A Novel TRAF6 Binding Site in MALT1 Defines Distinct Mechanisms of NF-κB Activation by API2-MALT1 Fusions*

Heidi Noels†§, Geert van Loo¶‖, Sofie Hagens§¶, Vicky Broeckx§¶, Rudi Beyaert¶‖, Peter Marynen§¶, and Mathijs Baens¶§¶

From the †Human Genome Laboratory, Department for Molecular and Developmental Genetics, Flanders Institute for Biotechnology (VIB), B-3000 Leuven, Belgium, the ‡Human Genome Laboratory, Molecular Genetics, Center for Human Genetics, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium, the ¶Unit of Molecular Signal Transduction in Inflammation, Department for Molecular Biomedical Research, Flanders Institute for Biotechnology (VIB), B-9052 Ghent, Belgium, and the ¶¶Unit of Molecular Signal Transduction in Inflammation, Department of Molecular Biology, Ghent University, B-9052 Ghent, Belgium

The recurrent translocation t(11;18)(q21;q21) associated with mucosa-associated lymphoid tissue (MALT) lymphoma results in the expression of an API2-MALT1 fusion protein that constitutively activates NF-κB. The first baculovirus IAP repeat (BIR) domain of API2 and the C terminus of MALT1, which contains its caspase-like domain, are present in all reported fusion variants and interact with TRAF2 and TRAF6, respectively, suggesting their contribution to NF-κB signaling by API2-MALT1. Also, the involvement of BCL10 has been suggested via binding to BIR1 of API2 and its interaction with the immunoglobulin domains of MALT1, present in half of the fusion variants. However, conflicting reports exist concerning their roles in API2-MALT1-induced NF-κB signaling. In this report, streptavidin pulldowns of biotinylated API2-MALT1 fusion variants showed that none of the fusion variants interacted with endogenous BCL10; its role in NF-κB signaling by API2-MALT1 was further questioned by RNA interference experiments. In contrast, TRAF6 was essential for NF-κB activation by all fusion variants, and we identified a novel TRAF6 binding site in the second immunoglobulin domain of MALT1, which enhanced NF-κB activation when present in the fusion protein. Furthermore, inclusion of both immunoglobulin domains in API2-MALT1 further enhanced NF-κB signaling via intramolecular TRAF6 activation. Finally, binding of TRAF2 to BIR1 contributed to NF-κB activation by API2-MALT1, although additional mechanisms involving BIR1-mediated raft association are also important. Taken together, these data reveal distinct mechanisms of NF-κB activation by different API2-MALT1 fusion variants with an essential role for TRAF6.

Extralobar marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) represents with 8% one of the most common types of non-Hodgkin B-cell lymphoma. Two translocations specific for MALT lymphoma are t(1,14)(p22; q32) and t(14,18)(q32;q21), which up-regulate the expression of the BCL10 and MALT1 gene, respectively, via their fusion to the enhancer of IgH (1–4). The most common genetic aberration is the translocation t(11;18)(q21;q21), where fusion of API2 to MALT1 results in the expression of an API2-MALT1 fusion protein (5).

BCL10 (6) and MALT1 (7, 8) play a crucial role in the signaling cascade from the antigen receptors to the transcription factor NF-κB. Upon T-cell receptor stimulation, CARMA1 (CARD-containing MAGUK protein-1) recruits the BCL10-MALT1-TRAF6 (9, 10) and the IKK (11) (IKKe-IKKB-IKKγ/NEMO) complexes to the lipid rafts (12–14) surrounding the receptor. Here it is thought that oligomerization of TRAF6 elicits its ubiquitin ligase activity, resulting in polyubiquitination of IKKγ, the regulatory subunit of the IKK complex (15, 16). This together with phosphorylation of IKKβ by the TAK1-TAB complex (16) fully activates the IKK complex, which then triggers phosphorylation and proteasome-mediated degradation of IkB-α, the inhibitor of NF-κB. In this way NF-κB is released as a free complex allowing it to translocate to the nucleus and elicit its transcriptional function.

There are several lines of evidence that the API2-MALT1 fusion protein somehow introduces a shortcut in the signaling pathway to NF-κB. Initial experiments in cell lines demonstrated that API2-MALT1 could induce constitutive NF-κB activity (17, 18). Later, this was linked to enhanced polyubiquitination of IKKγ in MALT1 lymphomas (19) and in transgenic mice (20). It is generally believed that the BIR domains of API2 mediate the oligomerization of the MALT1 moiety, which in turn triggers TRAF6 oligomerization activating its ubiquitin ligase activity. However, the interaction of TRAF6 with MALT1 via the two potential TRAF6 binding sites in its C terminus has

*This work was supported by Grants SCIE2003-09 from the “Belgische Federatie tegen Kanker” and 04-149 from the Association for International Cancer Research (to P. M.); by Grants 3G010505 from the Fonds voor Wetenschappelijk Onderzoek (FWO)-Vlaanderen and 01G06B6 from the Fonds “Geconcerteerde Onderzoeksacties” (to R. B.); and by the IAP6/18 network (to R. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Aspirant with FWO-Vlaanderen.
2 Postdoctoral research fellow with FWO-Vlaanderen.
3 To whom correspondence should be addressed: Human Genome Laboratory, Center for Human Genetics, Molecular Genetics, K.U. Leuven, Herestraat 49, B3000 Leuven, Belgium. Tel.: 32-16-33-01-30; Fax: 32-16-34-71-66; E-mail: Mathijs.Baens@Med.KULeuven.be.

4 The abbreviations used are: MALT, mucosa-associated lymphoid tissue; API2, apoptosis inhibitor-2; BCL10, B-cell CLL/lymphoma 10; BIR, baculovirus IAP repeat; DD, death domain; Ig, immunoglobulin; IkB, IkB-inhibitory protein; IKK, IκB-kinase; MEF, murine embryonic fibroblast; NF-κB, nuclear factor-kB; siRNA, small interfering RNA; TAK1, transforming growth factor-β-activated kinase-1; TAB, TAK1-binding protein; TRAF, tumor necrosis factor receptor-associated factor.

10180 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 282 • NUMBER 14 • APRIL 6, 2007
Role of TRAF6 in NF-κB Signaling by API2-MALT1 Fusions

thus far only been demonstrated via overexpression and colocalization studies (16, 21). Moreover, a recent report questioned a role for TRAF6 in NF-κB activation by API2-MALT1, as the mutation of both potential TRAF6 binding sites does not affect its signaling potential (19). In contrast, we observed a dominant negative effect on NF-κB activation for the C-terminal TRAF6 binding site mutation (E806A), suggesting an essential role for TRAF6 in NF-κB signaling by API2-MALT1 (20). Different API2-MALT1 fusion variants were used in these studies (19, 20), which might explain the observed differences.

In approximately half of the MALT lymphoma cases with t(11;18), the API2-MALT1 protein contains the three N-terminal BIR domains of API2 fused to the C-terminal caspase-like domain of MALT1. Depending on the breakpoint, however, the fusion variants have in addition one or two immunoglobulin (Ig) domains of MALT1, as observed in ~40 and 10% of the cases, respectively. The Ig domains interact with BCL10, which could explain the enhanced NF-κB activation associated with fusion variants carrying these domains (18). In addition, overexpressed BCL10 was reported to interact with the first BIR domain of API2 and to synergistically activate NF-κB with API2-MALT1 (22). The first BIR domain further interacts with TRAF1 and TRAF2, although this interaction then again is apparently not involved in NF-κB activation by API2-MALT1 (23).

To clarify the mechanisms by which API2-MALT1 activates NF-κB, we investigated the role of BCL10, TRAF2, and TRAF6 for the different fusion variants. Our data argue against a role for BCL10 in their NF-κB activating potential, although TRAF2 does contribute via its interaction with the first BIR domain of API2. Furthermore, we have demonstrated an essential role for TRAF6 in NF-κB activation by all fusion variants and identified a new TRAF6 binding site in the second Ig domain of MALT1. Both TRAF6 binding sites contribute to NF-κB signaling by MALT1 and API2-MALT1 and seem to enhance NF-κB activation by the API2-MALT1 fusion variant with both Ig domains of MALT1 via intramolecular TRAF6 activation.

EXPERIMENTAL PROCEDURES

Constructs—A vector enabling expression of biotinylated proteins was constructed by introducing oligonucleotides encoding the biotinylation (bio) sequence (GLNDIFEAQKIEWHE) described by Beckett et al. (24) downstream of the N-terminal FLAG epitope in the plasmid pcDNA3.1 (pcD-F-bio). A second vector contained the bio-tag preceded by a flexible linker (SGSGGGSSG) C-terminal of the multiple cloning site (pcD-F-bioC). These vectors were used to generate bio-con structs for MALT1 and the API2-MALT1 fusion variants A7M8, A7M5, and A7M3 (fusion of exon 7 of API2 with exons 8, 5, and 3 of MALT1, respectively; see Fig. 1A). The open reading frames of A7M8, A7M5, A7M3, and MALT1, and BCL10 were amplified by reverse transcription-PCR using long distance PCR (Roche Applied Science). PCR-based mutagenesis was applied to generate MALT1 fragments with deletion of exon 7 (M7d) or mutation of the TRAF6 binding sites (E313A/E316A or M7d and E653E and E806A, separately or combined), which were subcloned into the above mentioned constructs to generate the different mutants. The same strategy was used to generate API2-MALT1 constructs containing the E47A/R48A (ER-
Role of TRAF6 in NF-κB Signaling by API2-MALT1 Fusions

FIGURE 1. NF-κB activation by API2-MALT1 does not require BCL10. A, features of MALT1 and API2-MALT1 plus the domain content (solid bars) of the MALT1 deletion constructs and the A7M8, A7M5, and A7M3 fusion variants (fusion API2 exon 7 to MALT1 exons 8, 5, and 3, respectively). Deletions are depicted by dotted lines. B, inclusion of one or two Ig domains of MALT1 in A7M5 and A7M3, respectively, results in higher NF-κB-dependent luciferase activity in reporter assays compared with A7M8 (see “Experimental Procedures”). Equal expression levels were confirmed by immunoblot with luciferase/MALT1), H9251 mass standards are in kDa.

JOURNAL OF BIOLOGICAL CHEMISTRY

values were normalized for galactosidase values to correct for differences in transfection efficiency (plotted as luciferase/β-galactosidase).

FIGURE 1. NF-κB activation by API2-MALT1 does not require BCL10. A, features of MALT1 and API2-MALT1 plus the domain content (solid bars) of the MALT1 deletion constructs and the A7M8, A7M5, and A7M3 fusion variants (fusion API2 exon 7 to MALT1 exons 8, 5, and 3, respectively). Deletions are depicted by dotted lines. B, inclusion of one or two Ig domains of MALT1 in A7M5 and A7M3, respectively, results in higher NF-κB-dependent luciferase activity in reporter assays compared with A7M8 (see “Experimental Procedures”). Equal expression levels were confirmed by immunoblot with luciferase/MALT1), H9251 mass standards are in kDa.

JOURNAL OF BIOLOGICAL CHEMISTRY

values were normalized for galactosidase values to correct for differences in transfection efficiency (plotted as luciferase/β-galactosidase).

Bio-Immunoprecipitation and Western Blot Analysis—The bioimmunoprecipitation method is described by de Boer et al. (26). Briefly a protein of interest containing the biotinylation tag becomes biotinylated in vivo via co-expression of the E. coli BirA biotin protein ligase. After cell lysis in NDLB lysis buffer for 30 min on ice, the biotinylated protein complex is precipitated via binding to paramagnetic streptavidin beads (Dynabeads M-280, Invitrogen) for 2 h at 4°C. Protein precipitates were washed three times in lysis buffer and boiled for 10 min at 95°C in 1× SDS gel loading buffer (with a final concentration of 4% SDS and 300 mM β-mercaptoethanol).

Rat purifications were performed as described previously (20). Cell lysates and precipitated protein complexes were fractionated on 4–12% SDS-polyacrylamide gels (NuPage, Invitrogen) and transferred to polyvinylidene difluoride membranes (GE Healthcare). API2-MALT1 (A7M8, A7M5, and A7M3) and MALT1 were detected with α-FLAG (Sigma-Aldrich) or with a rabbit polyclonal antiserum raised against the C terminus (residues 731–824) of MALT1 (α-MALT1-C). Antibodies from Santa Cruz Biotechnology were used for immunodetection of BCL10 (sc-5273), TRAF6 (sc-7221), and TRAF2 (sc-876), respectively. The antibodies against UBC13 (4E11) and phosphorylated IκB-α (Ser32–Ser36, 5A5) was purchased from Invitrogen and Cell Signaling, respectively.

RESULTS

NF-κB Activation by API2-MALT1 Does Not Require BCL10—The API2-MALT1 fusion protein containing both Ig domains of MALT1 (A7M3 or case 2; see Fig. LA) possessed a higher NF-κB activating potential in 293 cells compared with the variant lacking these domains (A7M8 or case 1) (18). In MALT1, the Ig domains mediate the interaction with BCL10, which is essential for MALT1 to synergistically enhance NF-κB activation with BCL10 (17). Because the differences in potential of the fusion variants to activate NF-κB
correlated with their ability to interact with overexpressed BCL10, it was suggested that BCL10 contributes to NF-κB signaling by the A7M3 fusion variant via binding to its Ig domains (18). Transient expression of API2-MALT1 constructs with one (A7M5) or both (A7M3) Ig domains in HEK-293T cells indeed resulted in higher activity of the NF-κB luciferase reporter compared with the A7M8 variant (Fig. 1A and B). To investigate whether this correlated with their potential to recruit BCL10, we purified protein complexes from HEK-293T cells transiently expressing these API2-MALT1 fusion variants with a biotinylation tag. Co-expression of the E. coli biotin protein ligase BirA allowed in this way the pulldown of biotinylated API2-MALT1 with streptavidin-coated paramagnetic beads (26). Western blot analysis of purified API2-MALT1 complexes showed an interaction with endogenous TRAF2 and TRAF6. We could however not co-precipitate endogenous BCL10 with any of the fusion variants, in contrast to the efficient co-purification with a biotinylated MALT1 construct (Fig. 1C). Similarly, deletion of the N-terminal death domain (DD) in MALT1 abolished its interaction with BCL10 (Fig. 1D). As BCL10 did not co-precipitate with the bio-tagged DD alone, this suggested that the DD and the Ig domains of MALT1 cooperate for BCL10 recruitment (Fig. 1E). To exclude a cell type-specific effect, we generated SSK41 MALT lymphoma B-cells with stable expression of both the BirA biotin ligase and bio-tagged A7M8 and E653A and E806A mutants in HEK-293T cells showed that only the E653A mutation with the M7d deletion or the M7m mutation, respectively, completely blocked NF-κB reporter activation by A7M3, which correlated with a complete disruption of TRAF6 interaction (Fig. 2C, lanes 4 and 6).

It was reported previously that deletion of the C-terminal Ig-like domain of MALT1 in an A7M5 construct (del 1 (19)) disrupts its interaction with overexpressed UBC13 and abolishes NF-κB activation, suggesting a crucial role for binding of UBC13 to this Ig-like domain. A similar deletion prevented indeed NF-κB activation by an A7M3 construct (Fig. 2D); however, we could not detect endogenous UBC13 in the purified protein complexes from the different API2-MALT1 fusion variants (Fig. 2, B–D). We noticed however that this deletion not only prevented the interaction of TRAF6 with the C terminus of A7M3 but also disrupted its recruitment to the exon 7 binding site, which could explain the defective NF-κB signaling. Finally, when the A7M3 and A7M8 fusions were expressed in Traf6−/− MEFs, the absence of Traf6 function abolished API2-MALT1-mediated NF-κB activation (Fig. 2E).

According to the current model (20), TRAF6 interacts with the E653A and E806A mutants in HEK-293T cells showed that only the E806A mutant had lost its potential to interact with TRAF6 and to activate the NF-κB reporter (Fig. 2B), confirming the essential role of TRAF6 in A7M8-mediated NF-κB activation. A recent report, however, questioned the involvement of TRAF6 in NF-κB signaling by API2-MALT1 as mutation of both T6-C1 and T6-C2 (EA2) in A7M5 had no effect on NF-κB activation (19). We therefore wondered whether the EA2 mutation disrupted TRAF6 binding in the fusion variants containing one or two Ig domains, and we analyzed protein complexes purified with bio-tagged A7M5/3 and their EA2 mutant. Although we did observe a reduction in NF-κB activation, the EA2 mutation apparently did not affect binding of TRAF6 with A7M3 (Fig. 2C), suggesting either an indirect interaction or the existence of another TRAF6 binding site in the MALT1 moiety. Because the same results were obtained for A7M5 (data not shown) and in view of the dominant negative effect of the E806A mutation on TRAF6 binding to A7M8, we suspected the second Ig domain (lg2) of MALT1 to be involved in this additional interaction with TRAF6. Closr examination of its sequence revealed two partial TRAF6 binding consensus sequences just downstream of the lg2 (TDEAVE, MALT1 residues 311–316; and AVECTE, MALT1 residues 314–319), both located in exon 7 of MALT1 that encodes only 11 amino acids (Fig. 2A). Deletion of these 11 amino acids (M7d) or the double mutation E313A/E316A (M7m) did not inhibit TRAF6 binding but reduced the potential of A7M3 to activate NF-κB by half, suggesting an involvement in NF-κB signaling (Fig. 2C, lanes 3 and 5). A combination of the EA2 mutation with the M7d deletion or the M7m mutation, respectively, completely blocked NF-κB reporter activation by A7M3, which correlated with a complete disruption of TRAF6 interaction (Fig. 2C, lanes 3 and 5).
TRAF6 in HEK-293T and Jurkat T-cells (Fig. 1, C and F). Similar to A7M3 and A7M5, the interaction between TRAF6 and MALT1 could only be disturbed by the double M7m-E2A mutation, indicating that MALT1 also contains two functional TRAF6 binding sites (Fig. 3, A and B). To find out whether they were both involved in MALT1-mediated signaling to NF-κB, we analyzed the effect of their mutation on the synergism of MALT1 with BCL10 for NF-κB activation. As shown in Fig. 3C, mutation of either one of the TRAF6 binding sites in MALT1 had little effect on the synergism with BCL10, whereas a total block of TRAF6 binding to MALT1 via the double mutation completely abolished the effect of MALT1 co-expression and dropped NF-κB activation to the level of BCL10 expression alone. In conclusion, these data show two distinct TRAF6 binding sites in MALT1 that both contribute to NF-κB signaling.

Role for Lipid Raft Association in MALT1-mediated NF-κB Activation

Overexpression of MALT1 does not trigger NF-κB activation although it synergistically activates NF-κB with BCL10 (17), suggesting that BCL10 is required for the efficient oligomerization and activation of MALT1-TRAF6. Following antigen receptor stimulation, this oligomerization event is mediated by the recruitment of the BCL10-MALT1-TRAF6 complex to the lipid rafts at the receptor, which allows interaction with downstream signaling molecules and is essential for NF-κB activation. We therefore investigated whether raft-mediated oligomerization might be involved in the above mentioned overexpression experiments. As shown in Fig. 3D, overexpressed MALT1 is not associated with the lipid raft membrane fractions in HEK-293T cells, in contrast to overexpressed BCL10. Their co-expression, however, resulted in the redistribution of MALT1 to the upper, lower density fractions containing raft-associated proteins such
Role of TRAF6 in NF-κB Signaling by API2-MALT1 Fusions

Intramolecular TRAF6 Activation by the A7M3 Fusion Variant—In contrast to A7M8 and A7M5, the BIR1 deletion construct of A7M3 could still activate NF-κB by A7M3. In view of the experiments above but also for A7M5 oligomerization (23), this then implies an additional raft-independent mechanism for NF-κB activation by A7M3. In view of the two functional TRAF6 binding sites in MALT1, we questioned whether their fusion to API2 might trigger an intramolecular TRAF6 dimerization and activation event. As shown in Fig. 5, mutation of a single TRAF6 binding site (M7m or E2A) completely blocked NF-κB activation by A7M3-ΔB1, although interaction of TRAF6 with the other site was conserved, suggesting indeed that an intramolecular TRAF6 activation mechanism contributes to NF-κB signaling by A7M3. In contrast, the same did not hold for A7M5, where deletion of BIR1 completely abolished NF-κB activation, although both TRAF6 binding sites were still able to recruit TRAF6 (data not shown). In conclusion, these data indicate that intramolecular activation of TRAF6 contributes to NF-κB signaling by A7M3 but not A7M5.

Role for Lipid Raft Association in API2-MALT1-mediated NF-κB Activation—Previously we showed a role for lipid rafts in NF-κB signaling by A7M8, given that deletion of the first BIR domain (BIR1) of API2 inhibited not only raft association of A7M8 but also NF-κB activation (20). Similarly, deletion of BIR1 prevented recruitment of A7M5 (not shown) and A7M3 to the lipid rafts (Fig. 4, A and B), although this resulted only for A7M5 in a complete block of NF-κB activation: the BIR1-deletion construct of A7M3 could still partly activate NF-κB (Fig. 4C). Because the first BIR domain contains a TRAF1/2 binding site (25), we examined its role in NF-κB signaling by all fusion variants. As shown in Fig. 4C, the ER-AA mutation of the TRAF1/2 binding site in BIR1 abolished the interaction of all fusion variants with endogenous TRAF2 and inhibited NF-κB activation by A7M8, although it led only to reduced NF-κB activation by A7M5 and A7M3 (Fig. 4C). Purification of detergent-resistant membrane fractions showed that the ER-AA mutation did not inhibit the association of A7M5 (not shown) and A7M3 with the lipid rafts (Fig. 4B), which then might explain why they still activated NF-κB (Fig. 4C). Although raft association was also intact for A7M8 (not shown), the ER-AA mutation apparently disturbed its interaction with TRAF6, which was observed for the BIR1 deletion mutant of A7M8 as well and thus explains the complete block in NF-κB signaling (Fig. 4C).

Altogether these data indicate that lipid raft localization of all API2-MALT1 fusion variants is mediated by the first BIR domain of API2. Although for A7M8 and A7M5, raft localization is associated with their potential to signal to NF-κB, A7M3 can activate NF-κB via an additional raft-independent mechanism. Furthermore, TRAF2 binding to BIR1 contributes to NF-κB signaling by all fusion variants, although it is not required exclusively for their raft localization.

Intramolecular TRAF6 Activation by the A7M3 Fusion Variant—In contrast to A7M8 and A7M5, the BIR1 deletion construct of A7M3 could still activate NF-κB (Fig. 4C). Because BIR1 is essential not only for raft localization as shown in the experiments above but also for A7M5 oligomerization (23), this then implies an additional raft- and oligomerization-independent mechanism for NF-κB activation by A7M3. In view of the two functional TRAF6 binding sites in MALT1, we questioned whether their fusion to API2 might trigger an intramolecular TRAF6 dimerization and activation event. As shown in Fig. 5, mutation of a single TRAF6 binding site (M7m or E2A) completely blocked NF-κB activation by A7M3-ΔB1, although interaction of TRAF6 with the other site was conserved, suggesting indeed that an intramolecular TRAF6 activation mechanism contributes to NF-κB signaling by A7M3. In contrast, the same did not hold for A7M5, where deletion of BIR1 completely abolished NF-κB activation, although both TRAF6 binding sites were still able to recruit TRAF6 (data not shown). In conclusion, these data indicate that intramolecular activation of TRAF6 contributes to NF-κB signaling by A7M3 but not A7M5.

FIGURE 3. Both TRAF6 binding sites contribute to MALT1-mediated NF-κB activation. A, features of MALT1 plus the domain/TRAF6 binding site content (solid bars) of the series of mutation constructs. Mutated TRAF6 binding sites are depicted by an open circle. B, MALT1 contains two functional TRAF6 binding sites. Protein complexes purified with the respective bio-tagged constructs were immunoblotted with α-FLAG, α-TRAF6, α-TRAF2, and α-BCL10. Bio-IP, bio-immunoprecipitation. C, both TRAF6 binding sites of MALT1 are involved in the synergism with BCL10 for NF-κB activation. NF-κB reporter assays (see “Experimental Procedures”) of HEK-293T cells transfected with the indicated constructs. NF-κB-dependent luciferase activity is shown as -fold induction of vector-transfected cells and represents the mean ± S.D. of at least three independent experiments. The expression levels of the biotagged MALT1 constructs and BCL10 were determined by immunoblot with α-FLAG or α-BCL10, respectively. D, HEK-293T cells transiently transfected with MALT1 and BCL10, alone or in combination, were lysed, subjected to sucrose density gradient centrifugation, and immunoblotted with α-FLAG (for MALT1 and BCL10) and α-Lyn.
Role of TRAF6 in NF-κB Signaling by API2-MALT1 Fusions

DISCUSSION

Constitutive NF-κB activation induced by the chimeric API2-MALT1 protein is considered essential for B-cell transformation and progression of t(11;18)-positive MALT lymphomas. It is believed that self-oligomerization of API2-MALT1 triggers K63-linked polyubiquitination of IKKγ, and a role for the RING domain ubiquitin ligase TRAF6 herein was suspected (16). A recent report, however, suggested that API2-MALT1 might stimulate NF-κB activation independently of TRAF6 and attributed the ubiquitin ligase activity to MALT1 itself (15, 19). Also TRAF1 and TRAF2, adaptors that normally regulate API2 function, were shown not to contribute to API2-MALT1 signaling (23). In contrast, BCL10 was reported to synergistically activate NF-κB with API2-MALT1 via its interaction with the first BIR domain of API2 (22). In this report, we evaluated the role of BCL10, TRAF2, and TRAF6 for NF-κB activation by different API2-MALT1 fusion variants. Our data show that BCL10 does not interact with API2-MALT1 or contribute to its signaling to NF-κB. In contrast, all fusion variants bind either one or two TRAF6 molecules, and we have demonstrated that this interaction is essential for NF-κB activation. Finally, TRAF2 also was found to contribute to NF-κB signaling by API2-MALT1.

Both BCL10 and MALT1 have crucial roles in the antigen receptor signaling pathway leading to NF-κB activation (6–8). A role for BCL10 was also suggested in API2-MALT1-mediated NF-κB activation, as overexpression studies demonstrated an interaction between BCL10 and the fusion variants A7M3 or A7M8 via the Ig domains of MALT1 or the first BIR domain of API2, respectively (18, 22). However, we were unable to confirm the interaction between all tested API2-MALT1 variants and endogenous BCL10, conform the results that Hosokawa et al. (27) obtained with an A7M5 construct. A MALT1 construct lacking the DD, or the DD alone, did not interact with endogenous BCL10 in contrast to full-length MALT1 (Fig. 1, D and E), which suggests that the DD cooperates with the Ig domains of MALT1 for this interaction, possibly via an effect on its conformation. Our further observation that knockdown of BCL10 did not affect API2-MALT1-mediated NF-κB signaling therefore argues against its involvement in this process. This is further supported by the finding that the A7M5 variant activates NF-κB to the same degree in wild-type and Bcl10-deficient MEFs (7). In contrast, Hu et al. (22) reported that BCL10 knockdown silenced A7M8 signal propagation to NF-κB in HeLa cells. Whether this discrepancy reflects the different cell types used needs to be determined.

Constitutive NF-κB activation by the API2-MALT1 fusion protein was shown to be linked to enhanced polyubiquitination of IKKγ as well in cell lines (19) and in MALT lymphomas (19) and splenic lymphocytes of transgenic mice (20). The presence of two potential TRAF6 binding sites in the MALT1 C terminus hinted at a deregulation of the ubiquitin ligase activity of TRAF6, although thus far its interaction with MALT1 was confirmed only by overexpression studies. Here we show that the A7M8 fusion variant that lacks the Ig domains of MALT1 binds...
Role of TRAF6 in NF-κB Signaling by API2-MALT1 Fusions

TRAF6 solely via its C-terminal TRAF6 binding site T6-C2 (PVEITTD), which explains the dominant negative effect of the E806A mutation on its NF-κB signaling potential (20). In contrast, fusion variants A7M5 and A7M3, which contain one or both Ig domains, respectively, recruit TRAF6 via an additional binding site (T6-Ig) just downstream of the second Ig domain of MALT1. Both TRAF6 binding sites contribute to A7M3- and A7M5-mediated signaling to NF-κB. Simultaneous occupation of the two TRAF6 binding sites might therefore cause the increased signaling potential associated with these variants. However, this seems to contradict a recent report showing that TRAF6 knockdown does not affect the ability of A7M5 to activate NF-κB (19), which could be because of a partial knockdown of TRAF6 in these experiments. Indeed, lack of TRAF6 function abolishes NF-κB activation by the API2-MALT1 fusions (Fig. 2E).

Polyubiquitination of IKKγ by TRAF6 requires in addition an E2 ubiquitin-conjugating enzyme, and an essential role for UBC13 was hypothesized, as deletion of the Ig-like domain in the MALT1 C terminus of A7M5 abolished both the interaction with overexpressed UBC13 and NF-κB activation (19). We could not confirm an interaction between API2-MALT1 and endogenous UBC13; however, we noticed that a similar deletion in A7M3 completely abolished TRAF6 binding and NF-κB activation, possibly because of folding artifacts. The direct interaction between TRAF6 and UBC13 (28) suggests an interaction of UBC13 with MALT1 via TRAF6 rather than a direct binding to its Ig-like domain, further strengthening the essential role of TRAF6 for NF-κB activation by all fusion variants.

Our binding studies revealed preformed BCL10-MALT1-TRAF6 complexes in unstimulated HEK-293T and Jurkat cells, which was also suggested previously via colocalization studies (21). Recruitment and clustering of these complexes at the membrane rafts upon T-cell receptor triggering or their concentration in so-called POLKADOTS is thought to represent an essential step in T-cell receptor signaling propagation to NF-κB (10, 12–14, 21). This could not only facilitate the interaction with downstream effectors as the IKK complex, but it could also oligomerize TRAF6 and evoke its ubiquitin ligase activity resulting in polyubiquitination of IKKγ and NF-κB activation. Simply targeting the TRAF6 binding site of the C terminus of MALT1 to the lipid rafts is indeed sufficient to trigger IKKγ polyubiquitination (20). Similarly, recruitment of MALT1 to the lipid rafts in HEK-293T cells upon co-expression of BCL10 is linked to its potential to activate NF-κB with BCL10 synergistically. As the synergism also requires functional TRAF6 binding, this further strengthens the link between raft association of MALT1 and TRAF6 oligomerization.

Permanent raft association of the API2-MALT1 fusion was observed in splenic lymphocytes of A7M8 transgenic mice (20) and in A7M5-expressing BJAB-cells (29), coinciding with increased IKKγ polyubiquitination and NF-κB activation, respectively. We hypothesized that raft association of the A7M8-TRAF6 complex in this way could shortcut antigen receptor signaling (20). However, deletion of BIR1, which prevented raft recruitment of the fusion protein (20), abolished not only NF-κB signaling (20) but also TRAF6 binding to A7M8, thereby jeopardizing our hypothesis. Reduced NF-κB signaling by A7M8 after disruption of rafts via cholesterol depletion then again argues for a role of rafts in NF-κB signaling (20). This role was further confirmed by the BIR1 deletion constructs of A7M5 and A7M3 that also prevented their raft association. Failure to localize to the rafts blocked NF-κB activation by A7M5 without affecting the interaction with TRAF6. The residual NF-κB activity for the BIR1 deletion construct for A7M3 could be assigned to an intramolecular TRAF6 activation event, based on the necessity of two functional TRAF6 binding sites for NF-κB activation. The failure of the BIR1 deletion construct of A7M5 to activate NF-κB (even though it harbors two functional TRAF6 binding sites) therefore reflects either a conformational constraint or a role for the first Ig domain of MALT1 herein.

Because our data indicate that rafts play an important role in NF-κB activation by all fusion variants, the reduced signaling potential of A7M5/3 mutated for the TRAF1/2 binding site could result from reduced raft association mediated by TRAF1/2. Both adaptors bind to the cytosolic domain of tumor necrosis factor receptor (TNFR) family members of which some are reported to be associated with rafts without receptor stimulation (30). However a complementary
Role of TRAF6 in NF-κB Signaling by API2-MALT1 Fusions

TRAF2-independent mechanism of raft recruitment, mediated via BIR1, must also be present, as the ER-AA constructs could still be found in the rafts in contrast to the BIR1 deletion constructs. This further explains why the BIR1 deletion reduces NF-κB activation by A7M5/3 more efficiently than the TRAF1/2 binding site mutation. Furthermore, BIR1 also seems to be crucial for a more direct oligomerization of the fusion protein, in contrast to the TRAF1/2 binding site, which also has been implicated in NF-κB signaling by API2-MALT1 (23).

In conclusion, our analysis unravels distinct mechanisms of NF-κB activation by the API2-MALT1 fusion proteins that rely on the domains contributed by the MALT1 protein (Fig. 6). Inclusion of the second Ig domain of MALT1 in the fusion protein intensifies NF-κB signal strength, as it delivers additional TRAF6 binding potential. Further enhancement of NF-κB signaling is conveyed by the first Ig domain of MALT1 via an intramolecular TRAF6 activation event. Finally, the first BIR domain of API2 underlies the NF-κB activating potential of all fusion variants via its interaction with TRAF1/2 and a yet unknown mechanism required for their recruitment to the lipid raft membrane fractions. Purification of API2-MALT1 protein complexes and the identification of new partner proteins for this BIR domain will provide further insight into the mechanisms by which API2-MALT1 signals to NF-κB.

Acknowledgments—We thank Dr. Manolis Pasparakis, University of Cologne, Germany, for providing us with Traf6−/− MEF cells and Dr. Martin Dyer, University of Leicester, United Kingdom, for the SSK41 cells.

REFERENCES

1. Willis, T. G., Jadayel, D. M., Du, M. Q., Peng, H., Perry, A. R., Abdul-Rauf, M., Price, H., Karran, L., Majekodunmi, O., Wlodarska, I., Pan, L., Crook, T., Hamoudi, R., Isaacson, P. G., and Dyer, M. J. (1999) Cell 96, 35–45.
2. Zhang, Q., Siebert, R., Yan, M., Hinzmann, B., Cui, X., Xue, L., Rakestraw, K. M., Naeve, C. W., Beckmann, G., Weisenburger, D. D., Sanger, W. G., Nowotny, H., Vesely, M., Callet-Bauchu, E., Salles, G., Dixit, V. M., Rosenthal, A., Schlegelberger, B., and Morris, S. W. (1999) Nat. Genet. 22, 63–68.
3. Streubel, B., Lamprecht, A., Dierlamm, J., Cerroni, L., Stolte, M., Ott, G., Raderer, M., and Chott, A. (2003) Blood 101, 2335–2339.
4. Sanchez-Izquierdo, D., Buchonnet, G., Siebert, R., Gascoyne, R. D., Climent, J., Karran, L., Marin, M., Blesa, D., Horsman, D., Rosenwald, A., Staudt, L. M., Albertson, D. G., Du, M. Q., Ye, H., Marynen, P., Garcia-Conde, J., Pinkel, D., Dyer, M. J., and Martinez-Climent, J. A. (2003) Blood 101, 4539–4546.
5. Dierlamm, J., Baens, M., Wlodarska, I., Stefanova-Ouzounova, M., Hernandez, J. M., Hossfeld, D. K., Wolf-Peeters, C., Hagemeijer, A., Van den, B. H., and Marynen, P. (1999) Blood 93, 3601–3609.
6. Ruland, J., Duncan, G. S., Elia, A., del, B. I., Nguyen, L., Pyte, S., Millar, D. G., Bouchard, D., Wakeham, A., Ohashi, P. S., and Mak, T. W. (2001) Cell 104, 33–42.
7. Ruland, J., Duncan, G. S., Wakeham, A., and Mak, T. W. (2003) Immunity 19, 749–758.
8. Rueff-Brasse, A. A., French, D. M., and Dixit, V. M. (2003) Science 302, 1581–1584.
9. Gaide, O., Favier, B., Legler, D. F., Bonnet, D., Brissoni, B., Valitutti, S., Bron, C., Tschopp, J., and Thome, M. (2002) Nat. Immunol. 3, 836–843.
10. Che, T., You, Y., Wang, D., Tanner, M. J., Dixit, V. M., and Lin, X. (2004) J. Biol. Chem. 279, 15870–15876.
11. Stilo, R., Liguoro, D., Di Jeso, B., Formisano, S., Consiglio, E., Leonardi, A., and Vito, P. (2004) J. Biol. Chem. 279, 34323–34331.
12. Egawa, T., Albrecht, B., Favier, B., Sunshine, M. J., Michandchi, K., O’Brien, W., Thome, M., and Litman, D. R. (2003) Curr. Biol. 13, 1252–1258.
13. Hara, H., Bakal, C., Wada, T., Bouchard, D., Rottapel, R., Saito, T., and Penninger, J. M. (2004) J. Exp. Med. 200, 1167–1177.
14. Bidere, N., Snow, A. L., Sakai, K., Zheng, L., and Lenardo, M. J. (2006) Curr. Biol. 16, 1666–1671.
15. Zhou, H., Wertz, L., O’Rourke, K., Uitl, S., Seshagiri, S., Eby, M., Xiao, W., and Dixit, V. M. (2004) Nature 427, 167–171.
16. Sun, L., Deng, L., Li, C. K., Xia, Z. P., and Chen, Z. J. (2004) Mol. Cell 14, 289–301.
17. Lucas, P. C., Yonezumi, M., Inoza, N., Yamanaka, T., Aizawa, M. E., Chen, F. F., Yamaoka, S., Seto, M., and Nunez, G. (2001) J. Biol. Chem. 276, 19012–19019.
18. Uren, A. G., O’Rourke, K., Arvand, L. A., Pisabarro, M. T., Seshagiri, S., Koonin, E. V., and Dixit, V. M. (2000) Mol. Cell 6, 961–967.
19. Zhou, H., Du, M. Q., and Dixit, V. M. (2007) Cancer Cell 7, 425–431.
20. Baens, M., Fever, S., Sagaerts, X., Noels, H., Hagens, S., Broeckx, V., Billiau, A. D., De Wolf-Peeters, C., and Marynen, P. (2006) Cancer Res. 66,
Role of TRAF6 in NF-κB Signaling by API2-MALT1 Fusions

21. Rossman, J. S., Stoicheva, N. G., Langel, F. D., Patterson, G. H., Lippincott-Schwartz, J., and Schaefer, B. C. (2006) Mol. Biol. Cell 17, 2166–2176
22. Hu, S., Du, M. Q., Park, S. M., Alcivar, A., Qu, L., Gupta, S., Tang, J., Baens, M., Ye, H., Lee, T. H., Marynen, P., Riley, J. L., and Yang, X. (2006) J. Clin. Investig. 116, 174–181
23. Varfolomeev, E., Wayson, S. M., Dixit, V. M., Fairbrother, W. J., and Vucic, D. (2006) J. Biol. Chem. 281, 29022–29029
24. Beckett, D., Kovaleva, E., and Schatz, P. J. (1999) Protein Sci. 8, 921–929
25. Samuel, T., Welsh, K., Lober, T., Togo, S. H., Zapata, J. M., and Reed, J. C. (2006) J. Biol. Chem. 281, 1080–1090
26. de Boer, E., Rodriguez, P., Bonte, E., Krijgsve, J., Katsantoni, E., Heck, A., Grosveld, F., and Stroublouis, J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 7480–7485
27. Hosokawa, Y., Suzuki, H., Suzuki, Y., Takahashi, R., and Seto, M. (2004) Cancer Res. 64, 3452–3457
28. Wooff, J., Pastushok, L., Hanna, M., Fu, Y., and Xiao, W. (2004) FEBS Lett. 566, 229–233
29. Ho, L., Davis, R. E., Conne, B., Chappuis, R., Berczy, M., Mhawech, P., Staudt, L. M., and Schwaller, J. (2005) Blood 105, 2891–2899
30. Legler, D. F., Micheau, O., Doucey, M. A., Tschopp, J., and Bron, C. (2003) Immunity. 18, 655–664