Tyrosine Residues within the Intracellular Domain of the Erythropoietin Receptor Mediate Activation of AP-1 Transcription Factors*

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** Binding of erythropoietin (Epo) to the Epo receptor (EpoR) initiates a signaling cascade resulting in tyrosine phosphorylation of several proteins and induction of AP-1 transcription factor(s). While Epo is known to activate c-fos gene expression, the mechanism of AP-1 activation is unknown. Here we show that AP-1 activation by Epo requires tyrosine kinase activity and also de novo protein synthesis. Using a mutant EpoR containing no cytosolic tyrosine residues, and a set of eight mutants containing a single cytosolic tyrosine residue, we show that multiple EpoR tyrosines, thought to activate multiple intracellular signal transduction proteins, can mediate AP-1 activation. An EpoR containing only tyrosine 343 or tyrosine 464 supports a maximal level of AP-1 activation. We also show that AP-1 activation does not require maximal STAT5 activation and may occur via a STAT5-independent signaling pathway.

Erythropoietin (Epo)† is a kidney-produced hormone that regulates erythropoiesis by controlling the survival, proliferation, and differentiation of committed erythroid progenitors (1, 2). Epo signals through the Epo receptor (EpoR), a member of the cytokine receptor superfamily (3–5). Epo induces dimerization of cell surface receptors, leading to phosphorylation and activation of the receptor-associated protein tyrosine kinase JAK2 (6). JAK2, or another as yet unknown kinase, phosphorylates multiple tyrosine residues on the cytoplasmic domain of the EpoR, creating “docking sites” for the SH2 domains of signaling molecules such as protein tyrosine phosphatases SHP-1 (7) and SHP-2 (8), phosphatidylinositol 3-kinase (9, 10), and STAT5 (11–13). Several of these EpoR-docked proteins, including STAT5, then become tyrosine phosphorylated and activated, presumably by JAK2.

However, little is known of how these signal transduction proteins, activated by the receptor complex assembled at the plasma membrane, affect erythroid gene expression in the cell nucleus. Among the genes that are activated in response to Epo and other cytokines are those encoding transcription factors, such as AP-1, that can subsequently activate other genes in a transcriptional cascade.

The AP-1 complex is a transcriptional activator composed of members of the Jun and Fos families, including c-Fos, Fra1, Fra2, c-Jun, JunB, and JunD. These proteins form homo- or heterodimers that bind to a conserved DNA sequence, identified initially as TRE (TPA responsive element) (14), and activate transcription of target genes. While Jun-Jun homodimers are able to bind to TRE sites and activate transcription, Fos proteins are active only as heterodimers with Jun proteins; any Fos protein can form a complex with any Jun, allowing for generation of a large number of transcription factors. AP-1 activity is controlled by increased expression of both fos and jun genes (15, 16) and by phosphorylation of both Fos and Jun proteins (17). While Epo is known to activate expression of the c-fos gene (18–20), the mechanism of AP-1 activation via the EpoR remains largely unknown.

Here we studied Epo activation of AP-1 in Epo-dependent HCD57 erythroleukemia cells and in 32D myeloid cells that express wild-type and mutant Epo receptors. We found that ongoing protein synthesis is required for Epo activation of AP-1. We showed that tyrosine kinase activity is required for AP-1 activation by Epo, and that a mutant EpoR lacking all 8 tyrosines in the cytoplasmic tail is incapable of AP-1 activation. However, either of two tyrosine residues in the EpoR, Tyr-343 or Tyr-464, can support maximal activation of AP-1 DNA binding and also Epo-induced transcriptional transactivation of promoters containing the AP-1-binding site TRE. One of these tyrosine residues, Tyr-343, supports activation of STAT5 but another one, Tyr-464, does not. These and other results we present indicate that multiple EpoR-induced intracellular signal transduction cascades can lead to activation of AP-1, and that Epo activation of AP-1 can occur independently of STAT5 activation.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—The wild-type EpoR cDNA was introduced into the BamHI and EcoRI restriction sites of the retrovirus expression vector pBabe (puro) (21) as described in Ref. 12. Mutant EpoRs lacking all 8 tyrosine residues in the cytoplasmic domain (F8) or containing only one tyrosine (F7 YXXX) were generated by overlap extension and cloned into the ApoI and EcoRI restriction sites in pBabe-Epo-R (puro) as described in Ref. 12. The truncated EpoRs 1–374 and 1–414 were generated by introducing an in-frame stop codon after amino acid positions 374 or 414, respectively, and an EcoRI restriction site. The fragments were amplified by polymerase chain reaction and subcloned into the ApoI and EcoRI restriction sites of the expression vector pBabe-Epo-R (puro).

Selection of Stable Cell Lines—pBabe (puro) DNA encoding the wild-

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Fig. 1. AP-1 activation by Epo in HCD-57 and 32D-EpoR cells. Left panel, kinetics of AP-1 activation by Epo. Cells were starved for 24 h and then left untreated (0 h) or stimulated with 100 units/ml Epo for 3 and 6 h. Nuclear extracts were prepared and analyzed by EMSA using a 32P-TRE probe. Right panel, specificity of the AP-1 DNA-binding complex in Epo-stimulated cells. Nuclear extracts were prepared from Epo-induced HCD-57 or 32D-EpoR cells and preincubated with no addition (none) or with a 100-fold molar excess of unlabeled oligonucleotides containing the specific AP-1 binding site (TRE) or a mutated AP-1-binding site (mutant TRE). The extracts were analyzed by EMSA using a 32P-TRE probe.

**RESULTS**

Epo Activates AP-1 DNA Binding in HCD57 and 32D-EpoR Cells—Activation of AP-1 DNA binding was analyzed both in the Epo-dependent erythroid progenitor cell line HCD57, which expresses endogenous EpoRs, and in interleukin 3-dependent myeloid 32D-EpoR cells which express a transfected wild-type Epo and can proliferate in the presence of Epo. HCD57 and 32D-EpoR cells were starved of growth factors and serum for 24 h, and stimulated with Epo for 3 and 6 h. Nuclear extracts were prepared and tested for proteins that bind to a radiolabeled oligonucleotide probe containing a consensus AP-1-binding site, TRE, using an EMSA. In both cell lines Epo induced the formation of an AP-1-DNA complex (Fig. 1, left panel). Maximum activation occurs at 6 h; by 12 h the level of AP1 complexes has been reduced over 3-fold (data not shown.) As judged by the different mobilities of the AP-1 complexes in the two cell lines, their protein composition is expected to be different. Epo-induced AP-1 activity is specific for the TRE-binding site since preincubation of nuclear extracts with a 100-fold molar excess of unlabeled oligonucleotide containing the TRE site prevented AP-1 binding to the radiolabeled probe, while preincubation with a 100-fold excess of a mutant TRE with reduced specificity for AP-1 did not affect AP-1 binding (Fig. 1, right panel).

**AP-1 Activation by Epo Requires Ongoing Protein Synthesis**—Most of the increase in AP-1 activity occurred between 3 and 6 h after Epo addition (Fig. 1) and could involve activation of the genes encoding the components of the AP-1 complex as well as modification (e.g. phosphorylation) of pre-existing AP-1 proteins. To determine whether Epo activation of AP-1 requires de novo protein synthesis, we used cycloheximide, an inhibitor of protein synthesis. HCD57 cells were treated with CHX for various periods of time before and after stimulation with Epo and nuclear extracts were analyzed for AP-1 DNA binding (Fig. 2A). Treatment of cells with CHX for 0.5 h before stimulation with Epo completely inhibited induction of AP-1 DNA binding.

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activity (Fig. 2A, lane 3) while addition of CHX simultaneous with Epo or 0.5 and 1 h after Epo addition dramatically decreased induction of AP-1 DNA binding activity (Fig. 2A, lanes 4–6). The low level of AP-1 binding activity induced in the presence of CHX may reflect activation of pre-existing AP-1 complexes, presumably by phosphorylation, in response to Epo. Thus, the results in Fig. 2A indicate that most of the increase in AP-1 binding activity following Epo addition depends on de novo protein synthesis.

However, because the half-life of newly made Epo receptors is very short, less than 2 h, and because cell surface Epo receptors are degraded with a half-life of only 30–45 min (26, 27), we needed to show that the effects of CHX on AP1 induction were not due to a reduction in the level of functional EpoRs. The experiment in panel B of Fig. 2 shows that sufficient EpoRs remain after 6 h of CHX treatment to support normal Epo induction of STAT5 DNA binding activity. Here HCD57 cells were treated with CHX for up to 6 h before stimulation with Epo; after 10 min nuclear extracts were analyzed for STAT5 DNA binding activity. Clearly induction of STAT5 DNA binding activity is unaffected even by a 6-h pretreatment with CHX, showing that ongoing protein synthesis is not required for Epo activation of STAT5 binding. Thus, the effects seen in panel A are not due to a depletion in the numbers of functional EpoRs on the cells even after 6 h of CHX treatment. The requirement of ongoing protein synthesis for AP-1 activation by Epo suggests that Epo induces transcriptional (or translational) activation of AP-1 components.

**Tyrosine Kinase Activity Is Required for AP-1 Activation by Epo**—To examine the role of tyrosine kinase activation in Epo induction of the AP-1 complex, HCD57 cells were incubated with the tyrosine kinase inhibitor genistein and with the tyrosine phosphatase inhibitors zinc chloride (ZnCl₂) and sodium orthovanadate (Na₃VO₄) in the absence or presence of Epo. The results (Fig. 3) show that genistein completely inhibited AP-1 activation by Epo (lane 7), while zinc chloride and sodium orthovanadate augmented AP-1 activation of AP-1 (compare lane 4 with lanes 5 and 6). Addition of zinc chloride to cells not treated with Epo did not cause significant AP-1 activation (lane 2) while addition of sodium orthovanadate slightly increased AP-1 activity in the absence of EPO (lane 3). Similar results were obtained with 32D-EpoR cells (data not shown). Our results indicate that phosphorylation by a protein tyrosine kinase is essential for Epo activation of AP-1.

**Tyr-343 and Tyr-464 in the Cytoplasmic Domain of the EpoR Independently Support AP-1 Activation**—To determine the segment(s) of the cytosolic domain of the EpoR which mediate AP-1 activation, we first used 32D cells expressing mutant EpoRs missing 42 (EpoR 1–441), 69 (EpoR 1–414), or 109 (EpoR 1–374) C-terminal amino acids; EpoR 1–374 contains the minimal cytosolic domain essential for cell proliferation and erythroid differentiation (11, 28–30). We also employed EpoR F8, containing a full-length cytosolic domain but with all 8 cytosolic tyrosines mutated to phenylalanine; F8 cannot stimulate cell proliferation or erythroid differentiation (10, 31). EMSA analysis of nuclear extracts show that all truncated EpoRs are able to activate AP-1 binding with an efficiency comparable to that of the wild-type EpoR (Fig. 4). However, Epo activation of AP-1 binding is dramatically reduced in cells expressing mutant EpoR F8 (Fig. 4), demonstrating that tyrosine residue(s) of the EpoR are necessary for Epo activation of AP-1. Mutant EpoR 1–374 contains only tyrosine residue 343 and is able to support normal AP-1 activation. In contrast, EpoR 1–374 with a Y343F mutation is unable to support cell proliferation (30) and cannot support normal AP-1 activation (data not shown). Thus, we conclude that (phospho)Tyr-343 can stimulate cell proliferation or erythroid differentiation (10, 31). EMSA analysis of nuclear extracts show that all truncated EpoRs are able to activate AP-1 binding with an efficiency comparable to that of the wild-type EpoR (Fig. 4). However, Epo activation of AP-1 binding is dramatically reduced in cells expressing mutant EpoR F8 (Fig. 4), demonstrating that tyrosine residue(s) of the EpoR are necessary for Epo activation of AP-1. Mutant EpoR 1–374 contains only tyrosine residue 343 and is able to support normal AP-1 activation. In contrast, EpoR 1–374 with a Y343F mutation is unable to support cell proliferation (30) and cannot support normal AP-1 activation (data not shown). Thus, we conclude that (phospho)Tyr-343 can stimulate cell proliferation or erythroid differentiation (10, 31).

**To identify other tyrosine residues in the cytoplasmic domain of EpoR that can support AP-1 activation, we used 32D cells stably expressing mutant EpoRs containing only one tyrosolic tyrosine; these mutants are named F7 Yxxx according to the single tyrosine present. Pools of transfected 32D cells expressing comparable numbers of receptors were used for all experiments. The results (Fig. 5A) show that several tyrosine resi-
dues are individually able to mediate Epo activation of AP-1 binding. EpoR F7 Y343 and EpoR F7 Y464 supported the same level of AP-1 activation as did the wild-type receptor, indicating that either (phospho)Tyr-343 or (phospho)Tyr-464 are able to support maximal activation of AP-1 DNA binding activity.

We then determined whether these tyrosine residues in the cytoplasmic domain of the EpoR are sufficient not only for activation of AP-1 DNA binding activity but also for transcriptional transactivation of promoters containing the AP-1-binding site TRE. We again used 32D cells stably expressing the wild-type EpoR, EpoR F8, and all 8 mutant F7 YXXX EpoRs; these were transiently transfected with the 3TRE-luc plasmid containing 3 TRE sites linked to a reporter luciferase gene. The results (Fig. 5B) show that in cells expressing the wild-type EpoR, Epo induced a 7-fold increase in luciferase activity. In cells expressing mutant EpoR F8 Epo was unable to induce luciferase activity. However, cells expressing EpoR F7 Y343 and EpoR F7 Y464 supported normal Epo-induced activation of luciferase activity, and thus of the TRE promoter. Cells expressing EpoR F7 Y401 and EpoR F7 Y429 supported partial (~30%) Epo-induced activation of luciferase activity. Taken together with the ability of Epo-R F7 Y401 and Epo-R F7 Y429 to support partial activation of AP-1 DNA binding (Fig. 5A), this indicates that (phospho)Tyr-401 and (phospho)Tyr-429 can mediate partial AP-1 activation, presumably by binding one or more intracellular signal transduction proteins. Cells expressing other EpoRs containing single tyrosine residues were able to support little, if any, Epo-induced activation of luciferase activity. Thus, the ability of F7 Yxxx EpoRs to support activation of AP-1 DNA binding activity (Fig. 5A) correlates perfectly with their ability to transactivate a promoter containing the TRE AP-1-binding site.

Epo Activation of AP-1 Does Not Require Maximal STAT5 Activation—Our previous work showed that EpoR F8 supported activation of only ~10% the amount of STAT5 DNA binding activity as did wild-type EpoR. EpoR F7 Y343 supported maximal activity of STAT5, while EpoR F7 Y464 supported activation of STAT5 to the same low extent (~10%) as did EpoR F8 (12). Thus, activation of AP-1 DNA binding activity by mutant F7 YXXX Epo receptors (Fig. 5) did not correlate with activation of STAT5; if STAT5 were required, activation of only 10% of the maximal level would be sufficient. To test directly whether STAT5 is essential for AP-1 activation by Epo,
we expressed a dominant-negative STAT5 mutant in HCD57 cells (Fig. 6); in these HCD57 cells the dominant-negative STAT5, ΔSTAT5 (22), was under the control of a doxycycline-repressible promoter. Removing doxycycline from the cells induces the expression of ΔSTAT5. Induced expression of ΔSTAT5 resulted in a significant decrease in the level of Epo-activated STAT5 DNA binding activity (Fig. 6, lane 3), but had no effect on the level of Epo-induced AP-1 DNA binding activity (Fig. 6, lane 6).

**DISCUSSION**

**Specific Tyrosine Residues in the EpoR and the Activation of AP-1**—Within minutes of binding of Epo to the EpoR, a number of intracellular signal transduction proteins become activated: the protein tyrosine kinase JAK2, the transcription factor STAT5, the protein tyrosine phosphatases SHP-1 and SHP-2, phosphatidylinositol 3-kinase, and adapter proteins such as Shc and Grb2 that stimulate activation of the ras pathway (see Introduction for references). Here we showed that, in both HCD57 cells and 32D-EpoR cells, the DNA binding activity of AP-1 transcription factors becomes activated about 3 h after Epo addition and reaches the maximum level at 6 h. We showed that AP-1 activation by Epo is prevented by CHX and therefore depends on de novo protein synthesis. This presumably reflects Epo receptor activation of gene transcription and/or protein synthesis of Jun and Fos AP-1 complex components. Using a mutant EpoR (F8) containing no cytosolic tyrosine residues, and a set of eight mutants (EpoR F7 YXXX) containing a single cytosolic tyrosine residue, we showed that any of several tyrosines can mediate AP-1 activation to a significant extent. These studies also demonstrated that AP-1 activation does not require maximal STAT5 activation and may occur via a STAT5-independent signaling pathway.

First, we showed that Epo activation of AP-1 requires tyrosine kinase activity, since it was suppressed by a tyrosine kinase inhibitor genistein and was activated by zinc chloride and sodium orthovanadate, inhibitors of protein tyrosine phosphatases. Tyrosine kinase JAK2 becomes tyrosine phosphorylated and activated in response to Epo and is believed to phosphorylate a number of substrates including the EpoR. Tyrosine phosphorylation of the EpoR creates binding sites for the SH2 domains of many signal transduction proteins and is crucial for EpoR signaling. AP-1 activation by Epo was strongly reduced in cells expressing the EpoR mutant F8; neither induction of AP-1 proteins able to bind to TRE sequences nor transactivation of promoters containing AP-1 binding sites occurred after Epo addition. Thus, at least one cytosolic tyrosine in the cytosolic domain of the EpoR is essential for normal AP-1 activation.

Second, several lines of evidence indicated that individual tyrosine residues in the EpoR, thought to support activation of different intracellular signal transduction proteins, could mediate normal AP-1 activation. First, a truncated EpoR (1–374), containing only the single tyrosine 343, could activate AP-1 to the same extent as the wild-type EpoR. EpoR 1–374 is able to support Epo-dependent proliferation of Ba/F3 cells and also differentiation of fetal liver erythroid progenitors from the fetal livers of EpoR−/− mice (31). In contrast, EpoR 1–374 with a Y343F mutation is unable to support cell proliferation (30) and cannot support normal AP-1 activation (data not shown). Thus, we conclude that (phospho)Tyr-343 can support Epo activation of AP-1.

Additionally, an EpoR containing only tyrosine 343 (EpoR F7 Y343) or only tyrosine 464 (EpoR F7 Y464) supported maximal activation both of AP-1 DNA binding activity and of transcriptional transactivation of a promoter containing the AP-1-binding site TRE. An EpoR containing only tyrosine 401 (EpoR F7 Y401) or only tyrosine 429 (EpoR F7 Y429) was able to support partial activation of AP-1. Thus, there is marked redundancy in the abilities of individual tyrosine residues within the EpoR to mediate AP-1 activation. Presumably each of these phosphotyrosine residues binds the SH2 or PTB domain of one or more intracellular signal transduction proteins. The significance of this redundancy is made evident by Table I, which shows that individual tyrosine residues in the EpoR are known to support activation of different and sometimes overlapping sets of intracellular signal transduction proteins.

**The Role of STAT5 in AP-1 Induction**—As summarized in Table I, multiple tyrosine residues in the cytokotic domain of the EpoR are able to mediate activation of STAT5 by Epo. An EpoR containing only tyrosine 343 (EpoR F7 Y343) or only tyrosine 401 (EpoR F7 Y401) supported maximal STAT5 activation (11–13, 18). Tyrosine residues 429 and 431 can partially activate STAT5, but EpoR F8 and EpoR F7 Y464 are each able to support activation of only 10% the amount of STAT5 binding activity as does wild-type EpoR (12). Thus, activation of STAT5 does not correlate with AP-1 activation since our present results showed that EpoR F8 is unable to activate AP-1 while EpoR F7 Y464 supports maximal activation of AP-1. Additionally, expression of a mutant dominant-negative STAT5, which decreased both phosphorylation of STAT5 and Epo-induced activation of STAT5 DNA binding activity, did not affect the ability of Epo to induce AP-1. These results support our conclusion that maximal induction of STAT5 activity is unnece-
sary for maximal induction of AP-1 activity. However, since Tyr-343 is able to support maximal activation of both STAT5 and AP-1, we cannot exclude the possibility that different signaling pathways can result in the activation of AP-1: one supported by Tyr-464 and independent of STAT5, and another supported by Tyr-343 which may involve STAT5 activation.

Conflicting results have been reported regarding the involvement of STAT5 in the activation of the c-fos component of AP-1. In one study, the role of STAT5 was addressed using naturally occurring dominant-negative variants of STAT5 (32). Although expression of a dominant-negative STAT5 suppressed the interleukin 3 induction of genes normally induced via STAT5, such as oncostatin M (OSM) and CIS (22, 33), there was no effect on induction of the c-fos gene (32). In contrast, a different study showed that a truncated dominant-negative STAT5 suppressed interleukin 3-induced c-fos gene expression (22). The upstream region of the c-fos promoter contains several regulatory sequences, including a sis-inducible element which binds STAT1 and STAT3 proteins (34). Sis-inducible element was shown to be essential for c-fos activation by granulocyte macrophage-colony stimulating factor (35). However, the role of the SIE element in Epo activation of c-fos has not been determined.

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