COP9 signalosome controls the Carma1–Bcl10–Malt1 complex upon T-cell stimulation

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INTRODUCTION

The Carma1–Bcl10–Malt1 (CBM) complex connects T-cell receptor (TCR) signalling to the canonical IκB kinase (IKK)/NFκB (nuclear factor)-κB pathway. Earlier studies have indicated that the COP9 signalosome (CSN), a pleiotropic regulator of the ubiquitin/26S proteasome system, controls antigen responses in T cells. The CSN is required for the degradation of the NF-κB inhibitor IκBα, but other molecular targets involved in T-cell signalling remained elusive. Here, we identify the CSN subunit 5 (CSN5) as a new interactor of Malt1 and Carma1. T-cell activation triggers the recruitment of the CSN to the CBM complex, and CSN downregulation impairs TCR-induced IKK activation. Furthermore, the CSN is required for maintaining the stability of Bcl10 in response to T-cell activation. Taken together, our data provide evidence for a functional link between the evolutionarily conserved CSN and the adaptive immunoregulatory CBM complex in T cells.

Keywords: COP9 signalosome; signalling; T-cell activation

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RESULTS

We searched for new regulators of the CBM complex. To this end, we carried out a yeast-two-hybrid screen using the carboxy-terminal regions of Malt1 (amino acids 313–813) that comprise the paracaspase domain as well as the C-terminal TRAF6-binding motifs and ubiquitin acceptor sites. During the screen, we identified CSN5 as a new interaction partner for Malt1 (supplementary Fig S1 online). To analyse whether CSN5 and Malt1 associate in cells, we carried out immunoprecipitation (IP) experiments after co-transfection of human embryonic kidney 293 cells (Fig 1A). Indeed, Flag–Malt1 and His–CSN5 interacted in this overexpression system. As cellular Malt1 is associated with Bcl10 in unstimulated cells and Thome, 2008). The termination of TCR-induced CBM activity is accomplished by the post-inductive degradation of Bcl10 (Scharschmidt et al, 2004; Loby et al, 2007).

The multimeric CSN complex contains eight subunits and participates in the regulation of many fundamental processes that are controlled by the ubiquitin/26S proteasome system (Cope & Deshaies, 2003; Wolf et al, 2003; Schwechheimer, 2004). The best-characterized intrinsic activity of the CSN is the removal of the ubiquitin-like modifier NEDD8 from cullin proteins catalysed by the metalloprotease activity of the CSN5 subunit (Lyapina et al, 2001; Schwechheimer et al, 2001). Cycles of cullin neddylation and deneddylation are believed to be required for the activation of cullin–RING ubiquitin ligases, such as SCF (Skp1–cullin1–F-box protein), which are involved in controlling the proteasomal degradation of various cellular proteins, for example p27, β-catenin or IκBα (Nakayama & Nakayama, 2006). In the immune system, the CSN was shown to function at various stages of T-cell development, homeostasis and antigen responses (Perez et al, 2003; Monen et al, 2007; Panattoni et al, 2008). Genetic deletion of CSN5 in thymocytes causes a marked reduction in nuclear NF-κB and decreased expression of anti-apoptotic NF-κB target genes (Panattoni et al, 2008), indicating that CSN5 might be regulating T-cell activation. Here, we report that CSN interacts with CBM in activated T cells and that the CSN fine-tunes IKK activation by maintaining Bcl10 stability.

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Bcl10–Malt1 is recruited to Carma1 upon T-cell activation, the interaction of CSN5 with other CBM members was investigated. CSN5 did not directly bind to Bcl10, but it associated with the Malt1–Bcl10 module (Fig 1B). In addition, CSN5 bound to the Carma1 moiety of the CBM complex after overexpression, and by using a yeast two-hybrid screen, we verified a direct interaction between CSN5 and the C-terminus of Carma1 (supplementary Fig S1 online). To identify the CSN5 interaction surface on Malt1 and Carma1, we tested for the binding of CSN5 to deletion mutants (supplementary Figs S2 and S3 online). In the case of Malt1, CSN5 did not associate with the amino-terminus but interacted with a more extensive surface that comprises the caspase-like domain and is a part of the C-terminus. On Carma1, CSN5 interacted with the C-terminal fragments of the membrane-associated guanylate kinase region and did not associate with the N-terminal region. However, CSN5 interaction with the C-terminal Carma1 domain was much weaker than binding to full-length Carma1. The regions within the C-terminus contributed differentially to the association. We have found that the guanylate kinase and the PDZ–SH3 domain form an intramolecular association (data not shown), which probably contributes to the mode of CSN5–Carma1 interaction. Taken together, the mapping of the Malt1–CSN5 and Carma1–CSN5 interaction surfaces indicates that CSN5 does not simply recognize a linear amino-acid stretch but rather that it interacts with more complex structural features, a model that has also been suggested for the binding of CSN5 to other partners (Richardson & Zundel, 2005).

To address whether the CSN could be involved in TCR-induced CBM activity, we determined whether the endogenous CSN and CBM complexes associate in T cells (Fig 2). For this approach, an antibody against CSN1 was used that efficiently precipitates the entire CSN complex (Wei & Deng, 1998; da Silva Correia et al., 2007). We detected a strong interaction between the CSN and Malt1 on activation of Jurkat T cells or primary human T cells after phorbol 12-myristate 13-acetate/ionomycin (P/I) treatment or CD3/CD28 co-ligation (Fig 2A,B). By contrast, tumour-necrosis factor-α (TNFα) stimulation did not promote binding of the CSN to Malt1. By using anti-CSN1 IP, we could detect a slightly retarded Malt1 band that corresponds to the ubiquitin-modified form of Malt1 (Oeckinghaus et al., 2007), indicating that the CSN preferentially associates with the active CBM complex upon T-cell activation. To determine the compartment for the CSN–CBM interaction, we carried out cell-fractionation experiments. The association of the CSN with ubiquitinated Malt1 was located in the cytoplasm of activated Jurkat T cells (supplementary Fig S4 online). The recruitment of CSN to the activated CBM complex was further supported by time-course analysis of CSN–Malt1 and CSN–Carma1 interactions (Fig 2C,D). The association between the CSN and ubiquitin-modified Malt1 and Carma1 was maximal after 15–30 min of T-cell activation and gradually ceased thereafter. The kinetics of CSN–Malt1 and CSN–Carma1 interactions after T-cell stimulation correlated with the peak of the CBM formation, as monitored by Bcl10/Carma1 co-IP (Fig 2C, lower panel). In turn, CBM disassembly, which is mainly achieved by Bcl10 degradation, promotes the release of the CSN (Fig 2C; lower panel and compare with Scharschmidt et al., 2004). An association between the CSN and Carma1, in activated Jurkat T cells, was also seen after anti-CSN5 IP (Fig 2E). Furthermore, no association was observed between CSN and Malt1 in Carma1-deficient Jurkat-derived JPM50.6 cells (Wang et al., 2002), indicating that the CSN did not associate with Malt1 independently from Carma1 (Fig 2F).

An anti-CSN1 IP efficiently precipitates CSN5 as well as other CSN subunits (supplementary Fig S5 online), which indicates that, in T cells, Malt1 associates with CSN5 in the context of the CSN holocomplex. However, CSN5 subcomplexes have been described earlier (Fukumoto et al., 2005; Tomoda et al., 2005). To clarify whether the CSN holocomplex associates with the assembled CBM complex after stimulation, we separated cellular
Fig 2 | T-cell activation promotes association between the COP9 signalosome and the Carma1–Bcl10–Malt1 complex. (A) CSN–CBM interaction in Jurkat T cells. Jurkat T cells were stimulated with CD3/CD28, phorbol 12-myristate 13-acetate/ionomycin (P/I) or tumour-necrosis factor (TNF)-α for 30 min. If not stated otherwise, cells were lysed in co-immunoprecipitation (co-IP) buffer, and CSN1 antibody was used for IP in (A) and the following panels. (B) CSN–CBM interaction in human primary T cells. Peripheral T cells were stimulated as indicated. (C) Kinetic analysis of the CSN–Malt1 interaction and CBM complex formation. Jurkat T cells were stimulated with P/I for the indicated time points. The stimulus-dependent Bcl10–Carma1 interaction was shown by Bcl10 IP. (D,E) Stimulus-dependent recruitment of the CSN to Carma1. Jurkat T cells were stimulated with P/I for the indicated time points. The CSN–Carma1 interaction was shown by CSN1 IP (D) or CSN5 IP (E). (F) Carma1 is essential for the CBM–CSN interaction. Jurkat T cells and Carma1-deficient JPM50.6 cells were stimulated as indicated. (G) Stimulus-dependent recruitment of the CSN to the CBM complex. Extracts of Jurkat T cells (untreated or 20 min P/I-stimulated) were fractionated (fr.) by gel filtration chromatography. Elution profiles of Malt1, CSN1 and CSN5 were analysed by Western blotting. Molecular-weight standards depict the peak elution of marker proteins. Fractions containing either the CBM or the CSN complex were pooled and subjected to CSN1 IP. Note: Malt1 antibody detected an unspecific band after CSN1 IP (asterisk), which is also visible after isotype control IP (see supplementary Fig S4 online). CBM, Carma1–Bcl10–Malt1; CSN, COP9 signalosome; Ubi, ubiquitin.
Fig 3 | COP9 signalosomes subunits 2 and 5 are required for optimal T-cell receptor-induced IκB kinase activation. (A) Short interfering (si)RNA-mediated reduction of CSN2 resulted in a strong decrease of CSN3. Jurkat T cells were transfected with green fluorescent protein (GFP) control, CSN2 or CSN5 siRNAs. Lysates were analysed by Western blotting. (B, C) CD3/CD28-dependent IKK kinase activation requires the CSN. Jurkat T cells were transfected with GFP control, CSN2 (B) or CSN5 (C) siRNAs. Cells were stimulated with CD3/CD28 or tumour-necrosis factor (TNF)-α for 20 min. In vitro IKK kinase assays were carried out. Phosphorylation of recombinant GST–IκBα amino acids 1–53 was quantified by densitometric analysis. Quantification and standard deviation of three independent experiments are shown in the histogram; CSN, COP9 signalosomes; GST, glutathione S-transferase; IKK, IκB kinase; KA, kinase assay; TCR, T-cell receptor.

Protein complexes from extracts of Jurkat T cells by gel filtration (Fig 2G). Peak elution of CSN1-containing CSN holocomplex (fractions 19–23) was found to be distinct from a CSN5-containing complex (fraction 28). In addition, other CSN subunits (e.g. CSN2) eluted exclusively at the size of the CSN holocomplex (data not shown). The elution of the CSN holocomplex was shifted slightly toward higher molecular-weight fractions in response to P/I stimulation. More prominently, P/I stimulation induced the appearance of ubiquitinated Malt1 in CSN1- and CBM-containing fractions (20–22 and 10–12, respectively; compare with Oeckinghaus et al, 2007). To monitor the association, we carried out anti-CSN1 IPs from the pooled fractions 9–12 (elution of the CBM complex) and fractions 19–21 (elution of the CSN holocomplex; Fig 2G, lower panel). Indeed, P/I stimulation induced the association between small amounts of ubiquitinated Malt1 and CSN1 in the CSN holocomplex-containing fractions (lower panel; IP pool B). Furthermore, P/I stimulation promoted the co-elution of ubiquitinated Malt1 in the assembled CBM complex (fractions 10–12) with a small amount of CSN1 and CSN5 that could only be detected after enrichment by anti-CSN1 IP of the fractions (lower panel; IP pool A). In the same samples, anti-CSN1 IP also co-precipitated ubiquitin-modified Malt1 after stimulation. These data provide evidence for a weak association between CSN and ubiquitinated Malt1 outside the CBM complex. However, T-cell stimulation induces association of the CSN and CBM complexes to give a higher-order complex that elutes with an apparent molecular weight of more than 1,000 kDa.

To analyse the functional impact of the CSN on CBM activity, we carried out short interfering RNA (siRNA) knockdown in Jurkat T cells using two independent siRNAs against CSN2 and one against the CSN5 subunit of the CSN. It is known that the downregulation or deletion of one CSN component can affect the expression of other CSN subunits (Menon et al, 2007; Schweitzer et al, 2007; Fig 3A). As observed previously, targeting the CSN2 subunit by siRNAs induced a similar reduction in CSN2 and CSN5 protein amounts (Miyauchi et al, 2008). By contrast, siRNA-mediated reduction of CSN5 caused only a slight reduction in CSN2 protein amounts. CSN2 and CSN5 siRNAs weakly affected the expression of the CSN subunits 1, 3 and 8.

Perez et al (2003) have shown that a CSN5 antagonistic peptide reduces interleukin-2 expression in activated T cells. In agreement with this, knockdown of CSN2 or CSN5 resulted in a reduced induction of interleukin-2 messenger RNA in Jurkat T cells (supplementary Fig S6 online). However, as the CSN might be involved in the regulation of several and sometimes even opposing steps in NF-κB activation and gene induction, we chose to determine IKK activation as a direct CBM-downstream readout in CSN2 and CSN5 knockdown cells (Fig 3B, C). We carried out in vitro IKK activity assays after IP of the IKK regulatory subunit IKK-γ/NF-κB essential modulator (NEMO). Knockdown of CSN2 by two independent siRNAs reduced CD3/CD28-induced IKK activity by almost 60%. By contrast, TNFα-dependent IKK activation in Jurkat T cells was largely unaffected. Similarly, downregulation of CSN5 reduced the CD3/CD28-induced IKK activity by almost 60%. By contrast, TNFα-dependent IKK activation in Jurkat T cells was largely unaffected.
activation, even though the effect was slightly less pronounced than with the CSN2 knockdown. CSN2 and CSN5 knockdown specifically affected IKK activation, as we did not observe any changes in the CD3/CD28-induced c-Jun N-terminal kinase (JNK), extracellular-signal-regulated kinase (ERK) or p38 phosphorylation (supplementary Fig S7 online). The data show that the CSN is required for optimal TCR-induced IKK activation and thereby might promote antigen signalling upstream from the canonical NF-κB pathway.

The CSN has been intimately linked to the regulation of protein stability (Cope & Deshaies, 2003; Wolf et al., 2003). In T cells, CSN5 deficiency enhances the expression of some proteins such as β-catenin or p53, whereas the amounts of other proteins, for example, IκBα and p27kip1, are decreased (Panattoni et al., 2008). Proteolytic degradation of Bcl10 was shown to counteract CBM activity and to terminate NF-κB signalling, indicating that Bcl10 protein amounts are crucial for CBM function (Scharschmidt et al., 2004; Hu et al., 2006; Lobry et al., 2007; Wu & Ashwell, 2008). We have shown previously that Bcl10 partly localizes to lysosomal vesicles after P/I stimulation of Jurkat T cells (Scharschmidt et al., 2004). Indeed, Bcl10 degradation in Jurkat T cells was impaired after treatment with the protease inhibitors leupeptin and pepstatinA, which are effective inhibitors of cysteine proteases and aspartic proteases in the lysosomal compartment (supplementary Fig S8 online). We determined the Bcl10 protein amount after siRNA transfection of Jurkat T cells (Fig 4). Knockdown of CSN2 or CSN5 caused accelerated Bcl10 degradation in response to CD3/CD28 or P/I stimulation (Fig 4A–D). Neither Malt1 nor Carma1 stability was influenced by siRNA-mediated downregulation of the two CSN subunits. As ubiquitination of Bcl10 was shown to precede its degradation (Scharschmidt et al., 2004; Hu et al., 2006; Wu & Ashwell, 2008), we determined the kinetics of Bcl10 ubiquitination in CSN2- or CSN5-siRNA-transfected Jurkat T cells (Fig 4E,F). The peak of Bcl10 ubiquitination in CSN

Fig 4 | COP9 signalosomes subunits 2 and 5 regulate Bcl10 stability and ubiquitination. Jurkat T cells were transfected with green fluorescent protein (GFP), CSN2 (A,B) or CSN5 (C,D) short interfering (si)RNAs. Cells were stimulated with CD3/CD28 or phorbol 12-myristate 13-acetate/ionomycin (P/I) for the indicated time points. Relative Bcl10 levels were calculated by densitometric measurement. Quantification and standard deviation of three independent experiments are shown in the histograms. Jurkat T cells were transfected with GFP as a control, CSN2 (E) or CSN5 (F) siRNAs and stimulated with P/I. Cells were lysed and immunoprecipitation (IP) was carried out using anti-Bcl10 antibody. An unspecific band detected by the CSN2 antibody is marked in all experiments by an asterisk. CSN, COP9 signalosome.
knockdown cells was shifted to an earlier time-point after P/I stimulation, which again correlated with the faster degradation of Bcl10. We also investigated stimulus-dependent Malt1 ubiquitination in CSN2-siRNA-transfected Jurkat T cells. In contrast to Bcl10 ubiquitination, Malt1 ubiquitination was not influenced by CSN2 siRNA (supplementary Fig S9 online). Taken together, these data provide compelling evidence that the CSN influences Bcl10 stability. Thus, the CSN is involved in fine-tuning the CBM complex activity in activated T cells.

**DISCUSSION**

In this study, we show that in T cells, the CSN associates directly with the Carma1–Malt1 module and influences CBM-triggered IKK/NF-κB signalling. T-cell-specific ablation of CSN5 has been shown to affect several stages of T-cell development and to impair NF-κB activation in thymocytes (Panattoni et al, 2008). However, owing to the many functions of CSN5, the phenotype of CSN5-deficient T cells is complex and it is difficult to discriminate the molecular targets. By showing that the CSN holocomplex binds to CBM and affects Bcl10 stability, we were able to identify a new target for the CSN in T cells. In contrast to the genetic ablation of CSN5, the genetic ablation of the CSN8 subunit in T cells controls TCR-induced cell cycle progression but leaves NF-κB signalling intact (Menon et al, 2007). In CSN8−/− cells, CSN3, but not CSN2 or CSN5, expression was significantly reduced (Menon et al, 2007). As CSN3 and CSN8 can form a subcomplex and as we did not observe a strong decrease in CSN3 or CSN8 expression after CSN2 or CSN5 knockdown, we suppose that distinct CSN subunits exert specific biological functions that do not require the CSN holocomplex.

Several studies have shown that T-cell activation induces Bcl10 ubiquitination and degradation (Scharschmidt et al, 2004; Hu et al, 2006; Lobry et al, 2007; Zeng et al, 2007; Wu & Ashwell, 2008), but much less is known about how the degradation of Bcl10 is arranged. Lobry et al (2007) have shown an association between overexpressed β-transducin repeat-containing protein (βTRCP) and Bcl10, suggesting that SCFβTRCP might catalyse Bcl10 proteasomal degradation. However, at present there is no evidence that SCFβTRCP controls Bcl10 stability in activated T cells. In fact, we and others have shown that Bcl10 is degraded by a proteasome-independent pathway, and we found that Bcl10 localizes with lysosomal vesicles after T-cell activation (Scharschmidt et al, 2004; Zeng et al, 2007). In agreement with this, we now report that the inhibition of lysosomal peptidases stabilizes Bcl10. As the degradation of Bcl10 is enhanced in CSN2 and CSN5 knockdown T cells, our results indicate that the CSN might be involved in controlling the degradation of a non-proteasomal substrate. However, further studies on the regulation of Bcl10 stability are required to finally resolve whether the CSN influences proteasomal or lysosomal degradation.

Lysine residues within the CARD domain of Bcl10 are essential for triggering Bcl10 degradation (Wu & Ashwell, 2008), underscoring the importance of ubiquitination. In CSN2 or CSN5 knockdown cells, enhanced Bcl10 degradation coincides with increased ubiquitination of Bcl10 at early time-points of stimulation, suggesting that the CSN maintains Bcl10 stability by counteracting ubiquitination. The deubiquitinating enzyme ubiquitin-specific peptidase (USP) 15 purifies with CSN (Hettfield et al, 2005) and CSN-associated USP15 has been shown to enhance IkBα stability post-induction by deubiquitinating IkBα synthesized de novo (Schweitzer et al, 2007). Even though we did not find that siRNA-mediated knockdown of USP15 affects Bcl10 stability (supplementary Fig S10 online), it is tempting to speculate that other deubiquitinating enzymes might associate with the CSN to counteract the degradation of proteins such as Bcl10. Taken together, we provide evidence that the CSN, which is a pleiotropic regulator of protein neddylation, ubiquitination and degradation, associates with the CBM signalling module and regulates its activity in activated T cells.

**METHODS**

**Antibodies, reagents, siRNAs and plasmids.** The following antibodies were used: human CD3, human CD28, mouse IgG1 and mouse IgG2a (BD Bioscience, Heidelberg, Germany); Malt1 (H300, B12), Bcl10 (331.3), CSN5 (FL-334, B-7); CSN1 (K-19), p65 (c-20), lamin B (M-20), IKKγ (FL-419) and Actin (I-19) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Carma1 and CSN2 (Abcam, Cambridge, UK); Flag-M2 (Sigma, St Louis, MO, USA); ubiquitin (Covance, Richmond, CA, USA); CSN1 and CSN8 (Biomol (www.biomol.com)); phospho-IκBα, IκBβ (L35A5), IKKβ, phospho-p38, phospho-Erk1/2 and phospho-Jnk1/2 (Cell Signaling (www.cellsignal.com)); and CSN3 (Calbiochem, Darmstadt, Germany). The following reagents and siRNAs (100 nM) were used: PMA (200 ng/ml) and Ionomycin (300 ng/ml) (Calbiochem), r-IL-2 (20 U/ml), protease inhibitor cocktail (Roche, Mannheim, Germany); Dynal T-cell-negative isolation kit and Dynabeads CD3/CD28 T-cell expander (Invitrogen, Karlsruhe, Germany); si-GFP–control, siCSN5-1: AAGCTCAGATTCATCGTAAAA, siCSN5-2: GCACCTGAAAAAGATATTA, siCSN2-1: GAGCTGAAAAGATATTA, siCSN2-2: GATAGACTGTGGTTAAAG (Eurogentec, Seraing, Belgium). Al tagged Malt1 and Carma1 cDNAs were cloned in the pEF backbone vector (Invitrogen). His–CSN5 was cloned into pcDNA3 (Invitrogen).

**Cell culture.** Human embryonic kidney 293 cells were transfected using the standard calcium phosphate precipitation protocols. Standard cell culture, transfection and stimulation of Jurkat T cells (P/I or CD3/CD28 antibody co-ligation) were carried out as described (Wegener et al, 2006). For RNA interference, Jurkat T cells were transfected with 100 nM siRNA and Atufect transfection reagent (Silence Therapeutics, Berlin, Germany) and analysed after 72 h. Primary T cells were isolated from human blood by the Dynabead method (Invitrogen). For expansion, the Dynabeads CD3/CD28 T-cell expander and r-IL-2 were used according to the recommendations of the manufacturer.

Further methods are available as supplementary information online.

**Supplementary information** is available at EMBO reports online (http://www.emboreports.org).

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**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.
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