Maturation of Erythroid Cells and Erythroleukemia Development Are Affected by the Kinase Activity of Lyn

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ABSTRACT

This study examined the impact of the tyrosine kinase Lyn on erythropoietin-induced intracellular signaling in erythroid cells. In J2E erythroleukemic cells, Lyn coimmunoprecipitated with numerous proteins, including SHP-1, SHP-2, ras-GTPase-activating protein, signal transducers, and activators of transcription (STAT) 5a, STAT5b, and mitogen-activated protein kinase; however, introduction of a dominant-negative Lyn (Y397F Lyn) inhibited the interaction of Lyn with all of these molecules except SHP-1. Cells containing the dominant-negative Lyn displayed altered intracellular phosphorylation patterns, including mitogen-activated protein kinase, but not erythropoietin receptor, Janus-activated kinase (JAK) 2, or STAT5. As a consequence, erythropoietin-initiated differentiation and basal proliferation were severely impaired. Y397F Lyn reduced the protein levels of erythroid transcription factors erythroid Kruppel-like factor and GATA-1 up to 90%, which accounts for the inability of J2E cells expressing Y397F Lyn to synthesize hemoglobin. Although Lyn was shown to bind several sites on the cytoplasmic domain of the erythropoietin receptor, it was not activated when a receptor mutated at the JAK2 binding site was ectopically expressed in J2E cells, indicating that JAK2 is the primary kinase in erythropoietin signaling and that Lyn is a secondary kinase. In normal erythroid progenitors, erythropoietin enhanced phosphorylation of Lyn; moreover, exogenous Lyn increased colony forming unit-erythroid, but not burst forming unit-erythroid, colonies from normal progenitors, demonstrating a stage-specific effect of the kinase. Significantly, altering Lyn activity in J2E cells had a profound effect on the development of erythroleukemias in vivo; the mortality rate was markedly reduced and latent period extended when either wild-type Lyn or Y397F Lyn was introduced into these cells. Taken together, these data show that Lyn plays an important role in intracellular signaling in nontransformed and leukemic erythroid cells.

INTRODUCTION

The Src family of intracellular membrane-associated tyrosine kinases consists of eight members (1). v-Src was the prototype viral oncogene transduced from c-Src by Rous sarcoma virus. Lyn is a member of this family (2) and is expressed mainly in hemopoietic cells of myeloid (macrophages, monocytes, and platelets) and B-lymphoid origin. Lyn is involved in the transmission of signals from antigen receptors and activators of transcription (STAT) 5, STAT5, and the epo receptor itself (9, 17–20).

This study examined the impact of the tyrosine kinase Lyn on erythropoietin-induced intracellular signaling in erythroid cells. In J2E erythroleukemic cells, Lyn coimmunoprecipitated with numerous proteins, including SHP-1, SHP-2, ras-GTPase-activating protein, signal transducers, and activators of transcription (STAT) 5a, STAT5b, and mitogen-activated protein kinase; however, introduction of a dominant-negative Lyn (Y397F Lyn) inhibited the interaction of Lyn with all of these molecules except SHP-1. Cells containing the dominant-negative Lyn displayed altered intracellular phosphorylation patterns, including mitogen-activated protein kinase, but not erythropoietin receptor, Janus-activated kinase (JAK) 2, or STAT5. As a consequence, erythropoietin-initiated differentiation and basal proliferation were severely impaired. Y397F Lyn reduced the protein levels of erythroid transcription factors erythroid Kruppel-like factor and GATA-1 up to 90%, which accounts for the inability of J2E cells expressing Y397F Lyn to synthesize hemoglobin. Although Lyn was shown to bind several sites on the cytoplasmic domain of the erythropoietin receptor, it was not activated when a receptor mutated at the JAK2 binding site was ectopically expressed in J2E cells, indicating that JAK2 is the primary kinase in erythropoietin signaling and that Lyn is a secondary kinase. In normal erythroid progenitors, erythropoietin enhanced phosphorylation of Lyn; moreover, exogenous Lyn increased colony forming unit-erythroid, but not burst forming unit-erythroid, colonies from normal progenitors, demonstrating a stage-specific effect of the kinase. Significantly, altering Lyn activity in J2E cells had a profound effect on the development of erythroleukemias in vivo; the mortality rate was markedly reduced and latent period extended when either wild-type Lyn or Y397F Lyn was introduced into these cells. Taken together, these data show that Lyn plays an important role in intracellular signaling in nontransformed and leukemic erythroid cells.

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3 The abbreviations used are: epo, erythropoietin; JAK, Janus-activated kinase; STAT, signal transducers and activators of transcription; GAP, GTPase-activating protein; PI3, phosphatidylinositol 3'; MAP, mitogen-activated protein; CFU-E, colony forming unit-erythroid; BFU-E, burst forming unit-erythroid; EKLF, erythroid Kruppel-like factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
MATERIALS AND METHODS

Cell Culture. J2E (10) and J2E-NR (21) cells were maintained in DMEM with 5% FCS. Cells were stimulated with epo (5 units/ml), and hemoglobin production was detected by benzidine staining (22). Trititated thymidine uptake assays were performed essentially as we have described previously (11).

Amphotropic Viral Infection of J2E with Retroviral Lyn Constructs. The retroviral vector pRuthkNeo (23) was used to express wild-type (JRLyn) and dominant-negative Lyn (JR397), as described previously (8). J2E cell lines infected with the pMSCV-neo 2.2 vector (24) containing epo receptors that were truncated (J-Δ321) or had a mutated JAK2-binding site (J-W282R), have been described previously (25). Results are shown for representative subclones.

Cell Immunoprecipitation and Immunoblotting. Immunoprecipitations and immunoblotting have been described elsewhere (7, 13). Antibodies used were anti-epo receptor (189) (26); anti-Lyn (mouse; L05620; Transduction Laboratories, Lexington, KY); antiphosphotyrosine (05-321; Upstate Biotechnologies, Lake Placid, NY); anti-Lyn (rabbit), anti-STAT5a, anti-STAT5b, anti-phospho-MAP kinase, anti-p42 MAP kinase, anti-ras-GAP, anti-SHP-1, anti-SHP-2, anti-GATA-1, anti-raf, and anti-Epo-R (sc15, sc1081, sc835, sc7383, sc154, sc265, sc133, and sc697; Santa Cruz Biotechnology Inc., Santa Cruz, CA); anti-globin (no. 55447; Cappel Research, Organon Technika, Boxtel, The Netherlands) and anti-EKLF (27, 28). For immunoblotting, v-raf expression was used as a loading control. Lyn exokinase activity was determined by immunoprecipitation with anti-Lyn (rabbit; sc15; Santa Cruz Biotechnology) antibodies using [γ-32P]ATP and enolase as a substrate, as described previously (8). Lyn levels were detected using anti-Lyn (mouse) antibodies (L05620; Transduction Laboratories).

Protein Binding to Cellulose-bound Peptide. A cellulose-bound set of overlapping peptides (13mer, 10 amino acids overlapping) of the epo receptor cytoplasmic domain was prepared as described in detail elsewhere (29). Radiolabeled Lyn protein was in vitro translated using [35S]methionine (1000 Ci/mmol; Amersham) and the TNT-coupled Reticulocyte Lysate System (Promega). Incubation of peptide filters with radioactive Lyn preparations was performed essentially as described by Adam-Klages et al. (30) with the inclusion of tyrosine phosphatase inhibitor sodium vanadate (1 mm).

Northern Blot Analysis. Total cytoplasmic RNA was isolated from cells (31), then separated on agarose gels and hybridized with a 32P-labeled probes for c-myc (32), EKLF (27), GATA-1 (33), β-globin (34), or GAPDH (35).

Erythroleukemias. Mice received i.v. inoculations of cells as described previously (36).

Fetal Liver Assays. Murine fetal liver progenitor cells were obtained from 12-day-old fetuses and as described previously (37). Cell suspensions were harvested and either 5,000 (CFU-E) or 20,000 (BFU-E; 0.4 mg/ml G418) cells were plated per dish. Benzidine-positive colonies were counted 2 (CFU-E) or 7 (BFU-E) days later.

RESULTS

Dominant-Negative Lyn Alters Epo-induced Signaling. J2E-NR cells, which lack Lyn, had disrupted signaling, including a failure to activate MAP kinase, despite normal activation of JAK2 and phosphorylation of the epo receptor (14). Therefore, to further examine the role of Lyn in epo-induced signaling, parental J2E cells were infected with Y397F dominant-negative Lyn, which reduced the kinase activity by 80% (8). Significant alterations in intracellular tyrosine phosphorylation were observed in JR397 cells, even in the absence of epo stimulation (Fig. 1A). In particular, there was a marked reduction in tyrosine-phosphorylated Lyn in JR397 cells. However, epo-stimulated phosphorylation of its cognate receptor occurred normally in these cells (Fig. 1A and B). Furthermore, phosphorylation of STAT5 (Fig. 1B) and JAK2 (data not shown) was normal in JR397 cells after epo stimulation, whereas p42 MAP kinase was not activated in these cells (Fig. 1B). These results show that the dominant-negative Lyn had a significant effect on intracellular phosphorylation patterns, including MAP kinase, but did not influence epo receptor, JAK2, or STAT5 phosphorylation, observations that are remarkably similar to those in J2E-NR cells (14).

To identify molecules that bound to endogenous Lyn and may have been affected by the dominant-negative Lyn, a series of coimmunoprecipitation experiments were conducted. These studies demonstrated that Lyn coimmunoprecipitated with a surprising number of molecules that bound to Lyn (38). Interestingly, the levels of SHP-1 increased in J2E-NR cells (14). We examined the biological consequences of these alterations. JR397 cells were used to test whether the introduction of dominant-negative Lyn into J2E cells affected signaling cascades (Fig. 1), and the results are shown for representative subclones.

Fig. 1. Dominant-negative Lyn alters epi-induced signaling. J2E and JR397 cells were unstimulated or stimulated with epo (5 units/ml) and either immunoblotted (IB; A and C) lysates, 100 µg) or immunoprecipitated (IP; B and D) with the antibodies indicated. MAPK, MAP kinase; pY, phosphorytyrosine.
in the absence of serum (Fig. 2D). Therefore, altering Lyn in J2E cells retarded the basal rate of proliferation and inhibited hemoglobin synthesis.

**Dominant-Negative Lyn Alters Transcription Factor Expression.** Because the Y397F Lyn had a significant impact on epo-induced differentiation, we examined the effect of the dominant-negative mutant on erythroid-restricted transcription factors EKLF and GATA-1, which play a key role in stimulating globin synthesis (33, 38–41). The Northern blot presented in Fig. 3A shows that EKLF transcripts were unaltered by Y397F Lyn, whereas GATA-1 levels rose 2–3-fold. Although mRNA for EKLF was maintained and GATA-1 rose, β-globin mRNA transcripts fell appreciably (Fig. 3A); nuclear run-on assays revealed that this unexpected decrease was produced by reduced transcription (data not shown). Because EKLF and GATA-1 are crucial for globin gene transcription, the protein content of these DNA-binding proteins was ascertained. Despite the presence of considerable mRNA, a striking 80–95% decrease in both EKLF and GATA-1 proteins was detected in JR397 cells (Fig. 3B).

The effect of Y397F Lyn was specific for EKLF and GATA-1 proteins: STAT5a and STAT5b protein levels were unaffected by the dominant-negative Lyn. These data suggest that Y397F Lyn reduced EKLF and GATA-1 protein content by posttranscriptional means, which severely impaired globin synthesis.

c-myc is a transcription factor implicated in the regulation of growth factor-induced proliferation because it increases rapidly following mitogenic stimulation (42). Because c-myc has been shown to be downstream of the tyrosine kinase Src (43), the effect of Y397F Lyn on c-myc was examined. Epo stimulation of J2E cells produced a significant increase in c-myc mRNA (Fig. 3C) similar to the increase that we observed previously (11). However, in JR397 cells, c-myc transcripts did not increase after exposure to the hormone. This result suggests that (a) Lyn is involved in regulating mRNA levels of c-myc and (b) the transient increase in c-myc is dispensable for epo-induced proliferation because JR397 cells underwent a proliferative burst after hormonal stimulation (Fig. 2A).

**Lyn Is a Secondary Kinase for Epo Receptor and Has Multiple Binding Sites.** JAK2 associates with the epo receptor, and upon ligand binding it autophosphorylates and phosphorylates tyrosine residues in the receptor’s cytoplasmic domain (44). Because Lyn has also been shown to bind the epo receptor and is activated in response to epo (8, 9), it was important to determine whether the increase in enzymatic activity of Lyn was dependent on JAK2 activation. To address this issue, J2E cells expressing two mutated epo receptors were used: (a) J-W282R cells, which bear a receptor with an altered JAK2 binding site that prevents phosphorylation of JAK2 in response to epo; and (b) J-Δ321 cells, which contain a carboxy truncated receptor that retains a normal JAK2 binding domain but is hypersensitive to epo (25). We have shown elsewhere that epo-stimulated J282K phosphorylation is abolished in J-W282R cells and extended in J-Δ321 cells.4 Cell surface expression of epo receptors is increased 2–3-fold in cell lines expressing the mutant receptors, and we have proposed that heterodimeric complexes are formed between endogenous and mutated receptors following epo stimulation (25). Fig. 4A shows full-length, as well as truncated Δ321 receptors, coimmunoprecipitated with Lyn. Significantly, Lyn exokinase activity increased after exposure to epo in J2E and J-Δ321 cells, but not in J-W282R cells (Fig. 4B), indicating that Lyn activity depends on JAK2 activation.

To further define the site of Lyn interaction with the epo receptor, binding of radiolabeled Lyn to a series of cellulose-bound, overlapping peptides (13mer, 10 amino acids overlapping) of the cytoplasmic domain of the epo receptor was analyzed (Fig. 4C). Four major regions of interaction between Lyn and the epo receptor were observed in this assay: amino acids 299–317, 326–341, 350–362, and 407–419 (Fig. 4, C and D). The first two binding sites are consistent with the interaction between Lyn and the epo receptor truncated at amino acid 321 (Fig. 4A) and with the report of Chin et al. (9) that Lyn associates with the membrane-proximal 91 amino acids of the receptor. In addition, two new COOH-terminal regions of the epo receptor were identified as potential Lyn-interaction sites.

**Lyn Is Activated in Normal Erythroid Progenitor Cells.** In this and previous studies (8), the importance of Lyn to erythroid differentiation has been investigated in a variety of epo-responsive cell lines, immortalized at different stages of erythroid development. Therefore, the effects of Lyn on red cell maturation were extended using normal hemopoietic progenitor cells isolated from fetal livers. Fig. 5A shows that stimulation of these progenitor cells with epo

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4 Sarna et al., Differential regulation of SOCS genes in erythroid cell-Lyn downregulates SOCS-1 and SOCS-3, submitted for publication.
Lytes (3 mg) from unstimulated J2E cell lines with mutated epo receptors determined by immunoprecipitation with mouse anti-Lyn antibodies and g, full-length epo receptors and the Arrows with anti-epo receptor (189) antibodies, and then rabbit anti-Lyn antibodies. IB blotted (IP) with mouse anti-Lyn antibodies and immuno- 

Fig. 4. Lyn is a secondary kinase for epo receptor and has multiple binding sites. A, lysates (3 mg) from unstimulated J2E cell lines with mutated epo receptors A321 and W282R, were immunoprecipitated (IP) with mouse anti-Lyn antibodies and immuno- 

Proteins 1–76, Lyn exokinase activity was 

Fig. 5. Lyn in normal erythroid progenitor cells. A, fetal liver cells were stimulated with epo, immunoprecipitated (IP) with Lyn, and then immunoblotted with antiphosphotyrosine antibodies (pY IB). To optimally visualize the increase in phosphorylation, a short exposure is shown in which only p56 Lyn is visible. p53 Lyn became visible after a longer exposure (data not shown). The increase in tyrosine phosphorylation of p56 Lyn was quantitated by densitometry and expressed in arbitrary units. B and C, fetal liver cells were infected with retrovectors pRufNeo or pMSCV alone or with wild-type Lyn (R-Lyn) or M-Lyn, respectively). The cells were plated in methyl cellulose, and the number of benzidine-positive BFU-E (B) and CFU-E (C) were recorded. The results shown are the mean ± SD (bars; n = 3). 

produced a 2–3-fold increase in the tyrosine phosphorylation of p56Lyn. This epo-initiated increase in Lyn phosphorylation prompted us to raise Lyn expression in normal erythroid progenitors using retroviral vectors pRufNeo and pMSCV. Infected cells were plated in methylcellulose, and the number of hemoglobin-synthesizing BFU-E and CFU-E were enumerated. The data shown in Fig. 5, in methylcellulose, and the number of hemoglobin-synthesizing 

DISCUSSION

In this report we have demonstrated that Lyn is an important tyrosine kinase for erythroid maturation. Interference with Lyn activity via the dominant-negative, Y397F Lyn, suppressed epo-induced differentiation in the J2E cell line. Conversely, overexpression of Lyn in normal erythroid progenitors promoted maturation. Moreover, alterations in Lyn activity had a striking effect on the development of erythroleukemias.

J2E cells expressing Y397F Lyn were unable to manufacture hemoglobin in response to epo because the protein content of erythroid transcription factors EKLF and GATA-1 was reduced up to 90%. Because these DNA-binding proteins are essential for globin gene transcription (33, 38 – 41), the striking decrease in EKLF and GATA-1 resulted in markedly reduced globin gene transcription, mRNA, and protein content. Intriguingly, mRNA for these transcription factors did
not fall, indicating posttranscriptional regulation by Y397F Lyn. Because GATA-1 is susceptible to caspase-mediated breakdown (46), we are investigating whether the posttranscriptional regulation is attributable to reduced translation, increased degradation, or both.

In contrast to the inhibition of differentiation imposed by the dominant-negative Lyn in the J2E cell line, enforced expression of Lyn in nontransformed progenitors favored erythroid colony formation. However, this effect was stage specific: CFU-E numbers increased with ectopic Lyn expression, whereas BFU-E numbers were unaffected by exogenous Lyn. These data favor the notion that Lyn is associated with epi receptor-driven maturation because CFU-E have substantially more receptors than BFU-E and are more responsive to the hormone (47). Increased phosphorylation of Lyn following epo stimulation of fetal liver cells provided further evidence that it is involved in the maturation of normal erythroid cells.

The data presented here provide an important insight into the relative roles of JAK2 and Lyn in erythroid differentiation (8, 44). We previously have shown in J2E cells that reducing the levels of JAK2 or introducing the W282R epi receptor, which is unable to bind JAK2, abolished all three functions of epi, i.e., proliferation, differentiation, and viability (15, 16, 25). This study demonstrated that when JAK2 was not activated after epo stimulation of J-W282R cells, Lyn kinase activity did not increase. Therefore, the epo-induced Lyn activity is contingent on JAK2 activation. It was concluded that JAK2 is the primary kinase responsible for the activation of all epi-signaling pathways, whereas Lyn is a secondary kinase associated primarily with epi-stimulated differentiation.

Like JAK2, Lyn preassociates with the epi receptor (8, 9). Using a membrane-bound peptide approach, we have defined four sites on the receptor capable of binding Lyn (Fig. 4, C and D). Two of the sites (amino acids 299–317 and 326–341) fall within the membrane-proximal 91-amino acid domain for Lyn binding defined by Chin et al. (9). Furthermore, communoprecipitation of Lyn with a receptor truncated at amino acid 321 (Fig. 4A) confirms the existence of the binding region involving amino acids 299–317. It is significant that two novel COOH-terminal association domains were also identified by this method.

In addition to the epi receptor, Lyn has been shown to associate with numerous signaling molecules in J2E cells. These include HS-1 (48), STAT5a, STAT5b, ras-GAP, SHP-1, SHP-2, p42 MAP kinase (Fig. 1B), PI3-kinase, and Shc (data not shown). Although Lyn may interact directly with these proteins, it is also conceivable that Lyn is a central molecule in a large signaling complex within erythroid cells. Recently, multimeric complexes involving PI3-kinase, SHP-1, Grb2, Shc, c-Src, cbl, and STAT3 have been identified in association with the colony-stimulating factor 1 receptor in macrophages (49). Lyn has been implicated in the phosphorylation of several of these proteins (9, 17–20), and it is noteworthy that J2E cells lacking Lyn have markedly reduced tyrosine phosphorylation of ras-GAP, PI3-kinase, and p42 MAP kinase (14). Chin et al. (9) demonstrated that Lyn may also phosphorylate the epi receptor and STAT5. Although the epi receptor and STAT5 were phosphorylated with normal kinetics in J2E cells bearing the dominant-negative Lyn (Fig. 1B), it is possible that residual wild-type Lyn kinase activity may have contributed to the phosphorylation of these molecules. However, the association between Lyn and STAT5 in this study supports the proposition that Lyn may play a role in activation of the STAT5 pathway (9). Although the numerous interactions with Lyn may make a "Lyn pathway" difficult to define, HS-1 clearly is involved in the Lyn cascade because we recently showed that interference with HS-1 suppresses Lyn levels and prevents epi-induced differentiation (48).

The dominant-negative Lyn also inhibited the basal replication rate of J2E cells, but did not influence transient epi-initiated proliferation. This observation is consistent with reports that Lyn is involved in regulation of cell division and progression through the cell cycle (50–54). It also suggests that Lyn may be associated with other receptors in erythroid cells involved with proliferation (55). The failure of c-myc to increase in Y397F cells after epi indicates that Lyn, like Src (43), is upstream of this transcription factor. However, it also demonstrates that the increase in c-myc is not essential for epi-induced cell division to occur.

We recently reported that interference with JAK2 activation in J2E cells, via introduction of the W282R mutant epi receptor, severely reduced the leukemogenic potential of these cells (25). The data presented here show that altering the activity of Lyn also had a profound effect on erythroleukemia development. Both the dominant-negative Lyn and ectopically expressed wild-type Lyn reduced mortality substantially and extended the latent period 3-fold. The decreased leukemogenicity of JR397 cells was probably related to reduced replication rates (Fig. 2A), which supports recent attempts to inhibit signaling molecules Bcr/abl and JAK2, which slow the growth of leukemic cells (56, 57). In contrast, overexpression of Lyn promoted spontaneous differentiation (8) and also suppressed leukemia development. Thus, enhancing signaling pathways that favor maturation may provide an alternative means of differentiation therapy. In summary, altering the kinase activity of Lyn had a significant impact on the maturation of erythroid cells and the development of erythroleukemias.

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Fig. 6. Lyn activity affects the development of erythroleukemias. J2E (circles), JR397 (squares), and JRLyn (triangles) cells (106) were injected i.v. into three groups of 10 mice each (except J2E experiment 2, n = 9), and the emergence of erythroleukemias was monitored. The filled symbols represent experiment 1, and the open symbols represent experiment 2.
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