Three Tyrosine Residues in the Erythropoietin Receptor Are Essential for Janus Kinase 2 V617F Mutant-induced Tumorigenesis*

Received for publication, July 20, 2016, and in revised form, December 20, 2016 Published, JBC Papers in Press, December 20, 2016, DOI 10.1074/jbc.M116.749465

Fumihito Ueda1, Kenji Tago1, Hiroomi Tamura8, and Megumi Funakoshi-Tago†§

From the †Division of Hygienic Chemistry, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512 and the ‡Division of Structural Biochemistry, Department of Biochemistry, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi-ken 329-0498, Japan

Edited by Alex Toker

The erythropoietin receptor (EpoR) regulates development of blood cells, and its full activation normally requires the cytokine erythropoietin (Epo). In the case of myeloproliferative neoplasms (MPN), Epo-independent signaling through EpoR can be caused by a point mutation, V617F, in the EpoR-interacting tyrosine kinase Janus kinase 2 (JAK2). In cells expressing the JAK2 V617F mutant, eight tyrosine residues in the intracellular domain of EpoR are phosphorylated, but the functional role of these phosphorylations in oncogenic signaling is incompletely understood. Here, to evaluate the functional consequences of the phosphorylation of these tyrosine residues, we constructed an EpoR-8YF mutant in which we substituted all eight tyrosine residues with phenylalanine. Co-expression of EpoR-8YF with the JAK2 V617F mutant failed to induce cytokine-independent cell proliferation and tumorigenesis, indicating that JAK2-mediated EpoR phosphorylation is the reason for JAK2 V617F mutant-induced oncogenic signaling. An exhaustive mutational analysis of the eight EpoR tyrosine residues indicated that three of these residues, Tyr-343, Tyr-460, and Tyr-464, are required for the JAK2 V617F mutant to exhibit its oncogenic activity. We also showed that phosphorylation at these three residues was necessary for full activation of the transcription factor STAT5, which is a critical downstream factor of JAK2 V617F-induced oncogenic signaling. In contrast, Epo stimulation could moderately stimulate the proliferation of cells expressing wild type JAK2 and EpoR-8YF, suggesting that the requirement of the phosphorylation of these three tyrosine residues seems to be specific for the oncogenic proliferation provoked by V617F mutation. Collectively, these results have revealed that phosphorylation of Tyr-343, Tyr-460, and Tyr-464 in EpoR underlies JAK2 V617F mutant-induced tumorigenesis. We propose that the targeted disruption of this pathway has therapeutic utility for managing MPN.

The proliferation and differentiation of hematopoietic cells are regulated by various cytokines that act through their specific receptors (1, 2). Erythropoietin (Epo) is a pleiotropic cytokine that exhibits hematopoietic functions such as the development of erythrocytes through the erythropoietin receptor (EpoR) (3). Once Epo binds to EpoR, the non-receptor tyrosine kinase, Janus kinase 2 (JAK2), which interacts with EpoR, is activated. Activated JAK2 then immediately phosphorylates multiple tyrosine residues in the cytoplasmic domain of EpoR (4).

EpoR contains the following eight tyrosine residues in its intracellular domain that are phosphorylated by JAK2: Tyr-343, Tyr-401, Tyr-429, Tyr-431, Tyr-443, Tyr-460, Tyr-464, and Tyr-479. The JAK2-induced phosphorylation of these tyrosine residues in EpoR provides a platform for the recruitment and activation of signaling mediators and is critical for Epo-induced cellular proliferation (3, 5, 6). Previous studies clarified that the transcription factor, signal transducer and activator of transcription 5 (STAT5), bound to phosphorylated tyrosine residues at Tyr-343 in EpoR through its Src homology 2 (SH2) domain (7, 8). STAT3 is activated through the phosphorylated EpoR (9). In addition, EpoR associates with Grb2 and the p85α subunit of PI3K via phosphorylated Tyr-464 and Tyr-479, respectively (10, 11). Grb2 induces the activation of ERK1/2 through the Ras-Raf-MEK pathway (12), and PI3K induces the activation of Akt through the phosphorylation of phosphatidylinositol (13, 14). These signaling molecules are known to be involved in Epo-stimulated cell proliferation and erythroid differentiation (6–14). Conversely, the molecules that inhibit the Epo signaling pathway are also known to interact with phosphorylated EpoR. Cytokine-inducible SH2-containing protein (CIS) interacts with phosphorylated Tyr-401 in EpoR and suppressor of cytokine signaling 3 (SOCS3) with phosphorylated Tyr-429 and Tyr-431 in addition to Tyr-401. CIS and SOCS3 both act as negative regulators of Epo signaling by inhibiting the activation of the STAT5 and ERK pathways (15–17). Furthermore, SH2 domain-containing tyrosine phosphatase 1 (SHP1) has been shown to associate with EpoR at Tyr-429 and Tyr-431. SHP1 regulates the activity of JAK2 and STAT5.

1 To whom correspondence should be addressed. Tel.: 81-3-5400-2689; Fax: 81-3-5400-2689; E-mail: tago-mg@pha.keio.ac.jp.

2 The abbreviations used are: Epo, erythropoietin; EpoR, erythropoietin receptor; CIS, cytokine-inducible SH2-containing protein; MEK, murine embryonic fibroblast; MPN, myeloproliferative neoplasm; MSCV, murine stem cell virus; SH2, Src homology 2; SHP1, SH2 domain-containing tyrosine phosphatase 1; SOCS3, suppressor of cytokine signaling 3; qPCR, quantitative PCR.

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Role of EpoR in JAK2 V617F Mutant-induced Tumorigenesis

EpoR Was Phosphorylated by the JAK2 V617F Mutant in the Absence of Epo Stimulation—EpoR contains the following eight tyrosine residues in its cytoplasmic domain that are phosphorylated by JAK2 (Fig. 1A, EpoR): Tyr-343, Tyr-401, Tyr-429, Tyr-431, Tyr-443, Tyr-460, Tyr-464, and Tyr-479. To evaluate the importance of tyrosine phosphorylation of EpoR for the Epo-induced and oncogenic JAK2 V617F mutant-induced signaling pathways, we first generated the 8YF mutant, in which all eight tyrosine residues in the cytoplasmic domain of EpoR were substituted to phenylalanine (Fig. 1A, 8YF). EpoR and the 8YF mutants were co-expressed with wild-type JAK2 or the JAK2 V617F mutant tagged with HA on their C terminus in JAK2-deficient murine embryonic fibroblasts (MEFs), respectively (Fig. 1B). Using these cells, we first investigated the activation of JAK2 or JAK2 V617F mutant and their association with EpoR. The activation of JAK2 was evaluated by detection of the phosphorylation at Tyr-1007/1008 in the activation loop of immunoprecipitated JAK2. When co-expressed with EpoR or the 8YF mutant, EpoR stimulation induced the phosphorylation of wild-type JAK2 at Tyr-1007/1008 (Fig. 1C, see lanes 10 and 12 in p-JAK2). In contrast, the JAK2 V617F mutant was phosphorylated at Tyr-1007/1008 regardless of the absence of the Epo stimulation and expression of EpoR (Fig. 1C, see lanes 13 and 14 in p-JAK2). Furthermore, the co-expression of EpoR and 8YF enhanced the phosphorylation of the JAK2 V617F mutant (Fig. 1C, see lanes 15–18 in p-JAK2). We next investigated the interaction of EpoR with JAK2 and the phosphorylation of EpoR.

Both wild-type JAK2 and the JAK2 V617F mutant constitutively interacted with EpoR and 8YF regardless of Epo stimulation (Fig. 1C, see lanes 9–12 and 15–18 in HA), suggesting that the phosphorylation state of EpoR and activation state of JAK2 had no effect on their interaction. By utilizing JAK2-deficient MEFs, we clearly demonstrated that the phosphorylation of EpoR required the stimulation with Epo and expression of JAK2 (Fig. 1C, see lanes 9 and 10 in pY). However, the 8YF mutant failed to be phosphorylated by the Epo stimulation in the presence of wild-type JAK2 (Fig. 1C, see lanes 11 and 12 in pY). In JAK2-deficient MEFs expressing the JAK2 V617F mutant, the phosphorylation of EpoR was observed in the absence and presence of the Epo stimulation (Fig. 1C, see lanes 15 and 16 in pY). 8YF was not phosphorylated under all conditions (Fig. 1C, see lanes 17 and 18 in pY). Therefore, these results suggest that EpoR was phosphorylated not only by Epo-stimulated wild-type JAK2, but also by the constitutively active JAK2 V617F mutant. In addition, the phosphorylation state of EpoR had no effect on the Epo-induced activation of JAK2 or constitutive activation of the JAK2 V617F mutant.

Phosphorylation of EpoR Is Critical for JAK2 V617F Mutant-induced Cytokine-independent Cell Proliferation—To investigate the role of the phosphorylation of EpoR in JAK2 V617F mutant-induced cellular transformation, Ba/F3 cells were infected with various combinations of retroviruses such as the empty virus (–) and retroviruses, including EpoR, 8YF, wild-type JAK2 (WT), and the JAK2 V617F mutant. We established nine kinds of sub-cell lines: (A1) /–/ cells; (A2) –/EpoR cells; (A3) –/8YF cells; (A4) WT/– cells; (A5) WT/EpoR cells; (A6) WT/8YF cells; (A7) V617F/– cells; (A8) V617F/EpoR cells; and (A9) V617F/8YF cells (Fig. 2A). Both wild-type JAK2 and the JAK2 V617F mutant constitutively interacted with EpoR and 8YF regardless of the Epo stimulation (Fig. 2B, see lanes 4–9). In addition, when EpoR or 8YF was co-expressed in Ba/F3 cells, the stimulation with Epo caused the activation of wild-type JAK2, which is evidenced by the phosphorylation at Tyr-1007/1008; however, the JAK2 V617F mutant was constitutively phosphorylated at Tyr-1007/1008 regardless of Epo stimulation (Fig. 2C, see lanes 9–12 and 15–18). Although we also investigated the phosphorylation of JAK1 by immunoblotting, JAK1 was not phosphorylated in these cells. Therefore, it was indicated that either Epo stimulation or the expression of JAK2 V617F mutant failed to induce activation of JAK1 (Fig. 2D).

We then investigated the effects of the phosphorylation of EpoR on cell proliferation in the absence and presence of Epo or IL-3 using the WST assay (Fig. 2, E and F). Although only Ba/F3 cells expressing the JAK2 V617F mutant and EpoR exhibited cytokine-independent cell proliferation, the co-expression of 8YF with the JAK2 V617F mutant failed to confer growth factor independence on Ba/F3 cells (Fig. 2E, left graph). When stimu-
lated with Epo, all sub-lines of Ba/F3 cells expressing EpoR strongly proliferated. In contrast, the sub-lines of Ba/F3 cells expressing 8YF exhibited a partially suppressed proliferation rate regardless of the co-expression of the wild-type JAK2 and JAK2 V617F mutant (Fig. 2E, right graph). In the presence of IL-3, no significant difference was observed in the cell growth of each sub-line of Ba/F3 cells (Fig. 2F). To evaluate the cell proliferation, we counted the cell numbers of each sub-line in the absence and presence of Epo stimulation by trypan blue staining (Fig. 2G). We obtained similar results showing that the phosphorylation of EpoR played an indispensable role in cytokine-independent cell proliferation induced by the JAK2 V617F mutant but partially contributed to Epo stimulation-induced cell proliferation (Fig. 2G). We also evaluated the cell viability of each sub-line in the absence and presence of Epo stimulation (Fig. 2H). The Ba/F3 cells, which do not express EpoR such as (A1) −/− cells, (A4) WT−/− cells, and (A7) V617F−/− cells, died regardless of Epo stimulation. Whereas (A2) −/EpoR cells and (A5) WT+/EpoR cells survived when stimulated with Epo, the viability of (A3) −/8YF cells and (A6) WT+/8YF cells was slightly lower in the presence of Epo stimulation. Interestingly, although (A8) V617F+/EpoR cells survived regardless of Epo stimulation, (A9) V617F+/8YF died in the absence of Epo stimulation. However, cell death of the A9 sub-line was canceled by the stimulation with Epo (Fig. 2H, graph). We then investigated the populations of the cell cycle phases of these sub-lines in the absence and presence of Epo stimulation using flow cytometry (Fig. 2J). We observed significant increases in the sub-G1 phase, which is consistent with apoptotic cells, in all sub-lines of Ba/F3 cells, except (A8) V617F+/EpoR cells in the absence of Epo stimulation. In the presence of Epo stimulation, the decreases in the sub-G1 phase were observed in all sub-lines of Ba/F3 cells.

FIGURE 1. Phosphorylation of EpoR by the Epo stimulation or JAK2 V617F mutant. A, schematic diagram of the wild-type EpoR c-FLAG and 8YF mutant c-FLAG in which the eight tyrosine residues in the cytoplasmic region: Tyr-343, Tyr-401, Tyr-429, Tyr-431, Tyr-443, Tyr-460, Tyr-464, and Tyr-479, are substituted with phenylalanine. TM indicates the transmembrane region. JAK2 interacts with EpoR through Box 1 and Box 2 regions. B, JAK2-deficient MEFs were infected with an empty virus (−) or retroviruses expressing wild-type JAK2 c-HA (JAK2) or JAK2 V617F mutant c-HA (V617F). Cells were then sequentially infected with an empty virus (−) or retroviruses expressing wild-type EpoR (EpoR) or the 8YF mutant (8YF). Whole cell lysates were immunoblotted (IB) with an anti-HA antibody, anti-FLAG antibody, or anti-β-actin antibody. C, infected JAK2-deficient MEFs were incubated with DMEM containing 1% FBS for 24 h and stimulated with Epo (1 unit/ml) for 15 min. Cell lysates were immunoprecipitated (IP) with an anti-HA antibody or anti-FLAG antibody (described as IP: HA or IP: FLAG), and immunoblotted with an anti-phospho-JAK2 antibody (Tyr-1007/Tyr-1008) (described as IB: p-JAK2), anti-HA antibody, anti-phospho-tyrosine antibody (pY), or anti-FLAG antibody.
expressing EpoR or 8YF (Fig. 2i, graph). These results confirmed that the phosphorylation of EpoR was necessary for JAK2 V617F mutant-induced cytokine-independent cell proliferation and cell survival. In addition, it was also observed that unphosphorylated EpoR such as 8YF was responsible for Epo-induced cell proliferation and cell survival.

Phosphorylation of Multiple Tyrosine Residues in EpoR Is Required for JAK2 V617F Mutant-induced Cytokine-independent Cell Proliferation—To identify which tyrosine residues in EpoR need to be phosphorylated for JAK2 V617F mutant-induced cytokine-independent cell proliferation, we generated the following eight kinds of EpoR-7YF mutants retaining one

FIGURE 2. Significance of phosphorylation of EpoR on cytokine-independent cell proliferation induced by the JAK2 V617F mutant. Ba/F3 cells were infected with an empty virus (-), a retrovirus encoding JAK2 c-HA (WT) and JAK2 V617F mutant c-HA (V617F), and retroviruses encoding EpoR or 8YF. Each sub-line was numbered as A1–A9. A and B, transduced Ba/F3 cells were incubated with RPMI 1640 medium containing 1% FBS in the absence of IL-3 for 24 h. A, whole cell lysates were immunoblotted with an anti-HA antibody, anti-FLAG antibody, or anti-β-actin antibody. B, cell lysates were immunoprecipitated using an anti-FLAG antibody (described as IP: FLAG (EpoR)) and immunoblotted (IB) with an anti-HA antibody or anti-FLAG antibody. C and D, transduced Ba/F3 cells were incubated with RPMI 1640 medium containing 1% FBS in the absence or presence of Epo (1 unit/ml) for 1 day. C, cell lysates were immunoprecipitated using an anti-HA antibody (described as IP: HA) and immunoblotted with an anti-phospho-JAK2 antibody (Y1007/1008) (described as IB: p-JAK2) or anti-HA antibody. D, HEK293T cells were transfected with pRK5-JAK1 and incubated for 48 h and stimulated with IFNγ (100 ng/ml) for 15 min. Cell lysates were immunoblotted with anti-phospho-JAK1 antibody (Y1022/1023) or anti-JAK1 antibody. E and F, transduced Ba/F3 cells were incubated with RPMI 1640 medium containing 1% FBS in the absence or presence of Epo (1 unit/ml) or IL-3 (2 ng/ml) for 3 days. Cell proliferation was measured using the WST-1 assay. Values are the mean ± S.D. of four independent experiments. G and H, transduced Ba/F3 cells were incubated with RPMI 1640 medium containing 1% FBS in the presence and absence of Epo (1 unit/ml) or IL-3 (2 ng/ml) for 3 days. Cell viability was measured by trypan blue exclusion method. Values are the mean ± S.D. of three independent experiments. I, transduced Ba/F3 cells were incubated with RPMI 1640 medium containing 1% FBS in the presence and absence of Epo (1 unit/ml) for 2 days. Cells were fixed, treated with propidium iodide, and subjected to flow cytometry.
tyrosine residue each and then expressed them in Ba/F3 cells and Ba/F3 cells expressing the JAK2 V617F mutant (Fig. 3, A and D): (B1) 7YF-Y343; (B2) 7YF-Y401; (B3) 7YF-Y429; (B4) 7YF-Y431; (B5) 7YF-Y443; (B6) 7YF-Y460; (B7) 7YF-Y464; and (B8) 7YF-Y479. In the presence of the Epo stimulation, the expression of (B1) 7YF-Y343, (B2) 7YF-Y401, (B4) 7YF-Y431, (B6) 7YF-Y460, (B7) 7YF-Y464, and (B8) 7YF-Y479 partially recovered the proliferation of Ba/F3 cells, and their proliferation rates were slightly higher than that of the 8YF mutant, suggesting that Tyr-343, Tyr-401, Tyr-431, Tyr-460, Tyr-464, and Tyr-479 are involved in Epo-induced cell proliferation. However, (B3) 7YF-Y429 and (B5) 7YF-Y443 failed to regain the proliferation rate of Ba/F3 cells; their proliferation rate was similar to that of 8YF, suggesting that phosphorylation of Tyr-429 and Tyr-443 is dispensable for Epo-stimulated cell proliferation (Fig. 3E, graph). In addition, these cells underwent cell death upon Epo withdrawal (Fig. 3F, graph). These results suggest that the phosphorylation of only one tyrosine residue in EpoR is not sufficient for JAK2 V617F mutant-induced cellular transformation. In the presence of IL-3, no significant difference was observed in the proliferation of each cell (Fig. 3, G and H).

Tyr-343, Tyr-460, and Tyr-464 in EpoR Are Required for JAK2 V617F Mutant-induced Cytokine-independent Cell Proliferation—We previously reported that STAT5 plays a critical role in JAK2 V617F mutant-induced cytokine-independent cell proliferation (26). The phosphorylation of Tyr-343 has been shown to trigger the association of EpoR with STAT5 and positively regulates Epo-induced cell proliferation through the activation of STAT5 (7, 8). Therefore, we speculated that Tyr-343 in EpoR may play a critical role in JAK2 V617F mutant-
induced cell proliferation, and we constructed seven kinds of EpoR-6YF mutants harboring two intact tyrosine residues, including Tyr-343 and another tyrosine residue. We generated additional sub-lines of Ba/F3 cells expressing the JAK2 V617F mutant with each 6YF mutant as follows: (C1) 6YF-Y343/401; (C2) 6YF-Y343/429; (C3) 6YF-Y343/431; (C4) 6YF-Y343/443; (C5) 6YF-Y343/460; (C6) 6YF-Y343/464; and (C7) 6YF-Y343/479 (Fig. 4A), and we analyzed the proliferation rate and the viability of these cells (Fig. 4B, C, and D). The expression of (C5) 6YF-343/460 and (C6) 6YF-343/464 partially recovered the proliferation of Ba/F3 cells expressing the JAK2 V617F mutant in the absence of the cytokine (Fig. 4B, graph). In comparison with the viability of V617F/8YF cells, the viability of cells expressing (C5) 6YF-343/460 and (C6) 6YF-343/464 with JAK2 V617F mutant was significantly increased (Fig. 4C, graph), suggesting that Tyr-460 and Tyr-464 play pivotal roles in JAK2 V617F mutant-induced cell proliferation in addition to Tyr-343 (Fig. 4, B and C). To confirm this, we constructed EpoR-5YF

**FIGURE 3. Role of each tyrosine residue of EpoR in cell proliferation induced by the Epo stimulation and JAK2 V617F mutant.** Ba/F3 cells and Ba/F3 cells expressing the JAK2 V617F mutant were infected with an empty virus (−) or a retrovirus encoding Epo, 8YF-c-FLAG, or the 7YF mutants (7YF-Y343, 7YF-Y343, 7YF-Y401, 7YF-Y429, 7YF-Y431, 7YF-Y443, 7YF-Y460, 7YF-Y464, and 7YF-Y479). Each sub-line of Ba/F3 cells was numbered as B1–B8 and is indicated in figure. A and D, whole cell lysates were immunoblotted (IB) with an anti-FLAG antibody or anti-β-actin antibody. B and E, transduced Ba/F3 cells were incubated with RPMI 1640 medium containing 1% FBS in the presence of Epo (1 unit/ml) for 3 days. Cell proliferation was measured using the WST-1 assay. Values are the mean ± S.D. of four independent experiments. C and F, transduced Ba/F3 cells were incubated with RPMI 1640 medium containing 1% FBS in the presence of Epo (1 unit/ml) for 3 days. The cell viability was measured by trypan blue exclusion method. Values are the mean ± S.D. of three independent experiments. G and H, transduced Ba/F3 cells and Ba/F3 cells expressing the JAK2 V617F mutant were incubated with RPMI 1640 medium containing 1% FBS with IL-3 (2 ng/ml) for 3 days. Cell proliferation was measured using the WST-1 assay. Values are the mean ± S.D. of four independent experiments.
mutants harboring intact Tyr-343, Tyr-460, and one of the following tyrosine residues: (D1) 5YF-Y343/460/401; (D2) 5YF-Y343/460/429; (D3) 5YF-Y343/460/431; (D4) 5YF-Y343/460/443; (D5) 5YF-Y343/460/464; and (D6) 5YF-Y343/460/479, and we expressed them in Ba/F3 cells expressing the JAK2 V617F mutant (Fig. 4D). The expression of (D5) 5YF-Y343/460/464 induced the cytokine-independent proliferation of Ba/F3 cells expressing the JAK2 V617F mutant, similar to the expression of EpoR. However, the expression of (D1) 5YF-Y343/460/401, (D2) 5YF-Y343/460/429, and (D4) 5YF-Y343/460/443 failed to enhance the proliferation rate of these cells over that with (C5) 6YF-Y343/460 and the expression of (D3) 5YF-Y343/460/431.
and (D6) 5YF-Y343/460/479 resulted in a lower proliferation rate than with 6YF-Y343/460 (Fig. 4E, graph). In addition, compared with the viability of cells expressing (C5) 6YF-Y343/460 and the JAK2 V617F mutant, the viability of cells expressing the (D5) 5YF-Y343/460/464 and JAK2 V617F mutant was significantly increased (Fig. 4F, graph). No significant difference was observed in the proliferation ability of these cells in the presence of the IL-3 stimulation (Fig. 4G and H). We also generated EpoR-5YF mutants with Tyr-343, Tyr-460, and another tyrosine residue, and we analyzed their effects on JAK2 V617F mutant-induced cell proliferation (Fig. 5, A and B). The obtained results confirmed that Tyr-343, Tyr-460, and Tyr-464 were necessary for JAK2 V617F mutant-induced cell proliferation (Fig. 5, B and C). In addition, we constructed 6YF-Y460/464 and expressed it in Ba/F3 cells expressing the JAK2 V617F mutant (Fig. 6D). The expression of 6YF-Y460/464 failed to induce the proliferation of Ba/F3 cells expressing the JAK2 V617F mutant, suggesting that the phosphorylation of Tyr-343,
which may mediate the activation of STAT5, was essential for JAK2 V617F mutant-induced cell proliferation (Fig. 5, E and F). Furthermore, the co-expression with (C5) 6YF-Y343/460 or (C6) 6YF-Y343/464 reduced the population in the sub-G₁ phase and caused a slight increase in the population in the S phase over that in cells co-expressing 8YF (Fig. 5G). The alteration induced in the cell cycle population by (D5) 5YF-Y343/460/464 was similar to that by wild-type EpoR, suggesting that these three tyrosine residues are critically involved in JAK2 V617F-induced cell cycle regulation (Fig. 5G, graph).
Phosphorylation of Tyr-343, Tyr-460, and Tyr-464 Was Critical for JAK2 V617F Mutant-induced Cytokine-independent Cell Proliferation—

We examined whether the eight tyrosine residues in the intracellular domain of EpoR are actually phosphorylated in JAK2 V617F mutant-provoked transformed cells. We co-expressed the JAK2 V617F mutant and EpoR-7YF mutants in JAK2-deficient MEFs, and we analyzed whether the phosphorylation of the intact tyrosine residue in each EpoR-7YF mutant was detectable in an immunoblot analysis using an anti-phosphotyrosine antibody. As shown in Fig. 6A, although 8YF was not phosphorylated, the phosphorylation of the tyrosine residue in each 7YF mutant was detectable by the immunoblot analysis, indicating that all eight tyrosine residues are phosphorylated in cells expressing the JAK2 V617F mutant (Fig. 6A).

To further confirm the importance of the three tyrosine residues, Tyr-343, Tyr-460, and Tyr-464, in EpoR, we generated EpoR mutants in which these tyrosine residues were substituted with phenylalanine. The JAK2 V617F mutant was phosphorylated in cells expressing these EpoR mutants (Fig. 6B).
expressed with wild-type EpoR and the 8YF and YF mutants as follows: Y343F, Y460F, Y464F, Y343F/Y460F, Y343F/Y464F, Y460F/Y464F, and Y343F/Y460F/Y464F, in Ba/F3 cells (Fig. 6B), and the proliferation rate and the viability of these cells were analyzed (Fig. 6, C and D). The co-expression of Y343F, Y460F, and Y343F/Y460F partially reduced the proliferation rate of Ba/F3 cells expressing the JAK2 V617F mutant to lower than that with the co-expression of EpoR (Fig. 6C, graph). Furthermore, the co-expression of Y464F, Y343F/Y464F, and Y460F/Y464F caused a significant reduction in cell proliferation. The result of the point mutation at Tyr-464 suggested its more critical function over the other tyrosine residues Tyr-343 and Tyr-460. We then investigated the effects of mutants of all three tyrosine residues, Tyr-343, Tyr-460, and Tyr-464, on cell proliferation induced by the JAK2 V617F mutant. The Y343F/Y460F/Y464F mutant completely lost the ability to induce JAK2 V617F mutant-induced cell proliferation, similar to 8YF (Fig. 6C, graph). When EpoR mutants such as Y464F, Y343F/Y460F, Y343F/Y464F, or Y460F/Y464F were co-expressed with the JAK2 V617F mutant, cell viability was significantly decreased rather than the case of the cells co-expressing wild-type EpoR and the JAK2 V617F mutant. In addition, Ba/F3 cells expressing Y343F/Y460F/Y464F mutant and the JAK2 V617F mutant exhibited the comparable viability with the cells expressing 8YF and the JAK2 V617F mutant (Fig. 6D, graph).

We investigated alterations in the cell cycle population induced by these EpoR mutants. As shown in Fig. 6E, the ratio of the sub-G1 phase in cells co-expressing Y343F/Y464F and Y343F/Y460F/Y464F was markedly increased. Furthermore, the cell cycle distribution of cells co-expressing Y343F/Y460F/Y464F was similar to cells co-expressing 8YF and was characterized by a reduction in the S and G2/M phases (Fig. 6E, graph). These results clearly suggest that the phosphorylation of Tyr-343, Tyr-460, and Tyr-464 in EpoR is essential for JAK2 V617F mutant-induced cell proliferation.

JAK2 V617F Mutant Induces the Full Activation of STAT5 via Phosphorylated Tyr-343, Tyr-460, and Tyr-464 in EpoR—To further explore the properties of phosphorylated tyrosine residues, Tyr-343, Tyr-460, and Tyr-464 in EpoR, activation of downstream signaling events was examined. Because it has been reported that activated EpoR recruited a number of signaling molecules such as STAT5, CrkL, Grb2, and PI3K through the phosphorylated Tyr-343, Tyr-460, Tyr-464, and Tyr-479, respectively (7, 8, 10, 11, 27), we first investigated whether wild-type EpoR and EpoR mutants such as 8YF, (B1) 7YF-Y343, (B6) 7YF-Y460, (B7) 7YF-Y464, and (D5) 5YF-Y343/460/464 could recruit these molecules by immunoprecipitation. Wild-type EpoR and all EpoR mutants are associated with JAK2 V617F mutant (Fig. 7A, see lanes 2–7 in HA). Whereas wild-type EpoR associated with STAT5, CrkL, Grb2, and PI3Kp85, 8YF failed to associate with these molecules (Fig. 7A, see lanes 2 and 3 in STAT5, Grb2, and PI3Kp85). However, although CrkL was expressed in Ba/F3 cells expressing the JAK2 V617F mutant (Fig. 7A, IB: CrkL), EpoR failed to recruit CrkL (data not shown). Interestingly, not only EpoR but also EpoR mutants such as (B1) 7YF-Y343, (B6) 7YF-Y460, and (D5) 5YF-Y343/460/464 interacted with STAT5, suggesting that the phosphorylated Tyr-343 and Tyr-460 are critical for the recruitment of STAT5 in Ba/F3 cells expressing the JAK2 V617F mutant (Fig. 7A, see lanes 4, 5, and 7 in STAT5). Both (B6) 7YF-Y460 and
(D5) 5YF-Y343/460/464 interacted with Grb2, suggesting that the phosphorylation of Tyr-460 but not Tyr-343 or Tyr-464 seemed to be sufficient for the recruitment of Grb2 in Ba/F3 cells expressing the JAK2 V617F mutant (Fig. 7A, see lanes 5 and 7 in Grb2). Furthermore, these EpoR mutants failed to associate with PI3Kp85, suggesting that PI3K-Akt pathway seemed to be dispensable for JAK2 V617F mutant-induced oncogenic signals (Fig. 7A).

We next examined the influence of mutations in tyrosine residues, including Tyr-343, Tyr-460, and Tyr-464 in EpoR on JAK2 V617F mutant-induced activation of STAT5 and ERK. Although the co-expression of EpoR with the JAK2 V617F mutant significantly induced the phosphorylation of STAT5 at Tyr-694, 8YF failed to induce the JAK2 V617F mutant-induced tyrosine phosphorylation of STAT5 (Fig. 7B, see lanes 2 and 3 in p-STAT5 (Y694)). (B1) 7YF-Y343, (B6) 7YF-460, and (B7) 7YF-464 induced the phosphorylation of STAT5 at Tyr-694 slightly more strongly than 8YF (Fig. 7B, see lanes 3–6 in p-STAT5 (Y694)). (C5) 6YF-Y343/460 and (C6) 6YF-Y343/464 exhibited slightly stronger abilities to induce the tyrosine phosphorylation of STAT5 than the (B1) 7YF-Y343, (B6) 7YF-460, and (B7) 7YF-464 mutants; however, 6YF-Y460/464 failed to induce the tyrosine phosphorylation of STAT5 (Fig. 7B, see lanes 7–9 in p-STAT5 (Y694)). Moreover, (D6) 5YF-Y343/460/464 induced the phosphorylation of STAT5 at Tyr-694 to the same extent as EpoR (Fig. 7B, see lanes 2 and 10 in p-STAT5 (Y694)). Previously, Pircher et al. (28) reported that ERK directly interacted with STAT5α and phosphorylated STAT5α at serine residue 780 in the transactivation domain. However, the phosphorylation of STAT5α at Ser-780 was detected in Ba/F3 cells expressing JAK2 V617F mutant, and its phosphorylation level was not changed by the expression of EpoR and its mutants (Fig. 7B, see lanes 1–10 in p-STAT5 (S780)). In addition, whereas the co-expression of EpoR with the JAK2 V617F mutant significantly induced the phosphorylation of ERK1/2, the co-expression of 8YF, (B1) 7YF-Y343, (B6) 7YF-460, (B7) 7YF-464, and (C5) 6YF-Y343/460 failed to induce the phosphorylation of ERK1/2 (Fig. 6B, see lanes 3–7 in p-ERK1/2). In contrast, co-expression of (C6) 6YF-Y343/464, 6YF-Y460/464, and (D6) 5YF-Y343/460/464 induced the phosphorylation of ERK1/2 (Fig. 7B, see lanes 8–10 in p-ERK1/2). These results suggest that the phosphorylation of Tyr-464 is insufficient for the activation of ERK, but the phosphorylation of either Tyr-343 or Tyr-460 in addition to Tyr-464 could induce the activation of ERK in the cells expressing the JAK2 V617F mutant.

The effects of these EpoR mutants on the expression of the targeted genes of STAT5, such as il-2ra, cis, c-myc, and pim-1, were analyzed using real time PCR. The co-expression of (D6) 5YF-Y343/460/464 induced the expression of all series of STAT5 target genes tested in this study, such as the mRNA of il-2ra, cis, c-myc, and pim-1, to the same extent as that with the co-expression of EpoR (Fig. 7, C and D, graphs). Although (C5) 6YF-Y343/460 and (C6) 6YF-Y343/464 moderately induced the expression of il-2ra and cis mRNAs, other EpoR mutants did not affect their expression. However, the expression of c-myc and pim-1 exhibited different response patterns to the expression of EpoR mutants (Fig. 7D). (B6) 7YF-Y460 and (B7) 7YF-464 slightly induced the expression of c-myc mRNA, whereas (C5) 6YF-Y343/460 and (C6) 6YF-Y343/464 significantly induced its expression. In contrast, (B1) 7YF-Y343 and 6YF-Y460/464 failed to induce the expression of c-myc mRNA. In the case of the expression of pim-1 mRNA, (B1) 7YF-Y343 slightly induced the expression of pim-1. An additional intact tyrosine residue such as Tyr-464 (Tyr-343/464) but not Tyr-460 (Tyr-343/460) enhanced the expression of pim-1 more than Tyr-343 alone, and this was consistent with the phosphorylation of STAT5 (Fig. 7, B and D, graphs).

Furthermore, to examine whether the STAT family directly activated the promoters of these genes, chromatin immunoprecipitation (ChIP) assays were performed using (A7) V617F/− cells and (A8) V617F/EpoR cells with the specific antibodies against STAT3 and STAT5. Immunoprecipitated genomic DNA (gDNA) by antibodies against STAT3 or STAT5 was analyzed by quantitative PCR (qPCR) using primers specific for the locus of il-2ra, cis, c-myc, and pim-1 genes as shown in Fig. 8A. We detected that the binding of STAT5 onto the promoters of the il-2ra and cis genes (il-2ra-1, cis-1, and cis-2) was amplified by qPCR in the case of c-myc gene, several binding elements for STAT were found in its promoter region or enhancer region. Among them, two STAT-binding elements located in enhancers of c-myc gene (c-myc-2 and c-myc-3) were amplified by qPCR from immunoprecipitated gDNA with antibody against STAT5 in (A8) V617F/EpoR cells (Fig. 8B). Although Kiuchi et al. (29) have reported that STAT3 bound to the STAT-binding site in the promoter of the c-myc gene, the binding of STAT3 to the region was not detected (Fig. 8B, the graph of c-myc-1). In contrast, any STAT-binding elements in pim-1 promoter were not amplified by qPCR (Fig. 8B). Furthermore, we detected that phosphorylated STAT5 at Tyr-694 bound to the promoters of il-2ra and cis genes and the enhancers of the c-myc gene (Fig. 8C). We also confirmed that STAT3 was not phosphorylated in Ba/F3 cells expressing JAK2 V617F mutant and EpoR (Fig. 8, D and E). These observations clearly suggest that JAK2 V617F drastically induced the association of STAT5 onto the promoters of the il-2ra and cis genes and enhancers of the c-myc gene in the presence of EpoR.

Phosphorylation of Tyr-343, Tyr-460, and Tyr-464 in EpoR Is Required for JAK2 V617F Mutant-induced STAT5 Activation—To obtain further confirmation, we examined the effects of the EpoR YF mutants Y343F, Y460F, Y464F, Y343F/Y460F, Y343F/Y464F, Y460F/Y464F, and Y343F/Y460F/Y464F on the activation of STAT5 and ERK1/2. Y343F/Y460F/Y464F failed to induce the tyrosine phosphorylation of STAT5, similar to 8YF (Fig. 9A, see lanes 2, 3, and 10 in p-STAT5 (Y694)). Although Y343F, Y460F, Y464F, and Y460F/Y464F still moderately phosphorylated STAT5 at Tyr-694, Y343F/Y460F and Y343F/Y464F lost this ability (Fig. 9A, see lanes 4–9 in p-STAT5 (Y694)). Phosphorylation of STAT5 at Ser-780 was detected in Ba/F3 cells expressing JAK2 V617F mutant, and the expression of EpoR mutants failed to affect it (Fig. 9A, p-STAT5 (S780)). In addition, all YF mutants utilized failed to induce activation of ERK1/2 (Fig. 9A, p-ERK1/2).
Using these EpoR mutants, we investigated the mRNA expression of STAT5 target genes. The effects of these EpoR mutants on the expression of the mRNAs of STAT5 target genes exhibited different patterns for each target gene (Fig. 9, B and C). Y343F and Y460F/Y464F still strongly induced the expression of il-2rα mRNA, but its intensity was weaker than that induced by wild-type EpoR. However, other single tyrosine mutants of EpoR such as Y460F and Y464F exhibited a weaker...
ability to induce the expression of \( il-2r \) than Y343F. The multiple tyrosine mutants of EpoR, Y343F/Y460F/Y464F induced the loss of function to induce the expression of \( il-2r \); however, the intensity of their effects was similar to that with Y343F/Y460F and Y343F/Y464F (Fig. 9B, the graph of \( IL-2R \)). Moreover, the effects of these EpoR mutants on the expression of \( cis \) mRNA exhibited different patterns from their effects on the expression of \( il-2r \). In the case of \( cis \) expression, Tyr-343 appeared to be the most important for stimulating its transcription, and additional mutations at Tyr-460 and Tyr-464 did not appear to be effective when Tyr-343 was mutated. Y460F/Y464F exhibited similar suppressive effects with a single mutation at Tyr-343 (Fig. 9B, the graph of \( CIS \)). None of the YF mutants induced the expression of \( c-myc \) mRNA. Consistent with their ability to induce the phosphorylation of STAT5, Y343F, Y460F, Y464F, and Y460F/Y464F strongly induced the
expression of pim-1 mRNA. In contrast, Y343F/Y460F, Y343F/Y464F, and Y343F/Y460F/Y464F slightly induced its expression (Fig. 9C, the graph of pim-1). Although the contribution of Tyr-343, Tyr-460, and Tyr-464 of EpoR to the expression of STAT5 target genes differed, these results suggest that the phosphorylation of these three tyrosine residues in EpoR is necessary for the full activation of STAT5 by the JAK2 V617F mutant.

Phosphorylation of Tyr-343, Tyr-460, and Tyr-464 of EpoR Is Critical for JAK2 V617F Mutant-induced Tumorigenesis in Nude Mice—In an attempt to clarify the role of phosphorylated EpoR in JAK2 V617F mutant-induced tumorigenesis in vivo, transduced Ba/F3 cells expressing the JAK2 V617F mutant and EpoR mutants were inoculated into nude mice, and the induction of tumor formation was examined. Although nude mice inoculated with cells expressing only the JAK2 V617F mutant showed no changes, significant tumor formation was observed in nude mice inoculated with cells expressing the JAK2 V617F mutant with wild-type EpoR or (D6) 5YF-Y343/460/464. However, tumor formation was markedly suppressed in mice inoculated with cells co-expressing 8YF, (B7) 7YF-Y464, and 6YF-Y460/464. This is consistent with the results obtained from tissue culture experiments; the inoculation of cells expressing (B1) 7YF-Y343 and (B6) 7YF-Y460 caused slight tumor formation, and cells expressing (C5) 6YF-Y343/460 and (C6) 6YF-Y343/464/464 exhibited greater tumorigenicity (Fig. 10, A and B). Furthermore, 13 days after the inoculation, the spleen, liver, and lymph nodes were abnormally larger in nude mice inoculated with cells co-expressing EpoR or (D6) 5YF-Y343/460/464 than in those inoculated with control cells. The spleen and lymph nodes were also slightly enlarged in mice inoculated with cells co-expressing (C5) 6YF-Y343/460 and (C6) 6YF-Y343/464 (Fig. 10, C and D).

To confirm the importance of Tyr-343, Tyr-460, and Tyr-464 in JAK2 V617F-induced tumorigenesis, we analyzed tumor formation in nude mice inoculated with cells expressing the EpoR mutants, in which the three tyrosine residues were substituted with phenylalanine. Although significant tumor formation was induced in mice inoculated with cells co-expressing Y343F, Y460F, Y464F, Y343F/Y460F, Y343F/Y464F, and Y460F/Y464F, slight tumor formation was observed in mice inoculated with cells co-expressing Y343F/Y460F/Y464F (Fig. 11, A and B). In nude mice inoculated with cells co-expressing

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FIGURE 10. Critical roles of Tyr-343, Tyr-460, and Tyr-464 in EpoR on JAK2 V617F mutant-induced tumorigenesis. Transduced Ba/F3 cells (1 x 10^7) were s.c. injected into nude mice. A, nude mice were photographed 12 days post-inoculation. B, after 13 days post-inoculation, mice were sacrificed. Tumors at injected sites were weighed. Values are the mean ± S.D. of three independent experiments. Data were analyzed using the Student’s t test. ** and *** indicate significant differences of p < 0.01 and p < 0.001, respectively. n.d., not detected. C, in the mice shown in B, the spleen, liver, and lymph nodes were eviscerated and then photographed. D, spleen, liver, and lymph nodes shown in C were weighed. Values are the mean ± S.D. of three independent experiments. Data were analyzed using Student’s t test. *, **, and *** indicate significant differences of p < 0.05, p < 0.01, and p < 0.001, respectively.
Y343F/Y460F/Y464F, the enlargement of the liver, spleen, and lymph nodes was not induced, which was similar to mice inoculated with cells co-expressing 8YF. The enlargement of the spleen, liver, and lymph nodes was significant in mice inoculated with cells expressing Y464F and Y460F/Y464F (Fig. 11, C and D). Therefore, these results revealed that the phosphorylation of Tyr-343, Tyr-460, and Tyr-464 in EpoR was required for the JAK2 V617F mutant to exhibit its oncogenic activity.

**Ruxolitinib Inhibits the Cell Proliferation Induced by Not Only the JAK2 V617F Mutant but Also Epo Stimulation**—A JAK2 inhibitor, ruxolitinib, showed significant therapeutic benefit for the patients with MPN (30, 31). We investigated the effect of ruxolitinib on the proliferation of (A2) +/EpoR cells, (A5) WT/EpoR cells, and (A8) V617F/EpoR cells in the absence and presence of Epo stimulation. Ruxolitinib inhibited the proliferation of not only (A8) V617F/EpoR cells but also (A2) +/EpoR cells and (A5) WT/EpoR cells in the presence of Epo stimulation (Fig. 12). Previous study reported that ruxolitinib causes hematologic adverse events, including anemia and thrombocytopenia (30, 31). In this study, our observations clearly showed that ruxolitinib inhibits the cell proliferation induced by not only the JAK2 V617F mutant but also Epo stimulation, and these data seem to clearly explain the reason why ruxolitinib causes these hematopoietic side effects.

**Discussion**

Several studies have reported the importance of homodimeric cytokine receptors such as EpoR for the activation of the JAK2 V617F mutant and its cellular transforming activity (23–26). The FERM domain of JAK2 is conserved in the JAK family and is necessary for the interaction of JAK2 with cytokine receptors (32, 33). We previously reported that Tyr-119 in the FERM domain of JAK2 was phosphorylated by JAK2 itself, and this phosphorylation reduced binding affinity between JAK2 and EpoR (34). When Tyr-119 in JAK2 V617F mutant was substituted with glutamic acid (JAK2 V617F/Y119E), this JAK2 mutant failed to be activated by EpoR and also lacked transforming activity (24). Furthermore, Wernig et al. (35) showed that an additional mutation in the FERM domain such as Y114A in the JAK2 V617F mutant led to the inability of the JAK2 mutant to be activated by EpoR, suggesting that the physical interaction with EpoR is indispensable for the JAK2 V617F mutant to induce the pathogenesis of MPN.
In this study, we first found that the co-expression of the JAK2 V617F mutant and EpoR 8YF mutant failed to accelerate cell proliferation under tissue culture conditions with 1% FBS; however, the ability to induce tumor formation in nude mice was preserved. Lu et al. (23) reported that Ba/F3 cells co-expressing the JAK2 V617F mutant and 8YF proliferated at slower rates. They performed experiments under different culture conditions, in which 10% FBS was included in the culture medium, and this may have caused the differences in the findings obtained between their experiments and ours. Although speculative with no direct evidence, 8YF may still harbor the ability to facilitate the JAK2 V617F mutant to induce cellular transformation. As shown in Fig. 2, 8YF inhibited the proliferation of not only V617F/EpoR cells but also WT/EpoR cells and 8YF/EpoR cells stimulated with Epo.

We demonstrated that three tyrosine residues in EpoR, Tyr-343, Tyr-460, and Tyr-464, are required for JAK2 V617F mutant-induced cellular transformation. In this study, the characteristic roles of each phosphorylation site such as Tyr-343, Tyr-460, and Tyr-464 in the JAK2 V617F mutant-induced signaling pathway were clarified (Fig. 13). The phosphorylation of these tyrosine residues contributed to the full activation of STAT5. We previously reported that the silencing of STAT5 expression markedly diminished JAK2 V617F mutant-induced cellular transformation. Furthermore, the enforced expression of the constitutively active mutant of STAT5 exhibited potent oncogenic activity, suggesting that STAT5 appears to be necessary for JAK2 V617F mutant-induced cellular transformation and sufficient for tumor formation by itself. The phosphorylation of Tyr-343 and Tyr-460 contributed to the recruitment of STAT5, and the phosphorylation of Tyr-343, Tyr-460, and Tyr-464 is necessary for the activation of STAT5. However, Grb2 is recruited to the phosphorylated Tyr-460 in EpoR, and the phosphorylation of each tyrosine residue such as Tyr-343, Tyr-460, and Tyr-464 is necessary for activation of ERK1/2.
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bound to Tyr-460 but not Tyr-464 in EpoR in the transformed cells by JAK2 V617F mutant (Fig. 7A). In addition, we confirmed that EpoR mutant such as Y460F failed to recruit Grb2 (data not shown). Interaction between phosphorylated tyrosine residue in EpoR and Grb2 indicates the involvement of Tyr-460 in EpoR-mediated activation of Ras through the recruitment of SOS, a GEF protein for Ras GTPase (12).

We also observed that both 6YF-Y343/Y464 and 6YF-Y460/Y464 could induce the activation of ERK1/2, suggesting the importance of phosphorylation at Tyr-464 in EpoR for the ERK activation. However, the phosphorylation of Tyr-464 in EpoR was not sufficient for ERK activation (Figs. 7 and 9).

Furthermore, we previously reported that a truncated mutant of EpoR is sufficient for JAK2 mutant-promoted tumorigenesis and the activation of STAT5 when Tyr-343 is intact (26). In the study, the JAK2 V617F mutant failed to exhibit oncogenic activity in the presence of EpoR-HM, which lacks the ability to activate STAT5. However, EpoR-HM still exhibited weak tumorigenesis in a mouse xenograft model. Combining these findings with the results of this study, two questions arise.

1) How do Tyr-460 and Tyr-464 contribute to the activation of STAT5? 2) What is the functional role of EpoR mutants such as EpoR-Y8F and EpoR-HM in tumorigenesis in a mouse xenograft model? These points need to be clarified in further investigations involving an analysis of protein complexes, including EpoR.

As shown in Figs. 7 and 9, different patterns of the combination of tyrosine phosphorylation in EpoR appear to be required to induce each STAT5 target gene. We still do not have a suitable explanation for why these STAT5 target genes require different patterns of the phosphorylation of tyrosine residues in EpoR. However, the different complex formation of STAT5 may lead to different gene expression patterns. Previous studies reported that activated STAT5 binds DNA as dimers to regulate gene expression and may also form tetramers via N-terminal domain-mediated interactions (37, 38). STAT5 tetramers have been shown to accumulate more than its dimers in various human leukemias, indicating that a relationship exists between STAT5 tetramers and leukemogenesis (39, 40). To date, cytokine-regulated genes, the expression of which requires STAT5 tetramers, have been identified using STAT5A-STAT5B double knock-in N-domain mutant mice with STAT5 proteins that form dimers and not tetramers. Of these, il-2ra was identified as one of the genes that was expressed through STAT5 tetramers (41). Among the STAT5 target genes analyzed, the expression pattern of il-2ra mRNA exhibited the closest relationship with the proliferation and tumorigenesis of cells expressing the JAK2 V617F mutant and EpoR mutants (Fig. 7 and 9), suggesting that STAT5 forms tetramers in cells expressing the JAK2 V617F and EpoR mutants. STAT5 is also known to be phosphorylated at not only tyrosine residues but also serine residues. Although the tyrosine phosphorylation of STAT5 is required for DNA binding activity and dimerization, its serine phosphorylation is also needed for maximal transcriptional activity (42, 43). Pircher et al. (28) demonstrated that ERK directly interacts with STAT5a and phosphorylates at Ser-780. However, in our observation, STAT5 was constitutively phosphorylated at Ser-780, and EpoR/JAK2 signal failed to affect it (Figs. 7B and 9A).

These observations suggest that other protein kinases may be involved in the phosphorylation of STAT5 at Ser-780, and this could be involved in cell proliferation. Friedbichler et al. (44) demonstrated that the phosphorylation of STAT5 at a serine residue (Ser-725 in STAT5A and Ser-779 in STAT5B) was a prerequisite for STAT5-mediated leukemogenesis but played a minor role in lineage repopulation. A previous study reported that STAT5 at serine residues was phosphorylated by group I p21-activated kinase in transformed cells by BCR-ABL (45). Therefore, it is important to analyze the effect of Tyr-343, Tyr-460, and Tyr-464 in EpoR on the tetramer formation of STAT5 and phosphorylation of STAT5 at serine residues except Ser-780. Furthermore, we need to analyze the protein complexes, including EpoR, to solve the questions left in this study.

As shown in Fig. 12, ruxolitinib inhibited the proliferation of not only (A8) V617F/EpoR cells but also (A2) −/−EpoR cells and (A5) WT/EpoR cells in the presence of Epo stimulation. Recently, Nakaya et al. (46) reported that a novel JAK2 inhibitor, NS-018, suppressed the growth of Ba/F3 cells expressing the JAK2 V617F mutant more strongly than that of cells expressing wild-type JAK2, suggesting that NS-018 is a more effective therapeutic drug. However, because it has been reported that NS-018 inhibited not only the JAK2 V617F mutant but also Src family kinases (46), NS-018 may have some kind of adverse effects. Collectively, the results of this study and previous findings indicate the importance of EpoR as a suitable target for therapeutic drugs against MPN and also that the functional role of EpoR in oncogenic signaling induced by the JAK2 V617F mutant needs to be elucidated in more detail.

Experimental Procedures

Reagents—Recombinant human Epo (ESPO® 3000) and recombinant murine IL-3 were purchased from Kirin Brewery Co. (Tokyo, Japan) and PeproTech (Rocky Hill, NJ), respectively. An anti-phospho-STAT5 antibody (Tyr-694), and anti-Pi3Kp85 antibody were purchased from Cell Signaling Technology (Danvers, MA). An anti-CrkL antibody and anti-phosphotyrosine antibody were purchased from Abcam (Cambridge, MA) and BD Biosciences, respectively. An anti-β-actin antibody, anti-lamin B antibody, anti-Grb2 antibody, anti-STAT5 antibody, and anti-phospho-STAT5 antibody (Ser-780) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). An anti-FLAG antibody (M2) and anti-HA antibody (3F10) were purchased from Sigma and Roche Applied Science, respectively. Peroxidase-conjugated swine anti-goat, rabbit anti-mouse, rabbit anti-rat, and goat anti-rabbit secondary antibodies were purchased from Dako (Glostrup, Denmark).

Plasmids—Murine JAK2 c-HA and murine EpoR c-FLAG were subcloned into the retroviral plasmids, murine stem cell virus (MSCV)-Hygro and MSCV-Puro (Clontech, CA), respectively. The substitution of amino acid residues V617F in JAK2 and Y343F, Y401F, Y429F, Y431F, Y443F, Y460F, Y464F, and Y479F in EpoR was performed using a site-directed mutagenesis kit (Stratagene, CA).

Cell Cultures—JAK2-deficient MEFs were infected with an empty virus (−) and retroviruses expressing wild-type JAK2 c-HA, JAK2 V617F mutant c-HA, wild-type EpoR c-FLAG, and...
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EpoR mutant c-FLAG as described previously (26). These cells were cultured in DMEM (Nacalai Tesque, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies, Inc.) and 1% penicillin/streptomycin mixed solution (Nacalai Tesque). To select infected JAK2-deficient MEFs, 2 μg/ml puromycin and 200 μg/ml hygromycin were used. Ba/F3 cells were infected with an empty virus (−) and retrovirus expressing wild-type JAK2 c-HA, JAK2 V617F mutant c-HA, wild-type EpoR c-FLAG, and EpoR mutant c-FLAG using RetroNectin® (TAKARA, Shiga, Japan) as described previously (25). These cells were cultured in RPMI 1640 medium (Nacalai Tesque) supplemented with 10% FBS, 1% penicillin/streptomycin mixed solution, and 2 ng/ml IL-3. To select infected Ba/F3 cells, 2 μg/ml puromycin and 200 μg/ml hygromycin were used.

Cell Proliferation Assay—Transduced Ba/F3 cells (1 × 10^5 cells/500 μl) were cultured with RPMI 1640 medium supplemented with 1% FBS and 1% penicillin/streptomycin mixed solution in the presence or absence of Epo (1 unit/ml) or IL-3 (2 ng/ml) in a 24-well plate. After 24-, 48-, and 72-h incubations, living cells were counted using a Beckman Coulter Vi-Cell method. In the water-soluble tetrazolium-1 (WST-1) assay, 10^4 cells/100 μl were transduced Ba/F3 cells (1 × 10^5 cells) in a 96-well plate in the presence or absence of Epo (1 unit/ml) or IL-3 (2 ng/ml). After 24-, 48-, and 72-h incubations, 10 μl of WST-1 solution was added to each well, and the cells were incubated at 37 °C in 5% CO2 for 2 h. Absorbance was measured at 450/690 nm using the microplate reader, Infinite 200 PRO (Tecan, Switzerland).

Immunoprecipitation and Immunoblotting—Transduced JAK2-deficient MEFS (5 × 10^5 cells) and transduced Ba/F3 cells (5 × 10^5 cells) were cultured with DMEM and RPMI 1640 medium supplemented with 1% FBS and 1% penicillin/streptomycin mixed solution for 24 h, respectively. Cells were harvested in ice-cold PBS and lysed in Nonident P-40 lysis buffer (50 mm Tris-HCl, pH 7.4, 10% glycerol, 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonident P-40, 200 mM NaF, 0.2 mM Na2VO4) supplemented with protease inhibitors. Cell lysates were centrifuged at 15,000 rpm at 4 °C for 15 min to remove debris. Regarding immunoprecipitation, the supernatants were incubated with the indicated antibody and protein G-Sepharose (Zymed Laboratories Inc.) at 4 °C for 4 h. Immune complexes were precipitated and washed five times with lysis buffer and then eluted with Laemmli buffer. For co-immunoprecipitation with EpoR and its mutants, the immunoprecipitates were washed with lysis buffer as described above and then eluted from the beads by lysis buffer containing FLAG peptide (Sigma). Eluted proteins were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). Membranes were probed using the designated antibodies and visualized with the ECL detection system (GE Healthcare, UK).

Cell Cycle Analysis—Cells were fixed with 70% (v/v) ethanol at −20 °C overnight. Cells were then centrifuged at 5000 rpm for 2 min and resuspended in PBS containing 10 μg/ml RNase A (Wako, Tokyo, Japan) and 100 μg/ml propidium iodide (Sigma). Following a 30-min incubation, cell cycle parameters were measured in a flow cytometry analysis using FACS Calibur (25). All data were recorded and analyzed using CellQuest software.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)—Transduced Ba/F3 cells were cultured with RPMI 1640 medium containing 1% FBS and 1% penicillin/streptomycin mixed solution for 24 h. Total RNA was extracted using TRIzol (Life Technologies, Inc.). RT was performed using an oligo(dT)20 primer and 1 μg total RNA for first-strand cDNA synthesis, as described previously (25). Quantitative real time PCR was performed using an iCycler detection system (Bio-Rad). PCR was performed in a volume of 10 μl with the KAPA SYBR® FAST qPCR kit (KAPA Biosystems, Wilmington, MA). PCR primer sequences were as follows: gapdh 5’-acctcactccagccaaatct-3’ (upstream) and 5’-cctcctccaggaagattt-3’ (downstream); il-2ra 5’-agaacacccagattctgg-3’ (upstream) and 5’-agctggcaccgctcattca-3’ (downstream); pim-1 5’-cctcggtcgtctactg-3’ (upstream) and 5’-ccgacccatctttcaca-3’ (downstream); cisc 5’-cccaagaaggtgacagag-3’ (upstream) and 5’-tagctgctcacaaggtgcac-3’ (downstream); c-my 5’-tgcgagcagaggaaagatt-3’ (upstream) and 5’-aaccgctccatacagttc-3’ (downstream).

Transplantation into Nude Mice—To investigate oncogenic potentials in vivo, transduced Ba/F3 cells (1 × 10^7 cells) were injected subcutaneously (s.c.) into female BALB/c nude mice aged 4 weeks. Thirteen days post-inoculation, animals were sacrificed, and the weights of the tumor, liver, lymph nodes, and spleen were recorded.

Chromatin Immunoprecipitation (ChIP)—ChIP was carried out by following previously reported procedures (47) using anti-STAT3 antibody, anti-STAT5a antibody, or anti-phospho-STAT5 antibody. Co-precipitated genomic DNA was analyzed by quantitative real time PCR using an iCycler detection system (Bio-Rad). Mouse il-2ra, c-my, and cis—specific primers have been described (Fig. 8A). The PCR primer sequences were as follows: il-2ra 5’-gctcatagatagatgtgctcatttca-3’ (upstream) and 5’-tgtcaggtggtttggttgta-3’; cis-1 5’-caactcttaggctccggcccagcc-3’ (upstream) and 5’-aaccctttgagcatgacagagacac-3’ (downstream); cis-2 5’-gtccaaagcatagcgtctg-3’ (upstream) and 5’-ttccgggaagctcattacct-3’ (downstream); c-my 5’-cctcggtcgtctcaggaagat-3’ (upstream) and 5’-gctcaggttgttctctctta-3’ (downstream); c-my 5’-gctcaggttgttctctctta-3’ (downstream); c-my 5’-gctcaggttgttctctctta-3’ (downstream); pim-1 5’-cctcggtcgtctcaggaagat-3’ (upstream) and 5’-gctcaggttgttctctctta-3’ (downstream).

Acknowledgment—We thank Dr. J. N. Ihle for the retroviral vectors of JAK2 and EpoR.

Author Contributions—F. U., K. T., and M. F. T. performed the experiments and F. U. and H. T. analyzed the data obtained. F. U., K. T., and M. F. T. wrote the manuscript.
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