NF-κB Suppression by the Deubiquitinating Enzyme Cezanne

A NOVEL NEGATIVE FEEDBACK LOOP IN PRO-INFLAMMATORY SIGNALING

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Transcription factors belonging to the NF-κB family regulate inflammation by inducing pro-inflammatory molecules (e.g. interleukin (IL)-8) in response to cytokines (e.g. tumor necrosis factor (TNF) α, IL-1) or other stimuli. Several negative regulators of NF-κB, including the ubiquitin-editing enzyme A20, participate in the resolution of inflammatory responses. We report that Cezanne, a member of the A20 family of the deubiquitinating cysteine proteases, can be induced by TNFα in cultured cells. Silencing of endogenous Cezanne using small interfering RNA led to elevated NF-κB luciferase reporter gene activity and enhanced expression of IL-8 transcripts in TNFα-treated cells. Thus we conclude that endogenous Cezanne can attenuate NF-κB activation and the induction of pro-inflammatory transcripts in response to TNF receptor (TNFR) signaling. Overexpression studies revealed that Cezanne suppressed NF-κB nuclear translocation and transcriptional activity by targeting the TNFR signaling pathway at the level of the IkB kinase complex or upstream from it. These effects were not observed in a form of Cezanne that was mutated at the catalytic cysteine residue (Cys209), indicating that the deubiquitinating activity of Cezanne is essential for NF-κB regulation. Finally, we demonstrate that Cezanne can be recruited to activated TNFRs where it suppresses the build-up of polyubiquitinated RIP1 signal adapter proteins. Thus we conclude that Cezanne forms a novel negative feedback loop in pro-inflammatory signaling and that it suppresses NF-κB activation by targeting RIP1 signaling intermediaries for deubiquitination.

The transcription factor NF-κB drives inflammatory responses by inducing pro-inflammatory molecules including adhesion proteins, cytokines, and chemokines (e.g. IL-8)2 (1). It is regulated by diverse stimuli including pro-inflammatory cytokines (e.g. TNFα, IL-1) (1) and mechanical forces (e.g. shear stress) (2) and plays a central role in numerous diseases including atherosclerosis, rheumatoid arthritis, and rejection of transplanted organs.

In unstimulated cells, NF-κB is sequestered in the cytoplasm by inhibitory IkB molecules that bind its nuclear localization sequence. Signaling through TNF receptors (TNFR) leads to the recruitment of several signaling intermediaries including RIP1, TRAF2/5, and NEMO (IKKγ), which control the activation of TAK1, a mitogen-activated protein kinase kinase kinase that can activate IKKs through phosphorylation. Activation of IKKβ triggers phosphorylation of IkBα, which is subsequently destabilized, thus releasing free NF-κB for nuclear translocation and stimulation of transcription (1).

Recent studies have revealed that pro-inflammatory signaling is regulated by ubiquitin, a small protein that can be covalently attached to lysine residues of cellular proteins through a chain reaction co-ordinated by E1, E2, and E3 proteins (3, 4). Multiple rounds of ubiquitination, where ubiquitin is itself ubiquitinated on lysine residues, generate isopeptide-linked branched chains that play a key role in signaling to NF-κB. Specifically, activation of NF-κB in response to TNF or IL-1/Toll-like receptor signaling relies on modification of RIP1 or TRAF6 signaling intermediaries, respectively, with a distinct structural form of polyubiquitin that contains ubiquitin Lys63 linkages (5–8). Pro-inflammatory signaling also leads to Lys63-polyubiquitination of NEMO, an adaptor protein that binds IKK and is essential for NF-κB activation (9). It has been suggested that Lys63 chains signal to NF-κB by interacting with TAB2 signaling intermediaries, thus triggering the oligomerization and activation of TAK1 and IKKβ (10). Polyubiquitin chains linked through Lys48 also play a vital role during NF-κB activation by targeting phosphorylated IkBα proteins for proteasomal degradation, thus liberating NF-κB for nuclear entry (1).

TNFα induces several negative regulators of NF-κB including A20, a protein that interacts with TRAFs (11, 12), NEMO (13), and IKKα (14) and inhibits the catalytic activity of IKKβ (15). Recent work demonstrated that A20 is a deubiquitinating cysteine protease that can cleave ubiquitin monomers from modified proteins (8, 16). Thus it has been suggested that A20 suppresses signaling to NF-κB by targeting signaling intermediaries for ubiquitin editing. We have previously described a of thermal cycles that were necessary to generate threshold amounts of product.

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novel protein called Cezanne that belongs to the A20 family of deubiquitinating enzymes and can modulate TNFα signaling when overexpressed (17, 18). Here we demonstrate that Cezanne can be induced by TNFα in cultured epithelial or endothelial cells. Gene silencing revealed that endogenous Cezanne attenuates NF-κB activation and the induction of pro-inflammatory transcripts in TNFα-treated cells, thus completing a novel negative feedback loop. Finally, we provide evidence that Cezanne suppresses NF-κB upstream of IKK activation by targeting RIP1 signaling intermediaries for deubiquitination.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Human recombinant TNFα was obtained commercially (R & D, Minneapolis, MN). Biotinylated human TNFα was kindly provided by Prof. G. Cohen (University of Leicester). Anti-RelA (p65) (sc372; Santa Cruz Biotechnology, Santa Cruz, CA), anti-1κBα (sc-847; Santa Cruz Biotechnology), anti-phospho1κBα Ser32/36 (Cell Signaling Technology), anti-RIP (BD Biosciences Pharmingen, Oxford, UK), anti-HA (Roche Applied Science), anti-GFP (Santa Cruz Biotechnology), anti-IKKα (BD Biosciences Pharmingen, Oxford, UK), anti-IKKγ (BD Biosciences Pharmingen, Oxford, UK), anti-RIP (BD Biosciences Pharmingen, Oxford, UK), anti-TNFFR (Invitrogen), and anti-tubulin (Sigma) were obtained commercially.

Rabbit polyclonal antibodies were raised against a peptide that was conserved between human and mouse versions of Cezanne but not encoded by other mammalian genes (C+/YSNGYREPPEPDGWA-COH2) and were subsequently affinity purified (Eurogentech, Liege, Belgium). Anti-Cezanne antibodies were validated by Western blotting of cytosolic lysates made from cells transfected with epitope-tagged forms of Cezanne (supplemental Fig. S1). Other reagents were purchased from Sigma-Aldrich unless otherwise stated.

**Plasmids**—Expression vectors encoding HA epitope-tagged Cezanne (pHM6-Cezanne), GFP-tagged Cezanne (pEGFP-Cezanne) and enzymatically inactive forms mutated at the catalytic cysteine (pHM6-Cezanne C209S and pEGFP-Cezanne C209S) have been described previously (17, 18). Expression plasmids containing HA-tagged ubiquitin or versions of ubiquitin containing single Lys residues (Lys48 or Lys63) were described previously (16).

**Endothelial Cells and Exposure to Flow**—Human umbilical vein endothelial cells (HUVEC) were collected using collagenase and cultured as described previously (19). Confluent HUVEC cultures were exposed to high shear (12 dynes/cm²) unidirectional laminar flow for 16 h using a parallel-plate flow chamber (Cytodyne, La Jolla, CA) as described previously (19).

**Cell Lines and Transfection**—HEK293 cells were cultured using Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, supplemented with 100 U/ml penicillin G/100 μg/ml streptomycin. Transient transfection of cells was achieved using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Stable expression of GFP or GFP-Cezanne in HEK293 cells was achieved by culturing cells transfected transiently using pEGFP or pEGFP-Cezanne, respectively, with neomycin. After 4 weeks, transfected cells expressing similar amounts of GFP or GFP-Cezanne were selected further by fluorescence-activated cell sorting and used in experiments.

**RNA Interference**—RNA interference was carried out using a specific siRNA that is known to silence Cezanne in HEK293 cells without altering the expression of related molecules.3 Cezanne-specific double-stranded siRNA oligonucleotides with 3′-dTdT (Cez 5′-GAATCTTATCTGCGCTTGA-3′; Dharmacon, Chicago, IL) or nontargeting scrambled controls (Silencer Negative control 1 siRNA; Ambion) were synthesized. Cell cultures that were 80–90% confluent were transfected with siRNA (final concentration, 10 nM) using Lipofectamine (Invitrogen) following the manufacturer’s instructions and then incubated in complete growth medium for 48 h before analysis.

**Comparative Real Time PCR**—Transcript levels were quantified by comparative real time PCR using gene-specific primers for Cezanne (sense, 5′-ACAATGTCCGATTTGGCCAGT-3′; antisense, 5′-ACAGTGAGGATGTCCTGATT-3′), IL-8 (sense, 5′-TGCAAAAGGATGCTAAAG-3′; antisense, 5′-CCTCCACAACCTCCTGAC-3′), and β-actin (sense, 5′-CTGGAAACGCTGTTGACA-3′; antisense, 5′-AAGGACTCTGTAACATGCA-3′). Total RNA was extracted and reverse transcribed as described previously (19). Real time PCR was carried out using the iCycler system and SYBR green master mix (Bio-Rad) according to the manufacturer’s instructions. The reactions were incubated at 95 °C for 3 min before thermal cycling at 95 °C for 10 s and 56 °C for 45 s. The reactions were performed in triplicate. Relative gene expression was calculated by comparing the number of thermal cycles that were necessary to generate threshold amounts of product (CT) as described previously (19). CT was calculated for the genes of interest and for the housekeeping gene β-actin. For each cDNA sample, the CT for β-actin was subtracted from the CT for each gene of interest to give the parameter DCT, thus normalizing the initial amount of RNA used. The amount of each target was calculated as 2−DDCT, where DDCT is the difference between the DCT of the two cDNA samples to be compared.

**Immunostaining to Detect Cezanne**—Immunostaining was performed by fixing cells using methanol for 10 min prior to the application of rabbit anti-Cezanne antibodies (1:50) and goat anti-rabbit Alexafluor488-conjugated secondary antibodies (1:500) followed by laser scanning confocal microscopy (LSM 510 META; Zeiss, Oberkochen, Germany).

**Immunostaining to Detect NF-κB and 1κB—Intracellular localization of RelA NF-κB and 1κB was assessed by immunostaining of paraformaldehyde-fixed cells using anti-RelA antibodies and Alexafluor 568-conjugated secondary antibodies followed by laser scanning confocal microscopy (LSM 510 META; Zeiss, Oberkochen, Germany). Image analysis was performed using Velocity software (Improvision, Coventry, UK) to calculate the ratio of RelA present in the nucleus compared with the cytoplasm. Levels of 1κBα were measured by immunostaining of paraformaldehyde-fixed cells using anti-1κBα antibodies and Alexafluor 568-conjugated secondary antibodies followed by laser scanning confocal microscopy (LSM 510 META; Zeiss, Oberkochen, Germany).

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3 H. Tran, F. Hamada, T. Schwarz-Romand, and M. Bienz, submitted for publication.
**Assay of NF-κB Transcriptional Activity—**NF-κB transcriptional activity was measured in HEK293 cells using an NF-κB reporter (pGL2) supplied by Dr. Martin Turner (The Babraham Institute, Cambridge, UK). The cells were co-transfected with pNF-luc and pRL-TK (encoding Renilla luciferase to normalize transfection efficiency) using Lipofectamine and incubated for 16 h. The cells were then treated with TNFα (10 ng/ml) for 16 h before measurement of NF-κB activity. Firefly and Renilla luciferase activity was assessed using a dual luciferase reporter assay kit (Promega, Madison, WI) and luminescence counter (Topcount Microplate Scintillation; Packard).

**IKK Kinase Assay—**Cultured cells were lysed using 50 mM Tris-HCl, pH 7.5, 10% glycerol, 250 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 3 mM EDTA, 3 mM EGTA. The debris was co-transfected with 1/10 of the NF-κB reporter (pEGFP-Cezanne or pEGFP-Cezanne C209S). Polyubiquitin and Cezanne were detected in cytosolic lysates by Western blotting using anti-HA (1:1000) and anti-GFP (1:1000) primary antibodies, respectively, horseradish peroxidase-conjugated secondary antibodies, and chemiluminescent detection.

**Precipitation of TNFR Complexes—**HEK293 cells were treated with biotinylated TNFα (200 ng/ml) for varying durations and then lysed using 50 mM Tris pH 7.6, 137 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 3 mM EDTA, 3 mM EGTA. The debris was removed from lysates by high speed centrifugation. IKKβ kinase was purified from lysates by immunoprecipitation using anti-IKKγ antibodies (BD Pharmingen, Oxford, UK), and its activity was assessed using glutathione S-transferase-IκBα as substrate as described previously (20).

**Precipitation of TNFR Complexes—**HEK293 cells were treated with biotinylated TNFα (200 ng/ml) for varying durations and then lysed using 50 mM Tris-pH 7.6, 137 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 3 mM EDTA, 3 mM EGTA, 5 mM EDTA, 10% glycerol, 0.1 mM Na3VO4, 10 μM N-ethylmaleimide, 20 mM MG132 plus a mixture of protease inhibitors (Roche Applied Science). The lysates were clarified by low speed centrifugation and pre cleared using protein G-Sepharose before precipitation of TNFR complexes using streptavidin-coated beads (Roche Applied Science). The beads were then washed extensively using lysis buffer. Precipitated material or lysates were analyzed by Western blotting using specific primary antibodies, horseradish peroxidase-conjugated secondary antibodies and chemiluminescent detection.

**Assay of Deubiquitination in Cultured Cells—**The effect of Cezanne on ubiquitinated proteins was assessed using HEK293 cells cultured in 60-mm dishes. They were transfected with 0.5 μg of mammalian expression vector containing HA-tagged wild-type ubiquitin or containing HA-tagged, mutated forms of ubiquitin that contained single Lys residues for polyubiquitin chain assembly i.e. Lys63 only or Lys48 only. Some cultures were co-transfected with 1 μg of pEGFP-Cezanne or pEGFP-Cezanne C209S. Polyubiquitin and Cezanne were detected in cytosolic lysates by Western blotting using anti-HA (1:1000) and anti-GFP (1:1000) primary antibodies, respectively, horseradish peroxidase-conjugated secondary antibodies, and chemiluminescent detection.

The effect of Cezanne on RIP1 ubiquitination was also measured. HEK293 cells were transfected with expression plasmids containing either wild-type or catalytically inactive forms of Cezanne using Lipofectamine (Invitrogen) following the manufacturer’s recommendations. After 48 h, the cells were treated with biotinylated TNFα (200 ng/ml) for varying durations before precipitation of TNFR complexes as above. Precipitated material or lysates were analyzed by Western blotting using specific primary antibodies, horseradish peroxidase-conjugated secondary antibodies, and chemiluminescent detection.

**Statistics—**The differences between samples were analyzed using a paired Student’s t test and analysis of variance (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

**RESULTS**

**Regulation of Cezanne Expression by Pro-inflammatory Stimuli—**We observed by comparative real time PCR that Cezanne transcripts were induced rapidly in response to TNFα in HEK293 cells (Fig. 1A). The expression of Cezanne was also assessed in HUVEC cultured either in static conditions or under physiological levels of laminar shear stress (12 dynes/cm²). Interestingly, although shear stress had relatively modest effects on Cezanne mRNA levels, it primed HUVEC for enhanced expression of Cezanne transcripts in response to TNFα (Fig. 1B). The effects of TNFα on Cezanne transcripts were paralleled by rapid and sustained increases in Cezanne protein levels in HUVEC as demonstrated by Western blotting (Fig. 1C) or immunostaining (Fig. 1D) using anti-Cezanne antibodies. Our observation that Cezanne can be induced in cultured cells by TNFα or shear stress suggests that it may regulate cellular responses to these stimuli.

**Cezanne Limits Pro-inflammatory Cellular Activation by Suppressing NF-κB—**We used RNA interference to assess the potential role of endogenous Cezanne in regulating pro-inflammatory responses to TNFα. We observed that Cezanne-specific siRNA suppressed Cezanne mRNA and protein by at least 70% in culture cells, whereas a nontargeting scrambled control had no effect (Fig. 2, A and B). Silencing of Cezanne in HUVEC had no effect on basal levels of IL-8 transcripts in unstimulated cells but enhanced the subsequent induction of IL-8 transcripts by TNFα (Fig. 2C, compare treatments 2 and 6), whereas a scrambled control sequence had no effect (compare treatments 2 and 4). Similarly, reporter gene assays revealed that NF-κB transcriptional activation in response to TNFα was significantly enhanced by Cezanne-specific siRNA in HEK293 cells but was not altered by scrambled control sequences (Fig. 2D, compare treatments 3 and 4). We observed that silencing of Cezanne using an alternative siRNA also enhanced NF-κB activity in TNFα-treated cells (Fig. 2D, compare treatments 3 and 5). Thus we conclude that endogenous Cezanne functions as a negative regulator of NF-κB activation and pro-inflammatory transcriptional responses in cells exposed to TNFα.

**The Deubiquitinating Activity of Cezanne Is Essential for NF-κB Suppression—**We determined whether the deubiquitinating cysteine protease activity of Cezanne is required for its inhibitory effects on NF-κB. Reporter gene experiments or assays of NF-κB localization were performed in HEK293 cells transfected transiently with expression plasmids containing either wild-type Cezanne or a catalytically inactive form in which the active site cysteine (Cys309) has been mutated to serine (Cezanne C209S). We observed that wild-type Cezanne reduced NF-κB transcriptional activity in a dose-dependent manner in HEK293 cells stimulated with TNFα (Fig. 3A, upper panel, compare bars 3–5 with bar 2) or IL-1 (lower panel, compare bars 3–5 with bar 2). Compared with wild-type Cezanne, the catalytically inactive form was ineffective in reducing NF-κB activity in response to TNFα (Fig. 3A, upper panel, com-
Ceazanne following 2 h of stimulation with TNFα (Fig. 3B, lower panel, compare treatments 4 and 7 and treatments 6 and 9). However, residual nuclear RelA was not exported from cells that overexpressed wild-type Ceazanne following 2 h of stimulation (Fig. 3B, lower panel, compare treatments 5 and 8), possibly because of the suppression by Ceazanne of NF-κB transcriptional activity, which is essential for RelA export. Collectively, our data indicate that the catalytic properties of Ceazanne are required for suppression of NF-κB nuclear localization and transcriptional activation in response to pro-inflammatory stimuli.

Ceazanne Suppresses Nuclear Translocation of NF-κB by Inhibiting IKK Activity—The level at which Ceazanne modulates TNFR signaling to NF-κB was assessed further by biochemical studies using HEK293 cells that stably expressed similar quantities of either GFP-Ceazanne or GFP alone. Western blotting of cytosolic lysates revealed that IkBα was entirely degraded by 15–30 min of TNFα treatment in untransfected cells (Fig. 4A, compare treatments 3 and 4 with treatment 1) and in cells that expressed GFP (compare treatments 7 and 8 with treatment 5), but it was largely preserved in cells that expressed GFP-Ceazanne (compare treatments 11 and 12 with treatments 3, 4, 7, and 8).

Given the role of IKKβ in phosphorylating IkBα, we assessed whether its activation by TNFα can be modulated by Ceazanne. We observed that IKK activity was elevated sharply by TNFα in control cells that expressed GFP, occurring maximally following 15 min of treatment (Fig. 4B). By comparison, IKK activation in response to TNFα treatment was significantly reduced in cells that expressed GFP-Ceazanne (Fig. 4B, compare treatments 8 and 3). We conclude therefore that Ceazanne targets the TNFR signaling pathway at the level of the IKK complex or upstream from it, thus suppressing NF-κB by stabilizing IkBα.

We determined whether the deubiquitinating activity of Ceazanne is required for its inhibitory effects on IkBα phosphorylation and degradation using cells transfected transiently with expression plasmids containing either wild-type Ceazanne or a catalytically inactive form (Ceazanne C209S). We observed by immunostaining and confocal microscopy that wild-type Ceazanne suppressed degradation of IkBα in response to TNFα, whereas overexpression of Ceazanne C209S had no effect (Fig. 4C, compare treatments 3 and 4 and treatments 5 and 6). Sim-
NF-κB Suppression by Cezanne

A

B

C

D

mixed Lys-polyubiquitin chains were reduced in HEK293 cells that stably expressed GFP-Cezanne compared with cells that stably expressed GFP alone (Fig. 5A, compare treatments 7–9 with treatments 4–8) and untransfected cultures (Fig. 5A, compare treatments 7–9 with treatments 1–3). In addition, the build-up of Lys63- or Lys48-polyubiquitin chains was suppressed in HEK293 cells that expressed wild-type Cezanne compared with cells that expressed a catalytically inactive version (Cezanne C209S) (Fig. 5B, compare treatments 1 and 2 and treatments 3 and 4). Collectively, these data imply that Cezanne has the capacity to deubiquitinate proteins modified with either Lys63- or Lys48-polyubiquitin in cultured cells.

Cezanne Is Recruited to the TNFR

Where It Targets RIP1 for Deubiquitination—Recent reports suggest that Lys63-polyubiquitination of RIP1 is essential for the activation of IKKβ in response to TNFα signaling. Given that Cezanne suppresses IKK activation, we assessed whether it could be recruited to the TNFR to alter RIP1 polyubiquitination. Wild-type or catalytically inactive Cezanne (C209S) was overexpressed in cultured cells, and its capacity to be recruited to the TNFR and its effects on polyubiquitinated RIP1 proteins were determined following purification of TNFR complexes using biotinylated TNFα. Western blotting revealed co-precipitation of Cezanne with biotinylated TNFα.

Cezanne Deubiquitinates Lys48- or Lys63-polyubiquitinated Cellular Proteins—We examined the effects of Cezanne on the modification of cellular proteins with Lys48- or Lys63-polyubiquitin chains, which are essential processes during NF-κB activation (Fig. 5). Polyubiquitin chains linked through Lys48, Lys63, or multiple Lys residues were generated in cells using expression vectors containing mutated, single Lys versions of ubiquitin or wild-type ubiquitin, respectively. We observed that the levels of cellular proteins modified with either Lys48-, Lys63-, or

FIGURE 2. Endogenous Cezanne regulates pro-inflammatory activation by suppressing NF-κB. A, HUVEC or HEK293 cells were transfected with Cezanne-specific siRNA (Cez) or with a scrambled control (Scr) as indicated. Levels of Cezanne transcripts were quantified from triplicate wells by comparative real time PCR and normalized by measuring β-actin transcript levels. The mean values calculated from duplicate measurements are shown with standard deviations. B, HUVEC transfected with siRNA or untransfected cells (mock) were stimulated with TNFα (10 ng/ml) for 2 h. Alternatively, cells were transfected with pHM6-Cezanne as a positive control for Cezanne expression. Expression levels of Cezanne proteins were determined by Western blotting of cytosolic lysates using anti-Cezanne or anti-tubulin antibodies (upper panels). Cezanne protein levels were quantified by densitometry and normalized by measuring tubulin protein levels. The mean values calculated from duplicate measurements are shown with standard deviations (lower panel). C, the effect of Cezanne silencing on the induction of IL-8 transcripts was assessed by comparative real time PCR. HUVEC transfected with siRNA or untransfected cells (mock) were treated with TNFα (10 ng/ml) for 2 h or remained untreated as indicated. Levels of IL-8 transcripts were quantified by real time PCR using gene-specific PCR primers and normalized by measuring β-actin transcript levels. The mean values calculated from triplicate measurements are shown with standard deviations. The data are representative of those from two experiments that gave closely similar results. D, the effect of Cezanne silencing on NF-κB transcriptional activity was assessed by reporter assay. HEK293 cells transfected with Cezanne-specific siRNA (Cez or Cez2) or untransfected controls (mock) were stimulated with TNFα (10 ng/ml) for 16 h or remained untreated as a control. The cell lysates were analyzed, and the ratio firefly/Renilla luciferase activity was calculated, which is a measure of NF-κB activity normalized for transfection efficiency. The mean values calculated from triplicate wells were pooled from four experiments and are shown with standard deviations.

Similarly, Western blotting revealed that wild-type Cezanne suppressed Ser phosphorylation of IκBα in response to TNFα, whereas Cezanne C209S did not (Fig. 4D, compare treatments 5 and 6). Thus we conclude that the deubiquitinating activity of Cezanne is essential for suppression of IκBα phosphorylation and degradation in activated cells.

Cezanne Deubiquitinates Lys48- or Lys63-polyubiquitinated Cellular Proteins—We examined the effects of Cezanne on the modification of cellular proteins with Lys48- or Lys63-polyubiquitin chains, which are essential processes during NF-κB activation (Fig. 5). Polyubiquitin chains linked through Lys48, Lys63, or multiple Lys residues were generated in cells using expression vectors containing mutated, single Lys versions of ubiquitin or wild-type ubiquitin, respectively. We observed that the levels of cellular proteins modified with either Lys48-, Lys63-, or
fected cells with Cezanne and a mutated version of ubiquitin that forms polyubiquitin chains linked exclusively through Lys63. Wild-type Cezanne suppressed the build-up of Lys63-polyubiquitin chains at the TNFR, whereas the catalytically inactive form did not (Fig. 6C, compare treatments 3 and 4). Collectively these data suggest that Cezanne can be recruited to the TNFR in response to TNFα/H9251 where it removes Lys63-polyubiquitin from RIP1.

DISCUSSION

Ubiquitination can be reversed by deubiquitinating enzymes that cleave ubiquitin from modified proteins, thus modulating their stability or activity (22). Until recently, deubiquitinating enzymes that specifically regulate signaling pathways have received little attention. Nevertheless, their potential importance in regulating fundamental and diverse physiological processes including inflammation, apoptosis (23), proliferation (24), and differentiation (25) has been demonstrated. Recent studies have identified the OTU family of deubiquitinating cysteine proteases, which includes Cezanne, A20, VCIP135, and Otubain 1. The OTU family regulates highly divergent physiological activities including NF-κB activity (A20 (8, 13, 14, 16)), Golgi-membrane fusion in mitosis (VCIP135 (26)), and T cell energy (Otubain 1 (27)). Previous overexpression studies using cultured mammalian cells have revealed that Cezanne can interact with TRAF signaling molecules and has the capacity to inhibit NF-κB transcriptional activity in response to TNFR or IL-1R signaling (17). However, the function of endogenous Cezanne has not been previously studied. Here we demonstrate by gene silencing that Cezanne can suppress NF-κB activation and the induction of pro-inflammatory molecules in cells exposed to TNFα/H9251. In contrast to a previous report from our group (17), we have now revealed using techniques with improved sensitivity that Cezanne can be induced in cultured cells by TNFα. Thus we conclude that Cezanne may contribute to the resolution of inflammation by forming a negative feedback loop in pro-inflammatory activation.

Signaling through TNFR leads to modification of RIP1 signaling intermediaries with Lys63-linked polyubiquitination chains, a process that triggers IKKβ activation (8, 10). Our study provides several lines of evidence to suggest that Cezanne suppresses TNFR signaling to NF-κB by targeting RIP1 for deubiquitination. First, Cezanne suppressed TNFR signaling at the level of the IKK complex or upstream from it. Second, Cezanne can be recruited to activated TNFRs where it can suppress polyubiquitinated forms of RIP1. Third, the capacity of Cez-
NF-κB Suppression by Cezanne

A

anti-IκBα
anti-GFP
anti-tubulin

TNFα (min) 0 1 2 3 4 5 6 7 8 9
untransfected pEGFP pEGFP-Cezanne




B

phospho-IκBα
anti-IκBα

TNFα (min) 0 1 2 3 4 5 6
pEGFP pEGFP-Cezanne




C

GFP IκBα merge
Cezanne
Cezanne C209S
Cezanne
Cezanne C209S

unreated


+ TNFα




D

phospho-IκBα

Cezanne

TNFα (min) 0 15 30
untransfected pEGFP pEGFP-Cezanne pEGFP-Cezanne C209S
NF-κB Suppression by Cezanne

Cezanne was also required for suppression of NF-κB activation in response to IL-1R signaling; however, the physiological substrate targeted for deubiquitination by Cezanne in this pathway remains uncertain.

Two other deubiquitinating enzymes, A20 and CYLD, function as negative regulators of NF-κB. A20, a close relative of Cezanne, also possesses ubiquitin ligase activity, which has been mapped to a zinc finger within the C-terminal half (8). It has been suggested that A20 uses both of its catalytic activities to suppress NF-κB activation by deubiquitinating and subsequently destabilizing pro-inflammatory signaling intermediaries such as RIP1 in the TNFR pathway and TRAF6 in the IL-1R/Toll-like receptor pathway (8, 28). CYLD, a member of the ubiquitin-specific protease family of deubiquitinating enzymes, was initially characterized as a tumor suppressor that is mutated in hereditary cylindromatosis (29–32). Subsequent studies revealed that CYLD also possesses anti-inflammatory functions by suppressing NF-κB activation in response to pro-inflammatory stimuli (33, 34). This property relies on the capacity of CYLD to cleave monoubiquitin from Lys48-polyubiquitinated signaling intermediaries. In macrophages, CYLD suppresses Toll-like receptor signaling by targeting IKKγ and TRAF6 for deubiquitination, but it does not target RIP1 in the TNFR pathway (34). By contrast, Cezanne and A20 can both target RIP1 for deubiquitination, and it is therefore surprising that both molecules are required for the negative regulation of TNFR signaling. However, it is possible that Cezanne and A20 have divergent specificities toward other TNFR signaling intermediaries, thus providing a potential explanation for their non-redundant functions. Future studies should now be conducted to characterize further the physiological substrates of Cezanne, A20, and CYLD in pro-inflammatory signaling pathways.

Laminar flow has profound effects on the physiology of endothelial cells and may protect arteries from the initiation of atherosclerosis by suppressing vascular inflammation (35–37). We have previously shown that exposure of cultured endothelial cells to laminar flow altered subsequent transcriptional responses to TNFα by suppressing the induction of pro-inflammatory (E-selectin, VCAM-1, IL-8) and anti-inflammatory (IκB, A20) transcripts (19). In contrast, we now report that laminar flow led to the induction of Cezanne in endothelial cells and also primed them for enhanced induction of Cezanne in response to TNFα. Thus it is likely that Cezanne regulates NF-κB activation in endothelial cells exposed to physiological conditions.

FIGURE 4. Cezanne stabilizes IκBα by inhibiting IKK activity. A and B, IκBα destabilization and IKK activation in response to TNFα were assessed in HEK293 cells transfected stably using pEGFP-Cezanne or pEGFP or in untransfected cells as a control. The cells were stimulated with TNFα (10 ng/ml) for varying times as indicated. A, expression levels of IκBα and GFP-tagged proteins were determined by Western blotting of cytosolic lysates using anti-IκBα, anti-GFP or anti-tubulin antibodies (lower panels). IκBα levels were quantified by densitometry and normalized by measuring tubulin protein levels. The mean values calculated from duplicate measurements were pooled from two experiments and are shown with standard deviations (lower panel). B, IKK activity was measured in cytosolic lysates, after immunoprecipitation of the IKK complex, by in vitro kinase assay using glutathione S-transferase-IκBα substrate (upper panels). Kinase activity was quantified by phosphorimaging. The data were normalized by measuring IKKα protein levels by Western blotting and densitometry. The mean values calculated from duplicate measurements were pooled from two experiments and are shown with standard deviations (lower panel). C, levels of IκBα were assessed in HeLa cells that were transfected with expression vectors encoding either native (pEGFP-Cezanne) or catalytically inactive (pEGFP-Cezanne C209S) versions of Cezanne. After 16 h, the cells were stimulated with TNFα (10 ng/ml) for varying durations as indicated. Immunostaining was performed using anti-IκBα antibodies followed by laser scanning confocal microscopy and image analysis. The images are shown that are representative of those from two closely similar experiments (upper panels). Mean levels of IκBα were calculated by measuring red channel fluorescence from multiple transfected cells and are shown with standard deviations (lower panel). D, serine phosphorylation of IκBα in response to TNFα was assessed in HeLa cells that were transfected transiently with pEGFP-Cezanne or pEGFP-Cezanne C209S. After 16 h, the cells were stimulated with TNFα (10 ng/ml) for 5 min or remained untreated as a control (as indicated). Cytosolic lysates were tested by Western blotting using anti-phospho-IκBα Ser32/36, anti-Cezanne, or anti-tubulin antibodies. Levels of phosphorylated IκBα were quantified by densitometry and normalized by measuring tubulin protein levels. The mean values calculated from triplicate measurements are shown with standard deviations (lower panel).

FIGURE 5. Cezanne targets cellular proteins modified with Lys48- or Lys63-polyubiquitin for deubiquitination. Assays of deubiquitination were carried out in cultured cells. A, HEK293 cells transfected stably using pEGFP-Cezanne or pEGFP or untransfected cells were co-transfected with expression vectors containing HA-tagged wild-type ubiquitin (WT) or mutated versions that contained single Lys residues for polyubiquitin chain assembly (Lys48 or Lys63). After 48 h, cultures transfected with WT or Lys63 ubiquitin were treated with 20 μM MG132 for 1 h, whereas cells transfected with Lys48 ubiquitin remained untreated. The cell lysates were tested by Western blotting using anti-HA antibodies to detect residual ubiquitinated proteins (top panels) or with anti-GFP antibodies to detect GFP-fusion proteins (center panels) or anti-tubulin antibodies (bottom panels). B, HEK293 cells were co-transfected transiently with expression vectors containing HA-tagged versions of ubiquitin (Lys48 or Lys63) and GFP-tagged versions of Cezanne (wild-type (pEGFP-Cez) or catalytically inactive (pEGFP-Cez C209S)). After 48 h, the cell lysates were tested by Western blotting using anti-HA antibodies to detect ubiquitinated proteins (upper panel) or with anti-GFP antibodies to detect Cezanne (lower panel).
NF-κB Suppression by Cezanne

FIGURE 6. Cezanne is recruited to the TNFR where it targets RIP1 for deubiquitination. RIP1 ubiquitination and its regulation by Cezanne were assessed by precipitation of TNFα-TNFR complexes from cultured cells using biotinylated TNFα. The cultures were stimulated with biotinylated TNFα for varying times before preparation of cytosolic lysates. Alternatively, cytosolic lysates were made from untreated cells as a control and then supplemented with biotinylated TNFα. Streptavidin-coated beads were then used to precipitate TNFα-TNFR complexes. A, cells were transfected with an expression vector encoding Cezanne (pEGFP-Cezanne). After 24 h, the cultures were stimulated with biotinylated TNFα for 5 min or remained untreated as a control. TNFα-TNFR precipitates were tested by Western blotting using anti-TNFR (top panel) or anti-Cezanne antibodies (center panel). The cytosolic lysates were tested by Western blotting using anti-tubulin antibodies (bottom panel). The images shown are representative of those from two closely similar experiments. B, cells were transfected with an expression vector encoding either wild-type (pEGFP-Cezanne) or catalytically inactive (pEGFP-Cezanne C209S) versions of Cezanne. After 24 h, the cultures were stimulated with biotinylated TNFα for varying times (as indicated) or remained untreated as a control. Precipitating proteins were tested by Western blotting using anti-RIP antibodies (upper panel). The cell lysates were tested by Western blotting using anti-RIP or anti-Cezanne antibodies (two lower panels). C, HeLa cells were co-transfected transiently with a HA-tagged version of ubiquitin that contained a single Lys residue at amino acid 63 (Ub K63) and with either wild-type (pEGFP-Cezanne) or catalytically inactive (pEGFP-Cezanne C209S) versions of Cezanne. After 24 h, the cultures were stimulated with biotinylated TNFα for 5 min or remained untreated as a control. TNFα-TNFR precipitates were tested by Western blotting using anti-HA antibodies to detect Lys-Ub polyubiquitin chains (upper panel). The cell lysates were tested by Western blotting using anti-Cezanne or anti-tubulin antibodies (two lower panels). The images shown are representative of those from four closely similar experiments.

flow in the absence of A20. Our findings are consistent with a previous study in which Cezanne transcripts were detected at high levels in porcine aortic endothelium exposed to high rates of laminar flow in the descending aorta, compared with endothelium exposed to low/oscillatory flow in the lesser curvature of the aortic arch (37). Gene targeting studies are now required to assess the potential importance of Cezanne in regulating vascular inflammation.

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