Bcl10 and MALT1, Independent Targets of Chromosomal Translocation in MALT Lymphoma, Cooperate in a Novel NF-κB Signaling Pathway*

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At least two distinct recurrent chromosomal translocations have been implicated in the pathogenesis of MALT lymphoma. The first, t(1;14), results in the transfer of the entire Bcl10 gene to chromosome 14 wherein Bcl10 expression is inappropriately stimulated by the neighboring Ig enhancer. The second, t(11;18), results in the synthesis of a novel fusion protein, API2-MALT1. Until now, no common mechanism of action has been proposed to explain how the products of these seemingly unrelated translocations may contribute to the same malignant process. We show here that Bcl10 and MALT1 form a strong and specific complex within the cell, and that these proteins synergize in the activation of NF-κB. The data support a mechanism of action whereby Bcl10 mediates the oligomerization and activation of the MALT1 caspase-like domain. This subsequently activates the IKK complex through an unknown mechanism, setting in motion a cascade of events leading to NF-κB induction. Furthermore, the API2-MALT1 fusion protein also strongly activates NF-κB and shows dependence upon the same downstream signaling factors. We propose a model whereby both the Bcl10-MALT1 complex and the API2-MALT1 fusion protein activate a common downstream signaling pathway that originates with the oligomerization-dependent activation of the MALT1 caspase-like domain.

B cell lymphomas of the mucosa-associated lymphoid tissue (MALT)1 were first described in 1983 by Isaacson and Wright (1). It is now recognized that these are the most common form of lymphoma arising in extranodal sites; most cases originate in the gastric mucosa where they are strongly associated with chronic Helicobacter pylori infection (2). However, MALT lymphomas may also occur in other sites such as the lung, salivary glands, and thyroid. Again, when the lymphoma arises in these sites, it is frequently associated with a prior chronic inflammatory process such as lymphoid interstitial pneumonia in the lung, Sjögren’s disease in the salivary glands, and Hashimoto’s thyroiditis. These findings have lead to the notion that, at least in early stages of tumor development, growth of the neoplastic cells is facilitated by antigen-driven T cells. Based on histopathologic criteria, MALT lymphomas are classified as either low- or high-grade. Nevertheless, it is likely that for most cases there is a gradual progression in the pathogenesis of the disease, such that untreated borderline cases progress to low-grade lymphomas which ultimately develop into high-grade, aggressive lymphomas. Consistent with this notion is the finding that many early cases of gastric MALT lymphoma can be effectively treated solely by eradication of H. pylori with antibiotics (3). More established, higher-grade lymphomas may require more aggressive treatment, as the progression of such tumors appears to no longer depend on a coexisting chronic inflammatory process.

The molecular events responsible for the development and progression of MALT lymphomas are still poorly understood. Several recurrent chromosomal abnormalities have been observed in these lymphomas, but no unifying molecular model has been proposed to explain how these distinct abnormalities may promote tumorigenesis. Perhaps the best characterized chromosomal abnormality is the recurrent t(1;14) (p22;q32) which was recently shown to result in the translocation of the entire coding region of the Bcl10 gene to chromosome 14, wherein its expression is inappropriately placed under the control of the strong Ig transcriptional enhancers (4, 5). We and others have shown that overexpression of Bcl10 (also called CIPER, mE10, c-CARMEN, CLAP, and c-E10) activates NF-κB, a potential pro-survival signal in B cells, and have suggested this to be a major mechanism whereby unregulated Bcl10 expression contributes to transformation and tumor progression (6–10).

A second major chromosomal translocation, seen in up to 50% of MALT lymphomas, is the t(11;18) (q22;q21). In this case, a fusion gene is created which encodes a chimeric protein consisting of the N-terminal portion of API2 (one of the inhibitors of apoptosis proteins, also known as c-IAP2, Hiap1, and MIHC) linked to the C terminus of a novel protein, MALT1 (MLT) (11–13). Although MALT1 contains a caspase-like domain in its C terminus, which is preserved in the API2-MALT1 chimera, no physiologic function has been ascribed to this protein. Although it is known that Bcl10 activates NF-κB, little is

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¶¶ The abbreviations used are: MALT, mucosa-associated lymphoid tissue; Ig, immunoglobulin; NF-κB, nuclear factor-κB; IkB, inhibitor of NF-κB; IKK, IkB kinase; B, B cell; TNF-α, tumor necrosis factor-α; TNFR1, tumor necrosis factor-α receptor 1; CARD, caspase recruitment domain; MEF, mouse embryonic fibroblast; FKBP, FK506-binding protein; PAGE, polyacrylamide gel electrophoresis.

chronic Helicobacter pylori infection (2). However, MALT lymphomas may also occur in other sites such as the lung, salivary glands, and thyroid. Again, when the lymphoma arises in these sites, it is frequently associated with a prior chronic inflammatory process such as lymphoid interstitial pneumonia in the lung, Sjögren’s disease in the salivary glands, and Hashimoto’s thyroiditis. These findings have lead to the notion that, at least in early stages of tumor development, growth of the neoplastic cells is facilitated by antigen-driven T cells. Based on histopathologic criteria, MALT lymphomas are classified as either low- or high-grade. Nevertheless, it is likely that for most cases there is a gradual progression in the pathogenesis of the disease, such that untreated borderline cases progress to low-grade lymphomas which ultimately develop into high-grade, aggressive lymphomas. Consistent with this notion is the finding that many early cases of gastric MALT lymphoma can be effectively treated solely by eradication of H. pylori with antibiotics (3). More established, higher-grade lymphomas may require more aggressive treatment, as the progression of such tumors appears to no longer depend on a coexisting chronic inflammatory process.

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known about the precise signaling pathway utilized by Bcl10 to accomplish this effect. In this study, we further characterized this pathway, and in so doing, discovered that MALT1 participates with Bcl10 in a novel mechanism for NF-κB activation. The data indicate that Bcl10 and MALT1 form a tight complex which serves to oligomerize and activate the caspase-like domain of MALT1. Through an unknown mechanism, this appears to subsequently activate the downstream IkB kinase (IKK) complex, leading to the induction of NF-κB. In similar fashion, we found that the API2-MALT1 fusion protein also potently activates NF-κB, again through activation of the IKK complex. These results provide a unifying model for the molecular pathogenesis of MALT lymphoma and also suggest a novel pathway for activation of NF-κB through the involvement of a caspase-like enzyme.

EXPERIMENTAL PROCEDURES

**Plasmids**—The expression plasmids pcDNA3-Myc (HA and -Flag), pcDNA3-p35, pEF1-BOS-β-gal, pcDNA3-Flag-IKKy, pcDNA3-RIP-(1–580)-Myc, pcDNA3-Myc-RICK, pcDNA3-Nod1-HA/Flag, pcDNA3-Apaf-1-(1–677)-Myc, pcDNA3-IKK-i, pcDNA3-IKK-γ, pcDNA3-IKK-β, pcDNA3-Flag-IKKi-(K38A), pcDNA3-Flag-IKKi-(K44A), pcDNA3-Flag-IKKi-(K42A), pc-TBK1-Myc, pcDNA3-TNFR1-Flag, pcDNA3-Flag-IKKi, and pcDNA3-Flag-IKKi-(K38A) have been described previously (6, 14–19). The plasmid pcDNA3-Bcl10-Myc (previously referred to as pcDNA3-CIPER-Myc) encodes the mouse Bcl10 protein with a C-terminal Myc tag, and has been described (6). pcDNA3-Bcl10Δ107–119 was created by adjoining two Bcl10 polymerease chain reaction fragments which together encompassed the entire coding region except for that corresponding to amino acids 107–119. The pcDNA3-MALT1-HA (and -Myc) vectors were constructed by inserting the coding region of MALT1 into the BamHI/Xhol sites of the parental tagged vectors using a polymerase chain reaction method. Site-directed mutagenesis of MALT1 that containing the Serine to Proline Quickchange Kit™ (Stratagene), directed as described by the manufacturer’s protocol. To construct pcDNA3-MALT1-Casp-FKBPx3, a polymerase chain reaction fragment encoding amino acids 324 to 813 of MALT1 was inserted into the Acc65/XhoI sites of pcDNA3-FKBPx3-Myc (20) which encodes three tandemly repeated FKBP dimerizer domains with a Myc tag. The pcDNA3-Flag-AP12-MALT1 plasmid was created by inserting the codon optimization of the entire MALT1 fusion gene into the XhoI/BamHI sites of pcDNA3-Flag to construct pcDNA3-Flag-AP12-MALT1. The full-length AP12-MALT1 insertion was digested with Acc65I and XhoI to clone the truncated fragment which was then subcloned into the parental tagged vector in the same fashion as the full-length cDNA. Likewise, pcDNA3-Flag-AP2-MALT1-(1–700) was constructed by digestion with Acc65I and SbfI, with subsequent subcloning into Acc65I/EcoRV-digested pcDNA3-Flag.

**Transfections and NF-κB Activation Assays**—2 × 10⁵ HEK293T cells were transfected with the reporter constructs pEF1-BOS-β-gal and pBVI-Luc, and indicated expression plasmids, using a calcium phosphate method as described (6, 14). NF-κB activation was assessed by measuring luciferase activity (normalized for β-galactosidase expression) in cell extracts after 24 h, as described (6, 14). pcDNA3-p35 was also transfected in all cases to prevent cell death. For transfections of the Rat-1 and 5R fibroblast cell lines, as well as the wild type and RelA-deficient MEFs, pcDNA3-TNFR1-Flag was transfected into the parental tagged vector as directed by the manufacturer’s protocol. To construct pcDNA3-MALT1-Casp-FKBPx3, a polymerase chain reaction fragment encoding amino acids 324 to 813 of MALT1 was inserted into the Acc65I/XhoI sites of pcDNA3-FKBPx3-Myc (20) which encodes three tandemly repeated FKBP dimerizer domains with a Myc tag. The pcDNA3-Flag-AP12-MALT1 plasmid was created by inserting the codon optimization of the entire MALT1 fusion gene into the XhoI/BamHI sites of pcDNA3-Flag to construct pcDNA3-Flag-AP12-MALT1. The full-length AP12-MALT1 insertion was digested with Acc65I and XhoI to clone the truncated fragment which was then subcloned into the parental tagged vector in the same fashion as the full-length cDNA. Likewise, pcDNA3-Flag-AP2-MALT1-(1–700) was constructed by digestion with Acc65I and SbfI, with subsequent subcloning into Acc65I/EcoRV-digested pcDNA3-Flag.

**Immunoprecipitations and Western Blotting**—HEK293T cells were harvested 24 h following transfection and lysed in 0.2% Nonidet P-40 lysis buffer (22). Immunoprecipitations were carried out using rabbit polyclonal anti-Myc antibody (Santa Cruz) as described (6, 14). The products were then resolved by 12% SDS-PAGE, and detected by Western blotting with mouse monoclonal anti-Myc (Santa Cruz) and Co-immunoprecipitated proteins were detected by probing blots with either mouse monoclonal anti-Flag (M2) (Sigma) or anti-HA 12CA5 (Roche Molecular Biochemicals). Total lysates were similarly analyzed for transgene expression by Western blotting, both in experiments where immunoprecipitations were performed and in experiments where NF-κB activity was being assessed.

**Gel Mobility Shift Assay**—Nuclear extracts were prepared following transfection of HEK293T cells by an established method (23). Gel shift assays were then performed as described (23) using a radiolabeled, double-stranded oligonucleotide containing a single xB site (14) as a probe. For competition experiments, the indicated amounts of unlabeled, double-stranded oligonucleotide, containing either the wild-type or mutant xB site (14), were included in the binding reactions. Super-shift analyses were performed by including anti-RelA antibody (Santa Cruz) in binding reactions.

**RESULTS AND DISCUSSION**

**Bcl10 Activates NF-κB by Signaling through the IKK Complex**—Many inducers of NF-κB are now known to signal through the IKK complex, which is composed of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit, IKKγ (NEMO/FIP3/IKKAP1) (Fig. 1A). Two related kinases, RIP and RICK, are prototypical examples of such inducers, and are now thought to activate the IKK complex through direct interaction with IKKγ (14, 24, 25). However, an emerging theme is that signaling pathways induced by other stimuli may bypass components of the IKK complex, particularly IKKγ, or even the entire IKK complex itself (Fig. 1A) (16, 17, 26–28). In order to delineate the downstream signaling pathway utilized by Bcl10, we analyzed the requirement for each step in the prototypical pathway mapped for inducers such as RIP and RICK. Starting with the most distal step in the signaling pathway, we first tested whether the well characterized functional activation of NF-κB by Bcl10 is dependent on one of the principle Rel family members, RelA (p65). Wild type and RelA-deficient MEFs were co-transfected with an expression plasmid encoding Bcl10 and an NF-κB-responsive luciferase reporter construct; while Bcl10 expression resulted in ~11-fold induction of NF-κB in the wild type MEF’s, almost no induction was seen in cells lacking RelA (Fig. 1B). As a control, activation of NF-κB by another inducer, Nod1 (14, 15), was also seen to depend upon RelA. In contrast, expression of Apaf-1, a protein involved in apoptosis signaling, had no effect on NF-κB signaling in either cell line. We next used the gel mobility shift assay to test whether expression of Bcl10 results in nuclear translocation of NF-κB and its binding to DNA containing the xB consensus sequence. Expression of Bcl10 in 293T cells resulted in the formation of a DNA-protein complex identical to that seen when cells were stimulated with TNF-α (Fig. 1C). Furthermore, formation of the complex was abolished by competition with unlabeled oligonucleotide containing the xB consensus, but not by oligonucleotide containing a mutant xB site. Finally, a specific supershift complex was seen when antibody to the p65 subunit of NF-κB was included in the binding reaction (Fig. 1C). Taken together, these results indicate that the activation of NF-κB by Bcl10 is indeed the result of enhanced nuclear translocation and DNA binding by the NF-κB transcription factor.

Next, Bcl10 was co-expressed with mutant forms of IkBα, IkKα, IkKβ, and IKKγ which have been shown to act as dominant inhibitors of their corresponding endogenous counterparts (14, 29). Expression of all four of these dominant negative mutants resulted in near complete inhibition of Bcl10-responsive NF-κB activation (Fig. 1D). Western blotting confirmed that none of these mutants interfered significantly with the expression of Bcl10, so that inhibition was likely to have occurred through functional interference with the Bcl10-responsive signaling pathway. As a control, a dominant negative mutant of IKK-β (IKKe) (16) had no effect on Bcl10-dependent NF-κB activation. These results support the notion that, similar to RIP and RICK, Bcl10 acts to stimulate a signaling pathway which depends upon the IKK complex.

Because IKKγ may represent a critical link between Bcl10 and downstream factors, we sought to more stringently test its
involvement in Bcl10 signaling. First, we performed a dose-response study to analyze the potency of the IKKγ dominant negative in inhibiting Bcl10 activity. This inhibitor blocked Bcl10 induction of NF-κB with precisely the same effectiveness as it blocked induction by RICK (Fig. 1E). In contrast, the IKKγ dominant negative had no effect on induction of NF-κB by IKK-ι; this was an expected finding, as it has been shown that IKK-ι appears to act as an IKK complex-independent 1B kinase (see Fig. 1A) (16, 26). Using a different methodology, we confirmed the requirement for IKKγ in Bcl10 signaling by testing NF-κB induction in the IKKγ-defective 5R cell line, a derivative of the Rat-1 fibroblast line (21). While in the parental Rat-1 cells, Bcl10 effectively induced NF-κB, we observed no Bcl10-responsive NF-κB activation in the IKKγ-deficient 5R cells (Fig. 1F). As a control, expression of RIP resulted in similar findings, consistent with the previously documented critical role for IKKγ in RIP signaling (14). To demonstrate that 5R cells can be capable of mounting a robust NF-κB response when transfected with factors downstream of IKKγ, we also expressed IKKβ in both cell lines and confirmed that this factor could activate NF-κB irrespective of the presence of IKK-ι (25).

Bcl10 Does Not Interact Directly with IKKγ, but Binds to MALT1—It has been previously shown that both the RIP and RICK signaling proteins must bind directly to IKKγ in order to stimulate the kinase activity of the IKK complex (14, 24, 25). To test if Bcl10 acts in a similar way, we first analyzed whether Bcl10 binds directly to IKKγ using a co-immunoprecipitation assay. Myc-tagged Bcl10 was co-expressed with Flag-tagged IKKγ in 293T cells, extracts were prepared, and Bcl10 was immunoprecipitated with anti-Myc antibody (Fig. 2A). Subsequent Western blotting failed to detect any significant co-immunoprecipitation of IKKγ. As a positive control, Flag-tagged IKKγ effectively co-immunoprecipitated with Myc-tagged RIP. These results suggest that there are one or more missing signaling factors which mediate the connection between Bcl10 and the IKK complex.

In a search to identify signaling proteins which may bridge the gap between Bcl10 and IKKγ, we decided to test the possible involvement of MALT1. MALT1 is a newly discovered protein of unknown function which contains a C-terminal caspase-like domain, including the universally conserved cysteine-histidine catalytic diad (11–13). The MALT1 gene was recently demonstrated to be the target of the recurrent t(11;18) (q21;q21) seen in a subset of MALT lymphomas. We therefore wondered whether the seemingly disparate translocations which target Bcl10 and MALT1 might, in fact, influence the same signaling pathway. To test for an interaction between Bcl10 and MALT1, we again performed co-immunoprecipitation experiments using Myc-tagged Bcl10 and HA-tagged MALT1. When co-expressed in 293T cells, MALT1 bound to Bcl10 but not to a series of other proteins involved in NF-κB signaling (Fig. 2B). Quantitative immunoprecipitation experiments demonstrated that the interaction is remarkably strong, with more than 20% of expressed MALT1 being co-immunoprecipitated in experiments where direct immunoprecipitation of Myc-Bcl10 is less than 50% efficient.2 These

2 P. C. Lucas and G. Núñez, unpublished results.
results indicate that Bcl10 and MALT1 form a tight and specific complex when co-expressed in cells.

**Bcl10 and MALT1 Synergize in the Activation of NF-κB**—To explore the functional consequence of the interaction between Bcl10 and MALT1, we tested whether MALT1 influences Bcl10-responsive NF-κB activation. In a dose-dependent manner, expression of Bcl10 alone resulted in NF-κB activation; a maximal level of ~35-fold induction was achieved with 75 ng of transfected expression plasmid (Fig. 3A). In contrast, transfection of up to 500 ng of MALT1 expression plasmid had absolutely no effect on NF-κB (Fig. 3B). However, when MALT1 was co-expressed with Bcl10, there was a remarkable synergy, with close to 150-fold induction of NF-κB achieved when maximal levels of Bcl10 were used (Fig. 3A). The effect of MALT1 was specific for Bcl10-responsive NF-κB activation, as a similar dose-response study showed no influence of MALT1 on RICK activity (Fig. 3B). Western blotting confirmed that neither Bcl10 nor RICK expression levels were affected by co-expression of MALT1, indicating that MALT1 was specifically influencing NF-κB activation; a maximal level of ~35-fold induction was achieved with 75 ng of transfected expression plasmid (Fig. 3A). In contrast, transfection of up to 500 ng of MALT1 expression plasmid had absolutely no effect on NF-κB (Fig. 3B). However, when MALT1 was co-expressed with Bcl10, there was a remarkable synergy, with close to 150-fold induction of NF-κB achieved when maximal levels of Bcl10 were used (Fig. 3A). The effect of MALT1 was specific for Bcl10-responsive NF-κB activation, as a similar dose-response study showed no influence of MALT1 on RICK activity (Fig. 3B). Western blotting confirmed that neither Bcl10 nor RICK expression levels were affected by co-expression of MALT1, indicating that MALT1 was specifically influencing the function of the Bcl10 signaling pathway (Fig. 3, C and D). Further specificity was demonstrated by testing the effect of MALT1 on either low or high levels of co-expressed IKK-1, TBK-1, or TNFR1 (Fig. 3, E and F). In all cases, MALT1 had no significant effect on the level of NF-κB induction achieved with these signaling proteins.

**Bcl10 Binds to the MALT1 Ig-like Domains through a Region Critically Important for NF-κB Activation**—To test whether the binding between Bcl10 and MALT1 is functionally related to the observed synergy in activation of NF-κB, we analyzed several mutants of each protein. First, we mapped the region of MALT1 which is responsible for binding Bcl10. Three truncation mutants were constructed which together encompassed the entire MALT1 protein, but which isolated the three known domains of the protein, the death domain (DD), two adjacent Ig-like domains, and the caspase-like domain (Fig. 4A). All mutants were HA-tagged and coexpressed with Myc-tagged Bcl10. Following immunoprecipitation with anti-Myc antibody, Western blots were probed with anti-HA antibody to identify co-immunoprecipitated MALT1 mutants. The results clearly showed that only the mutant containing both Ig-like domains could interact with Bcl10 (Fig. 4B). Identification of the two Ig-like domains as the composite Bcl10-binding domain is compatible with the known role of the Ig-like domain as a protein recognition motif (30).

Next, we made use of previously published reports which together implicated a short 20-amino acid region in Bcl10 as a critical component of the Bcl10 NF-κB activation domain. Our laboratory had shown that a truncated Bcl10 protein consisting of only the N-terminal CARD (amino acids 1–103) plus the adjoining 16 amino acids of C-terminal sequence (amino acids 104–119) was active at inducing NF-κB (6). Others, however, had shown that the CARD alone was ineffective (9). These results suggested that the region of Bcl10 between amino acids 104 and 119 is critically important for NF-κB activation. We therefore targeted this region by selectively deleting 13 amino acids from this domain, residues 107–119 (Fig. 4C). When expressed in 293T cells, this mutant indeed showed almost a complete loss of function with regard to NF-κB activation (Fig. 4D). Importantly, there was also complete absence of functional synergy between the mutant and MALT1. Despite the lack of activity, however, Western blotting confirmed that the mutant was expressed at least as well as the wild-type counterpart (Fig. 4D).
Transfections were carried out either in the presence or absence of 0.5 mM and analyzed for both NF-κB binding studies. Asterisk antibodies. The in the legend to Fig. 2, and Western blots of both total lysates and immunoprecipitated proteins were carried out using anti-Myc and anti-HA antibodies. The asterisk represents a supershifted MALT1 band which was occasionally seen; it is unclear whether this indicates phosphorylation or some other modification of MALT1. C, schematic representation of wild-type and mutant Bcl10 proteins used in the following functional and binding studies. D, 2 × 10^6 293T cells were transfected with either 75 ng of pcDNA3-Bcl10-Myc or 225 ng of pcDNA3-Bcl10Δ107–119-Myc. Transfections were carried out either in the presence or absence of 0.5 μg of added pcDNA3-MALT1-Myc, as indicated. Cell lysates were prepared and analyzed for both NF-κB activation and transgene expression as described previously. E, 2 × 10^6 293T cells were transfected with 2 μg of pcDNA3-Bcl10-Myc, 6 μg of pcDNA3-Bcl10Δ107–119-Myc, or pcDNA3 control. As indicated, transfections were carried out in the presence of 134 μg of added pcDNA3-MALT1-(1–330)-HA. Myc-tagged Bcl10 proteins were immunoprecipitated and both total lysates and immunoprecipitated products were analyzed by Western blot (WB) as described above.

To determine whether the loss of function produced by deleting these 13 amino acids correlated with a loss of MALT1 binding, we again used the co-immunoprecipitation assay. The MALT1 mutant containing the Ig-like domains (MALT1-(1–330)), which shows full binding to Bcl10, was used for the analysis because this truncated form of the protein can be expressed at significantly higher levels than the wild-type MALT1, and is thereby more amenable for analysis in immunoprecipitation experiments. Again, Bcl10 was seen to efficiently bind to this MALT1 construct, while Bcl10Δ107–119, when expressed at similar levels, showed absolutely no interaction with MALT1 (Fig. 4E). These results demonstrate a correlation between the Bcl10 residues required for NF-κB activation and those required for binding to MALT1. In summary, the analysis suggests that the binding observed between Bcl10 and MALT1 may be mechanistically related to the functional synergy seen with the two proteins.

Bcl10 Mediates the Oligomerization and Activation of the MALT1 Caspase-like domain, a Step Which Is Sufficient to Activate NF-κB—To explore the mechanistic relationship between Bcl10 and MALT1, we hypothesized that MALT1 may represent the downstream factor in a Bcl10-MALT1 signaling complex. The C-terminal domain of MALT1 shows homology to the proteolytic domain of caspases, and includes the universally conserved cysteine-histidine catalytic diad (31). For caspases such as caspase-9, activation is mediated through oligomerization directed by upstream regulators (Apaf-1 in the case of caspase-9). If MALT1 behaves in an analogous manner, then it is possible that binding to Bcl10 serves to oligomerize and activate the caspase-like domain of MALT1. This activated domain may then function as the effector in the NF-κB signaling pathway, at a point upstream of the IKK complex. To test this hypothesis, we first analyzed the role of the caspase-like domain in the synergistic enhancement of NF-κB by the combination of Bcl10 and MALT1. Two mutants were used for the analysis. For the first, MALT1-(1–330), we removed the entire caspase-like domain. This deletion completely abolished enhancement of Bcl10 activity (Fig. 5), indicating that the caspase-like domain is a critical component of the NF-κB signaling pathway. For the second mutant, the conserved cysteine (Cys453), which is critical for catalytic activity of traditional caspases (31), was mutated to alanine (Fig. 5). Importantly, in six separate experiments, the C453A mutant showed only an approximate 40% reduction (p = 0.01) in the synergistic enhancement of NF-κB (Fig. 5). These results indicate that while the caspase-like domain is essential for Bcl10/MALT1 signaling, this domain may differ significantly in its function from that of traditional caspases; either this caspase-like domain possesses a proteolytic active site that does not depend as critically on the cysteine residue, or there may be some other function of this domain that is important for this NF-κB signaling pathway, but which does not involve catalytic activity. Indeed, others have now suggested that MALT1 does not behave as a traditional caspase in that it does not induce apoptosis and cannot cleave a range of known caspase substrates (32). Structural modeling suggests that the active site may in fact show specificity toward an uncharged residue, rather than aspartic acid, in the substrate P1 position (32). Because of these properties, it may be most accurate to classify MALT1 under a broader category of cysteine proteases, and not as a caspase per se. Nevertheless, for lack of a better term, we shall continue to refer to the protease domain of MALT1 as a caspase-like domain.

Having established a role for the MALT1 caspase-like domain in NF-κB activation, we next tested whether Bcl10 is capable of inducing the oligomerization of MALT1. To this end,
we expressed both HA- and Myc-tagged versions of MALT1 in 293T cells, either in the presence or absence of several different Flag-tagged proteins (Fig. 6A). Myc-tagged MALT1 was immunoprecipitated and Western blots were probed for the presence of co-immunoprecipitated HA-tagged MALT1. While no oligomerization was observed when the MALT1 proteins were expressed by themselves, the presence of Bcl10 resulted in strong MALT1 oligomerization (Fig. 6A). As negative controls, coexpression of either IKKα or Nod1 was ineffective at promoting MALT1 oligomerization. The Nod1 control was particularly relevant, as this protein contains a CARD, as does Bcl10, and has been shown to mediate a similar oligomerization of the signaling protein RICK (14).

If oligomerization of the MALT1 caspase-like domain results in its activation, and this activated domain represents the effector portion of the Bcl10-MALT1 signaling complex, then we would expect that artificial oligomerization of the isolated caspase-like domain, in the absence of Bcl10 or other MALT1 domains, may be sufficient to activate NF-κB. To test this notion, we constructed a chimeric expression plasmid, encoding the MALT1 caspase-like domain linked to three tandemly repeated FKBP dimerization domains which can be oligomerized by the cell-permeable artificial ligand, AP1510 (Fig. 6B) (20). Expression of the resulting fusion protein, MALT1-Casp-FKBPx3, resulted in ligand-dependent activation of NF-κB, while neither the caspase-like domain alone nor the FKBP domains showed any ligand-responsive activity (Fig. 6B). Taken together, the above results support a model whereby Bcl10 promotes the oligomerization of MALT1, thereby activating MALT1 caspase-like domains whose activity in some way stimulates the IKK complex and sets in motion the downstream steps leading to NF-κB activation.

The API2-MALT1 Fusion Protein Activates NF-κB—As shown above, MALT1 was unable to activate NF-κB on its own, in the absence of its oligomerization which is specifically mediated by co-expressed Bcl10 (Figs. 3E and 6A). However, the possibility remained that the API2-MALT1 fusion protein, created as a consequence of the t(11;18) translocation, might represent a gain-of-function mutant which is active at inducing NF-κB, independent of Bcl10-mediated oligomerization. Several different breakpoints have been identified for this translocation, producing several variants of the fusion protein, the principle difference between the variants being the presence or absence of the Ig-like domains of MALT1. In all cases, however, the caspase-like domain of MALT1 is preserved and linked to the three BIR domains present in the N terminus of API2 (Fig. 7A). To test the hypothesis that the API2-MALT1 chimera may display gain-of-function activity, we transfected 293T cells with an expression plasmid encoding the chimera depicted in Fig. 7A, and measured NF-κB activation. Indeed, this fusion protein was able to induce a potent NF-κB response (Fig. 7B). Furthermore, this response was completely dependent on an intact caspase-like domain, as deletion mutants which removed the region corresponding to the small subunit of the domain showed no significant activity. Western blots confirmed that these mutants were expressed at levels comparable to that of the full-length fusion protein.3 To test whether the API2-MALT1 protein utilizes the same downstream signaling pathway as Bcl10, we analyzed the effect of co-expressing dominant negative mutants of the principle IKK complex components. Similar to the results obtained for Bcl10, dominant negative mutants of IKKα, IKKβ, and IKKγ all blocked API2-MALT1-responsive NF-κB activation (Fig. 7C). As a control, dominant negative mutants of two unrelated kinases, Ask1 and DAPK, had no effect. Finally, we confirmed the essential role of IKKγ in API2-MALT1 signaling by testing NF-κB activation in the IKKγ-deficient cell line, 5R. Again, similar to Bcl10, API2-MALT1 was unable to activate NF-κB in these cells while in the parental Rat-1 cells, the NF-κB response was fully intact (Fig. 7D).

A Unifying Model for the Pathogenesis of MALT Lymphoma—The data presented herein suggest that Bcl10 serves to oligomerize and activate the MALT1 caspase-like domain. Whether the caspase-like domain of MALT1 is capable of cleaving an unknown substrate that is then able to activate the IKK complex through direct or indirect interaction with IKKγ is an open question which must be tested further. However, in our hands we have not seen a direct interaction between MALT1 and IKKγ,2 suggesting that at least one signaling step may separate MALT1 from the IKK complex. Importantly, the MALT1 caspase-like domain may differ significantly in its function from that of traditional caspases, as site-directed mutagenesis of the conserved catalytic cysteine had only a modest effect on NF-κB activation. Thus, the MALT1 caspase-like domain may serve more than one function, such that the catalytic activity of the domain might not be required for NF-κB activation. Alternatively, active catalysis may not rely as much on the conserved cysteine as it does for traditional cysteine proteases.

A second major chromosomal translocation that is seen in up to 50% of MALT lymphomas is the t(11;18) which creates a fusion gene encoding the chimeric API2-MALT1 protein. We have now shown that this chimeric protein can efficiently activate NF-κB. As described previously, several variants of the API2-MALT1 fusion gene have been characterized, but the only domain from MALT1 which is consistently preserved in all API2-MALT1 variants is the caspase-like domain (11–13). This is consistent with the data that implicates the MALT1 caspase-like domain as the effector arm of the Bcl10-MALT1 signaling complex. However, unlike wild-type MALT1 which appears to depend upon an interaction with Bcl10 as a mechanism for oligomerization and autoactivation, the API2-MALT1 fusion protein may possess a mechanism for self-oligomerization; the

3 N. Inohara and G. Núñez, unpublished results.
three BIR domains contributed by the API2 portion of the chimera could fulfill this role. These domains are thought to represent protein interaction domains (33, 34), and a recent report has shown oligomerization of BIR-containing proteins through direct BIR-BIR interactions (35). Alternatively, the API2-MALT1 protein might be oligomerized through BIR-dependent interactions with a second, ubiquitous cellular component. Thus, the API2-MALT1 fusion protein may possess a mechanism for efficient, Bcl10-independent autoactivation of the MALT1 caspase-like domain.

In addition to creating a chimeric protein which potentially possesses a mechanism for self-activation, fusion of the API2 and MALT1 genes may result in a second advantage with regard to NF-κB signaling. The API2 gene promoter is known to be NF-κB responsive, so that expression of the API2-MALT1 fusion along with 125 ng of either pcDNA3 or expression plasmids encoding the dominant negative mutants of IKKα, IKKβ, Ask1, or DAPK. 24 h post-transfection, extracts were prepared and NF-κB induction was measured as described in the legend to Fig. 1. D, the Rat-1 and 5R cell lines were transfected as described in the legend to Fig. 1. Ig, Ig-like domain.

In summary, we have provided evidence that two independent targets of chromosomal translocation in MALT lymphoma, Bcl10 and MALT1, bind to one another and cooperate in the activation of NF-κB. As NF-κB is known to direct the expression of several survival-related genes, it is now thought that unregulated activation of this pathway may contribute to malignant transformation and/or progression (37). As this article was completed, Uren et al. (32) published a report which complements the findings described herein. These authors used a yeast two-hybrid system to screen for binding partners of the MALT1 protease. Multiple positive clones were identified and found to encode Bcl10; the validity of this interaction was further tested by demonstrating an interaction between endogenous MALT1 and Bcl10 in a number of cell lines (32). Although the functional significance of the interaction was not explored in their study, the authors did demonstrate that two API2-MALT1 fusion proteins, slightly different from that used in our studies, could activate NF-κB. Taken together, the results of their study and ours support a unifying model for understanding how two distinct translocations may impact the

FIG. 6. Oligomerization of the MALT1 caspase-like domain is sufficient for NF-κB activation. A, 2 × 10⁶ 293T cells were transfected as indicated with 6 µg of pcDNA3-MALT1-(1–330)-HA or -Myc, and 3 µg of pcDNA3-Bcl10-Flag, 4 µg of pcDNA3-IKKα-Flag, or 4 µg of pcDNA3-Nod1-Flag. Myc-tagged MALT1-(1–330) was immunoprecipitated and both total lysates and immunoprecipitated products were analyzed by Western blot as described previously. B, 2 × 10⁶ 293T cells were transfected with 1000 ng of pcDNA3-MALT1-Caspase-FKBPx3-Myc, 500 ng of pcDNA3-MALT1-Caspase-Myc, or 250 ng of the parental pcDNA3-FKBPx3-Myc vector. 6 h post-transfection, cells were treated with the indicated concentrations of the AP1510 dimerizer for the next 18 h, after which lysates were prepared and analyzed for NF-κB activation. Western blotting confirmed that treatment with the drug had no effect on expression of any of the FKBP/MALT1 transgenes (see Footnote 3).

FIG. 7. The API2-MALT1 fusion protein activates NF-κB through the IKK complex, in a caspase domain dependent fashion. A, schematic representation of an API2-MALT1 fusion protein and related deletion mutants. B, 1 × 10⁶ 293T cells were transfected with 50 ng of expression vector encoding the full-length API2-MALT1 fusion protein or 125 ng of expression vector encoding the two deletion mutants depicted in panel A. 24 h post-transfection, extracts were prepared and NF-κB induction was measured as described in the legend to Fig. 1. C, 293T cells were transfected with 50 ng of expression vector encoding the full-length API2-MALT1 fusion protein along with 125 ng of either pcDNA3 or expression plasmids for the dominant negative mutants of IKKα, IKKβ, Ask1, or DAPK. 24 h post-transfection, extracts were prepared and NF-κB induction was measured as described in the legend to Fig. 1. D, the Ig-like domain.
same signal transduction pathway, and thereby contribute to the development of a single clinicopathologic entity, MALT lymphoma.

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Bcl10 and MALT1, Independent Targets of Chromosomal Translocation in MALT Lymphoma, Cooperate in a Novel NF-κB Signaling Pathway

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