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## Detection and measurement of paracaspase MALT1 activity

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### Summary

The paracaspase MALT1 is a Cys-dependent, Arg-specific protease that plays an essential role in the activation and proliferation of lymphocytes during the immune response.

Oncogenic activation of MALT1 is associated with the development of specific forms of B-cell lymphomas. Through specific cleavage of its substrates, MALT1 controls various aspects of lymphocyte activation, including the activation of transcriptional pathways, the stabilization of mRNAs and an increase in cellular adhesion. In lymphocytes, the activity of MALT1 is tightly controlled by its inducible monoubiquitination, which promotes the dimerization of MALT1.

Here, we describe both, *in vitro* and *in vivo* assays that have been developed to assess MALT1 activity.

**Key words** : immunomodulation; NF-kB; lymphocyte activation; lymphoma

**Running head:** Detection of MALT1 activity

## 1. Introduction

The gene encoding MALT1 was initially identified as a target of a chromosomal translocation occurring in B-cell lymphomas of the mucosa-associated lymphoid tissue (MALT lymphomas), which results in the formation of an oncogenic IAP2-MALT1 fusion protein [1]. Independently, MALT1 was described as a protein with sequence homology to proteins of the caspase and metacaspase families, and named paracaspase to account for this fact [2]. Subsequent genetic and biochemical studies have revealed a key function for MALT1 in the adaptive immune response, and in the development of diffuse large B-cell lymphomas (DLBCL) of the activated B-cell (ABC) subtype [3-5]. The most important function of MALT1 in lymphocytes seems to be its capacity to promote the activation of the transcription factor NF- $\kappa$ B [6,7] via both, its scaffold and its enzymatic function [8]. The activation of NF- $\kappa$ B then drives the expression of genes that are relevant for cellular proliferation and survival [9].

MALT1 contains a caspase-like domain that has high structural similarity to the protease domain of caspases [10,11] and that shares the presence of a conserved active site, the Cys-His dyad, with caspases and metacaspases [2]. However, MALT1 showed no caspase-like activity [2,12]. Therefore, it was initially thought that MALT1 contributes to NF- $\kappa$ B activation mainly by its scaffold function, through physical recruitment of signaling proteins. This would in turn promote the activation of the IKK complex, which phosphorylates the NF- $\kappa$ B inhibitor I $\kappa$ B to induce its proteasomal degradation and to allow NF- $\kappa$ B to enter the nucleus and initiate transcription [9]. This concept is supported by the discovery that MALT1 binds the ubiquitin ligase TRAF6 [13] and, upon its TRAF6-mediated polyubiquitination, physically recruits the IKK complex [14]. Nevertheless, it was noticed in earlier studies that mutation of the conserved Cys residue in the caspase-like domain reduced the capacity of MALT1 or an oncogenic IAP2-MALT1 fusion protein to promote the activation of the NF- $\kappa$ B pathway [2,15]. In 2008, two groups then independently reported the MALT1-dependent cleavage of Bcl10 and A20 directly C-terminal to an Arg residue, identifying MALT1 as an Arg-specific protease [16,17]. The finding of an Arg-directed proteolytic activity of MALT1 is well compatible with prior studies reporting a similar Arg- or Lys-directed cleavage activity of

metacaspases [18]. Indeed, both metacaspases and the MALT1 paracaspase contain conserved negatively charged residues in the substrate-binding S1 pocket that explain their preferential affinity for substrates with a positively charged Arg (or Lys) residue [18].

Over the last few years, several substrates of MALT1 have been identified, and the distinct roles of the cleavage of these individual substrates in the antigen-driven activation and proliferation of lymphocytes have been dissected. An important function of MALT1 in lymphocytes is to promote the activation of the transcription factor NF- $\kappa$ B [6,7] via both, its scaffold and its enzymatic function [8]. The scaffold function of MALT1 is required for IKK-mediated NF- $\kappa$ B activation [19,20]. That the protease activity of MALT1 is indeed similarly relevant for the NF- $\kappa$ B-dependent activation of lymphocytes was then established by the development of a MALT1 inhibitor [16] and by expression of catalytically inactive mutants of MALT1 in lymphocyte cell lines [17]. The protease activity of MALT1 controls NF- $\kappa$ B activation by cleaving the NF- $\kappa$ B family member RelB [20], which acts as negative regulator of T-cell activation [21]. RelB binds to the NF- $\kappa$ B subunits RelA- and c-Rel in the cytoplasm of lymphocytes [22,20]. Therefore, MALT1-dependent cleavage of RelB and its subsequent proteasomal degradation are required to allow the DNA binding of RelA- or c-Rel-containing NF- $\kappa$ B complexes in the nucleus [20]. MALT1-dependent cleavage of the deubiquitinating enzyme A20 has been proposed as another way to promote NF- $\kappa$ B activation [17]. Since A20 can negatively regulate the activity of the IKK complex by deubiquitination of the IKK subunit NEMO [23], it was initially proposed that MALT1-dependent cleavage of A20 might serve to prolong the IKK-dependent NF- $\kappa$ B response [17]. However, pretreatment of cells with the MALT1 inhibitor does not affect IKK activity [19,20], suggesting that A20 cleavage must contribute to lymphocyte activation in a distinct manner that remains to be identified.

MALT1-dependent cleavage of the deubiquitinating enzyme Cyld [24] and the adapter protein Bcl10 [16] promote the activation of the AP-1 transcriptional pathway and the adhesiveness of T cells, respectively, to regulate two other important aspects of lymphocyte activation. Recently, MALT1 was shown to promote T-cell activation in yet an additional manner, by cleavage of the RNase Regnase-1 (also known as MCP1P1 or Zc3h12a) [25].

MALT1-dependent cleavage of MCPIP-1 leads to the stabilization of mRNAs of T-cell effector genes, such as growth-promoting cytokines [25]. Thus, MALT1 controls lymphocyte activation by transcription-dependent and –independent means.

The Ser/Thr kinase NIK has been identified as a specific substrate of the IAP2-MALT1 fusion protein that results from a chromosomal translocation found in aggressive forms of MALT lymphoma [26]. NIK is a protein with a short half-life that is normally rapidly turned over by proteasomal degradation. The cleavage of NIK by the IAP2-MALT1 fusion protein results in the generation of a stable C-terminal NIK fragment, containing the kinase activity [26]. The resulting active NIK fragment promotes the phosphorylation of the IKK subunit IKK $\alpha$ , which in turn drives the activation of the so-called alternative NF- $\kappa$ B pathway by the phosphorylation-dependent processing of the inactive NF- $\kappa$ B precursor p100 to form the biologically active p52 subunit. Thereby, the IAP2-MALT1 fusion seems to constitutively activate the alternative NF- $\kappa$ B pathway in MALT lymphomas [26].

In addition to its well-described role in the adaptive immune response, MALT1 is also required for NF- $\kappa$ B activation downstream of other immune receptors that activate NK cells, mast cells, dendritic cells or myeloid cells, and also downstream of certain G-protein coupled receptors or the receptor tyrosine kinase EGFR [3]. However, at present it remains largely unknown to which extent the protease activity of MALT1 contributes to these functions.

How can the enzymatic activity of MALT1 be monitored? One line of experimental evidence for MALT1 protease activity comes from the detection, by Western blotting, of the presence of cleaved MALT1 substrates in cellular lysates of activated lymphocytes or lymphoma cells [16,20,17,24,26]. In addition, based on the cleavage site LRSR'G present in Bcl10, an *in vitro* assay was developed that uses the cleavage of a fluorogenic Ac-LRSR-AMC substrate by purified MALT1 or cellular lysates containing active MALT1 as a means to monitor MALT1 activity [16]. This *in vitro* cleavage assay has been further improved by the systematic testing of tetrapeptides in which the three amino acids preceding the fixed C-terminal Arg residue were systematically varied [27]. Amongst the best tetrapeptide substrates identified with this approach were peptides containing a Ser residue in the P2

position preceding the Arg (P1) residue, which is a feature that is common to 5 of the 6 presently identified substrates [16,17,20,24,26]. The P4 position showed a clear preference for Leu, a feature that is also present in the MALT1 substrates Bcl10, RelB and Regnase-1 [20,16,25]. In order for purified recombinant MALT1 to be present in an active form, several approaches have been used that all favor dimerization or oligomerization of MALT1. In one study, recombinant GST-MALT1 protein was oligomerized by its binding to glutathione-sepharose/agarose beads [16]. Alternative approaches have been to generate fusion proteins of MALT1 with bacterial gyrase B or a leucine zipper domain [17,28], or to perform assays in presence of a kosmotropic salt such as ammonium citrate, which is known to activate certain caspases by favoring their dimerization [29,30]. Like caspases, MALT1 has a propensity to dimerize via its caspase-like domain, as evident from recently published crystallographic structures [10,11]. The comparison of MALT1 crystals obtained in the absence or presence of a substrate analog, the irreversible tetrapeptide inhibitor z-VRPR-fmk, has revealed that while both conditions yielded MALT1 dimers, the dimer structure undergoes extensive conformational changes upon inhibitor binding, which lead to changes in the contact sites of the dimerization interface and a change in the orientation of the protease domain towards the C-terminal immunoglobulin (Ig)-like domain [10]. Thus, MALT1 dimerization or the adoption of a precise conformation of the dimer may be induced upon substrate binding. *In vivo*, MALT1 activation and dimerization requires an inducible monoubiquitination of MALT1 on a Lys residue (K644) that is localized within the C-terminal Ig-like domain [31]. A monoubiquitination-deficient K644R mutant of MALT1 was unable to sustain the activation of T cells and the growth of cell lines derived from ABC DLBCL [31] that critically depend on oncogenic MALT1 activity [32,33]. In contrast, an in-frame fusion of MALT1 to a C-terminal ubiquitin moiety resulted in a hyperactive MALT1-Ub construct that constitutively dimerizes [31]. Thus, monoubiquitination of MALT1 most likely induces a conformational change that promotes the formation or stabilization of a constitutively active MALT1 dimer *in vivo*.

Below, we describe various methods that allow the detection of MALT1 activity, including the isolation of recombinant active MALT1 from bacteria, the detection of the cleavage of endogenous MALT1 substrates or of MALT1 monoubiquitination, and the quantification of MALT1 activity using fluorogenic substrate peptides or a FRET-based reporter construct.

## 2. Materials

Deionized water is used throughout. All reagents are prepared and stored at room temperature (unless indicated otherwise).

### 2.1 Reagents and materials needed for the purification of MALT1 from bacteria

1. Plasmid coding for GST-MALT1: generated by cloning the MALT1 sequence into a vector allowing bacterial protein expression (such as pGEX from GE Healthcare).
2. LB agar dishes and LB medium with 100 µg/ml ampicillin.
3. Chemically competent BL21 bacteria (e.g. One Shot from Invitrogen).
4. Isopropyl-b-D-thiogalactopyranoside (IPTG, Fluka, No.59740), PreScission protease (GE Healthcare, No.27-0843-01), Glutathione Sepharose 4B (GE Healthcare, No. 17-0756-01) and Poly-Prep columns (Bio-Rad, No. 731-1550).
5. Lysis buffer: 50 mM Hepes (pH 7.9), 300 mM NaCl, 1 mM EDTA, 0.1% (v/v) NP-40 and 5 mM DTT. Always add DTT freshly before use, the other components can be mixed in advance and stored at 4°C for a few weeks (see **Note 1**).
6. Wash buffer: 50 mM Tris-HCl (pH 7.6) and 150 mM NaCl. 5 mM DTT should be added before use.
7. SDS-PAGE reagents and Coomassie staining solution.
8. Bacteria incubator (37°C and 18°C), spectrophotometer (wavelength 600 nm), refrigerated centrifuge, French press, shaker or rotation wheel and SDS-PAGE components.

## **2.2 Reagents and materials for the MALT1 *in vitro* cleavage assay**

1. Tetrapeptide substrate (Ac-LVSR-AMC or Ac-LRSR-AMC, Peptides International).
2. Cleavage assay buffer: 50 mM MES (pH6.8), 150 mM NaCl, 0.1 % (w/v) CHAPS and 1 M ammonium citrate. 10 mM DTT has to be freshly added before use.
3. Black 96-well plates (e.g. OptiPlate-96F, Perkin Elmer).
4. Microplate reader capable to excite at 350-380 nm and measure the emission at 460 nm (e.g. Synergy microplate reader, BioTek).

## **2.3 Reagents and materials for the BCL10 and Malt1 Western Blot**

1. Acrylamide (30% w/v), bis-acrylamide (1% w/v), ammonium persulfate (10 %) and TEMED.
2. Resolving buffer: 1.5 M Tris-HCl pH 8.8.

## **2.4 Reagents and materials for the FRET-based MALT1 activity assay**

1. Reporter plasmid: constructed by linking eYFP and eCFP with the MALT1 cleavage recognition site LVSR, which originates from the MALT1 substrate RelB [31,20]. A non-cleavable form with the linker sequence LVSG can be used as a control. Both constructs were cloned into a vector derived from pCR3 (Invitrogen).
2. Dulbecco's Modified Eagle Medium (DMEM, e.g. No. 10565-018, Gibco) with 10% fetal calf serum (FCS) and antibiotics.
3. 6-well cell culture plate (e.g. No. 92006, TPP).
4. 2xHeBS buffer, prepared by dissolving 16.4 g NaCl, 11.9 g Hepes (acid form) and 0.2 g Na<sub>2</sub>HPO<sub>4</sub> in 800 ml water. Titrate precisely to pH 7.05 with 5 M NaOH. Complete to 1 l and sterilize by filtration (0.22 µm).
5. Sterile 2.5 M CaCl<sub>2</sub> solution.
6. Flow cytometry buffer: 1% FCS and 1 mM EDTA in PBS.



### 3. Methods

All incubation steps are at room temperature unless otherwise stated.

#### 3.1. Purification of active recombinant MALT1 from bacteria

1. To transform BL21 chemically competent E. coli with the GST-MALT1 (pGEX) plasmid by heat-shock, thaw the bacteria on ice, add 10 – 100 ng of the plasmid, mix by tapping gently (see **Note 2**) and incubate the vial on ice for 30 min.
2. Heat-shock the bacteria for 30 sec at 42°C in water bath without shaking.
3. Spread up to 200 µl from the transformation on a pre-warmed LB plate with 100 µg/ml ampicillin and incubate overnight at 37°C.
4. Resuspend one single colony in a 25 ml LB liquid culture with ampicillin (100 µg/ml ampicillin) to produce a starter culture by incubation overnight at 37°C with shaking.
5. Inoculate 500 ml LB media with antibiotic with 20 ml of the starter culture.
6. Incubate at 37°C with shaking until OD<sub>600</sub> reaches 0.6.
7. Place the bacteria culture on ice for 10 min.
8. Induce the expression of GST-MALT1 by adding 40 µM IPTG and incubate the bacteria overnight at 18°C with shaking.
9. Pellet the bacteria by centrifugation at 4000 rpm for 20 min at 4°C.
10. Resuspend the pelleted bacteria with 15 ml of lysis buffer, incubate for 20 min on ice and lyse the bacteria with a French Press at 4°C.
11. Dilute the lysate with one volume of PBS.
12. Transfer the lysate to 2 ml vials and centrifuge for 15 min at 16'000 g and 4°C in a table-top centrifuge.
13. Collect the supernatant into a falcon tube (see **Note 3**) and take 100 µl for Western Blot analysis of the lysate (see **Note 4**). For better storage of the lysate antimicrobial inhibitors can be added (see **Note 5**).

14. 300  $\mu$ l of Glutathione-(GSH)-Sepharose beads must be washed three times with PBS (see **Note 6**) and incubated with the lysis supernatants for 2.5 h at 4°C on a shaker or a rotation wheel.
15. Transfer the beads with the supernatant into a column.
16. Wait until only beads are left in the column (do not let it run dry).
17. Wash the beads three times with 15 ml cold washing buffer at 4°C.
18. Wait until the column stops dripping, close the column and add 1.2 ml washing buffer with 80 units/ml PreScission protease (see **Note 7**).
19. Incubate for 2 h at 4°C to remove MALT1 from the GSH-beads.
20. Collect the flow through (see **Note 8**) and load 10  $\mu$ l together with the lysate and a BSA standard (0.5, 1, 2, 5 and 10  $\mu$ g) on a SDS-PAGE and stain the gel with Coomassie Blue to estimate the concentration of MALT1 in the solution.
21. The flow through can now be used for further experiments, but also stored at -80°C.

### **3.2. *In vitro* MALT1 cleavage assay of fluorogenic peptides**

1. Prepare cleavage assay buffer and add the tetrapeptide substrate (Ac-LRSR-AMC or Ac-LVSR-AMC, see **Note 9**) at a final concentration of 25  $\mu$ M.
2. Add 95  $\mu$ l of the cleavage assay buffer with the substrate and 2  $\mu$ g of soluble MALT1 (in a volume of 5  $\mu$ l) into the well of a black 96 well plate.
3. Cleavage assay buffer with substrate only – no MALT1 added - serves as a negative control; we also include the protease inactive mutant (MALT1 C464A) as a negative control (see **Note 10**).
4. Remove air bubbles in the wells since they might interfere with measurement.
5. Measure the fluorescence (excitation 350-380 nm, emission 460 nm) over time (measure every 10 min for 2-4 h) at 30°C.
6. To get the MALT1 protease activity, calculate the slope of the fluorescence over time and normalize to the amount of MALT1 protein (see **Note 11**).

### **3.3. Detection of endogenous BCL10 cleavage or Malt1 monoubiquitination by high-resolution gels and Western Blot**

The detection of Malt1 substrate cleavage or Malt1 monoubiquitination can serve as a qualitative means to monitor Malt1 activity. The MALT1 mediated cleavage of A20, CYLD, RelB and Regnase-1 can easily be visualized by standard Western Blot [17,24,20,25]. Only the detection of cleaved human BCL10 is challenging since MALT1 removes only 5 amino acids from its C-terminus. Cleaved BCL10 can therefore only be detected using high-resolution gels [16] or an antibody specific for cleaved BCL10 [32]. Moreover, the 8 kDa shift caused by the attachment of a single ubiquitin to Malt1 is more easily detectable using high-resolution gels. Here, we describe only the preparation of the high-resolution gels [34] for detection of cleaved BCL10 and Malt1 monoubiquitination since the further procedure does not vary from standard SDS-PAGE and Western blot protocol.

1. To prepare a 500 ml stock solution of a high resolution 15% acrylamide resolving gel mix to detect cleaved BCL10, add 250 ml acrylamide (30% w/v), 43 ml bis-acrylamide (1% w/v), 125 ml resolving buffer and 82 ml water. For a 7,5% high-resolution acrylamide gel mix, which is optimal to detect Malt1 monoubiquitination, add 125 ml acrylamide (30% w/v), 97 ml bis-acrylamide (1% w/v), 125 ml resolving buffer and 150 ml water (see **Note 12**).
2. Add 50  $\mu$ l of 10% ammonium persulfate and 5  $\mu$ l of TEMED per 10 ml of mix. Cast the gels within minutes and gently overlay with isopropanol.
3. Proceed with standard SDS-PAGE and Western blot protocol.

### **3.4. FRET-based assay of protease activity**

To quantify MALT1 protease activity in intact cells we generated an eYFP–Leu-Val-Ser-Arg–eCFP expression construct as well as the respective non-cleavable negative control, eYFP–Leu-Val-Ser-Gly–eCFP [31]. Here we describe the use of the FRET-based cleavage assay in HEK293T cells, but it might be adapted to other cells.

1. Plate approximately 150.000 HEK293T cells in DMEM (with 10% FCS and antibiotics) in a 6-well cell culture plate.
2. After 24 h, transfect the sub-confluent HEK293T cells with 0.1 µg of the reporter construct together with 1 µg MALT1 and 0.2 µg BCL-10 using the *calcium phosphate transfection* method. Mix 125 µl water with 12.5 µl CaCl<sub>2</sub> (2.5 M), add the respective amount of plasmids and add 125 µl 2xHeBS dropwise while gently shaking on a vortex.
3. Incubate the mix for 10 min at room temperature. Distribute the transfection mix carefully and equally on the cells.
4. Change the media 6 to 14 h after transfection with fresh completed DMEM.
5. 24 h after onset of transfection, add 1 ml of PBS to the wells and detach the cells with a 1 ml pipette by washing the cells off (by physical force).
6. Spin the cell suspension for 1 min at 1'000 g in a table-top centrifuge and after removing the PBS, add flow cytometry buffer.
7. Filter the cells into suitable FACS tubes through a 70 µm mesh to remove cell clumps.
8. Analyse the cells with a suitable flow cytometer containing a 405 nm laser (e.g. LSR II, BD Biosciences). For measurement of the eCFP and FRET signal, the 405-nm laser excited the transfected cells with a standard 450/50 filter for collection of the eCFP fluorescence and a 585/42 filter for FRET fluorescence, respectively. For an example of the FACS analysis please see the reference by Pelzer et al., 2013.
9. The expression levels of all constructs and the proportion of reporter cleavage can be controlled by Western Blot (see **Note 12**).

#### 4. Notes

1. DTT prevents the oxidation of the cysteine, which is located in the active site of MALT1.
2. Do not mix the bacteria by pipetting.
3. At this step the lysate can be stored at -20°C for several months.
4. To confirm the induction of protein expression, the lysate can be loaded immediately on a SDS-PAGE gel followed by a Coomassie Blue staining. GST-MALT1 migrates at around 115 kDa.
5. To protect the supernatants from microbial growth add sodium azide (5% w/v stock solution, use 1:1000).
6. To wash the beads, add at least 1 ml PBS to the beads and centrifuge at 1'000 g for 1 min in a table-top centrifuge to remove the ethanol. Repeat two times.
7. To increase the yield of MALT1 protein, the beads can be washed several times with 1 ml washing buffer. The respective collected fractions will still contain MALT1 protease even if the activity is lower than the first fraction.
8. MALT1 activity can be induced by concentrating it on beads or by the use of kosmotropic buffers, which favor MALT1 dimerization. Therefore, MALT1 protease activity is also present when GST-MALT1 is bound to glutathione-coupled beads. This approach might be considered if the planned experiment is not compatible with the presence of kosmotropic salts.
9. The sequence of the Ac-LRSR-AMC peptide derives from the substrate BCL-10 [16], whereas Ac-LVSR-AMC originates from RelB [20]. MALT1 protease activity for the RelB- mimicking substrate is roughly four times higher than for Ac-LRSR-AMC [20]. Both peptides can be stored frozen at a concentration of 10 mM dissolved in DMSO.
10. In case protease inactive recombinant MALT1 is not available, the addition of 1  $\mu$ M of the MALT1 inhibitor Z-VRPR-FMK (Bachem) might be useful as additional negative control.

11. Only consider the linear part of the fluorescence increase for the calculation of the MALT1 activity. During the first 20 min there might be variations in the fluorescence data due to mixing effects.
12. The stock solutions can be stored at 4°C for a few weeks. Please note that the acrylamide/bis-acrylamide ratio is much higher than in standard commercial pre-mixes.
13. Using an anti-GFP antibody (e.g. ALX 210-199; Enzo LifeSciences) the full-length eYFP-LVSR-eCFP (around 48 kDa) but also the cleaved fragments (around 24 kDa) are detectable since both YFP and CFP are recognized by the antibody.

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