MALT1 and the API2-MALT1 fusion act between CD40 and IKK and confer NF-κB–dependent proliferative advantage and resistance against FAS-induced cell death in B cells

Liza Ho, R. Eric Davis, Béatrice Conne, Richard Chappuis, Margaret Berczy, Paulette Mhawech, Louis M. Staudt, and Juerg Schwaller

The most frequently recurring translocations in mucosa-associated lymphoid tissue (MALT) B-cell non-Hodgkin lymphoma, t(11;18)(q21;q21) and t(14;18)(q32;q21), lead to formation of an API2-MALT1 fusion or IgH-mediated MALT1 overexpression. Various approaches have implicated these proteins in nuclear factor κB (NF-κB) signaling, but this has not been shown experimentally in human B cells. Immunohistochemistry showed that MALT1 is predominantly expressed in normal and malignant germinal center B cells, corresponding to the differentiation stage of MALT lymphoma. We expressed MALT1 and apoptosis inhibitor-2 API2/MALT1 in human B-cell lymphoma BJAB cells and found both transgenes in membrane lipid rafts along with endogenous MALT1 and 2 binding partners involved in NF-κB signaling, B-cell lymphoma 10 (BCL10) and CARMA1 (caspase recruitment domain [CARD]–containing membrane-associated guanylate kinase [MAGUK] 1). API2-MALT1 and exogenous MALT1 increased constitutive NF-κB activity and enhanced IkB kinase (IKK) activation induced by CD40 stimulation. Both transgenes protected BJAB cells from FAS (CD95)–induced death, consistent with increases in NF-κB cytoprotective target gene expression, and increased their proliferation rate. Expression of a dominant-negative IkBα mutant showed that these survival and proliferative advantages are dependent on elevated constitutive NF-κB activity. Our findings support a model in which NF-κB signaling, once activated in a CD40-dependent immune response, is maintained and enhanced through deregulation of MALT1 or formation of an API2-MALT1 fusion. (Blood. 2005;105:2891-2899)

Introduction

Four recurrent chromosomal translocations have been described in non-Hodgkin B-cell lymphoma of the mucosa-associated lymphoid tissue (MALT) type. Two of them, t(14;18)(q32;q21) and t(1;18)(q21;q21), are found in 30% to 50% of extranodal MALT lymphomas. The former juxtaposes the MALT1 (MLTI or para-caspase 1) gene to the IgH promoter region, resulting in the deregulation of MALT1 expression, whereas the latter creates a fusion between MALT1 and the inhibitor of apoptosis gene API2 (cIAP2/HIAP1).1-6 Unlike the low-grade indolent MALT lymphomas that are dependent on the continuous presence of Helicobacter pylori, MALT lymphomas with translocations targeting the MALT1 gene can be found in cases without H pylori and are refractory to treatment against H pylori.7-8 The other 2 much rarer translocations, t(1;14)(p22;q32) and t(1;2)(p22;p12), target the BCL10 gene on the short arm of chromosome 1.9,10

MALT1 was independently identified as a member of the human paracaspase family and an interacting partner of B-cell lymphoma 10 (BCL10).11 In vitro, MALT1 synergizes with BCL10 to enhance nuclear factor κB activation,12 and the association of the 2 proteins has been shown to mediate IkB kinase (IKK) activation by facilitating the ubiquitinylation of the NF-κB essential modulator (NEMO) by the ubiquitin-conjugating enzyme UBC13,13 BCL10 also interacts with a group of proteins that contain an N-terminal caspase recruitment domain (CARD) domain and show overall structural homology to MAGUK (membrane-associated guanylate kinase) proteins.14-17 These proteins were subsequently termed CARMA (CARD-containing MAGUK) proteins, of which CARMA1 (CARD11) expression is predominantly lymphoid specific. It has been shown recently that MALT1 not only interacts with BCL10 directly but also associates with CARMA1, suggesting that a complex containing MALT1, CARMA1, and BCL10 plays an important role in lymphoid cell signaling.18 The critical function of MALT1 in lymphocyte signaling has been revealed by the analyses of MALT1-deficient mice,19,20 whose T cells fail to proliferate in response to CD3/CD28 costimulation or to phorbol myristate acetate (PMA) and ionomycin. Similarly, B cells of MALT1−/− mice fail to proliferate in response to immunoglobulin M (IgM), CD40, or lipopolysaccharide (LPS) stimulation. The lymphoid compartments of these mice showed severe reduction of marginal zone B cells and a lack of germinal center formation in spleens, as well as deregulated maturation of T-cell subsets in the thymus. The phenotype of MALT1-deficient mice closely resembles that of mice lacking Bcl10, in particular with respect to the lack of marginal zone B cells, and in the lack of response to T-
B-cell stimuli leading to NF-kB activation. Consistently, phenotypes of mice lacking CARMA1 resemble those of MALT1- and Be110-deficient mice. These observations confirm the in vitro data that MALT1 and CARMA1 both interact with BCL10 and show that this ternary complex is critical in B- and T-cell stimulation leading to NF-kB activation and downstream immunologic responses.

The above evidence strongly suggests that translocations involving BCL10 and MALT1 lead to elevated NF-kB activity in MALT B-cell lymphoma. In addition, it has been proposed that chronic antigenic stimulation caused by H pylori infection triggers T-cell-mediated B-cell expansion early in the disease process via activation of the CD40 and NF-kB pathway. We therefore investigated the involvement of MALT1 and its fusion gene product, apoptosis inhibitor-2 (API2)/MALT1, in NF-kB signaling in the presence and absence of CD40 stimulation. Genetic analyses of immunoglobulin gene hypermutation of MALT lymphoma cells suggest that the tumor has a germinal center/post–germinal center origin, and the development of MALT lymphoma may be caused by inhibition of germinal center B-cell apoptosis. Using a germinal center/post–germinal center–derived B-lymphoma cell line, we examined the effect of stably expressed MALT1 or API2-MALT1 on the NF-kB pathway and on apoptosis induced by FAS (CD95)/apoploproteine 1 (APO1), which is a mediator of cell death in the germinal center counterbalanced by CD40 signaling. This study is the first report addressing the functional consequence of MALT1 and API2-MALT1 overexpression in human B-cell lymphoma cells. Our results support a model of MALT lymphomagenesis in which cytoprotection mediated by NF-kB, once activated in a CD40-dependent immune response (eg, against H pylori), is maintained and enhanced through deregulation of MALT1 or formation of an API2-MALT1 fusion.

Material and methods

Immunohistochemical staining

Formalin-fixed, paraffin-embedded tissue blocks from 65 non-Hodgkin B-cell lymphomas and 12 normal lymphoid tissues were retrieved from the archives of the Department of Pathology at Geneva University Hospital. Lymphoid tumors were classified according to the Revised European-American classification of lymphoid neoplasms. After deparaffinization, sections were incubated for 10 minutes in methanol plus peroxide at room temperature, followed by washing with phosphate-buffered saline (PBS) solution. Slides were heated in citrate buffer using a pressure cooker for 3 minutes. Incubation with the primary antibody, monoclonal αMALT1 antibody directed to the C-terminal part of MALT1, was carried out for 1 hour at room temperature. Immuno-reactivity detection was done by using the Envision system (DAKO, Carpenteria, CA).

Images were visualized using a Nikon Eclipse E400 microscope equipped with Plan Fluor 40 × (main panels) or 100 × (insets) objective lenses and aCF110X eyepiece (Nikon, Tokyo, Japan). Images were captured with a Nikon CoolPix E995 camera (Nikon) and processed with Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA).

Cell lines and retroviral constructs

Epstein-Barr virus (EBV)–negative BJAB human B-cell lymphoma cell lines were grown in RPMI-1640 with 10% fetal bovine serum. The human high-grade B-cell lymphoma cell lines SU-DHL-6, OCI-Ly3, and OCI-Ly7 were maintained as described previously. Full-length MALT1 or API2-MALT1 joining nucleotides 1 to 2048 of cIAP2 to nucleotides 814 to 2475 of MALT1 with c-terminal myc followed by 6 × His epitope tags in pCDNA3.1 vector (Invitrogen, Carlsbad, CA) were subcloned into pMSCV-ires-EGFP retroviral vector. Following viral infection, enhanced green fluorescent protein (EGFP)–positive cells were selected by fluorescence-activated cell sorter (FACS)–assisted cell sorting. Selected single-cell clones were reinfected with empty retroviral vector vires-puromycin or vector carrying S32G/S36A mutant of IκB with N-terminal FLAG epitope tag. A similar retroviral vector (vires-hygromycin) carrying MALT1 or API2-MALT1 cDNA and hygromycin resistance was also generated. Infected cells were selected with 3 μg/mL puromycin (Sigma, St Louis, MO) or 500 μg/mL hygromycin (Invitrogen). The IκBα-luciferase reporter cDNA was assembled in pBlueScript, excising from plcBl-EF7G (Clontec, Palo Alto, CA) its Kozak and IκBα sequence (which lacks a stop codon and is followed by a BamHI site) and inserting in-frame the luciferase coding region from pGL3 (Promega, Madison, WI), and transferred to vires-puramycin. The study was performed in accordance with ethical standards of the Helsinki Declaration of the World Medical Association and conformed with Geneva University Hospital ethical committee guidelines at the time of commencing the study.

Sucrose gradient raft fractionation

An established protocol for raft isolation was used. Briefly, 50 × 10⁶ cells were washed once in cold buffer A (50 mM Tris-HCl, pH 7.4; 25 mM KCl; 5 mM MgCl₂; and 1 mM EGTA [ethyleneglycoltetraacetic acid]), then lysed in 680 μL buffer A supplemented with 1% Triton TX-100 and protease inhibitors. This lysate was incubated on ice for 1 hour before mixing with 820 μL buffer A containing 73% (wt/vol) sucrose to give a final volume of 1.5 mL in 40% sucrose. This is transferred to a SW41 centrifugation tube and overlaid with 6 mL buffer A containing 35% sucrose and then 3.5 mL buffer A containing 5% sucrose. The gradient is centrifuged at 250 000g for 16 hours at 4°C. Following centrifugation, 11 fractions, of 1 mL each, were collected from the top of the centrifugation tube. All fractions were immunoblotted for LCK/YES–related novel tyrosine kinase (LYN; Santa Cruz Biotechnology, Santa Cruz, CA) to confirm the presence of lipid rafts in fractions 3 to 5. The pooled fractions were diluted in TKM and further centrifuged at 250 000g for 2 hours. The pellet containing the Triton-resistant membrane proteins was boiled in denaturing sample buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

SDS-PAGE and immunoblotting

Thirty to 50 μg of protein from cell lysates was analyzed by SDS-PAGE and immunoblotted with the appropriate antibodies: rabbit polyclonal anti-CARMA1 antibody against the N-terminus of CARMA1 (AL-220; Alexis Biochemicals, Lausanne, Switzerland) and monoclonal anti-BCL10 antibody (A-6; Alexis Biochemicals); rabbit anti–human BclXL antibody (A-3553; DAKO); polyclonal antibodies against API2 and actin (H-85; Santa Cruz Biotechnology).

IKK activity and nuclear NF-kB measurements

BJAB cells stably infected with vires-puramycin vector containing the IκBα-luciferase reporter alone or reinfected with vires-hygromycin vector carrying either MALT1 or API2-MALT1 cDNA were analyzed. Briefly, triplicates of 150 000 cells per well were either incubated in medium only (controls) or in the presence of 10 ng/mL tumor necrosis factor α (TNFα; R&D Systems, Minneapolis, MN), 40 ng/mL PMA with 2 μM ionomycin (Sigma), or 0.1 μg/mL CD40L (Alexis Biochemicals) cross-linked with 2.5 μg/mL M2 anti-FLAG antibody (Sigma) for 4 hours before analysis. Collected cells were lysed with 50 μL of Glo Lysis buffer (Promega) and mixed with 50 μL of Bright Glo stable substrate (Promega), and emission was measured in a luminoimeter. The values obtained for untreated controls were taken as 100%, and the stimulated samples were expressed as percentage of the corresponding controls.

Enzyme-linked immunosorbent assay (ELISA)–based measurement of nuclear NF-kB subunits was performed according to the manufacturer’s protocol (Active Motif, Rixensart, Belgium). Briefly, 3 μg of BJAB nuclear extract was used per sample point in triplicates in the 96-well plate precoated with consensus oligonucleotides for all NF-kB subunits (p50,
p65/RelA, RelB, p52, and c-Rel). After washing to remove nonspecific binding, specific antibodies to NF-κB subunits were added. After antibody binding, the plate was washed again before adding a horseradish peroxidase–conjugated secondary antibody. The peroxidase substrate was added and colorimetric change was measured at an optical density of 450 (OD450).

**FACS analyses for FAS and CD83 expression**

For FAS or CD83 expression, 5 × 10^5 to 10 × 10^5 BJAB cells were incubated in 100 μL FACS buffer (PBS/1% bovine serum albumin [BSA]/0.05% sodium azide) with 1 μg/mL 2R2 anti-FAS antibody (Alexis Biochemicals), the recommended concentration of anti-CD83 antibody (Pharmingen, San Diego, CA), or the corresponding isotype control IgG (Jackson ImmunoResearch, West Grove, PA). The cells were washed twice and incubated in 1:700 dilution of phycoerythrin (PE)– or fluorescein isothiocyanate (FITC)–conjugated anti–mouse IgG (Jackson ImmunoResearch) for 30 minutes. The cells were washed again and resuspended in 300 μL of the same buffer for FACS analyses for the presence of FAS or CD83 using FacsScan (Becton Dickinson, San Jose, CA).

**Cytotoxic assays**

The cytotoxic assays were performed using the tetrazolium compound MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Promega) and phenazine methosulfate (PMS; Sigma-Aldrich, St Louis, MO), as described previously. Briefly, 50,000 cells in a volume of 100 μL were subjected to serial dilutions of soluble FAS-L (gift of Pascal Schneider, Lausanne, Switzerland). Where stated, CD40L-FLAG (Alexis Biochemicals) cross-linked with anti-FLAG antibody (Sigma) or anti-CD154 monoclonal antibody (Pharmingen, San Diego, CA) was added to the medium with various concentrations of FAS-L. After overnight incubation, cell viability was determined by incubation with MTS/PMS reagent for 2 hours, followed by measuring the absorbance at 490 nm. In experiments with BJAB cells expressing the IκBα superrepressor, FAS-L at indicated concentrations was used in triplicate wells under the same experimental conditions.

[^H]-thymidine incorporation

Exactly 5000 cells per well were plated in triplicates in a 96-well culture plate. The cells were incubated for 24 hours before pulsing with 0.5 μCi/well (0.0185 MBq/well) [^H]-thymidine (Amersham, Arlington Heights, IL) for another 12 hours. Cells were harvested with a cell harvester (Inotech, Rockville, MD) and the counts per minute (cpm) counted with a Wallac Trilux scintillation counter (Perkin Elmer, Shelton, CT).

**Quantitative PCR and RNA preparation**

Total RNA was isolated from cells with Trizol reagent (Invitrogen) according to the manufacturer’s instructions. To eliminate DNA contamination, the final preparations were treated with RNAse-free DNase (Roche, Indianapolis, IN). Reverse transcription of 0.5 to 1 μg of total RNA was carried out using the cDNA first-strand synthesis kit (Roche), and 1 μL of a 100-fold dilution of this reaction was used to proceed real-time polymerase chain reaction (PCR) in iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Expression of mRNA was evaluated by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) analysis using an iCycler (Bio-Rad). For each target, PCR reactions were performed in triplicate in 2 independent experiments. The primers used are listed in Table 1. Selected primers detect only endogenous API2 but not the API1-MALT1 fusion, whereas MALT1 primers detect all species (endogenous, overexpressed MALT1, and the API2-MALT1 fusion). Expression levels of target mRNAs were normalized to the endogenous mRNA levels of GAPDH gene (ACT method). The thermal cycling conditions for all reactions were 95°C for 10 minutes followed by 40 cycles of 95°C 15 seconds and 60°C 1 minute.

**Results**

**Expression of MALT1 in normal and malignant lymphoid tissues**

To investigate the localization of MALT1 in human normal and neoplastic lymphoid tissues, we performed immunohistochemical studies using a monoclonal antibody against the C-terminal part of MALT1. MALT1 is expressed predominantly in B cells in the germinal center (white pulp) and marginal zone of the spleen, the germinal center of tonsils, and the medulla of the thymus. In contrast, mantle zone B cells and interfollicular T cells of tonsils express relatively low amounts of MALT1 (Figure 1A–C). We also stained 6 cases of MALT lymphoma, which showed strong staining in infiltrating neoplastic lymphocytes in the gastric mucosa (Figure 1D). MALT1 was strongly expressed in large and small follicular cells in all 20 cases of follicular lymphomas examined (Figure 1E). Overall, these findings are consistent with a germinal center/marginal zone origin for MALT lymphoma and with deficiencies in marginal zone B cells and germinal centers in MALT1-deficient mice.

**MALT1 and API2-MALT1 are components of lipid rafts of malignant B cells**

Detergent-resistant lipid-rich microdomains, or rafts, on the plasma membrane are believed to be signaling platforms harboring molecular complexes that are essential for signal transduction. The in vitro binding partner of MALT1, BCL10, is recruited to rafts by CARMA1 upon T-cell receptor (TCR) stimulation. We therefore examined several B-cell lymphoma cell lines established from DLBCL patients for MALT1 localization. We found that a pool of MALT1 was strongly expressed in large and small follicular cells in all 20 cases of follicular lymphomas examined (Figure 1E) and in large B-cell blasts in all 20 cases of diffuse large B-cell lymphoma (DLBCL; Figure 1F). Overall, these findings are consistent with a germinal center/marginal zone origin for MALT lymphoma and with deficiencies in marginal zone B cells and germinal centers in MALT1-deficient mice.

**Table 1. RT-PCR primers used for quantification of putative cytoprotective NF-κB target genes**

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<tr>
<th>Genes</th>
<th>Forward primers</th>
<th>Reverse primers</th>
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<td>5′-aat tgc cgt cac cgt tcc t-3′</td>
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<td>5′-atg cta aat gtc atc cag gct gc-3′</td>
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<td>5′-cac agg tga gaa gga gcc agg ga-3′</td>
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<td>5′-agg ctt tgg acc gtt cct g-3′</td>
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<td>5′-agt cat cca ggc aat tta agg ttc-3′</td>
</tr>
<tr>
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<td>5′-gga ccc atc aag att atc cca e-3′</td>
</tr>
<tr>
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<tr>
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<td>5′-ggc aat ttc cag tat atg g-3′</td>
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lymphoma cells. Figure 2. Constitutive expression of MALT1 in lipid rafts of human B-cell lymphoma (DLBCL; panel D), follicular lymphoma (panel E), and diffuse large B-cell lymphoma (DLBCL; panel F). Images were acquired as "immunohistochemical staining."

Effects of exogenous MALT1 and API2-MALT1 on constitutive and CD40-mediated NF-κB activity

We examined the effect on NF-κB activity of API2-MALT1 or MALT1 overexpression in BJAB cells using an ELISA-based assay to quantify NF-κB subunits (p50, p65/RelA, RelB, p52, and c-Rel) in nuclear extracts. In unmodified BJAB cells there were relatively high amounts of p50 and c-Rel compared with p65 (Figure 3) and other subunits (data not shown). After stimulation of CD40, which is known to activate the IKK complex, nuclear c-Rel was relatively unchanged but there were increases in p50 and p65/RelA (Figure 3A-C). Analysis of unstimulated cells showed that basal NF-κB activity was increased by API2-MALT1 (p50 and p65/RelA) or exogenous MALT1 (p50 only). The response to CD40 stimulation was also increased by API2-MALT1 (p50, c-Rel, and p65/RelA), and MALT1 produced a modest but significant rise in nuclear c-Rel after CD40 stimulation.

MALT1 and API2-MALT1 act in the CD40-inducing NF-κB pathway upstream of IKK complex activation

To address at which point the transgenes affect the CD40 pathway to NF-κB activation, we created a cell-based assay for IKK activity in BJAB cells by stably expressing an IκBα-luciferase fusion reporter gene. The assay derives from observations that the exogenous IκBα-reporter protein (like endogenous IκBα) is degraded as a result of IKK activity, but its transcription (unlike that of endogenous IκB) is relatively independent of NF-κB activity (ie, its production can be regarded as constitutive). Therefore, the level of the reporter protein (easily measured as luciferase activity) can serve as an indicator of IKK activity, falling as IKK activity increases and vice versa. As expected, BJAB cells stably expressing the reporter alone showed a substantial decline in the reporter level after stimulation by PMA and ionomycin, indicating an acute increase in IKK activity with less of a response to TNFα (Figure 3D). BJAB cells also expressing MALT1 or API2-MALT1 showed a similar pattern and degree of response to these agents, indicating that these transgenes had no effect on the respective pathways by which these agents increase IKK activity. In contrast, it was clear that these transgenes further augmented the increase in IKK activity caused by CD40 signaling in BJAB cells. The fact that API2-MALT1 was the more effective transgene, despite similar levels of expression shown by Western blots, is in agreement with NF-κB activation abilities observed in 293T cells (data not shown).
API2-MALT1 and MALT1 expression protect BJAB cells from FAS-mediated apoptosis

Our results so far indicate that MALT and API2-MALT1 can function in the CD40 signaling pathway, which blocks apoptosis induced by FAS (APO1) in B-cell lines and primary cells from the germinal center stage.33,40 We found that BJAB cells expressing MALT1 or API2-MALT1 were more resistant to FAS-induced apoptosis than were parental mock-infected cells (Figure 4A). This difference in FAS sensitivity was not due to differences in expression of FAS, which was essentially the same between the 3 cell lines (Figure 4D). Wild-type (WT) cells were more resistant to FAS in the presence of CD40 ligand (CD154; Figure 4B), approaching the resistance displayed by unstimulated cells expressing either transgene. The specificity of protection caused by CD40L (CD154) on WT cells was further confirmed when the sensitivity to FAS-L shifted back to that of untreated cells in the presence of an anti-CD154 antibody (Figure 4C).

Constitutive NF-κB activation is critical for MALT1- and API2-MALT1-mediated FAS resistance and proliferation

It is perhaps surprising that CD154 did not further increase the FAS resistance of cells expressing MALT1 or API2-MALT1 (Figure 4B), since these cells respond to CD40 stimulation in several other measures (eg, in the ELISA assay for nuclear subunits) and are in fact even more responsive than WT cells. However, this finding suggests that FAS resistance imparted by the transgenes is due to their impact on constitutive NF-κB activity, an effect shown by the ELISA assay in the absence of stimulation. To examine this, BJAB cells expressing no transgene, MALT1, or API2-MALT1 were secondarily infected with a “superrepressor” (IκBα-SR) form of IκBα, bearing mutations in the 2 serine phosphorylation sites (S32G, S36A). This mutant cannot be phosphorylated by IKK and consequently degraded and acts thus as a dominant negative in the NF-κB pathway.36 MALT1- and API2-MALT1–expressing cells were resistant to FAS-mediated apoptosis even at 2 μg/mL FAS-L. In contrast, the corresponding lines coexpressing SR were significantly less resistant, similar to their parental cells (Figure 5A). In the parental cells, the superrepressor had no effect on sensitivity to FAS-L. Given that all the SR+ lines express similar levels of IκBα-SR (Figure 5D), these results indicate that MALT1- or API2-MALT1–mediated resistance to FAS-induced apoptosis is dependent on their induction of constitutive NF-κB activity.

Because NF-κB activation in malignant lymphomas has been shown to increase proliferation as well as survival,41 we examined the effect of MALT1 and API2-MALT1 on proliferation as measured by incorporation of [3H] thymidine into DNA. Both transgenes conferred a modest but significant growth advantage to pools and single clones expressing the transgenes (Figure 5B). Because this advantage was largely eliminated by IκBα-SR (Figure 5C), it can be attributed to constitutive NF-κB activation. In contrast, expression of IκBα-SR did not affect proliferation of the parental BJAB cells.

MALT1 and API2-MALT1 specifically enhance CD40-stimulated NF-κB activation

The effect of the superrepressor (SR) as an effective inhibitor of NF-κB activation in these lines was further demonstrated by measuring surface expression of CD83, a known direct target of NF-κB.42,43 Among cell lines infected in parallel with the empty second vector, and in keeping with the results so far, the CD83 increase after 4 hours of CD40 stimulation was augmented by the transgenes (Figure 6A), although they did not affect the response to
Many NF-κB target genes protect against apoptotic stimuli in B cells and other cell types; in particular, CD40 signaling increases B-cell survival through NF-κB–mediated up-regulation of BclXL and Bfl1/A1. We therefore compared expression of a series of known antiapoptotic NF-κB target genes (API1, API2, BclXL, hA20, Bfl1/A1, and Bcl2) in BJAB cells expressing MALT1, API2-MALT1, or vector only (Figure 7A). While resting mRNA levels of API2 and hA20 were significantly higher in MALT1-expressing cells than in others, that of Bcl2 was significantly higher in API2-MALT1–expressing cells, suggesting that there may be differences in how MALT1 and API2-MALT1 produce constitutive NF-κB activation. CD40 stimulation of these lines produced significant elevations in mRNA levels of most targets (Figure 7B), but BclXl rose significantly only in the presence of API2-MALT1 or overexpressed MALT1, confirming the previous suggestion that both transgenes are involved in CD40-mediated NF-κB activation. The protein levels of BCLXl and API2 rose significantly following 24 hours of CD40 stimulation, consistent with the rise in their mRNA levels (Figure 7C). In the same experiment, no change due to transgenes and/or CD40 stimulation was found in the mRNA levels of several other known cytoprotective NF-κB target genes (cFLIP, hXIAP, SURVIVIN, and Gadd45β; data not shown), showing that these do not mediate the observed changes in FAS resistance in BJAB cells and that target gene expression is influenced by cell type.

**Discussion**

It has been known for some time that a small set of recurring chromosomal translocations is associated with MALT B-cell lymphomas, but the functional consequences of these genetic alterations have only recently come to light. Chromosomal translocations t(11;18)(q21;q21) and t(14;18)(q32;q21), found in up to 50% of MALT lymphomas, lead to overexpression of MALT1 or formation of an API2-MALT1 fusion protein. Although there was reason to believe that these would lead to NF-κB activation in PMA/ionomycin (Figure 6B). A similar pattern of enhancement by transgenese was seen over a spectrum of lower doses and shorter duration of CD40 stimulation and in experiments using transient transfection of an NF-κB–driven luciferase reporter plasmid (data not shown). In sharp contrast, all SR lines were completely unresponsive to CD40 stimulation (Figure 6A), i.e., CD83 remained virtually undetectable and lower than in the non-SR lines without stimulation (Figure 6C). The effect of the transgenese on CD40 signaling was not explained by baseline CD83 levels or levels of surface CD40 itself, which were little affected by the transgenese and only modestly reduced by IκBα-SR (Figure 6D). Furthermore, reduced CD40 was unlikely to be the only effect of the IκBα-SR on NF-κB activation by the CD40 pathway, since the CD83 response to PMA/ionomycin stimulation was also markedly reduced in SR lines.

**Figure 5.** NF-κB activation by MALT1 and API2-MALT1 is necessary for resistance to FAS-mediated apoptosis and proliferation. (A) BJAB cells expressing no primary transgene (WT) or expressing MALT1 or API2-MALT1 were secondarily infected with empty vector or IκBα superrepressor (SR) and incubated in triplicates with soluble FAS-L. Cell viability was assayed after 16 hours. The results are representative of 2 independent experiments. (B) Cell proliferation was measured by incorporation of [3H]-thymidine measured in single clones or pools of BJAB cells expressing no transgene (WT), MALT1, or API2-MALT1. Results are shown as cpm, with error bars representing standard deviation obtained from triplicate samples. (C) The same BJAB cells as in panel A with empty second vector (○) or IκBα superrepressor (●) were assayed for [3H]-thymidine incorporation as described above. The data shown are 1 of 3 independent experiments. (D) Lysates of equal numbers of the above BJAB cells were immunoblotted against the N-terminal FLAG epitope tag of SR-IκBα to show specific and equal expression.

**Figure 6.** Effect of MALT1 and API2-MALT1 on NF-κB activation as measured by CD83 expression. FACS measurement of surface markers in BJAB lines with no transgene (dotted line), MALT1 (thin line), or API2-MALT1 (bold line) secondarily infected with empty vector (top panels) or IκBα superrepressor (bottom panels). Histograms show CD83 expression after 4 hour incubation with cross-linked CD40L (A), PMA+ionomycin (B), or medium alone (C). Histogram (D) shows resting CD40 expression in the 6 lines.
MALT B-cell lymphoma, and immunostains of primary MALT lymphomas have shown evidence of NF-κB activation, this has not been shown experimentally in human B cells. Here we demonstrate that both MALT1 and API2-MALT1 confer constitutive NF-κB activity in a human B-cell line, resulting in increased proliferation and resistance to FAS-induced apoptosis. Furthermore, both transgenes act upstream of the IKK complex to enhance NF-κB activation induced by CD40. Many lymphoid malignancies have been associated with constitutive NF-κB activation and poor prognosis, and elevated NF-κB activity in MALT B-cell lymphoma would favor malignant B-cell proliferation and survival.41,46-48 Our results are consistent with recent findings that NF-κB activation by API2-MALT transforms NIH 3T3 cells and inhibits p53-induced cell death.49

Based on previous studies and our results presented here, we propose the following model for the role of MALT1 and API2-MALT1 in MALT lymphomagenesis (Figure 8). In early stages, the immune response triggered by H pylori infection induces NF-κB activation in B cells via CD40/CD40L interaction with T cells, increasing the expression of specific cytoprotective genes. The acquisition of recurring chromosomal translocations results in the expression of API2-MALT1 or overexpression of MALT1, producing constitutive NF-κB activation and enhancing the response to CD40 stimulation. Both of these are potentially advantageous to lymphoma cells, but constitutive NF-κB activity would confer independence from the immune response to H pylori, and we have shown that it alone may be sufficient for enhanced proliferation and resistance to FAS-induced apoptosis. Finally, because the API2-MALT1 fusion is itself regulated by NF-κB, a positive feedback loop may be maintained that further supports bacterium- and CD40-independent NF-κB activation, leading to progression of MALT lymphoma.7,8 It should be noted that at least parts of this model may not be restricted to gastric MALT lymphoma or H pylori infection, although those have generated much of the research implicating CD40 and antigen drive in MALT lymphoma. MALT1 translocations are found in tumors from other sites, and recently similar causal associations have been found between Chlamydia psittaci and MALT lymphoma of the orbit and between Campylobacter jejuni and a MALT lymphoma–like immunoproliferative lesion of the small intestine.50,51

Constitutive NF-κB activation by API2-MALT1 or overexpressed MALT1 is a primary feature of the proposed model. Normal mature B cells require some degree of constitutive NF-κB activity for survival, as shown by their disappearance from mice after conditional deletion of NEMO or IKKβ.52 Deficiencies in germinal centers and splenic marginal zone B cells are features of mice with germ line inactivation of members of the NF-κB family,53 as they are of mice with germ line inactivation of MALT1, BCL10, or CARMA1. Since there is in vitro evidence that these proteins can produce IKK activation, it is plausible that they normally function in a common pathway contributing to constitutive NF-κB activity in B cells. Using a human B-cell line corresponding to the germinal center stage of B-cell differentiation, we have shown that API2-MALT1 or overexpressed MALT1 produces increased constitutive NF-κB activity, resulting in increased proliferation and resistance to FAS-induced apoptosis. These benefits are plausible given that many NF-κB target genes have antiapoptotic functions and some (eg, cyclin D2) are associated with cell-cycle progression. However, this may not fully model the consequences of increased constitutive NF-κB activity in MALT lymphoma or other types of lymphoma in which it is found. In particular, while IκBα-SR merely abrogates the effects attributable to API2-MALT or overexpressed MALT1 in BJAB cells, returning them to a state comparable to that of wild-type cells, it is lethal to cell lines from lymphoma types characterized by

![Figure 7. Constitutive and CD40L-enhanced transcription of cytoprotective genes.](image)

![Figure 8. Model of the role of MALT1 and API2-MALT1 in the NF-κB activation pathway leading to MALT lymphoma.](image)
increased constitutive NF-κB activity. In other words, increased constitutive NF-κB activity is not merely advantageous but an essential feature of the biology of these types of lymphoma; whether this is the case for MALT lymphoma remains unknown.

The mechanisms of constitutive IKK activation by API2-MALT and overexpressed MALT1 may be multifaceted and somewhat different, related to our findings that the 2 transgenes had both similar and different effects by several measures. In vitro studies showed that IKK activation by MALT1 or BCL10 requires both proteins and this was supported by studies using embryonic fibroblasts from MALT1 knockout mice. Using purified proteins, Sun et al. successfully reconstituted the pathway from BCL10 to IKK activation, in which oligomerization of MALT1 by a BCL10/CARMA1/MALT1 complex leads to the recruitment of a ubiquitin ligase (TNF receptor–associated factor 6 [TRAF6]), followed by activation of a kinase (transforming growth factor-β [TGFβ]–activated kinase 1 [TAK1]), which activates the IKK complex and NF-κB. In contrast, BCL10 is not required for IKK activation by API2-MALT, and BCL10 was not among several proteins found to bind to API2-MALT in one study. API2-MALT is more stable than MALT1 or API2 because it lacks domains of those proteins that promote proteosomal degradation. Subcellular localization is also likely to be a major factor affecting the function of API2-MALT and MALT1. We found that endogenous MALT1 in several DLBCL cell lines and exogenously expressed MALT1 and API2-MALT in BJAB cells are constitutively present in lipid raft fractions together with BCL10 and CARMA1. This is contrary to previous studies using primary T cells that showed that BCL10 is only recruited into rafts upon TCR stimulation. Studies using cells from DLBCL patients have shown that preassembled signalosomes, containing molecules of the CD40 signaling pathway and may be responsible for elevated constitutive NF-κB activity. These observations suggest that MALT1 and API2-MALT may be preassembled into so-called signalosomes, contributing to their ability to activate IKK constitutively and/or enhance CD40 signaling. However, it is likely that there are additional regulating factors since DLBCL cell lines with similar findings on lipid raft analysis for MALT1, CARMA, and BCL10 (Figure 2A) have substantially different levels of constitutive NF-κB activity and a starkly different response to IκBα-SR expression.

A second key feature of our proposed model is that both API2-MALT and overexpressed MALT1 enhance the effect of CD40 stimulation on IKK activation. This is consistent with one study of germ line inactivation of MALT1, which found that MALT1 was required for the proliferative response of mouse B cells to CD40 stimulation. CD40 signaling has previously been implicated to play an essential role in MALT lymphomagenesis and is still subject to regulation by NF-κB activity. To study the roles of MALT1 and its fusion protein API2-MALT1 in lymphomagenesis in vivo, transgenic mouse models overexpressing these proteins would be informative. Due to the role of NF-κB as a common downstream mediator of a large number of signal transduction pathways, its targeted therapeutic inhibition may be difficult to achieve without untoward side effects. Identification and functional characterization of the upstream activators of NF-κB in particular types of lymphoma could provide more specific therapeutic targets. Since germ line inactivation of the MALT1 gene in mice seems almost exclusively to affect the lymphoid system, blocking MALT1 function may be associated with controllable side effects.

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MALT1 and the API2-MALT1 fusion act between CD40 and IKK and confer NF-κB-dependent proliferative advantage and resistance against FAS-induced cell death in B cells

Liza Ho, R. Eric Davis, Béatrice Conne, Richard Chappuis, Margaret Berczy, Paulette Mhawech, Louis M. Staudt and Juerg Schwaller