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# Tgat, a Rho-specific guanine nucleotide exchange factor, activates NF-κB via physical association with IκB kinase complexes

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**Abstract**

Constitutive activity of NF-κB is associated with various human cancers including adult T-cell leukemia (ATL). In this study, we have found *Tgat* that activates NF-κB by screening a cDNA expression library derived from ATL cells. We previously identified *Tgat* as the oncogene, which consists of the Rho-guanine nucleotide exchange factor (Rho-GEF) domain and the unique C-terminal region, as a consequence of alternative splicing of the *Trio* transcript. Tgat activated the IKK activity by binding with the IκB kinase (IKK) complex. The Tgat mutants lacking the C-terminal region failed to associate with the IKK complex suggesting an essential role of the unique sequence. The mutation causing the loss of GEF activity also abolished the NF-κB activation. Moreover, co-expressed p100 was efficiently processed into p52 in the Tgat-expressing cells, suggesting the co-involvement of non-canonical pathway.


*Keywords:* NF-κB; Tgat; Rho-GTPase; Trio; HTLV-1; Tax; ATL

Accumulating evidences have indicated a role of NF-κB in tumorigenesis. A number of other viral proteins might be oncogenic interacting with IKK complex and cause NF-κB activation. For instance, the Epstein–Barr virus (EBV), implicated in lymphoid and epithelial malignancies such as Burkitt's lymphoma and Hodgkin's disease, post-transplant lymphoma, gastric carcinoma, and nasopharyngeal carcinoma, induces persistent NF-κB activation mediated by latent membrane protein 1 (LMP-1) and EBV nuclear antigen-2 (EBNA-2) [1,2]. Kaposi's sarcoma-associated herpes virus (KSHV), the causal agent of Kaposi's sarcoma, primary effusion B cell lymphomas, and multicentric Castleman's disease, has been shown to transform the infected cells through p21-activated kinase 1 (Pak 1) mediated NF-κB activation [3]. Another example is human T-lymphotropic virus type-1 (HTLV-1), which causes adult T-cell leukemia (ATL). The *pX* region of HTLV-1 encodes a transcriptional transactivator Tax, which activates expression of HTLV-1 long terminal repeat (LTR) through a DNA element that resembles the cellular cyclic AMP-regulated enhancer (CRE) [4]. The Tax also activates NF-κB, and the activation has been shown to be indispensable for transformation of rat fibroblasts *in vitro* [5]. However, Tax is barely detectable in ATL cells freshly isolated from patients. Tax expression in ATL cells is often abolished by genetic mutation in the *tax* gene, deletion of the 5′ LTR [6,7] or hyper-methylation of the promoter/enhancer region in 5′ LTR [8]. Moreover, the NF-κB subunits activated in the leukemic cells is different from those in

*Abbreviations:* NF-κB, nuclear factor-κB; HTLV-1, human T-lymphotropic virus type 1; ATL, adult T-cell Leukemia; Tgat, trio-related transforming gene in ATL tumor cells; GEF, guanine nucleotide exchange factor; IKK, IκB kinase.

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Tax-expressing cell lines; the NF-κB binding activity in ATL cells consists of p50/p65 heterodimer and p50/p50 homodimer, whereas Tax-expressing T-cell lines mostly of p50/c-Rel [9]. The molecular mechanism responsible for the constitutive NF-κB activation in ATL cells remains unknown.

In this study, we identify Tgat as the molecule with the potential to activate NF-κB by screening cDNA expression library derived from freshly isolated ATL cells. This report also demonstrates that the features of NF-κB activation by Tgat mimic those observed in ATL cells.

## Materials and methods

*DNA constructs.* Methods of cDNA library construction and *Tgat* mutants (*Tgat*ΔC, *Tgat*PH2, and *Tgat*GEFdead) generation were as described previously [10]. For mutants construction, PCR-amplified DNA fragments encoding Rho-GEF domain were ligated into pDON-AI containing PH2 coding sequence of *Trio* gene. Site-directed mutagenesis was applied to construct *Tgat*GEFdead that lacked Rho-GEF activity. Nucleotide sequence of each construct was confirmed by DNA sequencing. pIL-2Rα-Luc contains the luciferase gene under the control of five tandem copies of the NF-κB binding sequence of the IL-2Rα gene (GGGAATCTCC).

*Transient transfection of 293T cells and the NF-κB reporter gene assay.* 293T cells, seeded at $5 \times 10^4$ cells per well 24 h prior to transfection, were transfected in 24-well plates by calcium phosphate co-precipitation. To analyze expression of the NF-κB-dependent reporter gene, pIL-2Rα-Luc or pNF-κB-Luc reporter plasmid, pDON-*Tgat* or its derivatives and RL-TK-Luc plasmid were co-transfected into 293T cells.

*Oligonucleotides.* The sequence of the oligonucleotide corresponding to the κB element from IL-2Rα gene was 5′-CAGTTGAGGGGAATCTCC CAGGC-3′. For competition study, oligonucleotide-containing mutant κB was used, and the sequence was 5′-CAGTTGAGatctATCTCCCA GGC-3′.

*Electrophoretic mobility shift assay.* To examine the NF-κB activity in 293T cells, an aliquot of the nuclear extracts was incubated in a reaction buffer (10 mM Hepes, pH 7.6, 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 0.25 mM PMSF, 10% glycerol, and 100 μg/ml poly(dI–dC)). In some cases, a 20-fold molar excess of unlabeled double-stranded oligonucleotide was added as a competitor. After a 10-min incubation on ice, an end-labeled double-stranded oligonucleotide containing the consensus NF-κB binding sequence was added to the reaction, which was then incubated for an additional 30 min at room temperature. The same oligonucleotide in the unlabeled form was used as the wild-type competitor. And the mutant competitor previously described was used. In some cases, the reactions were further incubated with anti-p50, anti-p65, anti-c-Rel, anti-p52 or anti-RelB Abs at room temperature for 30 min. The samples were analyzed by electrophoresis in a 5% non-denaturing polyacrylamide gel with 0.5× TBE buffer. The gels were dried and analyzed by autoradiography.

*Immunoprecipitation and kinase assay.* Cytoplasmic extracts prepared from equivalent number of cells were subjected to immunoprecipitation with anti-FLAG M2 affinity gel in TNT buffer (20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1% Triton X-100, 0.5 mM PMSF, 100 μM $Na_3VO_4$, 20 mM β-glycerophosphate, and one-hundredth volume of a protease inhibitor mixture). Immunoprecipitates were then washed three times with TNT buffer and three times with kinase reaction buffer (20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 100 μM $Na_3VO_4$, 20 mM β-glycerophosphate, 2 mM DTT, 20 μM ATP). Kinase reactions were performed for 30 min at 30 °C using 5 μCi of [$\gamma$-$^{32}$P]ATP and GST-IκBα (amino acids 1–72) as substrates. The reaction products were separated on 12% SDS polyacrylamide gels and revealed by autoradiography [11].

*Immunoblotting (IB) and co-immunoprecipitation (Co-IP) assays.* 293T cells were transfected with a HA-tagged *Tgat* construct or its mutants (5 μg) along with a IKK1 (5 μg) by calcium phosphate co-precipitation. At 48 h post-transfection, the cells were washed with 5 ml PBS and whole-cell lysates were harvested using radioimmuno-precipitation buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and one-hundredth volume of a protease inhibitor mixture). The cell lysates were then incubated with anti-IKKs Abs for 1 h and with 20 μl of protein G-Sepharose beads for another 2 h. The beads were washed three times with radioimmuno-precipitation buffer and bound proteins were eluted in 1× SDS sample buffer and subjected to SDS–PAGE and Western blot analysis using anti-HA mouse monoclonal Abs.

## Results

### *Identification of Tgat capable of activating NF-κB in 293T cells by expression cDNA cloning*

We sorted 293T-κB-EGFP cells showing NF-κB activity after transfection with a cDNA library derived from ATL cells and isolated the cDNAs capable of activating NF-κB (Supplementary Fig. S1). Two rounds of FACS enrichment and subsequent sib selection finally identified 15 positive clones. The clone 33-44-57-34 conferring fluorescence-activation on the transfected cells, was *Tgat* (trio-related transforming gene in ATL tumor cells), which we previously reported as a novel transforming gene activated by alternative RNA splicing between a part of Trio gene encoding Rho-GEF and a novel exon located downstream of the last exon of *Trio* [10]. Sequence analysis revealed that others, including clone 33-44-57-12 which gave very intense fluorescent signals in transfected 293T cells, encoded EGFP under the CMV promoter probably due to the artificial homologous recombination between the integrated κB-EGFP sequence and the promoter region of the transfected vector.

### *Tgat activates both canonical and non-canonical pathways*

To confirm the activation NF-κB by *Tgat*, the cDNA was co-transfected with a reporter plasmid with the luciferase gene under the κB enhancer. To rule out selective failure due to the subtle sequence heterogeneity of κB enhancers, we used two independent reporter plasmids, pIL-2Rα-Luc containing the κB enhancer from IL-2Rα chain (Fig. 1A) and pNF-κB-Luc (Supplementary Fig. S2). As shown in Fig. 1A, the level of luciferase activity detected in *Tgat*-transfected cells co-transfected with pIL-2Rα-Luc was about 2.5 times higher than that of empty vector-transfected cells with statistical significance ($p < 0.01$, ANOVA). The level of pNF-κB-Luc transactivation was less than that by HTLV-I Tax, but Tgat transactivated pIL-2Rα-Luc at similar level as Tax. Next the nuclear extracts of cells were subjected to EMSA. A small amount of NF-κB-specific protein/DNA complexes detectable in nuclear extracts of vector-transfected cells drastically increased in *Tgat*-transfected cells. The specificity of the binding was confirmed by competition with an excess amount of wild-type but not mutant oligonucleotides (Fig. 1B). NF-κB involved in the DNA binding in the *Tgat*-transfected cells predominantly consisted of p50 and

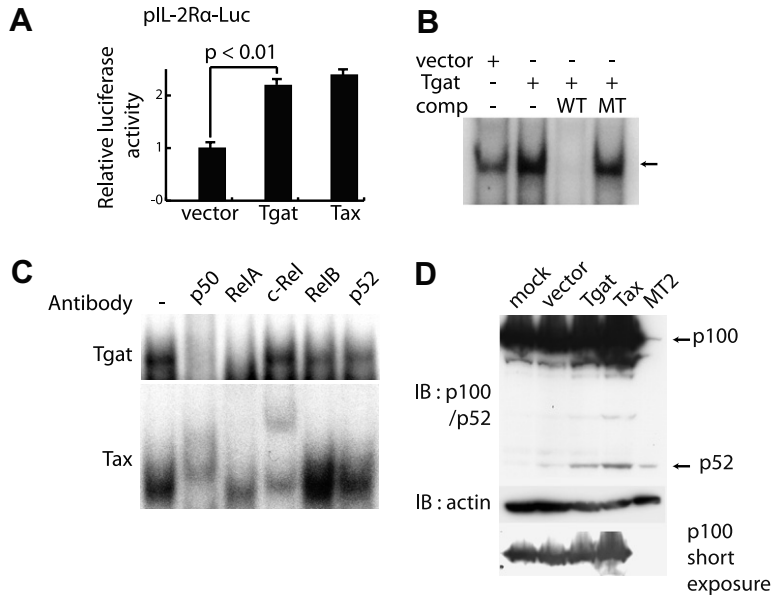


Fig. 1. Tgat and Tax express NF-κB-related activities. (A) 293T cell lines were transfected with 50 ng of a reporter plasmid containing the luciferase gene fused to five repeats of the κB motif of the IL-2Rα gene and enhancerless promoter of thymidine kinase (pIL-2Rα-Luc) along with combinations of 10 ng of the cDNA expression plasmids and 0.5 ng of the internal control plasmid (pRL-TK-Luc). After 48 h, relative luciferase activity was determined. The results shown are averages of three independent experiments with SE bars. The relative luciferase activities in *Tgat* or *tax*-transfected cells were increased more than 2.5-fold compared to control ($P < 0.01$). The data was analyzed statistically by the Scheffe's $F$ test. (B) Nuclear extracts (10 μg) from 293T cell line were assessed for activation of NF-κB by EMSA. The nuclear extracts were incubated with a $^{32}$P end-labeled oligonucleotide, followed by gel electrophoresis. The black arrow indicates the NF-κB-containing complex. Nuclear extracts from 293T transfected with *Tgat* were incubated with the labeled NF-κB probe, in the presence of competitors. The unlabeled oligonucleotide (lane 3) or the mutant NF-κB oligonucleotide (lane 4) was added as a competitor in a 20-fold molar excess to the binding reaction. (C) Nuclear extracts (10 μg) from 293T cell transfected with *Tgat* (top) or *tax* (bottom) were pre-incubated with NF-κB subunit-specific antibody, against p50, RelA, c-Rel, RelB or p52 as indicated above each lane, before the addition of radiolabeled probe. (D) 293T cells were transfected with 3.3 μg of pDON/SfiI vector, pDON-*Tgat* or pDON-*tax* together with 3.3 μg of pCn100, and lysed in 1× SDS sample buffer at 15 h post-transfection. Whole cell extracts were subjected to immunoblot analysis with anti-p52 antibody. The same quantity of the same extracts was used for detection of actin by immunoblotting.

RelA subunits, because antibodies against p50 and RelA efficiently inhibited formation of the complex (Fig. 1C). Moreover, treatment of anti-RelB and anti-p52 antibodies to a lesser extent but significantly decreased the complex in the *Tgat*-transfected cells, suggesting that Tgat may activate NF-κB through not only canonical but also non-canonical pathway. On the other hand, activated NF-κB in the *tax*-transfected cells were mainly composed of p50 and c-Rel (Fig. 1C), as previously reported. Non-canonical pathway of NF-κB activation involves the processing of NF-κB2 p100 to generate p52 [11]. To confirm the involvement of Tgat in p100 processing, we expressed p100 in 293T cells together with *Tgat* or *tax*. Polyclonal antibodies to p100/p52 visualized p52 on a Western blot of whole cell lysates of *Tgat*-transfected cells (Fig. 1D, lane 3) as well as Tax-transfected cells and Tax-positive HTLV-1-transformed lymphoid cells, MT-2, but p52 was barely detectable in those of untransfected and vector-transfected 293T cells.

### *Both Rho-GEF activity and the C-terminal unique sequence of Tgat are necessary for NF-κB activation*

Tgat protein contains the Rho-GEF domain followed by the unique 15 amino acid sequence at the carboxyl terminus. In order to elucidate the role of each region, transactivation potentials of the Tgat mutants, TgatΔC andTgatPH2 lacking the C-terminal unique region and TgatGEFdead encoding non-functional GEF [10], were examined. As shown in Fig. 2, luciferase activity detected in the cells transfected with *Tgat*ΔC, *Tgat*PH2, and *Tgat*GEFdead was decreased to the level similar to that of vector-transfected cells. Treatment of *Tgat*-transfected cells with 20 μM Y-27632, a pharmacological inhibitor of ROCK (Rho-associated kinase), also reduced the luciferase activity at the level similar to the control or *Tgat* mutants-transfected cells.

### *IKK1, IKK2, and IκBα mediate Tgat activation of NF-κB*

In order to elucidate mechanisms of the Tgat-induced activation of canonical pathway, we examined the effects dominant-negative mutants of IκBα and IKKs. The elevated luciferase activities in *Tgat* or *tax*-transfected cells were significantly inhibited by co-expression of IκBαM (Fig. 3A) as well as dnIKK1 and dnIKK2 (Fig. 3B). We next assessed the kinase activity of IKK complex in the *Tgat*-transfected cells using *in vitro* kinase assays. Flag-epitope-tagged IKK2 was expressed alone or in combination with *Tgat* or *tax*. Then Flag-IKK2 was immunoprecipitated

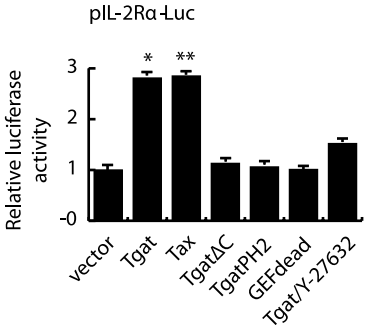


Fig. 2. NF-κB transcriptional activity of Tgat and its mutants in 293T cell line. Cells were co-transfected with pIL-2Rα-Luc (50 ng) and 10 ng *Tgat* or its mutant expression vector, together with 5 ng of the vector control plasmid (pRL-TK-Luc). After 48 h, relative luciferase activity was determined. The results shown are averages of three independent experiments with SE bars ($^{*,**}P < 0.01$).

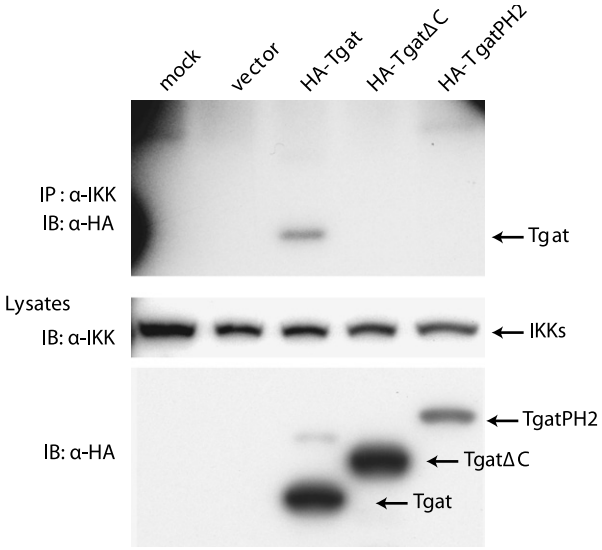


Fig. 4. Tgat interacts with IKK complex by its C-terminal region. 293T cells were transfected with a HA-tagged *Tgat* construct or its mutants. Polyclonal anti-IKKs antibody was used for immunoprecipitation (IP) and the immunoprecipitates were subjected to SDS–PAGE followed by Western blot analysis using anti-HA antibody.

from the cell lysates, one portion of the immunoprecipitates was used for *in vitro* phosphorylation assays with GST-IκBα as a substrate and the remainder was subjected to immunoblotting for detection of immunoprecipitated Flag-IKK2. The anti-Flag antibody precipitated roughly equivalent amounts of Flag-IKK2 from all the cell lysates (Fig. 3C, bottom). On the other hand, the levels of phosphorylation of GST-IκBα and autophosphorylation of Flag-IKK2 by *in vitro* kinase assay were about five times more in both *Tgat*- and *tax*-transfected cells, compared with those of the control cells (Fig. 3C, top).

### *Unique C-terminal region of Tgat is indispensable to the physical interaction with the IKK complex*

To investigate whether the Tgat protein would physically interact with the IKK complex, Co-IP assay was performed (Fig. 4). The polyclonal anti-IKKs antibody precipitated endogenous IKKs together with HA-tagged Tgat but not TgatΔC and TgatPH2 from the lysates of transfected 293T cells. Flag-tagged IKKs were detectable in the anti-myc immunoprecipitates from the myc-Tgat-expressing cell lysates, but not from those of myc-TgatΔC and -TgatPH2 expressor (Supplementary Fig. S3). Interestingly, Flag-IKKs were precipitated with another Tgat mutant, TgatGEFdead, which preserves the C-terminal region but lacks GEF activity. These results suggest that

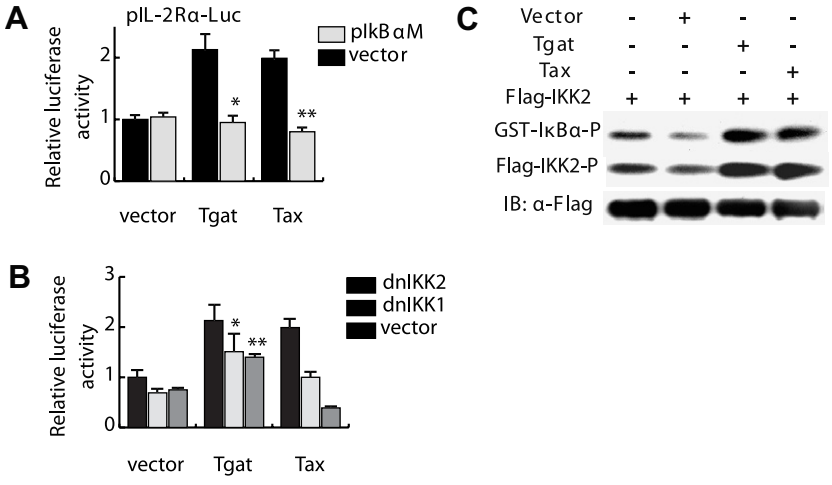


Fig. 3. IκBαM, DN-IKK1, and DN-IKK2 impaired Tgat activation of NF-κB in 293T. (A) 293T cells transfected with vector, pDON-*Tgat* (Tgat) or pDON-*tax* (Tax) and pIκBαM along with pIL-2Rα-Luc and pRL-TK-Luc. (B) 293T cells transfected with vector, pDON-*Tgat* (Tgat) or pDON-*tax* (Tax) and pDN-IKK1 or pDN-IKK2 along with pIL-2Rα-Luc and pRL-TK-Luc. The cells were harvested and lysed in lysis buffer 48 h after transfection. The cellular extracts were subjected to reporter gene assay. The κB-dependent luciferase activity was normalized based on the *Renilla* luciferase activity. The values shown are means from three separate transfections with SE bars (*,** $p < 0.01$). (C) Cytoplasmic extracts prepared from equivalent numbers of cells were immunoprecipitated with a FLAG specific monoclonal antibody and subjected to *in vitro* kinase assay using GST-IκBα as a substrate or to immunoblotting for detection of FLAG-IKK2 in the precipitates with a FLAG-specific monoclonal antibody.

the Tgat protein physically associates with IKKs via its carboxyl terminus and activates NF-κB.

## Discussion

In this study, we identified Tgat as a potent stimulator of NF-κB by screening ATL-derived cDNA library. Both Rho-GEF activity and C-terminal unique region of Tgat were required for the NF-κB activation. Tgat physically associates with the IKK complex and its mutants lacking the unique C-terminal region but preserving the potential to activate cellular Rho-GTPase [10] failed to associate with IKKs and activate NF-κB. Dominant-negative mutants of IKK-1 and -2 abolish the NF-κB activation, suggesting the essential role of the interaction between Tgat and IKK complex. Interestingly, Tgat enhances the processing of p100 to p52, suggesting the co-involvement of non-canonical pathway of NF-κB activation. The alternative form of Rho-GEF, Tgat, would provide novel mechanisms to link the small G-protein cascade to transcription factor(s).

*Tgat* was originally identified in a cDNA library derived from fresh ATL cells. The *Tgat* mRNA was detectable in ATL cells and tissues, in contrast to the undetectable level in normal lymphocytes, suggesting some roles of Tgat in the malignant phenotype of ATL. Constitutive NF-κB activity is considered to be essential for the survival of ATL cells because pharmacological inhibitors of NF-κB or super-repressor form of IκBα induces apoptosis of the cells [11]. A number of investigations about oncoprotein Tax encoded by HTLV-1 have been performed to understand the mechanism by which HTLV-1 transforms human T-cells or contributes to the establishment of HTLV-1-associated inflammatory disorders. Tax is a well-known NF-κB activator and responsible for trans-activation or trans-repression of a variety of cellular genes coding cytokines and regulators of cell cycles, DNA repair or apoptosis. However, Tax is hardly implicated to the constitutive NF-κB activity in ATL cells, since Tax expression is usually undetectable in fresh ATL cells.

It has been reported that Tax binds to not only NF-κB members such as RelA, p50, and p52 but also IκBs including IκBα, p105, and p100 [12–15]. Tax is, however, incapable of direct activation of NF-κB via the interaction with NF-κB or IκB members. It has become apparent after the identification of IKK that Tax activation of NF-κB is mediated by IKK leading to IκB degradation and nuclear translocation of NF-κB [16–19]. Moreover, O'Mahony et al. has reported that IKK1 and IKK2 play the essential role for Tax-induced NF-κB activation; the former is involved in phosphorylation of RelA, the latter in the nuclear translocation of the canonical NF-κB [20]. We demonstrate here that Tgat likewise activates IKK2 followed by the IκBα degradation and RelA/p50 nuclear translocation. It is also suggested that IKK1 contributes to the Tgat activation of NF-κB judging from the result of reporter assay using dominant negative form of IKK1. This is supported by the facts that small amounts of RelB/p52 can be detected in the activated NF-κB and Tgat induces p100-processing leading to p52 production. Thus, Tgat seems to target the multiple axes of the NF-κB signaling network by stimulating different IKK components as well as Tax. Interestingly, however, RelA/p50 nuclear translocation is mainly induced by Tgat whereas c-Rel/p50 by Tax [21]. It was reported that NF-κB DNA-binding activity in primary ATL cells and Tax-negative T-cell lines contained RelA/p50 [9]. Aberrant expression of p52 was also demonstrated in Tax-negative ATL cell lines [11]. In this regard, the features of NF-κB activation provoked by Tgat rather than Tax are close to those observed in ATL cells.

It is now clear that NF-κB plays pivotal roles for survival of ATL cells and the constitutive NF-κB activity is thus a potential target to develop therapeutics of ATL. On the other hand, NF-κB plays a variety of physiological functions, in particular, in the immune system. Drugs, whose effects are restricted to a cancer-specific pathway of NF-κB activation, if present, are ideal for clinical application. An agent that blocks the physical interaction between Tgat and IKK complex may be beneficial in therapy of ATL, although the mechanisms of Tax-independent NF-κB activation in ATL cells should be investigated more extensively.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.01.147.

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