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AKT1 induces caspase-mediated cleavage of the CDK inhibitor p27Kip1 during cell cycle progression in leukemia cells transformed by FLT3-ITD

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Abstract

p27Kip1 cleavage and caspase-3 regulate cell cycle in human myeloma cells and B cells however regulation of p27Kip1 cleavage during the cell cycle is not known. In BaF3-FLT3-ITD cells, p27Kip1 undergoes C-terminal cleavage. Inhibition of the PI3K/AKT pathway is associated with decreased cleavage of p27Kip1 and G1 phase arrest. The caspase-3 inhibitor reduces p27Kip1 cleavage and inhibits cell proliferation. Knockdown shRNA against AKT1 reduces cleavage of p27Kip1, inhibits caspase-3 activation, and is associated with a delay in cell cycle progression. Taken together, these findings indicate that AKT1 induces caspase-mediated cleavage of p27Kip1, required for G1-S progression in FLT3-ITD cells.

Keywords

FLT-ITD; AKT1; PI3K; Caspase 3; p27Kip1; cell cycle; AML

Introduction

Leukemogenesis is in part based on deregulation of one or more pathways mediating normal proliferation, apoptosis or self-renewal. The presence of a FLT3 ITD mutation, present in 25% of patients with AML, promotes clonal proliferation and is associated with an adverse outcome in acute myeloid leukemia (AML) patients treated with standard chemotherapy [1,2]. Understanding the downstream effects of FLT3-ITD mediated signals could lead to the development of new therapeutic agents. The PI3K/AKT pathway is constitutively activated by FLT3-ITD mutations [3,4]. AML patients with up-regulated activity of PI3K/AKT pathway have a relatively poor prognosis [5,6]. Pharmacologic inhibition of PI3K by LY294002 results in growth arrest of AML cells [7]. Our previous studies also show that

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Authorship Contributions

X.Y. and S.L. contributed equally to this work; S.K. and R.M.S. provided the conception and design of the study and drafted the article for important intellectual content; X.Y. and S.L. performed experiments; X.Y., S.K. and R.M.S. wrote manuscript and performed data analysis and all authors approved final version submitted.

Conflict of interest

There is no potential conflict of interest to declare.

inhibition of the PI3K/AKT pathway leads to cell cycle arrest but only has a minimal effect on apoptosis in FLT3-ITD transduced BaF3 (BaF3/FLT3-ITD) leukemic cells [8].

The AKT1-dependent phosphorylation and cytoplasmic mislocalization of p27Kip1 may account for proliferation mediated by an activated oncogene in cancer cells [9-11]. Previous studies show that the PI3K pathway is crucial in regulating the cyclin-dependent kinase (CDK) inhibitor p27Kip1 during G1/S progression [12]. The CDK inhibitor p27Kip1 forms complexes with cyclin D-CDK4/6 and cyclin E-CDK2, and thus inhibits CDK activity which is required for G1/S transition [13,14]. The amount of p27Kip1 is generally up-regulated in quiescent cells and is down-regulated upon cell cycle entry. Down-regulation of p27Kip1 expression is associated with aggressive tumor behavior and poor clinical outcome in cancers [15]. The down-regulation of p27Kip1 in cell cycle is mainly via decreased translation [16] and increased degradation [14,17]. Proteasome-dependent degradation of nuclear p27Kip1 requires phosphorylation at T187 by CDK2 [18-20]. Phosphorylation-mediated nuclear export of p27Kip1 represents another aspect of p27Kip1 regulation [21-23]; cytoplasmic retention of p27Kip1 is found in cancers [12,24,25]. Cytoplasmic retention of p27Kip1 may involve phosphorylation of S10 by hKIS [22,26], through phosphorylation of T157 and T198 by AKT [9,25-29], and via binding to 14-3-3 in cytoplasm.

Despite the aforementioned convincing evidence that p27Kip1 cleavage is critical for cell cycle regulation in cancer cells, the interaction of this moiety with apoptosis-promoting caspase 3 or caspase 3-like proteases [30,31] remains unclear. Furthermore, the regulation of p27Kip1 cleavage during the cell cycle requires elucidation in leukemia cells. We demonstrate that the PI3K/AKT pathway promotes caspase-3 activation and p27Kip1 cytoplasmic cleavage leading to G1-S progression consequent to the presence of FLT3-ITD. The cleavage of p27Kip1 to p23Kip1 removes the nuclear localization signal (NLS) and thus prevents the protein from entering the nucleus. PI3K/AKT pathway inhibition is associated with inhibition of caspase 3 inhibition limiting p27Kip1 cleavage. Taken together, the AKT-caspase 3-p27Kip1 pathway is involved in FLT3-ITD-mediated cell cycle regulation and could represent a therapeutic target in AML.

Material and Methods

Cell culture, treatments and reagents

FLT3-ITD transduced BaF3 stable cell lines (BaF3/FLT3-ITD) were maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM L-Glutamine and 400mg/ml G418. The FLT3 inhibitor PKC412 was obtained from Novartis; FLT3 inhibitor AG1296, PI3K inhibitor LY294002 and caspase-3 inhibitor Z-VAD-fms were obtained from Calbiochem-Novabiochem Corp (San Diego, CA). BaF3/FLT3-ITD cells were cultured at a starting density of 2×10^5 cells/ml in RPMI 1640 for 24 hours before cells were treated. For drug treatments, the FLT3 inhibitors PKC412 (5 nM) or AG1296 (5 μ M), the PI3K inhibitor LY294002 (15 μ M) or the caspase-3 inhibitor Z-VAD-fmk (50 μ M) were added to the medium.

Antibodies

Anti-p27Kip1 rabbit polyclonal antibody and monoclonal antibody, anti-cyclin D1 monoclonal antibody, anti-cyclin D2 rabbit polyclonal antibody, anti-cyclin D3 rabbit polyclonal antibody, anti- β -Tubulin monoclonal antibody, anti- β -actin monoclonal antibody, anti-Lamin B rabbit polyclonal antibody, anti-phospho-pRb rabbit polyclonal antibody and anti-caspase-3 rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Inc., (Waltham, MA). Anti-phospho-AKT (S473) polyclonal

antibody and anti-AKT mouse monoclonal antibody were procured from Cell Signaling Technology (Danvers, MA).

Analysis of cell cycle

The cells were grown and treated with different inhibitors for varying intervals of time as described above. The cells were fixed and stained with propidium iodide (PI) and were analyzed using flow cytometry.

Silencing of AKT1 by RNA interference

Five different clones of control or AKT1 shRNA lentiviral plasmids (vector: pLKO.1-puro) were purchased from Sigma-Aldrich. The AKT1 shRNA or control shRNA lentiviral plasmids were introduced into target cells (BaF3/FLT3-ITD cells) by electroporation using GenePulse II (Biorad) following the manufacturer's instructions. The cells were submitted to selection by both G418 (400 µg/ml, selection for transduction of FLT3-ITD plasmid) and puromycin (3 µg/ml, selection for transduction of shRNA plasmids). The knockdown of AKT1 was verified by Western blot.

Nuclear and cytoplasmic extraction

Cells were harvested and rinsed with ice-cold phosphate buffered saline (PBS). The nuclear and cytoplasmic extraction was done using NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Fisher Scientific Inc. Rockford, IL) following the manufacturer's instructions.

Immunoprecipitation and immunoblot analysis

Cells were harvested and rinsed with ice-cold phosphate buffered saline (PBS). Ice-cold lysis buffer (20 mmol/L Tris, pH7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na₃VO₄, 1 mg/ml leupeptin, 1 mmol/ml PMSF) was added to 1×10⁷ cells and sonicated on ice four times for five seconds each followed by microcentrifugation for 10 minutes at 4°C. Cell lysate (200 µl at 1 mg/ml) was pre-cleaned by incubating with 20 µl of 50% bead slurry of Protein-A agarose beads (Upstate) at 4°C for 30–60 minutes. Primary antibodies (1 µg) were added to 200 µl of the cell lysate and incubated with gentle rocking at 4°C for 2 hours. Protein A agarose beads (20 µl of 50% beads slurry) were added and incubated with gentle rocking at 4°C for additional 2 hours followed by microcentrifugation for 30 seconds at 4°C. The beads were washed five times with the ice-cold PBS. The proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blotted with primary antibodies and then with secondary antibodies. The protein bands were then detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). The same blots were stripped and re-probed with desired antibodies to confirm equal loading of proteins in various lanes. The blots were also analyzed by densitometric scanning and the relative values were normalized to actin. In each case the western blot depicted is a representative example from one of three independent experiments.

Immunostaining of cells and confocal microscopy

The cells were washed with growth medium, prefixed in growth medium containing 3.7% formaldehyde at 37°C for 15 minutes, washed with PBS, permeabilized in PBS containing 0.2% Triton X-100 at 25°C for 5 minutes, washed with PBS, post fixed in PBS containing 3.7% formaldehyde at 25°C for 5 minutes. Following washing with PBS, the cells were blocked with 10% goat serum at 25°C for 1 hour, stained with anti-p27Kip1 mouse monoclonal Ab (Santa Cruz, CA) at 25°C for 1.5 hour, washed with PBS, incubated with

FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 25°C for 40 minutes, washed with PBS, and incubated with 2 μ M TO-PRO3 (Molecular Probes Inc., Eugene, OR) at 25°C for 10 minutes. After mounting the coverslips, images were captured with a LSM510 confocal microscope (ZEISS).

Results

PI3K pathway regulates cleavage of p27Kip1

Cell proliferation and cell cycle were analyzed in BaF3/FLT3-ITD cells treated with the FLT3 inhibitor AG1296 or PI3K inhibitor LY294002 (Figure 1a). In concert with previous findings, treatment of BaF3/FLT3-ITD cells with the FLT3 inhibitor AG1296 was associated with growth inhibition (Figure 1a); cell cycle arrest (Figure 1b) was noted with AG1296 as well as the FLT3 inhibitor in clinical development, PKC412. Treatment with LY294002 also resulted in growth inhibition (Figure 1a) and G1 arrest (Figure 1b). PI3K inhibition only resulted in minimal apoptosis compared with FLT3-ITD inhibition (Figure 1b). The PI3K/AKT pathway was also inhibited by either the FLT3 inhibitor AG1296 or by the PI3K inhibitor LY294002 (Figures 1c and d).

To further study the effect of the PI3K/AKT pathway on the regulation of p27Kip1, BaF3/FLT3-ITD cells were treated with LY294002 (15 μ M) and the protein level of p27Kip1 was evaluated by western blot. Using an N-terminal p27Kip1 antibody, we identified a full-length form and a 23kDa clipped form (Figure 2a and b). Only the full length was detected using C-terminal p27Kip1 antibody (Figures 2a and b), implying cleavage at the C-terminal. LY294002 treatment was associated with a time-dependent accumulation of full-length p27Kip1 (Figure 2a) and a decrease of the p23Kip1 clipped-form (Figure 2a), suggesting that PI3K/AKT pathway is involved in the inhibition of the cleavage of p27Kip1.

We investigated the cleavage of p27Kip1 during cell cycle progression of cells that were released from G1 arrest. Following the exposure of cells to LY294002 (15 μ M) for 12 hours, cells were synchronized at G1 via washing and re-seeding in fresh media without LY294002. Removing LY294002 from the media was associated with down-regulation of the full-length p27kip1 (Figure 2b); and furthermore, the accumulation of the 23kDa clipped form of Kip1 was up-regulated over time-dependent fashion (Figure 2b). A caspase-3 cleavage site is located near the C-terminal (Figure 2c), suggesting that the cleavage may be carried out by caspase-3 or a caspase-3 like enzyme.

The cleavage of p27Kip1 is mediated by caspase-3 or caspase-3 like enzymes

We determined the activation of caspase-3 in BaF3/FLT3-ITD cells in the presence or absence of the PI3K/AKT pathway inhibitor LY294002. The activation of caspase-3 can be assessed by detecting the cleavage of its precursor (caspase-3 FL; full length) into active proteolytic fragments (caspase-3 CF; cleaved fragments). Caspase-3 is activated in BaF3/FLT3-ITD cells as detected by its cleaved fragments under normal culture conditions (Figure 3a, control) despite an expectation that such a proapoptotic step would be inhibited in FLT3-ITD cells. Inhibiting the PI3K/AKT with LY294002 was associated with substantial down regulation of caspase-3 activation (Figure 3a). Caspase-3 inhibitor (carbobenzoxycarbonyl-Val-Ala-Asp-fluotomethyl ketone [Z-VAD-fmk]) treatment of BaF3/FLT3-ITD cells released from LY294002 inhibition inhibited the p27Kip1 cleavage (Figure 3b). Taken together, these findings indicate that, in BaF3/FLT3-ITD cells, constitutive activation of PI3K/AKT pathway activates caspase-3.

AKT1 is upstream to caspase-3 and regulates cleavage of p27Kip1

To insure that AKT1 regulates caspase-mediated cleavage of p27Kip1 in BaF3/FLT3-ITD cells, we introduced a lentiviral-vector expressing shRNA against AKT1 (Figure 4a). Specific knockdown of AKT1 by RNA interference was associated with significant inhibition of caspase 3 activation (Figure 4b). Additionally, the cleavage of p27Kip1 during G1-S progression was reduced in cells where AKT1 expression was **down-regulated** (Figure 4c). Total p27Kip1 was also reduced in AKT1-knockdown cells compared with control cells (Figure 4c), possibly due to the ubiquitin-mediated nuclear degradation of p27Kip1 [32,33]. Furthermore, the progression from G1 was delayed in AKT1 knockdown cells (Figure 4d). These results demonstrated that AKT1 was specifically required for caspase-3 activation, p27Kip1 cleavage and G1 progression.

Cleavage of p27Kip1 occurs in cytoplasm resulting in nuclear exclusion

To identify the localization of p27Kip1 in cells during LY294002 treatment and after removal of LY294002 inhibition, BaF3/FLT3-ITD cells were stained using a p27Kip1 antibody that binds both the full-length and clipped form of this protein; a FITC conjugated second antibody was used for visualization with under a confocal microscopy. After treatment with LY294002 for 24 hours, p27Kip1 was localized in both cytoplasm and nucleus (Figure 5a). However, after removal of LY294002 from the cell culture for 30 minutes, p27Kip1 was delocalized from nucleus to cytoplasm (Figure 5b). In order to investigate the localization of full-length p27Kip1 and its clipped form p23, nuclear and cytoplasmic extractions were carried out on cells collected at different time intervals after the release of G1 arrest by LY294002 removal. Levels of p27Kip1 were reduced and levels of p23 were increased in BaF3/FLT3-ITD cells after release from G1 arrest (Figure 5c). The clipped form p23Kip1 was only detected in the cytoplasmic extract, while the full length p27Kip1 was detected in both nuclear and cytoplasmic extracts (Figure 5c).

Discussion

Previous studies have shown that the PI3K/AKT pathway phosphorylates p27Kip1 and delocalizes it from nucleus [23,29], in part by cytoplasmic signals via 14-3-3 [10,11]. In investigating the FLT3-ITD mediated factor independent growth in leukemia cells, we found that the downstream PI3K pathway mediates caspase 3 activation without causing apoptosis. Such AKT1-mediated caspase 3 activation facilitates cleavage of p27Kip1 during G1-S progression. The cytoplasmic cleavage of p27Kip1 prevents it from entering the nucleus where p27Kip1 can bind cyclic-CDK complexes to inhibit CDK activity and thus prevent cell cycle progression.

Although caspase-3 is well known to be involved in apoptosis, caspase-3 activation is not sufficient for apoptosis [30,34,35]. Caspase-3 can be involved in apoptosis and proliferation depending on the cellular context. Our results demonstrate that caspase-3 is activated in normally growing BaF3/FLT3-ITD cells (Figure 3a); inhibiting the PI3K/AKT pathway pharmacologically or genetically leads to substantial down regulation of caspase 3 activation (Figure 3a) and delay in G1-S progression.

Our results are consistent with the hypothesis that caspase 3-mediated cleavage of p27Kip1 in cells transformed by FLT3-ITD allows cell cycle progression in this oncogenic context. Further our AKT1 knockdown experiments suggest that this member of the PI3K/AKT pathway is upstream of caspase 3. Downregulation of AKT1 results in decreased caspase 3 cleavage, increase in nuclear localized full length p27Kip1 and decreased cell cycle progression.

These results suggest that inhibition of AKT may represent a therapeutic strategy in mutant FLT3 AML. Trials involving putative direct FLT3 inhibitors as single agent therapy in patients with advanced mutant FLT3 AML have shown biological activity but unimpressive disease remitting activity [36,37]. Reasons proposed for the lack of major activity include pharmacologic issues (not enough drug at the target for a long enough time), elaboration of survival factors in the protected stem cell niche where the leukemic colony forming cells resides, or activation of alternative or downstream leukemogenic pathways. Combining tyrosine kinase inhibitors with down stream pathway inhibitors such as the mTOR inhibitor rapamycin [38] or the STAT3 inhibitor CDDO-Me [39] represents a strategy which could synergistically inhibit FLT3-ITD mediated activation of proliferation. Our recent experiments suggest that inhibiting the elaboration of AKT1 protein or its function could alone (or in combination with FLT3 inhibitors) be developed as a therapeutic strategy in mutant FLT3 AML.

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d

Figure 1.

Arrest of BaF3/FLT3-ITD cells in G1 by inhibiting PI3K/AKT pathway. **a**, Cells under indicated treatments were counted on D0 (start time), D1 (1 day after seeding), D2 (2 days after seeding), D3 (three days after seeding): X-axis represents time; Y-axis represents cell counts for viable cells. The results are expressed as Mean + SE from three independent experiments. **b**, Cells were collected for cell cycle analysis after indicated treatments for 24 hours: the Y-axis represents cell counts and the X-axis represents DNA amount; the percentages of cells in G1 phase (G1) are shown in heavily shaded, S phase (S) in striped areas and apoptosis (AP) in lightly shaded areas. The experiments were repeated twice. Columns: mean of triplicate data points from one of two independent experiments; bars: SE (bottom panel). **c-d**, BaF3/FLT3-ITD cells were starved for at least 8 hours in medium containing 1% FBS and then treated with the indicated concentrations of FLT3 inhibitor AG1296 or the PI3K inhibitor LY294002 for 90 minutes. Western blotting was done to detect phospho-AKT (Ser-473). The growth factor IL-3 was used as a positive control for stimulation of AKT phosphorylation. The membranes were stripped and re-probed with anti-human AKT antibodies to show equal loading.

Figure 2.

Inhibition of PI3K/AKT pathway reduces the cleavage of p27Kip1. **a**, BaF3/FLT3-ITD cells were harvested after treatment with LY294002 for the indicated times and cell lysates were analyzed by immunoblotting with two kinds of anti-p27Kip1 antibodies: one recognizes both full-length and clipped form (upper panel) and the other only binds to the full length p27Kip1 (middle panel). The membranes were stripped and re-probed with anti-Actin antibody to demonstrate equal loading. **b**, BaF3/FLT3-ITD cells were synchronized at G1 by treatment with LY294002 for 12 hours and cells were harvested after removal of LY294002 for the indicated times. Total cell lysates were then analyzed by immunoblotting with two

kinds of anti-p27Kip1 antibodies (as described above): one recognizes both full-length and clipped form (upper panel) and the other only binds to the full length p27 Kip1 (middle panel). The membranes were stripped and re-probed with anti-Actin antibody to demonstrate equal loading (bottom panel). **c**, A capase-3 cleavage site is shown in mouse p27Kip1 protein. Cleavage at this site by caspase-3 or caspase-3 like enzymes generates clipped form p23 (removing C-terminal) which lacks nuclear localization signal (NLS).

Figure 3.

Inhibition of caspase-3 reduces the cleavage of p27Kip1. **a**, Inhibition of PI3K/AKT pathway by LY294002 reduces activation of caspase-3. BaF3/FLT3-ITD cells were harvested after treatment with LY294002 for the indicated times and cell lysates were then analyzed by immunoblotting with anti-caspase-3 antibody (upper panel). The membranes were stripped and re-probed with anti-Actin antibody to demonstrate equal loading (bottom panel). **b**, BaF3/FLT3-ITD cells were synchronized at G1 by treatment with LY294002 for 12 hours and cells were seeded in fresh medium containing ZVAD-fms after removal of LY294002 for the indicated times. Cell lysates were then analyzed by immunoblotting with anti-p27Kip1 antibody which recognizes both full-length and clipped form (upper panel).

The membranes were stripped and re-probed with anti-Actin antibody to demonstrate equal loading (bottom panel). The experiments were repeated three times. The amounts of p27Kip1 (normalized to actin, shown as mean + SE) are depicted in the graph (squares represent p27Kip1 from cells not treated with Z-VAD-fms and the diamonds represent cells treated with Z-VAD-fms).

a **b**

C

d

Figure 4.

Akt1 knockdown by RNAi inhibits caspase-3 activation and cleavage of p27kip1. BaF3/FLT3-ITD cells were transfected with lentiviral-AKT1-shRNA plasmids and 6 stable clones were picked. Results with one of the 6 stable clones are shown. Cells were arrested with treatment of LY294002 and then released from LY294002 inhibition for the indicated times.

a, Cell lysates were analyzed by immunoblotting with Western blot using anti-AKT1 antibody to determine AKT1 knockdown (upper panel). **b**, Cell lysates prepared were also analyzed by immunoblotting with caspase-3 antibody which recognizes both full-length caspase-3 precursor (Casp-3 FL) and activated form (Casp-3 CF). The membranes were stripped and re-probed with anti-Actin antibody to demonstrate equal loading (bottom panel). **c**, Cell lysates were also analyzed by immunoblotting with anti-p27Kip1 antibody which recognizes both full-length and clipped form. The membranes were stripped and re-probed with anti-Actin antibody to demonstrate equal loading. The experiments were repeated three times. The actin-normalized p27Kip1 and p23Kip1 levels are depicted in the graph (diamonds: the amount of p27Kip1 in a control shRNA-treated cell line; squares: p23Kip1 in control shRNA-treated cells; triangles: p27Kip1 in AKT1 shRNA cell line; cross: p23Kip1 in AKT1 shRNA cells). The graph shows the results from one of the three independent experiments. **d**, Cells were collected and analyzed for S phase entry. Two different stable transfected AKT1 shRNA-treated clones were used in triplicate experiments. Columns indicate the mean + SE of triplicate data points. (Lightly shaded: AKT1 shRNA clone 36; Highly shaded: AKT1 shRNA clone 37; White: control shRNA).

Figure 5.

The cleavage of p27Kip1 occurs in cytoplasm and the clipped form p23Kip1 unable to enter nucleus. **a-b**, BaF3/FLT3-ITD cells after treatment with LY294002 for 24 hours (5a) or after removal of LY294002 for 30 minutes (5b), were stained with anti-p27Kip1 antibody and analyzed by confocal microscopy. **c**, BaF3/FLT3-ITD cells were synchronized at G1 by treatment with LY294002 for 12 hours, and cells were harvested for nuclei extraction after removal of LY294002 for the indicated times. Nuclear and cytoplasmic extracts were then analyzed by immunoblotting with two kinds of anti-p27Kip1 antibodies: one recognizes both full-length and clipped form (upper panel). Levels of β-tubulin (cytoplasm marker) and lamin B are shown to demonstrate purity of the fractions (bottom panel).