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A20 Negatively Regulates T Cell Receptor Signaling to NF-κB by Cleaving Malt1 Ubiquitin Chains

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The Carma1-Bcl10-Malt1 signaling module bridges TCR signaling to the canonical IκB kinase (IKK)/NF-κB pathway. Covalent attachment of regulatory ubiquitin chains to Malt1 paracaspase directs TCR signaling to IKK activation. Further, the ubiquitin-editing enzyme A20 was recently suggested to suppress T cell activation, but molecular targets for A20 remain elusive. In this paper, we show that A20 regulates the strength and duration of the IKK/NF-κB response upon TCR/CD28 costimulation. By catalyzing the removal of K63-linked ubiquitin chains from Malt1, A20 prevents sustained interaction between ubiquitinated Malt1 and the IKK complex and thus serves as a negative regulator of inducible IKK activity. Upon T cell stimulation, A20 is rapidly removed and paracaspase activity of Malt1 has been suggested to cleave A20. Using antagonistic peptides or reconstitution of Malt1−/− T cells, we show that Malt1 paracaspase activity is required for A20 cleavage and optimal IL-2 production, but dispensable for initial IKK/NF-κB signaling in CD4+ T cells. However, proteasomal inhibition impairs A20 degradation and impedes TCR/CD28-induced IKK activation. Taken together, A20 functions as a Malt1 deubiquitinating enzyme and proteasomal degradation and de novo synthesis of A20 contributes to balance TCR/CD28-induced IKK/NF-κB signaling. The Journal of Immunology, 2009, 182: 7718–7728.

The transcription factor NF-κB is a key regulator of the adaptive immune response and controls lymphocyte activation, proliferation, and survival. Much progress has been made concerning our understanding of the signaling mediators and regulatory mechanisms that govern NF-κB activation initiated by TCR engagement on T lymphocytes (1, 2). TCR/CD28 coligation induces canonical NF-κB signaling, which involves phosphorylation and degradation of small cytosolic IκB inhibitors (e.g., IκBα) and subsequent nuclear translocation and DNA binding of NF-κB. IκB phosphorylation is catalyzed by the IκB kinase (IKK)1 complex, which consists of the catalytic subunits IKKα/IKKβ and the regulatory component IKKγ (also known as NEMO) (3). In T cells, activation of the atypical protein kinase C (PKC)θ has been shown to provide a critical link between TCR/CD28 proximal signaling events and the NF-κB pathway (4). Active PKCθ phosphorylates the lymphocyte-specific caspase recruitment domain (CARD)-containing adapter protein Carma1 (also known as CARD11). Phosphorylation imposes a conformational change in Carma1 that triggers the recruitment of the CARD protein Bcl10 and the paracaspase Malt1, resulting in the assembly of the Carma1-Bcl10-Malt1 (CBM) complex (5). Genetic ablation in mice revealed that every single component of the CBM complex is essential for TCR-initiated activation of the IKK complex (6–11). In addition, association of PDK1 and Caspase8 to the PKCθ-CBM signaling module has been shown to facilitate TCR-induced NF-κB activation (12, 13).

Recent data indicated that regulatory ubiquitination plays an important role in triggering IKK activation downstream of the CBM complex after T cell activation. Conditional ablation of UBC13/Uev1a, an E2 enzyme that catalyzes the covalent attachment of lysine (K) 63-linked ubiquitin chains to target molecules, results in defective TCR-induced IKK/NF-κB signaling in thymocytes (14). Further, the E3 ubiquitin ligase TRAF6 associates with Malt1 in response to T cell activation and small interfering RNA (siRNA)-mediated down-regulation of TRAF6 causes impaired IKK activation in Jurkat T cells (15–17). Because NF-κB activation is not defective in T cells that lack TRAF6, one or more other E3 ligases must compensate for the loss of TRAF6 (18). IKKγ and Malt1 have been identified as two potential substrates for TRAF6-mediated attachment of K63-linked ubiquitin chains upon T cell activation (16, 17). Whereas the replacement of the putative IKKγ ubiquitin acceptor lysine (K399) results in a mild reduction of NF-κB activation (19, 20), mutation of Malt1 ubiquitin acceptor sites strongly impairs T cell activation (16). Malt1 ubiquitin chains promote the association between the CBM and IKK complex by interacting with the ubiquitin binding domain of IKKγ after T cell activation. Similarly, ubiquitin modifications in the CARD of Bcl10 have been shown to trigger Bcl10-IKKγ binding and NF-κB activation in T cells (21).
The identification of deubiquitinating enzymes (DUBs), such as A20 and cylindromatosis (CYLD), that disassemble ubiquitin chains from cellular proteins strongly supported the concept that reversible ubiquitination of signaling mediators represents a critical step in the NF-κB signaling pathway (22–24). Both A20 and CYLD have been shown to function as negative regulators of T cell activation (24). CYLD seems to be primarily involved in counteracting constitutive NF-κB signaling activity (24). A20 can contribute to the decrease in A20 levels after T cell stimulation. However, whereas Malt1 paracaspase activity is required for initial IKK/NF-κB activation after PMA/Ionomycin stimulation, proteasomal degradation of A20 can contribute to the decrease in A20 levels after T cell stimulation. However, whereas Malt1 paracaspase activity is dispensable for initial IKK/NF-κB activation after CD3/CD28 costimulation.

**Materials and Methods**

**Abs and reagents**

The following Abs were used: human CD3, human CD28, mouse IgG1, mouse IgG2a, and IKKα (all purchased from BD Biosciences); Isocitrate dehydrogenase (CI; Sigma-Aldrich); MG132 (Calbiochem); IL-2 (20 U/ml; Roche); [32P]-ATP (PerkinElmer); Dynabeads CD4 and DetachA bead mouse CD4 (Dynal Bio- tech); and Z-VRPR-FMK (75 μM) (Alexis Biochemicals). The following reagents were used: MG132 (25 μM), PMA (200 ng/ml), and Ionomycin (300 ng/ml) (all purchased from Calbiochem); IL-2 (20 U/ml; Roche); [32P]-α- and [32P]-γ-ATP (PerkinElmer); Dynabeads CD4 and DetachA bead mouse CD4 (Dynal Bio-tech); and Z-VRPR-FMK (75 μM) (Alexis Biochemicals). IL-2 in the supernatant of Jurkat cells was measured by ELISA according to the manufacturer’s protocol (eBioscience). The following siRNAs were used: siA20 (59A426), and Thy1.1-allophycocyanin, IL-2-FITC (all purchased from BD Biosciences); IκBα (C-20) and IκBβ (FL419) (all purchased from Santa Cruz Biotechnology); Flag M2 and Flag M2-FITC (both purchased from Sigma-Aldrich); ubiquitin (FK2; BIOMOL); phospho-IκBα, IκBβ, and phospho-IKKα (Ser180)/IKKβ (Ser181) (all purchased from Cell Signaling Technology); A20 (59A426), and Thy1.1-allophycocyanin, IL-2-FITC (all purchased from eBioscience); and K48-linked (Apu2.07) and K63-linked (Apu3.A8) polyubiquitin Abs (Genentech). The following reagents were used: MG132 (25 μM), PMA (200 ng/ml), and Ionomycin (300 ng/ml) (all purchased from Calbiochem); IL-2 (20 U/ml; Roche); [32P]-α- and [32P]-γ-ATP (PerkinElmer); Dynabeads CD4 and DetachA bead mouse CD4 (Dynal Bio-tech); and Z-VRPR-FMK (75 μM) (Alexis Biochemicals). IL-2 in the supernatant of Jurkat cells was measured by ELISA according to the manufacturer’s protocol (eBioscience). The following siRNAs were used: siA20 (59A426), and Thy1.1-allophycocyanin, IL-2-FITC (all purchased from eBioscience); and K48-linked (Apu2.07) and K63-linked (Apu3.A8) polyubiquitin Abs (Genentech). The following reagents were used: MG132 (25 μM), PMA (200 ng/ml), and Ionomycin (300 ng/ml) (all purchased from Calbiochem); IL-2 (20 U/ml; Roche); [32P]-α- and [32P]-γ-ATP (PerkinElmer); Dynabeads CD4 and DetachA bead mouse CD4 (Dynal Bio-tech); and Z-VRPR-FMK (75 μM) (Alexis Biochemicals). IL-2 in the supernatant of Jurkat cells was measured by ELISA according to the manufacturer’s protocol (eBioscience). The following siRNAs were used: siA20 (59A426), and Thy1.1-allophycocyanin, IL-2-FITC (all purchased from eBioscience); and K48-linked (Apu2.07) and K63-linked (Apu3.A8) polyubiquitin Abs (Genentech). The following reagents were used: MG132 (25 μM), PMA (200 ng/ml), and Ionomycin (300 ng/ml) (all purchased from Calbiochem); IL-2 (20 U/ml; Roche); [32P]-α- and [32P]-γ-ATP (PerkinElmer); Dynabeads CD4 and DetachA bead mouse CD4 (Dynal Bio-tech); and Z-VRPR-FMK (75 μM) (Alexis Biochemicals). IL-2 in the supernatant of Jurkat cells was measured by ELISA according to the manufacturer’s protocol (eBioscience). The following siRNAs were used: siA20 (59A426), and Thy1.1-allophycocyanin, IL-2-FITC (all purchased from eBioscience); and K48-linked (Apu2.07) and K63-linked (Apu3.A8) polyubiquitin Abs (Genentech). The following reagents were used: MG132 (25 μM), PMA (200 ng/ml), and Ionomycin (300 ng/ml) (all purchased from Calbiochem); IL-2 (20 U/ml; Roche); [32P]-α- and [32P]-γ-ATP (PerkinElmer); Dynabeads CD4 and DetachA bead mouse CD4 (Dynal Bio-tech); and Z-VRPR-FMK (75 μM) (Alexis Biochemicals). IL-2 in the supernatant of Jurkat cells was measured by ELISA according to the manufacturer’s protocol (eBioscience). The following siRNAs were used: siA20 (59A426), and Thy1.1-allophycocyanin, IL-2-FITC (all purchased from eBioscience); and K48-linked (Apu2.07) and K63-linked (Apu3.A8) polyubiquitin Abs (Genentech). The following reagents were used: MG132 (25 μM), PMA (200 ng/ml), and Ionomycin (300 ng/ml) (all purchased from Calbiochem); IL-2 (20 U/ml; Roche); [32P]-α- and [32P]-γ-ATP (PerkinElmer); Dynabeads CD4 and DetachA bead mouse CD4 (Dynal Bio-tech); and Z-VRPR-FMK (75 μM) (Alexis Biochemicals). IL-2 in the supernatant of Jurkat cells was measured by ELISA according to the manufacturer’s protocol (eBioscience). The following siRNAs were used: si
Expression Plasmids

All A20 constructs were cloned with three N-terminal Flag epitope in pEF vector (Invitrogen). Mutagenesis was performed by standard PCR. Flag-TRAF6 and Myc-Malt1 constructs were expressed from pRK5 vector (Invitrogen). Retroviral Flag-Malt1 constructs were cloned using the Gateway system (Invitrogen) into pMSCV-Thy1.1 that couples Thy1.1 and Malt1 expression via an internal ribosome entry site (IRES) sequence.

Cell cultures and treatment

HEK293 and Phoenix packaging cells were cultured in complete Dulbecco’s modified Eagle Medium (supplemented with 10% FBS, 100 U of penicillin-streptomycin per ml, and 1 mM of sodium pyruvate) and transfected using standard calcium phosphate precipitation protocols. Cell culture, transfection, and stimulation of Jurkat T cells (PMA/Ionomycin (P/I) or CD3/CD28 Ab coligation) were performed as previously described (30).

For the inhibition of Malt1 paracaspase activity, cells were incubated with 75 nM Z-VRPR-FMK (dissolved in water) for the times indicated in the figure legends and during P/I or CD3/CD28 stimulation. Control cells were incubated with water. For RNA interference, Jurkat T cells were transfected with Atufect transfection reagent (Silence Therapeutics) and 100 nM siRNA oligos and analyzed after 72 h. Human T cells were isolated from peripheral blood and expanded using a T cell negative isolation kit and CD3/CD28 Dynabeads (both purchased from Dynal Invitrogen). Positive selection of murine wt and Malt1−/− CD4+ T cells was conducted with Dynabeads. Primary T cells were cultured in RPMI 1640 supplemented with 1% Pen/Strep, 1% glutamine, 10% FCS, and 0.1% mercaptoethanol. Retroviral infection of CD4+ T cells and FACS analysis were performed as described previously (16).

Co-immunoprecipitation (IP), cellular ubiquitination, determination of ubiquitin chains linkage, and kinase assay

For binding studies, cells were lysed in co-IP buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 0.2% Nonidet P-40, 10% glycerol, 1 mM DTT, 10 mM sodium fluoride, 8 mM β-glycerophosphate, 20 μM sodium vanadate, and protease inhibitor mixture (Roche)). Precipitations were conducted overnight at 4°C, and after washing IPs were boiled and analyzed by Western blotting. For detection of Malt1 ubiquitination, lysis buffer contained 1% SDS. For IPs, extracts were diluted 10-fold with co-IP buffer. For detection of ubiquitinated Bcl10, cells were lysed in ubiquitin lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 30 mM N-ethylmaleimide (NEM), 1 mM DTT, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride, 8 mM β-glycerophosphate, and protease inhibitor mixture. For the immunoprecipitation of polyubiquitinated proteins with linkage-specific Abs (directed against K48- or K63-linked ubiquitin chains), Jurkat T cells were lysed at room temperature in buffer containing 1% Triton-X-100, 6 M urea, and 2 mM NEM. Before overnight IP at room temperature, extracts were diluted to 4 M Urea. Ubiquitin linkage-specific Abs (3 μg) were used for IP and after washing IPs were boiled and analyzed by Western blotting. For the detection of ubiquitinated IκBα, cells were incubated with MG132 (25 μM) for 60 min before stimulation. For IKK kinase assays, siRNA-transfected or MG132-treated Jurkat cells were stimulated with P/I or anti-CD3/CD28, lysed in co-IP buffer, and subjected to either Malt1 IP (Fig. 5B) or IKKγ IP. Precipitates were washed and used for kinase assays using GST IκBα (aa 1–53) as substrate as described previously (30).

Electrophoretic mobility shift assay (EMSA) and deubiquitination assay

Whole cell extracts, Western blotting, and EMSA were performed essentially as described previously (31). For deubiquitination assays, Jurkat T cells (untreated or stimulated with P/I) were lysed in ubiquitin lysis buffer and subjected to Malt1 IP. Immunoprecipitates were washed seven times with co-IP buffer and two times with DUB buffer (25 mM HEPES (pH 7.4) 5 mM MgCl2, 1 mM DTT). In vitro deubiquitination reactions (12 μl total volume) were conducted in DUB buffer with 10 μM recombinant His-A20 (1–370, wt or C103A, expressed in Escherichia coli) for 2 h at 37°C. Finally, precipitates were washed twice with co-IP buffer and subjected to SDS-PAGE and Western blotting.

RESULTS

A20 is a negative regulator of IKK/NF-κB signaling in T cells

A20 has been suggested to counteract NF-κB activity and IL-2 production in T cells (27, 28). Congruently, we found that siRNA-mediated down-regulation of A20 augments IL-2 induction in Jurkat T cells in response to either P/I or anti-CD3/CD28 treatment (Fig. 1A). These data suggest that A20 can balance T cell activation. However, the direct molecular mechanisms by which A20 influences TCR-induced NF-κB activation have not been established.

A20 is removed shortly after stimulation, but reappears and is even enhanced in response to prolonged T cell stimulation (Fig. 1, B and C). Removal of A20 could be a possible way to damp its negative regulatory function and to allow optimal NF-κB activation. To test this hypothesis and to analyze at what stage A20 regulates NF-κB activation, we performed further A20 knock-down experiments in Jurkat T cells. Down-regulation of basal and induced A20 protein amounts by two different siRNAs caused an increased and prolonged NF-κB DNA binding activity in response to either P/I stimulation or CD3/CD28 coligation (Fig. 1, B and C). Further, sustained NF-κB DNA binding activity in A20 knock-down cells correlated with enhanced phosphorylation of resynthesized IκBα.

To determine whether A20 affects the signaling pathway upstream of the IKK complex, we monitored IKK activation in A20 knock-down cells using IKK kinase assays after anti-IKKγ IP. Reduction of A20 expression augmented and sustained IKK
activity after CD3/CD28 as well as after P/I stimulation (Fig. 1, D and E). Correlating with a sustained IKK activity, we detected an increased phosphorylation status of the IKK subunits α and β after A20 reduction in P/I-stimulated Jurkat T cells (Fig. 1F). The data suggest that A20 functions as a negative regulator of IKK activation and thereby controls the strength and the duration of canonical NF-κB signaling upon T cell activation.

Because NF-κB activation in response to CD3/CD28 costimulation is augmented by A20 down-regulation, we asked whether decreased A20 amounts might sensitize T cells to CD3 ligation in the absence of a costimulatory signal (Fig. 2). Indeed, CD3 stimulation in the absence of any costimulus induced an equivalent degree of IκBα phosphorylation, degradation, and NF-κB activation in A20 knock-down Jurkat T cells when compared with CD3/CD28 costimulation in control transfected cells. Thus, removal of A20 abolishes the need of CD28 costimulation to obtain a strong NF-κB response.

DUB activity of A20 cleaves K63-linked ubiquitin chains from Malt1

We have shown that C-terminal ubiquitination of Malt1 provides a critical link between the CBM and IKK complexes after T cell activation (16). Because A20 associates with Malt1 in response to T cell activation (28), we asked whether Malt1 ubiquitin chains could be a substrate for the DUB activity of A20 to balance NF-κB signaling postinduction. In 293 cells, expression of TRAF6 induced ubiquitination of Malt1 (Fig. 3A; compare with Ref. 16). Because association between Malt1, TRAF6, and A20 is disrupted after lysis in 1% SDS (data not shown), immunoprecipitates and lysates were analyzed by Western blotting for the presence of Malt1 (left) or IκBα (right). For the detection of IκBα ubiquitination, Jurkat T cells were pretreated for 1 h with MG132 before stimulation to inhibit proteasomal degradation. Data are representative of three (B and C) or two (A and D) independent experiments.
A20 counteracts Malt1 ubiquitination and CBM-IKK association

To address whether endogenous A20 is involved in counteracting stimulus-dependent Malt1 ubiquitination in T cells, we investigated ubiquitination of Malt1 after A20 depletion in time course experiments (Fig. 4). Indeed, A20 knock-down correlated with a prolonged ubiquitination of Malt1 after P/I stimulation (Fig. 4A) or CD3/CD28 coengagement (Fig. 4B). Also, initial Malt1 ubiquitination was enhanced when A20 levels were reduced, which was especially evident after P/I stimulation of Jurkat T cells.

Because Bcl10 ubiquitination was recently suggested to promote CBM-induced IKK activation (21), we analyzed the influence of A20 on Bcl10 ubiquitination (Fig. 4C). Given the fact that cellular lysis in 30 mM NEM disrupts Bcl10/Malt1 association (data not shown), only Bcl10 ubiquitination is detectable in this experiment. The overall level and the pattern of Bcl10 ubiquitination were not altered in A20 knock-down cells, indicating that Bcl10 ubiquitin chains are not targeted by A20 in vivo. Using the K63 or K48 ubiquitin linkage-specific Abs, we were unable to determine the linkage of ubiquitin chains added to Bcl10 (data not shown), which may be due to technical constraints (e.g., low abundance) or indicate the assembly of non-K63/K48-linked ubiquitin chains on Bcl10. Further, IKKγ ubiquitination has been implicated in IKK activation after T cell stimulation (17, 20) and might therefore represent another potential target for A20 in TCR signaling. However, we only detected weak induction of IKKγ ubiquitination after stimulation of Jurkat T cells and A20 knock-down did not change the pattern of stimulus-induced IKKγ ubiquitination (data not shown).

Previously, we demonstrated that IKKγ associates with Malt1 ubiquitin chains upon T cell activation and thereby recruits the IKK to the CBM complex (16). To test whether down-regulation of A20 affects the association of IKKγ and ubiquitinated Malt1, we performed co-IPs using an anti-IKKγ Ab in siA20-transfected Jurkat T cells (Fig. 5A). Reduced levels of A20 enhanced and prolonged the interaction between IKKγ and
ubiquitin-modified Malt1 upon P/I stimulation. To investigate whether the sustained Malt1-IKK interaction correlates with a prolonged association of active IKKs with Malt1, we performed the reverse experiment and determined Malt1-associated IKK activity after Malt1 IP with GST-IkBα (aa 1-53) as a substrate (Fig. 5B). In control cells, the recruitment of active IKK complexes to Malt1 after A20 knock-down. A20 siRNA-transfected Jurkat T cells or control cells were treated with P/I as indicated. After Malt1 IP, IKK kinase assays (KA) were performed using GST-IkBα (aa 1-53) as a substrate. Graphs represent a quantification of GST-IkBα phosphorylation. Data are representative of two independent experiments.

FIGURE 6. Stimulus-dependent removal of A20 coincides with maximal NF-κB activation and Malt1 ubiquitination. A, Removal of A20 correlates with IkBα degradation and maximal NF-κB DNA binding activity. Cell extracts from P/I- or CD3/CD28-stimulated Jurkat T cells were analyzed by Western blotting (WB) and EMSA. B, A20 protein levels correlate with Malt1 ubiquitination intensities. Jurkat T cells were stimulated with P/I for the indicated times and lysed in co-IP buffer supplemented with 1% SDS. Ubiquitination was detected following Malt1 IP and Western blotting. In a parallel experiment, A20 was immunoprecipitated to detect the A20 cleavage product (A20p37). Data are representative of three independent experiments.

As noted earlier, T cell activation induces a rapid decrease and subsequent reappearance of A20 (see Fig. 1, B and C; (28)). The disappearance of A20 coincided with maximal IkBα degradation and NF-κB activation in response to P/I or CD3/CD28 costimulation (Fig. 6A), suggesting that A20 removal could be a crucial step in eliminating the negative regulatory activity of A20 on IKK upstream signaling. CD3 ligation alone was not sufficient to induce A20 degradation, indicating that this process requires CD28 costimulation (compare Fig. 2). Congruent with the stimulus-dependent degradation and de novo synthesis of A20, maximal Malt1 ubiquitination was detectable after 15 to 30 min of stimulation. To investigate whether the sustained Malt1-IKK interaction correlates with a prolonged association of active IKKs with Malt1, we performed the reverse experiment and determined Malt1-associated IKK activity after Malt1 IP with GST-IkBα (aa 1-53) as a substrate (Fig. 5B). In control cells, the recruitment of active IKK activity to Malt1 is a transient process that peaks after 15 min of stimulation. However, A20 reduction resulted in an enhanced and prolonged binding of active IKK complexes to Malt1. Hence, our results provide evidence that A20 counteracts sustained IKK activation by mediating the dissociation of CBM and IKK complexes through catalysis of Malt1 deubiquitination.

Malt1 paracaspase activity is required for strong IL-2 production but dispensable for initial NF-κB signaling

FIGURE 5. A20 prevents sustained Malt1-IKK association. A. Enhanced and sustained interaction of IKKγ with ubiquitin-conjugated Malt1 in A20 depleted T cells. Jurkat T cells were transfected with control or A20 specific siRNA. Cells were stimulated with P/I and lysed in co-IP buffer. After IKKγ IP, coimmunoprecipitated ubiquitin-modified Malt1 was detected by Western blotting using an anti-Malt1 Ab. Migration of an unspecific cross-reaction in IKKγ IPs is marked by an asterisk (see also Ref. 16). B. Sustained recruitment of active IKK complexes to Malt1 after A20 knock-down. A20 siRNA-transfected Jurkat T cells or control cells were treated with P/I as indicated. After Malt1 IP, IKK kinase assays (KA) were performed using GST-IkBα (aa 1-53) as a substrate. Graphs represent a quantification of GST-IkBα phosphorylation. Data are representative of two independent experiments.

ubiquitin-modified Malt1 upon P/I stimulation. To investigate whether the sustained Malt1-IKKγ interaction correlates with a prolonged association of active IKKs with Malt1, we performed the reverse experiment and determined Malt1-associated IKK activity after Malt1 IP in an in vitro kinase assay using GST-IkBα (aa 1-53) as a substrate (Fig. 5B). In control cells, the recruitment of IKK kinase activity to Malt1 is a transient process that peaks after 15 min of stimulation. However, A20 reduction resulted in an enhanced and prolonged binding of active IKK complexes to Malt1. Hence, our results provide evidence that A20 counteracts sustained IKK activation by mediating the dis-
30 min postinduction, at times when A20 amounts were strongly decreased (Fig. 6B). Cleavage of A20 by the paracaspase Malt1 was suggested to contribute to the decrease in A20 (28) and we also detected an A20 cleavage product (A20p37) in response to P/I or CD3/CD28 stimulation (Fig. 6B and Fig. 7, A and B).

To investigate whether Malt1 paracaspase activity and A20 cleavage is required for primary NF-κB signaling, we used the cell-permeable inhibitory peptide Z-VRPR-FMK that was shown to inhibit Malt1 paracaspase activity in vitro and in vivo (29). We incubated Jurkat T cells with Z-VRPR-FMK prior to and during P/I or CD3/CD28 stimulation to analyze whether paracaspase inhibition also prevents A20 cleavage (Fig. 7, A and B). The antagonistic peptide inhibited T cell stimulation-induced appearance of A20p37 at early and late stages of T cell activation. However, treatment with Z-VRPR-FMK did not
FIGURE 9. Proteasomal inhibition impairs degradation of A20 and impedes IKK activation upon T cell stimulation. A and B, Stimulus-dependent degradation of A20 is sensitive to proteasomal inhibition. Jurkat T cells or human T cells were treated with MG132 1 h before stimulation either with P/I (A) or with anti-CD3/CD28 coligation (B) as indicated. Whole cell extracts were analyzed by Western blotting. C–E, Proteasomal inhibition delayed the phosphorylation of IKK. Jurkat T cells (C and D) or human T cells (E) were pretreated with MG132 for 1 h and stimulated by P/I (C and E) or CD3/CD28 coligation (D) as indicated. For the detection of IKK phosphorylation, IKKα and IKKβ were immunoprecipitated from cell lysates and analyzed by Western blotting with a phospho-specific Ab. Graphs in C represent a quantification of IKK phosphorylation. F and G, Delayed activation of the IKK complex after proteasomal inhibition. Jurkat T cells were treated with MG132 1 h before stimulation with either P/I (F) or CD3/CD28-antibodies (G). IKK activity was monitored after IKKγ IP from cell lysates in an in vitro kinase assay (KA) using GST- IκBα (aa 1-53) as a substrate. Graphs represent a quantification of IKK kinase activity. Data are representative of three (C and D) or two (A, B, E–G) independent experiments.
cause a detectable inhibition of A20 degradation or an altered A20 resynthesis. These findings indicate that additional mechanisms must contribute to the rapid stimulus-dependent removal of A20 (see also below). Because the antagonistic peptide was shown to reduce activation of an NF-kB reporter gene and IL-2 production (29), we asked whether this effect is actually due to an inhibition of initial IKK/NF-kB signaling. We monitored P/I- and CD3/CD28-induced NF-kB activation and IkBα degradation in the absence or presence of Z-VRPR-FMK (Fig. 7, A and B). Despite its effect on inducible A20 cleavage, IkBα phosphorylation and amounts, as well as NF-kB DNA binding, was not altered in Z-VRPR-FMK-treated cells. Also, in primary murine CD4+ T cells, Z-VRPR-FMK incubation did not inhibit IkBα degradation after P/I stimulation, as determined by intracellular staining of IkBα and subsequent FACS analysis (Fig. 7C). However, the antagonistic peptide led to a 50% reduction in IL-2-expressing cells, as well as to an overall reduction in the level of IL-2 expression after CD3/CD28 coengagement of CD4+ T cells (Fig. 7D). These findings are in support of the hypothesis that Malt1 paracaspase is required for optimal IL-2 production but dispensable for NF-kB signaling after T cell activation.

To obtain genetic evidence for the function of Malt1 paracaspase activity, we rescued Malt1−/− CD4+ T cells using either Malt1 wt, a Malt1 paracaspase defective mutant (Malt1 C464A) (28, 29), or empty vector control (Fig. 8). CD4+ T cells from Malt1−/− mice were retrovirally infected and expanded ex vivo (16). Coexpression of the surface marker Thy1.1 was used to identify infected cells by FACS analysis. Equivalent expression of Flag-Malt1 wt and Flag-Malt1 C464A was determined by Flag-FITC and Thy1.1-allophycocyanin costaining (Fig. 8A) and Malt1 detection after Flag-IP in a Western blot (Fig. 8B). Reconstitution of T cell activation in infected cells was determined by IkBα degradation and IL-2 production (Fig. 8, C and D). The requirement for Malt1 expression in the experimental system is evident from the strongly reduced IL-2 production and IkBα degradation in empty vector Thy1.1 control infected cells. Correlating with the data obtained with the antagonistic peptide, P/I-induced IkBα degradation was completely independent from the catalytic activity of Malt1 (Fig. 8C). Again, induction of IL-2 expression was reduced by ~50% in Malt1 C464A expressing cells when compared with Malt1 wt (Fig. 8D). Taken together, these results argue against a direct involvement of Malt1 paracaspase activity in initial NF-kB activation. Further, the data suggest that Malt1 paracaspase activity does not represent an essential trigger for the release from A20-imposed inhibition of NF-kB activity. Consequently, the catalytic activity of Malt1 must elicit its function on IL-2 induction by a yet unknown mechanism.

Proteasomal inhibition impairs A20 degradation and inhibits IKK activation

Because Malt1 cleavage activity is not required for initial NF-kB signaling and only affects a small portion of the cellular A20, we asked whether proteasomal degradation might contribute to the inducible reduction of A20 protein levels. Indeed, the proteasomal inhibitor MG132 inhibited P/I and CD3/CD28-induced degradation of A20 in Jurkat T cells as well as in primary human T cells (Fig. 9, A and B). Proteasomal inhibition reduced but did not completely abrogate A20 degradation, indicating that alternative mechanisms such as A20 cleavage may still function to decrease A20 protein amounts.

Given the rapid degradation of A20 after T cell stimulation, the data suggest that proteasomal activity may be required to counteract the inhibitory function of A20 and to promote IKK activation. Based on these considerations, we sought to monitor IKK phosphorylation and IKK activity at early time points after T cell activation in Jurkat T cells in the presence or absence of the proteasomal inhibitor MG132 (Fig. 9, C–G). Proteasomal inhibition did not only impede IkBα degradation upon T cell activation with either P/I or CD3/CD28 ligation, but also delayed the onset and reduced the overall amount of IKK T loop phosphorylation in Jurkat T cells (Fig. 9, C and D) as well as in primary human T cells (Fig. 9E). Congruently, IKK kinase assays after IP of the IKK complex using anti-IKKγ specific Abs confirmed that proteasomal inhibition delayed IKK activation in response to T cell activation (Fig. 9, F and G). Thus, proteasomal activity is not only required for IkBα degradation downstream of the IKK complex, but also contributes to the inducible degradation of the upstream regulator A20.

Discussion

Previous results have indicated that A20 exerts a negative regulatory effect on TCR-induced NF-kB activation and IL-2 production (27, 28). However, the role of A20 was not investigated and therefore the molecular basis remained elusive. In this paper, we show that A20 acts as a negative regulator that balances the strength and duration of IKK/NF-kB activation after T cell stimulation. After an initial reduction of cellular A20, de novo synthesis of A20 is responsible for the post-inductive shutdown of upstream processes that promote IKK activation in T cells. Consistent with this model, overexpression of A20 was shown to inhibit NF-kB signaling upon T cell activation (27). Interestingly, CD3 ligation alone was not sufficient to induce A20 degradation and down-regulation of A20 by siRNA nearly abolished the need for the CD28 costimulator. These data suggest that induction of A20 degradation by costimulation might serve as a possible explanation for the necessity of the CD28 coreceptor signal to obtain strong IKK/NF-kB activation.

A20 is a ubiquitin editing enzyme that can cleave K63-linked ubiquitin chains from substrates and at the same time is able to catalyze the attachment of K48-linked ubiquitin chains that target proteins for proteasomal degradation (33). DUB activity of A20 was required to inhibit P/I-induced NF-kB activation in Jurkat T cells (27) and with Malt1 we have identified a novel target for A20 DUB activity. Thereby A20 interferes with the ubiquitin-mediated association between Malt1 and IKKγ and fulfills its negative regulatory function by counteracting the CBM-driven IKK activation in T cells. A20 associates with Malt1 in activated T cells (28) and we show that the A20 DUB activity is required to remove K63-linked ubiquitin chains attached to Malt1 in vitro and in vivo, suggesting that ubiquitinated Malt1 is a direct substrate of A20. However, it cannot be completely excluded that A20 may modulate Malt1 ubiquitination indirectly, e.g., by regulating the stability of another factor involved in Malt1 ubiquitination.

A recent report suggested that Bcl10 ubiquitination at K31 and K63 can also provide a docking surface for the recruitment of the IKK complex to the CBM complex (21). Because A20 knockdown did not alter Bcl10 ubiquitination, it is unlikely that the negative regulatory function of A20 is mediated through modulating Bcl10 ubiquitination. Regarding the putative dual requirement for Malt1 and Bcl10 ubiquitination, it is possible that both proteins act in concert to form the CBM-IKK aggregate. Future studies are needed to evaluate the contribution of the different ubiquitination events under various stimulatory conditions.

In vitro DUB experiments have revealed that the A20 ovarian tumor domain catalyzes the disengagement of purified ubiquitin chains and does not display a high preference for K63- vs K48-linked ubiquitin chains (34, 35). This indicates that the A20 ovarian tumor itself does not possess a high selectivity for distinct
substrates. However, siRNA-mediated down-regulation of A20 impaired the removal of Malt1 ubiquitin chains without affecting Bcl10 or IKKγ ubiquitination, which proves that in vivo A20 targets distinct substrates. We have been unable to detect stimulus-dependent TRAF6 ubiquitination in T cells (data not shown), even though upon overexpression TRAF6 is strongly autoubiquitinated and ubiquitin chains are cleaved by A20 (data not shown and Ref. 35). In general, it is still unclear how specificity for A20 DUB activity is achieved, but recent findings on the role of A20 localization and the identification of other A20 interactors indicate that the recruitment to substrate molecules must be the critical event in this process (36–38). The targeting of A20 to the CBM complex upon T cell activation may provide the basis for the deubiquitination of Malt1 by A20 in vivo.

Maximal Malt1 ubiquitination and IKK/NF-κB activation coincided with a strong increase in A20 amounts, suggesting that removal of inhibitory A20 is a critical step to allow CBM-driven IKK activation. Even though we found that the TRC-induced cleavage of A20 was sensitive to paracaspase inhibition, only a minor fraction of A20 was cleaved in response to T cell activation. Further, IKK/NF-κB signaling was neither inhibited by the Malt1-antagonistic peptide nor impaired in reconstitution experiments using the Malt1 C464A paracaspase mutant. These results suggest that paracaspase activity is not directly modulating upstream IKK/NF-κB signaling. However, Malt1 paracaspase activity supported optimal IL-2 production in response to CD3/CD28 coligation (see also (29, 39)). At this stage, the molecular basis for the requirement of Malt1 paracaspase activity for IL-2 induction is unclear and it remains to be seen whether A20 cleavage is involved. Because the antagonistic peptide was shown to reduce activation of an NF-κB reporter, Malt1 paracaspase might modulate NF-κB transcriptional activity. However, Malt1-dependent processing of Bcl10 was shown to modulate TRC-induced cell adhesion, which indicates that alternative Malt1 targets might affect NF-κB independent mechanisms (29, 39). In addition, independent of its protease activity, the Malt1 paracaspase domain regulates caspase-8-induced cleavage and inactivation of c-FLIPL, which may provide an alternative mechanism to enhance signaling and downstream IL-2 expression (40).

Despite the appearance of an A20 fragment of a size consistent with cleavage by Malt1, we identified A20 as a novel target for proteasomal degradation in activated T cells. Thus, proteasomal degradation of A20 may account for the release of its negative regulatory function. Congruent with this model, proteasomal inhibition did not only impede IκBα degradation downstream of the IKK complex, but also impaired activation of the IKK complex itself upon T cell activation. To our knowledge this finding is the first example that proteasomal inhibition affected initial IKK activation in response to upstream signaling. Because the effect was also evident after PI1 treatment, proteasomal degradation must promote the depletion of effector molecules either at the level or downstream of PKCθ on the route to IKKs. A20 seems to be a good candidate for a proteasomal target upstream of the IKK complex. However, the kinetic analysis reveals a discrepancy between the onset of A20 degradation and the extent of IKK/NF-κB activation, which should be a secondary event. The weak temporal correlation could be due to the fact that initial degradation of A20 in the microenvironment of the CBM signaling complex may promote IKK activation at time points when the drop in the total cellular pool of A20 is not yet visible. In addition, other proteasomal targets upstream of the IKK complex may exist and A20 degradation may not be the only process involved in fine tuning the T cell response. It is noteworthy that A20 has been found to localize to lysosomal compartments, which hints that there are additional ways to decrease cytosolic A20 (37). To elucidate the functional role of A20 degradation or localization for initial T cell activation, future studies must determine the exact molecular mechanisms for the dynamic regulation of A20 in detail.

With Malt1 we have identified a novel physiologial target for A20 DUB activity in T cells and thereby have defined an important negative regulatory mechanism for T cell activation. It is likely that other DUB enzymes exist that target other ubiquitinated substrates (e.g., Bcl10) or display different activity profiles and act in conjunction with A20 to balance IKK activation. Similar to IκB proteins that determine activation and termination of nuclear NF-κB activity (3), degradation and resynthesis of such DUB enzymes apparently modulate the amplitude and duration of IKK activity depending on the cellular context and the activating stimuli.

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Disclosures

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References


