Constitutive NF-κB activation by the t(11;18)(q21;q21) product in MALT lymphoma is linked to deregulated ubiquitin ligase activity

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Summary

Mucosa-associated lymphoid tissue (MALT) lymphoma is a common type of lymphoma in extranodal sites. The most frequent chromosome translocation associated with MALT lymphoma is t(11;18)(q21;q21), which generates a chimeric protein of c-IAP2 and MALT1/paracaspase. The c-IAP2/MALT1 fusion protein activates the NF-κB pathway, which is considered critical to malignant B cell transformation and lymphoma progression. The mechanism by which this fusion protein activates NF-κB, however, remains unclear. Here we show that self-oligomerization of the c-IAP2/MALT1 protein causes deregulated ubiquitin ligase activity of MALT1/paracaspase. The chimeric protein targets NEMO for polyubiquitination and thereby activates NF-κB. Consistent with this finding, NEMO ubiquitination is increased in t(11;18)(q21;q21)-positive MALT lymphoma samples. Thus, t(11;18)(q21;q21) deregulates MALT1/paracaspase ubiquitin ligase activity, causing constitutive NF-κB activation and promoting tumorigenesis.

Introduction

Tumor development and progression are frequently associated with genetic lesions that impact cell proliferation, cell survival, angiogenesis, invasion, and metastasis (Hanahan and Weinberg, 2000). Many types of leukemias and lymphomas exhibit chromosome abnormalities, such as translocations and nonrandom gain and loss of chromosomes. Chromosome translocations, in particular, have provided important clues to understanding the genetic basis of lymphomagenesis. Prominent examples include t(14;18)(q32;q21) in human follicular lymphoma, t(8;14)(q24;q32) in Burkitt's lymphoma, and the t(9;22) Philadelphia chromosome in chronic myelogenous leukemia (CML) (Rowley, 1998). These translocations are generally associated with specific lymphoma subtypes, suggesting that they are causal to disease. Cloning of genes at the translocation breakpoints has greatly increased our understanding of these oncogenic events. In human follicular lymphoma, the t(14;18) translocation juxtaposes the antiapoptotic *bcl-2* gene adjacent to the immunoglobulin heavy chain (*IGH*) gene, resulting in increased Bcl-2 expression (Rowley, 1998). In Burkitt's lymphoma, the t(8;14) translocation juxtaposes the c-*myc* gene adjacent to the *IGH* gene, causing upregulated c-Myc expression (Dalla-Favera et al., 1983). The Philadelphia chromosome in CML places the *Abelson* (*ABL*) tyrosine kinase gene under the regulation of the promoter for the breakpoint cluster region (*BCR*) gene (de Klein et al., 1982). An in-frame chimeric gene encodes a BCR/Abl fusion protein that is produced only in malignant cells. Consistent with a causal role for this translocation in CML, inhibition of the deregulated tyrosine kinase activity of the BCR/Abl oncoprotein by imatinib (also termed Gleevec) has proved effective in treating t(9;22)-positive CML (Druker et al., 2001).

MALT lymphoma is the most common extranodal non-Hodgkin's lymphoma, arising in sites such as the stomach, lung, and thyroid (Isaacson and Du, 2004). These lymphomas are commonly derived from a background of chronic inflammation or autoimmune disease. For example, gastric MALT lymphoma is often preceded by infection with *Helicobacter pylori* (*H. pylori*), while thyroid MALT lymphoma is usually associated with Hashimoto's thyroiditis. These observations suggest that persistent inflammation may be linked to cell transformation. t(11;18)(q21;q21) is the most frequent chromosomal aberration associated with MALT lymphoma (Ye et al., 2003). This translocation generates a chimeric transcript fusing the inhibitor of apoptosis-2 (*c-IAP2*) gene on chromosome 11 to the *MALT1* (*m*ucosa-*a*ssociated *l*ymphoid *t*issue lymphoma translocation gene 1) gene on chromosome 18. Intriguingly, the c-IAP2/

SIGNIFICANCE

Chromosomal translocations are frequently associated with leukemias and lymphomas. Fusion oncoproteins that arise from chimeric genes generated by such translocations often play critical roles in tumorigenesis. It is important, therefore, to understand the function of the deregulated fusion protein in order to develop therapeutic interventions. The t(11;18)(q21;q21) is the most frequent chromosomal abnormality identified in MALT lymphoma. This translocation generates the c-IAP2/MALT1 fusion protein, which constitutively activates NF-κB. Here we show that aberrant activation of NF-κB by the fusion protein stems from its deregulated ubiquitin ligase activity. Thus, t(11;18)(q21;q21) represents a chromosomal translocation that produces a gain-of-function oncogenic ubiquitin ligase. The understanding of this pathway will be helpful for developing and testing molecularly targeted therapies.

MALT1 fusion protein can potently activate NF-κB in overexpression studies, but wild-type c-IAP2 and MALT1 do not (Akagi et al., 1999; Dierlamm et al., 1999; Uren et al., 2000; Lucas et al., 2001). c-IAP2 belongs to a family of IAP proteins characterized by the presence of one to three *b*aculovirus *I*AP *r*epeat (BIR) domains. c-IAP2 has three BIR domains, one *c*aspase-*a*ssociated *r*ecruitment *d*omain (CARD), and one RING domain (Figure 1A). MALT1 contains a death domain (DD), two immunoglobulin-like domains (Ig-like), and a region with homology to caspases (Figure 1A). This latter feature led to the naming of MALT1 as human paracaspase. No protease activity has been demonstrated for MALT1/paracaspase; rather, it participates in NF-κB signaling downstream of the antigen receptors by promoting ubiquitination of NEMO (Zhou et al., 2004; Snipas et al., 2004). The fusion junctions between c-IAP2 and MALT1/paracaspase have been well characterized (Figure 1A). The c-IAP2/MALT1 fusion protein always comprises the N-terminal portion of c-IAP2 with three BIR domains and the C-terminal region of MALT1/paracaspase containing an intact caspase-like domain (Akagi et al., 1999; Dierlamm et al., 1999; Baens et al., 2000; Liu et al., 2000; Liu et al., 2001; Liu et al., 2002; Ye et al., 2003). Selection for these domains of c-IAP2 and MALT1/paracaspase to form a functional fusion product strongly suggests their importance and synergy in tumor development.

The NF-κB family of transcription factors plays a key role in inflammatory and innate immune responses (Li and Verma, 2002). In addition to its importance in immunity, persistent NF-κB activation has been linked to the development of inflammation-associated tumors (Karin et al., 2002; Greten et al., 2004). Indeed, many human leukemias and lymphomas show constitutive NF-κB activation. The viral oncoprotein v-Rel, a member of the NF-κB family, is sufficient for cell transformation in vitro and in vivo (Gilmore, 1999). Oncogenic viruses, such as HTLV-1 and Epstein-Barr virus, also activate NF-κB (Karin et al., 2002). Significantly, the ability of BCR/Abl to activate NF-κB was shown to be essential for its oncogenic activity (Reuther et al., 1998). NF-κB factors themselves are also deregulated in human tumors; many hematopoietic and solid tumors are associated with chromosomal aberrations involving the human c-*rel*, *rela*, *nfkb1*, and *nfkb2* genes (Karin et al., 2002).

Constitutive NF-κB activation by the c-IAP2/MALT1 fusion protein is considered critical to malignant B cell transformation and lymphoma progression in t(11;18)-positive MALT lymphoma. The cellular mechanism by which this fusion protein activates NF-κB, however, has been unclear. Our data show that the fusion protein self-oligomerizes and has deregulated ubiquitin ligase activity. One target for ubiquitination by the c-IAP2/MALT1 fusion protein is NEMO (also called IKKγ, NEMO is the regulatory subunit of the IKK complex that has the catalytic subunits IKKα and IKKβ), which leads to increased NF-κB activity. Consistent with this notion, NEMO ubiquitination was increased in t(11;18)-positive MALT lymphoma samples. Taken together, these results suggest that the c-IAP2/MALT1 fusion protein promotes lymphomagenesis through deregulated ubiquitin ligase activity.

Results

The c-IAP2/MALT1 fusion protein induces ubiquitination and NF-κB activation

To investigate the biochemical basis for constitutive NF-κB activation by the c-IAP2/MALT1 fusion protein, we tested whether

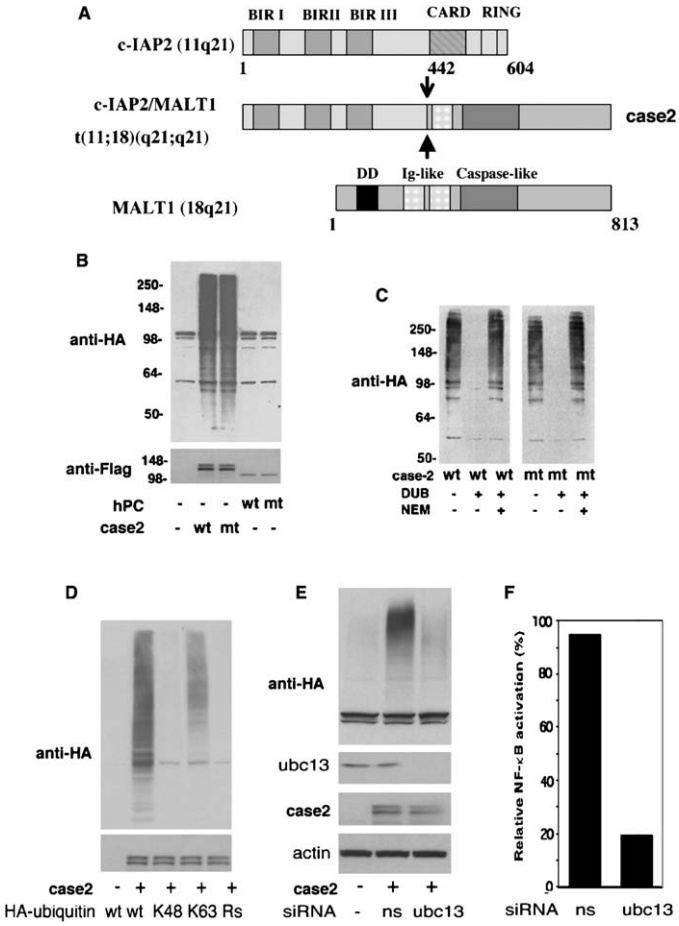

Figure 1. c-IAP2/MALT1 fusion protein induces ubiquitination

A: Schematic representation of c-IAP2, MALT1/paracaspase (hPC), and the cIAP2/MALT1 fusion protein (case2). BIR, baculoviral IAP repeat; CARD, caspase recruitment domain; RING, really interesting new gene domain; DD, death domain; Ig, immunoglobin-like domain.

B: Flag-case2, Flag-case2 mutant (C539A), Flag-hPC, or Flag-hPC mutant (C539A) were cotransfected with HA-ubiquitin into 293T cells as indicated. Total cell lysates were prepared and separated by SDS-PAGE. Total cellular protein ubiquitination was analyzed by HA immunoblotting (top). Case2 and hPC expression levels were confirmed by paracaspase immunoblotting (bottom).

C: Total cell lysates were incubated with or without USP2 in an in vitro deubiquitination (DUB) assay. NEM, N-ethylmaleimide, a deubiquitinating enzyme inhibitor.

D: Flag-case2 was transfected with HA-ubiquitin (wt), HA-ubiquitin (K48 only), HA-ubiquitin (K63 only), or HA-ubiquitin (all Rs) as indicated. K63- or K48-linked ubiquitination was determined by HA immunoblotting (top). Case2 expression was confirmed by paracaspase immunoblotting (bottom).

E: 293T cells pretreated with a control scramble siRNA (s) or siRNAs targeting Ubc13 (Ubc13) were transfected with Flag-case2 and HA-ubiquitin. Total cellular ubiquitination (top panel), Ubc13 protein expression (second panel), and case2 expression (third panel) was determined by immunoblotting with the antibodies indicated. Western blotting for actin is shown to demonstrate equal protein loading (bottom panel).

F: 293T cells were transfected with siRNAs, NF-κB reporter plasmids, and case2 as indicated. NF-κB activation under the indicated conditions was determined by NF-κB reporter assay.

the case2 form of the c-IAP2/MALT1 fusion protein (Figure 1A) regulates cellular protein ubiquitination. When 293T cells were cotransfected with case2 and hemagglutinin (HA)-tagged ubiquitin, case2 increased the overall level of ubiquitin conjugates in total cell extracts (Figure 1B). In contrast, wild-type MALT1/paracaspase or the C453A mutant of MALT1/paracaspase did not induce ubiquitination (Figure 1B). A case2 mutant that had the conserved cysteine residue in the caspase-like domain replaced with alanine induced total cellular ubiquitination similar to wild-type case2 (Figure 1B), so we conclude that the increased ubiquitination was not dependent on the hypothetical cysteine protease activity of MALT1/paracaspase. Case2 and the case2 mutant also stimulated equivalent NF-κB activation (data not shown). These results are consistent with recent findings that the MALT1/paracaspase C453A mutant enhances rather than decreases Bcl10-stimulated NF-κB activation (Lucas et al., 2001; Zhou et al., 2004; Sun et al., 2004). Thus, NF-κB activation by case2 or Bcl10 is not linked to any predicted cysteine protease activity of MALT1/paracaspase. Because case2 overexpression stimulates ubiquitination and activates NF-κB, whereas MALT1/paracaspase expression does neither, it is likely that these two events are mechanistically linked. We confirmed that the bands observed in Figure 1B were ubiquitin conjugates by incubating the cell lysates with the deubiquitinating enzyme Usp2. Purified Usp2 abrogated the bands, as revealed by anti-HA immunoblotting, indicating that ubiquitin was cleaved from ubiquitinated proteins. Usp2 failed to cleave the ubiquitin conjugates in the presence of NEM, an alkylating agent that inhibits deubiquitinating enzymes (Figure 1C).

Ubiquitination is a reversible posttranslational modification in which the C terminus of 76 amino acid ubiquitin is conjugated to a lysine residue in the target protein. The target protein can be modified by addition of either a single ubiquitin moiety or a polyubiquitin chain that has ubiquitins linked via specific lysine residues. The type of ubiquitin linkage often determines the fate of polyubiquitinated proteins. K48-linked polyubiquitination generally targets a protein for degradation in the proteasome, whereas K63-linked polyubiquitination often regulates protein function or protein-protein interactions (Pickart, 2001). For example, recent studies have indicated that assembly of signaling complexes required for activation of NF-κB involves K63-linked ubiquitination of proteins such as TRAF2, TRAF6, and NEMO (Burns and Martinon, 2004). To determine the predominant ubiquitin linkage induced by case2, 293T cells were cotransfected with case2 and either wild-type ubiquitin, ubiquitin that had all but one lysine (K48 or K63) mutated to arginine, or ubiquitin that had all of its seven lysines changed to arginine. Immunoblotting for the HA tag on the various ubiquitins revealed that case2 increased cellular ubiquitination only when K63 was available for conjugation (Figure 1D). K63-linked ubiquitination can be catalyzed, in part, by the ubiquitin-conjugating E2 enzyme Ubc13 in a heterodimeric complex with either Uev1a or Mms2 (Hofmann and Pickart, 1999; VanDemark et al., 2001). We tested whether Ubc13 is required for case2-induced ubiquitination and NF-κB activation by pretreating 293T cells with or without siRNAs targeting Ubc13. Knockdown of Ubc13 protein abrogated both case2-induced total cellular ubiquitination (Figure 1E) and NF-κB activation (Figure 1F). These data are consistent with the hypothesis that case2-induced ubiquitination is necessary for case2 to stimulate NF-κB activation.

The BIR I domain is essential for case2-induced NF-κB activation

To investigate the contribution of the BIR domains from c-IAP2 in case2-induced NF-κB activation, N-terminal deletion mutants of case2 were generated (Figure 2A). These mutants were transfected into 293T cells and tested for their ability to activate a NF-κB-dependent reporter gene. Case2 mutants lacking BIR I failed to activate NF-κB, whereas the case2 mutant that retained BIR I but lacked BIR II and BIR III stimulated considerable NF-κB activity (Figure 2B). These results indicate that the BIR I domain from c-IAP2 is both necessary and sufficient for NF-κB activation by the case2 fusion protein.

Previous studies have shown that Bcl10 promotes oligomerization of MALT1/paracaspase to activate NF-κB (Zhou et al., 2004; Sun et al., 2004). Because artificial crosslinking of the caspase-like domain of MALT1/paracaspase has been shown to activate NF-κB (Lucas et al., 2001; Zhou et al., 2004; Sun et al., 2004), we tested whether the BIR domains from c-IAP2, particularly BIR I, might oligomerize case2 and thereby activate NF-κB. Flag-tagged case2 and either HA-tagged case2 or HA-tagged case2 deletion mutants were cotransfected into 293T cells. HA-case2 and the HA-case2 mutant retaining BIR I coimmunoprecipitated with Flag-case2, whereas the HA-tagged case2 mutants lacking BIR I did not associate with Flag-case2 (Figure 2C). These results show that the BIR I domain can serve as an oligomerization motif and thus might regulate case2 function. Interestingly, the BIR domains of Op-IAP, another IAP family member, have also been shown to mediate Op-IAP oligomerization and regulate Op-IAP function (Hozak et al., 2000). The BIR I-only case2 mutant, but not those mutants with BIR I deleted, induced total cellular ubiquitination approaching that of full-length case2 (Figure 2D), indicating that BIR I-mediated oligomerization is essential for both ubiquitination and NF-κB activation by case2.

An Ig-like domain C-terminal to the caspase-like domain is required for NF-κB activation by case2

To investigate which domains from MALT1/paracaspase contribute to NF-κB activation by case2, C-terminal deletion mutants of case2 were generated (Figure 3A). Surprisingly, case2 del1 mutant, which has an intact caspase-like domain but lacks further C-terminal amino acids, could not activate expression of a NF-κB-dependent reporter gene (Figure 3B). Closer examination of the C terminus of MALT1/paracaspase revealed an Ig-like domain following the caspase-like domain. An additional 100 amino acids come after the Ig-like domain, but these do not resemble any known structural motif (Figure 3A). Retention of the Ig-like domain was found to be necessary for NF-κB activation, while the last 100 amino acids were dispensable (Figure 3B). These results indicate that the C-terminal Ig-like domain from MALT1/paracaspase is required for NF-κB activation by case2.

Recently, two consensus TRAF6 binding motifs were reported to be present in the C terminus of MALT1/paracaspase (Sun et al., 2004), and it was suggested that the Bcl10/MALT1/paracaspase complex engages TRAF6 to activate NF-κB. Both TRAF6 binding sites were deleted in the case2 del1 mutant, so we further investigated the contribution of these two sites to

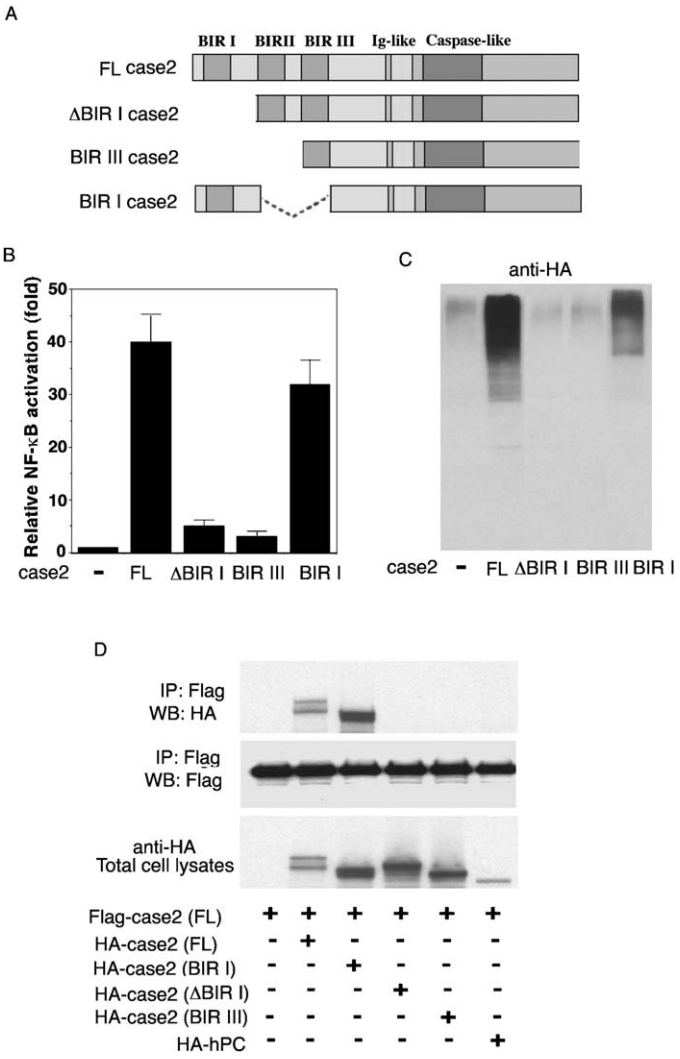

Figure 2. BIR I domain is essential for case2-induced NF-κB activation

A: Schematic representation of case2 and case2 deletional mutants. Full-length (FL) case2 was compared to deletion mutants lacking either the BIR I domain alone (ΔBIR I case2), both BIR I and BIR II (BIR III only case2), or BIR II and BIR III domains (BIR I only case2).

B: 293T cells were transfected with NF-κB reporter plasmids together with case2 or case2 mutants as indicated. NF-κB activation fold was determined by NF-κB reporter assay relative to the vector control. Data represent mean ± SEM of three independent experiments. ΔBIR I case2 failed to stimulate NF-κB activation, whereas BIR I only case2 still activated NF-κB.

C: Flag-case2 and either HA-case2 or HA-tagged case2 deletion mutants were transfected into 293T cells. Interactions between Flag-case2 and HA-tagged case2 deletion mutants were determined by immunoprecipitation with Flag monoclonal antibody followed by immunoblotting with antibody to HA.

D: HA-case2 or HA-case2 deletional mutants were transfected with Flag-ubiquitin into 293T cells. Ubiquitination was determined by immunoblotting for Flag.

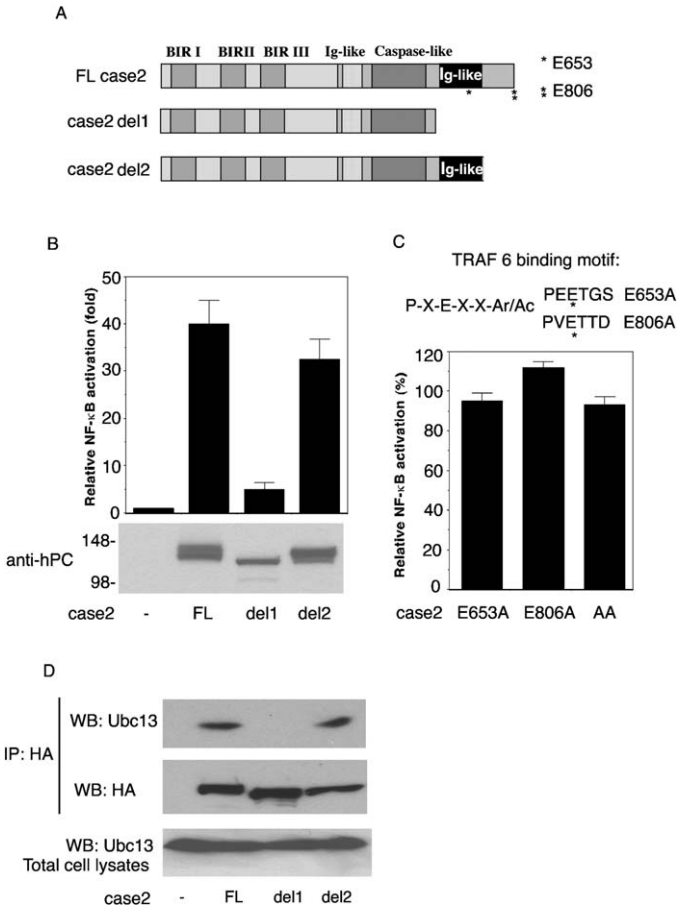

Figure 3. An Ig-like domain in the C terminus of MALT1/paracaspase is required for NF-κB activation by case2

A: Schematic representation of case2 and case2 deletional mutants. FL: full length case2; case2 /caspase-like domain; case2/caspase-like domain plus Ig-like domain.

B: 293T cells were cotransfected with NF-κB reporter plasmid and either case2 or case2 deletions as indicated. NF-κB fold activation was determined by NF-κB reporter assay relative to the vector control. Data represent mean ± SEM of three independent experiments.

C: 293T cells were transfected with NF-κB reporter plasmids together with case2, case2 (E653A), case2 (E806A), or case2 (E653A/E806A; AA) as indicated. Data are expressed relative to case2 control and represent the mean ± SEM of three independent experiments.

D: Ubc13 and HA-case2 or HA-tagged case2 deletion mutants were transfected into 293T cells. Association between Ubc13 and HA-tagged case2 deletion mutants was determined by immunoprecipitation with HA monoclonal antibody followed by immunoblotting with anti-Ubc13 antibody.

case2-induced NF-κB activation by generating full-length case2 point mutants E653A, E806A, and E653A/E806A (Figure 3C). All three case2 mutants activated NF-κB (Figure 3C), indicating that these potential TRAF6 binding sites may not be required for case2 to activate NF-κB.

Because Ubc13 is required for case2-induced NF-κB activation (Figure 1E), and because we have shown previously that the C-terminal half of MALT1/paracaspase catalyzes ubiquitination together with Ubc13/Mms2 (Zhou et al., 2004), we tested whether the C-terminal deletions of case2 impacted E2 binding. HA-case2 or HA-tagged case2 deletion mutants were cotransfected with Ubc13 into 293T cells. Ubc13 coimmunoprecipitated with full-length case2 and the case2 del2 mutant, which retains the Ig-like domain. No interaction was observed between Ubc13 and the case2 del1 mutant lacking the Ig-like domain (Figure 3D). The importance of this Ig-like domain in case2 for the recruitment of Ubc13 offers an explanation for

why deletion of this domain renders case2 unable to either activate NF-κB (Figure 3B) or increase total cellular ubiquitination (data not shown).

Case2 induces NEMO ubiquitination

To determine whether case2 activates NF-κB in a fashion similar to Bcl10 (Zhou et al., 2004; Sun et al., 2004), we tested whether case2 increased ubiquitination of cotransfected NEMO in 293T cells. Detection of ubiquitin conjugates was facilitated by transfection of HA-tagged ubiquitin. Case2 did induce NEMO ubiquitination, as determined by immunoblotting with NEMO and HA (Figure 4A). NEMO has a highly conserved lysine residue (K399) within its zinc finger, which, when mutated to arginine, markedly decreased NEMO ubiquitination in response to case2 (Figure 4A). The significance of polyubiquitination on K399 within NEMO to case2-induced NF-κB activation was assessed by transfecting case2 into NEMO-deficient 5R cells. Consistent with a previous study, case2 did not activate NF-κB in NEMO-deficient 5R cells (Lucas et al., 2001; Figure 4B). Significantly, 5R cells reconstituted with wild-type NEMO activated NF-κB in response to case2, but activation of NF-κB was much weaker when cells were reconstituted with the NEMO K399R mutant (Figure 4B). These results indicate that ubiquitination of NEMO on K399R is important for full NF-κB activation by case2. The contribution of ubiquitination to case2-induced NF-κB activation was confirmed using the deubiquitinating enzyme CYLD; case2-induced NF-κB activation in 293T cells was suppressed effectively by CYLD (Figure 4C), and this suppression correlated with a severe reduction in NEMO ubiquitination (Figure 4D).

NEMO ubiquitination is increased in t(11;18) MALT lymphoma tumor samples

Next, we examined the ubiquitination status of NEMO in t(11;18)-positive MALT lymphoma samples that transcribed c-IAP2/MALT1 fusion genes. c-IAP2/MALT1 fusion proteins could be detected by Western blotting in two t(11;18)-positive MALT lymphoma samples (G5661 and G6071), but, as expected, were not detected in human tonsil samples (Figure 4E). NEMO ubiquitination in these samples was examined by immunoprecipitation with NEMO antibody followed by immunoblotting with anti-ubiquitin antibody (Figure 4E). More polyubiquitinated NEMO was observed in the two MALT lymphoma samples than in the normal tonsil samples (Figure 4E), while total NEMO levels in the four samples were equivalent (Figure 4E). These data are consistent with the notion that the c-IAP2/MALT1 fusion protein drives NEMO ubiquitination in MALT lymphoma cells.

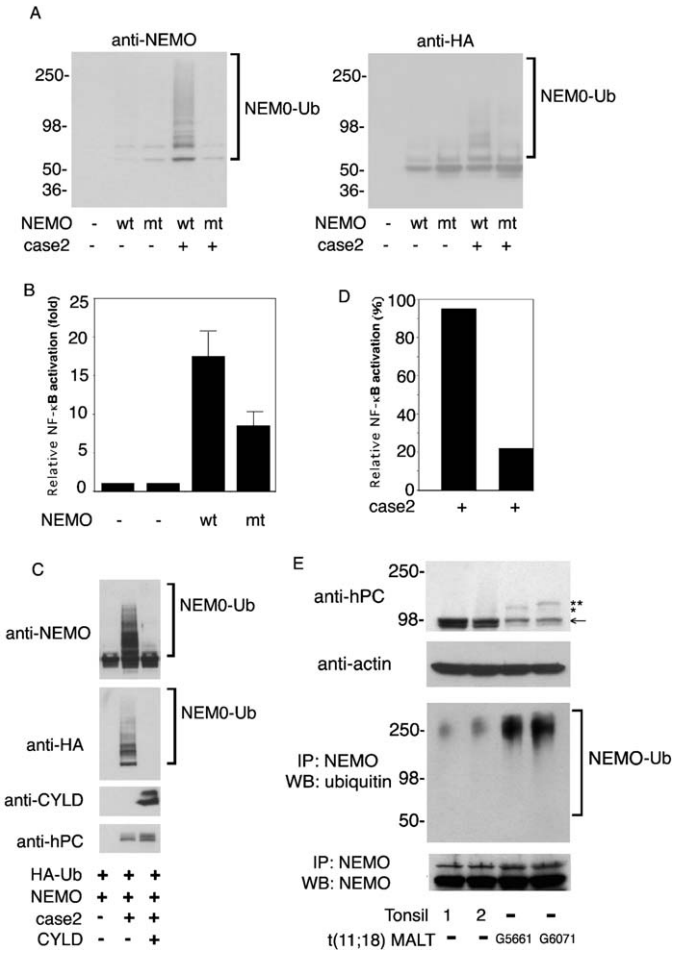

Figure 4. Case2 induces NEMO ubiquitination

A: HA-ubiquitin and Myc-case2, wild-type NEMO (wt), or NEMO mutant K399R (mt) were transfected into 293T cells. NEMO ubiquitination was determined by immunoblotting for NEMO (left) and HA (right).

B: NEMO-deficient 5R cells were cotransfected with case2 and NEMO (wt) or NEMO mutant K399R (mt). Case2-stimulated NF-κB activation was determined by NF-κB reporter assay. Data represent the mean ± SEM of three independent experiments.

C: A NF-κB reporter plasmid and case2 were transfected into 293T cells with or without CYLD. NF-κB activation was determined by NF-κB reporter assay.

D: HA-ubiquitin, NEMO, and case2 were transfected into 293T cells with or without CYLD. NEMO ubiquitination was detected by immunoblotting with anti-HA and anti-NEMO antibodies.

E: Expression of c-IAP2/MALT1 fusion proteins in two t(11;18)-positive MALT lymphoma samples (G5661, G6071) was determined by immunoblotting with anti-paracaspase antibody (top panel). Normal human tonsil samples were used as negative controls (Ton1, Ton2). Ubiquitination of NEMO, which was first immunoprecipitated from denatured lysates (Zhou et al., 2004), was determined by immunoblotting with anti-ubiquitin antibody as indicated.

Discussion

A causal relationship between inflammation and cancer has been suspected for many years, but the link between the two remains poorly understood. Chronic infection and inflammation might affect cells directly or indirectly to promote their malignant transformation. In gastric MALT lymphoma, the t(11;18) translocation is closely linked to infection with CagA-positive strains of *H. pylori* (Ye et al., 2003), which elicit significant inflammatory responses. It is tempting to speculate that the t(11;18) translocation occurs in premalignant lesions as a consequence of persistent B cell activation during chronic infection, and that expression of the c-IAP2/MALT1 fusion protein then drives progression to MALT lymphoma. Unfortunately, attempts to generate transgenic mice expressing the c-IAP2/MALT1 fusion protein in the B cell lineage have been unsuccessful (V.M.D., unpublished data), so it remains to be seen whether expression of the c-IAP2/MALT1 fusion protein alone promotes tumor development.

Two other rare, but recurrent, chromosomal translocations found in MALT lymphoma are t(1;14)(p22;q32) (Isaacson and Du, 2004) and t(14;18)(q32;q21) (Sanchez-Izquierdo et al., 2003; Streubel et al., 2003). t(1;14)(p22;q32) juxtaposes the *bcl10* gene to the immunoglobin (Ig) locus, resulting in upregulated Bcl10 expression. t(14;18)(q32;q21) places the *MALT1* gene under the control of the enhancer region of the *IGH* gene and elevates MALT1/paracaspase expression. Both Bcl10 and MALT1/paracaspase are required for antigen receptor-stimulated NF-κB activation and lymphocyte proliferation (Ruland et al., 2001, 2003; Ruefli-Brasse et al., 2003; Zhou et al., 2004). In fact, Bcl10 and MALT1 form a complex and synergize in the activation of NF-κB-dependent gene transcription (Lucas et al., 2001). Given that the c-IAP2/MALT1 fusion protein also activates the NF-κB pathway, it seems likely that all three distinct chromosomal translocations contribute to MALT lymphoma pathogenesis by allowing antigen-independent NF-κB activation to promote growth and expansion of the lymphoma clone.

Recently, it was suggested that MALT1/paracaspase recruits TRAF6 to activate NF-κB signaling (Sun et al., 2004). T cells from TRAF6-deficient mice, however, are reported to proliferate normally in response to crosslinking antibodies to CD3 and CD28 (Lomaga et al., 1999). By contrast, Bcl10- or MALT1/paracaspase-deficient T cells neither activate NF-κB nor proliferate upon TCR engagement (Ruland et al., 2001, 2003; Ruefli-Brasse et al., 2003). These observations argue against an essential role for TRAF6 downstream of Bcl10 and MALT1/paracaspase in antigen receptor-stimulated NF-κB activation. Mutation of the proposed TRAF6 binding sites in case2 did not attenuate case2-induced NF-κB activation (Figure 3C), nor could we detect the association of TRAF6 with case2 (Supplemental Figure S1). In addition, siRNA-mediated knockdown of TRAF6 did not inhibit case2-stimulated NF-κB activation (Supplemental Figure S2). These results suggest that the fusion protein engages NF-κB in a TRAF6-independent manner, although it cannot be ruled out that another member of the TRAF family might substitute for TRAF6.

Overexpressed case2, like Bcl10 (Zhou et al., 2004), induces ubiquitination of NEMO (Figure 4A), and this is required for full activation of NF-κB. It remains to be determined whether ubiquitination of NEMO contributes to IKK complex activation by recruiting an upstream activating kinase or by altering IKK complex conformation to cause autoactivation. The observation that a NEMO mutant defective in ubiquitination supports NF-κB activation, albeit to a lesser degree, indicates that additional molecules are likely involved. In a physiological setting, antigen receptor stimulation triggers assembly of the Bcl10-containing signaling complex, which oligomerizes paracaspase and promotes ubiquitin ligase activity (Zhou et al., 2004). Based on our current findings, we propose that the c-IAP2/MALT1 fusion protein generated by the t(11;18) translocation is constitutively active and bypasses the normal requirement for Bcl10-mediated oligomerization of MALT1/paracaspase. The observation that the c-IAP2/MALT1 fusion protein stimulates NF-κB activation in Bcl10-deficient cells would support this notion (Ruland et al., 2003). t(11;18) is often associated with a more aggressive clinical behavior and is used to predict the therapeutic response of gastric MALT lymphoma to *H. pylori* eradication. t(11;18)-negative MALT lymphoma often regresses in response to antibiotic therapy, while t(11;18)-positive MALT lymphoma is *H. pylori*-independent and does not respond to antibiotics, suggesting that c-IAP2/MALT1 activates NF-κB sufficiently to stimulate antigen-independent growth.

In summary, our study provides an example of a chromosomal translocation that contributes to a human malignancy by impacting the activity of an ubiquitin ligase. Given that the ubiquitin/proteasome (UPS) system is involved in multiple aspects of cell proliferation, differentiation, and survival, it is likely that UPS deregulation will emerge as a critical factor in tumorigenesis. Drugs such as the general proteasome inhibitor Velcade, which has been approved for multiple myeloma therapy, will hopefully exploit this possibility. For t(11;18)-positive MALT lymphoma, specific inhibition of c-IAP2/MALT1 ubiquitin ligase activity might be beneficial.

Experimental procedures

Cells, cell culture, antibodies, plasmids, and MALT lymphoma samples

Human kidney epithelial (HEK) 293T cells were grown in DMEM supplemented with 10% FBS at 37°C in the presence of 10% CO_2. All constructs for mammalian protein expression were generated in the pRK5B vector with a Flag- or HA-epitope tag placed at the NH2-terminal of the protein as indicated. The breakpoint in the cIAP2 gene from both G5661 and G6071 is nucleotide 2048. The breakpoint in the MALT1 gene in G5661 and G6071 is nucleotide 1123 and 814, respectively.

RNA interference

HEK 293T cells were transfected with double-stranded siRNAs using Lipofectamine 2000 (Invitrogen). 21-bp RNA oligonucleotides targeting Ubc13 were synthesized by Dharmacon as described previously (Zhou et al., 2004). 36 hr after adding siRNAs, cells were transfected with the indicated expression plasmids and cultured for an additional 36 hr before harvesting.

NF-κB reporter assay

293T and 5R cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen). 36 hr posttransfection, NF-κB luciferase activity was measured using the Promega Dual Luciferase Reporter Assay system (normalized to control luciferase activity) as previously described (Zhou et al., 2004).

Western blotting and immunoprecipitation

24 to 36 hr after transfection, cells were lysed in 1% NP-40 lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 2 mM DTT, 1% NP40, 1 mM EDTA) and immunoprecipitated with anti-FLAG M2 agarose (Sigma). The total cell lysates and immunoprecipitates were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies.

Supplemental data

Supplemental data for this article can be found at http://www.cancercell.org/cgi/content/full/7/5/425/DC1/.

Acknowledgments

We thank Kim Newton for critical reading and editorial assistance, Karen O'Rourke for excellent technical assistance, and members of the Dixit lab for helpful discussion.

Received: January 21, 2005
Revised: March 18, 2005
Accepted: April 13, 2005
Published: May 16, 2005

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