Activation of the Erythropoietin Receptor by the Friend Spleen Focus-Forming Virus gp55 Glycoprotein Induces Constitutive Protein Tyrosine Phosphorylation

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The erythropoietin receptor (EPO-R) can be activated to signal cell growth by binding either EPO or gp55, the Friend spleen focus-forming virus (SFFV) glycoprotein. EPO binding induces tyrosine kinase activity and rapid tyrosine phosphorylation of several cellular substrates. To test for gp55-induced tyrosine kinase activity, we performed immunoblots on two murine cell lines that stably express EPO-R and gp55. Stimulation of the parental cell line, Ba/F3, with murine interleukin-3 (IL-3) resulted in rapid, dose-dependent tyrosine phosphorylation of a 97-Kd substrate. Stimulation with IL-3 or EPO of the Ba/F3 cells expressing the recombinant EPO-R (Ba/F3-EPO-R) resulted in tyrosine phosphorylation of the same p97 substrate. These latter cells, when transformed to grow factor-independence by the Friend spleen focus-forming virus (SFFV). These latter cells, when transformed to grow factor-independence by the Friend spleen focus-forming virus (SFFV), were constitutively phosphorylated in CTLL-2-EPO-R-gp55 cells. In conclusion, a 97-Kd protein found in two murine cell lines is tyrosine-phosphorylated in response to multiple growth factors and viral oncogenes, and appears to be a central phosphoprotein in signal transduction.

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The erythropoietin receptor (EPO-R) is a member of the cytokine receptor superfamily, which includes the receptors for interleukin-2 (IL-2; p75 subunit), IL-3, IL-4, granulocyte-macrophage colony-stimulating factor (GM-CSF), and several other cytokines.1-2 Although the signal transduction mechanism by these receptors is poorly understood, one of the most rapid biochemical changes occurring after receptor binding is protein tyrosine phosphorylation.3-4 The induction of tyrosine kinase activity by some growth factors, such as GM-CSF and IL-3, correlates with the promotion of growth factor-induced cell proliferation.5-6 One cytokine receptor, the IL-2R (p75), was recently found to physically associate with lck, a member of the src family of tyrosine kinases.7-8

While several cellular proteins are rapidly tyrosine-phosphorylated after cytokine receptor activation, only a few specific substrates, such as raf, have been identified.9-10 There are currently two approaches for identification of physiologically important tyrosine-phosphorylated substrates. One approach uses a coimmunoprecipitation strategy, in which a candidate substrate specifically coimmunoprecipitates with an activated growth factor receptor.11 A second approach uses the identification of phosphotyrosyl proteins that are common to several different receptors and that are found in several cell types. In this way, Li et al12 recently identified a common 57-Kd protein that is tyrosine-phosphorylated by several independent pathways. Recent reports have analyzed cellular substrates that are specifically tyrosine-phosphorylated in response to EPO. In B6SUT cells, Quelle and Wojcikiewicz8 observed rapid phosphorylation of 74-Kd, 93-Kd, 100-Kd, and 140-Kd substrates. In DA-3 cells, Miura et al15 observed EPO-dependent and IL-3-dependent phosphorylation of common 56-Kd, 70-Kd, and 92-Kd substrates. In addition, EPO uniquely induced phosphorylation of a 72-Kd substrate that has been identified as the EPO-R polypeptide itself.16

The EPO-R can be activated to signal cell growth by binding either EPO or the gp55 glycoprotein of the Friend spleen focus-forming virus (SFFV).17 The EPO-R is activated by EPO at the cell surface, whereas it is activated by gp55 at an undefined intracellular compartment.18 Transfection with the EPO-R cDNA confers EPO-dependent growth on various murine IL-3–dependent cell lines, including Ba/F317 and FDCP-1.19 Ba/F3 is an early pro-B-lymphocyte cell line that has an absolute dependence on murine IL-3 for growth. Ba/F3–EPO-R cells are stably transfected with the EPO-R cDNA and grow in the presence of either EPO or IL-3. Ba/F3–EPO-R–gp55 cells are transfected with the EPO-R cDNA and infected with a retrovirus encoding the gp55 glycoprotein of Friend SFFV17; they grow constitutively without added growth factor.

The purpose of the current work was to identify common phosphotyrosyl protein substrates that are activated by EPO or other growth factors and viral oncogenes. We identified a substrate of 97 Kd that is rapidly tyrosine-phosphorylated in response to EPO, IL-3, and IL-2. Interestingly, this same polypeptide is constitutively phosphorylated when Ba/F3 or CTLL-2 cells coexpress gp55 and EPO-R.

MATERIALS AND METHODS

Cytokines. Highly purified recombinant human EPO was from Genetics Institute (Cambridge, MA). Murine IL-3 was from Biogen (Cambridge, MA). Human IL-2 was from Genzyme (Boston, MA).

Cell culture. The murine IL-3–dependent cell line, Ba/F3, has been previously described.20 CTLL-2 cells (a generous gift from Dr B. Bierer, Dana-Farber Cancer Institute, Boston, MA) were grown...
in RPMI 1640 (JRH Biosciences, Lenexa, KS) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and supplemented with either 0.6 mmol/L of IL-2 or with 0.18 U/mL of human recombinant EPO. Ba/F3 and CTLL-2 cells were grown in a 5% CO2 and 95% air humidified incubator at 37°C.

**MTT reduction assay.** The MTT (dimethylthiazol diphenyltetrazo-lum bromide) reduction assay measures cell viability and proliferation. Cells to be tested were washed twice in RPMI medium without fetal calf serum (FCS) and adjusted to 0.3 x 10^6 cells/mL in RPMI medium + 10% FCS (plain culture medium). Cell suspensions (0.05 mL) were added in duplicate to threefold dilutions of growth factor in the same culture medium in 96-well microtiter plates (Falcon, Oxnard, CA). After 36 hours of incubation at 37°C, 5% CO2, 0.02 mL of MTT in phosphate-buffered saline (PBS) solution (2.5 mg of MTT/mL; Sigma, St Louis, MO) was added to the wells. After a further 4 hours of incubation, 0.15 mL of propanol-2 HCl 0.04 N was added to the same wells. Absorbance of individual wells was then read in an enzyme-linked immunosorbent assay (ELISA) reader (Whittaker ELISA reader, model MA310; Whittaker Bioproducts, Walkersville, MD) at 600 nmol/L.

**Stimulation of cells with growth factors.** For each experiment, cells were collected at 1,000g, resuspended in RPMI + 10% FCS (HyClone), and incubated in RPMI + 10% FCS for 4 hours at 37°C. Starvation of these cells for greater than 6 hours results in apoptosis (A. D’Andrea, unpublished observation). The cells were pelleted at 1,000g and resuspended in 1 mL RPMI + FCS. The cells were stimulated with media containing either recombinant human EPO (1 mU/mL; Genetics Institute), murine IL-3 (10% WEHI-conditioned media), phorbol ester phorbol 12-myristate 13-acetate (PMA; 12 ng/mL; Sigma), or RPMI alone at 37°C for the indicated times.

**Cell lysis, gel electrophoresis, and immunoblotting.** Total cell lysates were prepared as described. Briefly, the cells were spun at 1,000g at 4°C and resuspended in 10 mL of PBS wash buffer containing 0.4 mmol/L orthovanadate, 5 mmol/L EDTA, 10 mmol/L NaF. The cells were resuspended in 300 mL lysis buffer containing 50 mmol/L Tris, pH 7.6, 150 mmol/L NaCl, 0.5% Triton, 10 mg/mL aprotinin, 10 mg/mL leupeptin, 10 mg/mL iodoacetamide, 1 mmol/L orthovanadate, 5 mmol/L EDTA, 10 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, and 4 mmol/L phenylmethylsulfonyl fluoride (PMSF). The cells were incubated on ice for 45 minutes and spun at 14,000g to make a cleared lysate. An aliquot of each cleared lysate was saved for protein determination using the BioRad (Richmond, CA) assay, according to vendor specifications.

Total cell lysates were electrophoresed on 6% to 12% gradient sodium dodecyl sulfate (SDS)-polyacylamide gels. One hundred fifty micrograms of cleared total cellular protein was electrophoresed and electroblotted to nitrocellulose. The nitrocellulose blots were processed and incubated with antisera, as described. Briefly, the blots were incubated with a 1:7,500 dilution of an antiphosphotyrosine monoclonal antibody (MoAb). This MoAb (4G10) does not cross-react with phosphoserine or phosphothreonine. The primary antibody was removed and then incubated with a 1:15,000 dilution of the second antibody, a goat antimouse alkaline phosphatase-conjugated IgG (Promega Biotech, Madison, WI). The secondary antibody was removed and the blot was then incubated in 100 mL Tris, pH 9.5, 100 mmol/L NaCl, 5 mmol/MgCl2, containing 165 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Promega Biotech) and 330 mg/mL nitro blue tetrazolium (NBT; Promega Biotech). The enzymatic color development was stopped by rinsing the filters in water.

**Two-dimensional electrophoretic analysis.** From total cellular lysates, phosphotyrosyl proteins were affinity purified with the antiphosphotyrosine MoAb and analyzed by two-dimensional gel electrophoresis. Five hundred micrograms of extracted whole cell protein was precipitated with the antiphosphotyrosine MoAb and applied to a 4% isoelectric focusing gel as the first dimension. The gel was applied to 8% SDS-polyacrylamide gels as a second dimension (details described in manuscript in preparation; B. Druker). The proteins from the second dimension gel were transferred to nitrocellulose and immunoblotted with the antiphosphotyrosine MoAb as described above.

**Transfection of Ba/F3 and CTLL-2 cells.** The EPO-R cDNA was electroporated into Ba/F3 and CTLL-2 cells as previously described. The cells were resuspended in 0.8 mL of PBS (without calcium or magnesium) to a density of 7 x 10^6 cells/mL. Ten micrograms of linearized EPO-R cDNA (p+psv2neo)23 was added to the 0.8 mL of cells in a sterile conical tube and kept on ice for 10 minutes. The cells and DNA were transferred to a Biorad cuvette (0.4 cm electrode gap) and electroporated using a Biorad Gene pulser set at 960 microfarads and 350 V. After electroporation, the cells were kept on ice for 10 minutes. The cells were resuspended to a density of 0.5 x 10^6 cells/mL in complete medium containing IL-3 (or IL-2 for CTLL-2 cells).

After 3 days, Ba/F3 cells (or CTLL-2 cells) expressing the EPO-R were selected in media containing G418 (1 mg/mL) plus IL-3 (or IL-2) to select for cells expressing the neo-resistance marker. By a similar approach, the expression vector pSG5-EPO, containing the full-length human EPO cDNA, was electroporated into Ba/F3–EPO-R cells to generate an autocrine cell line. Assay of heterologous protein expression was confirmed by immunoprecipitation. The gp55 polypeptide was introduced into Ba/F3 or CTLL-2 cells using a high-titer SFFV supernatant previously described.

**RESULTS**

To investigate the presence of phosphotyrosyl proteins in cells stably transfected with the EPO-R, we performed immunoblots of lysates obtained from these cell lines using an antiphosphotyrosine MoAb. The three Ba/F3 subclones were grown in RPMI medium with 10% FCS (no supplemental growth factor) for 4 hours, followed by stimulation for 15 minutes with the indicated growth factor. Cells were rapidly lysed in the presence of phosphatase and protease inhibitors, and phosphotyrosine-containing proteins were detected by immunoblotting. The parental Ba/F3 cells, induced with IL-3 (Fig 1A, lane 7), showed tyrosine phosphorylation of a 97-Kd and a 38-Kd protein. Other bands seen in lane 7 of Fig 1A are either nonspecific proteins interacting with the antiphosphotyrosine MoAb or phosphotyrosyl proteins with long half-lives whose phosphorylation persists beyond the 4-hour starvation period.

For the Ba/F3–EPO-R cells (Fig 1A, lanes 4 through 6), which proliferate in response to EPO or IL-3, the 97-Kd and 38-Kd substrates were tyrosine-phosphorylated when stimulated with growth factor. For the Ba/F3–EPO-R-gp55 cells (Fig 1A, lanes 1 through 3), the 97-Kd and 38-Kd proteins were tyrosine-phosphorylated even when the cells were cultured with control media without added growth factor (Fig 1A, lane 3). This blot is representative of at least three independent experiments.

The Ba/F3–EPO-R cells (Fig 1A, lanes 4 through 6) were found to have two additional tyrosine-phosphorylated proteins of 55 and 58 Kd compared with the parental Ba/F3 and the factor-independent Ba/F3–EPO-R–gp55. These
Fig 1. Tyrosine phosphorylation of a 97-Kd and a 38-Kd protein in Ba/F3 cells in response to growth factor or viral oncoprotein stimulation. (A) Ba/F3-EPO-R-gp55 cells (lanes 1 through 3), Ba/F3-EPO-R cells (lanes 4 through 6), or Ba/F3 cells (lanes 7 through 9) were grown in RPMI media (plus 10% FCS without supplemental growth factor) for 4 hours. Cells were washed and then treated for 10 minutes with murine IL-3 (lanes 1, 4, and 7), EPO (lanes 2, 5, and 8), or no supplemental growth factor (lanes 3, 6, and 9). Cells were lysed, 150 μg of extracted protein was loaded per lane, the SDS-denatured proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose, and immunoblotting was performed with an antiphosphotyrosine MoAb. (B) A similar experiment as in (A) except that 100 μg of extracted protein was loaded per lane. Ba/F3-EPO-R-gp55 cells (lanes 1 through 3), Ba/F3-EPO-R cells (lanes 4 through 6), or Ba/F3 cells (lanes 7 through 9) were grown in RPMI media (plus 10% FCS without supplemental growth factor) for 4 hours. Cells were washed and then treated for 10 minutes with murine IL-3 (lanes 1, 4, and 7), EPO (lanes 2, 5, and 8), or no supplemental growth factor (lanes 3, 6, and 9).

Fig 2. Time course of EPO-dependent tyrosine phosphorylation of the 97-Kd substrate in Ba/F3-EPO-R cells. Ba/F3-EPO-R cells were grown in RPMI media (plus 10% FCS without supplemental growth factor) for 4 hours. Cells were washed, treated with recombinant human EPO for the indicated time periods, and lysed. Fifty micrograms of extracted protein was loaded per lane. Proteins were transferred to nitrocellulose and immunoblotted with the PY20 (ICN) MoAb followed by radiiodinated antimouse IgG.

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Fig 3. Increased tyrosine phosphorylation of a 97-Kd substrate in Ba/F3 cells expressing a hypersensitive (truncated) EPO-R polypeptide. Ba/F3-EPO-R cells (lanes 1 through 4) or Ba/F3-EPO-R(-91 mutant) cells (lanes 5 through 8) were grown for 2 hours in the absence of supplemental growth factor. Cells were washed and then treated for 10 minutes with no growth factor (lanes 1 and 5), murine IL-3 (lanes 2 and 6), EPO (lanes 3 and 7), or phorbol ester (lanes 4 and 8). Cells were lysed, 150 µg of extracted protein was loaded per lane, the SDS-denatured proteins were resolved by SDS-PAGE and transferred to nitrocellulose, and immunoblotting was performed with an antiphosphotyrosine MoAb.

We next examined the time course of tyrosine phosphorylation of the 97-Kd substrate. Ba/F3-EPO-R cells were starved for 4 hours and then treated with EPO for 0 to 40 minutes (Fig 2). Effects on p97 were evident at 0.5 minutes and maximal at 5 minutes. The p97 phosphoprotein also has the same mobility by one-dimensional gel electrophoretic analysis as a p93 substrate from IL-3-induced MO-7 cells described previously (B. Drukter, unpublished observation).

IL-3 induced the tyrosine phosphorylation of p97 and p38 more strongly than EPO (compare band intensity in Fig 1A, lanes 7 v lanes 4 and 5). We therefore examined p97 and p38 tyrosine phosphorylation in an EPO-dependent cell line expressing a hypersensitive EPO-R mutant (Fig 3). This truncated EPO-R mutant polypeptide lacks 91 amino acids from the carboxy terminus. Although the EPO-induced tyrosine phosphorylation of p97 and p38 is greater for the hypersensitive cells [Ba/F3-EPO-R(-91)] (compare Fig 3,
lanes 7 and 3), IL-3 stimulates a more intense p97 and p38 signal in both cell types. Also, for both Ba/F3-EPO-R and Ba/F3-EPO-R (−91) cell lines, the 55-Kd and 58-Kd tyrosine-phosphorylated substrates were detected.

We next used a two-dimensional electrophoretic analysis (Fig 4) to confirm that the IL-3–induced and EPO–induced p97 tyrosine-phosphorylated substrate are the same polypeptide. Ba/F3–EPO-R cells were again stimulated with no growth factor (Fig 4A), IL-3 (Fig 4B), or EPO (Fig 4C). EPO and IL-3 induced the tyrosine phosphorylation of a p97 doublet (Fig 4B and C). The differences in the tyrosine-phosphorylated substrates in the 50-Kd range (Fig 4B v C) may reflect differences in the EPO–induced versus IL-3–induced differentiation pattern of the cells, as previously described.15

To demonstrate common tyrosine-phosphorylated substrates that exist in other murine cell lines, we next generated stable EPO-R transfectants of the murine IL-2–dependent T-cell line, CTLL-2.24 We reasoned that expression of the EPO-R would confer EPO-dependent growth on CTLL-2 cells because amino acid similarity exists between the EPO-R and the IL-2R (p75).25 The growth characteristics of the Ba/F3–EPO-R subclone and of the CTLL–EPO-R subclone were compared using an MTT reduction assay (Fig 5). Ba/F3–EPO-R cells demonstrated dose-dependent growth in EPO and IL-3, but did not grow in the presence of IL-2 (Fig 5A). CTLL-2 EPO-R cells demonstrated dose-dependent growth in EPO and IL-2, but did not grow in the presence of IL-3 (Fig 5B).

We next compared the induced protein tyrosine phosphorylation in Ba/F3–EPO-R cells and in CTLL–EPO-R cells (Fig 6). As before, stimulation of Ba/F3–EPO-R cells with either EPO or IL-3 resulted in tyrosine phosphorylation of p97 and (weakly) of p38 (Fig 6, lanes 2 and 3). In contrast, stimulation of CTLL–EPO-R with EPO or IL-2 resulted in tyrosine phosphorylation of p97 only (Fig 6, lanes 6 and 7). The p97 appears as a doublet in lanes 2, 3, 6, and 7 in Fig 6; however, only the lower band is inducible with growth factor, and the upper band is noninducible. Interestingly, stimulation of either cell line with phorbol ester (PMA, 12 ng/mL) resulted in tyrosine phosphorylation of p38, but not of p97 (Fig 6, lanes 1 and 3). The background (nongrowth factor–inducible) pattern of tyrosine phosphorylation in the Ba/F3–EPO-R and CTLL–EPO-R is different, further demonstrating the unrelatedness of these cell lines. For instance, the CTLL–EPO-R cells demonstrated an intense nongrowth factor–inducible band at 60 Kd.

To demonstrate whether gp55 would activate EPO-R in other cell types, we infected CTLL-2–EPO-R cells with the SFFV retrovirus encoding gp55 and isolated a factor-independent CTLL–EPO-R–gp55 cell line. Parental CTLL-2 cells, also transfected with gp55, remained dependent on IL-2 for growth (data not shown), demonstrating that gp55 does not activate the endogenous IL-2R in these cells. We next examined the tyrosine phosphorylation of p97 in the various subclones (Fig 7). CTLL–EPO-R–gp55 cells, despite 4 hours of starvation, have a constitutively activated p97 phosphorylation (Fig 7, lane 4). The signal intensity was less than that of IL-2– or EPO–induced phosphorylation (Fig 7, lanes 2 and 3) or that of gp55–induced phosphorylation in Ba/F3–Epo-R–gp55 cells (Fig 1, lane 3). Two other cell lines (CTLL and CTLL–EPO-R) had no p97 signal when these cells were starved and then stimulated (Fig 7, lanes 8 and 12). Again, no p38 tyrosine phosphorylation was observed for CTLL-2 cells except when PMA was used as inducer (Fig 7, lanes 1, 5, and 9). PMA induced the tyrosine phosphorylation of p38 in all CTLL-2 subclones, but did not induce p97 phosphorylation.

Ba/F3 cells can be converted to factor-independent growth by expression of various growth factors, growth factor receptors, or viral oncoproteins. Expression of a
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Fig 6. Tyrosine phosphorylation of a common 97-Kd protein in Ba/F3 cells and in CTLL-2 cells. Ba/F3-EPO-R cells (lanes 1 through 4) or CTLL-EPO-R cells (lanes 5 through 8) were grown in RPMI media for 4 hours. Cells were washed and then treated for 10 minutes with phorbol ester (lanes 1 and 5), murine IL-3 (lanes 2 and 6), EPO (lanes 3 and 7), or no supplement (lanes 4 and 8). One hundred fifty micrograms of extracted protein was loaded per lane, and the SDS-denatured proteins were resolved on an 8% to 12% acrylamide gradient gel and transferred to nitrocellulose for immunoblotting with the antiphosphotyrosine MoAb.

Fig 7. The activation of EPO-R by the Friend virus gp55 glycoprotein induces p97 tyrosine phosphorylation and factor-independent growth of CTLL-2 cells. CTLL-2-EPO-R/gp55 cells (lanes 1 through 4), CTLL-2-EPO-R cells (lanes 5 through 8), or CTLL-2 cells (lanes 9 through 12) were grown in RPMI media for 4 hours. Cells were treated for 10 minutes with phorbol ester (lanes 1, 5, and 9), recombinant human IL-2 (lanes 2, 6, and 10), human EPO (lanes 3, 7, and 11), or no growth factor (lanes 4, 8, and 12). One hundred fifty micrograms of extracted protein was loaded per lane and the SDS-denatured proteins were resolved on a 10% acrylamide gel and transferred to nitrocellulose for immunoblotting with an antiphosphotyrosine MoAb.

constitutive EPO-R polypeptide (EPO-R “C”), containing an arg to cys point mutation at amino acid residue 129, results in constitutive Ba/F3 cell growth. The expression of \( v-abl \), a viral oncprotein containing its own tyrosine kinase catalytic domain, results in constitutive Ba/F3 cell growth. We have also generated a third growth factor-independent subclone of Ba/F3 cells, called Ba/F3-EPO-R-EPO (autocrine). This subclone was stably transfected, first with the EPO-R cDNA and then with the human EPO cDNA (see Materials and Methods). Ba/F3–EPO-R–EPO (autocrine) cells grew independently of exogenous EPO in the media. The growth of these cells was not inhibited by an anti-EPO neutralizing antibody (A. D’Andrea, unpublished observation). The growth characteristics of the various Ba/F3 subclones were compared using an MTT reduction assay (Fig 8). Again, Ba/F3–EPO-R cells demonstrated dose-dependent growth in EPO, whereas the three constitutive cell lines grew independently of exogenous EPO.

To determine the phosphotyrosyl pattern of proteins in these three constitutive Ba/F3 cell lines, we performed
antiphosphotyrosine immunoblots. First, factor-independent Ba/F3–v-abl cells (Fig 9A, lanes 1 through 3) or growth factor-dependent Ba/F3–EPO-R cells (Fig 9A, lanes 4 through 6) were stimulated with no growth factor, EPO, or IL-3. EPO and IL-3 induce tyrosine-phosphorylation of p97 (Fig 9A, lanes 5 and 6). Ba/F3–v-abl cells had multiple tyrosine-phosphorylated substrates of 43 Kd, 55 Kd, 97 Kd, 180 Kd, and 210 Kd, as previously described.27 The number of tyrosine-phosphorylated substrates and the signal intensity in lanes 1 through 3 of Fig 9A demonstrates that these cells have increased tyrosine kinase activity. The intensity per microgram of extracted protein is much greater in the Ba/F3–v-abl cells than in the various Ba/F3 of CTLL-2 cells (Fig 9A). Also, in these constitutively growing Ba/F3–v-abl cells, the addition of EPO or IL-3 did not change the pattern of tyrosine-phosphorylated substrates or the intensity of the bands on the immunoblot (Fig 9A, lanes 2 and 3 v lane 1). The prominent tyrosine-phosphorylated triplet around 50 Kd in this immunoblot was weak (in Ba/F3 cells, Fig 9A, lanes 4 through 6), compared with the tyrosine phosphorylation pattern in Fig 1 (lanes 4 through 6). These variations probably represent differences in the level of tyrosine phosphorylation and in the development time of the blots.

Secondly, p97 was constitutively tyrosine-phosphorylated in Ba/F3–EPO-R–EPO cells (Fig 9B, lane 1) and in Ba/F3–EPO-R “C” cells (Fig 9B, lane 2). Interestingly, in these constitutively growing Ba/F3 cells, the 55-Kd and 58-Kd tyrosine-phosphorylated substrates were also detected. The gp55-activated phosphorylation of p97 may be due to kinase activation of protein tyrosine phosphatase inhibition.

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DISCUSSION

A common tyrosine-phosphorylated substrate of 97 Kd has been identified in Ba/F3 (B cell) and CTLL-2 (T cell) lines activated by several growth factors including EPO, IL-2, and IL-3. Other investigators have detected 93- to 97-Kd proteins that are tyrosine-phosphorylated in response to growth factor. This protein therefore appears to be a common target for growth factor-induced tyrosine phosphorylation in several different cellular systems. Importantly, the constitutive activation of the Ba/F3 cells or CTLL-2 cells by the coexpression of EPO-R and gp55 results in the constitutive phosphorylation of p97. These results argue strongly for a correlation between 97-Kd substrate phosphorylation and mitogenic signaling.

For these reasons, the further identification and cloning of the p97 substrate will be important for the elucidation of early signaling events by these cytokine receptors. Because proteins of approximately 97 Kd have been reported to be phosphorylated on tyrosine in response to a number of growth factors, it will be important to determine whether the growth factor-induced tyrosine phosphorylation of the p97 is a common feature. Also, our results indicate that IL-3 and EPO induce p38 phosphorylation in Ba/F3 cells but not in CTLL-2 cells, suggesting that p38 is not common among the cell systems described or is not required for mitogenic signaling.

Increased cellular protein tyrosine phosphorylation can result from either the activation of protein tyrosine kinases, the inhibition of phosphotyrosine phosphatases (PTPases), or both of these processes. The current studies have not distinguished among these possibilities. Interestingly, the pattern of tyrosine-phosphorylated substrates differs for EPO-stimulated EPO-R versus gp55-stimulated EPO-R. These differences may reflect the different cellular compartments of receptor activation. EPO activates the EPO-R at the cell surface, whereas gp55 activates the EPO-R in an undefined intracellular compartment, perhaps the endoplasmic reticulum.18 Alternatively, different tyrosine kinases or phosphotyrosine phosphatases may be activated by the two different ligands. Regardless of the mechanism, these differing pathways of tyrosine phosphorylation are the first evidence that the EPO and gp55 signaling mechanisms differ. Recent studies demonstrate that EPO and IL-3 stimulate the tyrosine phosphorylation of common substrates as well as unique, ligand-specific substrates.15 Common substrates may be important to the mitogenic pathway induced by both EPO and IL-3. Unique substrates may be important to the factor-specific effects, such as the ability of EPO to induce erythroid differentiation and globin production. Other recent studies demonstrate additional tyrosine-phosphorylated substrates in EPO- and IL-3–responsive cell lines. Although our studies show only a p97 and p38 substrate, these differences may be accounted for by the different antiphosphotyrosine MoAbs and immunoblotting protocols used.

The current work characterizes several new constitutive cell lines that should be of interest to laboratories studying EPO-R signal transduction. It is interesting that a p97 tyrosine-phosphorylated substrate is activated when Ba/F3 cells are transformed with the viral oncoprotein, v-abl, or when the cells express EPO-R “C” or coexpress EPO-R and EPO. Unlike the cytokine receptors, v-abl is an oncoprotein that has its own tyrosine kinase catalytic domain.26 Further analysis will be required to determine whether the p97 is identical in all cell types. In addition, v-abl induces tyrosine phosphorylation of several new substrates not found in cells whose growth is mediated by a cytokine receptor, such as the EPO-R or IL-3R.

Expression of the EPO-R polypeptide alone in CTLL-2 cells confers EPO dependence on these cells. In contrast, the expression of the human GM-CSF receptor polypeptide in CTLL-2 cells does not confer GM-CSF responsiveness (data not shown). CTLL-2 cells must be transfected with the GM-CSF cDNA and the AIC2B cDNA for GM-CSF signaling to occur.29 These results suggest that the EPO-R does not require the AIC2B as a second subunit for EPO signaling. Both Ba/F3 and CTLL-2 cells have intact signal transduction mechanisms in which EPO-R expression alone confers EPO responsiveness.

This report has identified a 97-Kd protein as an important common substrate of tyrosine phosphorylation induced by several growth factors and transforming viral oncoproteins. The elucidation of the function of this protein may therefore be important to analysis of the early events of cytokine receptor signaling.

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