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Malt1 and cIAP2-Malt1 as effectors of NF- κ B activation: Kissing cousins or distant relatives?

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Abstract

Malt1 is a multi-domain cytosolic signaling molecule that was originally identified as the target of recurrent translocations in a large fraction of MALT lymphomas. The product of this translocation is a chimeric protein in which the N-terminus is contributed by the apoptosis inhibitor, cIAP2, and the C-terminus is contributed by Malt1. Early studies suggested that Malt1 is an essential intermediate in antigen receptor activation of NF- κ B, and that the juxtaposition of the cIAP2 N-terminus and the Malt1 C-terminus results in deregulation of Malt1 NF- κ B stimulatory activity. Initial experimental data further suggested that the molecular mechanisms of Malt1- and cIAP-Malt1-mediated NF- κ B activation were quite similar. However, a number of more recent studies of both Malt1 and cIAP2-Malt1 now reveal that these proteins influence NF- κ B activation by multiple distinct mechanisms, several of which are non-overlapping. Currently available data suggest a revised model in which cIAP2-Malt1 induces NF- κ B activation via a mechanism that depends equally on domains contributed by cIAP2 and Malt1, which confer spontaneous oligomerization activity, polyubiquitin binding, proteolytic activity, and association with and activation of TRAF2 and TRAF6 at several independent binding sites. By contrast, emerging data suggest that the wild-type Malt1 protein uniquely contributes to NF- κ B activation primarily through the control of two proteolytic cleavage mechanisms. Firstly, Malt1 directly cleaves and inactivates A20, a negative regulator of the antigen receptor-to-NF- κ B pathway. Secondly, Malt1 interacts with caspase-8, inducing caspase-8 cleavage of c-FLIP_L, initiating a pathway that contributes to activation of the I κ B kinase (IKK) complex. Furthermore, data suggest that Malt1 plays a more limited and focused role in antigen receptor activation of NF- κ B, serving to augment weak antigen signals and stimulate a defined subset of NF- κ B dependent responses. Thus, the potent activation of NF- κ B by cIAP2-Malt1 contrasts with the more subtle role of Malt1 in regulating specific NF- κ B responses downstream of antigen receptor ligation.

Keywords

Malt1; cIAP2-Malt1; NF- κ B; signal transduction; T cell; B cell; MALT lymphoma; Carma1; Bcl10; caspase-8; IKK; ubiquitination

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1. Introduction

Nearly 10 years have passed since Malt1 was first identified as a gene frequently involved in chromosomal translocations in MALT lymphomas³. The novel fusion protein produced by this translocation, cIAP2-Malt1, was shown to be a potent activator of NF- κ B. cIAP2-Malt1-mediated NF- κ B activation was attributed to spontaneous oligomerization of the chimeric protein, due to self-association of the N-terminal cIAP2-derived sequence. Data suggested that oligomerization activates the C-terminal Malt1-derived region, stimulating ubiquitin ligase activity that targets and activates the I κ B kinase (IKK) complex. Although the originally postulated mechanism of oligomerization-dependent NF- κ B activation by cIAP2-Malt1 has withstood the test of time, more recent data have shown that the molecular mechanism of activation of NF- κ B by cIAP2-Malt1 is more complex than initially appreciated.

Initial descriptions of the activity of the wild-type Malt1 protein were based in large part upon analogy with the mechanism of NF- κ B signaling by cIAP2-Malt1. Early models postulated that the wild-type Malt1 protein becomes oligomerized and ubiquitinates the IKK complex following antigen receptor engagement, and that this ubiquitination activity of Malt1 was both required for and uniquely responsible for antigen receptor activation of NF- κ B. However, closer examination of older data, combined with more recent experimental results, suggests that the role of the wild-type Malt1 in TCR- and BCR-mediated signal transduction differs from what was suggested by early data. In this review, we re-evaluate and integrate the current body of data regarding the mechanisms whereby Malt1 and the cIAP2-Malt1 fusion protein effect NF- κ B activation. The synthesis of available data strongly suggests that, while there are clearly some key similarities in mechanisms of IKK activation by each molecule, there are also important distinctions between how Malt1 and cIAP-Malt1 effect NF- κ B activation.

2. Malt1 and cIAP2-Malt1 proteins as activators of NF- κ B

2.1 Historical perspective

Malt1 was initially identified as a component of an oncogenic protein fusion commonly expressed in a subset of MALT lymphomas. Several reports identified a chromosomal translocation, t(11;18)(q21;21), which was found to involve the gene encoding the cIAP2 anti-apoptotic protein, and the novel MLT gene [1–3]. The resulting protein fusion product combined the C-terminal domain of the MLT protein (hereafter referred to as Malt1 [4]) with the N-terminus of the cIAP2 protein, a member of the cellular inhibitor of apoptosis protein family (Fig. 1). This protein fusion was found to potently activate NF- κ B, a transcription factor of central importance in many biological processes [5]. Initially, it was unclear how the wild-type Malt1 protein activated NF- κ B, as transfection of cells with full-length Malt1 alone did not cause measurable NF- κ B activation [5]. However, early studies revealed that Malt1 binds to the protein Bcl10 [6], and that over-expressed Bcl10 spontaneously oligomerizes in a manner dependent upon the Bcl10 caspase recruitment domain (CARD) [7]. Furthermore, induced

³Abbreviations used: MALT – mucosa associated lymphoid tissue; cIAP2 – cellular inhibitor of apoptosis 2; IKK complex – inhibitor of κ B kinase; TCR – T cell receptor; BCR – B cell receptor; Bcl10 – B cell lymphoma 10; CARD – caspase associated recruitment domain; BIR – baculovirus IAP repeats; CARMA – CARD containing MAGUK protein; CBM – Carma1/Bcl10/Malt1 complex; Ig – immunoglobulin; Ubc13 – ubiquitin conjugating enzyme 13; TRAF – TNF receptor associated factor; UBA – ubiquitin associated domain; LUBAC – linear ubiquitin chain assembly complex; TNF – tumor necrosis factor; I κ B α – inhibitor of κ B α protein; SCF^{I κ B} – Skp1, Cullins, F box proteins, targeting I κ B α ; BAFF – B cell activating factor; NIK – NF- κ B inducing kinase; HOIL-1L – haem-oxidized IRP2 ubiquitin ligase-1 long form; HOIP – HOIL-1L interacting protein; NFAT – nuclear factor of activated T cells; AP-1 – activator protein 1; PDK1 – phosphoinositide dependent kinase 1; PKC θ – protein kinase C θ ; PKC β – protein kinase C β ; MAGUK – membrane associated guanylate kinase; FRET – Förster resonance energy transfer; POLKADOTS – punctate and oligomeric killing and activating domains transducing signals; Uev1A – ubiquitin conjugating enzyme variant 1A; UBD – ubiquitin binding domain; UBAN – UBD in ABIN proteins and NEMO; TAK1 – transforming growth factor-beta activated kinase 1; TAB2 – TAK1 binding protein 2; TAB3 – TAK1 binding protein 3; PMA – phorbol 12-myristate 13-acetate; c-FLIP_L – c-FLICE inhibitory protein, long form; MEF – mouse embryonic fibroblast

oligomerization of the Malt1 C-terminal domain was shown to stimulate potent NF- κ B activation [6]. Additional data demonstrated that cIAP2-Malt1 exhibits spontaneous multimerization in a manner dependent on the N-terminal BIR domains, contributed by cIAP2 [8]. Together, these data suggested a model in which very similar mechanisms of NF- κ B activation are employed by cIAP2-Malt1 and wild-type Malt1: namely, spontaneous multimerization of cIAP2-Malt1, and TCR- and Bcl10-dependent multimerization of wild-type Malt1, stimulate NF- κ B activation [6].

Further experimental evidence suggested that Bcl10 and Malt1 cooperate with Carma3 [9] or Carma1 [10] to form a trimeric protein complex (the CBM), implicated in NF- κ B activation. Other data suggested that Malt1 multimerization is Bcl10-dependent, and that formation of Bcl10/Malt1 multimers is required for antigen-receptor mediated activation of NF- κ B [11, 12]. Mechanistically, the model of TCR- and BCR-induced multimerization of the Bcl10/Malt1 complex for transducing NF- κ B activating signals is both similar to and consistent with the model of NF- κ B activation by steady-state oligomerization of the cIAP2-Malt1 fusion protein [6,13]. However, given that the activity of the cIAP2-Malt1 is independent of any upstream activators, while the activity of the wild-type Malt1 protein is strictly dependent on upstream signaling through Bcl10, there is reason to speculate that the mechanisms by which these two molecules effect NF- κ B activation may differ significantly. Indeed, accumulating evidence suggests that Malt1 and cIAP2-Malt1 utilize quite distinct mechanisms to regulate NF- κ B signal transduction.

2.2 Functional domains of the Malt1 and cIAP2-Malt1 proteins

Malt1 is a member of a family of caspase-like cysteine proteases called paracaspases, which are found in organisms ranging from the slime mold, *Dictyostelium*, to man [5]. As shown in Figure 2A, several distinct functional domains have been identified in the Malt1 protein. The N-terminal region of the protein is involved in interaction with Bcl10, with both the Ig-like domains [6] and the death domain [14,15] contributing to this association. Downstream of the Bcl10-interacting region is the caspase-like “paracaspase” domain, which has homology to the caspase and metacaspase families of cysteine proteases, particularly in regions essential for proteolytic activity [5]. Although this caspase-like module was recognized shortly after the initial identification of the Malt1 gene, a proteolytic function was only recently documented [16,17]. The caspase-like domain of Malt1 (aa 326–567) is sufficient for binding to caspase-8, with Leu359 being essential for biochemical association between Malt1 and caspase-8 [18]. Malt1 has also been shown to interact with the coiled-coil domain of Carma1, via an undefined sub-domain within the C-terminal two-thirds of the molecule (aa 333–824) [10]. A binding site for A20, a negative modulator of NF- κ B signaling and a target for Malt1 proteolysis, is also within this same region of Malt1 [16]. C-terminal to the caspase-like domain is another Ig-like domain, which has been reported to bind to the ubiquitin conjugating enzyme, Ubc13 [8]. The C-terminal region of Malt1 contains two binding sites for TRAF6 and TRAF2, centered around Glu653 and Glu806 [12], although some data suggest that only the most C-terminal TRAF binding site is functional [15,19]. A third TRAF6 binding site was recently identified in the second Ig-like domain in the N-terminus of Malt1, encompassing Glu313 and Glu316 [15].

Although the cIAP2-Malt1 fusion lacks the N-terminal Bcl10-binding portion of the wild-type protein, it contains the caspase-like domain, the C-terminal Ig-like domain, and the three TRAF2/6 binding sites described above (Fig 2B). The presence of the Malt1 N-terminal Ig-like domains in the cIAP2-Malt1 fusion is variable, depending on the exact translocation breakpoint (see Fig. 1B). The cIAP2-contributed N-terminus of the fusion protein contains three BIR domains and one ubiquitin-associated (UBA) domain. Among the three BIR domains, only the first, BIR1, is essential for NF- κ B activation by cIAP2-Malt1 [8,20]. BIR1

has been shown to contain an additional TRAF2/TRAF6 binding site, as well as a region that interacts with the Malt1-contributed C-terminus in a heterotypic oligomerization interaction that contributes to NF- κ B activation [13,20]. An intact UBA is present in roughly 98% of cIAP2-Malt1 fusions, and a functional UBA is required for efficient cIAP2-Malt1-mediated NF- κ B activation, although the precise molecular mechanism has yet to be established [21]. The N-terminus of cIAP2-Malt1 (aa 1–441) has also been reported to interact with Bcl10, perhaps further enhancing NF- κ B activation by the cIAP2-Malt1 chimera [22].

The C-terminal domain of cIAP2-Malt1 has been shown to interact with Ubc13 and A20 [8, 16] via the same domains used by wild-type Malt1. Presumably, cIAP2-Malt1 can also interact with Carma1 and caspase-8, as the defined binding domains [10,18] are present in the fusion protein. However, there are currently no published data in which association between the cIAP2-Malt1 chimera and either Carma1 or caspase-8 was directly tested. Data have shown that cIAP2-Malt1 functions independently of upstream activators, suggesting that any association with Carma1 is unlikely to contribute to NF- κ B activation. A potential role for caspase-8 in NF- κ B activation by cIAP2-Malt1 seems more likely, as Malt1 acts upstream of caspase-8 in TCR activation of NF- κ B [18].

2.3 NF- κ B activation pathways

The activation of NF- κ B is a complex biological process, involving numerous independent signaling cascades that activate NF- κ B via a number of mechanistically distinct pathways. There are many complexities involved in the activation of NF- κ B, many of which remain to be fully understood. In order to understand how Malt1 and cIAP2-Malt1 contribute to NF- κ B activation, it is useful to first present an overview of general pathways of NF- κ B signal transduction.

NF- κ B is not a single entity, but rather a family of transcription factors that are capable of forming homo or heterodimers. The five members of this family are RelA (p65), RelB, cRel, p100/p52, and p105/p50. The term 'NF- κ B' is typically used to refer to the RelA-p50 heterodimer, which appears to be the most common pairing involved in gene activation. NF- κ B proteins are ubiquitously expressed, and NF- κ B heterodimers control the expression of genes involved in cellular proliferation, survival, inflammation, stress responses, and a number of other cellular processes. Until recently, there were two known mechanisms by which receptor ligation triggers NF- κ B activation, referred to as the canonical and non-canonical pathways. Emerging data now indicate the existence of a third NF- κ B activation mechanism, referred to as the linear ubiquitin chain assembly complex (LUBAC) pathway. Although each of these pathways converge on the IKK complex to effect activation of NF- κ B, the mechanisms leading to IKK activation are distinct in each case.

The canonical pathway of NF- κ B activation is triggered by the engagement of diverse receptors, including T and B cell receptors, the TNF receptor, IL-1R/Toll-like receptors, and a variety of other ligand-activated receptors (reviewed in [23,24]). While the receptor-proximal signaling events involved in canonical NF- κ B activation depend upon which receptor is engaged, the terminal events in the signaling cascade are well-conserved [23,24]. Under resting conditions, the NF- κ B heterodimer associates with the I κ B α protein. This interaction masks the nuclear localization domains of the NF- κ B heterodimer, retaining the complex in the cytoplasm. Upon activation, the IKK complex is activated. The IKK complex consists of 3 subunits: the regulatory IKK γ subunit, and the enzymatically active IKK α and IKK β subunits. During canonical signaling, the IKK β subunit phosphorylates the I κ B α protein on conserved serine residues, an event which triggers the SCF^{I κ B} E3 ubiquitin ligase-mediated poly-ubiquitination of the I κ B α protein on lysine residues. This modification targets the I κ B α protein for proteasomal degradation, freeing the NF- κ B heterodimer to enter the nucleus and control the expression of target genes [23,24].

The alternative NF- κ B pathway appears to be triggered by only a small subset of receptors, including CD40, the BAFF receptor, and the lymphotoxin- β receptor. These receptors activate the kinase NIK, which in turn phosphorylates and activates the IKK α subunit in an IKK γ -independent manner. IKK α then phosphorylates the NF- κ B precursor protein, p100, resulting in partial proteolytic processing of p100 to the mature p52 NF- κ B subunit. These events promote the nuclear translocation of p52/RelB NF- κ B heterodimers, which transactivate target genes [25].

The LUBAC-regulated NF- κ B pathway was recently identified as an important regulator of TNF α - and IL-1 β -mediated NF- κ B activation [26]. Investigators have so far demonstrated that a complex of two enzymes, HOIL-1L and HOIP, catalyze the addition of linear head-to-tail linked polyubiquitin chains to target molecules [27]. Recently, IKK γ was identified as a target of the LUBAC pathway, and linear polyubiquitination of IKK γ was shown to activate the IKK complex, leading to NF- κ B activation. Currently, there is no published information regarding how the TNF α and IL-1 β receptors transduce signals to the HOIL-1L/HOIP complex. Similarly, it is not known whether the LUBAC pathway may sometimes function as a standalone NF- κ B signaling cascade that is coupled to specific activators, or if it always functions as an enhancer or amplifier of NF- κ B activation that operates side-by-side with the canonical NF- κ B cascade, as appears to be the case with the TNF α and IL-1 β receptors [26]. Indeed, given the very similar mechanisms of signal transmission employed by the canonical and LUBAC pathways, it is possible that many receptor-triggered pathways of NF- κ B activation currently labeled as “canonical” may in fact be entirely or partially dependent on the LUBAC system.

2.4 Antigen receptor activation of the canonical NF- κ B cascade

In lymphocytes, T and B cell receptor engagement triggers the rapid tyrosine phosphorylation of a large array of receptor subunits, signaling adaptors, and kinases. These early tyrosine activation events initiate several distinct downstream signaling pathways that activate specific transcription factors, including NFAT, AP-1, and NF- κ B, which promote broad changes in gene expression and concomitant lymphocyte activation (reviewed in [28]). Precisely how these initial tyrosine phosphorylation events are connected to the activation of NF- κ B is not fully understood.

Better characterized are several different serine/threonine phosphorylations and lysine polyubiquitinations, for which there is strong evidence of a mechanistic connection to antigen receptor activation of NF- κ B. The modification of proteins by polyubiquitin plays an essential role in the transduction of signals that ultimately lead to NF- κ B activation (reviewed in [29, 30]). There are several distinct forms of polyubiquitin chains, with distinct biological properties. K63-linked polyubiquitin consists of individual ubiquitin monomers concatemerized via the lysine at position 63. This linkage results in a relatively linear polyubiquitin conformation, and generally contributes to a change in the function of the modified protein. In contrast, concatemerization of ubiquitin monomers via lysine 48 results in a more globular structure, and marks proteins for proteasomal degradation (reviewed in [31–33]). For example, in the canonical NF- κ B cascade, the proteasomal degradation of I κ B α is triggered by K48 polyubiquitination of I κ B α (reviewed in [30]) (Fig. 3).

Currently, the earliest known event in a dedicated pathway of antigen receptor-mediated NF- κ B activation is the auto-phosphorylation of phosphoinositide-dependent kinase 1 (PDK1) (Fig. 3). Recent studies have shown that the coordinated engagement of the TCR and the T cell co-stimulatory receptor, CD28, leads to the PI-3-kinase-dependent auto-phosphorylation of PDK1 on Thr513. In T cells, phosphorylated PDK1 binds PKC θ , activating this kinase via phosphorylation of Thr538 [34,35]. Presumably, an analogous pathway in B cells results in phosphorylation and activation of PKC β , but this has yet to be demonstrated experimentally.

Next, the MAGUK protein, Carma1, is phosphorylated at numerous sites in the “linker” region by either PKC β in B cells or PKC θ in T cells [36,37]. Data are consistent with a model in which this phosphorylation event triggers a conformational change in Carma1, allowing the Carma1 caspase recruitment domain (CARD) to interact with the CARD of Bcl10. Because Bcl10 and Malt1 are constitutively associated in the cytoplasm [38], the Carma1-Bcl10 interaction also brings Malt1 into the complex, thereby forming the CBM complex (Fig. 3) [36,37]. Data suggest that the CBM is further stabilized by a direct interaction between the coiled coil domain of Carma1 and the C-terminal portion of Malt1 [10] (Fig. 2). Analysis of Carma1^{-/-} [39–42], Bcl10^{-/-} [43,44], and Malt1^{-/-} [45,46] mice showed very similar defects in lymphocyte proliferation and function, further supporting the hypothesis that these molecules interact and transduce antigen signals to NF- κ B.

Coincident with or downstream of formation of the CBM complex, a proportion of cellular Bcl10 and Malt1 becomes oligomerized, as demonstrated by biochemical methodology [12] and FRET microscopy [11], and it is these oligomeric species that are active in NF- κ B signal transduction [12]. Microscopic examination of activated T cells has shown that oligomerized Bcl10 and Malt1 are found preferentially in punctate cytosolic structures called POLKADOTS, which may be vesicular in nature [11,47]. FRET analyses further demonstrated that POLKADOTS are enriched in Bcl10 in close association with Malt1, as well as other signaling partners, including Carma1 (Fig 3). Because POLKADOTS are only formed by signaling-competent forms of Bcl10 and Malt1, it is likely that they are intermediates in and/or immediate by-products of signal transmission to NF- κ B [11].

The active/oligomeric forms of Bcl10 and Malt1 are also associated with specific ubiquitin ligases, particularly TRAF6 [11,12] (Fig. 3). TRAF6 is an ubiquitin ligase that adds K63-linked polyubiquitin chains to target molecules [48]. The ubiquitin conjugating enzyme, Ubc13, and its catalytically inactive cofactor, Uev1A, are also involved in TRAF6-mediated ubiquitination events stimulated by the CBM [12,49,50]. The C-terminal Malt1 Ig-like domain (Fig. 2) and/or the TRAF6 RING domain may serve as essential docking sites for Ubc13 [8,51].

TRAF6 is activated by oligomerization [52], which stimulates auto-ubiquitination on Lys 124. Auto-ubiquitination increases the catalytic activity of TRAF6, enabling TRAF6-mediated K63-polyubiquitination of specific protein targets [53]. Data suggest that activated TRAF6 polyubiquitinates Malt1 on multiple C-terminal lysines (Fig. 2) and Bcl10 on Lys31 and Lys63 [54,55]. Mutation of all C-terminal lysines in Malt1 reduced NF- κ B activation and IL-2 production, suggesting that Malt1 polyubiquitination is mechanistically involved in TCR activation of NF- κ B [54]. Interestingly, K63-polyubiquitination of Bcl10 is substantially enhanced by the presence of Malt1 [55], suggesting that Malt1-associated TRAF6 may contribute substantially to K63-polyubiquitin modification of Bcl10 in the CBM complex.

Data from one group suggests that K63-polyubiquitination of Bcl10 is essential for CBM association with the IKK complex and for consequent activation of NF- κ B [55], while another group reports an inability to detect K63-polyubiquitination of CBM-associated Bcl10 [56]. Overall, data are consistent with a model proposing that IKK γ is able to bind to K63-polyubiquitin chains on both Bcl10 and Malt1, and that blockade of either of these interactions impairs NF- κ B activation, by preventing efficient physical association between the CBM and IKK complexes. However, it is also formally possible that ubiquitin modification of Bcl10 and/or Malt1 affects the function of one or both proteins in other as yet unidentified ways that are crucial for maximal activation of the IKK complex and do not contribute directly to physical association with the CBM.

The IKK complex appears to be recruited to the CBM complex through a recently identified ubiquitin binding domain (UBD) in the IKK γ protein. Data suggest that this specialized UBD,

called the UBA1 (UBD in ABIN proteins and NEMO), facilitates association between the IKK complex and the K63-polyubiquitinated CBM complex [55,57,58]. The association of the CBM and IKK complexes then allows TRAF6 (in cooperation with Ubc13/Uev1A) to add K63-polyubiquitin chains to the C-terminal Zn-finger domain of IKK γ at Lys399 and possibly additional lysines [12,49,59]. Biochemical studies suggest that K63-polyubiquitination of IKK γ enables physical association with the TAK1 kinase/TAB2/TAB3 complex, via the UBD of TAB2. This association allows TAK1 to phosphorylate and activate the IKK β catalytic subunit [12,60] (Fig 3). However, a more recent T cell study has provided compelling evidence that the phosphorylation of IKK β is independent of CBM-mediated signaling and IKK γ ubiquitination, suggesting that the mechanism of TAK1 association with the IKK complex remains incompletely defined [61]. Regardless of the details by which the IKK complex is modified, data from both groups strongly suggest that the combination of IKK γ ubiquitination and IKK β phosphorylation is required for activation of IKK β kinase activity. [12,60,61]. IKK β then phosphorylates I κ B α , triggering the terminal activation events of the canonical NF- κ B activation cascade, as described above (reviewed in [30]) (Fig. 3).

2.5 Challenges to the current model of antigen receptor-stimulated NF- κ B activation

Although the above model (or close variants of this model) of antigen receptor-mediated NF- κ B activation is generally accepted, there are various experimental observations that appear to be at odds with the currently accepted paradigm. Firstly, in conditional knockouts of Ubc13, which specifically inactivated K63-polyubiquitination pathways in B or T lymphocytes, ligand-mediated activation of NF- κ B was diminished, but not abolished [62,63]. These data suggest that the LUBAC pathway (or a distinct K63-polyubiquitin-independent pathway) may be able to partially compensate for the absence of K63-polyubiquitination activity [26,64]. Additionally, another recent report has shown that murine T cells, particularly CD8⁺ T cells, deficient in PKC θ , Bcl10, or Malt1 still successfully activate NF- κ B and NF- κ B dependent functions in response to modest or strong stimulation through the TCR. These observations argue that there is an NF- κ B activation pathway that is independent of PKC θ and the CBM complex [65], which again suggests that the LUBAC pathway may be an important component of antigen receptor signaling to NF- κ B. Characterization of antigen receptor signaling to NF- κ B in HOIL-1^{-/-} mice [26] may help establish the relative importance of these distinct polyubiquitin-dependent pathways in TCR activation of NF- κ B.

2.6 Spontaneous activation of NF- κ B by the cIAP2-Malt1 fusion protein

Three recurrent translocations have been identified in Malt lymphoma tumors: The t(11;18)(q21;q21) cIAP2-Malt1 translocation, which is the most common; less common are the t(1;14)(p22;q32) and t(14;18)(q32;q21) translocations, which place the Ig heavy chain enhancer upstream of the Bcl10 and Malt1 genes, respectively, causing de-regulated expression of each protein (reviewed in [66]). Patients with the cIAP2-Malt1 translocation have a poorer clinical prognosis than patients harboring other translocations [67–73]. These clinical data may reflect stronger NF- κ B activation induced by expression of cIAP2-Malt1 fusions in comparison to over-expression of either Bcl10 or Malt1.

Indeed, initial studies of Malt1 demonstrated that the wild-type protein has no independent ability to activate NF- κ B and shows only modest enhancement of Bcl10-mediated NF- κ B activation, despite direct physical association with Bcl10 [5,6]. In contrast, over-expression of either of the other two members of the CBM complex, CARMA1 and Bcl10, results in potent NF- κ B activation [6,74–77]. The weak activation of NF- κ B by Malt1 is even more perplexing given data showing that both the cIAP2-Malt1 fusion and an artificially oligomerized Malt1 C-terminus show robust NF- κ B activation [5,6]. To begin to explain these apparently conflicting observations, it is perhaps most useful to first ask why the expression of the cIAP2-Malt1 fusion protein results in dramatic induction of NF- κ B activation independent of external

activation signals, while over-expression of the wild-type protein seems only capable of modestly enhancing NF- κ B activation by Bcl10.

There are several plausible mechanisms that may account for the behavior of the cIAP2-Malt1 protein fusion. Recent data have shown that cIAP2-Malt1 oligomerizes in a manner dependent on the BIR1 domain [8], with the BIR1 domain of one protein heterotypically interacting with an undefined site in the Malt1-contributed C-terminal region of another fusion protein [13] (Figs. 2 and 4). Consistent with early data demonstrating that forced oligomerization of the C-terminal portion of Malt1 (including the caspase-like domain and all downstream sequences) can trigger NF- κ B activation [6], spontaneous cIAP2-Malt1 oligomerization makes a measurable contribution to NF- κ B activation [13].

However, recent data indicate that binding to TRAF2 and/or TRAF6 is also essential for NF- κ B activation by the cIAP2-Malt1 molecule. A novel TRAF2 binding site was recently identified within BIR1, in a subsequence distinct from the region required for cIAP2-Malt1 multimerization (Fig. 2). Mutation of this binding site or over-expression of a TRAF2 null mutant protein resulted in a striking decrease in cIAP2-Malt1-dependent NF- κ B activation [13,20]. Interestingly, fusion protein mutants lacking the BIR1 TRAF binding site but retaining the capacity to oligomerize showed a considerable defect in NF- κ B activation [13,20]. Similarly, mutation of the TRAF6 binding sites in the Malt1-derived C-terminal region also impaired cIAP2-Malt1-mediated NF- κ B activation [15]. These data suggest that both the N-terminal and C-terminal TRAF binding sites are required for cIAP2-Malt1-mediated NF- κ B activation. While these data do not conclusively demonstrate TRAF2/TRAF6 activation as a consequence of interaction with the cIAP2-Malt1 fusion protein, they suggest that the ability of the fusion protein to activate NF- κ B is primarily dependent on binding to TRAF2/TRAF6 and triggering downstream signaling events. The aggregate data therefore suggest that the oligomerization of the Malt1 C-terminal domain and multiple TRAF2/TRAF6 binding sites are critical determinants of robust NF- κ B activation by the cIAP2-Malt1 chimeric protein (Fig. 4).

In addition to binding TRAF2/TRAF6, evidence suggests that the cIAP2-Malt1 protein has further activities that can augment signals to NF- κ B. For example, some data suggest that cIAP2-Malt1 itself possesses ubiquitin ligase activity that is capable of K63-polyubiquitinating IKK γ and triggering NF- κ B activation [8]. Also, recent data indicate that the cIAP2-Malt1 fusion protein binds to K63-polyubiquitinated IKK γ via the cIAP2-contributed UBA domain [21] (Fig. 2). It is possible that this interaction protects the IKK γ protein from de-ubiquitination, thereby prolonging activation of the IKK complex. Another possibility is that the UBA-IKK γ interaction stabilizes the interaction between cIAP-Malt1 and the IKK complex, increasing the efficiency of IKK γ K63-polyubiquitination by cIAP-Malt1-associated TRAF2 and TRAF6. Additionally, since the cIAP2-Malt1 UBA is capable of binding both K63-polyubiquitin and linear (head-to-tail) polyubiquitin [21,78], an intriguing possibility is that cIAP2-Malt1 mediates IKK activation in part via interaction with the LUBAC pathway, binding to IKK γ that has been modified by linear polyubiquitin chains.

There is also evidence that cIAP2-Malt1 mediates proteolytic cleavage of A20, a negative modulator of NF- κ B signaling. A20 binds to an unknown sequence within the Malt1-contributed C-terminus of the chimera, and proteolysis of A20 is mediated by the caspase-like domain (Figs. 2 and 4) [16]. A point mutation that disrupts this proteolytic activity decreases cIAP2-Malt1 NF- κ B activation by approximately 3-fold [5]. Thus, the proteolytic activity of cIAP2-Malt1 appears to augment NF- κ B activation by interfering with normal homeostatic mechanisms that serve to limit NF- κ B activation. These data are discussed in more detail in section 3, below.

Finally, Bcl10 has been reported to interact with the cIAP2-Malt1 protein fusion and synergistically enhance NF- κ B activation in the absence of appropriate stimuli [22]. In this study, data suggested that Bcl10 interacts with a subfragment of cIAP2 (1–441), which contains the three BIR domains and the UBA domain [21] (Fig. 2). It is unclear at this time precisely which amino acids of this subregion encode the putative Bcl10 binding site. Bcl10 interaction with the fusion protein may enhance NF- κ B activation, as cIAP2-Malt1 expression in the absence of Bcl10 resulted in a 4-fold decrease in NF- κ B activation [22]. Since Bcl10 is thought to act upstream of Malt1 in receptor-mediated signaling to NF- κ B, it is unclear how Bcl10 might enhance the ability of the fusion protein to activate NF- κ B. It is possible that the Bcl10-fusion protein interaction stabilizes the fusion protein and prevents its degradation, or that Bcl10 prolongs the interactions of the fusion protein with downstream mediators such as TRAF2/TRAF6, thereby extending signaling to NF- κ B. However, it is also important to note that at least one report disputes both the physical association of Bcl10 with cIAP2-Malt1 and the enhancement of cIAP2-Malt1-mediated NF- κ B activation by Bcl10 [15], underscoring the need for further studies in this area.

Taken together, the above data strongly suggest that IKK activation by cIAP2-Malt1 is based on cooperative activities resident in both the cIAP2- and Malt1-contributed regions of the chimeric protein. Additionally, these observations suggest a plausible model of cIAP2-Malt1 activation of NF- κ B (Fig. 4). It is likely that the constitutive oligomerization of the cIAP2-Malt1 fusion protein contributes to its ability to interact with and oligomerize downstream ubiquitin ligases, such as TRAF2/TRAF6. As in the canonical pathway of NF- κ B activation, oligomerization of TRAF2/TRAF6 activates the ubiquitin-ligase function of these proteins. In combination with the ubiquitin conjugating enzyme complex, Ubc13/Uev1A, TRAF2 and TRAF6 trigger the K63-polyubiquitination of the cIAP2-Malt1 fusion and the K63-autoubiquitination of the TRAFs, themselves. The polyubiquitinated cIAP-Malt1 complex is then bound by the IKK γ subunit of the IKK complex, via the UBAN of IKK γ [57,58]. This molecular association then allows the TRAFs associated with cIAP2-Malt1 to K63-ubiquitinate IKK γ , recruiting the TAK1/TAB2/TAB3 complex [12], and triggering the terminal events in the canonical NF- κ B activation cascade. Presumably, steady-state oligomerization of the cIAP2-Malt1 fusion protein, perhaps in conjunction with protection of polyubiquitin chains on IKK γ by the cIAP2-Malt1 UBA domain and cleavage of A20 by the caspase-like domain, prevents the termination of IKK activation.

Thus, the chromosomal translocation that generates the cIAP2-Malt1 fusion protein may most accurately be described as a ‘gain of function’ mutation [6,13], with the resulting combination of protein domains creating novel functions that neither individual full-length protein possesses. More specifically, the combination of essential functional domains from the N-terminus of cIAP2 and the C-terminus of Malt1 generates a chimeric protein that constitutively signals to IKK and NF- κ B, and is apparently refractory to the signal termination mechanisms that normally confine NF- κ B activation to a temporally limited period.

3. A more complex picture of the function of Malt1

3.1 Malt1 as a protease

Although Malt1 contains a C-terminal caspase-like domain [5], Malt1 was initially not thought to function as a protease, due to the lack of any known substrate [79]. Recently, however, Malt1 was found to modify two proteins via a proteolytic process. One target of Malt1 proteolytic activity is Bcl10, which is cleaved at a C-terminal arginine residue (Arg228) following T cell receptor ligation [17] (Fig. 5). This cleavage was found to be dependent on the catalytic cysteine at position 464 of the Malt1 caspase-like domain (Fig. 2), as deletion of this domain or amino acid substitution (C464A) abrogated Bcl10 cleavage. Additionally, target cleavage was reduced in a dose-dependent manner by z-VRPR-fmk, a peptide-based inhibitor designed to

specifically target the caspase-like domain of Malt1. Interestingly, proteolytic processing of Bcl10 is apparently not required for NF- κ B activation, but rather is necessary for T cell interaction with fibronectin and regulation of T cell adhesion [17,80]. In this same report [17], Malt1 proteolytic activity was shown to contribute to NF- κ B activation by an unknown mechanism, in agreement with earlier studies [6].

A mechanistic connection between Malt1 caspase-like activity and NF- κ B activation was demonstrated by the discovery that Malt1 cleaves A20, an inhibitor of NF- κ B (Fig. 5) [16]. A20 possesses both de-ubiquitinating and ubiquitin ligase functions, acting to inhibit signaling to NF- κ B by binding to K63-polyubiquitinated proteins such as TRAF6 and subsequently removing the ubiquitin chains. In addition, A20 mediates the K48-ubiquitination of proteins, targeting them for proteasomal destruction [81,82]. These activities of A20 result in the termination of the activating signal, thereby down-modulating NF- κ B activation.

Consistent with these data, over-expression of increasing concentrations of A20 resulted in a decrease in Bcl10-mediated NF- κ B activation [16], and siRNA-mediated knock-down of A20 resulted in prolonged NF- κ B activation in response to CD3/CD28 ligation or PMA/ionomycin stimulation [56]. Malt1 was found to cleave human A20 at Arg439, generating two protein fragments which, when expressed either individually or together, did not inhibit NF- κ B activation, suggesting that cleaved A20 is unable to inhibit NF- κ B activation. Interestingly, the human A20 cleavage site is not conserved in murine A20. Although murine A20 was also found to be cleaved by Malt1, the cleavage site was distinct from human A20, yielding a larger N-terminal fragment. Definitive identification of the murine A20 cleavage site will be of importance, facilitating the more precise characterization of the sequence specificity of the Malt1 caspase-like domain.

Overall, the above data suggest that the interaction of Malt1 with Bcl10 and Carma1 positions Malt1 to act on its two identified protein substrates, Bcl10 and A20. Malt1 and Bcl10 directly interact, making Bcl10 readily available for proteolytic cleavage. Additionally, the K63-polyubiquitination of TRAF6 and the Malt1 C-terminus creates substrates for A20 cleavage and recruits A20 to the CBM complex [16,56]. Following association with the CBM, A20 is either cleaved by Malt1 (Fig. 5A) [16] or A20 specifically removes K63-polyubiquitin chains from TRAF6, Malt1, and presumably other K63-polyubiquitinated proteins in the CBM complex, such as Bcl10 (Fig. 5B) [56,81,82]. In this model, the K63-polyubiquitination of Malt1 plays a non-essential role in the recruitment of the IKK complex and concomitant NF- κ B activation, due to redundancy with other K63 polyubiquitination sites within the CBM complex and associated proteins (e.g., Bcl10, TRAF6).

To date, there is not a clear mechanism that explains the selective cleavage of A20 and Bcl10 by Malt1. Interestingly, studies suggest that Bcl10 [16] or Bcl10 and Carma1 [17] are required to induce the proteolytic function of Malt1. Combined with the observation that Malt1 proteolytic activity is activated by TCR stimulation [16], these data suggest that Malt1 proteolytic activity requires the participation of upstream signaling intermediates. Furthermore, Malt1 proteolytic activity may be confined to activated (and perhaps oligomeric) CBM complexes within the cell, reverting to an inactive form upon signal termination. Future studies will be required to determine how upstream activators, other interaction partners, and protein oligomerization contribute to activation of the Malt1 caspase-like domain.

3.2 Malt1 as an activator of caspase-8

Several studies have documented a physical and functional association between the CBM complex and caspase-8, with a recent report defining the Malt1 caspase-like domain as the site of caspase-8 interaction. The initial link between caspase-8 and Malt1 was provided by a study demonstrating that caspase-8 is required for NF- κ B activation from antigen receptors and

several additional receptor types. In this report, caspase-8 was also shown to facilitate interaction between the Bcl10-Malt1 complex and the IKK complex by an unidentified mechanism [38] (Fig. 6A). Subsequent data showed that caspase-8 [83], via association with TRAF6 [84], mediates the redistribution of the Bcl10-Malt1 complex to lipid rafts. Raft-associated active caspase-8 was also found associated with many additional NF- κ B signaling molecules, including Carma1, IKK $\alpha/\beta/\gamma$, and TRAF2. Furthermore, data showed that c-FLIP_L is recruited to the raft-associated caspase-8 complex, where it is cleaved to the active FLIP(p43) form (Fig 6B) [83]. Numerous studies have demonstrated that c-FLIP plays a role in T cell activation, specifically in TCR-dependent NF- κ B activation and production of the autocrine cytokine, IL-2 (reviewed in [85]). Thus, the connection between Malt1, caspase-8, and c-FLIP activation suggests a distinct mechanism whereby Malt1 contributes to NF- κ B activation.

Further molecular details of the connection between the CBM complex and caspase-8 were provided by a recent publication showing that Malt1 activates caspase-8 in a manner dependent on the presence of the Malt1 caspase-like domain but independent of Malt1 proteolytic activity. Biochemical analyses suggested that Malt1 induces partial auto-proteolytic cleavage of caspase-8. Furthermore, Malt1-activated caspase-8 efficiently cleaves and activates the NF- κ B-inducer, c-FLIP_L, but inefficiently cleaves and activates the apoptosis initiator, caspase-3 [18], explaining how caspase-8 is activated in a manner that does not lead to apoptosis (Fig. 6C).

The molecular mechanism accounting for partial processing of caspase-8 is not yet apparent. Data from this study [18] would appear to rule out an 'induced proximity' model [86], in which caspase-8 is directly cleaved and activated by Malt1. By contrast, the limited activation of caspase-8 might be explained by an 'induced conformation' model [87], in which association between the paracaspase domain of Malt1 and caspase-8 induces a conformational change in caspase-8 that specifically triggers partial auto-processing, yielding an active enzyme with high specificity for cleavage of c-FLIP_L.

3.3 NF- κ B activation in the absence of Malt1

While the prevailing model holds that Malt1 plays an essential role in antigen receptor-mediated signaling to NF- κ B, a growing body of published data cannot be easily explained by this model. Recent data strongly suggest that Malt1 is not required for Carma1- and TRAF6-dependent activation of the IKK complex. In Jurkat cells, TRAF6 was found to inducibly associate with Carma1 following PMA/ionomycin stimulation [88], demonstrating that TRAF6 can interact with more than one protein in the CBM complex. To further characterize the interaction of Carma1 with adaptor proteins, investigators removed the inhibitory domain of Carma1 (Carma1 Δ ID); this modification results in the association of Carma1 with downstream proteins in the absence of stimulation. In Jurkat cells expressing Carma1 Δ ID, shRNA-mediated knockdown of Malt1 expression did not interfere with TRAF6 binding to Carma1, and only slightly reduced the association of Carma1 Δ ID and the IKK complex [88]. These data therefore suggest that under stimulatory conditions, Malt1 is not required for the recruitment of TRAF6 or the IKK complex to Carma1, which, by extension, suggests that Malt1 is dispensable for NF- κ B activation. Data from primary B cells lend independent support to this interpretation, as the absence of Malt1 does not affect recruitment of the IKK complex to lipid rafts following B cell receptor ligation [89].

Additional data from Malt1^{-/-} cells also provide evidence that Malt1 is dispensable for NF- κ B activation. Malt1^{-/-} MEF cells transfected with Bcl10 showed only a 2-fold reduction in NF- κ B activation compared to Malt1^{+/+} controls [45], suggesting that while Malt1 optimizes NF- κ B activation, it is not required. In contrast, Bcl10^{-/-}, Carma1^{-/-}, and Carma3^{-/-} cells showed serious deficits in NF- κ B activation, indicating these CBM constituents are of much

more importance in signaling to NF- κ B [39,40,43,90]. Consistent with these results, recent data showed that Malt1^{-/-} T cells, particularly the CD8⁺ subset, displayed almost wild-type levels of NF- κ B activation, especially after strong stimulation through the T cell receptor [65]. Furthermore, in a side-by-side comparison of T cell function in Malt1^{-/-}, Bcl10^{-/-}, and PKC θ ^{-/-} knockout models, Malt1^{-/-} T cells were minimally impaired with regard to T cell proliferation and expression of cell surface activation markers, whereas Bcl10^{-/-} and PKC θ ^{-/-} T cells showed a more striking reduction in these parameters. In addition, evaluation of IL-2 production and I κ B α phosphorylation by Malt1^{-/-}, Bcl10^{-/-}, and PKC θ ^{-/-} T cells showed that while all knock-out T cells exhibited reduced IL-2 production and impaired I κ B α phosphorylation, Malt1^{-/-} T cells consistently produced more IL-2 and showed higher levels of phospho-I κ B α than Bcl10^{-/-} and PKC θ ^{-/-} T cells [65]. Taken together, these data highlight the importance of Bcl10 and PKC θ in T cell function, and suggest that Malt1 is not absolutely required for NF- κ B activation and NF- κ B-dependent functions in T cells. Finally, analysis of Malt1 function in B cells showed that Malt1^{-/-} B cells display only a slight reduction in NF- κ B activation following B cell receptor ligation, as measured by the degradation of the NF- κ B inhibitor protein I κ B α [89].

Taken together, the data from analysis of Malt1^{-/-} cells indicate that NF- κ B activation still occurs, albeit with somewhat reduced efficiency, in the absence of Malt1. These data therefore suggest that Malt1 plays a less important and perhaps less direct role in NF- κ B activation than other proteins in the pathway, such as PKC θ , Carma1/Carma3, and Bcl10. Because the loss of Malt1 appears to only modestly reduce the magnitude of NF- κ B activation, it seems likely that the TRAF6 binding activity of Malt1 and the K63-polyubiquitination sites in the Malt1 C-terminus are redundant with other TRAF6 and K63-polyubiquitination sites on proteins such as Carma1 and Bcl10, respectively. The aggregate data thus suggest that a more important (and probably non-redundant) function of Malt1 is to relieve inhibition of the NF- κ B activation pathway via proteolytic cleavage of A20 (Fig. 5).

3.4 Evidence for a specialized role for Malt1 in IL-2 production and c-Rel activation

Although Malt1 appears to play a non-essential, augmentary role in NF- κ B activation, several independent studies indicate that Malt1 has a major role in IL-2 production. Malt1^{-/-} T cells or T cells expressing Malt1 mutants (Malt1-C464A caspase mutant or Malt1 ubiquitin mutants) show a consistent defect in IL-2 production, compared to WT controls [16–18,45,46,54,56, 65]. In side-by-side comparisons of Malt1^{-/-}, Bcl10^{-/-}, and PKC θ ^{-/-} knockout T cells, although the Malt1^{-/-} T cells produced more IL-2 than the other knockout T cells, they still produced only about 10% of wild-type levels of this cytokine in response to robust TCR stimulation [65]. In contrast, there was no difference between wild-type and Malt1^{-/-} T cells when expression of cell surface activation markers such as CD25 and CD44 were assayed at the same concentration of anti-TCR [65].

The IL-2 promoter region contains NF- κ B binding sites, indicating the importance of NF- κ B activation in transcription of the IL-2 gene (reviewed in [91]). Malt1 may contribute to IL-2 signaling by preventing inhibition of NF- κ B activation via cleavage of A20, as described above. One possible explanation for the discordance between IL-2 expression and the expression of cell surface activation markers in Malt1^{-/-} T cells is that maximal activation of the IL-2 gene may require more sustained NF- κ B activation than is necessary for maximal expression of the CD25 and CD44 markers. In this model, Malt1-mediated cleavage of A20 may serve to prolong robust NF- κ B activation, thereby allowing for sustained activation of the IL-2 promoter. Another possibility is that Malt1 enzymatically cleaves a protein or proteins involved in the epigenetic modification of the IL-2 promoter. In this model, the absence of Malt1 would prevent the demethylation of the IL-2 promoter, an event required for IL-2 expression [92].

An additional possibility is that IL-2 expression is activated by specific NF- κ B heterodimer combinations, and Malt1 plays a role in regulating which NF- κ B subunits are activated after TCR ligation. Previous data have shown that c-Rel deficient T cells are deficient in IL-2 production [93]. Furthermore, data from Malt1^{-/-} B cells showed that Malt1 is specifically required for activation of NF- κ B complexes containing c-Rel, but not for activation of complexes containing RelA. This finding was in contrast to data from Bcl10^{-/-} mice, showing that Bcl10 contributes to activation of both c-Rel- and RelA-containing NF- κ B heterodimers [89]. Together, these data suggest a model in which Malt1 specifically activates forms of NF- κ B containing c-Rel, which is required for optimal IL-2 expression. The molecular mechanism accounting for c-Rel-specific activation by Malt1 is currently unknown. One possibility is that the activation of caspase-8 and concomitant cleavage of c-FLIP_L activates the IKK complex in a manner that is somehow specific for c-Rel. Alternatively, the Malt1 caspase-like domain or the Malt1-caspase-8 complex may cleave c-Rel or a c-Rel-associated protein in a manner required for nuclear translocation of c-Rel.

4 The role of Malt1 in NF- κ B activation: an emerging model

Overall, accumulating data suggest that the role of Malt1 in signaling to NF- κ B is considerably more complex than the mechanisms suggested by early observations of the behavior of the cIAP2-Malt1 chimera and the oligomerized Malt1 C-terminus. Based on the above analysis of currently available data, we propose that the primary function of Malt1 in the regulation of NF- κ B signaling is to proteolytically cleave A20 (Fig. 5) and to specifically direct the proteolytic functions of caspase-8 to c-FLIP_L to enhance signaling to NF- κ B (Fig. 6). Other activities of Malt1, such as binding to TRAF6 and serving as a docking site for IKK γ (following K63-polyubiquitination of the Malt1 C-terminus) are redundant with other proteins in the CBM complex, such as Carma1 and Bcl10, respectively.

5 Unresolved questions regarding the role of Malt1 in antigen receptor-mediated NF- κ B activation

While the model in Figure 5 is consistent with the body of published data, there remain several gaps in our understanding. For example, it is currently unclear what regulates the balance between the cleavage of A20 by Malt1 (Fig 5A) and the A20-mediated removal of K63-polyubiquitin chains from the CBM complex (Fig. 5B). However, based on the observation that Malt1 plays a more essential role in T cell activation at low levels of TCR stimulation [65], it seems likely that Malt1-mediated cleavage of A20 predominates under conditions of weak TCR stimulation, whereas A20-mediated removal of K63-polyubiquitin from the CBM complex is the dominant activity under conditions of strong stimulation. The counterbalancing of these two regulatory activities may serve to amplify weak signals and down-modulate strong signals, thereby ensuring a consistent level of NF- κ B activation in response to a broad range of upstream signal strengths. More extensive studies evaluating the precise roles and activities of Malt1 and A20 under various stimulation conditions will be required to better understand how these molecules regulate each other.

Similarly, the relationship between IKK activation induced by K63-polyubiquitination of the CBM complex (Fig. 3) and IKK activation stimulated by the Malt1-, caspase-8-, and c-FLIP_L-regulated pathway (Fig. 6) is nebulous, at best. Do these two mechanisms represent components of a single pathway of IKK activation, or are these pathways molecularly distinct entities that activate the IKK complex with differing kinetics and/or efficiencies, perhaps even effecting distinct programs of NF- κ B activation (as discussed in Section 3.4).

Additionally, presently available data do not address whether the ubiquitination of Malt1 affects the activity of the caspase-like domain. As described above, substitution of all 11 C-

terminal Malt1 ubiquitination sites reduces NF- κ B activation [54]. Is this due to conformational changes in the C-terminus that reduce activation of the caspase-like domain, or is ubiquitination a required modification for Malt1 proteolytic activity? The latter is a distinct possibility, given the recent observation that A20 specifically de-ubiquitinates Malt1 [56]. The authors of this study provided evidence that A20-mediated de-ubiquitination of Malt1 reduces the association between the CBM and IKK complexes. However, it is also possible that Malt1 de-ubiquitination reduces the proteolytic activity of Malt1, thereby allowing more robust de-ubiquitination of the CBM complex by A20.

Indeed, there are currently no data offering a molecular mechanism to explain the activation or regulation of activity of the Malt1 caspase-like domain. Is Malt1 a constitutively active protease, with target cleavage regulated only by substrate availability? If an activation step is required, is the oligomerization of the C-terminus necessary and/or sufficient for activation? For many caspases, proteolytic processing is required to convert the inactive, zymogen form to the active form (reviewed in [86]). Are other proteases such as caspase-8 required to activate the Malt1 caspase-like domain via an unidentified cleavage of a binding partner or Malt1 itself? There are also currently no data that address how substrate modification may contribute to cleavage by the Malt1 caspase-like domain. For example, do modifications, such as phosphorylation (a well documented modification of Bcl10 [94]), affect interaction with or cleavage by Malt1? More intensive study of the regulation of cleavage of known substrates, as well as the potential identification of novel Malt1 substrates should facilitate a more complete understanding of regulation of the Malt1 protease domain.

6. Unresolved questions regarding the mechanism of NF- κ B activation by the cIAP2-Malt1 fusion

Although a number of details regarding the mechanism of NF- κ B activation by the cIAP2-Malt1 fusion are now reasonably well understood (Fig. 4), there is not yet a precise description of the mechanism that leads to association between cIAP2-Malt1 and the IKK complex. For example, there are currently no data defining the relative roles of K63-polyubiquitination by associated TRAFs and of ubiquitin binding by the UBA domain in the initiation and maintenance of the association between cIAP2-Malt1 and IKK. Additionally, sites of K63-polyubiquitination on the cIAP2-Malt1 fusion remain to be defined. As a result, it is unclear to what degree TRAF ubiquitination of the cIAP2-Malt1 molecule (vs. binding partners) is important in IKK association and activation.

The role of A20 in regulating cIAP2-Malt1 activity is also still uncertain. Over-expressed A20 is capable of inhibiting NF- κ B activation mediated by the cIAP2-Malt1 fusion, indicating that the cIAP2-Malt1 fusion can be recognized by A20. Indeed, A20 co-immunoprecipitates with both wild-type Malt1 and with the cIAP2-Malt1 fusion, suggesting that there is an A20 binding site in the C-terminus of Malt1 (Fig. 2). Additionally, data have shown that A20 can be cleaved and inactivated by cIAP2-Malt1 when both proteins are transiently over-expressed [16,56]. Moreover, mutation of the catalytic cysteine in cIAP2-Malt1 reduces NF- κ B activation by approximately 3-fold in transient transfection reporter assays, suggesting that the caspase-like domain may contribute to NF- κ B activation by the fusion protein [5]. Based on currently available data, it is unclear if A20 serves to down-modulate NF- κ B activation by cIAP2-Malt1 in MALT lymphoma cells, or if the majority of A20 is cleaved by the cIAP2-Malt1 caspase-like domain. There are also no data to indicate whether or not the caspase-like activity of cIAP2-Malt1 requires any co-factors or if it is regulated in any manner in Malt lymphoma cells. Thus, future investigation of the caspase-like activity of cIAP2-Malt1 may also contribute to elucidating the molecular mechanisms controlling activation of the Malt1 caspase-like domain.

Finally, there is no information regarding the role of caspase-8 in NF- κ B activation by cIAP2-Malt1. As the caspase-like domain of Malt1 is sufficient for binding to caspase-8 [18], existing data suggest that cIAP2-Malt1 fusion proteins are able to bind to caspase-8. Further experiments will be required to document such an association and to examine whether forms of cIAP2-Malt1 that cannot associate with caspase-8 show any reduction in NF- κ B induction.

7 Conclusions

As described in detail in this review, accumulating data suggest that Malt1 and the cIAP2-Malt1 fusion protein regulate NF- κ B activation in distinct ways and with markedly different potencies. The strong and constitutive activation of the IKK complex by cIAP2-Malt1 is dependent upon oligomerization, TRAF2/TRAF6 binding, proteolysis of A20, and a recently identified polyubiquitin binding domain (Figs. 2 and 4). In contrast, the wild-type Malt1 protein modestly enhances NF- κ B activation in response to antigen receptor ligation, and data suggest that the primary non-redundant activities of Malt1 are proteolytic cleavage of the A20 inhibitor and interaction with caspase-8, inducing proteolysis of c-FLIP_L and enhanced activation of the IKK complex (Figs. 5&6). The K63-polyubiquitination of Malt1 is probably redundant with other proteins in the CBM complex that also become K63-polyubiquitinated (e.g., Bcl10). These recent findings have helped resolve discordant data regarding NF- κ B activation by Malt1 and cIAP2-Malt1, and they have opened up new and intriguing avenues of research regarding the mechanisms by which signals are transduced to the IKK complex and to NF- κ B.

Clearly, full-length Malt1 was originally implicated in NF- κ B activation due to the potent induction of NF- κ B by the cIAP2-Malt1 fusion protein, and due to its direct association with Bcl10, a known transducer of signals from antigen receptors to NF- κ B. However, combined data from several different studies now strongly suggest that the cIAP2-Malt1 translocation product represents a gain of function mutation, in which equal and essential contributions to NF- κ B activation are made by the cIAP2-derived N-terminus and the Malt1-derived C-terminus. In contrast, accumulated data from many groups argues that Malt1 plays a limited role in antigen receptor activation of NF- κ B, serving to augment and/or “fine-tune” the activation signal transmitted by more crucial activators of NF- κ B, such as PKC θ , Carma1, and Bcl10. Additional data, which require considerable further investigation, suggest that Malt1 may serve as a signaling branch point, activating specific NF- κ B subunits, such as c-Rel, which may in turn control the activation of a limited subset of NF- κ B regulated targets, such as the IL-2 gene. Given that the proteolytic activity of Malt1 and Malt1 stimulation of caspase-8 activity were only recently identified, we anticipate the future identification of many novel substrates and biological activities of the Malt1 caspase-like domain and the Malt1-caspase-8 complex.

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Figure 1. Domain structure of cIAP2, Malt1, and the cIAP2-Malt1 chimera

A) Schematic representation of domains in the cIAP2 and Malt1 proteins. Abbreviations used: BIR = baculovirus IAP repeats; UBA = ubiquitin associated domain; CARD = caspase associated recruitment domain; RING = really interesting new gene domain; DD = death domain; Ig = immunoglobulin-like domain. **B)** The cIAP2-Malt1 chimera is composed of cIAP2 N-terminal domains fused to Malt1 C-terminal domains. Arrows indicate the distribution of sites of fusion between cIAP2 and Malt1 in individual MALT lymphoma tumors.

A: MALT1 **B: cIAP2-MALT1**

Figure 2. Protein-protein interaction sites within Malt1 and the cIAP2-Malt1 fusion protein
 Binding sites for interaction partners of Malt1 and cIAP2-Malt1 are indicated above the respective domain maps, with brackets designating the limits of the smallest fragment demonstrated to bind to each partner. Approximate locations and specific amino acid sequences of TRAF2 and TRAF6 binding sites are shown below the domain maps. **A)** Bcl10 interacts with the N-terminus of Malt1 via the DD and two Ig-like domains. Carma1 and A20 have been shown to interact with the C-terminus of Malt1, but precise binding sites have not yet been described. Proteolytic activity of Malt1 is mediated by the caspase-like domain, with the catalytic cysteine, C₄₆₄, being essential for cleavage of targets. The caspase-like domain is also the site of interaction with caspase-8. Ubc13 interacts with an Ig-like domain downstream of the caspase-like domain. TRAF6 has been shown to ubiquitinate multiple lysines within the Malt1 C-terminus, in the region downstream of the caspase-like domain. Locations of TRAF6 binding sites are shown beneath the domain map, with glutamic acids critical for TRAF6 binding indicated by amino acid numbers. **B)** The cIAP2-Malt1 fusion protein contains the three BIR domains and the UBA domain of cIAP2, plus the functional domains of the Malt1

C-terminus (see A, above). The two N-terminal Malt1 Ig-like domains are variably present (see Fig. 1B). A20 and Ubc13 binding to cIAP2-Malt1 has been directly demonstrated, whereas caspase-8 and Carma1 binding is inferred, based on presence of the respective binding sites in the fusion protein. The first BIR domain (BIR1) interacts with an undefined site in the Malt1-contributed C-terminal region, mediating cIAP2-Malt1 oligomerization. The BIR region has also been reported to bind to Bcl10, although these data are disputed in the literature. The UBA domain has been shown to bind K63- and linear-polyubiquitin chains, perhaps facilitating association between cIAP2-Malt1 and IKK γ . Four distinct TRAF2/TRAF6 binding sites have been defined in the cIAP2-Malt1 fusion, and specific binding sequences are shown below the domain map. The three most C-terminal TRAF6 binding sites are contributed by the Malt1 portion, and the most N-terminal binding site is located within the BIR1 domain, which is contributed by cIAP2.

Figure 3. Current model of TCR mediated NF-κB activation

Crucial events in the TCR-to-NF-κB activation cascade are depicted from left to right. The TCR is activated by interaction with the major histocompatibility complex (MHC) plus peptide antigen, and the CD28 costimulatory receptor is activated by interaction with B7, as shown in the upper left. TCR- and CD28-dependent signals lead to activation of phosphatidylinositol-3-kinase (PI-3-K), which phosphorylates the membrane lipid, PIP₂, yielding PIP₃. Production of PIP₃ leads to membrane recruitment and activation of phosphoinositide-dependent kinase 1 (PDK1). Activated PDK1 phosphorylates PKCθ, which in turn phosphorylates CARMA1. Phosphorylated CARMA1 undergoes a conformational change and interacts with a pre-existing complex of Bcl10 and Malt1 to form the CBM complex, which is thought to oligomerize into punctate signaling structures (POLKADOTS) within the cytosol. The ubiquitin ligase, TRAF6, in conjunction with the ubiquitin conjugating enzyme complex, Ubc13/Uev1a, mediates the K63-linked ubiquitination of TRAF6, itself, and other proteins in the CBM complex, including Malt1 and Bcl10. Ubiquitination of the CBM complex recruits the IKK complex, via the ubiquitin binding domain (UBAN) of IKKγ. TRAF6 then K63-polyubiquitinates IKKγ. The oligomerized CBM and IKK complexes are recruited to the plasma membrane, and the IKKβ subunit is phosphorylated by TAK1. The combination of IKKγ polyubiquitination and IKKβ phosphorylation leads to activation of the IKK complex and subsequent phosphorylation of the IκBα protein by IKKβ. Phosphorylated IκBα is K48-polyubiquitinated by the SCF^{IκB} E3 ubiquitin ligase (not depicted), which targets IκBα for degradation by the proteasome. Degradation of IκBα unmasks the NF-κB nuclear localization sequence, allowing the NF-κB heterodimer to enter the nucleus and stimulate transcription of target genes.

Figure 4. Molecular mechanisms contributing to receptor-independent cIAP2-Malt1 mediated NF-κB activation

The cIAP2-Malt1 fusion proteins oligomerize via heterotypic interactions between BIR1 of the cIAP2-derived region and the Malt1-contributed C-terminal domain (see Fig. 2B). TRAF2 and/or TRAF6 interact with oligomeric cIAP2-Malt1, triggering Ubc13/Uev1a-dependent K63-polyubiquitination of the TRAFs, themselves, and cIAP2-Malt1. The IKK complex then associates with the ubiquitinated cIAP2-Malt1 oligomers, via the UBAN of IKKγ. This association leads to Ubc13/Uev1a-dependent K63-polyubiquitination of IKKγ. The IKKβ is then phosphorylated by TAK1 or an unknown kinase. The combination of IKKγ polyubiquitination and IKKβ phosphorylation triggers and the terminal NF-κB activation

events as shown in the diagram and described in the legend for Figure 3. Data suggest that the cIAP2-Malt1 chimera also overcomes mechanisms that normally serve to limit cellular NF- κ B activation by cleaving and inactivating the A20 inhibitor protein.

Figure 5. Reciprocal regulation of NF-κB activation Malt1-mediated A20 cleavage and A20-mediated inactivation of polyubiquitinated signaling proteins

Opposing activities of Malt1 and A20 coordinately regulate NF-κB activation. A) In response to TCR signaling, CBM-associated Malt1 cleaves Bcl10 and A20. Bcl10 cleavage yields a truncated protein that regulates cellular adhesion, while cleavage of the A20 protein relieves inhibition of CBM-mediated activation of the IKK complex. Thus, this activity of Malt1 contributes to TCR activation of NF-κB. B) A20 inhibits NF-κB signaling by de-ubiquitinating the CBM complex and TRAF6. This activity prevents the association of the CBM and IKK complexes, thereby blocking activation of the IKK complex and consequent activation of NF-κB.

Figure 6. Physical interaction between Malt1 and caspase-8 contributes to NF- κ B activation

At least three distinct mechanisms have been proposed whereby caspase-8 interaction with the CBM complex facilitates TCR-dependent activation of NF- κ B. **A)** Caspase-8 interacts with the CBM complex, enhancing the recruitment of the IKK complex through an undefined mechanism. The enhanced association leads to improved CBM-dependent activation of the IKK. **B)** CBM-associated caspase-8 binds to TRAF6. The association with TRAF6 leads to the redistribution of the CBM complex to lipid rafts, where it can interact with other NF- κ B signaling proteins. One consequence of redistribution to lipid rafts is enhanced caspase-8-dependent cleavage of c-FLIP_L to FLIP(p43). FLIP(p43) then initiates a signaling cascade (not depicted) that leads to NF- κ B activation and IL-2 production. **C)** TCR signaling leads to formation of the CBM complex. Malt1 then binds to caspase-8, which associates with and cleaves c-FLIP_L, yielding the active FLIP(p43). FLIP(p43) then recruits TRAF2, which cooperates with the CBM/TRAF6 complex to polyubiquitinate IKK γ , contributing to activation of the IKK complex.