Activation of the Mitogen-activated Protein Kinase Pathway by the Erythropoietin Receptor*

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The erythropoietin receptor (EpoR) belongs to the cytokine receptor family, members of which lack a tyrosine kinase domain. Recent studies, however, have shown that a cytoplasmic tyrosine kinase, JAK2, interacts with the cytoplasmic domain of the EpoR and becomes activated upon binding of Epo to the receptor. Epo has also been shown to stimulate activation of Ras and Raf-1. The present studies were undertaken to examine the possible involvement of Epo-induced tyrosine phosphorylation in activation of the Ras/mitogen-activated protein kinase (MAP kinase) pathway and to determine its significance on the growth signaling from the EpoR. In an interleukin (IL)-3-dependent cell line expressing the transfected wild-type EpoR, Epo, or IL-3 induced tyrosine phosphorylation of Shc and its association with Grb2. These cytokines also induced tyrosine phosphorylation and activation of MAP kinase isofoms ERK1 and ERK2. A mutant EpoR with a carboxyl-terminal deletion of 108 amino acids (H mutant), which is mitogenically functional but lacks tyrosine phosphorylation sites in the carboxyl-terminal region, showed markedly diminished abilities to induce tyrosine phosphorylation of Shc and to phosphorylate and activate MAP kinases. A mutant receptor (PM4 mutant) inactivated by a point mutation, Trp2*2 to Arg, which abrogates the interaction with JAK2, failed to induce any effect on Shc or MAP kinases. In cells expressing a mutant EpoR that is constitutively activated by a point mutation, Arg389 to Cys, in the extracellular portion of the receptor, neither tyrosine phosphorylation of Shc nor activation of MAP kinases by phosphorylation was detectable without stimulation with Epo or IL-3. These results suggest that the carboxyl-terminal region of EpoR may play a crucial role in activation of MAP kinases through the Ras signaling pathway which may be activated by tyrosine phosphorylation of Shc and its association with Grb2. The activation of MAP kinases, however, failed to correlate with the mitogenic activity of mutant EpoRα and thus may not be required for growth signaling from the EpoR.

Erythropoietin (Epo) is a hematopoietic growth factor that regulates the growth and differentiation of erythroid progeni-

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‡ The abbreviations used are: Epo, erythropoietin; EpoR, erythropoietin receptor; IL, interleukin; SH2, Src homology 2; SH3, Src homology

3 MAP kinases, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinase; MEK, MAP kinase/ERK-activating kinase; MDP, myelin basic protein; GM-CSF, granulocyte colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; PAG, polyacrylamide gel electrophoresis.
autophosphorylated receptor tyrosine kinases through its SH2 domain (22, 23) and simultaneously associates through its SH3 domain with mSos1, a guanine-nucleotide-releasing protein that activates Ras by inducing exchange of GDP for GTP on Ras (24). Grb2 thus repositions mSos1 adjacent to Ras, which is located at the plasma membrane. The Shc gene encodes three overlapping proteins of 46, 52, and 66 kDa which possess a carboxyl-terminal SH2 domain and a glycine/proline-rich region but no obvious catalytic domain (25). Shc proteins become tyrosine-phosphorylated upon activation of a variety of receptor tyrosine kinases (25–27) and are phosphorylated constitutively in cells transformed by the v-Src or v-Fps tyrosine kinases (28). Shc has been implicated in activation of Ras, the preparation and properties of rabbit polyclonal antiserum against ERK2 and a monoclonal antibody (4G10) and rabbit antisera against ERK1 (erk1-CT) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). A rabbit antisemur against ERK2 and a monoclonal antibody against Grb2 were purchased from Transduction Laboratories (Lexington, KY). Recombinant human Epo was kindly provided by Sankyo Pharmaceutical Co. Ltd. (Tokyo, Japan).

**Immunoprecipitation and Immunoblotting—**For stimulation with Epo or IL-3, cells were starved for 12 h without IL-3 in complete medium. The cells were then left unstimulated as a negative control or stimulated with a saturating concentration of Epo or IL-3 at 37 °C for 10 min. The cells were lysed in a lysis buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin and leupeptin. For immunoprecipitation of MAP kinases, 1 × 10^6 cells were lysed in 100 μl of boiling lysis buffer containing 1% SDS and 10 μl Tris-HCl (pH 7.4) and boiled for another 5 min. The lysate was then diluted 10-fold with the Triton X-100 lysis buffer and denatured MAP kinases were immunoprecipitated with anti-phosphoryrosine monoclonal antibody 4G10. B, the relevant portion of the membrane was stripped and reprobed with anti-Shc to demonstrate equivalent loading of Shc. The molecular mass markers are indicated and given in kilodaltons. The positions to which p150, JAK2, the tyrosine-phosphorylated form of EpoR (EpoR-PY), and Shc migrated are indicated with arrows. The background band around 50 kDa is the immunoglobulin heavy chain from the immunoprecipitation.

**activation of MAP kinases. However, activation of MAP kinases failed to correlate with the mitogenic activity of mutant EpoRs.**

**MATERIALS AND METHODS**

**Cells and Reagents—**A clone of 32D cells, an IL-3-dependent cell line originally isolated from long term bone marrow cultures, has been previously described (37). 32D clones expressing the wild-type or various mutant EpoRs and IL-3-dependent DA3 cells expressing the wild-type EpoR were also described previously (10, 13) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 10% WEHI-3-conditioned medium as a source of IL-3.

An expression plasmid for activated mutant EpoR with an Arg^{129} to Cys mutation was constructed by primer-mediated mutagenesis using the polymerase chain reaction method as described previously (10). Transfection of the plasmid into DA3 cells and isolation of a clone expressing the mutant EpoR were also carried out as described previously (13). In brief, DA3 cells were transfected with 19 μg of the expression plasmid and 1 μg of the pSV2neo plasmid by electroporation and selected in medium containing G418. Six clones were isolated by limiting dilution and confirmed to grow in medium containing neither IL-3 nor Epo. These clones were then analyzed by ^{32}P-Epo binding assays, and the clone that bound the highest radioactivity was used for the subsequent studies.

For measurement of the cell number increase, cells were cultured in 25-cm^2 culture flasks at a density of 1 × 10^5 cells/ml of culture in 10% fetal calf serum-containing RPMI 1640 medium supplemented with 10% WEHI-3-conditioned medium or with 4 units/ml human recombinant Epo. Culture media were changed every 2 days. Viable cell counts were determined by trypsin blue staining.

The preparation and properties of rabbit polyclonal antiserum against the cytoplasmic portion of recombinant murine EpoR (38) or against synthetic peptides from Jak1 and Jak2 (39) have been described. Anti-phosphoryrosine monoclonal antibody (4G10) and rabbit antiserum against Shc and ERK1 (erk1-CT) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). A rabbit antisemur against ERK2 and a monoclonal antibody against Grb2 were purchased from Transduction Laboratories (Lexington, KY). Recombinant human Epo was kindly provided by Sankyo Pharmaceutical Co. Ltd. (Tokyo, Japan).

For stimulation with Epo or IL-3, cells were starved for 12 h without IL-3 in complete medium. The cells were then left unstimulated as a negative control or stimulated with a saturating concentration of Epo or IL-3 at 37 °C for 10 min. The cells were lysed in a lysis buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin and leupeptin. For immunoprecipitation of MAP kinases, 1 × 10^6 cells were lysed in 100 μl of boiling lysis buffer containing 1% SDS and 10 μl Tris-HCl (pH 7.4) and boiled for another 5 min. The lysate was then diluted 10-fold with the Triton X-100 lysis buffer and denatured MAP kinases were immunoprecipitated with anti-
MAP kinase antibodies.

For immunoprecipitation, a relevant antibody was added to the lysates along with protein A-Sepharose beads and incubated for 4 h at 4 °C. The beads were washed extensively, and the proteins bound to the beads were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, immunoblotted with the indicated antibody, and developed by the enhanced chemiluminescence (ECL) system (Amer sham Corp.). Aliquots of the cell lysates were also subjected to immunoblotting after directly mixed with equal volumes of 2 x Laemmli's sample buffer and heated at 100 °C for 5 min. For reprobing with a different antibody, the membranes were treated at 50 °C for 30 min with stripping buffer containing 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7).

For determination of the stoichiometry of tyrosine phosphorylation of Shc and MAP kinases, 2 x 10^5 cells stimulated with 75 units/ml Epo for 10 min were lysed in 1 ml of the lysis buffer. After clarification, 50 ml of the lysate was mixed with the equal volume of 2 x SDS buffer and boiled for 5 min, whereas 500 ml of the lysate was immunoprecipitated with 10 μg of the anti-phosphotyrosine 4G10 antibody conjugated with agarose beads and subsequently eluted with 50 μl of 1 x SDS buffer by boiling for 5 min. The whole cell lysate and the immunoprecipitate were diluted appropriately and varying amounts of the samples were subjected to immunoblot analysis using antibody against phosphorytrosine, Shc, or MAP kinases.

**Kinase Assays of Anti-MAP Kinase Immunoprecipitates in Myelin Basic Protein (MBP)-containing Gels after SDS-PAGE—** Determination of MAP kinase activity in MBP-containing gel was carried out essentially as described (40). In brief, anti-ERK1 immunoprecipitates were prepared as described above and electrophoresed on an SDS-12% polyacrylamide gel containing 0.5 mg/ml MBP. After removing SDS with buffer containing 20% 2-propanol, the gel was denatured with 6 M guanidine HCl and then renatured in a 0.04% Tween 40-containing buffer. Phosphorylation of MBP was carried out by incubating the gel at 22 °C for 1 h in 40 mM HEPES (pH 7.5), 0.1 mM MgCl2, 20 μM ATP, and 25 μCi of [γ-32P]ATP. After incubation, the gel was washed extensively with 5% trichloroacetic acid, 1% pyrophosphate solution, dried, and subjected to autoradiography. The radioactivity of phosphorylated MBP was also quantified by Bio-Imaging Analyzer BAS2000 (Fuji Film, Tokyo).

**RESULTS**

**Epo Induces Tyrosine Phosphorylation of Shc and Its Association with Grb2—** To explore the signal transduction pathways from the EpoR, we first examined whether Epo stimulation induces tyrosine phosphorylation of Shc, which has been implicated in coupling receptor tyrosine kinases to the Ras signaling pathways. As shown in Fig. 1A, Epo stimulation induced tyrosine phosphorylation of 150-, 130-, 97-, 92-, 72-, 70-, and 52-kDa proteins in the 32D cells expressing the wild-type EpoR (32D/EpoR-Wt). IL-3 stimulation induced an almost identical pattern of tyrosine phosphorylation except that the 72-kDa protein was not phosphorylated after IL-3 stimulation. In accordance with our previous results (13, 18, 19), anti-phosphotyrosine blotting of immunoprecipitates obtained with relevant antisera showed that the 72- and 130-kDa substrates were the EpoR and JAK2, respectively, whereas it was revealed that JAK1 was not phosphorylated after stimulation with these cytokines in the 32D cells examined.

Anti-phosphotyrosine blotting of anti-Shc immunoprecipitates revealed the presence of tyrosine-phosphorylated 52- and
49-kDa proteins in cells stimulated with Epo or IL-3 (Fig. 1A). These proteins were identified as Shc by reprobing with anti-Shc (Fig. 1B). In addition, tyrosine phosphorylated 210- and 150-kDa proteins were coimmunoprecipitated with Shc after Epo or IL-3 stimulation.

To address the functional significance of tyrosine phosphorylation of Shc, we next examined previously characterized 32D clones expressing various mutant EpoRs (10, 13). 32D/EpoR-H cells express the truncated H mutant lacking the carboxyl-terminal 108 amino acids. Although this mutant lacks the tyrosine phosphorylation sites in the carboxyl-terminal region and fails to associate with PI 3-kinase (15), it retains the abilities to activate JAK2 (18, 19) and to transduce a mitogenic signal (10, 13). The mitogenic response of 32D/EpoR-H, which was selected by the G418 resistance, to Epo was thus comparable with that to IL-3 as determined by [3H]thymidine incorporation (10) or by the growth curves (Fig. 2). 32D/EpoR-PM4 cells express the PM4 mutant, which contains a mutation, Trp282* to Arg (W282R), in a membrane proximal region of the cytoplasmic domain that shows homology with other members of the cytokine receptor superfamily (10). Having lost the ability to associate with JAK2 to activate its kinase activity (19), the PM4 mutant fails to induce tyrosine phosphorylation of cellular substrates, including the receptor itself, and to elicit a mitogenic response (10, 13). 32D/EpoR-PM4, thus, failed to grow in response to Epo, as shown in Fig. 2.

The abilities of these mutant EpoRs to induce tyrosine phosphorylation of cellular proteins were first examined by anti-phosphotyrosine blotting of total cell lysates. As shown in Fig. 3A, Epo induced tyrosine phosphorylation of p130 (JAK2) and p92 in 32D/EpoR-H cells. In most of the repeated experiments, the tyrosine phosphorylation of JAK2 in these cells was enhanced as compared with that in the 32D/EpoR-Wt cells, whereas p70 showed variable degrees of tyrosine phosphorylation in response to Epo. On the other hand, the tyrosine phosphorylation of p62 and p49, which correspond to Shc, after Epo stimulation in 32D/EpoR-H was constantly and remarkably diminished as compared with that observed after IL-3 stimulation in this cell line or after Epo stimulation in 32D/EpoR-Wt. The PM4 mutant failed to induce any detectable tyrosine phosphorylation of cellular substrates in response to Epo (Fig. 3A).

The tyrosine phosphorylation of Shc in these cells was then directly examined by anti-phosphotyrosine blotting of anti-Shc immunoprecipitates. Results shown in Fig. 3B confirmed that the Epo-induced tyrosine phosphorylation of Shc was severely impaired or abolished in 32D/EpoR-H or 32D/EpoR-PM4, respectively.

Because tyrosine-phosphorylated Shc has been shown to activate the Ras signaling pathway by physically associating with the Grb2-mSos1 complex through the SH2 domain of Grb2, the anti-Shc immunoprecipitates were reprobed with anti-Grb2 to examine the association of Grb2 with Shc in Epo-stimulated cells. As shown in Fig. 3D, in 32D/EpoR-Wt, Grb2 was shown to coimmunoprecipitate with Shc after stimulation with Epo or IL-3. 32D/EpoR-H cells also showed the Epo-induced association of Grb2 with Shc. However, the amount of Grb2 associated with Shc was markedly decreased in accordance with the decrease in tyrosine phosphorylation of Shc. Epo failed to induce the association of Grb2 with Shc in 32D/EpoR-PM4 (Fig. 3D).

Tyrosine Phosphorylation and Activation of MAP Kinases Induced by Epo Stimulation—Recent studies have revealed that activation of MAP kinases by a variety of receptor tyrosine kinases is mediated by Ras (31, 32). Since Epo has been shown to stimulate the activation of Ras (34, 35), we next examined whether Epo stimulation induces tyrosine phosphorylation and activation of MAP kinases, which are activated by phosphorylation on both tyrosine and serine/threonine residues (31, 32).

To examine tyrosine phosphorylation of MAP kinases, immunoprecipitates obtained with anti-ERK1 (erk1-CT), which also recognizes ERK2, were examined by anti-phosphotyrosine blotting. As shown in Fig. 4A, 42- and 44-kDa species of MAP kinases, which should correspond to ERK2 and ERK1, respectively, were found to be tyrosine-phosphorylated after Epo or IL-3 stimulation in 32D/EpoR-Wt. However, tyrosine phosphorylation of MAP kinases induced by Epo stimulation was remarkably reduced in 32D/EpoR-H (Fig. 4A). Reprobing of the membrane with anti-ERK1 confirmed that the 42- and 44-kDa species of tyrosine-phosphorylated proteins are directly recognized by anti-ERK1 and demonstrated equal loading of samples (Fig. 4A, center panel). The anti-ERK1 immunoprecipitates were then subjected to the MAP kinase assay in MBP-
Fig. 5. Stoichiometry of tyrosine phosphorylation of Shc and MAP kinases. Increasing amounts of whole cell lysate from 32D/EpoR-Wt cells stimulated with Epo for 10 min (lanes 1-6) and anti-phosphotyrosine immunoprecipitate from unstimulated (lane 7) or Epo-stimulated (lane 8) cell lysate were subjected to anti-phosphotyrosine blotting (left panels) or immunoblotting with anti-Shc (A) or anti-MAP kinase (B). Whole cell lysate from 32D/EpoR-Wt cells unstimulated (lane 9) or stimulated with Epo (lane 10) was also subjected to anti-phosphotyrosine blotting. The amount of sample applied to each lane is represented by the corresponding volume of original cell lysate (2 × 10⁶ cells/ml) and indicated under the panels.

containing gel. As shown in Fig. 4A (lower panel), Epo stimulation was found to stimulate the kinase activities of both ERK1 and ERK2. However, although the effect of Epo on the MAP kinase activity was about half as much as that of IL-3 in 32D/EpoR-Wt, Epo showed only a marginal effect on the MAP kinase activity in 32D/EpoR-H (Fig. 4, A and B). In 32D/EpoR-PM4 cells, Epo failed to induce any detectable tyrosine phosphorylation or activation of MAP kinases (data not shown).

Stoichiometry of the Tyrosine Phosphorylation of Shc and MAP Kinases—We next tried to determine what fraction of Shc or MAP kinases is tyrosine-phosphorylated in response to Epo stimulation. For this purpose, phosphotyrosyl proteins were immunoprecipitated with the 4G10 antibody from Epo-stimulated 32D/EpoR-Wt cell lysate and, along with varying amounts of whole cell lysate, subjected to immunoblot analyses. First, the efficiency of immunoprecipitation with 4G10 was evaluated by immunoblotting with this antibody. The amount of tyrosine-phosphorylated Shc or MAP kinases in the 4G10 immunoprecipitate was then estimated by immunoblotting with anti-Shc or anti-MAP kinase, respectively. As shown in Fig. 5, the efficiency of immunoprecipitation with 4G10 was rather low and varied significantly with each phosphotyrosyl protein, which may be at least partly because some phosphotyrosyl tyrosine residues are involved in intramolecular or intermolecular interaction with the SH2 domains and thus may not bind with 4G10. Densitometric analysis of the results shown in Fig. 5, representative of three repeated experiments, revealed that the efficiency of 4G10 immunoprecipitation of tyrosine-phosphorylated Shc or ERK-1 was ~8 or ~5%, respectively, whereas ~3 or ~0.5% of the total cellular Shc or ERK-1, respectively, was shown to be present in the 4G10 immunoprecipitates. From these results, it was calculated that ~38 or ~10% of total cellular Shc or ERK-1, respectively, undergoes tyrosine phosphorylation in response to Epo in 32D/EpoR-Wt cells. Thus, Epo induced significant but not near-stoichiometric tyrosine phosphorylation of Shc and MAP kinases. The rather low stoichiometry of tyrosine phosphorylation, particularly of MAP kinases, is compatible with our results that only a barely detectable activity of MAP kinases was observed in Epo-stimulated 32D/EpoR-H cells, in which Epo-induced tyrosine phosphorylation of Shc and MAP kinases is markedly diminished as compared with that in 32D/EpoR-Wt.

Cells Expressing a Constitutively Activated EpoR Mutant Show Epo-independent Growth without Tyrosine Phosphorylation of Shc or Activation of MAP Kinases—Previously, a single point mutation, resulting in an Arg to Cys change at residue 129 of the extracellular domain of EpoR, has been shown to activate the receptor independent of Epo binding (41, 42). The R129C mutant was confirmed to abrogate the factor-dependence of DA3 cells, because six subclones of transfected cells, selected by the G418 resistance due to the cotransfected pSV2neo plasmid, grew in the absence of IL-3 or Epo; the clone used in this study grew comparably in medium with or without Epo (Fig. 6). We examined the tyrosine phosphorylation states of Shc and MAP kinases as well as the activity of MAP kinases in a DA3 clone that expresses this mutant receptor and thus grows without added growth factors. As shown in Fig. 7, anti-phosphotyrosine and anti-Grb2 blotting of anti-Shc immunoprecipitates showed that tyrosine phosphorylation of Shc and
its association with Grb2 was dependent on Epo stimulation in these cells showing the Epo-independent growth. Tyrosine phosphorylation and activation of MAP kinases were also dependent on stimulation with Epo or IL-3 (Fig. 8). These cells were thus found to grow without showing tyrosine phosphorylation of Shc or activation of MAP kinases in growth factor-deficient medium.

DISCUSSION
The present studies demonstrated that Shc and MAP kinases are among the substrates of tyrosine phosphorylation induced by Epo stimulation. In addition, Epo induced physical association of Shc with Grb2 and activated the catalytic activity of MAP kinases. However, Epo did not have any effect on these signaling molecules in cells expressing the PM4 mutant EpoR, which has a point mutation, Trp282 to Arg, abolishing the ability of EpoR to couple with JAK2 to transduce a mitogenic signal. The effects of Epo on Shc and MAP kinases were markedly diminished in cells expressing the mitogenically functional, truncated H mutant, which has lost tyrosine phosphorylation sites in the carboxyl-terminal region. In cells expressing the mutant EpoR that is constitutively activated by a point mutation, Arg129 to Cys, tyrosine phosphorylation of Shc and its association with Grb2 as well as tyrosine phosphorylation and activation of MAP kinases were not observed without stimulation with Epo or IL-3. Thus, in cells expressing these mutant EpoRs, activation of MAP kinases correlated with tyrosine phosphorylation of Shc and its association with Grb2. Taken together with previous reports showing that Epo activates Ras and Raf-1 (34-36), the present results suggest that Shc and Grb2 may mediate activation of the Ras/MAP kinases pathway from the EpoR and that the carboxyl-terminal region of the receptor may play a major role in activation of this pathway. Activation of MAP kinases, however, may not be required for the transduction of growth signal from the EpoR, because the activity of MAP kinases did not correlate with proliferation of cells expressing the mutant EpoRs.

Recent studies also demonstrated that Epo or IL-3 induces tyrosine phosphorylation of Shc and its association with Grb2 (43-45). Damen et al. (43) also observed that tyrosine-phosphorylated Shc associates with the EpoR in a human MO7 cell line expressing transfected murine EpoR. However, association of Shc with the EpoR was not observed in DA3 transfectants in their studies (43). We also failed to observe any significant association of the EpoR with Shc in the 32D or DA3 clones examined. A tyrosine-phosphorylated protein of 145 kDa (43, 44) or 140 kDa (45), which should correspond to the 150-kDa protein in the present studies, were also found to be associated with Shc in cells stimulated with Epo or IL-3. Although the identity of this species remains unknown, we found in a previous study that this 150-kDa protein coimmunoprecipitated with the EpoR or JAK2 in digitonin lysates and underwent tyrosine phosphorylation in vitro (19). It is thus possible that the EpoR-JAK2 complex physically associates with the 140-kDa protein, Shc, and Grb2, although the association may be transient or unstable. Although the mechanism of how the EpoR activates Ras has remained elusive, inhibition of the Epo-induced activation of Ras by tyrosine kinase inhibitors suggested that it may be mediated through tyrosine phosphorylation (34). In a previous study (34), GTPase-activating protein was implicated in the Epo-induced activation of Ras, because Epo induced tyrosine phosphorylation of Ras GTPase-activating protein in a human erythroleukemia cell line (HEL). On the other hand, it was demonstrated in the present study and also in previous studies (43-45) that Epo stimulation induces tyrosine phosphorylation of Shc and its association with Grb2. Since Shc is strongly

FIG. 7. Tyrosine phosphorylation of Shc and its association with Grb2 in cells expressing the constitutively activated mutant EpoR. DA3 cells expressing the wild-type EpoR (WT) or the constitutively activated mutant (R129C) were cultured in the absence of IL-3 or Epo for 12 h and either left unstimulated (-) or stimulated (+) for 10 min with Epo (Ep) or IL-3 (IL3). The cells were lysed and subjected to immunoprecipitation with anti-Shc. The immunoprecipitates were resolved by 5-20% SDS-PAGE followed by immunoblotting with anti-phosphotyrosine (aPY) and reprobing with anti-Grb2 (aGrb2). The positions of p150, Shc, Grb2, and the heavy chain of immunoglobulin (IGH) are indicated.

FIG. 8. Tyrosine phosphorylation and activation of MAP kinases in cells expressing the constitutively activated mutant EpoR. DA3 cells expressing the wild-type EpoR (WT) or the activated mutant (R129C) were left unstimulated or stimulated with Epo (Ep) or IL-3 (IL3) for 10 min as indicated. The cells were lysed and the lysates were immunoprecipitated with anti-ERK1 (aERK1-CT). The immunoprecipitates were resolved by 8.4% SDS-PAGE and subjected to immunoblotting with anti-phosphotyrosine (aPY) followed by reprobing with anti-ERK1 (aERK1). Aliquots of the immunoprecipitates were also subjected to the MAP kinase assay in MBP-containing gel (IVK) as described under "Materials and Methods." The positions of 44-kDa ERK1 and 42-kDa ERK2 are indicated.
implicated in the activation of Ras through its interaction with Grb2 and indirectly with mSos1 (24, 26). Epo-induced activation of Ras may be mediated through tyrosine phosphorylation of Shc. In accordance with this idea, in cells expressing the mutant EpoRs, tyrosine phosphorylation of Shc and its association with Grb2 correlated with activation of MAP kinases, which are major targets of the Ras signaling pathway (31, 32). Very recently, we found that Epo also induces tyrosine phosphorylation of Vav (46), which has also been implicated in the Ras signaling pathway (47). However, the tyrosine phosphorylation of Vav induced by Epo did not show correlation with the activation of MAP kinases in cells expressing the various mutant EpoRs (46). Therefore, a role, if any, of Vav in the Epo-induced activation of Ras remains to be known.

Recent studies have demonstrated that activation of MAP kinases is involved in signaling from most of the members of cytokine receptor family. However, the functional significance of MAP kinase activation in regulation of growth and differentiation of hematopoietic cells by these cytokines remains elusive. In fibroblast, the MAP kinase activity was shown to be required for proliferation (48). Correlation of proliferative response with activation of MAP kinases has also been shown in hematopoietic cells stimulated with G-CSF; G-CSF activates MAP kinases in cell lines that proliferate in response to G-CSF, whereas neither G-CSF-induced granulocytic differentiation of 32D cells nor nonproliferative response of mature neutrophils to G-CSF was associated with activation of Ras, Raf-1, and MAP kinases. Activation of MAP kinases is involved in signaling from most of the members of the receptor family of tyrosine kinases, which has also been implicated in the cell translocation from the cytoplasm to the nucleus and form DNA-binding complexes which recognize the sis-inducible element in the c-fos promoter (54). A similar Ras-independent signaling pathway directly activating DNA-binding proteins that recognize the sis-inducible element is also utilized by receptors for various growth factors, cytokines, and interferons (55, 56). Importantly, the JAK family of tyrosine kinases, which includes JAK2, is strongly implicated in this pathway (55, 56). It is thus tempting to speculate that JAK2, which is activated by the H mutant, may activate a latent transcription factor in the cytoplasm through tyrosine phosphorylation and thus leads to activation of the promoters of the c-fos gene and other genes involved in cellular proliferation.

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