The Bcl10/paracaspase signalling complex is functionally conserved since the last common ancestor of planulozoa.

Jens Staal[1,2], Yasmine Driege[1,2], Paco Hulpiau[1,2], Rudi Beyaert[1,2]

[1] Inflammation Research Center, VIB, Ghent, Belgium

[2] Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium

Abstract

A single MALT1, cleavage and CLAP : an old story. The type 1 paracaspases are defined by their domain composition with an N-terminal Death-domain, immunoglobulin domains and a caspase-like (paracaspase) domain. Type 1 paracaspases originated in the edicarian geological period before the last common ancestor of bilaterans and cnidarians (planulozoa). Most organisms have a single type 1 paracaspase, except vertebrates where a triplication occurred in the ancestral jawed vertebrate. Mammals have lost two of the paralogs and only contain a single paracaspase (paracaspase-1, MALT1). Cnidarians have several paralog type 1 paracaspases, type 2 paracaspases, and a homolog of Bcl10 (CLAP). Notably in bilaterans, lineages like nematodes and insects lack Bcl10 whereas other lineages such as vertebrates, annelids, molluscs and acorn worms do contain Bcl10. There seems to be a correlation where invertebrates with Bcl10 have type 1 paracaspases which are more similar to the paracaspases found in vertebrates. A proposed evolutionary scenario includes two ancestral type 1 paracaspase paralogs in the bilateran last common ancestor, where one paralog usually is dependent on Bcl10 for its function. Functional analyses of invertebrate type 1 paracaspases and Bcl10 homologs support this scenario and indicate an ancient origin of the Bcl10/paracaspase signalling complex.

Introduction

The paracaspase MALT1 (PCASP1) was originally identified in humans as an oncogenic fusion with IAP2 in low-grade antibiotic-resistant MALT lymphomas [1]. Later, it was discovered that MALT1 is a critical component in T and B cell antigen receptor signalling as part of the CARMA1-Bcl10-MALT1 (CBM) complex[2 3 4]. More studies made it clear that MALT1 plays a role in several different CARD*-Bcl10-MALT1 complexes in many different types of signalling pathways, where currently known CARD* components are CARD9, CARD11 (CARMA1), CARD14 (CARMA2) and CARD10 (CARMA3). The use of the different CARD proteins in the CBM complexes is most likely mostly dependent on cell-type specific expression [5]. Despite being identified as a “paracaspase” due to sequence similarity with the true caspases[6], it was not until much later that a proteolytic activity of MALT1 was established [7 8]. In contrast to true caspases, but similar to metacaspases, the paracaspase MALT1 cleaves substrates specifically after an arginine residue [9 10 11]. Lately some protein substrates have been identified which are cleaved after a lysine by the API2-MALT1 oncogenic fusion [12]. The proteolytic activity of MALT1 was found to also be critical for another type of B-cell lymphoma, ABC-DLBCL [13]. MALT1 cleaves itself [14] and its interacting adaptor protein Bcl10 [8], the anti-inflammatory deubiquitinases A20 [7] and CYLD [15], the NF-κB member RelB [16], the ubiquitin ligase HOIL-1 [17 18], and the specific RNA degrading enzymes Regnase [19] and Roquin [20]. The anti-inflammatory role of many of the known protease substrates coupled with the critical role for MALT1 in inflammatory signalling has sparked an interest in targeting MALT1 protease activity as a therapeutic strategy treatment of autoimmune diseases. Although MALT1 has been clearly associated to NF-kB activity, its protease activity plays a more subtle role being specifically required for c-Rel activation [21 22 16 14]. There are mounting evidence that MALT1 also regulate other pathways, such as JNK/AP-1 [15], mTORC1 [23] and possibly WNT [24]. MALT1 belongs to the

type 1 paracaspase family, which consist of an N-terminal Death domain, immunoglobulin domains and a paracaspase domain [25]. The type 1 family of paracaspases originated sometime during the edicarian geological period, preceding the last common ancestor of bilaterans and cnidarians [25] [26]. Importantly, some organisms such as *C. elegans* contain a conserved type 1 paracaspase but lack NF-κB [27], which indicate that other roles or mechanisms might be responsible for the conservation of the general domain organization of the type 1 paracaspases [25]. Apart from functional studies of MALT1 in human and mouse models, investigating the evolutionary history of the type 1 paracaspases and its interacting proteins in alternative model systems could provide important clues to yet-unknown roles and functions of MALT1[25]. Finding those alternative functions of MALT1 could be important for future MALT1 inhibitor-based therapies [28].

Materials & Methods

Sequences of type 1 paracaspases and Bcl10
Protein sequences of type 1 paracaspase and Bcl10 homologs were retrieved from NCBI (https://www.ncbi.nlm.nih.gov), Ensembl (http://www.ensembl.org), JGI (http://genome.jgi.doe.gov/) and ICMB (https://imcb.a-star.edu.sg) using BLASTP [29]. All sequences used in the analyses can be found in supplemental material.

Sequence alignment and phylogenetic analysis
Sequence alignment was performed on the full sequence, using the T-coffee (http://www.tcoffee.org/) [30] algorithm. Phylogenetic analysis was performed with the MrBayes (http://mrbayes.sourceforge.net/) [31] method. Both alignments and phylogenetic analyses were performed using UGENE (http://ugene.net/) [32] on Arch (http://www.archlinux.org) Linux [33]. The PCASP2 branch was manually rotated in the scalable vector graphics (svg) file without changing any branch lengths using inkscape (https://inkscape.org) to avoid overlapping lines in the tree.

Cloning of Bcl10 and paracaspase homologs
The starlet sea anemone (*Nematostella)* type 1 paracaspase paralog “A” (LMBP:) and zebrafish PCASP3 (LMBP: 9573) were cloned previously[25]. The *Nematostella* type 1 paracaspase paralogs “A” (LMBP:) and “B” (LMBP: xxxx) and pacific oyster (*C. gigas*, LMBP: xxxx) were cloned behind the human ETV6 HLH domain for dimerization-induced activation as described previously[25]. Human (LMBP: 9637), zebrafish (LMBP: 9665), pacific oyster (LMBP: 9666) and *Nematostella* (LMBP: xxxx) Bcl10 were cloned in the pCAGGS vector with an N-terminal E-tag.

Cell culture, transfection and expression analysis
MALT1 KO HEK293T cells (clone #39)[25] were grown under standard conditions (DMEM, 10% FCS, 5% CO_2, 37 ^oC) and transfected with the calcium phosphate method [34]. For evaluation of the conservation of cleavage activity, the HLH-fused paracaspase constructs of were co-transfected with wild-type CYLD (LMBP: 6613) or uncleavable CYLD-R324A (LMBP: 6645) mutant. For evalutation of conservation of NF-kB induction, the three HLH paracaspase fusions were co-transfected with a NF-kB luciferase reporter (LMBP: 3249) and actin promoter-driven β-galactosidase (LMBP: 4341) as transfection control. For evaluation of the functional conservation of the Bcl10 homologs, the Bcl10 homologs were transfected with the luciferase reporter and beta-galactosidase in the MALT1 KO HEK293T cells with or without reconstitution with human MALT1 (LMBP: 5536).

Results & Discussion

Correlation vertebrate-like type 1 paracaspases and presence of Bcl10.

Alignment of several type 1 paracaspases show that type 1 paracaspases from species that contan Bcl10 (molluscs, annelids, hemichordates) cluster closer to the vertebrate paracaspases (Figure 1), indicating a conserved common Bcl10-dependent ancestor. In contrast, Bcl10 sequences appear to evolve in a manner similar to how the species have diverged throughout evolution (Figure 2).

Functional conservation of invertebrate type 1 paracaspases and Bcl10

Based on BLASTP, mollusc paracaspases were identified as the non-deuterostome homologs most closely resembling vertebrate type 1 paracaspases, and the pacific sea oyster (*C. gigas*) was used as a model for the molluscs. Conversely, the most distantly related species where type 1 paracaspases and Bcl10 could be found, the starlet sea anemone (*Nematostella vecterensis*) was used as a source for as divergent proteins as possible. In order to investigate the functional conservation of invertebrate type 1 paracaspases, we evalutated artificially activated paracaspases fused to the ETV6 HLH domain. As positive control, the currently most distantly related paracaspase with conserved activity (zebrafish PCASP3) was used. In an NF-κB luciferase assay, only the activated zebrafish PCASP3 could induce the reporter, indicating that the pacific oyster (CgPCASP) and the two starlet sea anemone type 1 paracaspase paralogs (NvPCASP-t1A, NvPCASP-t1B) could not recruit critical downstream signalling components such as TRAF6 (Figure 3A). In contrast, evaluation of protease activity revealed that the pacific oyster paracaspase specifically cleaves human CYLD at R324, just like vertebrate paracaspases (Figure 3B). The “A” paralog from starlet sea anemone could not cleave CYLD at all and the “B” paralog appeared to cleave CYLD, but at a different residue.
To further investigate the functional conservation of the Bcl10/paracaspase co-evolution, we transfected human, zebrafish, pacific oyster and starlet sea anemone Bcl10 in MALT1 KO HEK293T cells with or without reconstitution with human MALT1. Strikingly, the starlet sea anemone Bcl10 could induce human MALT1-mediated NF-kB induction and appears to be modified and possibly cleaved, which is similar to human and zebrafish Bcl10 (Figure 3C). In contrast, the pacific oyster Bcl10 failed to induce any NF-κB reporter activity, which might be due to its small size. It will be interesting to see if future annotations of the mollusc genomes will establish a longer transcript encoding for a functional Bcl10 homolog.

Future challenges

Previous studies has shown that the MALT1-like activities are conserved at least as far back as the last common ancestor of the three vertebrate type 1 paracaspase paralogs[25]. Similarly, also Bcl10 has been shown to be functionally conserved as far back as zebrafish [35]. We have now shown that Bcl10 and MALT1-like activities from type 1 paracaspases are considerably older. The observation that invertebrate type 1 paracaspases from organisms that also contain Bcl10 are more similar to the vertebrate paracaspases provides a new interesting perspective on the functional evolution of MALT1. We still don’t know how far back that MALT1-like activities such as TRAF6 interaction and NF-kB induction, protease activity and specificity are conserved. With the observation that mollusc paracaspases have conserved protease activity and specificity, but fail to induce NF-κB in a human cellular background, we are starting to unravel the sequence of evolutionary events leading to the current MALT1 activities in humans. The observation that cnidarian Bcl10 can activate human MALT1 indicates a highly conserved interaction surface between the two proteins. This type of conservation could be used to further model the interaction surfaces using evolutionary data [36]. A major future challenge will be to collect and functionally evaluate more invertebrate type 1 paracaspase and Bcl10 homologs to verify the proposed correlation of a Bcl10-dependent ancestral type 1 paracaspase paralog with MALT1-like activity. There are several aspects that are yet not clear, for example can no Bcl10 homolog currently be found in lancelets, which clearly have a PCASP3 ortholog.

The current model proposes an ancient parallel evolution of a Bcl10-dependent and a Bcl10-independent paracaspase. An alternative scenario is that Bcl10-independence has evolved several times independently. In order to further clarify this, more invertebrate sequences from informative phyla are needed[37]. Several proteins associated to MALT1 in humans are conserved as far back as cnidarians, such as Bcl10, TRAF6, TRAF2 and CYLD[25]. Investigating early-diverging biological systems such as the cnidarian model organism *Nematostella* [38] for protein interactions and signal transduction mechanisms could further pin-point the original and most conserved functions.

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Supplemental material
Supplemental text 1 : Species key : 2-letter abbreviations
Supplemental text 2 : FASTA sequences of type 1 paracaspases used in phylogeny
Supplemental text 3 : FASTA sequences of Bcl10 homologs used in phylogeny

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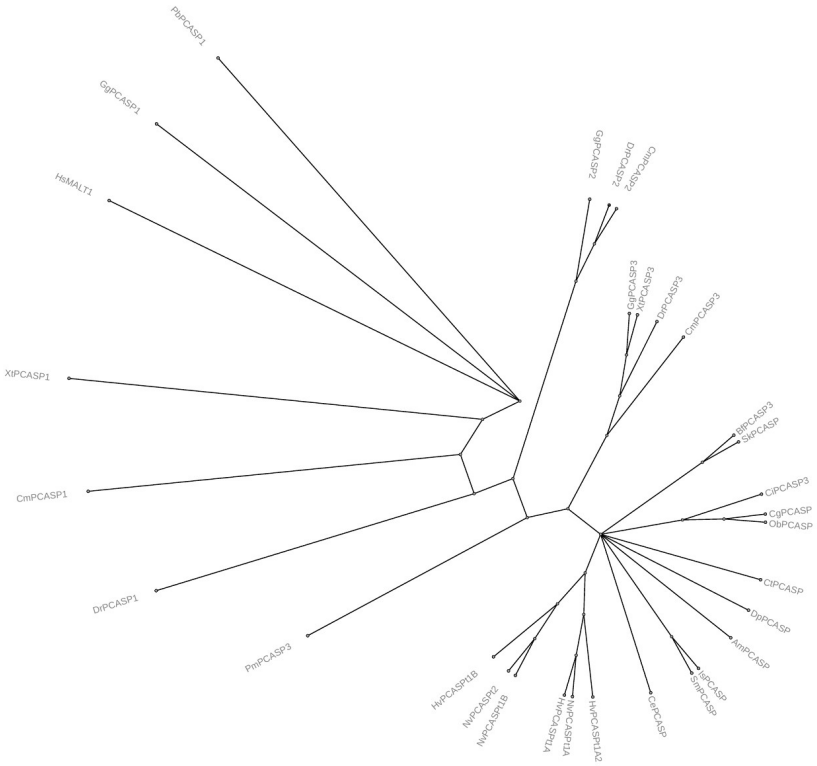
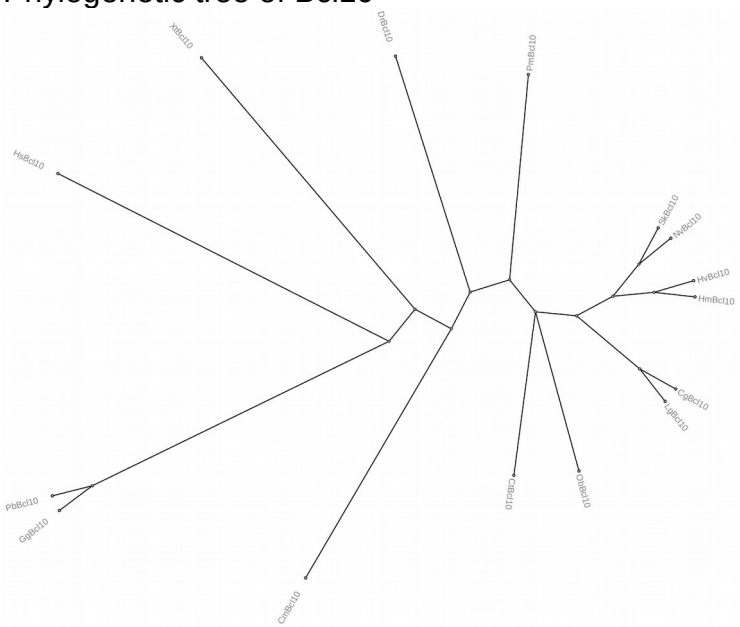
Figure 1
Phylogenetic tree of the type 1 paracaspases.



Bcl10-containing species like molluscs and hemichordates have vertebrate-like type 1 paracaspases, and cluster with lancelets and tunicates. The paracaspase from the Bcl10-containing Annelid could not be resolved with the currently available sequence information.

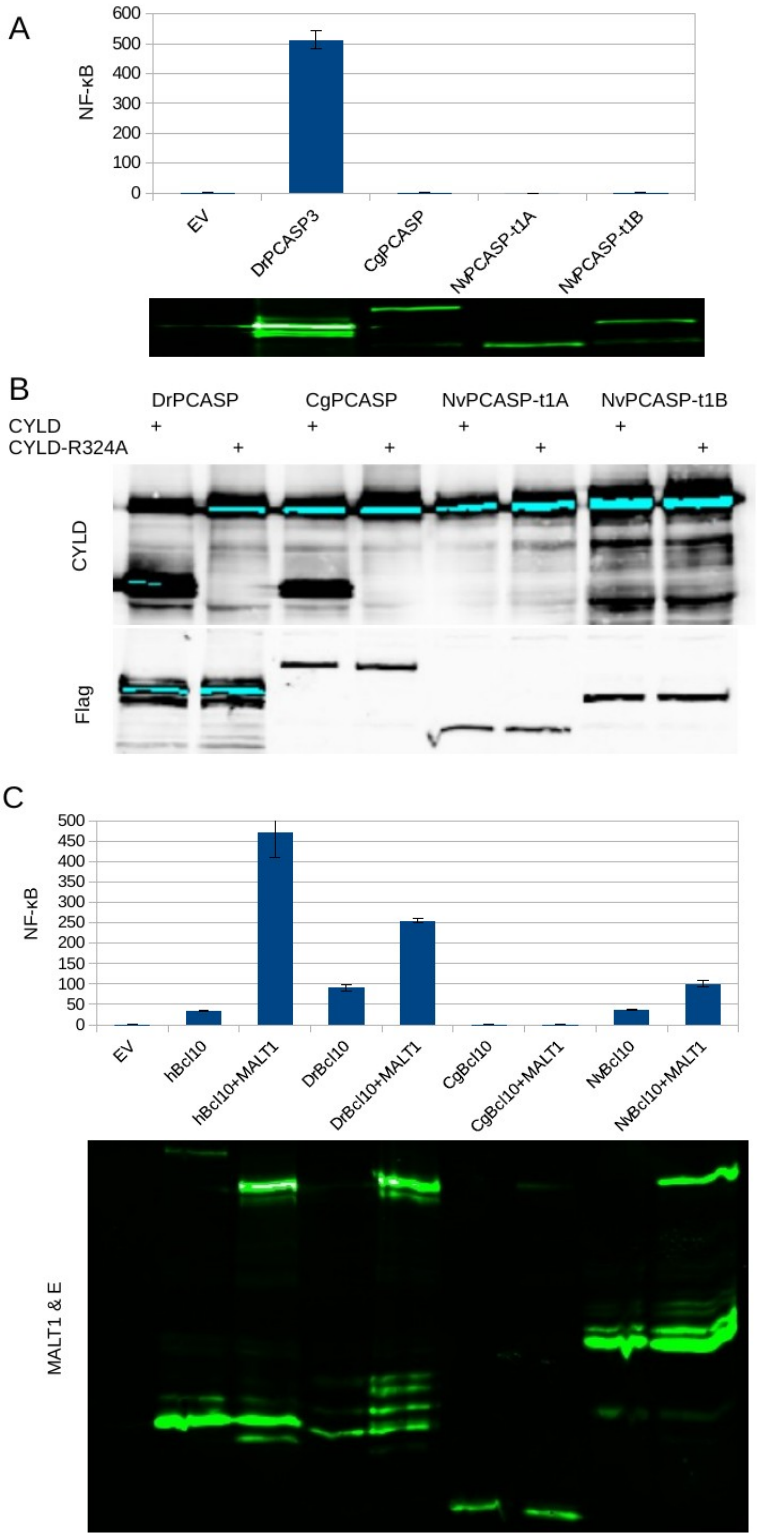
Figure 2
Phylogenetic tree of Bcl10



The hemichordate acorn worm cluster with the other invertebrates. Vertebrate Bcl10 roughly follows the evolutionary relationship at species-level. Annelid Bcl10 similar to mollusc Bcl10.

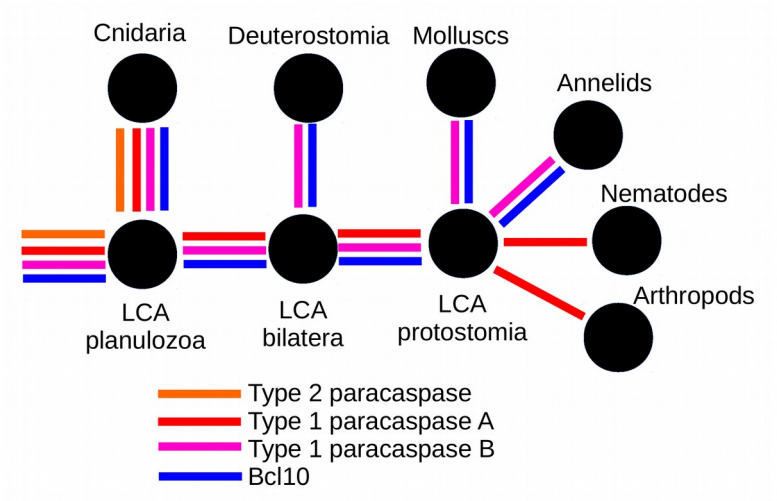
Figure 3

Functional conservation of invertebrate paracaspase and Bcl10



A) NF-κB induction by activated HLH-paracaspase fusions **B**) CYLD cleavage by activated HLH-paracaspase fusions **C**) human MALT1-dependent NF-κB induction by different Bcl10 homologs.

Figure 4
Proposed model



A model that proposes 2 ancient type 1 paracaspases, one Bcl10-dependent and one Bcl10-independent. The Bcl10-dependent type 1 paracaspase shows MALT1-like activities. We don't know when the paralogs originated but it needs to have been before or at the planulozoan last common ancestor (LCA). Deuterostomia (including vertebrates and hemichordates like the acorn worm), annelids and molluscs inherited the Bcl10-dependent type 1 paracaspase (B) whereas most other bilateran invertebrates kept the Bcl10-independent type 1 paracaspase (A). The model is based on currently available reliable sequence information and might change with additional data.