Thyroid Hormone Receptor-interacting Protein 1 Modulates Cytokine and Nuclear Hormone Signaling in Erythroid Cells*

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Erythropoietin (Epo) and thyroid hormone (T₃) are key molecules in the development of red blood cells. We have shown previously that the tyrosine kinase Lyn is involved in differentiation signals emanating from an activated erythropoietin receptor. Here we demonstrate that Lyn interacts with thyroid hormone receptor-interacting protein 1 (Trip-1), a transcriptional regulator associated with the T₃ receptor, providing a link between the Epo and T₃ signaling pathways. Trip-1 co-localized with Lyn and the T₃ receptor in the cytoplasm/plasma membrane of erythroid cells but translocated to discrete nuclear foci shortly after Epo-induced differentiation. Our data reveal that T₃ stimulated the proliferation of immature erythroid cells, and inhibited maturation promoted by erythropoietin. Removal of T₃ reduced cell division and enhanced terminal differentiation. This was accompanied by large increases in the cell cycle inhibitor p27Kip1 and by increasing expression of erythroid transcription factors GATA-1, EKLF, and NF-E2. Strikingly, a truncated Trip-1 inhibited both erythropoietin-induced maturation and T₃-initiated cell division. This mutant Trip-1 acted in a dominant negative fashion by eliminating endogenous Lyn, elevating p27Kip1, and blocking T₃ response elements. These data demonstrate that Trip-1 can simultaneously modulate responses involving both cytokine and nuclear receptors.

Erythropoiesis, the process of generating red blood cells, is controlled by hormones that bind to cytokine receptor and nuclear hormone receptor families (1, 2). Two well characterized molecules that strongly influence erythropoiesis are erythropoietin (Epo) and thyroid hormone (T₃). Epo binds to a cell surface receptor of the cytokine receptor family (3), initiating an intracellular signaling cascade that has been deciphered gradually over the past decade (reviewed in Refs. 4–7). Numerous signaling proteins are activated by Epo, including JAK2, STAT5, Ras, phosphatidylinositol 3-kinase, phospholipase Cγ, and MAP kinase (5, 7); activation of negative regulators such as SHP1, SOCS1, and CIS also occurs after receptor engagement (8–10).

T₃ binds to the intracellular thyroid hormone receptor (TR), a member of the nuclear hormone receptor family, which regulates gene expression (11). T₃ has a potent effect on erythropoiesis, especially in hypothyroid patients who are often anemic; however, erythroid hyperplasia can occur in individuals with hyperthyroidism (12, 13). It is noteworthy that the TRα isoform is expressed preferentially in differentiating erythroid cells (14) and that the v-erbA oncogene involved in avian erythroleukemia represents a mutated form of TRα (15, 16). Studies with whole animals have indicated that T₃ stimulates erythropoiesis (17), whereas in vitro assays have shown that T₃ inhibits colony formation by erythroid progenitors (18, 19). The elegant studies of Beug and colleagues (19–24) have demonstrated that the balance between proliferation and differentiation can be altered by the introduction of exogenous TRα (c-erbA) or v-erbA into immature avian red blood cells.

We have examined Epo-initiated signaling in J2E erythroid cells as they proliferate, remain viable, produce hemoglobin, and undergo morphological maturation in response to Epo (25–27). Following Epo stimulation of these cells, phosphorylation changes to the Epo receptor, JAK2, STAT5, Ras-GAP, phosphatidylinositol 3-kinase, phospholipase Cγ, and MAP kinase are identical to the kinetics reported in other cell systems (28). The tyrosine kinase Lyn is crucial for Epo-induced differentiation of immature J2E and R11 cell lines (27). Lyn associates with the Epo receptor and can phosphorylate the receptor and STAT5 in vitro (28, 29). As the most abundant Src family kinase in red blood cells (30), Lyn also phosphorylates key erythrocyte membrane proteins (31). Our recent data indicate JAK2 is the primary kinase that initiates Epo signaling and that Lyn acts as a secondary kinase to promote differentiation (32). Significantly, the erythroid progenitor compartment is altered in Lyn−/− mice.²

² M. Hibbs, personal communication.

The abbreviations used are: Epo, erythropoietin; T₃, thyroid hormone; TR, thyroid hormone receptor; Trip-1, thyroid hormone receptor-interacting protein 1; tTrip-1, truncated Trip-1; SH2 and -3, Src homology 2 and 3; S, Src homology 2; CFU-E, erythroid colony-forming units; BFU-E, burst-forming units; GST, glutathione S-transferase; JAK, Janus kinase; STAT, signal transducers and activators of transcription; MAP, mitogen-activated protein; NF-E2, nuclear factor E2; COUP-TFI, chicken ovalbumin upstream promoter transcription factor-II; EKLF, erythroid Kruppel-like factor; ORCA, orphan receptor coactivator.

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In this study we attempted to identify downstream effectors of Lyn in erythroid cells using a yeast two-hybrid screen. HS1, a known target for Src family kinases including Lyn, was identified in this screen and its effects on erythroid differentiation demonstrated (33). Here, we report on the interaction between Lyn and thyroid hormone receptor-interacting protein 1 (Trip-1), a transcriptional regulator that associates with TRα (34–36). The Lyn/Trip-1 association, therefore, provides a link between the Epo and T₃ signaling pathways.

EXPERIMENTAL PROCEDURES

Cell Culture—Cells were grown in Dulbecco's modified Eagle's medium, 5% fetal calf serum, or serum depleted of T₃ (24). T₃ could not be detected in depleted sera by radioimmunooassay. The Epo-responsive J2E (25) and R11 (37) cell lines were derived from murine fetal liver cells transduced with retroviruses expressing v-Raf-Myc (J2E) or v-Raf/Raf (R11). Differentiation of J2E and R11 cell lines was initiated with Epo (5 units/ml). Nuclear hormones were used at a final concentration of 1 μM. Viability was determined by eosin dye exclusion and hemoglobin synthesis by benzidine staining (28). Cell morphology was examined following cytocentrifugation onto glass slides and Wright-Giemsa staining (26). Proliferation was assayed by [³H]thymidine incorporation (26).

Fetal liver cells were plated in methylcellulose for CFU-E and BFU-E assays as described previously (38) before benzidine-positive colonies were enumerated. All graphical data are represented as the mean ± S.D. (n ≥ 3).

Yeast Two-hybrid Analysis—The yeast two-hybrid system (33, 39) utilized the S. cerevisiae L40 strain. Wild-type Lyn (Lyn) and a dominant negative Lyn (Y397F) cDNAs were used to screen a yeast two-hybrid library derived from the lymphohemopoietic progenitor cell line EML C.1 (40) as described previously (33). The plasmids expressing VP16 fusions of full-length Trip-1 (pVP16-Trip-1), amino acids 1–171 (pVP16-Trip-1-CC-A), and the coiled-coil domain, amino acids 50–100 (pVP16-Trip-1-CC) were generated by ligating yeast cDNA reaction fragments into pVP16.

In Vitro Binding Assays—Plasmids expressing Glutathione S-transferase (GST) fusion proteins of Lyn (pGEX-Lyn) and Trip-1 (amino acids 1–171) (pGEX-Trip-1–171) were generated by ligating yeast cDNA reaction fragments into pGEX-2T. GST fusion proteins were purified as described previously (33). Binding experiments were performed by the addition of purified soluble Trip-1 (100 ng) to GST, GST-Lyn, GST-LynY397F, or GST-LynUn (500 ng) attached to glutathione-agarose beads in buffer (33) and then incubated at 4 °C for 2 h. Bound Trip-1 was detected by SDS-polyacrylamide electrophoresis and immunoblotting using an anti-Trip-1 antibody (36).

Immunoprecipitation and Immunoblotting—Cells were lysed as described previously (33), and proteins were co-immunoprecipitated with antibodies (anti-Trip-1 (41), anti-EpoR (no. 187), anti-Lyn; SC-15G, anti-STAT5; SC-772, Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 2 h at 4 °C and then collected with protein A-Sepharose beads for 16 h before analysis by immunoblotting. Additional antibodies used in immunoblotting were directed against Lyn, Lck, Src, Hck, Fyn, Syk, MAPK, v-Raf, phosphotyrosine and GATA-1 (SC-15, SC-13, SC-19, SC-72, SC-65, SC-573, SC-154, SC-133, SC-7020, and SC-285, Santa Cruz). Antibodies to EKLF, NF-E2, globin (catalog no. 55447, Cappel Research, Organon Technika, Boxtel, The Netherlands), and p27Kip1 and p57Kip2 (catalog no. 12331A and 65021A, respectively, PharMingen, San Diego, CA) were also used for immunoblotting. Secondary antibodies were coupled to horseradish peroxidase and detected by enhanced chemiluminescence (Amer sham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK).

Indirect Immunofluorescence Microscopy—Cells were cytocentrifuged onto slides, fixed in 50% methanol, 50% acetone, and then stained with indirect immunofluorescence using anti-Lyn, anti-Trip-1 (41), or anti-TRα antibodies and AlexaFluor-conjugated anti-rabbit and anti-mouse secondary antibodies (Molecular Probes, Eugene, OR). DNA was counterstained with Hoechst 33258. Slides were mounted in 50 mM Tris-HCl, pH 8.0, 50% glycerol, 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 (Cs) and antisense (C) tTrip-1 cDNAs encoding amino acids 1–171 were generated by polymerase chain reaction, and the fragments were subcloned into the pMSCV2.2neo vector (42). Amphotropic and ecotropic retroviruses expressing the Cs and C constructs were used to infect erythroid cells as described previously (33). The efficiency of fetal liver cell infection was at least 50% (43). Numerous independent clones were isolated, and representative clones are shown.

RESULTS

Trip-1 Associates with Lyn and TRα—To identify specific binding partners of Lyn, a yeast two-hybrid screen was conducted using wild-type Lyn and the kinase-inactive Y397F mutant (33); Fig. 1A shows the association of Lyn with Trip-1. In addition to transcriptionally regulating TRα (34–36), Trip-1 also possesses intrinsic helicase activity, and independently it has also been described as SUG1, a component of the 26-S proteasome (41, 45).

In vitro studies with purified proteins revealed that Lyn and Trip-1 interacted directly (Fig. 1B) in a phosphorytrosine-independent manner (Fig. 1, A–C). Trip-1 also bound the kinase-inactive Y397F mutant of Lyn (Fig. 1, A and B), indicating that the enzymatic activity of Lyn was not required for this association. Deletion analyses showed that the SH2 and, to a lesser extent, the SH3 domain of Lyn was responsible for Trip-1 binding in vitro (Fig. 1, B and C); these observations were confirmed using lysates from erythroid cells (data not shown). The regions of Trip-1 that bound Lyn were then analyzed. Fig. 1D shows that the amino-terminal of Trip-1 but not the coiled-coil domain alone was required for Lyn binding. This region is distinct from the highly conserved ATPase/DNA helicase AAA (ATPase associated with a variety of cellular activities) domain of Trip-1 needed to bind the TRα (41).

FIG. 1. Lyn associates with Trip-1. A, Lyn and Trip-1 interact in the yeast two-hybrid system. Hisα assays of yeast co-expressing LexA fusions of Lyn, LynY397F (kinase inactive Lyn), or HLS7 (negative control) (43) with a VP16 fusion of Trip-1. B, purified Lyn and Trip-1 interact in vitro. Immunoblot analysis of a binding assay with purified Trip-1 (amino acids 1–171) and GST fusions of Lyn, LynY397F, or the Unique plus SH3 domain (LynUN-SH3). C, the SH2 and SH3 domains of Lyn bind Trip-1. Yeast co-expressing LexA fusions of Lyn, LynY397F, Lyn243, LynUn (Unique), LynSH3, or LynSH2 and a VP16 fusion of Trip-1 were assayed for β-galactosidase (β-gal) activity. D, the coiled-coil (CC) containing amino-terminal domain of Trip-1 binds Lyn. Yeast expressing pVP16 fusions of Trip-1, Trip-1-CC-A, or Trip-1-CC and Lyn were assayed for β-galactosidase activity. E, Lyn, Trip-1, and TRα interact in vivo. J2E and R11 cells were lysed, and then Trip-1 (or TRα) was immunoprecipitated, and co-immunoprecipitation of Lyn, Trip-1, or TRα was detected by immunoblotting.
Co-immunoprecipitation experiments performed on lysates from erythroid cell lines show that Lyn does indeed associate with Trip-1 in vivo (Fig. 1E). Similarly, Trip-1 and TRα co-immunoprecipitated in these cells. These studies show that Trip-1 is able to bind both Lyn and TRα in erythroid cells, thereby connecting discrete signaling pathways involving Epo and T3.

**Trip-1 Co-localizes with Lyn and TRα in the Cytoplasm of Erythroid Cells**—Having established a biochemical interaction between Lyn and Trip-1, the subcellular localization of these proteins was ascertained in uninduced erythroid cells. Lyn was found primarily in the cytoplasm of erythroid cells, with significant concentration at the plasma membrane (Fig. 2A). As Trip-1 was also distributed in the cytosol and plasma membrane, appreciable co-localization between Lyn and Trip-1 was observed (Fig. 2A). As described by Zhu et al. (46), TRα was detected in both the cytoplasm and nucleus. Consequently, Trip-1 co-localized with TRα in the cytoplasm, but not the nucleus, of these unstimulated erythroid cells (Fig. 2B). Comparable results were obtained with other erythroid cells (data not shown). These experiments demonstrate that the association of Trip-1 with Lyn and TRα occurs in the cytoplasm/plasma membrane of erythroid cells.

**T3 Inhibits Epo-induced Differentiation**—As both Epo and T3 affect erythropoiesis (1, 2), and Trip-1 was shown to associate with Lyn and TRα (Figs. 1 and 2), biological evidence for interplay between these pathways was sought. To this end, T3 and Epo concentrations were manipulated and cellular responses monitored. Fig. 3A shows that the removal of T3 enhanced Epo-induced hemoglobin production, whereas the addition of T3 severely impeded synthesis of the oxygen carrier. These effects were T3-specific, as reverse T3 (Fig. 3A) and a variety of nuclear hormones (data not shown) had no effect on differentiation. The inhibitory effects of T3 on hemoglobin synthesis were concentration-dependent with an IC_{50} of 100 pM. Furthermore, morphological maturation was severely retarded in the presence of T3; nuclear condensation, cytoplasmic acidophilia, reduced cell size, and enucleation were all restricted by T3 (Fig. 3B). Conversely, removal of T3 accelerated the appearance of erythroid cells with a more mature phenotype, in particular orthochromatic erythroblasts and reticulocytes. Identical results were obtained with other Epo-responsive cell lines (data not shown).

In marked contrast with the inhibition of Epo-initiated differentiation, T3 promoted [3H]thymidine uptake (Fig. 3C). When T3 was removed from cultures, DNA synthesis almost ceased; however, replication resumed upon re-introduction of T3. This observation was supported by monitoring cell numbers in these cultures (data not shown).

To extend the analysis of erythroid cells beyond cell lines, the Epo/T3 axis was examined in red cell progenitors from murine fetal livers. Fig. 4A shows that the inhibitory effects of T3 on differentiation were not restricted to immortalized cells, as increasing the T3 concentration reduced Epo-induced colonies from normal progenitors. However, the effect was more pronounced on erythroid colony-forming units (CFU-E) than erythroid burst-forming units (BFU-E). These data indicate that T3 also had an inhibitory effect on Epo-induced differentiation of normal erythroid cells.

To determine whether T3 affected fetal liver erythroid progenitors before exposure to Epo, cells were pre-incubated with T3 and then treated with Epo. Intriguingly, when T3 was added prior to Epo, both the BFU-E and CFU-E numbers rose (Fig. 4B). This expansion of the erythroid progenitor compartment by pre-incubation with T3 indicates that the effects of T3 are stage-specific. Taken together these results show that T3 promotes proliferation and the expansion of immature erythroid...
cells at the expense of maturation, whereas Epo favors terminal differentiation toward a nonreplicating state.

**Trip-1 Affects Erythroid Transcription Factors and p27Kip1**—To identify the biochemical mechanism for the effects of T3 on erythroid proliferation and differentiation, an immunoblot analysis was performed on key transcription factors and cell cycle regulators. The effects of T3 were quite striking as the addition of T3 to J2E cells resulted in a 20-fold increase in p27Kip1, a cell cycle inhibitor important for reticulocyte differentiation and facilitates red cell maturation, whereas elevated p27Kip1 enables T3-induced proliferation was severely impeded (Fig. 6, filled bars). An asterisk indicates a significant increase in colony number with T3 pre-incubation ($p < 0.01, n = 3$).

**Trip-1 Affects Responsiveness to Epo and T3**—To determine whether Trip-1 could simultaneously regulate differentiation signaling by Epo and proliferation promoted by T3, a truncated Trip-1 (tTrip-1) encompassing the Lyn-binding domain (Fig. 1D) was introduced into J2E cells. Numerous independently isolated transfectants were termed JCs cells, whereas the antisense controls were labeled JC−. Significantly, tTrip-1 blocked erythroid differentiation and proliferation of normal as well as immortalized erythroid cells to Epo and T3.

**Trip-1 Modulates Epo/T3 Cross-talk**—The biochemical mechanisms by which tTrip-1 inhibited Epo and T3 action were then investigated. The inhibition of Epo-induced differentiation by tTrip-1 coincided with the elimination of Lyn but not other tyrosine kinases such as Fyn, Src, Syk, Lck, and Hck (Fig. 8A and data not shown). Conversely, the elevated levels of p27Kip1 in JCs cells correlated with reduced proliferation (Fig. 8A). However, the inability to differentiate was not caused by a reduction in GATA-1, EKLF, NF-E2, globins, or endogenous STAT5 (data not shown). It is noteworthy that the antisense control did not affect Trip-1 protein levels, nor was it due to restricted phosphorylation of the Epo receptor or STAT5 (data not shown). It was concluded from these experiments that Trip-1 plays an important role in regulating the responses of normal as well as immortalized erythroid cells to Epo and T3.

**Trip-1 Affects Lyn and p27Kip1 Levels**—The biochemical mechanisms by which tTrip-1 inhibited Epo and T3 action were then investigated. The inhibition of Epo-induced differentiation by tTrip-1 coincided with the elimination of Lyn but not other tyrosine kinases such as Fyn, Src, Syk, Lck, and Hck (Fig. 8A and data not shown). Conversely, the elevated levels of p27Kip1 in JCs cells correlated with reduced proliferation (Fig. 8A). However, the inability to differentiate was not caused by a reduction in GATA-1, EKLF, NF-E2, globins, or endogenous Trip-1 (Fig. 8A), nor was it due to restricted phosphorylation of the Epo receptor or STAT5 (data not shown). It is noteworthy that the antisense control did not affect Trip-1 protein levels, validating its use as an additional control (Figs. 6 and 7).

To determine whether tTrip-1 also interfered with T3-induced transcription, activation of T3 response elements was examined in JCs cells. The cells were transfected with either direct repeats or inverted palindromes of the T3 response elements and were then exposed to T3. Significantly tTrip-1 negated the T3 responsiveness of both elements (Fig. 8B and data not shown). It was concluded from this series of experiments that tTrip-1 blocked erythroid differentiation and proliferation.
by suppressing endogenous Lyn, increasing p27Kip1 and interfering with the transcriptional activity of T3 response elements.

**Trip-1 Translocates to the Nucleus during Erythroid Differentiation**—Trip-1 localized primarily in the cytoplasm of uninduced erythroid cells (Fig. 2). The subcellular localization of this protein was then examined in cells stimulated with Epo or when T3 was withdrawn from culture. Strikingly, cytoplasmic Trip-1 translocated to discrete nuclear regions after 30 min of exposure to Epo or removal of T3 (Fig. 9, A and B). Altering the compartmental balance of Trip-1, therefore, coincided with enhanced differentiation and reduced replication. Trip-1 also relocated to the nucleus in JCs cells (data not shown), indicating that the inhibitory effects of tTrip-1 were not due to impaired nuclear translocation.

**DISCUSSION**

In this article we have demonstrated that Trip-1, a transcriptional regulator of Tα (34, 36), associates with the tyrosine kinase Lyn in erythroid cells. The interaction between Lyn and Trip-1 was initially identified by a yeast two-hybrid screen and was confirmed by in vitro binding, co-immunoprecipitations, and intracellular co-localization (Figs. 1 and 2). Because Lyn is involved in the Epo signaling cascade (27, 29, 32), Trip-1 provides a link between pathways mediated by cytokine receptors and nuclear hormone receptors.

The parallels between the Trip-1 association with Lyn and the interaction of p62ORCA with Lck and COUP-TFII are striking. Like Trip-1 and Lyn, p62ORCA associates with Lck (the closest Src kinase family member to Lyn) through phos-
Trip-1 subcellular localization changes with differentiation. A, J2E cells were incubated with or without T3 (1 µM) before analysis of Trip-1 localization by confocal microscopy. B, J2E cells were incubated with Epo (5 units) for the indicated times before analysis of Trip-1 subcellular localization.

The dominant negative effects of the truncated Trip-1 were quite profound, as it simultaneously inhibited Epo-induced differentiation in immortalized and normal erythroid cells and suppressed T3-mediated proliferation. The introduction of tTrip-1 resulted in a complete loss of endogenous Lyn, increased p27Kip1, and an inability to activate T3 response elements. As Trip-1 is also a component of the 26-S proteasome (45), perhaps the truncated Trip-1 fosters proteasomal degradation of Lyn. The importance of Trip-1 to the T3 pathway was illustrated by the suppression of T3 response elements with the truncated Trip-1.

It is noteworthy that Trip-1 localized with Lyn and TRα in the cytoplasm/plasma membrane of uninduced erythroid cells and then translocated to discrete nuclear foci within 30 min of Epo stimulation or after withdrawal of T3. To our knowledge this is the first description of Trip-1 relocation between subcellular compartments correlating with enhanced differentiation. Studies are currently under way to determine the nature of these nuclear structures and their function during erythroid maturation.

Epo and T3 had major effects on erythroid maturation in both cell lines and normal progenitors. Whereas Epo promoted differentiation (manifest by hemoglobin synthesis and morphological maturation), T3 stimulated proliferation at the expense of terminal differentiation. Similarly, it has been shown that T3 prevents hemoglobin production by NFS-60 cells and increases the erythrocyte yield from erythroblasts (18, 20). Our data with normal erythroid progenitors confirm that T3 inhibits colony formation (18). However, we also demonstrated that T3 is able to expand the erythroid progenitor compartment prior to Epo stimulation, which supports the notion that the effects of T3 may be stage-specific (18, 19, 24).

Withdrawal of T3 from erythroid cells produced large increases in erythroid-restricted transcription factors GATA-1, EKLF, and NF-E2, which have been strongly implicated in the control of red cell maturation, especially in hemoglobin production (52–56). Increasing the concentration of these transcription factors, therefore, promotes differentiation. Cross-regulation of these nuclear proteins may also be important because TRα and COUP-TFI together suppress GATA-1 transcription (57), whereas TRα is able to associate directly with NF-E2 (58). Altering the concentration, activity, or combinations of these DNA proteins can have a major impact upon expression of genes required for red cell maturation. Removal of T3 also caused a marked elevation in p27Kip1, which is significant because entry of erythroid cells into the noncycling, terminally differentiated state involves increasing p27Kip1 levels (48). Thus, terminal differentiation was enhanced by the combination of cell cycle exit and elevated transcription factor levels.

It is conceivable that the Epo/T3 axis provides a complementary mechanism for expanding erythroid progenitors and increasing cell numbers before terminal differentiation to generate the correct number of functionally mature red blood cells. As TRα has been proposed to act as a switch between proliferation and differentiation (24), Trip-1 may be a vehicle for coordinating the biological responses initiated by T3 and Epo.

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