Identification of the Erythropoietin Receptor Domain Required for Calcium Channel Activation*

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Erythropoietin (Epo) activates a voltage-independent Ca\textsuperscript{2+} channel that is dependent on tyrosine phosphorylation. To identify the domain(s) of the Epo receptor (Epo-R) required for Epo-induced Ca\textsuperscript{2+} influx, Chinese hamster ovary (CHO) cells were transfected with wild-type or mutant Epo receptors subcloned into pTracer-cytomegalovirus vector. This vector contains an SV40 early promoter, which drives expression of the green fluorescent protein (GFP) gene, and a cytomegalovirus immediate-early promoter driving expression of the Epo-R. Successful transfection was verified in single cells by detection of GFP, and intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]) changes were simultaneously monitored with rhod-2. Transfection of CHO cells with pTracer encoding wild-type Epo-R, but not pTracer alone, resulted in an Epo-induced [Ca\textsuperscript{2+}] increase that was abolished in cells transfected with Epo-R F8 (all eight cytoplasmic tyrosines substituted). Transfection with carboxyl-terminal deletion mutants indicated that removal of the terminal four tyrosine phosphorylation sites, but not the tyrosine at position 479, abolished Epo-induced [Ca\textsuperscript{2+}] increase, suggesting that tyrosines at positions 443, 460, and/or 464 are important. In CHO cells transfected with mutant Epo-R in which phenylalanine was substituted for individual tyrosines, a significant increase in [Ca\textsuperscript{2+}] was observed with mutants Epo-R Y443F and Epo-R Y464F. The rise in [Ca\textsuperscript{2+}] was abolished in cells transfected with Epo-R Y460F. Results were confirmed with CHO cells transfected with plasmids expressing Epo-R mutants in which individual tyrosines were added back to Epo-R F8 and in stably transfected Ba/F3 cells. These results demonstrate a critical role for the Epo-R cytoplasmic tyrosine 460 in Epo-stimulated Ca\textsuperscript{2+} influx.

Erythropoietin is a hematopoietic growth factor that regulates the proliferation, differentiation, and viability of erythroid progenitors and precursors (1). The erythropoietin receptor is a member of the superfamily of cytokine receptors (2, 3), which is characterized by the conservation of cysteines and a WSXWS motif in the extracellular domain (1–8), as well as limited similarities in the cytoplasmic domain, including Box 1 and Box 2 in the membrane proximal region (2, 3). No kinase or other enzyme motif has been identified in the cytoplasmic domains of members of the cytokine receptor superfamily. For erythropoietin, interaction with its receptor rapidly induces dimerization/oligomerization (9, 10), which results in increased affinity for a member of the Janus family of cytoplasmic tyrosine kinases, JAK2 (9–12). JAK2 is activated by transphosphorylation of its active site and phosphorylates some or all of the eight tyrosine residues in the intracellular domain of the Epo\textsuperscript{1} receptor. JAK2 also is activated by other cytokine receptors, including thrombopoietin, prolactin, and growth hormone (2, 3, 13). Phosphorylation of the intracellular tyrosines attracts other intracellular proteins that bind to Epo-R via their Src homology 2 (SH2) domains; many are in turn tyrosine-phosphorylated (14).

Identification of the phosphorylated tyrosines of the erythropoietin receptor to which specific SH2-containing proteins bind is important in defining the pathways involved in erythropoietin stimulated proliferation and differentiation. Tyrosine at position 343 or 401 of the murine Epo-R mediates maximal STAT5 activation (15–20). The proliferation stimulating SH2-containing tyrosine phosphatase SHP2 is phosphorylated by JAK2 after binding at position 401 (21, 22). The proliferation inhibiting SH2-containing tyrosine phosphatase SHP1 is recruited to the Epo receptor following the phosphorylation of tyrosine 429, resulting in inactivation of JAK2 and termination of proliferative signals (23, 24). Phosphatidylinositol 3-kinase directly associates with the murine erythropoietin receptor at amino acids 479 and 343 (25–27). Other kinases/signal transducers that are tyrosine-phosphorylated in response to erythropoietin include GAP (28), Shc (14), Vav (29), c-fps/fes (30), Lyn (31), phospholipase C-γ1 (32), and c-Blk (33, 34). It is not known whether these transducers are recruited directly to the Epo-R or bind indirectly via other adaptor proteins. Ligand binding of the Epo-R causes an increase in intracel-
lular Ca\(^{2+}\), and it is believed that this is part of the signaling mechanisms controlling proliferation/differentiation of human (35–37) and murine (38–42) erythroid progenitors and precursors. The mechanism of regulation of calcium by erythropoietin has been examined at the single cell level using normal human burst-forming units-erythroid-derived cells at defined stages of differentiation and fluorescence microscopy coupled to digital video imaging (35, 36, 43, 44), patch clamp (44, 45), and microinjection (46). Our laboratory has shown that erythropoietin induces a dose-dependent increase in [Ca\(^{2+}\)] in single normal human BFU-E-derived erythroblasts modulated through a voltage-independent ion channel permeable to calcium (44, 45) and dependent on tyrosine phosphorylation (47). Here, we identified the domain of the Epo receptor required for Epo-induced calcium influx using a system in which single cells that expressed transfected wild-type or mutant erythropoietin receptors were identified by detection of green fluorescent protein (GFP) with digital video imaging and cytosolic Ca\(^{2+}\) changes simultaneously measured in transfected cells.

**EXPERIMENTAL PROCEDURES**

**Transfection of Wild-type and Mutant Epo Receptors into CHO Cells—**CHO cells were cultured in DMEM with 10% fetal calf serum at 37 °C in 5% CO\(_2\). All constructs were based on the pTracer-CMV vector (Invitrogen). This mammalian expression vector contains an SV40 early promoter that drives expression of the GFP gene fused to a zeocin resistance gene and a CMV immediate-early promoter driving expression of a second cDNA. A panel of Epo-R deletion mutants (see Fig. 2) was generated as described previously (48). Briefly, a region from nucleotide 1083 to the specific 3′-end of each construct was amplified via polymerase chain reaction. After subcloning the polymerase chain reaction fragment into pCR-Script, the fragment was digested with SphI and EcoRI and subcloned into SphI-EcoRI-digested pCDNA3-EPO-R. The Epo-R (221) construct was subcloned from pXM into pCDNA3 (48). Epo-R tyrosine mutants were generated via overlap extension polymerase chain reaction. These included a series of single tyrosine mutants in which phenylalanine was substituted for different tyrosine residues in the Epo receptor (see Fig. 3) and a series of add back mutants to an Epo receptor devoid of tyrosine residues. Oligonucleotide primers were selected that produced either a phenylalanine at amino acid position 443, 460, or 464, or a tyrosine at amino acid position 443, 460, or 464. Polymerase chain reaction was performed using either pBSK-Epo-R or pBSK-Epo-R F8 (all eight cytoplasmic tyrosines converted to phenylalanine) to generate a SphI-EcoRI fragment in pCR-Script. The fidelity of all constructs was confirmed by sequencing both strands of the 443-base pair fragment. Each SphI-EcoRI fragment was subcloned into SphI-EcoRI-digested pBSK-Epo-R. The Epo-R cDNA was subcloned into pCDNA3 or pTracer-CMV vector sites in the pTracer vectors.

CHO cells were transfected with transfection solution containing pTracer-CMV vectors (6 μg/ml) and LipofectAMINE\(^{TM}\) (Life Technologies, Inc.; 16 μg/ml) for 5 h at 37 °C. Successful transfection of CHO cells was verified by detection of expression of green fluorescent protein by digital video imaging (44–46). The excitation peaks of GFP are 495 and 478 nm, and the emission peak is 507 nm. Cells were excited at 380 nm, and emission was detected at 510 nm. The optimal time for expression of transfected pTracer-CMV Epo-R was found to be 48–72 h after transfection by Western blotting, and this time interval posttransfection was selected to examine the response of transfected CHO cells to Epo. At this time interval, 40–50% of CHO cells expressed GFP.

**Measurement of [Ca\(^{2+}\)] with Digital Video Imaging—**A fluorescence microscopy coupled digital video imaging system was used to measure [Ca\(^{2+}\)] (35, 36, 43–46). To study changes in intracellular calcium in transfected cells, we were not able to use Fura-2 as the detection fluorophore because its excitation and emission wavelengths overlap with those of GFP. Instead, we used the fluorescence indicator rhod-2 (Molecular Probes, Eugene, OR) (49, 50). Fluorescence of GFP does not directly compare because we were unable to calibrate rhod-2 in CHO cells treated with or without physiological calcium (0.7 mM) (36). Pertussis toxin was obtained from List Biological Laboratory (Campbell, CA) and was heat-inactivated by incubating at 95 °C for 20 min (51).

**Immunoblotting—**Whole cell lysates were prepared, and ECL was performed as described previously (52). Membranes were incubated with rabbit anti-Epo-R antibody (EpoTech, Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:100). Donkey anti-rabbit antibody (1:1500) was used as the secondary antibody.

**RESULTS**

**Transfection of CHO Cells with pTracer-CMV Epo-R—**To determine the domains of the Epo-R required for erythropoietin regulation of calcium channels, we established a system in which single cells transfected with wild-type or mutant Epo-R could be identified by GFP fluorescence, and [Ca\(^{2+}\)] was measured simultaneously in the identical cells with digital video imaging. CHO cells were used for these transfections, because they lack endogenous Epo-R and have been shown to contain all the necessary transducers required for growth hormone-induced [Ca\(^{2+}\)] increase (54). In Fig. 1, five CHO cells are shown 24 h after transfection with Epo-R subcloned into the pTracer-CMV vector. Successfully transfected CHO cells were detected by fluorescence from GFP (Fig. 1B). [Ca\(^{2+}\)] was detected by intracellular rhod-2 fluorescence. In Fig. 1C, a significant increase in [Ca\(^{2+}\)] was observed in CHO cells stimulated with Epo following transfection with pTracer-CMV mEpo-R (Table I, p < 0.02). The rate of rise in intracellular calcium was similar to that previously observed in BFU-E-derived cells (35, 36). The magnitude of [Ca\(^{2+}\)] changes cannot be directly compared because we were unable to calibrate rhod-2 signals into [Ca\(^{2+}\)], and rhod-2 was observed to have a smaller dynamic range than Fura-2. No change in [Ca\(^{2+}\)] was observed in CHO cells treated with Epo alone (data not shown). No increase in [Ca\(^{2+}\)] was observed in Epo treated CHO cells transfected with vector alone. Nontransfected CHO cells (e.g. the other four cells in Fig. 1C) did not demonstrate a significant change in percentage of Fv/fo over the experimental period (20 min), indicating that problems due to leakage and photobleaching of rhod-2 are minimal (no decrease in Fv/fo), and that the increase in [Ca\(^{2+}\)] in successfully transfected CHO cells was specific for Epo.

The Ca\(^{2+}\) response to erythropoietin was characterized in CHO cells. The rise in intracellular Ca\(^{2+}\) was dependent on extracellular calcium because no response to erythropoietin was seen in medium without calcium (data not shown). Pretreatment with pertussis toxin, but not heat-inactivated pertussis toxin, significantly inhibited the increase in percentage of Fv/fo observed in Epo-stimulated pTracer-CMV mEpo-R transfected CHO cells (Table I, p < 0.002). These results demonstrated that the influx of calcium in CHO cells is...
with wild-type or mutant Epo receptors subcloned into pTracer-CMV vector. We initially selected a mutant receptor in which phenylalanine was substituted for all eight tyrosines (F8), because tyrosine phosphorylation is required for the Epo-modulated calcium increase (47). Transfection with the Epo-R F8 mutant abolished the Epo-induced intracellular calcium increase (Table III). CHO cells were then transfected with carboxyl-terminal deletion mutants to narrow the number of tyrosine sites that might be involved (Fig. 2). CHO cells transfected with the mEpo-R carboxyl-terminal deletion mutants −221 (−8 tyrosines), −99 Y343F (−8 tyrosines), −99 (−7 tyrosines), −69 (−6 tyrosines), and −43 (−4 tyrosines) all displayed no calcium influx in response to erythropoietin stimulation (Table III). The Epo-induced increase in [Ca], in mEpo-R −14 (−1 tyrosine) transfected cells was similar to the wild-type mEpo-R. These results suggested that tyrosines at position 443, 460, and/or 464 are necessary for Epo-induced calcium influx.

Identification of the Tyrosine(s) Involved in Epo-Receptor Mediated Calcium Influx—CHO cells were then transfected with mutant mEpo-R in which phenylalanine was substituted for individual tyrosines at positions 443, 460, or 464 (Fig. 3). In cells transfected with mEpo-R Y443F and mEpo-R Y464F, the significant increase in [Ca], in response to Epo stimulation was similar to that observed with the wild-type mEpo-R (Table IV). In contrast, the increase in percentage of F/Fo was abolished in cells transfected with mEpo-R Y460F.

To confirm that a single tyrosine residue was sufficient to mediate Epo-R modulated Ca\(^{2+}\) influx, CHO cells were transfected with pTracer expressing mutants in which single tyrosines were added back to a mEpo-R devoid of tyrosine residues (Epo-R F8). A significant increase in [Ca], in response to Epo was observed in cells transfected with mEpo-R F7 Tyr-460 but not in cells transfected with mEpo-R F7 Tyr-443 or F7 Tyr-464 (Table IV). These results demonstrate that the Epo-R cytoplasmic tyrosine at position 460 is necessary and sufficient for Epo-stimulated Ca\(^{2+}\) influx.

Western blotting was performed on lysates of transfected CHO cells and demonstrated that all tyrosine substitution and add back mutants were expressed (Fig. 4A). As observed for wt mEpo-R, receptor expression was greater at 48 than 72 h after transfection. The deletion mutant mEpo-R-14, shown here as a negative control, was not recognized by this antibody, which was raised against the carboxyl-terminal 20 amino acids.

Role of mEpo-R Tyr-460 in Hematopoietic Cells—To confirm these findings in hematopoietic cells, the ability of erythropoietin to modulate [Ca], in Ba/F3 cells stably transfected with wild-type or mutant mEpo-R was examined. Subclones were isolated via limiting dilution and individual subclones were selected that expressed comparable levels of Epo-R expression (Fig. 4B). Stable transfection of mEpo-R was maintained by antibody selection, and the level of receptor expression in a single cell could not be assessed.

A significant increase in F/Fo was observed following Epo stimulation in Ba/F3 cells transfected with mEpo-R, but not in parental cells (Fig. 5). No increase in F/Fo was found when measurements were obtained at 10 s intervals for the first minute. Cells transfected with the mEpo-R deletion mutant −43 showed no Epo-induced [Ca], increase, whereas cells transfected with mEpo-R −14 displayed a significant calcium rise. Furthermore, no increase in [Ca], was observed in Ba/F3 cells transfected with mEpo-R Y7 Phe-460, but the calcium response to Epo was specifically restored in an add back mutant to the F8 mEpo-R in which tyrosine was added back at position 460 (Fig. 5). All cell lines tested activated the tyrosine

FIG. 1. Detection of GFP and rhod-2 in five CHO cells transfected with the pTracer-CMV vector. A, white light image of CHO cells. B, only one of these five CHO cells successfully expressed GFP (excitation, 380 nm; emission, 510 nm). C, rhod-2 fluorescence of the same CHO cells (excitation, 540 nm; emission, 590 nm).

regulated by a pertussis toxin-sensitive G protein. CHO cells transfected with pTracer-CMV mEpo-R were then pretreated with the i-type Ca\(^{2+}\) channel blocker nifedipine. Nifedipine blocked the increase in [Ca], seen in response to erythropoietin, but only at doses higher than typically required to block voltage-sensitive i-type calcium channels (Table II). These data confirm that the Epo-R in transfected CHO cells behaved similarly to the endogenous Epo-R on BFU-E-derived cells (44–46, 51).

Identification of Erythropoietin Receptor Domains Involved in Calcium Channel Activation Using Receptor Deletion Mutants—To determine erythropoietin receptor domains required for Epo-induced calcium influx, CHO cells were transfected...
**Epo-R Tyr-460 Mediates Epo-dependent Calcium Channel Activation**

**TABLE I**

| Preincubated with | pTracer vector | Fo | Ft | Ft/Fo | n |
|-------------------|----------------|----|----|-------|---|
| 5 µg/ml PT        | CMV mEpo-R     | 42 ± 4 | 62 ± 5 | 151 ± 9* | 6 |
| 5 µg/ml HI PT     | CMV mEpo-R     | 50 ± 5 | 52 ± 5 | 105 ± 3 | 11 |
| None              | CMV mEpo-R     | 42 ± 4 | 64 ± 6 | 153 ± 8* | 13 |
|                   | None           | 50 ± 7 | 53 ± 6 | 110 ± 4 | 6 |

*Significant increase in percentage of Ft/Fo above cells not transfected with vector (p < 0.05), which had no detectable GFP fluorescence.

**TABLE II**

| Pretreated with | Stimulated with | Fo | Ft | Ft/Fo | n |
|-----------------|-----------------|----|----|-------|---|
| IMDM            | Epo             | 51 ± 5 | 74 ± 8 | 146 ± 7* | 6 |
| 1 µM Nif        | Epo             | 41 ± 7 | 80 ± 13 | 214 ± 24* | 8 |
| 10 µM Nif       | Epo             | 41 ± 9 | 58 ± 14 | 154 ± 29* | 6 |
| 50 µM Nif       | Epo             | 54 ± 8 | 55 ± 8 | 104 ± 3 | 4 |
| IMDM            | IMDM            | 56 ± 7 | 59 ± 9 | 103 ± 3 | 4 |

*Significant increase in percentage of Ft/Fo above cells not transfected with vector (p < 0.05).

**TABLE III**

| mEpo-R          | Fo | Ft | Ft/Fo | n |
|-----------------|----|----|-------|---|
| mEpo-R wt       | 51 ± 1 | 78 ± 3 | 154 ± 3* | 73 |
| None            | 54 ± 1 | 66 ± 1 | 102 ± 1 | 70 |
| mEpo-R F8       | 56 ± 2 | 60 ± 2 | 103 ± 3 | 24 |
| mEpo-R Y460F    | 52 ± 3 | 58 ± 3 | 113 ± 3 | 9 |
| −221            | 48 ± 1 | 55 ± 2 | 114 ± 2 | 13 |
| −99             | 53 ± 2 | 59 ± 3 | 113 ± 4 | 22 |
| −69             | 54 ± 2 | 61 ± 4 | 112 ± 5 | 21 |
| −43             | 49 ± 2 | 56 ± 3 | 115 ± 3 | 32 |
| −14             | 54 ± 3 | 73 ± 5 | 138 ± 8* | 13 |

*Significant increase in percentage of Ft/Fo above cells not transfected with vector (p < 0.05).

phosphorylation of JAK2, confirming that the Epo-R constructs were expressed on the cell surface (Fig. 6). This is expected because all constructs have an intact Box 1/Box 2 region. These data demonstrate that the mEpo-R Y460F is functional in activating JAK2 but unable to transmit the calcium signal.

**DISCUSSION**

We have transfected CHO cells with wild-type and mutant mEpo receptors to study the erythropoietin signal transduction pathway involved in calcium channel activation. Digital video imaging was used to identify GFP fluorescence in single cells transfected with the pTracer vector, which expresses both the GFP and Epo receptor genes. The pTracer vector possesses the theoretical advantage that the gene of interest is not expressed at the single cell level by fluorescence emissions from GFP, a co-transfected gene. Our method can be applied to many receptor/channel systems.

We previously demonstrated that erythropoietin modulates a voltage-independent calcium channel on normal erythroid precursors (35–36, 44–45) that is dependent on tyrosine kinase activation (47). The results reported here, demonstrating the importance of the tyrosine at position 460 in calcium channel activation, are consistent with our previous data. Signal transducers that interact with Tyr-460 and the subsequent signaling pathway(s) involved have not yet been identified. One potential transducer, Ras, has been shown to couple to calcium channel entry in other systems. Insulin-like growth factors I and II have been shown to activate a calcium-permeable channel by a mechanism involving a pertussis toxin-sensitive GTP-binding protein, Gia2 (55). Ras activation is required for insulin-like growth factor II receptor and Gia2 coupling, which regulates the IGF-II calcium signaling pathway in 3T3 cells (55). Because the same pertussis toxin-sensitive protein (Gia2) has been shown to be involved in erythropoietin-mediated calcium channel opening on erythroid precursors (46, 51), Ras may be in-
involved in the erythropoietin-modulated pathway and activated by interaction of one of its transducers with the receptor at Tyr-460. Another transducer that may interact at this site is the Crk family of adaptor proteins, including CrkI, CrkII, and CrkL (34). The CrkI proteins, through SH3-dependent interactions, associate with C3G, a guanine nucleotide release factor that displays specificity for the activation of the Ras related gene Rap1. Indeed, Rap1 activation has been shown to be associated with calcium signaling in platelets and neutrophils (56, 57).

Erythropoietin has previously been shown to induce tyrosine phosphorylation and activation of phospholipase C-γ1 (PLC-γ1).
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in UT-7/Epo cells, resulting in an increase in intracellular calcium (32). In rat glomerular mesangial cells, erythropoietin promotes the interaction of phospholipase C-γ1 with the erythropoietin receptor as well as stimulation of a Ca2+-activated, Ca2+-permeable channel that is dependent on tyrosine phosphorylation and activation of PLC-γ1 (58). Many of the characteristics of the Epo-mediated rise in intracellular calcium in normal erythroid and mesangial cells are similar, including the magnitude of the rise in intracellular calcium, the 20 min peak of intracellular Ca2+ response, the dependence on tyrosine phosphorylation, and the regulation of voltage-independent Ca2+ channel activity (58). However, the PLC-γ1 cascade results in a biphasic rise in intracellular Ca2+ with an initial increase from inositol 1,4,5-trisphosphate-sensitive intracellular Ca2+ stores followed by influx of extracellular Ca2+ (58).

Recent studies suggest that phosphatidylinositol 3,4,5-trisphosphate is an activator of calcium channel activity in mast cells (59, 60). The SH2 inositol 5'-phosphatase SHIP has been shown to abrogate receptor-induced phosphatidylinositol 3,4,5-trisphosphate accumulation regulated by PLC-γ2, resulting in the blockade of calcium influx. Although no reports have implicated PLC-γ2 in Epo-mediated signaling, SHIP is a target of Epo-dependent tyrosine phosphorylation (61). Gene targeting experiments reveal that SHIP knockout mice have decreased CFU-E in 4- and 8-week-old animals (60). SHIP may regulate several different signal transduction cascades, including activation of Erk, PKB/Akt and/or calcium entry. This matter will be resolved by an analysis of EPO-dependent signaling in primary cells isolated from the SHIP nullizygous mice.

Two other members of the cytokine superfamily, growth hormone (54) and prolactin (62), mediate a slow increase in intracellular free calcium. The Ca2+ response to growth hormone, like that of Epo, is dependent on external calcium but growth hormone appears to activate a voltage-dependent L-type Ca2+ channel in the plasma membrane and the response is not dependent on JAK2 (54). The Ca2+ response to prolactin is of the same magnitude as that of erythropoietin but is dependent on mobilization from intracellular stores as well as external calcium entry (62). The specific transducers involved in each of these cascades need to be determined so that shared and receptor-specific pathways can be identified.

The role of intracellular calcium in regulation of cell proliferation and differentiation is becoming better defined. Calcium has been linked to expression of proto-oncogenes (63), transcription factor phosphorylation, and the appearance of a nuclear Ca2+,-Mg2+-dependent endonuclease capable of generat-
ing single strand breaks in chromosomal DNA (64). In the murine erythroleukemia cell line ELM-1, the Ca2+-/calmodulin-dependent serine/threonine-specific phosphatase calcineurin (65) mediated Ca2+-induced down-regulation of c-Myb expression and an increase in hemoglobin synthesis. Calcium is also involved in inhibition of DNA binding of basic helix-loop-helix transcription factors (66) and in the differential activation of NF-κB factors (67). Both of these families of transcription factors are involved in regulation of erythropoiesis (68–71). Here, we have demonstrated the tyrosine site on the erythropoietin receptor required for activation of the signaling cascade that leads to calcium channel activation. Our next goals will be identification of the transducers that bind to that site and their subsequent targets and determination of the specific nuclear events that are modulated by the nuclear/cyttoplasmic calcium gradient (43) that results.

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