Distinct Signaling from Stem Cell Factor and Erythropoietin in HCD57 Cells*

(Received for publication, August 7, 1996, and in revised form, January 15, 1997)

Sarah M. Jacobs-Helber, Kalyani Penta, Zhenhong Sun, Amy Lawson, and Stephen T. Sawyer

From the Department of Pharmacology/Toxicology, Medical College of Virginia/Virginia Commonwealth University, Richmond, Virginia 23298 and the Department of Medicine, Division of Cardiology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-2257.

A recent report (Wu, H., Klingmuller, U., Besmer, P., and Lodish, H. F. (1995) Nature 377, 242–246) documents the interaction of the erythropoietin (EPO) receptor (EPOR) with the stem cell factor (SCF) receptor (c-KIT) and suggests that SCF acts through the EPOR. To elucidate the ability of SCF to affect the erythropoietin signaling pathway, we studied the effect of SCF on EPOR phosphorylation, SHC/ERK-1 activity, and cell proliferation and apoptosis in EPO-dependent HCD57 cells.

Treatment of these cells with SCF resulted in phosphorylation and apoptosis in EPO-dependent HCD57 cells. However, SCF-dependent phosphorylation of the EPOR did not initiate an EPO-like intracellular signal. SCF-induced proliferation and apoptosis was observed in these cells. These data may illustrate two distinct pathways for erythroid cell proliferation and prevention of apoptosis in response to EPO, thereby providing a system to discriminate these intracellular signals.

Erythropoietin (EPO) is the glycoprotein necessary for the proliferation, cell survival, and differentiation of immature erythroid cells (1). EPO acts on erythroid cells by binding the EPO receptor (EPOR), a member of the cytokine receptor superfamily (1). Although the EPOR contains an intrinsic tyrosine kinase activity, ligand binding to the receptor results in the rapid activation of the Janus kinase JAK2 and phosphorylation of the EPOR (2, 3). STAT5 binds to the activated receptor, becomes phosphorylated, and translocates to the nucleus where it binds DNA (4). Colony-forming units-erythroid and proerythroblasts are absolutely dependent on EPO for their survival; withdrawal of these cells from EPO results in apoptosis or programmed cell death (5, 6). The mechanism by which erythroid cells survive apoptosis is currently under investigation; JAK2 has been implicated in this pathway (7). However, the ability of EPO-dependent cell lines to survive without EPO through signaling via other cytokines has currently come under debate. A physical interaction between the receptor for EPO and the receptor for stem cell factor (SCF) was recently demonstrated in HCD57 cells (8).

In the absence of EPO, the EPOR in HCD57 cells treated with SCF but not in SCF/ml. Twenty indicated above from cells treated with no cytokine, EPO, or 1000 ng of SCF/ml, or left untreated; alternatively, the cells were incubated in EPO for 2 h and then treated with no additional growth factor or 100 ng of SCF/ml for 5 min.

Materials and Methods

Cell Culture and Immunoprecipitation—HCD57 cells were cultured in Iscove's modified Dulbecco's medium (Life Technologies, Inc.), 25% fetal calf serum, 10 ng/ml gentamicin at 37°C in a 5% CO₂ environment. All EPO treatment of cells was at 10 units of EPO/ml of media. For each immunoprecipitation time point, 1 × 10⁵ HCD57 cells were washed three times in serum-free media and cultured for 18 h in the absence of EPO. The cells were then treated for 2, 5, or 10 min as indicated in the figure legends with either EPO, 100 ng of SCF/ml, or left untreated; alternatively, the cells were incubated in EPO for 2 h and then treated with no additional growth factor or 100 ng of SCF/ml for 5 min.

Cell extracts were subjected to immunoprecipitation as described previously (11) with either anti-phosphotyrosine (UBI), anti-SHC (UBI), 1 μg of anti-ERK-1 (Santa Cruz), or anti-EPOR receptor (raised against a 15-amino acid peptide corresponding to the C-terminal of the murine receptor) (10). Competition of EPOR immunoprecipitation was conducted with excess amounts of this C-terminal peptide (10 μg). Western blot analysis was conducted using anti-phosphotyrosine (UBI) antibody. Western blots were visualized using enhanced chemiluminescence (Amersham). For the detection of STAT5 activation, 1 × 10⁵ HCD57 cells were deprived of EPO as described above. Cells were then left untreated or treated with EPO, 100 ng of SCF/ml, or 5 ng interferon-γ/ml (as a control of STAT1 activation). Nuclear extracts were prepared as described previously (11), and 20 μg of protein were subjected to Western blot analysis with either anti-STAT5 (Transduction Research) or anti-phosphotyrosine (UBI) antibody. Western blots were visualized using enhanced chemiluminescence (Amersham). For the cell viability studies, cells were deprived of EPO as indicated above and cultured at 1 × 10⁵ cells/ml in the absence of cytokines or in the presence of EPO and/or SCF as indicated in the figure legend. Cell viability was determined by counting cells on a hemocytometer in the presence of 0.2% trypan blue.

STAT5 DNA Binding Studies—Nuclear extracts were prepared as indicated above from cells treated with no cytokines, EPO, or 1000 ng of SCF/ml. Twenty μg of nuclear extract were incubated with a γ²³²PITPL-label DNA fragment corresponding to the STAT5 binding site in the rat β-casein promoter, the prolactin-inducible element (12), and subjected to electrophoresis as described previously (5, 13).

In vitro Kinase Assay—Anti-ERK-1 immunoprecipitated proteins were concentrated into 25 μl of lysate buffer and subjected to an in vitro kinase assay (14). Briefly, 1 μl of immunoprecipitate was incubated in 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM NaF, 20 mM KHEPES, pH 7.4, 15 mM magnesium acetate, 1 mM KEGTA, 1

This paper is available on line at http://www-jbc.stanford.edu/jbc/
Distinct Signaling from SCF and EPO

6851

mat dithiothreitol containing 1 μg/ml each chymotrypsin, leupeptin, aprotinin, pepstatin, and soybean trypsin inhibitor, 0.5 μM PKI protein kinase inhibitor peptide (TTYADFIASGRTGIRNAHID), 0.5 mg/ml myelin basic protein, 50 μM ATP, and 5 μCi of [γ-32P]ATP for 15 min at 30°C. The reaction was stopped with the addition of 90°C 2 × SDS sample buffer, and proteins were resolved on a 15% SDS-polyacrylamide gel electrophoresis gel. The gel was fixed and dried in vacuo, and phosphorylated myelin basic protein was visualized with autoradiography.

Apoptosis Studies—Cells were cultured in the absence of cytokine or in the presence of 100 ng of SCF/ml or EPO. Cells were harvested at 24-h intervals, and genomic DNA was isolated from the cells (15). Ten μg of genomic DNA were resolved on 2.25% agarose, 1 × TAE (40 mm Tris acetate, pH 8.0, 1 mx EDTA), 300 ng of EtBr/ml of gel. DNA laddering indicative of apoptosis was visualized using ultraviolet light.

RESULTS

Treatment of HCD57 cells with either EPO or SCF or without additional cytokines revealed that numerous proteins are tyrosine-phosphorylated in response to EPO and SCF (Fig. 1, lanes A, B, and C). Although the phosphorylation patterns were quite different for each growth factor treatment, tyrosine-phosphorylated bands similar in molecular mass to a number of proteins in the EPO signal transduction pathway were observed in both the EPO- and SCF-treated lanes (Fig. 1, lanes B and C); these include the EPOR (78 kDa) (10) and SHC (47 and 52 kDa) (17). A tyrosine-phosphorylated band was also detected which was similar to the molecular mass of JAK2 (130 kDa) (16); however, re-probing this blot of Tyr(P)-containing proteins with anti-JAK2 antibody revealed that JAK2 was only phosphorylated in response to EPO (Fig. 1A, lane E); no phosphorylation of JAK2 was observed in cells treated with SCF (Fig. 1A, lane F). Immunoprecipitation of JAK2 and Western blot analysis with anti-phosphotyrosine confirmed this lack of effect of SCF on JAK2 phosphorylation (Fig. 1B, lane 10).

Immunoprecipitation of HCD57 cellular proteins with anti-EPOR antibody and Western blotting with anti-phosphotyrosine antibody demonstrated that the EPOR was phosphorylated in response to SCF (Fig. 1B, lanes 2, 5, and 6); this phosphorylation was greatest at 5 min but maintained for 10 min or longer. However, this was 10% or less of the level of phosphorylation in response to EPO (Fig. 1B, lane 8). Immunoprecipitation of this band could be prevented by the addition of competitor C-terminal EPOR peptide (Fig. 1B, lanes 3 and 9), confirming its identity as the EPOR.

Immunoprecipitation using anti-SHC antibody revealed that both the 47- and 52-kDa SHC proteins were phosphorylated in response to either SCF or EPO (Fig. 1C, lanes 4 and 5). However, the level of p52SHC phosphorylation was increased after EPO treatment as compared with SCF treatment. The p47SHC phosphorylation was similar after either SCF or EPO treatment. Culturing the cells in EPO down-regulated the cell surface EPOR (10) such that EPO no longer activated SHC (Fig. 1C, lane 1); however, SCF was still able to activate SHC phosphorylation (Fig. 1C, lane 2). Since SHC has been implicated in the activation of the Ras/MAPK cascade (18), this difference in SCF activation of the SHC/MAPK pathway was tested by assessing ERK-1 activation. SCF-dependent ERK-1 activation paralleled SHC activation; SCF activated ERK-1 to a lesser extent than EPO (Fig. 1D) and was able to activate ERK-1 to a

FIG. 1. Effect of EPO and SCF on tyrosine phosphorylation in HCD57 cells. A, HCD57 cells were deprived of EPO and then treated with EPO (lanes B and E), SCF (lanes C and F), or left untreated (lanes A and D). Cell extracts were immunoprecipitated with anti-phosphotyrosine and then probed with either anti-phosphotyrosine (lanes A, B, and C) or anti-JAK2 (lanes D, E, and F). Arrows indicate molecular mass markers. B, tyrosine phosphorylation of EPOR and JAK2 protein kinase. HCD57 cells were cultured in the absence of cytokines (C), SCF (S), or EPO (E) for the time indicated. Cell extracts were immunoprecipitated with anti-EPOR (lanes 1–9) or anti-JAK2 (lanes 10–12) and Western-blotted with anti-phosphotyrosine (lanes 1–12). The EPOR C-terminal peptide was used as a competitor for EPOR immunoprecipitation (lanes 3 and 9). Top and bottom arrows indicate position of JAK2 and EPOR proteins, respectively. C, tyrosine phosphorylation of SHC proteins. HCD57 cells were cultured in the presence of EPO for 2 h and treated with no further cytokines (lane 1) or 100 ng/ml SCF (lane 2) or were deprived of EPO for 18 h and incubated in the absence of cytokines (C), SCF (S), or EPO (E). Upper panel, proteins were immunoprecipi-
lesser degree when the EPOR was down-regulated (Fig. 1D, lane 2).

Treatment of HCD57 cells with EPO results in the phosphorylation, nuclear localization, and DNA binding activity of STAT5, a DNA-binding phosphoprotein (4). Western blot analysis of nuclear extract preparations of cells treated with EPO or SCF revealed that STAT5 was translocated to the nucleus in response to EPO but not SCF (Fig. 2A). A 95-kDa band corresponding to STAT5 was tyrosine-phosphorylated in response to EPO (Fig. 2B, lane 2) but not SCF (Fig. 2B, lane 3), and DNA binding to a STAT5 DNA element was observed only in EPO-treated cells (Fig. 2C, lane 2).

HCD57 cells cultured in the presence of EPO, SCF, or no growth factor revealed that SCF had a proliferative effect on these cells between days 1 and 3 compared with culture in the absence of additional cytokines (Fig. 3, left panel). However, this effect was short lived when compared with proliferation in the presence of EPO; the number of viable cells decreased rapidly after a 4-day culture in SCF. This effect was seen despite culture in high doses of SCF (Fig. 3, center and right panels). SCF was synergistic with EPO only at suboptimal levels of EPO.

As a more direct measure of cell survival, the apoptotic state of these cells was assessed by isolation and examination of genomic DNA for the typical DNA cleavage seen in cells undergoing programmed cell death (Fig. 4). EPO completely protected these cells from undergoing apoptosis (Fig. 4, right panel), but SCF was unable to prevent apoptosis in these cells (Fig. 4, middle panel).

**Discussion**

The data presented here confirm the observation by Wu et al. (8) that SCF enhanced proliferation and induced phosphorylation of the EPOR. We report here that the EPOR was phosphorylated in response to SCF to 10% or less than that observed in response to EPO.

We have further investigated the signal transduced by EPO and SCF to test the hypothesis raised by Wu et al. (8) that the phosphorylation of the EPOR by c-KIT allows SCF to promote cell proliferation and survival in the absence of EPO. Our observation that treatment of these cells with SCF enhanced proliferation but did not prevent apoptosis strongly suggests that the intracellular signal transduced by SCF is distinct from that transduced by EPO. Studies in other laboratories using human erythroid cells also have shown differential signaling by SCF as compared with EPO (19, 20). The fact that treatment with SCF does not activate JAK2 reinforces this notion of dual signaling pathways. A recent study, however, reported a slight and transitory increase in JAK2 activation by SCF in human and murine cells (21). We saw no such increase in JAK2 activation within the first 2–5 min following SCF stimulation in our system (data not shown). The study by Weiler et al. (21) did not assess STAT5 activation or other downstream activity in response to SCF. We see no STAT5 phosphorylation, nuclear translocation, or DNA binding activity in HCD57 cells in response to SCF. This result confirms that SCF does not transduce the same intracellular signal as EPO. The division of the proliferative and apoptotic properties of these cells in response to EPO also suggests that activation of the JAK-STAT pathway may be necessary for the prevention of apoptosis in these cells. Recent observations that a dominant negative JAK2 protein can block EPO-dependent inhibition of apoptosis in the murine myeloid cell line FDC-P1 support this hypothesis (7). Alternatively, EPO may be selectively activating a signal transduction pathway other than JAK-STAT that is necessary for the prevention of apoptosis.

These experiments suggest that a pathway for proliferation distinct from the control of apoptosis is activated by both SCF and EPO. The fact that both EPO and SCF treatments resulted in phosphorylation of the 47- and 52-kDa SHC proteins and
activation of ERK-1 suggests that the proliferative response may be transduced through the SHC/Ras/MAPK pathway, as has been suggested for the EPO- (18) and SCF-responsive cells (22). However, the data suggest that SCF is not acting solely through the EPO signaling pathway. SCF appeared to activate SHC and ERK-1 independent of the EPOR (in cells where the EPOR was down-regulated and EPO treatment no longer activated SHC and ERK-1), as has been previously demonstrated (22). The experiments shown in Fig. 1, C and D, suggest that the presence of cell surface EPOR facilitates the SCF-dependent SHC/ERK-1 pathway. Since the proposed region of association between c-KIT and the EPOR, the extended box 2 region, is essential for mitogenesis but not for JAK2 activation (23), it is possible that c-KIT is tapping into the EPO pathway via this region. SCF-dependent phosphorylation of the EPOR may recruit SHC and other substrates to the cell surface where these substrates may be phosphorylated by c-KIT. If this were the case, the substrates recruited could transduce a proliferative signal but would be unable to transduce a signal to prevent programmed cell death. Treatment of cells with SCF results in phosphorylation of only the 78-kDa form of the EPOR, which has been shown to be the fully processed, functional form of the receptor (4, 10). JAK2 is also associated solely with this more highly glycosylated, serine-phosphorylated form of the EPOR in HCD57 cells (4). It is interesting to speculate that the modifications that allow the 78-kDa EPOR to interact selectively with JAK2 also facilitate its interaction with c-KIT. Further studies are needed to verify these hypotheses. Therefore, although c-KIT can phosphorylate the EPOR in SCF-treated cells, SCF clearly cannot replace EPO in the survival of these cells.

Acknowledgment—We thank Dr. Robert Tombes for his assistance with the in vitro kinase assay.

REFERENCES

1. Koury, M. J., and Bondurant, M. C. (1992) Eur. J. Biochem. 210, 649–663
2. Miura, O., Nakamura, N., Quelle, F. W., Witthuhn, B. A., Ile, J. N., and Aoki, N. (1994) Blood 84, 1501–1507
3. Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Yi, T., Tang, B., Miura, O., and Ile, J. N. (1993) Cell 74, 227–236
4. Sawyer, S. T., and Penta, K. (1996) J. Biol. Chem. 271, 32430–32437
5. Ruff-Jamison, S., Chen, K., and Cohen, S. (1993) Science 261, 1733–1736
6. Sawyer, S. T., and Penta, K. (1994) Hematol. Oncol. Clin. N. Am. 8, 895–911
7. Zhuang, H., Niu, Z., He, T.-C., Patel, S. V., and Wojcikowski, D. M. (1995) J. Biol. Chem. 270, 14500–14504
8. Wu, H., Klingmuller, U., Besmer, P., and Lodish, H. F. (1995) Nature 377, 245–246
9. Russetti, S. K., Janesch, N. J., Chakraborti, A., Sawyer, S. T., and Hankins, W. D. (1990) J. Virol. 64, 1057–1062
10. Sawyer, S. T., and Hankins, W. D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6849–6853
11. Penta, K., and Sawyer, S. T. (1995) J. Biol. Chem. 270, 31282–31287
12. Gouilleux, F., Wakao, H., Mundt, M., and Groner, B. (1994) EMBO J. 13, 4361–4369
13. Ruff-Jamison, S., Chen, K., and Cohen, S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4215–4218
14. Pelte, S. L., Tombes, R. M., Meijer, L., and Krebs, E. G. (1988) Dev. Biol. 130, 26–36
15. Maniatus, T., Fritsch, E. F., and Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. Argetsinger, L. S., Campbell, G. S., Yang, X., Witthuhn, B. A., Silvennoinen, O., Ile, J. N., and Carter-Su, C. (1993) Cell 74, 237–244
17. Damen, J. E., Liu, L., Cutler, R. L., and Krystal, G. (1993) Blood 82, 2296–2303
18. Miura, Y., Miura, O., Ile, J. N., and Aoki, N. (1994) J. Biol. Chem. 269, 29962–29969
19. Muta, K., Krantz, S. B., Bondurant, M. C., and Dai, C. H. (1995) Blood 86, 572–580
20. Muta, K., Krantz, S. B., Bondurant, M. C., and Wickrema, A. (1994) J. Clin. Invest. 94, 34–43
21. Weiler, S. R., Mou, S., DeBarry, C. S., Keller, J. R., Russetti, F. W., Ferris, D. K., Lengo, D. L., and Linnekin, D. (1996) Blood 87, 3698–3699
22. Matsuguchi, T., Salgia, R., Hallek, M., Eder, M., Druker, B., Ernst, T. J., and Griffin, J. D. (1994) J. Biol. Chem. 269, 5016–5021
23. He, T. C., Jiang, N., Zhuang, H., Quelle, D. E., and Wojcikowski, D. M. (1994) J. Biol. Chem. 269, 18291–18294