overall decrease in tyrosine phosphorylation within JDs cells (Fig. 5*B*).

As erythroid transcription factors are crucial for the development of red blood cells, several DNA-binding proteins known to be involved in erythroid maturation were studied. Data presented in Fig. 5*C* show that transcription factors EKLF and NF-E2 were not markedly affected by the introduction of tHS1. In contrast, GATA-1, a key regulator of erythropoiesis, was barely detectable in the JDs cells. Globin protein content was not significantly effected by the tHS1 construct. Therefore, tHS1 affected the amount of Lyn and GATA-1 in J2E cells, as well as the phosphorylation status of the cells. In addition, tHS1 inhibited endogenous HS1 association with Lyn (Fig. 5*D*), which supports the proposition that tHS1 plays a dominant negative role.

To investigate the effect of tHS1 on epo receptor and STAT5 activation, cells were stimulated with epo and phosphorylation of the receptor, and STAT5 was assessed. Fig. 6 demonstrates that, despite reduced surface receptor numbers (Fig. 3*D*), the epo receptor was phosphorylated with typical kinetics. However, the activation of STAT5 was delayed in JDs cells. These

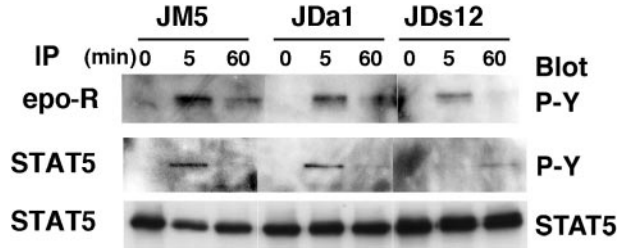

FIG. 6. **Truncated HS1 retards epo-induced STAT5 phosphorylation.** Anti-phosphotyrosine immunoblot of epo-R and STAT5 immunoprecipitates from JM5, JDa1, and JDs12 cells stimulated with epo for the indicated times.

results suggest that tHS1 did not affect receptor activation but did alter the kinetics of STAT5 phosphorylation.

Epo-mediated Proteolysis of Endogenous HS1 and Lyn in JDs Cells—It has been suggested that HS1, with its helix-turn-helix features, may translocate to the nucleus following ligand activation of receptors (24). To determine whether this occurred in erythroid cells, the JM5, JDa, and JDs lines were exposed to epo and subjected to indirect immunofluorescence and confocal microscopy (Fig. 7*A*). However, no translocation from the cell membrane to the nucleus was observed. Significantly, HS1 protein virtually disappeared from JDs cells 60 min after epo stimulation (Fig. 7*A*). Immuno-blot analysis was then conducted to measure the diminution of HS1 in these cells. Fig. 7*B* confirmed that the protein content of endogenous HS1 in JDs cells decreased markedly following ligand binding, but this was not evident in the other cell lines. Furthermore, the levels of Lyn were also diminished 120 min after epo activation.

The fine punctate appearance of HS1 in epo-treated JDs cells (Fig. 7*A*) indicated that the protein might be degraded in endosomes/lysosomes. To examine this possibility, JDs cells were treated with NH_4Cl/chloroquine to inhibit endosomal/lysosomal degradation. Fig. 7*C* shows that, in the presence of the inhibitors, HS1 degradation was impeded dramatically. These data demonstrate that tHS1 facilitates endosomal/lysosomal degradation of its endogenous counterpart following exposure to epo.

DISCUSSION

In this manuscript we have shown that the known Src kinase substrate HS1 associates with Lyn in erythroid cells. We have also demonstrated that a truncated HS1 (tHS1) markedly interferes with the phenotype of erythroid cells and impairs their ability to proliferate and differentiate. By disrupting the Lyn/HS1 interaction, we have generated a cascade of events which had a profound effect on erythroid maturation. These data indicate that HS1 plays a pivotal role in regulating intracellular signaling within erythroid cells and support the recent prediction by Nagata *et al.* (29) that " . . . HS1 is likely to be involved in erythroid proliferation and differentiation. . . . "

Introduction of a truncated form of HS1 into erythroid cells produced significant morphological, biochemical, and functional perturbations. The truncated mutant spanned the carboxyl-terminal, Lyn-binding region of HS1 that is sequentially phosphorylated by kinases (49), but it did not include the amino-terminal Hax1-binding region (50). Strikingly, cells expressing tHS1 were smaller, more basophilic, and replicated much more slowly. In addition, epo-induced differentiation was almost totally blocked as the cells failed to mature morphologically and did not synthesize hemoglobin. These data demonstrate that tHS1 acted in a dominant negative fashion and emphasize the importance of a fully functional HS1 to erythroid maturation.

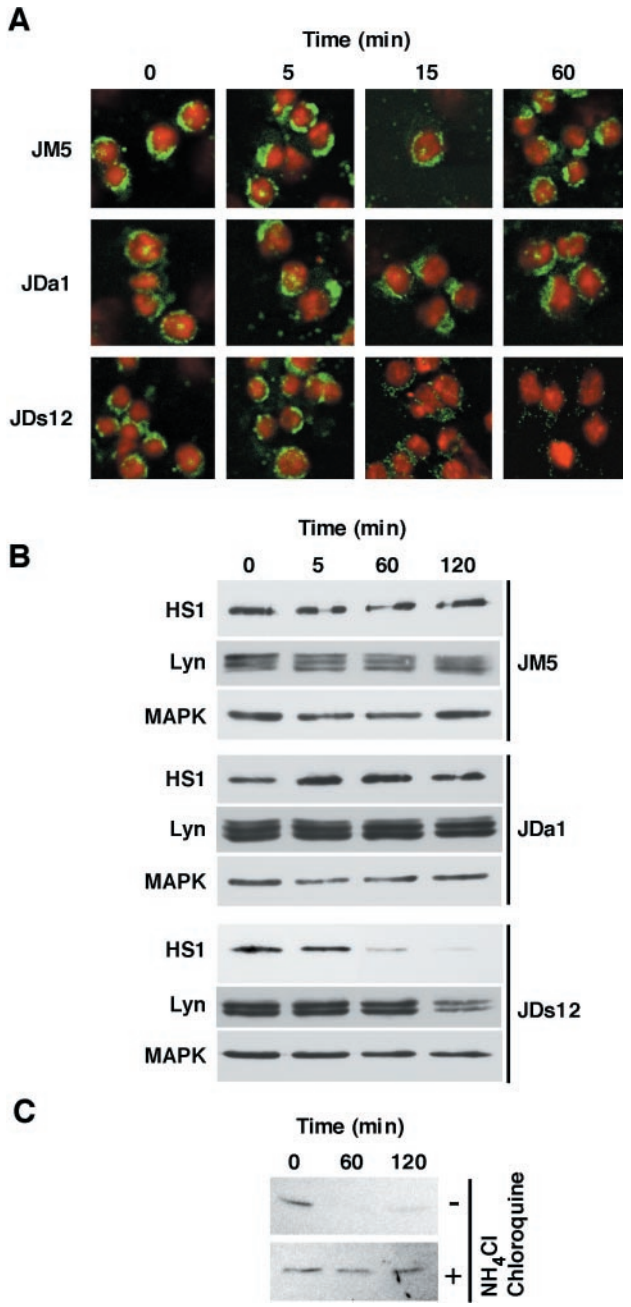

FIG. 7. **Epo-mediated proteolysis of endogenous HS1 and Lyn in JDs cells.** *A*, indirect immunofluorescence analysis of HS1 in JM5, JDa1, and JDs12 cells stimulated with epo over 60 min. Cell nuclei were stained with Hoechst 33258 (*red*), and HS1 was identified with FITC-conjugated antibodies (*green*). *B*, immunoblot analysis of HS1, Lyn, and MAP-kinase (*MAPK*) protein levels in JM5, JDa1, and JDs12 cells stimulated with epo for 120 min. Protein loading of JDs12 lysates was adjusted 10-fold to compensate for the lower level of Lyn. *C*, immunoblot analysis of HS1 levels in JDs12 cells stimulated with epo for 120 min with, or without, the endosome/lysosome inhibitors NH_4Cl (5 mM) and chloroquine (5 mM).

The tHS1 mutant had several biochemical effects on the erythroid cells, which may account for its dominant negative activity. First, it greatly reduced the level of Lyn protein within the cells, and as a consequence a marked decrease in tyrosine phosphorylation of intracellular proteins was detected. This result is compatible with our previous observation that the J2E-NR subclone expressed low levels of Lyn, and tyrosine phosphorylation of proteins was substantially reduced (5). As Lyn associates with the epo receptor (5, 6), transmission of signals from the receptor via this kinase would be significantly diminished in the transfected cells. Second, the GATA-1 content fell dramatically in these cells. GATA-1 is a key transcription factor involved with erythroid development (51, 52), and in its absence erythroid precursors arrest at the proerythroblast stage (53). Thus, the loss of GATA-1 protein may play a significant role in the inability of cells bearing tHS1 to mature morphologically or to produce hemoglobin.

In addition to reducing the levels of Lyn and GATA-1, tHS1 had a significant effect on endogenous HS1. While the epo receptor was phosphorylated normally in cells containing the mutant HS1 (Fig. 6), endogenous HS1 was degraded rapidly in endosomes/lysosomes after exposure to epo (Fig. 7), indicating that the truncated mutant promoted this proteolysis. It is likely that degradation of endogenous HS1 prevented transmission of signals by this molecule. Thus, tHS1 uncovered an unexpected mechanism for dominant negative action. This observation warrants further investigation with the recent association of signaling molecules with degradation *viz*. the SOCS family of negative regulators of cytokine action target Janus kinases for degradation by associating with elongins (54, 55), and c-cbl regulates receptor ubiquitination and endocytosis (56, 57).

In this study HS1 was identified as a Lyn-binding protein through a yeast two-hybrid screen, which was confirmed by direct association *in vitro*, together with co-immunoprecipitation *in vivo*. In addition to intracellular co-localization within erythroid cells, the proline-rich region of HS1 was shown to bind the SH3 domain of Lyn, similar to the Lck/HS1 interaction reported previously (21). Lyn has been shown to associate with HS1 in B and T cells (45), but this is the first report that we are aware of where Lyn interacts with HS1 in erythroid cells. Although HS1 and Lyn were constitutively associated in erythroid cells, the interaction increased appreciably after epo activation (Fig. 2*C*), which is consistent with previous observations in T cells where the SH3 domain of Lck, or Lyn, binds to HS1 in the absence of stimulation, then the SH2 domain of these kinases associates with HS1 upon receptor activation, increasing the interaction (25).

HS1 is linked with several kinases. Here we demonstrated HS1 co-immunoprecipitated with Lyn, and two other Src kinases (Lck and Fyn) in J2E cells (Fig. 2*B*); tHS1 could potentially interfere with signaling from these molecules. HS1 also associates with the novel hemopoietic kinase HPK1 in the erythroid SKT6 cell line (29). In addition, Takemoto *et al.* (45) suggested that the HS1 association with the SH3 domain of Grb2 may regulate the Grb2 and Src signaling pathways. Together these results suggest that HS1 may mediate signals emanating from several kinases and play a crucial role in transmitting intracellular signals within erythroid cells.

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HS1 Interacts with Lyn and Is Critical for Erythropoietin-induced Differentiation of Erythroid Cells

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