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API2-MALT1 fusion protein induces transcriptional activation of the API2 gene through NF-κB binding elements: Evidence for a positive feed-back loop pathway resulting in unremitting NF-κB activation ☆

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Abstract

t(11;18)(q21;q21) is a characteristic as well as the most frequent chromosomal translocation in mucosa-associated lymphoid tissue (MALT) type lymphoma, and this translocation results in a fusion transcript, *API2-MALT1*. Although API2-MALT1 has been shown to enforce activation of NF-κB signaling, the transcriptional target genes of this fusion protein remains to be identified. Our analyses of the API2-MALT transfectants suggested that one of the target genes may be the apoptotic inhibitor *API2* gene. Luciferase reporter assays with deletion and mutational constructs of the *API2* promoter and electrophoretic mobility shift assays established that API2-MALT1 induces transcriptional activation of the *API2* gene through two NF-κB binding elements. Moreover, supershift experiments indicated that these elements are recognized by the NF-κB p50/p65 heterodimer. Taken together, our results strongly indicated that API2-MALT1 possesses a novel mechanism of self-activation by up-regulating its own expression in t(11;18)(q21;q21)-carrying MALT lymphomas, highlighting a positive feedback-loop pathway resulting in unremitting NF-κB activation.

Keywords: MALT lymphoma; NF-κB; Apoptosis inhibitor; Apoptosis; MALT1; Transcriptional regulation

Malignant lymphoma of mucosa-associated lymphoid tissue (MALT) was first described by Issacson and Wright [1,2]. Subsequently, the definition of extranodal lymphomas originating from the MALT was extended to include a number of extranodal low grade B-cell lymphomas. These lymphomas are now recognized and defined as extranodal marginal zone lymphomas of MALT type in the revised European-American classification of lymphoid neoplasms (REAL) and the recently published World Health Organization (WHO) classification of malignant lymphomas [3,4]. MALT lymphomas, accounting for about 8% of all non-Hodgkin's lymphomas (NHLs), represents one of the most common NHLs [5]. Most of them occur in the stomach, but they may affect the other anatomic sites, including

☆ *Abbreviations:* MALT, mucosa-associated lymphoid tissue; REAL, revised European-American classification of lymphoid neoplasms; WHO, World Health Organization; API2, apoptosis inhibitor 2; NF-κB, nuclear factor-κB; EMSA, electrophoretic mobility shift assays; IκB, inhibitor of NF-κB; *H. pylori*, *Helicobacter pylori*; CARD, caspase recruitment domain; DD, death domain; CLD, caspase-like domain; TNF, tumor necrosis factor.

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the ocular adnexa, lung, salivary glands, thyroid, skin, and intestine, where they share overlapping morphological and immunophenotypic features. An important feature of MALT lymphomas is the presence of lymphoepithelial lesions formed by invasion of individual mucosal glands or other epithelial structures by lymphoma cells [2,4]. These lymphomas often originate from various types of chronic inflammation, such as *Helicobacter pylori* (*H. pylori*) gastritis or autoimmune diseases including Sjögren's syndrome and Hashimoto's disease, and tend to remain as a localized disease, mostly exhibiting an indolent clinical course.

Despite its well-recognized clinical and pathological characteristics, the cytogenetic features of MALT lymphoma have only recently begun to be understood. The recurrent abnormalities of MALT lymphomas include trisomies of chromosomes 3, 7, 12, and 18, as well as the presence of t(1;14)(p22;q32), t(11;18)(q21;q21), and, more recently, of t(14;18)(q32;q21) [6–12]. The *BCL10* gene was isolated from the breakpoint of the t(1;14) translocation in MALT lymphoma [13,14], and was also isolated as a caspase recruitment domain (CARD)-containing protein by several groups using the protein databases [15–17]. Overexpression of BCL10 was found to result in NF-κB activation through CARD-mediated self-oligomerization. On the other hand, the t(11;18) translocation is reported to be one of the most frequent and specific chromosomal translocations in MALT lymphomas [18], and a novel gene, known as *MALT1* or *MLT*, was cloned by us as well as others from the breakpoint of the t(11;18)(19–21), while it has also been demonstrated that this aberration results in the fusion of two genes, the *API2* (also known as *c-IAP2*) at 11q21 and the novel gene, *MALT1* at 18q21, thus generating the API2-MALT1 fusion protein. API2 belongs to the inhibitor of the apoptosis protein (IAP) gene family, which includes X-IAP, API1 (c-IAP1), API2 (c-IAP2), ML-IAP, and survivin, and contains N-terminal baculovirus IAP repeat (BIR) domains, one CARD, and a C-terminal RING finger domain, which is known to possess ubiquitin ligase activity. MALT1 is a novel protein that contains an N-terminal death domain (DD), two Immunoglobulin (Ig)-like domains, and a C-terminal caspase-like domain (CLD) [19–22]. We as well as others have provided evidence that BCL10 and MALT1 form a physical complex and that these two proteins synergize to enhance NF-κB activation [22,23]. API2-MALT1 can also induce NF-κB activation through its homo-dimerization mediated via the N-terminal API2 portion, whereas full-length API2, MALT1 or their truncated forms cannot. Recent genetical and biochemical studies have indicated that BCL10 and MALT1, while having the disparate translocations, affect a common signaling pathway, since both are essential for antigen receptor stimulated-NF-κB activation in T and B lymphocytes [24–30]. More recently, BCL10 and MALT1 have been shown to mediate IκB kinase (IKK) activation by facilitating the K63-linked polyubiquitination of NEMO, a regulatory subunit of IKK complex, although the upstream ubiquitin ligase for NEMO remains controversial [31,32]. It can be assumed that API2-MALT1 bypasses the usual BCL10/MALT1 cellular signaling pathway, thereby inducing antigen receptor-independent events of lymphocytes [33,34]. Given that several apoptotic inhibitors were shown to be up-regulated by NF-κB activation, it can be expected that API2-MALT1-induced NF-κB activation contributes to anti-apoptosis through up-regulation of such apoptotic inhibitor genes. To the best of our knowledge, however, direct experimental evidence supporting such speculation has yet to be substantiated.

In this study, we tried to identify the downstream target genes of API2-MALT1 through NF-κB activation. The results of our gene expression profiling of the API2-MALT transfectants suggested that such target genes may include an impressive list of genes involving anti-apoptosis, cell migration and metastasis, as well as cell growth and differentiation. Indeed, one of the up-regulated genes has been found to be the *API2* gene. Here we demonstrate that API2-MALT1-induced transactivation of the *API2* gene is mediated through two NF-κB binding sites, which strongly suggests that a novel positive feedback-loop mechanism may operate in t(11;18)(q21;q21)-carrying MALT lymphomas.

Materials and methods

Cell culture and plasmids. 293T cells were maintained in Iscove's medium supplemented with 10% FBS in a 5% CO_2 incubator at 37 °C. HeLa cell transfectants were maintained in the same medium containing 0.5 mg/ml geneticin. The plasmid encoding Flag-API2-MALT1 (pcDNA3-Flag-API2-MALT1) has been described elsewhere [35]. The pBIIX-Luc reporter plasmid and the DN-IκBα (S32A and S35A) expression vector were also described [23]. The pRL-TK, *Renilla* luciferase expression vector was obtained from Promega (Promega, Madison, WI).

Gene expression profiling. Total RNA was extracted from the HeLa cell transfectants by means of homogenization in guanidinium thiocyanate and centrifugation through cesium chloride. Cy5 or Cy3 labeled complementary RNA (cRNA) was generated from total RNA by using low RNA Input Linear Amplification Kit (Agilent Technologies, Palo Alto, CA) according to the manufacturer's protocol. The labeled cRNA probes were then mixed and simultaneously hybridized overnight to Agilent Human 1A Oligo Microarray (Agilent Technologies) with the aid of an in situ Hybridization Kit Plus (Agilent Technologies) according to the manufacturer's protocol. For the first experiment, RNA from API2-MALT1 transfectants was labeled for Cy5, and RNA from mock transfectants was labeled for Cy3, and for the second experiment, the labeling was reversed. Fluorescent images of hybridized microarrays were obtained with the aid of Agilent Scanner, and analyzed with Feature Extraction software (Agilent Technologies) to generate fluorescence ratios. DNA spots were automatically segmented, and the local background was subtracted to obtain the signal intensities. Next, the ratios of the signal intensity of two dyes (Cy3 intensity/Cy5 intensity) were calculated for each spot, and converted into log10 ratios on an Excel sheet. The ratios were then normalized and as

reported in these files finally transformed into fold change. Finally, the average in fold change from the two experiments was calculated, and the top 50 genes in API2-MALT1 transfectants listed, which are summarized in Table 1.

RT-PCR. Total RNA was isolated from HeLa cell lines in the log-growth phase by means of homogenization in guanidinium thiocyanate and centrifugation through cesium chloride. First-strand cDNA was generated with 5 μg of total RNA, 200 U of superscript reverse transcriptase (Invitrogen Corporation), and 200 ng of random primer (Boehringer–Mannheim, Germany) in a total volume of 50 μl. Following first-strand cDNA synthesis, the PCR was performed in a total volume of 50 μl containing 1 μl of the cDNA and 1 U of Ex *Taq* (TaKaRa, Ohtsu, Japan), 2.0 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, and 0.32 μmol/L of each primer. The primers used were as follows. For API1: sense primer, 5′-TTGACTCTACATTGTATAAG-3′ and antisense primer, 5′-GCTAGTAAGTAGCAAATC-3′. For API2: sense primer, 5′-AAGCTGAAGCTGTGTTATATGAGC-3′ and antisense primer, 5′-TAAGGAAACCAAATTAGGATGAAAG-3′. For G6PDH: sense primer, 5′-CATGGTGCTGAGATTTGCC AAC-3′ and antisense primer, 5′-TCAACACCTTGACCTTCTCATC

Table 1
Genes up-regulated in API2-MALT1-expressing HeLa cells

Accession No.	Protein name and function	Fold change
M38685	Protein containing a formin homology 2 domain	50.0
Y12653	FAT10, diubiquitin	38.4
AF303134	Aldehyde dehydrogenase 8 family member A1, ALDH8A1	31.0
BC015516	Secretory granule proteoglycan 1, serglycin	29.9
NM_147150	PALM2-AKAP2	28.1
D90226	Pleiotrophin, a heparin binding protein	23.0
AJ242015	ADAM28, disintegrin and metalloproteinase domain 28	22.9
NM_016235	G protein-coupled receptor family C group 5, member B, GPRC5B	22.3
AJ320534	Protein of unknown function	18.7
BC010115	Friend leukemia virus integration 1	17.6
BC011802	Six transmembrane epithelial antigen of the prostate	15.6
AF288814	Protein with high similarity to guanylate binding protein 1, GBP4	15.2
BC002642	Cathepsin S	13.5
BC023981	Carbonic anhydrase 12	13.2
AK024562	Member of the enoyl-CoA hydrase or isomerase family	12.7
NM_021076	Neurofilament, heavy polypeptide 200 kDa	12.3
BC032003	Protein containing a Kazal-type serine protease inhibitor domain	12.2
M97925	Defensin α5	12.1
AF070674	Baculoviral IAP repeat-containing 3, c-IAP2	11.7
AK026248	Member of the ADP-ribosylation factor (ARF) family	11.5
U55258	Neuronal cell adhesion molecule	11.3
U07225	Purinergic receptor P2Y2	10.7
BC009694	G0-G1 switch gene 2	10.1
NM_152680	Hypothetical protein FLJ32028	10.1
M10050	Fatty acid binding protein 1	9.8
J03132	Intercellular adhesion molecule 1, ICAM1	9.8
AA894564	Lu5 *Homo sapiens* cDNA clone	9.3
BC029112	Protein containing a SAM (sterile α motif) domain	9.1
U37028	Protein with high similarity to integrin α X (human ITGAX)	8.6
X91171	Laminin α 4	8.4
AF288815	Protein with high similarity to guanylate binding protein 1, GBP5	8.3
X54925	Matrix metalloproteinase 1, MMP1	8.2
BC029804	Protein of unknown function	8.1
Z48804	Ocular albinism 1 (Nettleship-Falls)	8.0
AF134891	Nuclear factor erythroid-derived 2 like 3	7.9
AK092503	*Homo sapiens* cDNA FLJ35184	7.9
BC009200	Aplysia Ras homolog GDP dissociation inhibitor beta	7.5
AF251057	Protein containing a type 1 thrombospondin domain	7.4
AK000614	Tescalcin, a calcium-binding protein with an EF-hand domain	7.3
AA911334	EST, NCI_CGAP_GC4 *Homo sapiens* cDNA clone, IMAGE1476620	7.3
M83248	Secreted phosphoprotein-1, osteopontin	7.3
AF247788	Protein containing a C-type lectin domain	7.2
NM_018599	*Homo sapiens* hypothetical protein PRO1430 (PRO1430)	7.2
BC004107	Follistatin	7.2
U09278	Fibroblast activation protein	7.1
X67016	Syndecan 4, amphiglycan, ryudocan	7.1
AA977218	EST, NCI_CGAP_GC4 *Homo sapiens* cDNA clone, IMAGE1587417	7.0
BC020861	Salivary amylase alpha 1A, 1,4-α-D-glucan glucanohydrolase	6.9
BE221726	EST, NCI_CGAP_Kid11 *Homo sapiens* cDNA clone, IMAGE3132684	6.9
AF385400	Potassium channel subfamily K member 10	6.7

AC-3′. The PCR was performed with precycling at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 45 s. The identity of the RT-PCR products were confirmed by direct DNA sequencing.

Transfection and luciferase assays. A series of reporter plasmids containing 5′-deletion and mutational constructs of the human *API2* promoter was described previously [36]. In brief, the deleted DNA fragments were PCR amplified with various forward primers and a common reverse primer, followed by subcloning into the basic luciferase reporter plasmid, pGL2. The −247LUC constructs with mutations in potential NF-κB sites were produced by using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The reporter plasmids used in this study include −900LUC, −447LUC, −247LUC, −200LUC, −174LUC, −93LUC, −247(mκB1)LUC, −247(mκB2)LUC, −247(mκB3)LUC, and −247(mκB1and 3)LUC. Transient transfection was performed by using the ESCORT V transfection reagent (Sigma–Aldrich, St. Louis, MO) according to the manufacturer's instructions. 6×10^5 293T cells were placed in six-well plates, washed the following day, and then transfected with 2.0 μg of Flag-API2-MALT1 expression plasmid along with 0.5 μg of each reporter luciferase constructs and 0.1 μg of pRL-TK, an internal control *Renilla* luciferase expression vector. The total amount of transfected DNA was kept constant with the plasmid pcDNA3-FLAG. At 24 h after transfection, the cells were lysed in 0.5 ml of lysis buffer (Promega, Madison, WI) and 20 μl of the cell lysates was assayed for both firefly and *Renilla* luciferase activity by using the Dual Luciferase Assay systems (Promega) in a Lumat model LB9501 luminometer (Berthold Technologies, Bad Wildbad, Germany). The firefly luciferase activity was then normalized to the *Renilla* luciferase activity.

Nuclear extracts preparation and electrophoretic mobility shift assays (EMSA). 293T cells were transfected by means of Effectene (Qiagen K.K., Tokyo, Japan) with either a Flag-API2-MALT1 expression plasmid or an empty plasmid, and after 24 h, the nuclear extracts were prepared as follows. The cells were washed with PBS and suspended in hypotonic buffer (10 mM Hepes [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM DTT) supplemented with protease inhibitors cocktail (Roche Diagnostics). The cells were lysed with three freeze–thaw cycles in a dry/ethanol bath, and a soluble and an insoluble fraction were separated by a centrifugation at 14,000 rpm for 20 s. The nuclear pellet was resuspended in extraction buffer (20 mM Hepes [pH 7.9], 420 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and 25% glycerol) supplemented with protease inhibitors. After a 30 min incubation on ice with occasional agitation, DNA pellets were eliminated by centrifugation at 14,000 rpm for 1 min. The supernatant was recovered as nuclear extract, and the protein concentration was determined by BCA protein assay kit (Pierce Biotechnology, Rockford, IL) and then stored at −80 °C. The oligonucleotide probes for EMSA corresponded to the three potential NF-κB binding sites in the *API2* promoter (NF-κB site 1: sense, 5′-gATGGAAATCCCCGA-3′ and antisense, 5′-gTCGGGGATTTCCAT-3′; NF-κB site 2: sense, 5′-gAGTGGGTTTGCCAG-3′ and antisense, 5′-gCTGGCAAACCC ACT-3′; NF-κB site 3: sense, 5′-gGCTGGAGTTCCCCT-3′ and antisense, 5′-gAGGGGAACTCCAGC-3′). Lowercase letters represent G residues added for efficient labeling purposes with T4 polynucleotide kinase. Two oligonucleotides complementary to each other were annealed to generate each of the double-stranded probe. Labeling was accomplished by treatment with T4 polynucleotide kinase in the presence of [γ^{32}P]dATP. Ten micrograms of nuclear extracts was preincubated in a total of 10 μl of the binding buffer (10 mM Tris–HCl, pH 7.5, 60 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, and 4% glycerol) containing 0.05 mg/ml of poly(dI–dC) (Roche Molecular Biochemicals, Indianapolis, IN), followed by addition of approximately 1 ng of the labeled probes. After incubation for 20 min at room temperature, the DNA–protein complex was separated on a 5% non-denaturing PAGE with 0.5× TBE buffer (40 mM Tris borate, 1 mM EDTA, pH 8.0). The gel was vacuum-dried and subjected to autoradiography. To examine specificity of the oligonucleotide probes, we preincubated 100-fold molar excess of unlabeled competitor oligonucleotide with nuclear extracts for 10 min at room temperature before the addition of the labeled probe. The mutant oligonucleotide probes were the follows: mutant NF-κB site 1: sense, 5′-gAT GGAAATAGCCGA-3′ and antisense, 5′-gTCGGCTATTTCCAT-3′; mutant NF-κB site 3: sense, 5′-gGCTGGAGTTAACCT-3′ and antisense, 5′-gAGGTTAACTCCAGC-3′. To identify NF-κB proteins in the DNA–protein complex in EMSA, we used specific antibodies against NF-κB proteins including p50, p65, p52, or c-Rel (Santa Cruz Biotechnology, Santa Cruz, CA) to elicit a supershift of the DNA–protein complex. For supershift experiments, nuclear proteins were preincubated with 0.2 μg of these antibodies for 30 min at room temperature before the addition of the labeled probe.

Results

NF-κB activation and API2 gene transactivation in API2-MALT1 transfectants

We recently established HeLa cell lines stably expressing API2-MALT1 and demonstrated for the first time that API2-MALT1 can indeed exert an anti-apoptotic action [35]. This anti-apoptotic action appeared to be mediated, in part, by the direct interaction with apoptotic regulators including Smac. As shown Fig. 1A, API2-MALT1 can confer resistance against UV-induced apoptosis in HeLa cells, consistent with our recent results [35]. We next performed NF-κB reporter assay to examine whether the API2-MALT1 transfectants exhibit elevated NF-κB activity. As shown in Fig. 1B, the reporter assay indicated that the API2-MALT1 transfectants show a 9.7-fold increase in NF-κB activity, when compared with mock transfectants. Expression of API2-MALT1 protein was confirmed by Western blot analysis.

Given that several apoptotic inhibitors have been shown to be up-regulated by NF-κB activation, it can be expected that API2-MALT1-induced NF-κB activation may contribute to anti-apoptosis through NF-κB-mediated up-regulation of apoptotic inhibitor genes. However, direct experimental evidence in support of such hypothesis has yet to be presented. As a first step to address this issue, we used gene expression microarrays to identify the downstream target genes of API2-MALT1. Total RNAs extracted from the HeLa cell transfectants stably expressing API2-MALT1 or a mock vector were labeled by Cy5 and Cy3, and labeled cRNAs probes were then mixed and hybridized to Agilent Human 1A Oligo Microarray. Microarray experiments were performed twice independently by substituting Cy5 for Cy3 as labeling RNAs. Since our aim was to search for the target genes of API2-MALT1 through NF-κB activation, we first focused on the highly up-regulated genes, although we did not necessarily exclude as candidate target genes those showing less induction. A number of up-regulated genes (the top 50

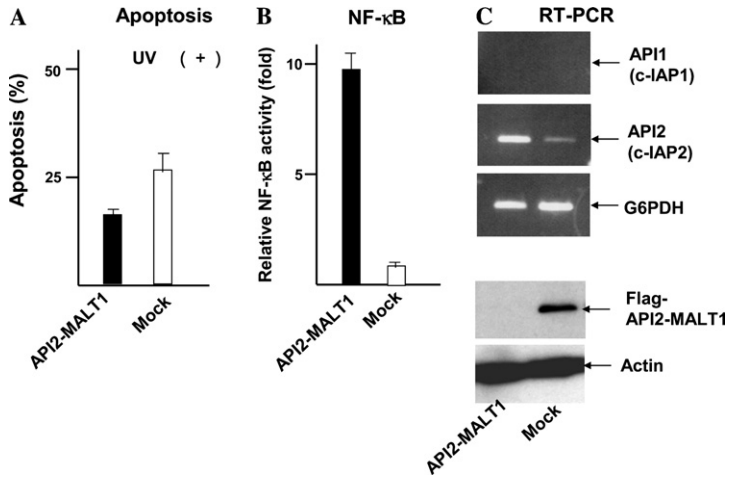

Fig. 1. Anti-apoptosis, NF-κB activation, and RT-PCR analyses in API2-MALT1 transfectants. (A) HeLa cells, stably transfected with a Flag-tagged API2-MALT1 plasmid or an empty plasmid were stimulated with UV irradiation (200 J/m^2). After 6 h, apoptosis was assessed by the presence of condensed chromatin. Data represent mean values ± SD of triplicate samples. (B) NF-κB activity was measured by reporter assay. 3×10^5 HeLa cells were transfected with 0.2 μg of pBIIX-Luc for NF-κB activity and 0.02 μg of pRL-TK for control *Renilla* luciferase activity. After 24 h, the cells were lysed in 1× passive lysis buffer and the cell lysates was then assayed for both firefly and *Renilla* luciferase activity using the Dual Luciferase Assay systems. (C) Up-regulation of endogenous *API2* mRNA in API2-MALT1 transfectants. Expression of *API1*, *API2*, and *G6PDH* mRNAs was evaluated by means of semi-quantitative reverse-transcriptase polymerase chain reaction assay. Whole cell lysates were analyzed with immunoblotting using anti-Flag mAB or anti-β actin mAB.

genes) in API2-MALT1 transfectants are summarized in Table 1. These include an impressive list of genes involving anti-apoptosis, cell adhesion, cell migration, and metastasis, as well as cell growth and differentiation. For example, the gene identified as apoptosis inhibitor is *API2*, also known as *c-IAP2*. However, up-regulation of the *API1* gene was not detected in the expression profiling. The genes involved in cell adhesion include ICAM1 and lamin α4, and those involved in cell migration and metastasis include ADAM28, cathepsin S, and matrix metalloproteinase. Of special interest was the second highest up-regulation of the ubiquitin-like protein FAT10 (also known as diubiquitin or ubiquitin D) by API2-MALT1. FAT10 is a ubiquitin-like modifier that was previously shown to be highly up-regulated in hepatocellular carcinoma and other gastrointestinal and gynecological cancers, to interact with the MAD2 spindle-assembly checkpoint protein, and to be expressed in dendritic and mature B cells [37–39]. In addition, the computer search resulted in the detection of the presence of two typical NF-κB binding sites in the 5′-proximal flanking region of the human *FAT10* gene (data not shown), thus warranting further studies to explore the mechanism underlying transactivation of this gene.

We first focused on apoptotic inhibitors for the candidate target genes, and as mentioned earlier, one of these genes listed in this study was the apoptotic inhibitor, *API2*. Since the N-terminal API2 portion is ectopically expressed in the API2-MALT1 transfectants, we examined whether the endogenous *API2* gene is actually up-regulated. For this purpose, we performed semi-quantitative RT-PCR for the *API2* gene and, for comparison, of the *API1* gene by using oligonucleotide primers designed for the C-terminal portions of these two inhibitors. The RT-PCR was performed three times and the similar result was obtained. The findings thus obtained strongly suggested that the endogenous *API2* mRNA level was actually up-regulated in the API2-MALT1 transfectants (Fig. 1C).

Luciferase reporter analysis of deletion constructs of human API2 promoter driven by API2-MALT1

It was previously demonstrated that the *API2* gene is transcriptionally activated by tumor necrosis factor (TNF)-α, CD40, and Epstein–Barr virus latent membrane protein 1 [36]. According to an earlier report, we arbitrary assigned the sequence number 1 for the 5′-end nucleotide G of an *API2* cDNA (GenBank Accession No. AF070674). The computer search also suggested several potential regulatory *cis*-elements [36]. In particular, three putative NF-κB binding sites were also detected at positions −147, −197, and −210. In addition to these NF-κB binding sites, a cAMP-responsible element (at −49) and a glucocorticoid responsible element (at −514) were shown to be functionally relevant for the *API2* gene expression [40,41]. To investigate whether API2-MALT1 induced transcriptional activation of the human *API2* gene, we first performed luciferase reporter assays with a series of constructs of its 5′-flanking region. In order to identify the *cis*-regulatory elements present within the 5′-flanking region, which confer API2-MALT1-responsiveness, we transiently transfected 293T cells with the reporter constructs of serially deleted sequences of the *API2* promoter linked to the *luciferase* gene. It was found that no significant

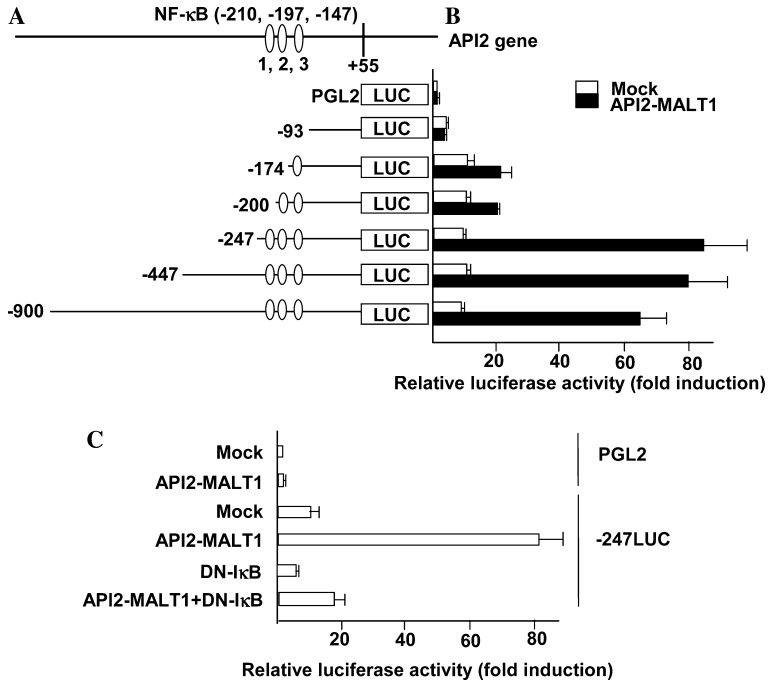

Fig. 2. *API2* gene transactivation by API2-MALT1. (A) Diagram summarizing the positions of potential regulatory elements relative to the structure of six deletion constructs used in the experiments, whose 5′-deletion boundaries are indicated. (B) Summary of the results of luciferase assays carried out with extracts of 293T cells transfected with 0.5 μg of each of the deletion construct and 0.02 μg of pRL-TK, an internal control *Renilla* luciferase expression plasmid together with the API2-MALT1 expression plasmid or mock plasmid. After 24 h, cell lysates were prepared and their luciferase activities were measured by using the Dual Luciferase Assay systems. Data represent mean values ± SD of triplicate samples. (C) Dominant-negative (DN) IκB suppresses *API2* gene transactivation by API2-MALT1. Transactivation of *API2* gene by API2-MALT1 was suppressed by DN-IκBα. 293T cells were transfected with 0.5 μg of −247LUC and 0.02 μg of pRL-TK control plasmid with or without API2-MALT1 expression plasmid or DN-IκB plasmid. Data represent mean values ± SD of triplicate samples.

induction in luciferase activity caused by API2-MALT1 was evident in the construct −93LUC (Fig. 2B). However, the construct −174LUC, which includes the proximal NF-κB site 3, showed a 2.1-fold increase in response to API2-MALT1 in luciferase activity. The −200LUC construct, which includes an NF-κB site 2, showed a similar fold increase in luciferase activity to that in the −174LUC construct. The −247LUC construct, which includes all the potential NF-κB sites 1, 2, and 3, showed a significant 9.2-fold increase in luciferase activity after API2-MALT1 transfection, compared with the findings for the −174LUC and −200LUC constructs. With the longer −900LUC and −447LUC constructs, the luciferase activity did not show any apparent difference from that observed with the −247 construct. Taken together, these results indicate that the regulatory region critical for transactivation of the *API2* gene by API2-MALT1 is located between positions −247 and −93, which include the potential NF-κB binding sites.

Dominant-negative (DN)-IκBα suppressed the API2 gene transactivation by API2-MALT1

To further examine the involvement of NF-κB activation by API2-MALT1 in *API2* gene transactivation, we used the DN-IκBα for the reporter assay. The DN-IκBα possesses mutations at Ser32 and Ser36, which inhibit IκBα degradation through ubiquitin-26S proteasome pathway. As shown in Fig. 2C, the *API2* gene transacitvation by API2-MALT1 was drastically suppressed by more than 95% in the cells transfected with the DN-IκBα. This result further indicates that the *API2* gene transactivation by API2-MALT1 is mediated via the classical NF-κB pathway.

Mutagenesis analysis of NF-κB binding sites in the API2 gene transactivation by API2-MALT1

Since our reporter assays raised the possibility that API2-MALT1-inducible transactivation of the *API2* gene may be accomplished through NF-κB binding elements present in the 5′-flanking region between −247 and −93, we tried to determine which of the potential NF-κB sites were important for such activation. To this end, we used four different −247LUC constructs in which site-directed mutations were introduced, each of which included one or two mutated NF-κB sequences (Fig. 3). The NF-κB mutant at site 2 showed API2-MALT1-induced transactivation almost identical to that obtained with the wild-type construct, whereas the double mutation at both sites 1 and 3 showed no evidence of inducibility, thus indicating that the NF-κB site 2 is not

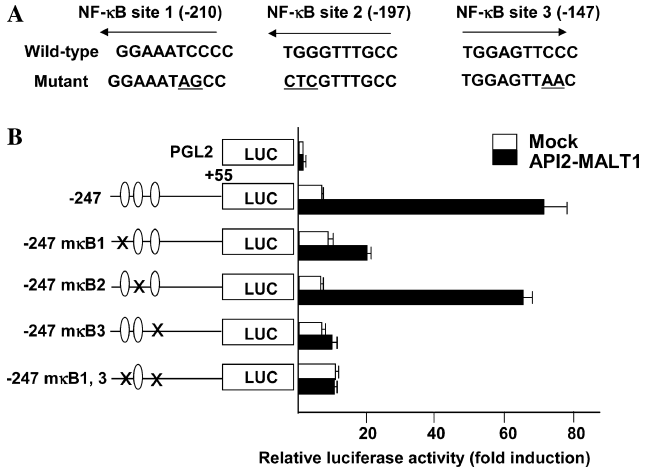

Fig. 3. Site-directed mutational analysis of the NF-κB regulatory elements. (A) Site-directed mutational NF-κB sequences used in this study are shown together with their corresponding wild-type sequences. (B) Each construct linked to the *luciferase* gene was transfected together with the API2-MALT1 expression plasmid or a mock plasmid into 293T cells. After 24 h, cell lysates were prepared and their luciferase activities were measured by using the Dual Luciferase Assay systems. Data represent mean values ± SD of triplicate samples.

important for API2-MALT1-induced transactivation. In contrast, mutations of the NF-κB elements at sites 1 and 3 resulted in a significant loss of promoter activity by 71% and 86%, respectively. Taken together, these results indicate that both NF-κB sites 1 and 3 are functionally relevant for *API2* promoter activity induced by API2-MALT1 and that they cooperate in regulating the expression of the *API2* gene.

Gel mobility shift assays: API2 gene transactivation by API2-MALT1 is mediated through two NF-κB sites bound by the NF-κB p50/p65 heterodimer

Since the reporter assays of the *API2* promoter demonstrated that API2-MALT1 activates transcription through NF-κB sites 1 and 3, we next sought to identify the nuclear factors that can bind to these sites. Specific oligonucleotides containing the NF-κB elements at sites 1, 2, and 3 were designed and used as probes for the EMSA with nuclear extracts from API2-MALT1-transfected 293T cells. As shown in Fig. 4A, nuclear extracts from the 293T cells transfected with API2-MALT1 resulted in the induction of DNA–protein complexes that bound to NF-κB sites 1 and 3 but not to the site 2. These complexes were judged to be specific to the NF-κB sites 1 and 3, because competition assays using excess unlabeled oligonucleotides of sites 1 and 3 resulted in elimination of the DNA–protein complexes, whereas the mutant oligonucleotides exerted almost no effect. These results are highly consistent with those obtained from the reporter assays.

We further sought to characterize the DNA–protein complexes bound to the NF-κB sites 1 and 3. To determine which NF-κB members bind to sites 1 and 3, we performed the EMSA using antibodies against members of the NF-κB family including p50, p52, p65, and c-Rel. As shown in Fig. 4B, preincubation with the p52 or c-Rel antibody did not affect the complexes, but the complexes were supershifted by the addition of the p50 or p65 antibody, while the NEMO antibody had almost no effect. Our results therefore indicate that the NF-κB p50/p65 heterodimeric complex binds to the NF-κB sites 1 and 3 in the API2-MALT1-transfected 293T cells.

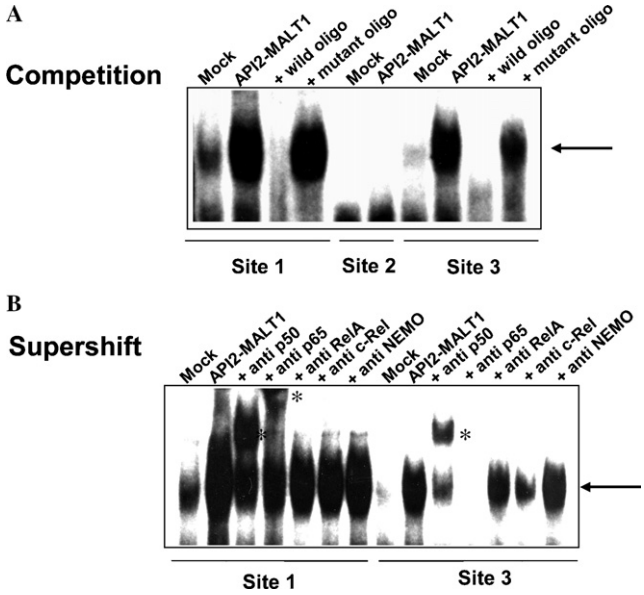

Fig. 4. Electrophoretic mobility shift assays (EMSA). (A) EMSA with site-specific oligonucleotides for nuclear protein NF-κB binding activity. End-labeled oligonucleotides spanning NF-κB sites 1, 2, and 3 within the *API2* promoter were incubated with 10 μg of nuclear extracts from HeLa cells transiently transfected with API2-MALT1 or with a mock vector in the absence or in the presence of the corresponding 100-fold excess unlabeled oligonucleotides (wild oligo) or mutant oligonucleotides (mutant oligo) used as competitors. After separating the protein–DNA complex by nondenaturing PAGE, the shifted bands were visualized by means of autoradiography. The protein–DNA complex is indicated by an arrow. The labeled NF-κB oligonucleotides used for EMSA are indicated at the bottom. (B) Supershift experiments with antibodies against NF-κB proteins. Antibody supershifting experiments were performed by preincubating the nuclear extracts from HeLa cells transiently transfected with API2-MALT1 or a mock vector with 0.2 μg of the antibody against p50, p65, Rel A, c-Rel, or NEMO. The supershifted bands are shown by asterisks. The labeled NF-κB oligonucleotides used for EMSA are indicated at the bottom.

Discussion

Recent genetic and biochemical studies have definitely established that BCL10 and MALT1 form a physical and functional complex and are both essential for antigen receptor stimulated-NF-κB activation in T and B lymphocytes [24–30]. We as well as others have demonstrated that API2-MALT1 enforces NF-κB activation

through its homo-dimerization via the N-terminal BIR domains [22,23]. It can thus be assumed that API2-MALT1 can bypass this usual BCL10/MALT1 cellular signaling pathway linked to NF-κB activation [33,34]. Given that several apoptotic inhibitors have been found to be up-regulated by NF-κB activation [42,43], it can be also expected that API2-MALT1-induced NF-κB activation contribute to anti-apoptotic action through up-regulation of these apoptotic inhibitor genes. To the best of our knowledge, however, unequivocal evidence supporting this hypothesis is still missing.

In the study presented here, we demonstrated that API2-MALT1 in fact induces transcriptional activation of the *API2* gene through the two NF-κB binding elements. To the best of our knowledge, ours is the first study to definitely identify the downstream target genes of API2-MALT1 through NF-κB activation. Since expression of API2-MALT1 is under the control of the *API2* promoter in t(11;18)-carrying MALT lymphomas, our data strongly suggested that this fusion protein may possess a novel mechanism of self-activation by up-regulating its own expression, thus highlighting a positive feedback-loop pathway resulting in unremitting NF-κB activation (Fig. 5). We recently examined the protein stability of exogenously expressed API2, MALT1, and API2-MALT1 by using Western blot analysis of the cell lysates with or without treatment of MG132, a proteasome inhibitor [44]. This analysis showed that MALT1 is rapidly degraded via the ubiquitin–26S proteasome pathway, as is the case with API2. Upon completion of the fusion, API2-MALT1 became readily detectable even without MG132, thus suggesting that this fusion protein becomes stable against the ubiquitin–26S proteasome pathway [44]. Such an increase in the protein stability of API2-MALT1 can be expected to result in NF-κB activation, so that API2-MALT1 appears to possess two novel mechanisms for self-activation, one at the transcriptional level and the other at the post-translational level.

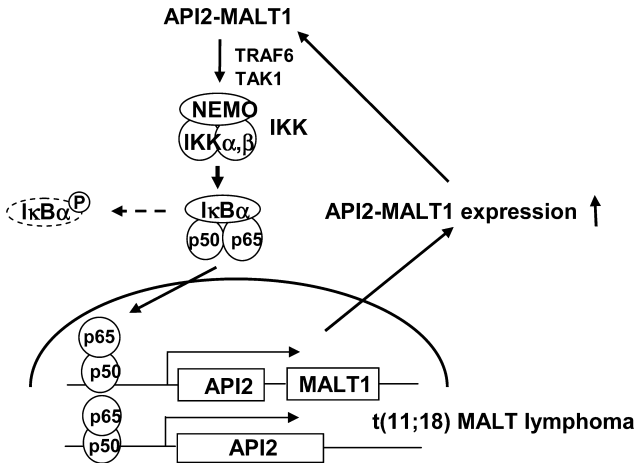

Fig. 5. Schematic representation of the positive feedback-loop pathway through NF-κB activation by API2-MALT1. API2-MALT1 enforces activation of the IKK complex probably through TRAF6/TAK1 signaling pathway [32]. The activated IKK complex then phosphorylates IκB proteins and targets these inhibitors for degradation by the ubiquitin–26S proteasome pathway, thus allowing NF-κB to enter the nucleus to turn on target genes, including the *API2* gene. Expression of the *API2-MALT1* transcript is under the control of the *API2* promoter in t(11;18)-carrying MALT lymphoma. Thus, API2-MALT1 possesses a novel mechanism of self-activation by up-regulating its own expression in t(11;18)-carrying MALT lymphoma.

Several lines of evidence presented here confirm that the apoptosis inhibitor API2 is a target of NF-κB activity induced by API2-MALT1. First, findings derived from gene expression profiling of the API2-MALT transfectants and semi-quantitative RT-PCR analysis suggested that one of the target genes of API2-MALT1 may be the *API2* gene. Second, both the luciferase reporter assays with deletion and mutational constructs of the *API2* promoter region and the competition and supershift experiments in EMSA established that API2-MALT1 induces transactivation of the *API2* gene through two NF-κB binding elements that can be recognized by the NF-κB p50/p65 heterodimer. Third, API2-MALT1-induced transactivation was almost completely eliminated by the DN-IκBα, a strong inhibitor of NF-κB.

Our gene expression profiling of the API2-MALT transfectants suggested that the candidate target genes of API2-MALT1 make for an impressive list of genes involved in anti-apoptosis, cell migration and metastasis, as well as cell differentiation. It should be noted that some of these genes have already been shown to be up-regulated by NF-κB activation. A recent study provided evidence that API2-MALT1 contributes to transformation of cells by activating the NF-κB activity and can inhibit DNA damaged-induced apoptosis [45], and the latter finding is also borne out by our recent results [35]. In their gene expression profiling, several cluster genes including immunoreceptors and cell surface receptors, stress responsive genes, transcription factors, genes that regulate extracellular matrix, and genes involved in cell growth were reported to be regulated by the fusion protein, API2-MALT1 [45]. However, these genes are not always the same as those obtained in our gene expression profiling. The reason for this discrepancy is currently unclear, although it may be due to the differences in the cellular context of cell lines used (NIH3T3 cells versus HeLa cells). In this respect, it will be of particular importance to further investigate the candidate target genes listed here by analyzing patient samples as well as by using the reporter and EMSA assays. In addition, we are currently trying to establish the stable transfectants expressing API2-MALT1 in lymphoid cell lines derived from germinal-center B cells. Hopefully, further studies to identify the target genes induced by API2-MALT1 as well as to define the mechanism underlying transactivation are expected to provide a molecular

rationale for the pathophysiology of MALT lymphomas carrying this fusion protein.

Our reporter assays with serial deletion constructs indicated that the regulatory region between positions −247 and −93 in the *API2* promoter is essential for API2-MALT1-induced transactivation. Within this region, there are three potential NF-κB binding sites. The findings obtained from the reporter assays with mutational constructs in NF-κB binding sites indicated that NF-κB binding site 3 (at −210) and, to a somewhat lesser degree, NF-κB binding site 1 (at −147) are important for API2-MALT1-induced transactivation. The competition and supershift experiments demonstrated that the NF-κB p50/p65 heterodimeric complex specifically binds to the two NF-κB sites, thereby inducing the *API2* gene transactivation.

To summarize, we have provided experimental evidence that API2-MALT1 possesses a novel positive feedback-loop mechanism of self-activation by up-regulating its own expression in t(11;18)(q21;q21)-carrying MALT lymphomas, thereby resulting in unremitting NF-κB activation (Fig. 5). We recently demonstrated that API2-MALT1 possesses anti-apoptotic action, in part, through its direct interaction with apoptotic regulators including Smac. We, therefore, hypothesize that anti-apoptotic action by API2-MALT1 may be mediated by in one way its interaction with apoptotic regulators as well as by in another way its self-activation resulting in unremitting NF-κB activation. The continuous and sustained anti-apoptotic stimuli driven by API2-MALT1 are most likely to play major roles in the pathogenesis of MALT lymphomas. It is hoped that further studies will provide further insights into the contribution of API2-MALT1 to the pathophysiology of MALT lymphoma, and ultimately may lead to new diagnostic and therapeutic advances in the treatment of this lymphoma.

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