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Provisional chapter

The Roles of AMP-Activated Protein Kinase-Related Kinase 5 as a Novel Therapeutic Target of Human T-Cell Leukaemia Virus Type 1-Infected T-Cells

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Additional information is available at the end of the chapter

1. Introduction

HTLV-1 (human T-cell leukemia virus type 1) is a human retrovirus and the causative agent of ATL (adult T-cell leukemia), which is an aggressive and fatal T cell malignancy characterized by dysregulated proliferation of CD4-positive T cells [1-3]. HTLV-1 causes ATL in 3-5% of infected individuals after a long latent period of 40-60 years [4]. The prognosis of patients with aggressive ATL remains poor with a median survival time of less than 1 year despite advances in both chemotherapy and supportive care [5, 6]. Infiltration of leukemic cells into various organs, such as lymph nodes, liver, spleen, lung, skin and intestinal tract, is a frequent manifestation of ATL. This type of cell infiltration often poses serious clinical problems for ATL patients, affecting the disease profile and prognosis. Because tumor cell survival and growth are maintained by nutrients, especially glucose and oxygen supplied by blood vessels, angiogenesis is considered to be essential for tumor malignancy [7].

Currently, the molecular mechanism of malignant transformation by HTLV-1 remains undefined. However, Tax, the 40-kDa transactivator protein encoded by HTLV-1, plays a crucial role in T cell transformation and leukemogenesis. Tax triggers viral transcription as well as induction of cellular genes involved in cell proliferation and anti-apoptotic signaling. In addition to activation of transcription, Tax transforms the infected cells by some mechanisms due to protein-protein interaction between Tax and other proteins [8, 9]. Moreover, one key feature of ATL is aneuploidy and chromosomal instability. Tax also contributes transformation of the cells by inducing aneuploidy and inactivating chromosomal instability checkpoint [10]. Indeed, it immortalizes primary human T cells derived from peripheral blood or cord blood [11, 12] and induces tumors and leukemia in transgenic mice [13, 14].

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NF-κB (nuclear factor κB) is a major survival signaling pathway activated by HTLV-1. This pathway is constitutively active in HTLV-1-transformed T-cells and primary ATL cells [15, 16].Tax can activate NF-κB pathway by associating with various signaling molecules in this pathway. For example, Tax binds IKKγ (also known as NEMO) and triggers the phosphorylation of IKKα and IKKβ, which form a complex with IKKγ [15]. Subsequently the IKK complex phosphorylates IκBα, leading to its proteasome-mediated degradation, which frees IκBα-sequestered cytoplasmic NF-κB to migrate into the nucleus where it activates the transcription of NF-κB-responsive genes [15]. Tax can also stimulate an alternative NF-κB pathway through the IKKα-dependent processing of the NF-κB p100 precursor protein to its active p52 form [17]. The NF-κB signaling pathways are activated in ATL cells that do not express Tax, although the mechanism of activation remains unknown [16]. One of the potential mechanisms by which ATL cells could develop resistance to apoptosis is through the activation of NF-κB. From this point of view, NF-κB has become an attractive target for therapeutic intervention.

AMPK (AMP-activated protein kinases) are a class of serine/threonine kinases that are activated by increased intracellular concentrations of AMP. ARK5 is a fifth member of the AMPK catalytic subunit family [18-20], and involved in tumor invasion and metastasis [21], and also known to induce cell survival during nutrient starvation or death receptor activation [22, 23]. ARK5 promoter contains two putative MARE (Maf-recognition element) sequences [24]. The *maf* proto-oncogene is identified within the genome of the avian musculoaponeurotic fibrosarcoma virus, AS42 [25]. The products of the Maf family share a conserved bZip motif that mediates dimer formation and DNA binding to the MARE [26]. Transcription of *ARK5* gene is regulated by the large Maf-family proteins including c-Maf and MafB. ARK5 is induced when a c-Maf or MafB expression vector is introduced into non-ARK5-expressing colon cancer cells [24]. Deregulated expression of ARK5 is also associated with Maf-transforming activity in human angioimmunoblastic T-cell lymphoma and in Maf-driven T-cell lymphoma in transgenic mice [27]. In multiple myeloma cells, over expression of ARK5 correlates with the expression of c-Maf and MafB and exhibits increased invasiveness [24]. ARK5 mRNA expression in colon cancer is stage-associated and liver metastatic foci of colon cancer express very high levels of ARK5 mRNA [28, 29]. In this study, we focused on ARK5 and analyzed its expression and role on the growth of HTLV-1-infected T-cells.

2. Materials and methods

2.1. Reagents

Bay 11-7082 and LY294002 were purchased from Calbiochem. D-(+)-glucose was purchased from Nakalaitesque.

2.2. Cell lines

The HTLV-1-uninfected T-cell leukemia cell lines MOLT-4 and CCRF-CEM, the HTLV-1-infected T-cell lines MT-2 [30], MT-4 [31],C5/MJ [32], SLB-1 [33], HUT-102 [1], MT-1 [34] and TL-OmI [35]were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (Sigma-Aldrich) at

37˚C in 5% CO_2. MT-2, MT-4, C5/MJ and SLB-1 are HTLV-1-transformed T-cell lines which were established by an *in vitro* coculture protocol. MT-1 and TL-OmI are leukemic T-cell lines derived from patients with ATL. HUT-102 was established from a patient with ATL, but its clonal origin is unclear. TY8-3 is an IL-2-dependent cell line established from a thymoma specimen of a myasthenia gravis patient. TY8-3/MT-2 cells were established from TY8-3 cells by coculture with mitomycin C-treated HTLV-1-infected MT-2 cells in the presence of IL-2 [36]. JPX-9 and JPX/M (kindly provided by Dr. M. Nakamura, Tokyo Medical and Dental University, Tokyo, Japan) are subclones of Jurkat cells that express Tax wild type and Tax mutant protein defective in its some abilities including activation of NF-κB, respectively, under the control of the metallothionein promoter [37]. Expression of Tax was induced by addition of $CdCl_2$ to a final concentration of 20 μM.

2.3. RT (reverse transcriptase)-PCR

Total cellular RNA was extracted from cells using Trizol reagent as described by the supplier (Invitrogen). First-strand cDNA was synthesized in a 10-μl reaction volume using RNA-PCR kit (TAKARA BIO) with random primers. Thereafter, cDNA was amplified for ARK5 and c-Maf. The oligonucleotide primers used were as follows: for ARK5; sense, 5′- GAGTCCACTC-TATGCATC-3′ and antisense, 5′- ATGTCCTCAATAGTGGCC-3′; for c-Maf; sense, 5′-TGCACTTCGACGACCGCTTCT C-3′ and antisense, 5′- CGCTGCTCGAGCCGTTTTCTC-3′. Product sizes were 256-bp for ARK5 and 327-bp for c-Maf. The amplification programs were follows: denaturing at 94˚C for 2 min, an annealing step at 55˚C for 30 s and an extension step at 72˚C for 30 s. Amplification cycles were 35 cycles for ARK5 and c-Maf, 25 cycles for β-actin. The PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining.

2.4. Real-time RT-PCR

Total RNA was extracted from cells with Trizol reagent. Total RNA was reverse transcribed to obtain single-strand cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR was carried out in a total volume of 25 μl of reaction mixture containing 1 μl of diluted cDNA, 12.5 μl of Brilliant SYBR® Green QPCR Master Mix (Stratagene), and 100 nM of each primer with a Mx3000P® Real-Time PCR System (Stratagene). For precise quantitative determination of the transcripts, we assessed the expression levels of GAPDH as an internal control. PCR conditions were set according to the instructions supplied by the manufacturer. The real-time PCR assay of each sample was conducted in triplicate, and the mean value was used as the mRNA level. The PCR primer pairs used in this study for ARK5 and c-Maf are listed above and those for Tax and GAPDH were as follow: for Tax; sense, 5′-CCCACTTCCCAGGGTTTGGACAGA-3′ and antisense, 5′- CTGTAGAGCTGAGCCGA-TAACGCG-3′; for GAPDH; sense, 5′-GAGTCAACGGATTTGGTCGT-3′ and antisense, 5′-GACAAGCTTCCCGTTCTCAG-3′.

2.5. Western blot analysis

Western blot analysis was performed as described previously [38]. In brief, cells were lysed in sodium dodecyl sulfate (SDS) sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. The lysates were resolved by electrophoresis on polyacrylamide gels and then electroblotted onto polyvinylidene difluoride membranes (Millipore). The membranes were incubated overnight with the appropriate primary antibody, as indicated, at 4°C. After washing, the blots were exposed to the appropriate secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. The reaction products were visualized using enhanced chemiluminescence reagent (GE Healthcare) according to the instructions provided by the manufacturer. We used primary antibodies against Tax (Lt-4) [39], phosphorylated IκBα (Ser32/36), phosphorylated AKT(Ser473), AKT, NF-κB (p65) (Cell Signaling Technology), IκBα, (Santa Cruz Biotechnology) and actin (Lab Vision). Horseradish-peroxidase-conjugated secondary antibodies were purchased from GE Healthcare.

2.6. Plasmids

The reporter assay construct for ARK5 promoter was described previously [24]. In brief, based on the results of a Genomic BLAST Search, primers with the NheI (upstream primer) or XhoI (downstream primer) site were synthesized, and PCR was then performed with the primers for genomic DNA extracted from PANC-1 cells. The PCR fragment digested with NheI and XhoI was ligated into pGL2-basic.A series of expression vectors for Tax (Tax WT) and mutants thereof (Tax M22 and Tax 703) were described previously [40, 41]. IκBα ΔN and IκBβ ΔN are deletion mutants of IκBα and IκBβ lacking the N-terminal 36 amino acids and 23 amino acids, respectively. IKKβ K44A and NEMOΔC are the dominant negative mutants of IKKβ and NEMO, respectively [42, 43]. The expression vector for mouse c-Maf was described previously [44]. NF-κB (p65) expression plasmid was described previously [45].

2.7. Transfection and luciferase assay

Transfections were performed in CCRF-CEM cells by electroporation with Microporator MP-100® (Digital Bio Technology) according to the instructions supplied by the manufacturer for optimization and use. In all cases, the reference plasmid phRL-TK, which contains the *Renilla* luciferase gene under the control of the Herpes simplex virus thymidine kinase promoter, was cotransfected to correct for transfection efficiency. Then cells were collected by centrifugation and lysed in reporter lysis buffer (Promega). Luciferase assays were performed by using the Dual-Luciferase Reporter System (Promega), in which relative luciferase activities were calculated by normalizing transfection efficiency according to the *Renilla* luciferase activities.

2.8. siRNA (small interfering RNA)

To knockdown ARK5 and c-Maf expression, predesigned double-stranded siRNAs (siGENOME SMART pool Human ARK5 and Human MAF;Dharmacon) were used. The siCON-

TROL non-targeting siRNA pool (Dharmacon) was used as a negative control. siRNAs were transfected into MT-2 cells by electroporation with MicroporatorMP-100®.

2.9. EMSA (electrophoretic mobility-shift assay)

Nuclear extracts were prepared from cells and DNA-binding activity was analyzed by EMSA, as described previously [16]. Briefly, 5 μg of nuclear extracts were pre-incubated in a binding buffer containing 1 μg poly-deoxy-inosinic-deoxy-cytidylic acid (Amersham Biosciences), followed by addition of [α-^{32}P]-labeled oligonucleotide probe. These mixtures were incubated for 15 min at room temperature. The DNA-protein complexes were separated on 4% polyacrylamide gels and visualized by autoradiography. The probes or competitors used were prepared by annealing the following sense and antisense synthetic oligonucleotides: NF-κB binding sites ARK5 κB A and ARK5 κB B derived from the ARK5 gene promoter 5′- gatcCTCTTGGGGTTCTCCTGGAC-3′ and 5′-gatcAGGTGGGGGAAGCCCTGGCT-3′, respectively. Mutants ARK5 κB A and ARK5 κB B are 5′-gatcCTCTTGGCCACGAGCTGGAC-3′and 5′-gatcAGGTGGGCCTCCAGCTGGCT-3′, respectively. To identify NF-κB proteins in the DNA-protein complex identified by EMSA, we used antibodies specific for various NF-κB family proteins, including p50, p65, c-Rel, RelB and p52 (Santa Cruz Biotechnology), to elicit a supershift DNA-protein complex formation. These antibodies were incubated with the nuclear extracts for 45 min at room temperature before incubation with radiolabeled probes.

2.10. Cell proliferation assay

The cells transfected with siRNA were incubated for 12 h, then seeded into 24-well plates at 1×10^5 viable cells per well, and incubated in glucose-containing or non-containing medium for the indicated time periods. The number of viable cells was determined every 24 h by counting trypan blue-excluding cells in a hemocytometer.

2.11. Statistical analysis

Data were expressed as mean ± SD. Differences between groups were analyzed by the unpaired Student's *t*-test. A *p* value less than 0.05 denoted the presence of a statistically significant difference.

3. Results

3.1. ARK5 and c-Maf are highly expressed in HTLV-1-infected T-cell lines

Expression of ARK5 and c-Maf mRNA was examined in 6 HTLV-1-infected (MT-2, MT-4, C5/MJ, SLB-1, HUT-102, MT-1 and TL-OmI) and 2 HTLV-1-uninfected (MOLT-4 and CCRF-CEM) T-cell lines. ARK5 mRNAs were detectable in all HTLV-1-infected T-cell lines, but not in uninfected T-cell lines (Figure 1A, left panel). c-Maf expression was relatively higher in HTLV-1-infected T-cell lines than in HTLV-1-uninfected T-cell lines (Figure 1A, right panel).

The high expression of MafB was detected only in HTLV-1-infected MT-2 cells, but no differences in expression were noted between other infected and uninfected T-cell lines (results not shown). Although Tax protein was not detectable in ATL-derived T-cell lines (Figure 1C), Tax mRNA was expressed in all HTLV-1-infected T-cell lines by real time RT-PCR, which is more sensitive method than Western blot (Figure 1B). These results suggest a close association between HTLV-1 infection and induction of ARK5 and c-Maf mRNA expression.

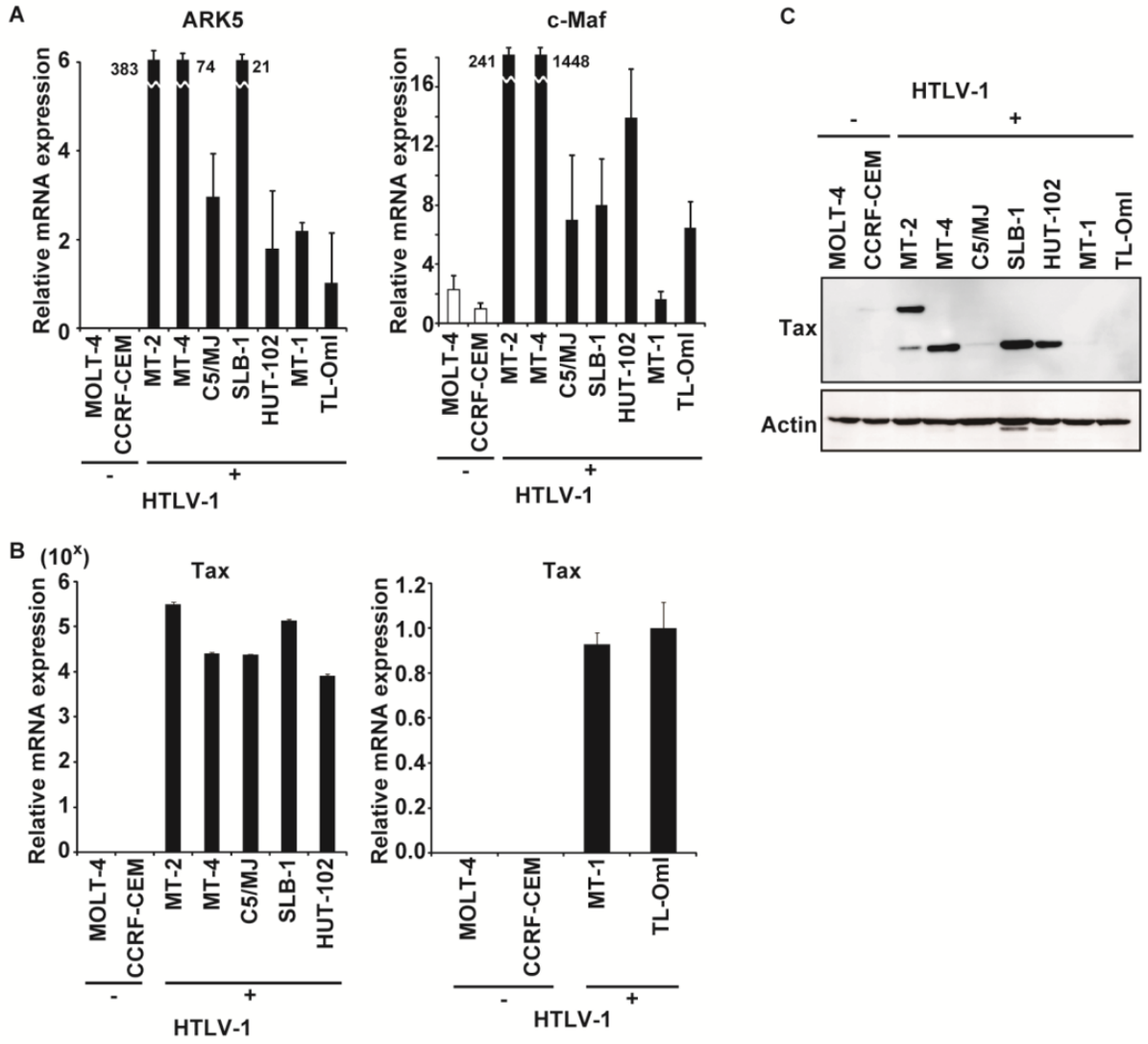


Figure 1. Overexpression of ARK5 and c-Maf in HTLV-1-infected T-cell lines. The expressions of ARK5, c-Maf (A) and Tax (B) mRNAs were analyzed in HTLV-1-infected (HTLV-1; +) and uninfected (HTLV-1; -) T-cell lines by real time RT-PCR. Results are shown as fold change of mRNA expression relative to that of TL-Oml (ARK5 and Tax) or CCRF-CEM (c-Maf). Real time RT-PCR data were obtained using the ΔΔCt method, with normalization to the reference GAPDH mRNA. Data are mean ± SD of triplicate experiments. Numbers on MT-2, MT-4, and SLB-1 represent the actual values. (C) Western blotting was used to determine the expression of Tax protein. Actin was a loading control.

3.2. HTLV-1 Tax induces ARK5 and c-Maf expression in T cells

To examine the direct association between ARK5 or c-Maf mRNAs induction and HTLV-1 infection, we used HTLV-1-infected TY8-3/MT-2 cells, which were established from TY8-3 cells by cocultivation with HTLV-1-infected MT-2 cells [36]. Although MafB expression level was slightly increased in TY8-3/MT-2 cells (results not shown), the expression of ARK5 and c-Maf mRNAs was clearly higher in TY8-3/MT-2 cells than parental TY8-3 cells (Figure 2A). Because Tax induces various cellular genes, we next examined whether this includes the expression of ARK5 and c-Maf mRNAs in T cells. We used JPX-9 cells, which stably carry Tax expression plasmid, in which Tax expression is induced by the addition of $CdCl_2$ [37]. The expression of ARK5, c-Maf, and Tax mRNAs was analyzed by real time RT-PCR (Figure 2B). The addition of $CdCl_2$ to the culture medium of JPX-9 cells induced the expression of Tax within 2 h, which persisted until 72 h after treatment. A concomitant increase of ARK5 mRNA within 10 h of treatment with $CdCl_2$ was observed in JPX-9 cells. Rapid expression of c-Maf mRNA was also observed within 2 h, and peaked after 10 h of treatment with $CdCl_2$. The induction of ARK5 or c-Maf could not be attributed to $CdCl_2$ treatment, since ARK5 or c-Maf expression was not induced in JPX/M cells, which express Tax mutant protein, after treatment with $CdCl_2$ (results not shown). These results indicate that Tax can increase the expression of ARK5 and c-Maf in T cells.

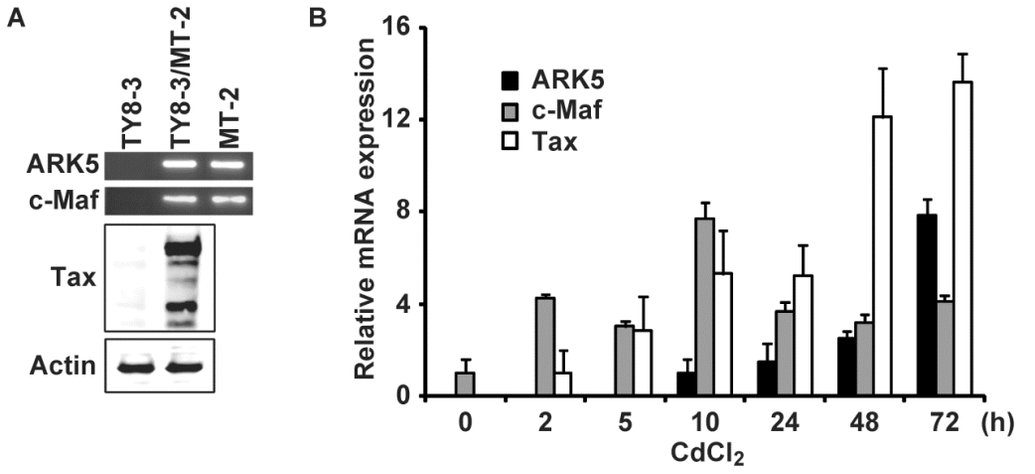


Figure 2. HTLV-1 Tax induces the expression of ARK5 and c-Maf mRNAs. (A) Infection of HTLV-1 induced ARK5 and c-Maf mRNA expression. The expression levels of ARK5 and c-Maf mRNAs in TY8-3 cells and HTLV-1-infected TY8-3 cells established by coculture with MT-2 cells were analyzed by RT-PCR. MT-2 was a positive control. Representative results of three experiments with similar results. (B) Tax expression was induced by adding $CdCl_2$ (20 μM) in JPX-9 cells. Cells were harvested at the indicated time points. ARK5, c-Maf, and Tax mRNA expression levels were analyzed by real time RT-PCR. Results are shown as fold change of mRNA expression relative to that at 0 h (c-Maf), 2 h (Tax), and 10 h (ARK5). Real time RT-PCR data were obtained using the ΔΔCt method, with normalization to the reference GAPDH mRNA. Data are mean ± SD of triplicate experiments.

3.3. c-Maf does not alter ARK5 expression in T cells

ARK5 gene promoter contains two putative MARE sequences [24]. c-Maf and MafB induce the gene transcription through interaction with MARE on the promoter region of target gene [26, 46]. ARK5 is induced when a c-Maf expression vector is introduced into non-ARK5-expressing colon cancer cells [24]. Our findings of a strong correlation between ARK5 and c-Maf expression in HTLV-1-infected T-cell lines and Tax inducible JPX-9 cells suggest that ARK5 could be regulated by c-Maf which is induced by Tax in these cells. However, transient transfection of c-Maf expression plasmid into ARK5-negative CCRF-CEM cells did not induce ARK5 mRNA expression (Figure 3A). Furthermore, c-Maf did not induce transcriptional activation of *ARK5* gene promoter reporter plasmid (Figure 3B) and knockdown of c-Maf expression in MT-2 cells by siRNA did not affect the expression level of ARK5 mRNA (Figure 3C). These results suggest that c-Maf does not contribute to induction of ARK5 transcription in T lymphocytes.

3.4. Tax activates ARK5 transcriptional activity through NF-κB pathway

Next, we investigated whether Tax could directly enhance the activity of *ARK5* promoter. CCRF-CEM cells were transiently transfected with a reporter gene construct containing the *ARK5* promoter together with Tax. Tax enhanced the transcriptional activity of this reporter (Figure 4A). We analyzed the nucleotide database and found two putative NF-κB sites on the promoter of *ARK5* gene. Tax stimulates transcription through distinct transcription factors, such as NF-κB and CREB (cyclic AMP response element-binding protein). Therefore, we tested two mutant forms of Tax; Tax M22 and Tax 703 [40, 41], to investigate whether Tax-mediated activation of NF-κB signaling pathway was required for induction of the *ARK5* promoter activation in T cells. Tax M22 activates CREB but does not affect NF-κB, while Tax 703 activates NF-κB but does not affect CREB [41]. In the present experiments, Tax 703, but not Tax M22, activated the *ARK5* promoter reporter (Figure 4A). Blocking NF-κB signaling pathway using various dominant negative forms of these signaling molecules reduced Tax-induced activation of *ARK5* promoter (Figure 4B). The nuclear extracts from HTLV-1-infected T-cell lines showed high NF-κB DNA-binding activity by EMSA using both NF-κB binding sites; denoted as ARK5 κB A and B sites, in the *ARK5* promoter as probes. In contrast, no significant DNA-binding activity was detected in extracts of HTLV-1-uninfected T-cell lines (Figure 4C). Competition and supershift assays showed that the observed DNA-protein complexes were specific for either ARK5 κB A or B site and included NF-κB components; p50, p65 or c-Rel proteins (Figure 4D). Transient transfection of NF-κB p65 expression plasmid in CCRF-CEM cells showed that overexpression of NF-κB p65 induced promoter activity of *ARK5* gene (Figure 4E) and expression of ARK5 mRNA (Figure 4F). These results suggest that NF-κB activation directly contributes to induction of the *ARK5* gene expression by Tax.

3.5. NF-κB inhibitor suppresses ARK5 expression in an HTLV-1-infected T-cell line

NF-κB is constitutively activated not only in HTLV-1 transformed T-cell lines but also in ATL-derived T-cell lines and primary ATL cells [16]. We analyzed the effects of an NF-κB inhibitor Bay11-7082, an inhibitor of phosphorylation of IκBα, on the expression of ARK5 in an HTLV-1-infected T-cell line. The expression of ARK5 mRNA in MT-2 cells was reduced

by treatment with Bay11-7082 (Figure 5A, left panels). Inhibition of phosphorylation of IκBα and stabilization of IκBα protein were confirmed by Western blotting (Figure 5A, upper right panels). LY249002, a PI3K (phosphatidyl inositol3-kinase)/AKT inhibitor, did not affect the expression of ARK5 (Figure 5A, left panels). Using Western blotting, we also confirmed inhibition of phosphorylation of AKT by LY294002 (Figure 5A, lower right panels). Inhibition of NF-κB DNA-binding activity by Bay11-7082 was also detected by EMSA using oligonucleotide probes of ARK5 κB A and B sites (Figure 5B). These results support out findings in Figure 4 that indicate the contribution of NF-κB signaling to induction of *ARK5* gene expression in HTLV-1-infected T-cells.

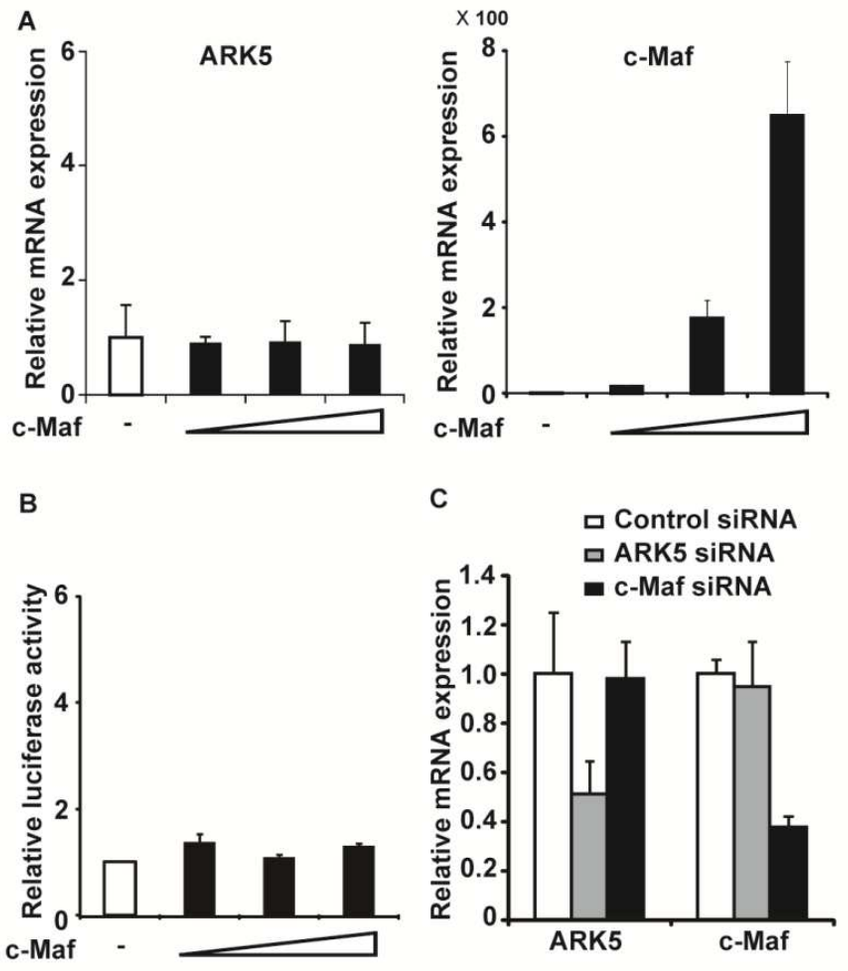


Figure 3. Maf does not affect ARK5 expression in T-cells. (A) c-Maf does not induce ARK5 mRNA expression in HTLV-1-negative T cells. ARK5 mRNA expression in CCRF-CEM cells 48 h after transfection with increasing amounts of c-Maf expression plasmids (0, 0.1, 0.5 and 1 μg) were analyzed by real time RT-PCR (left panel). Transfected c-Maf mRNA expression was confirmed by real time RT-PCR (right panel). (B) c-Maf does not induce *ARK5* promoter activity. CCRF-CEM cells were transfected with increasing amount of c-Maf expression plasmid together with *ARK5*promoter reporter plasmid. Cells were harvested 48 h after transfection and luciferase activity was analyzed. Data are mean ± SD of triplicate experiments. (C) Knockdown of c-Maf did not reduce ARK5 expression in HTLV-1-infected T cells. MT-2 cells were transfected with either ARK5, c-Maf or control siRNA (100nM). The expressions of c-Maf and ARK5 mRNAs were analyzed by RT-PCR. β-actin was a loading control. Representative results of triplicate experiments with similar results.

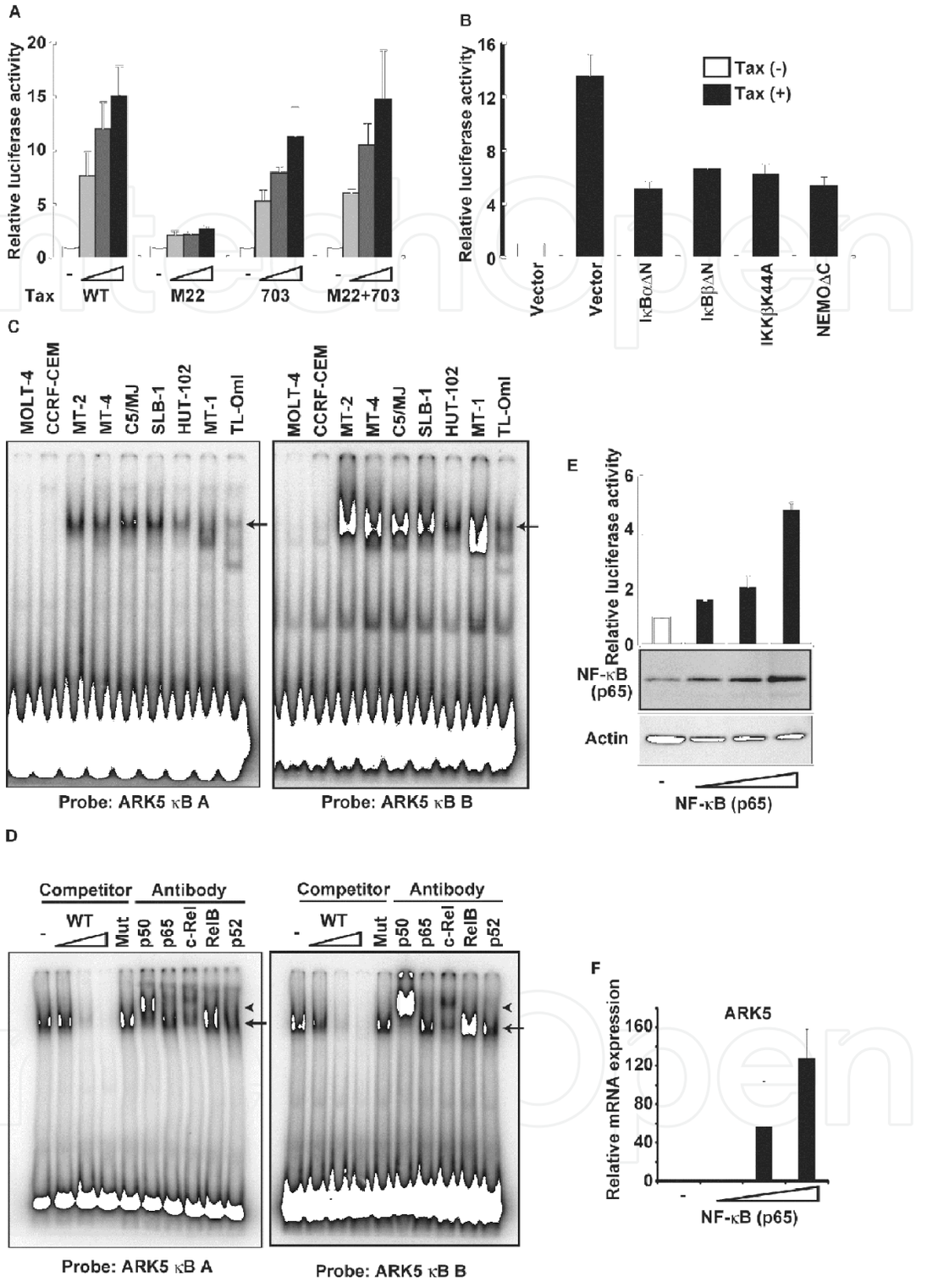


Figure 4. Tax activates ARK5 promoter activity via NF-κB signaling pathway. (A) CCRF-CEM cells were transfected with increasing amounts (0, 0.1, 0.5or 1 μg) of Tax wild type (WT) or mutant (M22 and 703: deficient in NF-κB and CREB signaling activation, respectively) expression plasmids together with ARK5 gene promoter reporter plasmid. Cells were collected 48 hr after transfection and luciferase activity was analyzed. Data are mean ± SD of triplicate experiments. The activity was expressed relative to that of cells transfected with reporter plasmid alone, which was defined as 1. (B)

CCRF-CEM cells were transfected with various dominant negative forms of NF-κB signaling proteins (0.1 μg) and Tax expression plasmid (1 μg) together with ARK5 reporter plasmid. Cells were harvested 48 h after transfection and luciferase activity was analyzed. Data are mean ± SD of triplicate experiments. The activity was expressed relative to that of cells transfected with reporter plasmid alone, which was defined as 1. (C) DNA-binding of NF-κB proteins to ARK5 gene promoter in HTLV-1-infected T-cell lines. DNA-binding of NF-κB proteins to ARK5 promoter was analyzed by EMSA using the ARK5 κB A (*top*) andARK5 κB B (*bottom*) oligonucleotide probes containing the NF-κB-binding sites from ARK5 gene. (D) NF-κB subunit specificity was determined using nuclear extracts from MT-2 cells and antibodies to NF-κB components p50, p65, c-Rel, RelB and p52, resulting in super shift. Cold competition using 1, 10 or 100-fold excess of unlabeled probes (wild type probe; WT) or 100-fold excess mutated probe(mutant probe; Mut) demonstrated the specificity of the protein-DNA-binding complex. Arrows indicate specific complexes of NF-κB with ARK5 κB A or ARK5 κB B oligonucleotides, and arrowheads indicate super shift of the bands by antibodies against p50, p65, or c-Rel. (E) NF-κB p65 activates *ARK5* promoter activity. CCRF-CEM cells were transfected with increasing amounts (0, 0.1, 0.5 or 1 μg) of NF-κB p65 expression plasmid together with *ARK5* promoter reporter plasmid. Cells were harvested 48 h after transfection and luciferase activity was analyzed. Data are mean ± SD of triplicate experiments. The activity was expressed relative to that of cells transfected with reporter plasmid alone, which was defined as 1. The expression of NF-κB p65 was confirmed by Western blotting (lower panel). (F) The expression of ARK5 mRNA induced by NF-κB p65 was analyzed by real time RT-PCR.

3.6. ARK5 maintains tolerance to glucose starvation in HTLV-1-infected T-cells

Finally, we investigated the role of ARK5 on the growth of HTLV-1-infected T-cells. Knockdown of ARK5 expression in MT-2 (Figure 6A, upper panels) and HUT-102 (Figure 6A, lower panels) cells did not affect growth of cells in the complete medium, which contained 2000 mg/mL glucose (Figure 6A, left panels). In contrast, knockdown of ARK5 expression reduced the cell growth in the glucose-free medium (Figure 6A, right panels). The knockdown efficiency was analyzed by real-time RT-PCR and almost equal knockdown efficiency was detected between with and without glucose conditions in both cell lines (Figure 6B). These results suggest that ARK5 maintains tolerance to glucose starvation in HTLV-1-infected T-cells.

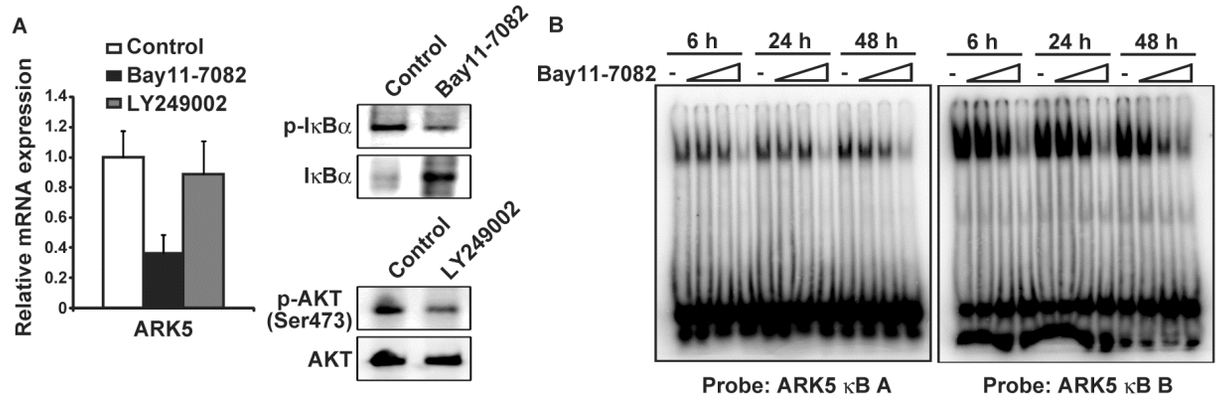


Figure 5. NF-κB inhibitor suppresses ARK5 expression in an HTLV-1-infected T-cell line. (A) MT-2 cells were treated with IκBα phosphorylation inhibitor Bay11-7082 (10 μM) or PI3K inhibitor LY249002 (20 μM) for 24 h. ARK5 expression was analyzed by real time RT-PCR (left panel). Inhibition of phosphorylation and stabilization of IκBα protein by treatment with Bay11-7082 and inhibition of phosphorylation of AKT by treatment with LY249002 were confirmed by Western blotting (right panels). (B) NF-κB inhibitor reduces DNA-binding of NF-κB protein to *ARK5* gene promoter in an HTLV-1-infected T-cell line. MT-2 cells were treated with increasing amounts of Bay 11-7082 (0, 1, 5 or 10 μM) for the indicated time periods. DNA-binding of NF-κB proteins to ARK5 promoter was analyzed by EMSA using the ARK5 κB A (*top*) andARK5 κB B(*bottom*) oligonucleotide probes containing the NF-κB-binding sites from *ARK5* gene.

4. Discussion

Some tumor cells have a strong tolerance to nutrient starvation; tolerance to glucose starvation can be induced by hypoxia. AKT and AMPK appear to be involved closely in the mechanism of tolerance [47-49]. ATL cells often invade the lung, liver, bone, intestine and nerves. Invading leukemia cells might be under nutrient-starvation condition. Therefore, we investigated the roles of ARK5, which is a member of the AMPK family and downstream target of AKT in leukemogenesis by HTLV-1. The results of this study showed high expression of ARK5 and c-Maf in HTLV-1-infected T-cells and that such expression was induced by HTLV-1 Tax (Figure 1 and 2). The promoter region of *ARK5* gene has MARE site where c-Maf binds and activates transcription [24]. Unexpectedly, c-Maf induced neither transcriptional activity of *ARK5* promoter nor expression of ARK5 mRNA in T lymphocytes (Figure 3), suggesting that transactivation of *ARK5* promoter through MARE by c-Maf is cell type-dependent. What is the important transcription factor that induces*ARK5* gene expression? We analyzed the nucleotide database and found two putative NF-κB sites on the promoter of *ARK5* gene. Tax induced the transcriptional activity of *ARK5* gene promoter through activation of NF-κB signaling pathway (Figure 4). This is the first report showing the involvement of NF-κB in the transcription of *ARK5* gene.

NF-κB signaling pathway is not only activated by Tax but also constitutively activated in primary ATL cells which express little amount of Tax [16]. Therefore, NF-κB inhibitors are promising therapeutic agents for ATL. At present, several trials are being conducted using the Bay11-7082 [50] and the proteasome inhibitor PS-341 [51] for treatment of ATL. Recently, a new NF-κB inhibitor, dehydroxy-methyle poxy-quinomicin, has been found to inhibit NF-κB signaling pathway induced by Tax as well as the constitutive NF-κB activation in primary ATL cells, without affecting normal peripheral blood mononuclear cells [52, 53]. In the present study, we demonstrated that Bay11-7082 reduced ARK5 expression in an HTLV-1-infected T-cell line (Figure 5), suggesting that NF-κB inhibitors may modulate ATL cells invasion into multiple organs.

Another important finding in this study is that ARK5 is necessary for the growth of HTLV-1-infected T-cells during glucose starvation (Figure 6). Previously, we and others have demonstrated activation of PI3K/AKT signaling in HTLV-1-infected T-cells and Tax-expressing cells [54]. These findings are important because PI3K/AKT signaling is required for malignant growth of HTLV-1-infected T-cells [55, 56]. However, there are numerous other downstream targets of PI3K/AKT [57]. ARK5, one of the downstream targets of PI3K/AKT signaling, contains the consensus sequence of the AKT phosphorylation at amino acids 595-600, and is directly activated by AKT [21, 23]. We propose that Tax has dual roles as an accelerator to induce glucose tolerance in HTLV-1-infected T-cells (Figure 7); 1) induction of ARK5 expression through NF-κB activation (present study), and 2) activation of PI3K/AKT signaling pathway [55, 56].

The molecular mechanisms of induction of tolerance to glucose starvation by ARK5 in HTLV-1-infected T-cells are not elucidated in this study. Previous studies showed that during glucose starvation, survival of human hepatoma HepG2 cells is induced by ARK5 and

activation of ARK5 by AKT is necessary for this effect [22, 23]. Glucose tolerance induced by ARK5 in HTLV-1-infected T-cells may also require phosphorylation and activation of ARK5 by AKT. However, we did not analyze the phosphorylation levels or activity of ARK5 in HTLV-1-infected T-cell lines, because a suitable antibody that can recognize phosphorylated ARK5 is not available commercially at present time. ARK5 also negatively regulates death receptors, such as Fas ligand-, TNF-and TRAIL-mediated cell death [22, 58]. When Fas is activated by the ligation of Fas ligand, intracellular interaction of the Fas-death domain, FADD and caspase-8 (death-inducing signaling complex (DISC) recruitment) is initiated for the activation of executioner caspase [59], and c-FLIP is the inhibitor of DISC recruitment. ARK5 directly inactivates caspase-6 through the phosphorylation at Ser^{257}, resulting in c-FLIP preservation, which in turn suppresses DISC formation [58]. Although cell death during glucose starvation is independent of death receptor, DISC recruitment is needed to induce cell death [22]. In this way, ARK5 may prevent cell death during glucose starvation.

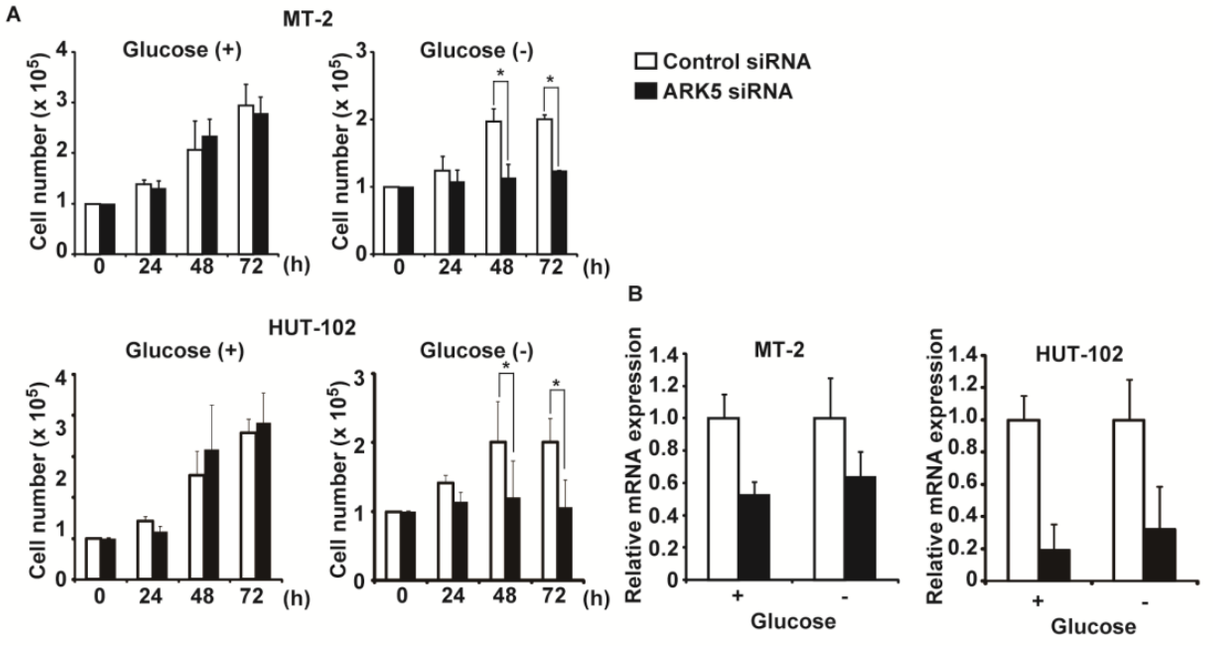


Figure 6. ARK5 maintains tolerance to glucose starvation in HTLV-1-infected T-cell lines. (A) MT-2 cells were transfected with either ARK5 siRNA (solid bars) or control siRNA (open bars) at final concentration of 100 nM. Cells were incubated in glucose containing (+; 2000 mg/L) or glucose-free (-) RPMI for the indicated time points. The effect of siRNA on cell growth was examined by counting the number of viable cells in triplicate by trypanblue dye-exclusion method. Data are mean ± SD of triplicate experiments (*p<0.05). (B) Efficacy of knockdown by ARK5 siRNA in either glucose-containing or glucose-free medium was determined by detecting the expression of ARK5 mRNA by real-time RT-PCR. Results are shown as fold change of mRNA expression relative to that in control siRNA transfected cells. Real-time RT-PCR data were obtained using the ΔΔCt method, with normalization to the reference GAPDH mRNA. Data are mean ± SD of triplicate experiments.

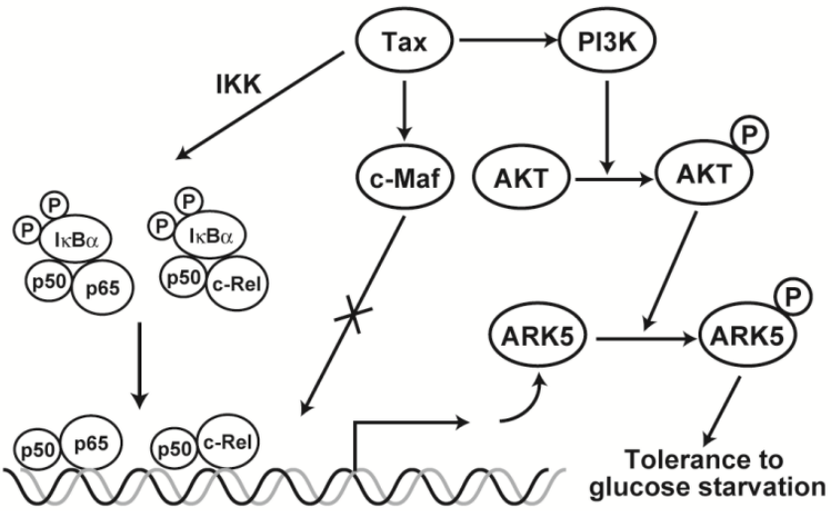


Figure 7. Schematic representation of the effects of Tax on ARK5 expression and AKT activation. Tax induces ARK5 expression by activating NF-κB signaling pathway. AKT is phosphorylated and activated through Tax-induced activation of PI3K. c-Maf is also induced by Tax, but it does not induce ARK5 expression. Activated AKT could phosphorylate and activate ARK5, resulting in tolerance to glucose starvation.

The results showed that c-Maf is highly expressed in HTLV-1-infected T-cells and induced by Tax in T cells (Figure 1 and 2). A previous study showed that c-Maf is expressed in ATL cells in lymph nodes of patients [27]. c-Maf transgenic mice develop T-cell lymphoma and ARK5 is upregulated in c-Maf transgenic thymocytes and T lymphoma cells [27]. In contrast, we found that c-Maf did not activate ARK5 promoter transcription in T-cells. However, c-Maf encodes a Th2-specific transcription factor that activates the expression of IL-4 and IL-10 in T cells [60]. In this regard, a subpopulation of ATL cells produces Th2-associated cytokines [61]. Taken together, it is of interest to identify other downstream target genes responsible for the actions of c-Maf that might contribute to malignant transformation of T cells. For example, some of the target genes of c-Maf, such as those that encode cyclin D2 and integrin β7, have deregulated expression in c-Maf transgenic mice [27]. It might be interesting to investigate the role of c-Maf in the regulation of expression of these genes in ATL cells.

5. Conclusion

We demonstrated overexpression of ARK5 in HTLV-1-infected T-cells and that Tax induced *ARK5* expression by activating the NF-κB signaling pathway. The results also indicated that ARK5 enhanced the growth of HTLV-1-infected T-cells during glucose starvation. The PI3K/AKT pathway, because of its central roles in cell survival, is a target for induction of cell death in HTLV-1-infected T-cells. Thus, ARK5, a downstream target of this pathway, becomes an attractive target in treatment of ATL with invasion of leukemia cells into multiple tissues.

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Abbreviations

ATL, adult T-cell leukemia;

AMPK, AMP-activated protein kinases;

ARK5, AMP-activated protein kinase-related kinase 5;

CREB, cyclic AMP response element-binding protein;

DISC, death-inducing signaling complex;

HTLV-1, human T-cell leukemia virus type 1;

EMSA, electrophoretic mobility-shift assay;

MARE, Maf-recognition element;

NF-κB, nuclear factor-kappa B;

PI3K, phosphatidyl inositol 3-kinase;

RT, reverse transcriptase;

SDS, sodium dodecyl sulfate;

siRNA, small interfering RNA;

WT, wild type.

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