Erythropoietin Activates Raf1 by an Shc-Independent Pathway in CTLL-EPO-R Cells

By Dwayne L. Barber, Cristin N. Corless, Kai Xia, Thomas M. Roberts, and Alan D. D’Andrea

Stimulation of the erythropoietin receptor (EPO-R) or the interleukin-2 receptor (IL-2-R) by their respective ligands has been reported to activate tyrosine phosphorylation of the cytoplasmic protein, Shc. We have recently characterized a cell line, CTLL-EPO-R, that contains functional cell-surface receptors for both EPO and IL-2. Although stimulation with IL-2 or IL-15 resulted in the rapid, dose-dependent tyrosine phosphorylation of Shc, stimulation with EPO failed to activate Shc. EPO, IL-2, and IL-15 activated the tyrosine phosphorylation of the adaptor protein, Shp2, and the association of Shp2/Grb2/cytokine receptor complexes. In addition, EPO, IL-2, and IL-15 activated Raf1 and ERK2, demonstrating that the Raf1/MEK/MAP kinase pathway was activated. These results indicate that multiple biochemical pathways are capable of conferring a mitogenic signal in CTLL-EPO-R. EPO can activate the Raf1/MEK/MAP kinase pathway via Shc-dependent or Shc-independent pathways, and Shc activation is not required for EPO-dependent cell growth in CTLL-EPO-R.

© 1997 by The American Society of Hematology.
DNA transfection. Ba/F3-EPO-R cells and DA-3-EPO-R cells were transfected by electroporation with pLXSN-EPO-R cDNA. Selection with G418 (1.0 mg/mL) in IL-3 medium was initiated 48 hours after electroporation. Similarly, CTLL-EPO-R cells were generated by co-electroporation with pXM-EPO-R cDNA and pSV2-neo. Selection was performed with G418 (1.0 mg/mL) in IL-2 medium. Subclones were cultured in RPMI 1640 medium supplemented with 10% FCS and 0.5 U/mL murine recombinant EPO per milliliter.

Analysis of Shc activation and identification of associated proteins. For starvation assays, cell lines were incubated in RPMI 1640/10% FCS (no supplementary growth factor) for a 4-hour period and then stimulated for various periods with either no factor, IL-2, IL-3 (Kirin Brewery), IL-4 (Genzyme, Cambridge, MA), IL-15 (generously provided by Immunex Corp, Seattle, WA), or EPO (Kirin Brewery). Cell lysates were prepared in 50 mmol/L Tris-HCl (pH 8.0) 150 mmol/L NaCl, 0.1% Triton X-100 plus phosphatase and protease inhibitors as previously described.41 Immunoprecipitations were performed with either a Shc polyclonal antibody (Transduction Laboratories, Lexington, KY), a Grb2 polyclonal antibody (Santa Cruz Biotechnology), a Grb2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or an anti-N-terminal EPO-R polyclonal antibody as previously described.42 For in vitro binding analysis, 5 µg of each fusion protein was incubated with 2 µg of lysate from CTLL-EPO-R cells stimulated with no factor, 50 U/mL IL-2, or 50 U/mL EPO. The glutathione-S-transferase (GST) fusion proteins expression the SH2 domains of Grb2, Shp2, and Shc were produced in bacteria as described.43 For in vitro binding analysis, 5 µg of each fusion protein was incubated with 2 µg of lysate from CTLL-EPO-R cells stimulated with no factor, 50 U/mL IL-2, or 50 U/mL EPO. The glutathione-S-transferase (GST) fusion proteins expressing the SH2 domains of Grb2, Shp2, and Shc were produced in bacteria as described. For in vitro binding analysis, 5 µg of each fusion protein was incubated with 2 µg of lysate from CTLL-EPO-R cells stimulated with no factor, 50 U/mL IL-2, or 50 U/mL EPO. The glutathione-S-transferase (GST) fusion proteins expression the SH2 domains of Grb2, Shp2, and Shc were produced in bacteria as described.43

Glutathione-S-transferase (GST) fusion proteins expressing the SH2 domains of Grb2, Shp2, and Shc were produced in bacteria as described.43 For in vitro binding analysis, 5 µg of each fusion protein was incubated with 2 µg of lysate from CTLL-EPO-R cells stimulated with no factor, 50 U/mL IL-2, or 50 U/mL EPO. The glutathione-S-transferase (GST) fusion proteins expression the SH2 domains of Grb2, Shp2, and Shc were produced in bacteria as described.43

After electrophoretic transfer of proteins to nitrocellulose, the membrane was blocked and incubated with the antiphosphotyrosine MoAb 4G10, washed in 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.1% Triton X-100, 10 mmol/L NaPO4, 10 mmol/L NaF, 5 mmol/L EDTA, 1 mmol/L NaVO4, and prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously.41

Shc tyrosine phosphorylation, we examined several EPO-described previously.41 For in vitro binding analysis, 5 µg of each fusion protein was incubated with 2 µg of lysate from CTLL-EPO-R cells stimulated with no factor, 50 U/mL IL-2, or 50 U/mL EPO. The glutathione-S-transferase (GST) fusion proteins expression the SH2 domains of Grb2, Shp2, and Shc were produced in bacteria as described.43 For in vitro binding analysis, 5 µg of each fusion protein was incubated with 2 µg of lysate from CTLL-EPO-R cells stimulated with no factor, 50 U/mL IL-2, or 50 U/mL EPO. The glutathione-S-transferase (GST) fusion proteins expression the SH2 domains of Grb2, Shp2, and Shc were produced in bacteria as described.43

Results

EPO-induced mitogenesis does not require Shc activation in CTLL-EPO-R cells. Previous studies showed that Shc activation was observed after stimulation of EPO-dependent hematopoietic cells.18,21,41 To further test for EPO-induced Shc tyrosine phosphorylation, we examined several EPO-dependent hematopoietic cell lines (Fig 1). The 52- and 46-kD Shc proteins were tyrosine phosphorylated by IL-3 in Ba/F3 (Fig 1, lane 2), IL-3 or EPO in Ba/F3-EPO-R (Fig 1, lanes 5 and 6) and in DA-3-EPO-R (see Fig 4) transfectants as previously reported.18,41 HCD-57,41 a murine cell line expressing EPO-R, also activated Shc tyrosine phosphorylation upon EPO stimulation (Fig 1, lane 8). IL-2 activated Shc in CTLL (Fig 1, lane 10) CTLL-EPO-R (Fig 1, lane 13) cells. Surprisingly, EPO-dependent Shc phosphorylation was not observed after stimulation of CTLL-EPO-R cells (Fig 1, lane 14). Similar amounts of Shc are expressed in the various cell lines as revealed by reprobing the blot for Shc (Fig 1, Shc immunoblot). Tyrosine phosphorylation of Shc at Y317 results in the binding of the adapter protein Grb2 to Shc.15

To examine this association, the blot was reprobed for Grb2 (Fig 1, Grb2 Immunoblot). Grb2 binding correlated with tyrosine phosphorylation of Shc.

To verify the absence of EPO-dependent Shc activation in CTLL-EPO-R cells, a dose-response experiment was performed. Stimulation with increasing concentrations of IL-2 resulted in dose-dependent Shc tyrosine phosphorylation (Fig 2A, lanes 2 through 6) after a 5-minute incubation. In contrast, EPO failed to activate Shc at concentrations as high as 100 U/mL (Fig 2A, lanes 8 through 13). Equivalent amounts of Shc were immunoprecipitated with each stimulation (Fig 2A, Shc immunoblot). Grb2 was shown to bind to Shc only after stimulation in an IL-2-dependent manner (Fig 2A, Grb2 immunoblot).

A time-course experiment was next performed to determine the kinetics of Shc activation in CTLL-EPO-R cells (Fig 2B). IL-2 rapidly induced Shc tyrosine phosphorylation in CTLL-EPO-R cells within 1 minute (Fig 2B, lanes 2 through 6) whereas 50 U/mL EPO failed to activate Shc phosphorylation at any time point for two independent
Shc-INDEPENDENT EPO SIGNALING

Fig 1. EPO fails to activate Shc tyrosine phosphorylation in CTLL-EPO-R cells. Ba/F3 (lanes 1 through 3), Ba/F3-EPO-R (lanes 4 through 6), HCD-57 (lanes 7 and 8), CTLL (lanes 9 through 11), and CTLL-EPO-R (lanes 12 through 14) cells were depleted of cytokine for 4 hours and stimulated with no factor (lanes 1, 4, 7, 9, and 12), 50 U of murine IL-3 (lanes 2 and 5), 50 U of murine IL-2 per milliliter (lanes 10 and 13), or 50 U of human EPO per milliliter (lanes 3, 6, 8, 11, and 14) for 10 minutes. After cell lysis, an immunoprecipitation was performed with an Shc polyclonal antibody. Immune complexes were resolved by SDS-PAGE and blotted to nitrocellulose. The immunoblot was probed with HRP-conjugated antiphosphotyrosine (pTyr) MoAb RC20. The blot was then stripped and reprobed with an Shc polyclonal antibody (Shc immunoblot) or a Grb2 MoAb (Grb2 immunoblot).

CTLL-EPO-R subclones (Fig 2B, lanes 8 through 12 and 14). Other substrates, such as EPO-R and JAK2, demonstrated time- and concentration-dependent activation in CTLL-EPO-R cells. Simlar amounts of Shc were immunoprecipitated in this experiment (Fig 2B, Shc Immunoblot). Again, Grb2 association with Shc was observed only upon IL-2-dependent Shc tyrosine phosphorylation (Fig 2B, Grb2 Immunoblot).

EPO activates the tyrosine phosphorylation of the EPO-R and the formation of EPO-R/Grb2 complexes. Because EPO did not activate Shc in CTLL-EPO-R cells, we reasoned that if the Grb2/mSOS/Ras pathway was activated, it would be mediated by an Shc-independent pathway. For example, previous studies have shown that the Grb2 binds EGF-R directly and thereby activates the Ras pathway. CTLL-EPO-R cells were starved and stimulated with various cytokines, and cellular proteins were immunoprecipitated with either anti-Grb2 or anti-EPO-R antibodies (Fig 3). EPO activated the tyrosine phosphorylation of EPO-R (Fig 3, lane 10), which co-immunoprecipitated as a 72-kD phosphoprotein with an anti-Grb2 antibody (Fig 3, lane 5). The Shc signal (pp52 and pp46) is somewhat overexposed in this experiment, because a long exposure was required to observe other associated proteins. Multiple phosphoproteins associated with Grb2 upon IL-2 and IL-15 stimulation, including Shc, a pp80-kDa protein (possibly IL-2-Rβ) and pp64, pp97, and pp145. A 145-kD protein has been observed to associate with the phosphotyrosine binding domain (PTB) of Shc after fibroblast growth factor (FGF) stimulation and has recently been described as an SH2-domain inositol 5-phosphatase (SHIP)26-28.

EPO and IL-2 activate tyrosine phosphorylation of Shp2 and activate formation of Shp2/Grb2 complexes. Previous studies have shown an EPO-dependent physical interaction between Shp2 and Grb2 and between Shp2 and EPO-R.29 We reasoned, therefore, that the 64-kD protein activated by IL-2, IL-15, and EPO in CTLL-EPO-R cells might be Shp2. To test this hypothesis, we performed an anti-Grb2 immunoprecipitation (Fig 4). As observed in Fig 3, multiple phosphoproteins were shown to associate with Grb2 after cytokine stimulation. A 64-kD phosphoprotein was observed after IL-2 (Fig 4, lane 2), IL-15 (Fig 4, lane 4), or EPO (Fig 4, lane 5) stimulation of CTLL-EPO-R cells. This protein was shown to be Shp2 after stripping and reprobing with a monoclonal Shp2 antibody. IL-4 failed to induce tyrosine phosphorylation and association of Shp2 with Grb2, as previously described (Fig 4, lane 8).

Association of tyrosine phosphorylated 52- and 46-kD proteins paralleled the pattern of Shc activation shown in Fig 1 (Fig 4, pTyr immunoblot, lanes 2, 4, and 5). Subsequent stripping and reprobing the membrane showed that these proteins were in fact Shc (Fig 4, Shc immunoblot). IL-15, like its related cytokine, IL-2 also resulted in tyrosine phosphorylation and Grb2 association (Fig 4, lane 9). Tyrosine phosphorylated EPO-R was shown to co-immunoprecipitate as a 72- to 78-kD phosphoprotein (Fig 4, lane 5). A 145-kD Shc-associated protein, presumably SHIP, co-immunoprecipitated with Grb2 (Fig 4, lanes 2, 4, and 5). Equal amounts of Grb2 were immunoprecipitated in this experiment. These data show that, although EPO does not tyrosine phosphorylate Shc in CTLL-EPO-R cells, this cytokine activates Grb2/Shp2 complexes in these cells.

The activated EPO-R binds to the SH2 domains of Grb2 and Shp2. In CTLL-EPO-R cells, the tyrosine phosphorylated EPO-R co-immunoprecipitates with Grb2 and Shp2, but not with tyrosine phosphorylated Shc. GST fusion proteins containing the SH2 domains of Grb2, Shp2, or Shc were used to verify that the binding of Grb2 and Shp2 to the EPO-R is mediated by SH2 domain interactions (Fig 5). CTLL-EPO-R cells were stimulated in the presence of no
added factor, IL-2, or EPO and incubated with various GST fusion proteins. Bound phosphoproteins were detected by antiphosphotyrosine immunoblotting. GST-SH2-Grb2 bound Shc (pp46 and pp52) only after IL-2 (Fig 5, lane 6), but not EPO (Fig 5, lane 10) stimulation, consistent with the immunoprecipitation data presented earlier. In addition, Shp2 (pp64) associated with GST-SH2-Grb2 after IL-2 (Fig 5, lane 6) or EPO (Fig 5, lane 10) stimulation. The EPO-R (pp72) was observed to associate with GST-SH2-Grb2 (Fig 5, lane 10) or GST-(N + C)-SH2-Shp2 (Fig 5, lane 11) after EPO stimulation. An additional, unidentified 60-kD phosphoprotein that is phosphorylated under conditions of cytokine deprivation is shown to associate with GST-(N + C)-SH2-Shp2 (Fig 5, lanes 3, 7, and 11). GST or GST-SH2-Shc fail to bind any phosphoproteins from CTLL-EPO-R cells.

EPO, IL-2, and IL-15 activate the Raf1 and MAP kinase pathway in CTLL-EPO-R cells. Other investigators have shown that effectors downstream of Ras including Raf1 and MAP kinase display EPO-dependent activation in...
Fig 3. EPO induces tyrosine phosphorylation and the formation of Grb2/EPO-R complexes. CTLL-EPO-R cells were depleted of cytokine for 8 hours and stimulated with no factor (lanes 1 and 6), 50 U of murine IL-2 per milliliter (lanes 2 and 7), 100 ng of murine IL-4 per milliliter (lanes 3 and 8), 100 ng of simian IL-15 per milliliter (lanes 4 and 9), or 50 U of human EPO per milliliter (lanes 5 and 10) for 10 minutes. After cell lysis, an immunoprecipitation was performed with either a Grb2 polyclonal antibody (lanes 1 through 5) or an anti-amino terminal EPO-R polyclonal antibody (lanes 6 through 10). Immune complexes were resolved by SDS-PAGE and blotted to nitrocellulose. The immunoblot was probed with 4G10 monoclonal anti-phosphotyrosine antibody followed by HRP-sheep anti-mouse IgG. The blot was then stripped and reprobed with a Grb2 polyclonal antibody. Molecular mass standards are indicated. Ab, antibody.

CTLL-EPO-R cells were depleted of cytokine for 8 hours in serum-free conditions before stimulation with the indicated cytokines. Raf1 was then immunoprecipitated from the lysates and in vitro kinase reactions were performed in the presence of kinase inactive purified MEK1 as an exogenous substrate. IL-2, IL-15, and EPO all led to an increase in MEK1 phosphorylation (Fig 6). The amount of IL-4–dependent Raf1 activation was increased over background, but less than that observed with other cytokines in this experiment. EPO stimulation resulted in a threefold stimulation of Raf activity, similar to IL-2 and IL-15 as monitored by phosphorimager detection (data not shown). These data are representative of three independent experiments. Equal amounts of Raf1 were immunoprecipitated in this experiment as revealed by probing the membrane with a Raf1 antibody (data not shown).

Next, the activation of ERK1 and ERK2 was examined (Fig 7). Lysates from CTLL-EPO-R–stimulated cells were immunoprecipitated with anti-ERK1 or anti-ERK2 peptide-specific antibodies. An in vitro kinase reaction was then performed using myelin basic protein as an exogenous substrate. IL-2, IL-15, and EPO all activated ERK2 as detected by enhanced phosphorylation of MBP (Fig 7). The level of IL-4–dependent MBP phosphorylation was enhanced over background. EPO, like IL-2 and IL-15, activated MBP phosphorylation twofold as determined by phosphorimager detection (data not shown). These data were found to be reproducible in three independent experiments. Similarly, IL-2, IL-15, and EPO stimulations resulted in tyrosine phosphorylation of ERK2 in immunoprecipitation/Western blotting experiments (data not shown). ERK1 kinase activity was not enhanced by any of the cytokines in CTLL-EPO-R cells, although ERK1 is expressed in CTLL-EPO-R cells (data not shown).

DISCUSSION

Previous studies have shown that Shc is rapidly tyrosine phosphorylated in response to EPO,18-21 IL-2,19,21,30 IL-3,19,21,41,52-54 IL-5,53,54 granulocyte-macrophage colony-stimulating factor (GM-CSF),52-54 growth hormone,55 and thrombopoietin.56,57 In the present study we have demonstrated that EPO activates Shc in some, but not all, EPO-dependent cell lines. CTLL-EPO-R cells display EPO-dependent proliferation31,32 and activation of JAK232,58 and STAT532 (Barber DL, unpublished observation). Here we show that EPO stimulates the tyrosine phosphorylation of the EPO-R and Shp2 which both can bind to the SH2 adaptor protein Grb2. Stimulation with either IL-2 or IL-15 results in dose- and time-dependent Shc tyrosine phosphorylation in CTLL-EPO-R cells. However, EPO failed to stimulate Shc tyrosine phosphorylation in any of these experiments.

Why EPO fails to activate Shc tyrosine phosphorylation in CTLL-EPO-R cells is not clear. CTLL-EPO-R have similar EPO-R receptor number, affinity, and EPO dose-dependent growth characteristics when compared to other EPO-dependent cell lines.32 The EPO-R may be differentially tyrosine phosphorylated in CTLL-EPO-R cells. Therefore, Shc may be unable to dock to the EPO-R and as a result is not tyrosine phosphorylated. Conversely, Shc may associate with the tyrosine-phosphorylated EPO-R but is not itself tyrosine phosphorylated. However, EPO-R failed to associate with Shc in...
CTLL-EPO-R (data not shown). JAK2 can phosphorylate Shc when analyzed by in vitro kinase reactions using lysates prepared with Bri2,21 EPO activates JAK2 in CTLL-EPO-R,21 therefore, the block in Shc activation does not reside in aberrant Janus kinase activation. It has been shown that IL-2 activates the lck25,61 and Syk protein tyrosine kinases.62 However, Syk is not expressed in CTLL cells (data not shown). lck phosphorlylates Shc in similar in vitro experiments.63 It is unknown which, if any, src family kinases are activated by EPO. Perhaps the failure of EPO to activate Shc in CTLL-EPO-R cells results from the inability of EPO-R to activate lck.

Several reports describe EPO-R expression in CTLL cells. One study indicated that EPO-R failed to generate an EPO-dependent response in CTLL cells.64 Overexpression of K-Ras generated an EPO-dependent growth response and was shown to be correlated with the IL-2 or EPO-dependent activation of 130- and 160-kD phosphoproteins.65 Another study demonstrated EPO-dependent growth of CTLL-EPO-R cells after a latent period of 4 to 15 days.66 We have characterized the growth and proliferative capacity of CTLL-EPO-R cells in earlier studies.31,52 Multiple reports have indicated that JAK2,32,67 STAT5,58,68 and EPO-R (this study) are all tyrosine phosphorylated, irrespective of EPO-growth dependency. The inability of certain CTLL-EPO-R subclones to proliferate in EPO therefore does not result from aberrant JAK-STAT signaling.

Despite prominent EPO-dependent Shc activation in Ba/F3-EPO-R, DA-3-EPO-R, and HCD-57, Shc failed to co-immunoprecipitate with the EPO-R from Triton X-100 lysates. Shc may bind to the EPO-R via its amino terminal phosphotyrosine binding domain (PTB),45,69 or the carboxy terminal SH2 domain. However, the EPO-R cytoplasmic tail does not contain any potential NPXY binding sites for PTB domains.13,30-72 The ability of the Shc SH2 domain to bind directly to the EPO-R under physiologically relevant conditions remains to be established. Alternatively, Shc could associate indirectly with the EPO-R through a bridging molecule such as SHIP, an inositol 5-phosphatase recently identified.46-48 The association between Shc and EPO-R in MO-7-EPO-R may result from overexpression of EPO-R in this cell line.18

Two other recent studies illustrate that cytokine-dependent Shc activation is not required for mitogenesis. Shc associates with the IL-3-R β chain at Y577.73 Mutation of this critical tyrosine does not adversely affect GM-CSF-R–dependent proliferation, JAK-STAT activation, or Raf1 activation.73 Mutation of Y388 in the IL-2-R β chain prevents association of Shc with the IL-2-R β chain.74 Furthermore, Shc was not tyrosine phosphorylated and Shc phosphorylation was not required for IL-2–dependent proliferation.74 The association of Shc with the IL-3-R β,75 or IL-2-R β chain76 is mediated by the phosphotyrosine binding domain of Shc.

The Grb2 SH2 domain binds to tyrosine phosphorylated proteins containing the motif YXX.77 A number of the proteins examined in these experiments contain this motif. Shc phosphorylated at Y317 (YTNV) binds Grb2,15 Shp2 contains two such motifs at Y542 (YTNI) and Y580 (YEVT).28 In addition, the EPO-R has a consensus Grb2 binding motif at Y463 (YENS).78 It is unknown if the EPO-R is tyrosine phosphorylated at this position. Therefore, Grb2 may bind directly to two or more substrates in these experiments.

An activated growth factor receptor may activate the Ras/Raf1/MAP kinase pathway via multiple alternative mechanisms. The tyrosine kinase receptor, PDGF-R, can activate Ras via Grb2 direct, Shc/Grb2, or SH-PTP-2/Grb2 mechanisms.26-28 Regarding EPO-dependent Ras activation, in Ba/F3-EPO-R, DA-3-EPO-R, and HCD-57 cells, all three mechanisms appear to be intact (data not shown). However, in CTLL-EPO-R, Grb2 associates with the EPO-R directly or through binding to Shp2, but without Shc activation. The level of Shp2 tyrosine phosphorylation varies among the cell lines examined in this study (data not shown). This implies that Shp2 may have a varying role in Grb2 recruitment.

EPO, IL-2, and IL-15 activated Raf1 (2.5- to 3.0-fold) and ERK2 (1.7- to 1.8-fold) kinase activity to a similar degree, as reported earlier for EPO.20,31 However, IL-4 displayed intermediate activation of Raf1 (1.8-fold) and ERK2 (1.2-fold) when compared with unstimulated controls. CTLL cells fail to proliferate in IL-4, but JAK1 and JAK3 are activated by this cytokine.79 Previous studies had failed to observe IL-4–dependent activation of the Ras signaling cascade.79,80
The activated EPO-R binds directly to the SH2 domains of Grb2 and Shp2. CTLL-EPO-R cells were depleted of cytokine for 8 hours and stimulated with no factor (lanes 1 through 4, and 13), 50 U of murine IL-2 per milliliter (lanes 5 through 8, and 14) or 50 U of human EPO per milliliter (lanes 9 through 12, and 15) for 10 minutes. Lysates were incubated with 10 μg of GST (lanes 1, 5, and 9), GST-SH2-Grb2 (lanes 2, 6, and 10), GST-(N+C)-SH2-Shp2 (lanes 3, 7, and 11) or GST-SH2-Shc (lanes 4, 8, and 12). The immunoblot was probed with 4G10 monoclonal anti-phosphotyrosine antibody followed by HRP-sheep anti-mouse IgG. A diffuse, constitutively phosphorylated 60-kD phosphoprotein associates with GST-(N+C)-Shp2 (lanes 3, 7, and 11). Lysate controls from CTLL-EPO-R cells are illustrated in lanes 13 through 15. The migration of EPO-R, Shp2, and Shc were determined by stripping and reprobing the membrane as shown in Fig 4 (data not shown). Molecular mass standards are indicated. Ab, antibody.

The biologic importance of Ras/Raf1/MAP kinase activation by the cytoplasmic tail of the EPO-R remains unknown. While the carboxy-terminus of the EPO-R activates this pathway and correlates with c-fos induction,21 this region of the carboxy terminus can be truncated without a loss of mitogenic signal in transfected Ba/F3 cells.21,22 When a truncated EPO-R lacking 108 amino acids is expressed in 32D cells, EPO-dependent Shc activation was not observed, despite normal JAK2 activation.20 However, in FDCP-1 transfectants, a truncated EPO-R lacking 154 amino acids displayed both Shc and JAK2 tyrosine phosphorylation and mitogenesis.21 The membrane proximal region of the EPO-R is required for mitogenesis and JAK2 activation.21,81 The differences observed in Shc activation in these cell lines suggest either multiple mechanisms of EPO-R-dependent Grb2 recruitment exist, or that EPO-dependent activation of additional tyrosine kinases occurs in selected hematopoietic...
cell lines. Although activation of the Ras/Raf1/MAP kinase may not be required for growth of immortalized cell lines, like 32D, it may be essential for growth of primary hematopoietic cells.

ACKNOWLEDGMENT

We thank David Cosman (Immunex Corp, Seattle, WA) for the gift of IL-15, Jim Griffin for GST-SH2-Shc, and Ben Neel for GST-SH2-Grb2 and GST-(N + C)-SH2-Shp2. We appreciate helpful comments on the manuscript from Martin Carroll, Cheryl Miller, Sonya Penfold, and Kodimangalam Ravichandran. We thank Anton Bennett, Ben Neel, Kodimangalam Ravichandran, Joanne Pratt, and members of the D’Andrea laboratory for helpful discussions throughout this work.

REFERENCES

17. Obrenovic A, Bradshaw RA, Seedorf K, Choaidas A, Schlessinger J, Ullrich A: Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLC-g1. EMBO J 13:1585, 1994
26. Kazlauskas A, Feng GS, Pawson T, Valius M: The 64-kDa protein that associates with the platelet-derived growth factor receptor beta subunit via Tyr-1009 is the SH2-containing phosphotyrosine phosphatase Sy. Proc Natl Acad Sci USA 90:6693, 1993
31. Showers MO, Moreau J-F, Linnekin D, Drucker B, D’Andrea AD: Activation of the erythropoietin receptor by the Friend spleen...


56. Drachman JG, Griffin JD, Kaushansky K: The c-Mpl ligand interacts with and activated by the IL-2 receptor: Possible link with the c-fms induction pathway. Immunity 2:89, 1995.


receptors with the cytoplasmic domain of the Interleukin-3 receptor β subunit (βIL3). J Biol Chem. 268:15833, 1993


80. Welham MJ, Duronio V, Leslie KB, Bowtell D, Schrader JW: Multiple hemopoietins, with the exception of interleukin-4, induce modification of Shc and mSos1, but not their translocation. J Biol Chem 269:21165, 1994


83. Witthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O, Ihle JN: JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell 74:227, 1993
Erythropoietin Activates Raf1 by an Shc-Independent Pathway in CTLL-EPO-R Cells

Dwayne L. Barber, Cristin N. Corless, Kai Xia, Thomas M. Roberts and Alan D. D’Andrea