Biochemical and Biophysical Research Communications **275,** 16–19 (2000)
doi:10.1006/bbrc.2000.3266, available online at http://www.idealibrary.com on IDEAL®

Activation of the Akt/FKHRL1 Pathway Mediates the Antiapoptotic Effects of Erythropoietin in Primary Human Erythroid Progenitors

Shahab Uddin, Samanthi Kottegoda, Danielle Stigger, Leonidas C. Platanias, and Amittha Wickrema

Section of Hematology/Oncology, University of Illinois at Chicago and West Side VA Medical Center, Chicago, Illinois 60607

Received July 17, 2000

Erythropoietin (Epo), stem cell factor (SCF), and insulin-like growth factor-1 (IGF-1) are key regulators of erythroid cell proliferation and differentiation. To understand the mechanisms of generation of signals by each of these growth factors, we determined the activation of the PI3-kinase/Akt pathway during proliferation and differentiation of primary human erythroid progenitors. Our results demonstrate that PKB/Akt is activated by Epo and SCF, but not by IGF-1 in human primary erythroid progenitors. In addition, Epo treatment of erythroid progenitors induces phosphorylation of a member of the Forkhead family (FH) of transcription factors FKHRL1, downstream of activation of the Akt kinase. Such Epo-dependent activation of FKHRL1 apparently regulates the generation of Epo-dependent antiapoptotic signals as evidenced by the induction of apoptosis of erythroid progenitors during treatment of cells with the PI3-kinase (PI3K) inhibitor LY294002. Thus, the PI3K/Akt/FKHRL1 pathway is essential for inhibition of apoptosis in response to Epo and SCF, while the IGF-1 receptor utilizes a different pathway.

PKB/Akt is a mediator of cell survival and lies directly downstream of the PI3-kinase in the antiapoptotic signaling pathway (1). Previous studies have shown erythropoietin induces phosphorylation PKB/Akt in erythroid cells, although it is unknown whether Akt targets transcription factors that may directly control the cell survival/apoptotic genes in erythroid cells (2, 3). The recent discovery of a family of Forkhead (FH) transcription factors has lead to the hypothesis that these proteins may be direct targets of Akt kinase similar to what has been observed in nematode *Caenorhabditis elegans* where FH homologue DA16 is regulated by PI3/Akt (4–6). The human FH family of proteins include FKHR, FKHRL1 and AFX that contain consensus Akt phosphorylation sites (7). Recent studies have shown both FKHR and FKHRL1 are phosphorylated by Akt in response to insulin and IGF −1 in human hepatoma and epithelial kidney cell lines (8–11). We sought to determine whether Epo, SCF, and IGF-1 activate the Akt kinase in primary human erythroid progenitors to generate the antiapoptotic signals. Furthermore, we investigated whether members of the FH family of proteins are involved in Epo signaling in primary erythroid progenitors.

Our studies demonstrate that FKHRL1 is phosphorylated in response to Epo treatment of erythroid progenitors and such phosphorylation requires upstream activation of the PI3-kinase. Inhibition of PI3-kinase activation using the specific inhibitor LY294002 suppresses the phosphorylation of FKHRL1 and triggers apoptosis in a caspase-dependent pathway.

This work was supported in part by American Cancer Society Illinois Division (to A.W.) by National Institutes of Health Grants CA73381 and CA77816 and a Merit Review grant from the Department of Veterans Affairs (to L.C.P.).

MATERIALS AND METHODS

Cell culture. Human $CD34^+$ cells were purchased from AllCells, LLC (Berkeley, CA) and had been isolated from mobilized peripheral blood collected from normal donors. Human primary erythroid progenitors, that are at the colony-forming unit-erythroid stage (CFU-E) were derived by culturing $CD34^+$ cells as previously described (12). Briefly, $CD34^+$ cells were cultured in a medium containing 15% fetal calf serum, 15% human AB serum, Iscove's modified Dulbecco's medium (IMDM), 500 units/ml penicillin, 40 μg/ml streptomycin, 10 ng/ml interleukin-3, 2 units/ml Epo, 50 ng/ml stem cell factor and 50 ng/ml insulin-like growth factor-1. Prior to activation by growth factors day 7 cells (CFU-E) were washed twice with IMDM and cultured in a serum-free media for 4 h without growth factors. Purity of cells for erythroid lineage were determined by measurement of the percentage of transferrin receptor (CD71) and glycophorin A. Eighty to 90% of these cells were positive for CD71 and glycophorin A as determined by flow cytometry.

0006-291X/00 $35.00

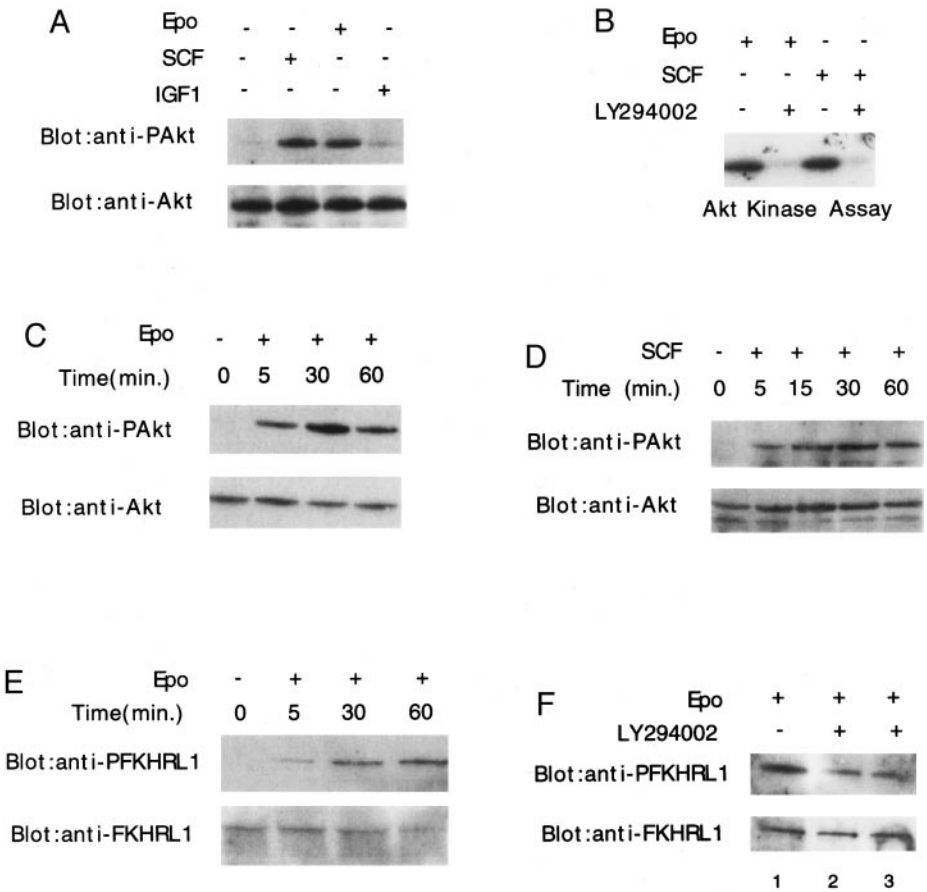

FIG. 1. Activation of PKB/Akt and FKHRL1 in primary erythroid progenitors. (A) Primary human erythroid progenitors at the CFU-E stage of differentiation were deprived of growth factors for 4 h and subsequently treated with Epo, SCF, and IGF-1 for 30 min. Cell lysates were analyzed by SDS–PAGE and immunoblotted an anti-phospho Akt antibody (top panel). The same blot was stripped and reprobed with an anti-Akt antibody. (B) Cells were treated with the indicated growth factors for 30 min, total lysates (15×10^6 cells per sample) were immunoprecipitated with anti-Akt antibody and immunoprecipitates were subjected to *in vitro* kinase assays. (C) Cells stimulated with or without Epo for different times as indicated and cell lysates were analyzed by SDS–PAGE and immunoblotted with anti-phospho Akt antibody (top panel). The same blot was stripped and reprobed with an anti-Akt antibody (bottom panel). (D) Cells stimulated with or without SCF for different times as indicated and cell lysates were analyzed by SDS–PAGE and immunoblotted with anti-phospho Akt (top panel). The same blot was stripped and reprobed with and an anti-Akt antibody (bottom panel). (E) Cells stimulated with or without Epo for different times as indicated and cell lysates were analyzed by SDS–PAGE and immunoblotted with anti-phospho FKHRL1 (top panel). The same blot was stripped and reprobed with and anti-FKHRL1 antibody (bottom panel). (F) Cells were stimulated with Epo for 30 min after incubation without LY294002 inhibitor (lane 1) or with 10 μM (lane 2) and 50 μM (lane 3) concentrations of the LY294002 inhibitor as indicated. Cell lysates were analyzed by SDS–PAGE, and immunoblotted with anti-phospho FKHRL1 (top panel). The same blot was stripped and reprobed with and anti-FKHRL1 antibody (bottom panel).

Immunoblotting and growth factor stimulation. Growth factor starvation, stimulation, cell collection and immunoblotting were performed as previously described (12). Anti-Phospho Akt (Ser473) and anti-Akt antibodies were purchased from New England Bio Labs. The FKHRL1 antibodies were purchased from Upstate biotechnology Inc. An anti-PARP antibody was purchased from Pharmingen Inc.

Apoptosis studies. Primary erythroid progenitors on day 8 of culture (CFU-E) were treated with either 50 μM LY294002 or 200 ng of rapamycin over a 24-h time period. In addition, a third sample was grown without growth factors in a serum-free media for the same length of time. The percentage of apoptotic cells were determined at 0, 6, and 24 h following treatment with the inhibitor by flow cytometry after staining with fluorescein conjugated annexin-V and propidium iodide.

Akt kinase assays. These assays were performed as previously described (13).

RESULTS AND DISCUSSION

We sought to determine whether the PKB/Akt signaling pathway is activated in response to Epo, SCF, and IGF-1 treatment of primary erythroid cells. The cells were serum starved and stimulated with each of the growth factors. The cells were subsequently lysed and analyzed by SDS–PAGE and immunoblotted with anti-phospho Akt antibody. Akt was phosphorylated in response to Epo and SCF but not by IGF-1 (Fig. 1A), while there was no difference in the amount of Akt protein in each of the lanes in the immunoblot (Fig. 1A). Thus, only Epo and SCF activate the Akt pathway in human erythroid progenitors. This is in agreement

with previous findings showing distinct roles for Epo, SCF, and IGF1 during erythroid development (14–16). We subsequently performed *in vitro* kinase assays that clearly indicated a high level of Akt kinase activity in cells treated with Epo and SCF (Fig. 1A). Such activity was abrogated by pretreatment of cells with the PI3-kinase inhibitor LY294002, confirming that Akt is directly activated downstream of the PI3-kinase (Fig. 1B). To determine the kinetics of Akt activation in primary erythroid progenitors in response to Epo and SCF, we performed time course studies. Both Epo and SCF induced phosphorylation of Akt within 5 min of treatment and the signal peaked at 30 min after growth factor stimulation (Figs. 1C and 1D, top panel). Reprobing of the immunoblots with anti-Akt antibody indicated equivalent levels of Akt protein in each of the lanes (Figs. 1C and 1D, lower panels).

We then sought to determine whether FKHRL1, a recently identified member of the Forkhead family of transcription factors is phosphorylated by Epo in primary erythroid cells. Epo treatment of cells resulted in phosphorylation of FKHRL1 (Fig. 1E) and such phosphorylation was partially inhibited by pretreatment of cells with the PI3-kinase inhibitor LY 294002, demonstrating that such phosphorylation/activation is PI3-kinase dependent. We subsequently sought to determine whether activation of Akt/FKHRL1 downstream of PI3-kinase is required for cell survival of primary erythroid progenitors. Expanded erythroid progenitors were either starved of growth factors or treated with the PI3-kinase inhibitor LY294002 in the presence of growth factors. Treatment of cells with the p70 S6 kinase inhibitor rapamycin, which does not inhibit Akt, was used as a control. Our data show that approximately 60% of the LY294002 treated cells were nonviable compared to 75% for growth factor starved cells (Fig. 2A), while rapamycin, treatment did not induce significant level of apoptosis in erythroid cells. Finally, we sought to determine whether apoptosis of erythroid progenitors during growth factor starvation and PI3-kinase inhibition occurs by a caspase dependent manner. Previous studies have shown the existence of two mechanisms depending on the stress stimuli (17). We determined the cleavage of caspase 3 substrate poly-(ADP ribose) polymerase (PARP) as indicator of caspase dependent apoptosis in our studies. Our data show growth factor starvation and LY294002 treatment triggers apoptosis in a caspase dependent manner (Fig. 2B).

Although further work is required to ascertain if FKHRL1 is translocated into the nucleus, as in fibroblasts cells (9) to regulate transcription of death genes in erythroid cells our present study establishes for the first time a critical role for Akt and FKHRL1 in mediating the antiapoptotic effects of Epo and SCF in primary erythroid progenitors.

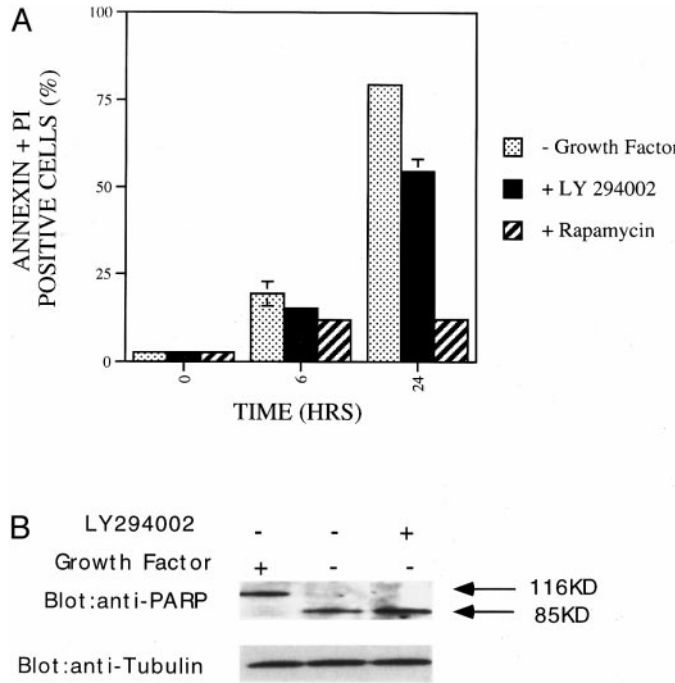

FIG. 2. Induction of apoptosis by inhibition of PI3-kinase pathway in primary erythroid progenitors. (A) Primary human erythroid progenitors at the CFU-E stage of differentiation were either deprived of growth factors or treated with LY294002 or rapamycin in the presence of growth factors in the medium for different times, as indicated. Cells were analyzed for apoptosis by determining the percentage of cells that are positive for annexin and propidium iodide (PI). The results are the mean of two separate experiments using different cell preparations. (B) Primary human erythroid progenitors at the CFU-E stage of differentiation were grown with or without growth factors or treated with LY294002 inhibitor for 24 h, and cell lysates subjected to immunoblot analysis with anti-poly (ADP) ribose polymerase (PARP) (top panel) and anti-tubulin antibodies (bottom panel).

ACKNOWLEDGMENTS

The authors thank Tony Clark for her technical assistance and Stephanie Kubilus for her assistance in preparation of the figures.

REFERENCES

1. Coffer, P. J., Jin, J., and Woodgett, J. R. (1998) *Biochem. J.* **335,** 1–5.
2. Bao, H., Jacobs-Helbor, S. M., Lawson, A. E., Penta, K., Wickrema, A., and Sawyer, S. T. (1999) *Blood* **93,** 3757–3773.
3. Haseyama Y., Sawada, K-I., Oda, A., Koizumi, H., Takano, H., Tarumi, T., Nishio, M., Handa, M., Ikeda, Y., and Koike, T. (1999) *Blood* **94,** 1568–1577.
4. Lin, K., Doman, J. B., Rodan, A., and Kenyon, C. (1997) *Science* **278,** 1319–1322.
5. Ogg, S., Paradis, S., Gottlieb, S., *et al.* (1997) *Nature* **389,** 994–999.
6. Paradis, S., and Ruvkun, G. (1998) *Genes Dev.* **12,** 2488–2498.
7. Anderson, M. J., Viars, C. S., Czekay, S., Cavenee, W. K., and Arden, K. C. (1998) *Genomics* **47,** 187–199.

8. Nakae, J., Park, B.-C., and Accilli, D. (1999) *J. Biol. Chem.* **274,** 15982–15985.
9. Brunet, A., Bonni, A., Zigmond, M. J., *et al.* (1999) *Cell* **96,** 857–868.
10. Rena, G., Guo, S., Cichy, S. C., Unterman,T. G., and Cohen, P. (1999) *B. J. Biol. Chem.* **274,** 17179–17183.
11. Guo, S., Rena, G., Cichy. S., He, X., Cohen, P., and Unterman, T. G. (1999) *J. Biol. Chem.* **274,** 17184–17192.
12. Wickrema, A., Uddin, S., Sharma, A., *et al.* (1999) *J. Biol. Chem.* **270,** 24459–24474.
13. Cichy, S. B., Uddin, S., Danilkovich, A., Guo, S., Klippel, A., and Unterman,T. G. (1998) *J. Biol. Chem.* **273,** 6482–6487.
14. Muta, K., Krantz, S. B., Bondurant, M. C., and Wickrema, A. (1994) *J. Clin. Invest.* **94,** 34–43.
15. Jacobs-Helber, S., Penta, K., Sun, Z., Lawson, A., and Sawyer, S. T. (1997) *J. Biol. Chem.* **272,** 6850–6853.
16. Boyer, S. H., Bishop, T. R., Rogers, O. C., Noyes, A. N., Frelin, L. P., and Hobbs, S. (1992) *Blood* **80,** 2503–2512.
17. Mooney, L., Otter, I., Oliver, R., *et al.* (1998) *J. Biol. Chem.* **273,** 6121–6131.