

A Member of Forkhead Transcription Factor FKHRL1 Is a Downstream Effector of STI571-induced Cell Cycle Arrest in BCR-ABL-expressing Cells*

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A member of the Forkhead transcription factor family, FKHRL1, lies downstream of the phosphatidylinositol 3-kinase-Akt activation pathway in cytokine signaling. Because the phosphatidylinositol 3-kinase-Akt activation pathway is required for BCR-ABL-mediated transformation and survival signaling in chronic myelogenous leukemia (CML), in this study we examined the involvement of FKHRL1 in the BCR-ABL-mediated signaling pathway. FKHRL1 was constitutively phosphorylated in BCR-ABL-expressing cell lines KCL22 and KU812, and its phosphorylation was inhibited by treatment with STI571, a specific inhibitor of BCR-ABL tyrosine kinase. Concomitantly, STI571 induced cell cycle arrest at the G₀/G₁ phase, accompanied by up-regulation of a cyclin-dependent kinase inhibitor p27/Kip1 in KCL22 cells. In addition, FKHRL1 was constitutively phosphorylated in the TF-1/bcr-abl cell line ectopically expressing BCR-ABL but not in the parent TF-1 cell line. Considering several lines of evidence that phosphorylated FKHRL1 has lost transcriptional activity and that p27/Kip1 expression is positively regulated by dephosphorylated “active” FKHRL1, BCR-ABL may down-regulate p27/Kip1 expression via the loss of FKHRL1 function as a transcription factor. To demonstrate this hypothesis, we generated a tamoxifen-inducible “active FKHRL1” FKHRL1-TM (a triple mutant of FKHRL1, in which all three Akt phosphorylation sites have been mutated), estrogen receptor system in the KCL22 cell line. The addition of tamoxifen inhibited the cell growth indicating that overexpression of FKHRL1 in the nucleus antagonized deregulated proliferation of CML cells. Collectively, FKHRL1 regulates the expression of p27/Kip1 as a downstream molecule of BCR-ABL signaling in CML cells. BCR-ABL-induced loss of FKHRL1 function may be involved in oncogenic transformation of CML partially via the down-regulation of p27/Kip1 proteins.

The *bcr-abl* fusion gene originates from a reciprocal translocation between the long arms of chromosomes 9 and 22, resulting in the formation of the Philadelphia chromosome (1). The resultant *bcr-abl* fusion gene encodes the chimeric BCR-ABL proteins of p230, p210, and p185 (230, 210, and 185 kDa). These fusion proteins have constitutively active tyrosine kinase activity and are implicated in the pathogenesis of chronic myelogenous leukemia (CML)¹ and Philadelphia-positive acute lymphoblastic leukemia (2). BCR-ABL exerts diverse actions on hematopoietic cells as follows: transformation, protection of apoptosis, cell cycle progression, altered cell migration, and adhesion to the extracellular matrix (3–10). The expression of BCR-ABL activates multiple signaling cascades, including the JAK/STAT, Ras, and phosphatidylinositol 3-kinase (PI3K) pathways (11). Among these, it was demonstrated that the activation of PI3K and the downstream Akt signaling pathways is required for not only BCR-ABL-mediated transformation but also cell survival of CML cells (12).

Recently, it was demonstrated that members of human Forkhead transcription factors FKHRL1, AFX, and FKHR are directly phosphorylated by activated Akt (13–17). We also reported that FKHRL1 is directly phosphorylated by activated Akt as one of the downstream molecules of the PI3K/Akt activation pathway in erythropoietin and thrombopoietin signalings (18, 19). More than 100 Forkhead family transcription factors have been identified in diverse species ranging from yeasts to humans (20). These transcription factors are related to embryogenesis, differentiation, and tumorigenesis. Interestingly, the three Forkhead transcription factors, FKHRL1, AFX, and FKHR, are human homologs of DAF-16, which is involved in lifespan extension of *Caenorhabditis elegans* (21, 22). The genes encoding these three molecules were originally identified in breakpoints of which chromosome translocations are recognized in human tumors (23–25).

FKHRL1 has three potential Akt phosphorylation sites (RXRXX(S/T)): Thr-32 (RPRSCT³²), Ser-253 (RRRAVS²⁵³), and Ser-315 (RSRTNS³¹⁵) (26). When cells are stimulated with serum or growth factors, FKHRL1 is phosphorylated by activated Akt and is exported from the nucleus to the cytoplasm, resulting in the inhibition of target gene transcription (13–17). In contrast, when cells are deprived of serum or growth factors, FKHRL1 becomes a dephosphorylated form, translocates into

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¹ The abbreviations used are: CML, chronic myelogenous leukemia; PI3K, phosphatidylinositol 3-kinase; ER, estrogen receptor; 4-OHT, 4-hydroxytamoxifen; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IMDM, Iscove’s modified Dulbecco’s medium; IREs, insulin-response elements; VHL, von Hippel-Lindau.

the nucleus, and activates the transcription of target genes. Thus, the transcriptional activity of FKHRL1 is negatively regulated via Akt-induced phosphorylation. Therefore, FKHRL1-TM, which is replaced at three amino acids, Thr-32, Ser-253, and Ser-315 with alanine, is predicted to be an active form as a transcription factor (13). Medema *et al.* (27) and we (19) found that FKHRL1-TM induced cell cycle arrest at the G₀/G₁ phase in a variety of cell lines. Furthermore, Dijkers *et al.* (28) reported that "active" FKHRL1 up-regulates the expression of cyclin-dependent kinase inhibitor p27/Kip1 at the transcriptional level, resulting in blockage of cell cycle progression. These findings strongly suggested that FKHRL1 controls cell cycling via regulating the p27/Kip1 expression. Taken together with several lines of evidence that BCR-ABL suppressed p27/Kip1 expression via the PI3k/Akt activation pathway (29–31), FKHRL1 may lie downstream of the BCR-ABL signaling pathway as a phosphorylated "inactive" form, leading to the down-regulation of p27/Kip1 expression. To address this notion, we examined the phosphorylation of FKHRL1 in a CML-derived cell line. To elucidate further the pathological role of FKHRL1 in CML, we generated a tamoxifen-inducible FKHRL1-TM:estrogen receptor (ER) system in a CML-derived cell line. We show here that FKHRL1 lies downstream of the BCR-ABL signaling pathway, and that this molecule has a negative effect on deregulated cell growth caused by BCR-ABL fusion protein. Loss of function of FKHRL1 may be involved in the oncogenic transformation of CML cells.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Fetal calf serum (FCS) and 4-hydroxytamoxifen (4-OHT) were purchased from Sigma. Polyclonal antibody against BCR was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-FKHRL1 (Thr-32) and phospho-c-Abl (Tyr-245) were purchased from Cell Signaling Technology Inc. (Beverly, MA). Antibody against native FKHRL1 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). An antibody against p27/Kip1 was purchased from BD Biosciences. The 2-phenylamino-pyrimidine derivative STI571 (molecular mass, 590 daltons) was developed and kindly provided by Novartis (Basel, Switzerland). The stock solutions of this compound were prepared at 1 mM with Me₂SO and stored at –20 °C. pcDNA3 containing human FKHRL1-TM-ER cDNA was kindly provided by Paul Coffey from the University Medical Center (Heidelberglaan, The Netherlands). To prepare the dominant-negative form of FKHRL1 (FKHRL1-DN), we restricted pcDNA3.1-containing human FKHRL1-TM cDNA with *Bst*1107 and *Eco*RV restriction enzymes, and we deleted the transactivation domain. Anti-Bim antibody was a gift from Toshiya Inaba (Hiroshima, Japan).

Cell Culture of CML-derived Cell Lines and Generation of Transfectants—KCL22, KU812 cell lines, and TF-1/bcr-abl cell lines were maintained in liquid culture with IMDM containing 10% FCS (32–34). TF-1 cells were maintained in liquid culture with IMDM containing 10% FCS with granulocyte-macrophage colony-stimulating factor (1 ng/ml). KCL22 cells were transfected with mammalian expression vector (pcDNA3; Invitrogen) containing human FKHRL1-TM-ER (27) or FKHRL1-DN cDNA by the Lipofectin method according to the manufacturer's instructions (Promega, Madison, WI). We selected three independent clones resistant to neomycin (1.0 mg/ml).

Colorimetric MTT Assay for Cell Proliferation—Cell growth was examined by a colorimetric assay according to Mosmann (35) with some modifications. Briefly, cells were incubated at a density of 1×10^4 /100 μ l in 96-well plates in IMDM containing 10% FCS. After 72 h of culture at 37 °C, 20 μ l of sterilized 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added to each well. Following 2 h of incubation at 37 °C, 100 μ l of 10% SDS was added to each well to dissolve the dark-blue crystal product. The absorbance was measured at a wavelength of 595 nm using a microplate reader (model 3550; Bio-Rad).

Preparation of Cell Lysates and Western Blotting—The cells were washed and suspended in lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1.7 ng/ml aprotinin, 50 μ g/ml leupeptin, 2 mM sodium orthovanadate, and 20 mM sodium fluoride. After 20 min of incubation

on ice, insoluble materials were removed by centrifugation at 15,000 \times g for 20 min. The supernatants were boiled for 5 min in SDS-PAGE sample buffer, resolved by SDS-PAGE, and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad). The blots were blocked with 5% skim milk in Tris-buffered saline for 1 h at room temperature and then incubated with the appropriate concentration of primary antibody overnight at 4 °C or for 1–2 h at room temperature. After washing with Tris-buffered saline containing Tween 20 (1:2,000), the blots were probed with a 1:10,000 dilution of anti-rabbit or anti-mouse horseradish peroxidase-conjugated second antibodies for 90 min at room temperature. After a second washing, the blots were incubated with an enhanced chemiluminescence substrate (ECL Western blot detection system; Amersham Biosciences) and exposed to Hyperfilm ECL (Amersham Biosciences) to visualize the immunoreactive bands. The blots were stripped with 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 μ M 2-mercaptoethanol at 50 °C for 30 min, washed, blocked, and reprobed.

Cell Cycle Analysis—Cell cycle analysis was performed by staining DNA with propidium iodide in preparation for flow cytometry with the FACScan/CellFIT system (BD Biosciences).

Luciferase Assay—Oligonucleotides containing an array of seven insulin-response elements (IREs) (three IRSA (CAAAACAA) and four IRSB (TTATTTTG)) were cloned into the *Kpn*I-*Hind*III site of pGL3 basic vector to generate pGL3-7xIREs. The pGL3-7xIREs and pECE vector containing FKHRL1-TM cDNA with or without pcDNA3.1 containing FKHRL1-DN cDNA were introduced into 293 cells by lipofection with the internal control plasmid pRL-TKLUC (Promega, Madison, WI). After transfection, the cells were cultured for 36 h and then harvested for dual luciferase assay according to the manual (Promega). In some experiments, a luciferase reporter containing the p27/Kip1 promoter was used.

RESULTS

STI571 Blocks Cell Cycle Progression at G₀/G₁ Phase in KCL22 Cells—In the following experiments, we mainly used the KCL22 cell line. This cell line was established from the bone marrow cells of a patient with blastic crisis of BCR-ABL-positive CML (32). Therefore, KCL22 would be a good model of CML cells for elucidating the biological function of FKHRL1. We initially examined whether or not STI571 inhibits the proliferation of KCL22 cells. KCL22 cells were exposed to various concentrations of STI571 (0.03–3 μ M) for 3 days. MTT reduction assay revealed that STI571 inhibited the proliferation of KCL22 cells in a dose-dependent manner (Fig. 1A). To clarify the mechanism by which STI571 inhibited the cell growth of KCL22 cells, we examined the effect of STI571 on cell cycling of the KCL22 cells. 1 μ M STI571 induced the G₀/G₁ arrest in KCL22 cells in a time-dependent manner. The G₀/G₁ ratio began to increase at 9 h and reached a plateau at 24 h upon STI571 treatment, inversely to S and G₂/M populations (Fig. 1B). The ratio of apoptotic cells did not increase during the observation periods up to 72 h (data not shown). As shown in Fig. 1C, a CDK inhibitor, p27/Kip1 protein was detected after 3 h of exposure to STI571, and the expression level was gradually increased up to 48 h.

STI571 Inhibits Phosphorylation of FKHRL1 Proteins in a Time- and Dose-dependent Manner in KCL22 Cells—We performed Western blotting with anti-phospho-c-Abl (Tyr-245) or anti-phospho-FKHRL1 (Thr-32) antibody in KCL22 cells. FKHRL1 and BCR-ABL fusion protein were constitutively phosphorylated in this cell line (Fig. 2A, lane 1). Next we examined whether or not STI571 inhibits the phosphorylation of these two proteins. KCL22 cells were exposed to STI571 (1 μ M) for the indicated periods of up to 8 h and then harvested. The phosphorylation of BCR-ABL fusion protein began to diminish after a 1-h exposure to STI571 (Fig. 2A). Moreover, KCL22 cells were exposed to increasing concentrations of STI571 (0.05–5 μ M) for 6 h. The phosphorylation of BCR-ABL significantly diminished at 1 μ M and completely disappeared at 5 μ M STI571 (Fig. 2B). Consistent with the kinetics of BCR-ABL phosphorylation, STI571 inhibited the phosphorylation of FKHRL1 in a dose- and time-dependent manner (Fig. 2, A and

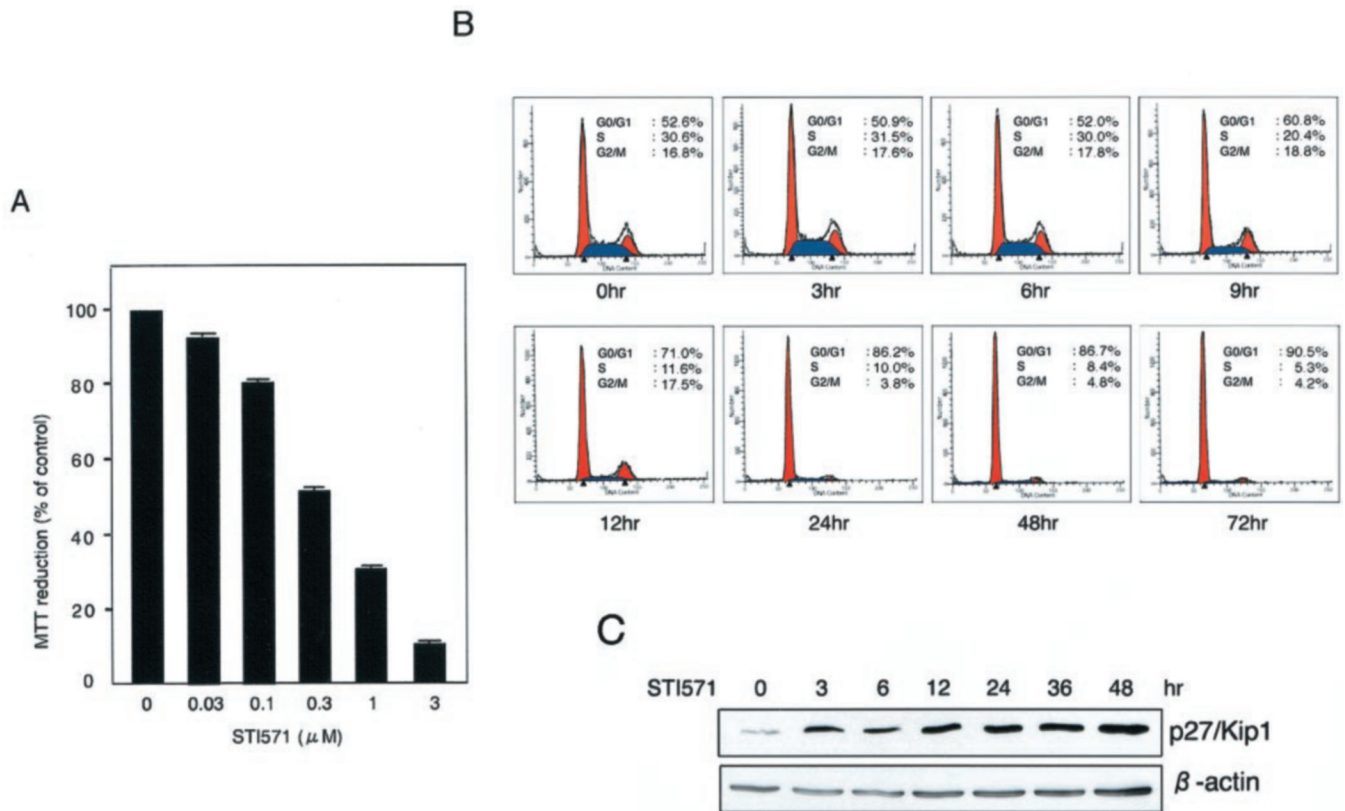


FIG. 1. **STI571 blocks cell cycle progression at the G₀/G₁ phase in KCL22 cells.** A, effect of STI571 on proliferation of KCL22 cells. KCL22 cells were plated at a density of 10,000 cells/well in IMDM supplemented with 5% FCS and cultured with various concentrations of STI571 (0.03–3 μ M). MTT reduction assay was performed after 3 days of culture. The values represent the mean \pm S.D. from triplicate cultures and are expressed as a percentage of untreated KCL22 cells. B, effect of STI571 on cell cycle. KCL22 cells were treated with STI571 (1 μ M), sequentially cultured for the periods indicated, and harvested for cell cycle analysis. C, up-regulation of p27/Kip1 protein after STI571 treatment. KCL22 cells were treated with STI571 (1 μ M), sequentially cultured for the periods indicated, and harvested for Western blotting analysis.

B). However, STI571 did not affect the expression level of BCR-ABL and FKHRL1 proteins (Fig. 2, A and B).

FKHRL1 Is Commonly Phosphorylated in BCR-ABL-positive Cell Lines—To exclude the possibility that phosphorylation of FKHRL1 is limited to KCL22, we examined the phosphorylation of FKHRL1 in another Bcr-Abl-positive cell line, KU812. BCR-ABL was constitutively phosphorylated, and its phosphorylation was inhibited by STI571 in this cell line (Fig. 3A). Concomitantly, the constitutive phosphorylation of FKHRL1 was inhibited by STI571 treatment. Collectively, these results suggested that FKHRL1 lies downstream of BCR-ABL signaling. To confirm this hypothesis, we used the TF-1/bcr-abl cell line ectopically expressing BCR-ABL (34). BCR-ABL was constitutively phosphorylated in TF-1/bcr-abl but not in the parent TF-1 cells (Fig. 3B, 1st and 2nd lanes), and its phosphorylation was inhibited after treatment with 1 μ M STI571 (Fig. 3B, 3rd lane), indicating that 1 μ M STI571 indeed inhibited the BCR-ABL activity in TF-1/bcr-abl cells. As expected, constitutive phosphorylation of FKHRL1 was observed in TF-1/bcr-abl but not in the parental TF-1 cells (Fig. 3B, 1st and 2nd lanes), and its constitutive phosphorylation was inhibited after STI571 treatment (Fig. 3B, 3rd lane). These results strongly supported our hypothesis that FKHRL1 lies downstream of BCR-ABL signaling. p27/Kip1 protein was down-regulated in TF-1/bcr-abl cells, compared with that in TF-1 cells (Fig. 3B, 1st and 2nd lanes). In addition, STI571 induced inhibition of the BCR-ABL activity, accompanied by up-regulation of p27/Kip1 protein in TF-1/bcr-abl cells (Fig. 3B, 2nd and 3rd lanes). These results strongly suggested that p27/Kip1 lies downstream of BCR-ABL signaling, as reported previously by other groups (29–31).

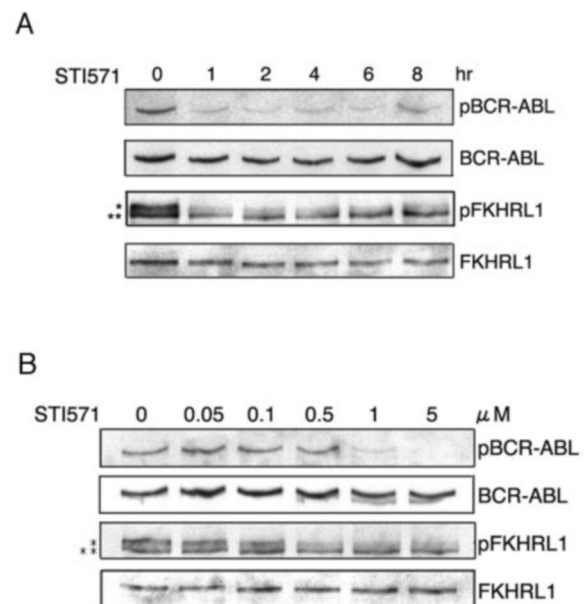


FIG. 2. **STI571 inhibits phosphorylation of FKHRL1 proteins in a time- and dose-dependent manner in KCL22 cells.** The cells were then stimulated with increasing concentrations of STI571 (0.05–5 μ M) for 6 h (A) or with STI571 (1 μ M) for the periods indicated (B). After solubilization, cell extracts were resolved by 7.5% SDS-PAGE and immunoblotted with the antibodies directed against phospho-Thr-32. The blot was reprobbed with anti-FKHRL1 antibody to confirm equal loading of protein. Anti-phospho-FKHRL1 antibody recognizes two bands; the upper band (*) is the phosphorylated form, the lower band (**) is nonspecific.

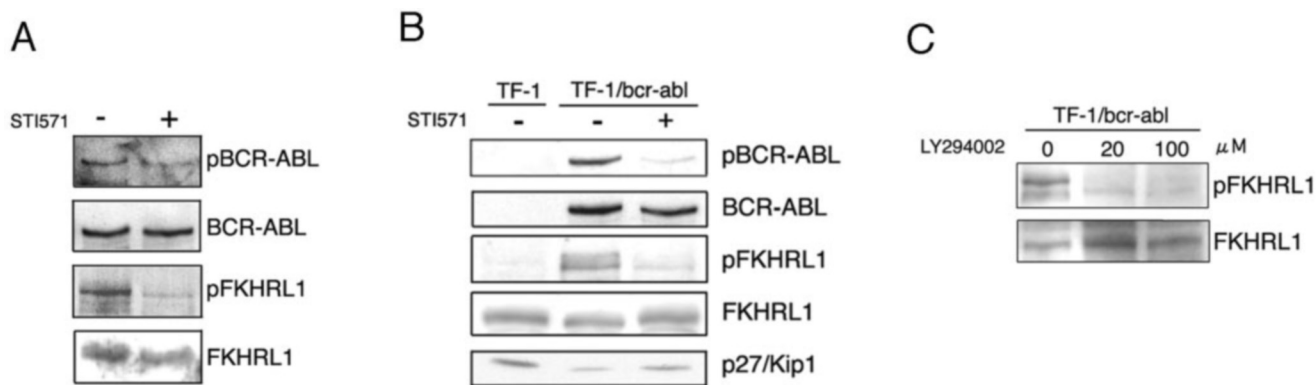


FIG. 3. **FKHRL1 is commonly phosphorylated in BCR-ABL-positive cell lines.** A, constitutive phosphorylation of FKHRL1 in KU812 cells. KU812 cells were stimulated with STI571 (1 μM) for 6 h. After solubilization, cell extracts were resolved by 7.5% SDS-PAGE and immunoblotted with the antibodies directed against phospho-FKHRL1 (Thr-32) antibody. The blot was reprobed with anti-FKHRL1 antibody to confirm equal loading of protein. B, BCR-ABL-induced phosphorylation of FKHRL1. Granulocyte-macrophage colony-stimulating factor-deprived TF-1 cells or TF-1/bcr-abl cells were treated with STI571 (1 μM) for 6 h. After solubilization, cell extracts were resolved by 7.5 or 15% SDS-PAGE and immunoblotted with the antibodies directed against phospho-ABL, phospho-FKHRL1 (Thr-32), or p27/Kip1. The blot was reprobed with anti-BCR or FKHRL1 antibody to confirm equal loading of protein. C, BCR-ABL-PI3K-dependent phosphorylation of FKHRL1. TF-1/bcr-abl cells were treated with LY294002 (20 and 100 μM) for 1 h. After solubilization, cell extracts were resolved by 7.5% SDS-PAGE and immunoblotted with the antibody directed against phospho-FKHRL1 (Thr-32). The blot was reprobed with FKHRL1 antibody to confirm equal loading of protein.

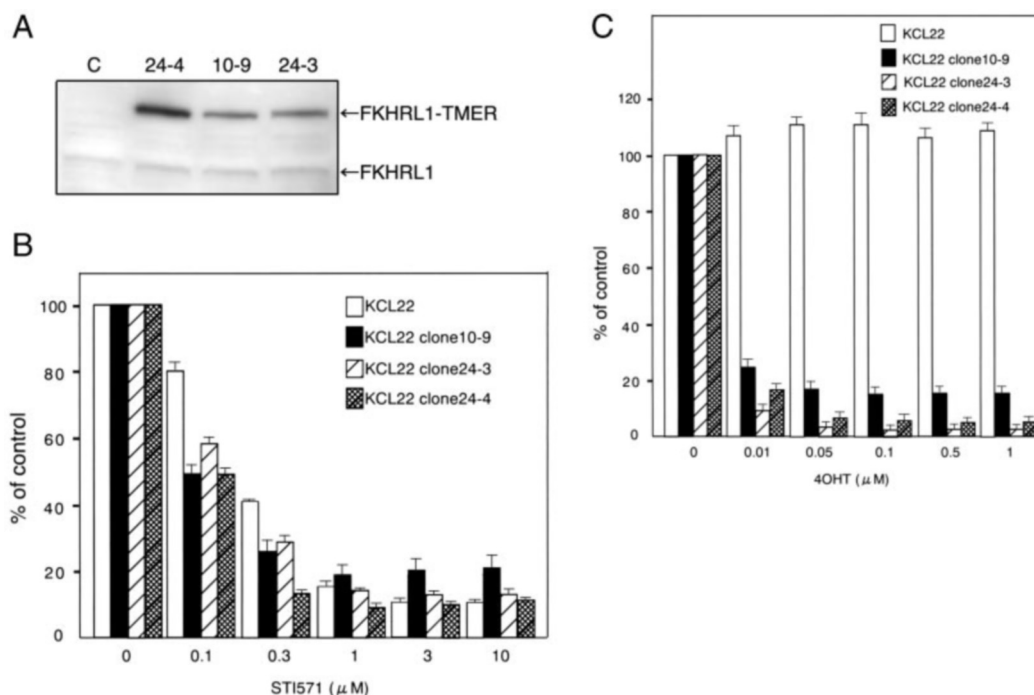
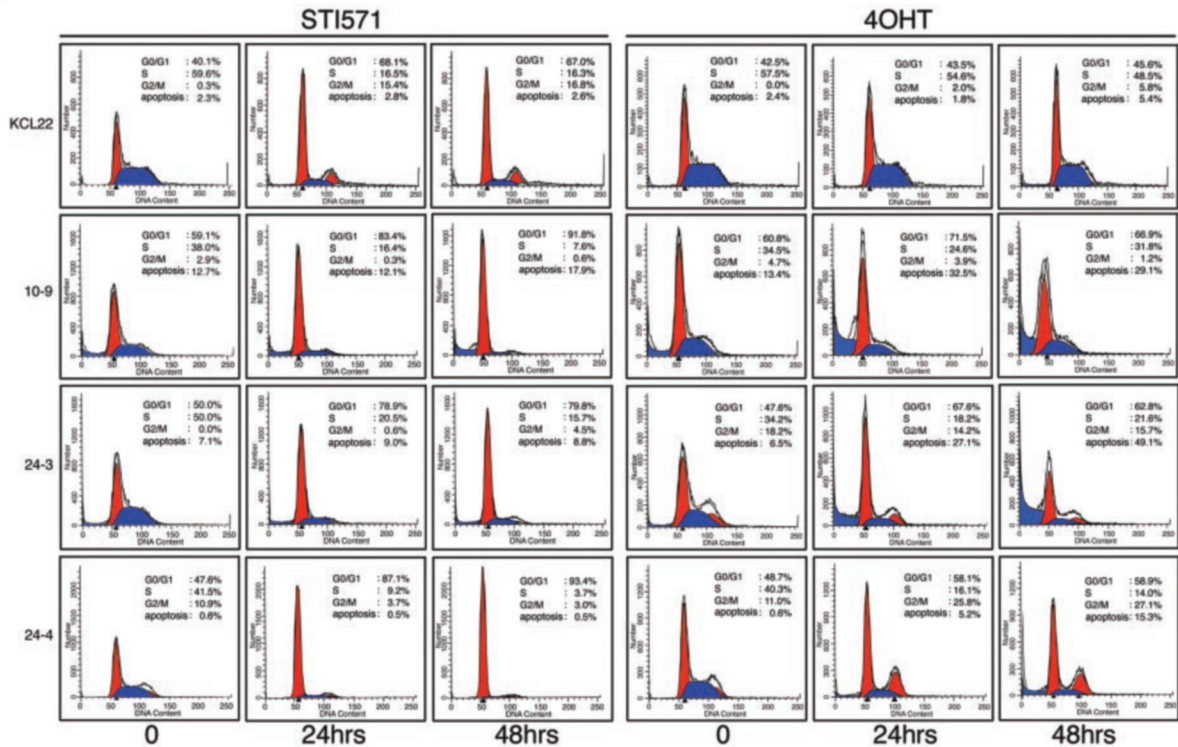


FIG. 4. **Active FKHRL1 (FKHRL1-TM-ER) inhibits the proliferation of KCL22 cells via up-regulation of p27/Kip1.** A, generation of transfectants expressing FKHRL1-TM-ER. After solubilization, cell extracts were resolved by 7.5% SDS-PAGE and immunoblotted with the antibodies directed against anti-FKHRL1 antibody. The parent KCL22 cells were used as a control (lane C). B, effect of STI571 on the proliferation of transfectants. The cells were plated at a density of 10,000 cells/well in IMDM supplemented with 10% FCS and cultured with various concentrations of STI571 (0.1–10 μM). MTT reduction assay was performed after 3 days of culture. The values represent the mean ± S.D. from triplicate cultures and are expressed as a percentage of untreated transfectant cells. The parental KCL22 cells were used as a control. C, effect of 4-OHT on proliferation of transfectants. The cells were plated at a density of 10,000 cells/well in IMDM supplemented with 10% FCS and cultured with various concentrations of 4-OHT (0.01–1 μM). MTT reduction assay was performed after 3 days of culture. The values represent the mean ± S.D. from triplicate cultures and are expressed as a percentage of untreated transfectant cells. The parental KCL22 cells were used as a control. D, effect of FKHRL1-TM on cell cycle. The transfectant cells and the parent cells were treated with STI571 (1 μM) or 4-OHT (0.5 μM), sequentially cultured for the periods indicated, and harvested for cell cycle analysis. E, induction of p27/Kip1 or Bim protein after STI571 and 4-OHT treatment. The parent KCL22 and the transfectant cells were treated with STI571 (1 μM) or 4-OHT (0.5 μM), sequentially cultured for the periods indicated, and harvested for Western blotting analysis with antibody against p27/Kip1 or Bim protein. The blot was reprobed with β-actin antibody to confirm equal loading of protein.

PI3K Activity Is Involved in BCR-ABL-dependent Phosphorylation of FKHRL1—We examined whether PI3K activity is involved in BCR-ABL-dependent phosphorylation of FKHRL1 using a specific PI3K inhibitor LY294002. As shown in Fig. 3C, 20 μM LY294002 completely suppressed the phosphorylation of FKHRL1, indicating that the phosphorylation of FKHRL1 is dependent on PI3K activity in BCR-ABL signaling.

Active FKHRL1 (FKHRL1-TM-ER) Inhibits the Proliferation of KCL22 Cells—Considering that FKHRL1 is constitutively phosphorylated in BCR-ABL-expressing cells, FKHRL1 may be in an “inactive state” in CML, leading to oncogenic transformation. To clarify this hypothesis, we generated transfectants expressing FKHRL1-TM-ER which became the active form after the addition of 4-OHT. We selected three independent pos-

D



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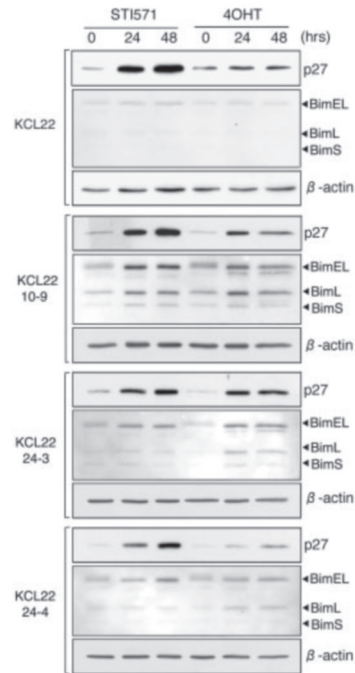


FIG. 4—continued

itive clones highly expressing ectopic FKHRL1-TM-ER (Fig. 4A). The transfectant cells or the parent KCL22 cells were treated with STI571 (1 μ M) or 4-OHT (0.5 μ M) for the indicated periods and then harvested for MTT incorporation assay or cell cycle analysis. As shown in Fig. 4B, STI571 inhibited the proliferation of the transfectant cells to the same degree as the parent KCL22 cells. These results suggested that the transfectant cells have still retained the sensitivity to STI571 to the same degree as the parent KCL22 cells. Induced activation of FKHRL1-TM by 4-OHT treatment led to the suppression of

MTT incorporation into the cells, whereas 4-OHT treatment had no effect on MTT incorporation into the parental control cells (Fig. 4C). These results suggest that FKHRL1-TM-ER is actually functional in our system. As shown in Fig. 4D, the percentage of cells in the G₀/G₁ phase was significantly elevated 24 h after 4-OHT treatment, suggesting that FKHRL1-TM-ER induced cell cycle arrest at the G₀/G₁ phase. Concomitantly, the expression level of p27/Kip1 protein was significantly elevated after 24 h of exposure to 4-OHT (Fig. 4E). As shown in Fig. 4D, the ratio of sub-G₁ population repre-

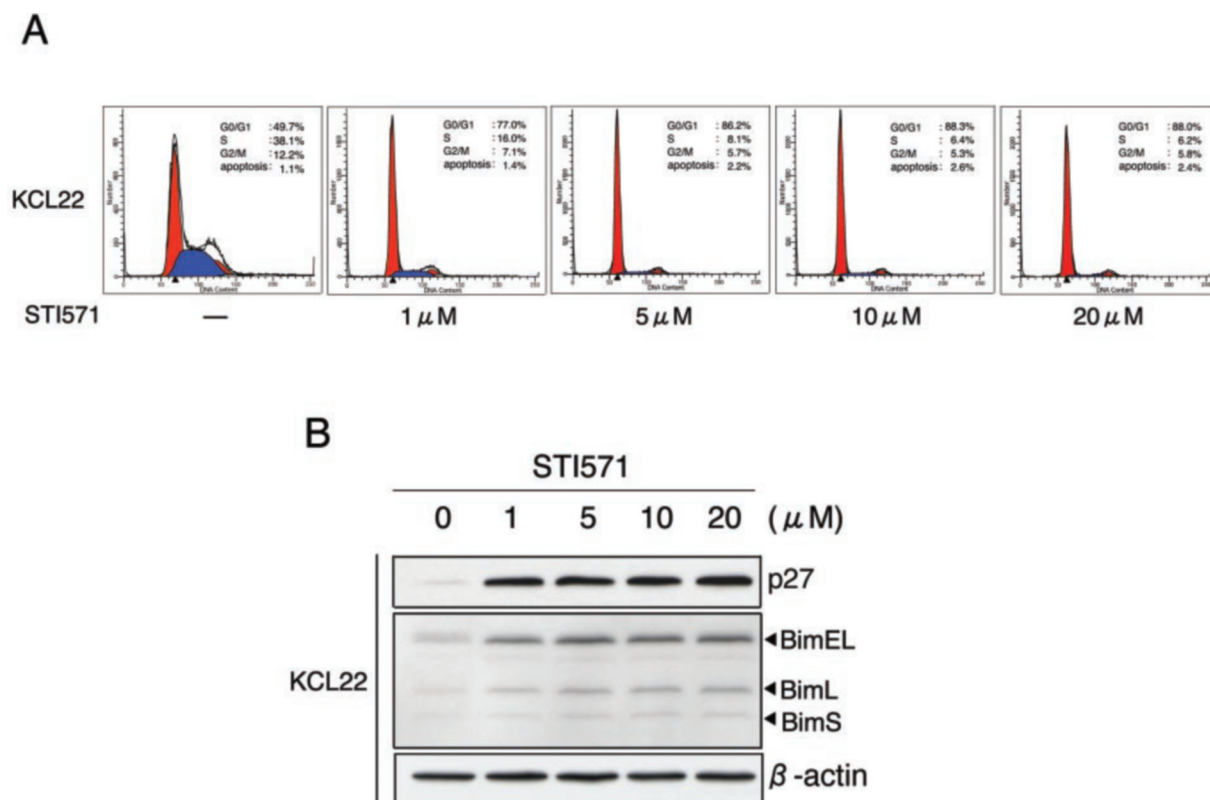


FIG. 5. High dose of STI571 induces cell cycle arrest at the G₀/G₁ phase and up-regulation of Bim protein but not apoptosis in KCL22 cells. KCL22 cells were cultured with various concentrations of STI571 (1–20 μM) for 48 h and then harvested for cell cycle analysis or Western blotting analysis. **A**, effect of high dose of STI571 on cell cycle. **B**, effect of high dose of STI571 on the expression of p27/Kip1 and Bim proteins. Western blotting analysis was performed with antibody against p27/Kip1 or Bim protein. The blot was reprobated with β-actin antibody to confirm equal loading of protein.

senting apoptosis was increased after 4-OHT treatment in these three clones, especially in clones 10-9 and 24-3. To elucidate the mechanism by which the transfectant cells underwent rapid apoptosis after the addition of 4-OHT, we focused our attention on the expression of Bim, a member of the BH3-only subfamily of cell death activators, because it was recently reported that Bim is transcriptionally regulated by FKHRL1 (36). As shown in Fig. 4E, the expressions of BimEL, BimL, and BimS proteins were slightly up-regulated 24 and 48 h after the addition of 4-OHT. A high dose of STI571 up to 20 μM induced cell cycle arrest at the G₀/G₁ phase but not apoptosis during the observation periods up to 72 h (Fig. 5A). However, up-regulation of Bim proteins was observed in the presence of STI571 in the range of 1–20 μM (Fig. 5B).

A Dominant-negative FKHRL1 (FKHRL1-DN) Enhances the Sensitivity of KCL22 Cells to STI571—To further elucidate the functional role of FKHRL1 in STI571-induced cell cycle arrest of KCL22 cells, we generated transfectants expressing FKHRL1-DN but lacking the transactivation domain. Initially, we examined whether FKHRL1-DN functions as a dominant-negative inhibitor. For this purpose, we used a pGL3-7xIREs construct containing seven consecutive FKHRL1-binding sites, and we introduced this construct into 293 cells with FKHRL1-TM cDNA and then assayed the luciferase activity. As illustrated in Fig. 6A, FKHRL1-TM induced promoter activity, which was completely inhibited by FKHRL1-DN. These results indicate that FKHRL1-DN functions as a dominant-negative inhibitor on the transactivation activity of FKHRL1-TM. Based on this result, we transfected KCL22 cells with pcDNA3.1 containing FKHRL1-DN and selected three independent positive clones expressing FKHRL1-DN protein (Fig. 6B). Cell cycle analysis demonstrated that STI571 treatment

significantly enhanced the ratio of the sub-G₁ population in the transfectants expressing FKHRL1-DN but not vector alone (Fig. 6C). The ratio of sub-G₁ population was increased in proportion to the expression level of FKHRL1-DN protein (Fig. 6, A and C). Consistent with this finding, cell viability of the transfectants was much lower than that of the parent cells (Fig. 6D). This suggested that the inhibition of endogenous FKHRL1 by FKHRL1-DN enhanced sensitivity to STI571 in KCL22 cells.

DISCUSSION

In this study, we demonstrated that FKHRL1 is located downstream to the BCR-ABL signaling pathway as an inactive phosphorylated form and functions as an effector when FKHRL1 is converted to the dephosphorylated form by STI571 treatment in BCR-ABL-expressing cells. We found that FKHRL1 is constitutively phosphorylated in KCL22, KU812, and TF-1/bcr-abl cells and that STI571 induces down-regulation of the BCR-ABL tyrosine kinase activity, dephosphorylation of FKHRL1, and cell cycle arrest at the G₀/G₁ phase in KCL22 cells. This was accompanied by up-regulation of p27/Kip1 expression. Moreover, 4-OHT-inducible FKHRL1-TM induced the cell cycle arrest and induced up-regulation of p27/Kip1 proteins in the KCL22-derived transfectant cells. Taken together, our results suggested that STI571 inhibited the cell growth of BCR-ABL-expressing cells, at least in part via dephosphorylated FKHRL1-mediated up-regulation of p27/Kip1 proteins. Therefore, constitutive phosphorylation of FKHRL1 induced by BCR-ABL fusion protein may be an important mechanism in BCR-ABL-mediated cell cycle signaling (29–31).

STI571 (1 μM) inhibited the proliferation of cells but did not induce apoptosis in KCL22 cells during the observation periods

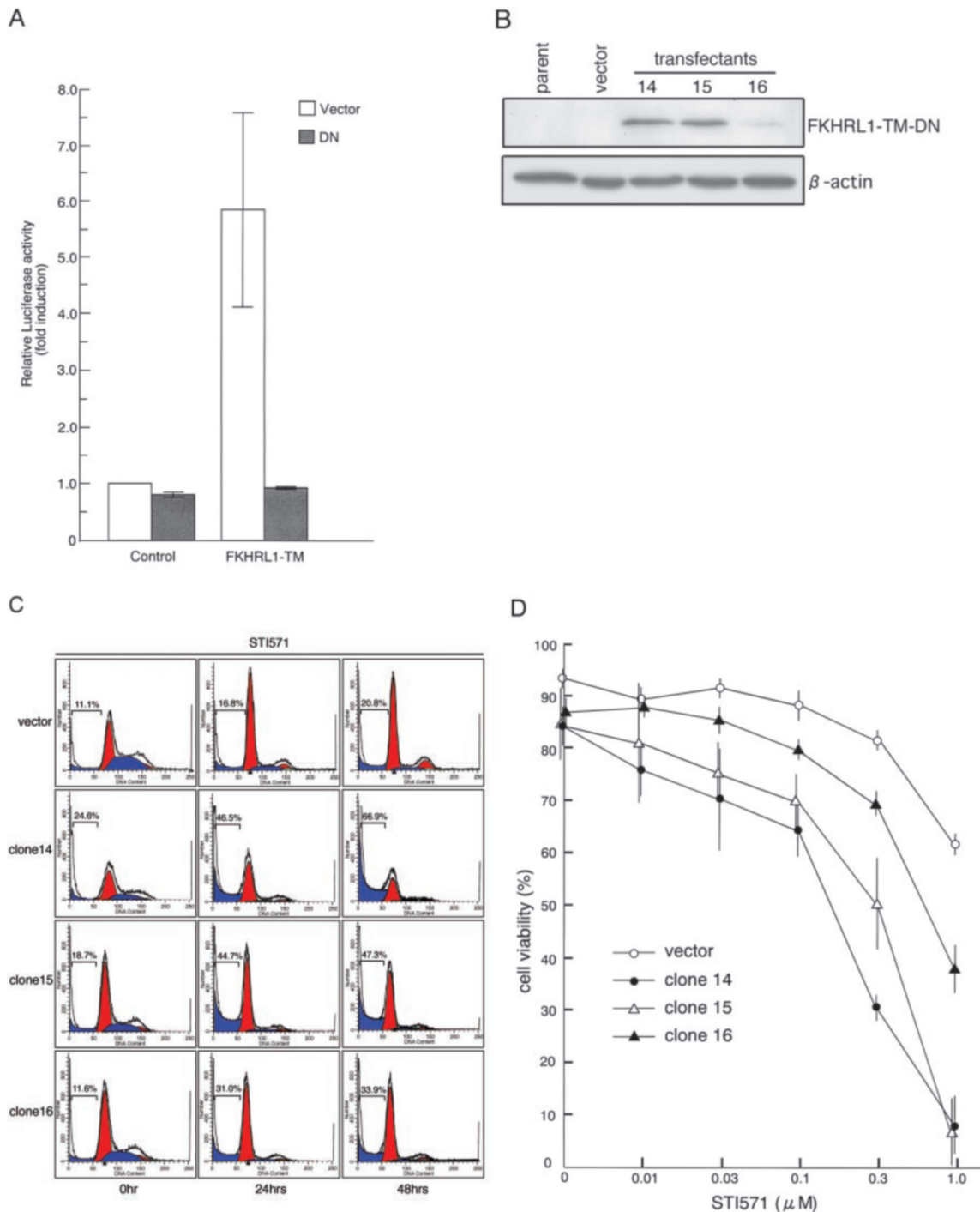


FIG. 6. A dominant-negative (DN) FKHRL1 (FKHRL1-DN) enhanced sensitivity to STI571 in KCL22 cells. *A*, a dominant-negative FKHRL1 suppresses the transcription activity of FKHRL1. Two hundred ninety three cells were co-transfected with pGL3-7xIREs vector and pcDNA3.1 or pcDNA3.1-FKHRL1-WT with or without pcDNA3.1-FKHRL1-DN expression vector. PRL-TK vector was used as an internal control. After a 36-h incubation, the cells were harvested for dual luciferase assay. The results are expressed in terms of the increased induction of luciferase activity from pcDNA3.1 vector alone. The values represent the mean \pm S.D. from triplicate experiments. *White column*, minus pcDNA3.1-FKHRL1-DN; *black column*, plus pcDNA3.1-FKHRL1-DN. *B*, expression of FKHRL1-DN proteins in the transfectants. After solubilization, cell extracts were resolved by 7.5% SDS-PAGE and immunoblotted with antibodies directed against anti-FKHRL1 antibody. *C*, effect of FKHRL1-DN on cell cycle. The transfectant cells and parent cells were treated with STI571 (1 μ M), sequentially cultured for the periods indicated, and harvested for cell cycle analysis. *D*, cell viability of transfectants and the parent KCL22 cells after STI571 treatment. Cells were treated with increasing concentrations of STI571 (0.01–1 μ M) for 4 days and then the cell viability was assessed by trypan dye exclusion ($n = 4$).

up to 72 h. Taken together with the previous reports that 1 μ M STI571 was sufficient to induce cell death in BCR-ABL-transformed cell lines and primary leukemia cells from CML patient samples in chronic phase, KCL22 cells appear to be less sensitive to STI571. This may be explained by several lines of evidence that KCL22 was established from a patient with CML

blastic crisis and that survival pathways other than BCR-ABL tyrosine kinase possibly operate in the blastic crisis cells. However, because phosphorylated FKHRL1 was detected in TF-1/bcr-abl but not the parental TF-1 cells, we concluded that FKHRL1 lies downstream of BCR-ABL tyrosine kinase.

The ectopic expression of active FKHRL1 did not induce cell

cycle arrest as robustly as STI571. In fact, in clones 10-9 and 24-4, the changes in the ratio of G₀/G₁ population was about 10%, whereas treatment with STI571 routinely elicited 20–40% changes. Therefore, besides FKHRL1, other cell cycle-associated molecules such as FKHR and AFX may be also involved in STI571-induced cell cycle arrest, because these Forkhead family members commonly induce p27/Kip1 (27, 37).

p27/Kip1 is generally regulated at the post-translational level via a proteasome-dependent degradation pathway (38–40). Consistent with this, it was previously shown that BCR-ABL fusion inhibited the p27/Kip1 expression through the PI3K-Akt pathway and its down-regulation mainly occurred through a proteasome-dependent degradation pathway (31). However, it was reported recently (28) that FKHRL1 elevated the p27/Kip1 promoter activity in Ba/F3 cells expressing a 4-OHT-inducible FKHRL1-TM-ER construct, suggesting that the transcriptional activity of FKHRL1 directly induced the p27/Kip1 gene expression. Thus, the mechanism of how FKHRL1 regulates p27/Kip1 expression was still controversial. To address this unresolved problem, we examined the mechanism by which FKHRL1 regulates the p27/Kip1 expression in KCL22 cells. In this study, we found that both STI571 treatment and ectopic expression of FKHRL1-TM induced the up-regulation of p27/Kip1 expression at the protein level but not at the mRNA level (Figs. 1D and 4E and data not shown). In addition, a luciferase assay revealed that 4-OHT treatment did not enhance the promoter activity of the p27/Kip1 gene in the KCL22 transfectants having 4-OHT-inducible FKHRL1-TM (data not shown). Although we cannot completely exclude the possibility that the discrepancy among these results was dependent on the different cell lines used, our results suggested that the regulation of p27/Kip1 expression by FKHRL1 does not occur at the transcriptional level, at least in KCL22-derived transfectant cells. If so, FKHRL1 may indirectly up-regulate the p27/Kip1 expression via an unidentified target molecule of FKHRL1. Therefore, it would be of interest to identify the target molecules of FKHRL1. Previously, it was reported that the von Hippel-Lindau (VHL) tumor suppressor molecule controls cell cycle progression by regulation of p27/Kip1 at both the mRNA and protein levels (41). However, our preliminary data showed that 4-OHT did not induce VHL proteins in the KCL22 transfectants expressing FKHRL1-TM-ER (data not shown). Therefore, it is unlikely that VHL is a target molecule for FKHRL1.

Bim is a member of the BH3-only subfamily that inhibits the function of anti-apoptotic Bcl-2 family members by binding to them, resulting in inhibition of release of cytochrome *c* from the mitochondria to the cytosol (42). Recently, Shinjyo *et al.* (43) reported that Bim lies downstream of cytokine signaling, and its down-regulation is a prerequisite for the survival of hematopoietic cells. In addition, Kuribara *et al.* (44) reported that BCR-ABL induced deregulated hematopoiesis via the down-regulation of Bim expression in CML. These findings suggested that Bim expression is critical for the regulation of hematopoiesis, and its deregulated expression may contribute to leukemogenesis such as CML. In this study, we found that 4-OHT induced apoptosis, accompanied by up-regulation of Bim proteins (BimEL, BimL, and BimS) in BCR-ABL-positive KCL22 cells ectopically expressing FKHRL1-TM-ER. However, even a high dosage of STI571 (20 μ M) did not induce apoptosis, irrespective of the up-regulation of Bim proteins (Fig. 5B). These results suggested that the expression of Bim proteins is required but not sufficient for the induction of apoptosis, at least in KCL22 cells. This notion is supported by the evidence that STI571 induced G₀/G₁ arrest and up-regulation of p27/Kip1

and Bim proteins but not apoptosis in clone 24-4 (Fig. 4, D and E).

It is noteworthy that although a dominant-negative form of FKHRL1 (FKHRL1-DN), though not completely, did significantly block the STI571-induced cell cycle arrest at the G₀/G₁ phase, this mutant drastically enhanced STI571-induced apoptosis in KCL22 cells. These results strongly suggest that active FKHRL1 could protect the KCL22 cells from apoptosis via the induction of cell cycle arrest at the G₀/G₁ phase. Considering the several lines of evidence that the *C. elegans* DAF2 pathway regulates a state of diapause called dauer, which is an arrested juvenile form triggered by food limitation, high temperature, and crowding, and that the DAF16 is the main target of the DAF2 pathway, it would not be surprising that active FKHRL1 protects cells from apoptosis via cell cycle arrest at the G₀/G₁ phase in the presence of STI571. If so, FKHRL1 may play an important role in the acquisition of “dormancy” in leukemia cells after exposure to anti-leukemic agents.

However, it should be stressed that 4-OHT-induced expression of FKHRL1-TM-ER also induced apoptosis in KCL22 cells. Previously, we established a tetracycline-inducible system in a human TPO-dependent cell line UT-7/TPO and showed that induced expression of FKHRL1-TM lacking ER led to cell cycle arrest at G₀/G₁ phase but not to apoptosis in this cell line (19). Although we cannot exclude the possibility that this discrepancy was due to the different cell lines used in the experiments, overexpression of FKHRL1-TM-ER fusion protein may in part act as a dominant-negative inhibitor of Akt activity. This notion can be supported by several lines of evidence that overexpressed FKHRL1 binds to Akt (45) and that overexpression of the FKHR mutant replacing Ser-256 with alanine strongly suppressed the phosphorylation of the endogenous Akt substrates FKHRL1 and GSK3 α (46). Alternatively, overexpression of FKHRL1-TM may deprive endogenous FKHRL1 of cofactors prerequisite for the transcription activity, and as a result, FKHRL1-TM might function as a dominant-negative inhibitor against anti-apoptotic effect of endogenous FKHRL1, although this concept is speculative at present. Thus, as proposed by Woods and Rena (47), caution may be needed when considering any results of overexpression experiments with FKHRL1-TM. Very recently, Kops *et al.* (48) reported that glucose deprivation-induced apoptosis is significantly blocked by a PI3K inhibitor LY294002 in a human colon carcinoma cell line. However, our preliminary experiments revealed that LY294002 up to 20 μ M did not prevent 4-OHT-induced apoptosis in the KCL22 transfectant expressing FKHRL1-TM-ER. Therefore, it is unlikely that constitutive activation of PI3K activity by BCR-ABL tyrosine kinase enhanced 4-OHT-induced apoptosis in the FKHRL1-TM-ER-expressing cells.

The data presented here demonstrate that FKHRL1 is important in the oncogenic transformation of CML. PTEN acts as a tumor suppressor, at least in part, by antagonizing PI3K-Akt signaling (49). Recently, it was reported (37) that Forkhead transcription factors including FKHRL1 are critical effectors of PTEN-mediated tumor suppression, suggesting that FKHRL1 also functions as a tumor suppressor. Taken together with our results that FKHRL1 lies downstream of BCR-ABL signaling as an inactive phosphorylated form, and that FKHRL1-TM suppressed the cell growth of CML-derived cell line KCL22, active FKHRL1 may be a potent anti-proliferative molecule against BCR-ABL-induced tumorigenesis. In addition, our finding that FKHRL1-DN caused apoptosis in STI571-treated KCL22 cells suggests that FKHRL1 represents an attractive target for therapeutic manipulation in CML.

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