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Zinc-finger protein A20, a regulator of inflammation and cell survival, has de-ubiquitinating activity

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Ubiquitination regulates the stability and/or activity of numerous cellular proteins. The corollary is that de-ubiquitinating enzymes, which ‘trim’ polyubiquitin chains from specific substrate proteins, play key roles in controlling fundamental cellular activities. Ubiquitin is essential at several stages during the activation of NF- κ B (nuclear factor κ B), a central co-ordinator of inflammation and other immune processes. Ubiquitination is known to cause degradation of the inhibitory molecule I κ B α (inhibitor of κ B). In addition, activation of TRAF (tumour-necrosis-factor-receptor-associated factor) and IKK γ (I κ B kinase γ)/NEMO (NF- κ B essential modifier) signal adaptors relies on their modification with ‘non-classical’ forms of polyubiquitin chains. Ubiquitin also plays a key role in determining cell fate by modulating the stability of numerous pro-apoptotic or anti-apoptotic proteins. The zinc-finger protein A20 has dual functions in inhibiting NF- κ B activa-

tion and suppressing apoptosis. The molecular mechanisms of these anti-inflammatory and cytoprotective effects are unknown. Here we demonstrate that A20 is a de-ubiquitinating enzyme. It contains an N-terminal catalytic domain that belongs to the ovarian-tumour superfamily of cysteine proteases. A20 cleaved ubiquitin monomers from branched polyubiquitin chains linked through Lys⁴⁸ or Lys⁶³ and bound covalently to a thiol-group-reactive, ubiquitin-derived probe. Mutation of a conserved cysteine residue in the catalytic site (Cys¹⁰³) abolished these activities. A20 did not have a global effect on ubiquitinated cellular proteins, which indicates that its activity is target-specific. The biological significance of the catalytic domain is unknown.

Key words: A20, apoptosis, de-ubiquitinating enzyme, nuclear factor κ B (NF- κ B), ovarian tumour (OTU), ubiquitin.

INTRODUCTION

Ubiquitination regulates the activities of numerous cellular proteins [1]. Covalent attachment of ubiquitin is catalysed by E1, E2 and E3 ubiquitin ligases, which collectively determine substrate specificity. Multiple rounds of ubiquitination, where ubiquitin is itself ubiquitinated on Lys⁴⁸, generate an isopeptide-linked branched chain. Proteins thus modified are targeted to the proteasome, where they are degraded. Non-classical forms of ubiquitination, such as mono-ubiquitination or assembly of chains linked through Lys⁶³, regulate internalization of transmembrane proteins and also influence the function of signal adaptors [1]. De-ubiquitinating enzymes are important regulators of these processes. They play an essential role in generating a pool of ubiquitin monomers and in proteasome function [2,3]. In addition, some ubiquitin hydrolases remove ubiquitin from specific cellular proteins and thus modulate their activity. For example, Liquid facets protein is the specific target of Fat facets, a de-ubiquitinating enzyme involved in *Drosophila* eye development [4].

The transcription factor NF- κ B (nuclear factor κ B) is a central co-ordinator of inflammatory responses [5]. It is sequestered in the cytoplasm of unstimulated cells through binding to I κ B (inhibitor of κ B) molecules. Activated TNF (tumour necrosis factor) receptor 1 (TNFR1) recruits several signal adaptor molecules, including RIP (receptor-interacting protein), TRAF2

(TNF-receptor-associated factor) and IKK γ (I κ B kinase γ)/NEMO (NF- κ B essential modifier). These molecules co-ordinate recruitment of I κ B kinases (IKK α and IKK β), which are responsible for phosphorylation of I κ B. Phosphorylated I κ B is then modified with Lys⁴⁸-linked polyubiquitin chains and is subsequently degraded, thereby promoting translocation of NF- κ B to the nucleus. Recent studies have revealed a requirement for ubiquitination at several other levels during pro-inflammatory signalling. Signal transduction through TRAF molecules and through IKK γ depends on their modification with Lys⁶³-linked polyubiquitin chains [6–8]. Engagement of TNFR1 also leads to ubiquitination of RIP and TNFR1 itself, but the significance of these modifications is unknown [9].

The zinc-finger molecule A20 is an important negative regulator of inflammation [10]. It uses an unknown mechanism to suppress NF- κ B activity. Transcription of the A20 gene is induced by NF- κ B, suggesting that A20 comprises a negative feedback loop in pro-inflammatory signalling [11]. Studies using embryonic fibroblasts cultured from A20-deficient mice revealed that A20 is required to prevent persistent IKK activity and subsequent I κ B α degradation following stimulation with TNF α [10]. This suggests that A20 functions at the level of the IKK complex or upstream from it. This is consistent with biochemical studies demonstrating that A20 interacts with both TRAF and IKK molecules. The C-terminal half of A20, which contains seven zinc fingers (Figure 1),

Abbreviations used: AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; C209S, Cys²⁰⁹ $\rightarrow$ Ser; DTT, dithiothreitol; E-tagged, GAPVYPDPLEPR-epitope-tagged; FLAG (epitope), Asp-Tyr-Lys-Asp-Asp-Asp-Lys (DYKDDDDK); HA, haemagglutinin; HRP, horseradish peroxidase; I κ B, inhibitor of κ B; IKK, I κ B kinase; NF- κ B, nuclear factor κ B; NEMO, NF- κ B essential modifier; OTU, ovarian tumour; pam3Cys (S)-[2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-*N*-palmitoyl-(*R*)-Cys(S)-Ser(S)-Lys(4)-OH trihydrochloride; RIP, receptor-interacting protein; TNF, tumour necrosis factor; TNFR, TNF receptor; TRAF, TNF-receptor-associated factor.

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Figure 1 Domain composition of A20

Amino acid sequences were analysed using BLAST (<http://www.ncbi.nlm.nih.gov>), PFAM (<http://www.sanger.ac.uk>) and ProSite (<http://ca.expasy.org>) programs. The position of recognized domains is represented. The catalytic domain and zinc fingers (ZF) are indicated. Truncated versions of A20 used in this study are also depicted.

interacts with IKKs [12,13] and also promotes self-association of A20 [14]. The N-terminal region of A20 is known to bind TRAF molecules [15]. A20 also functions as a negative regulator of apoptosis. It is essential for the survival of thymocytes or fibroblasts exposed to TNF α [10]. A20 is also cytoprotective against several other pro-apoptotic stimuli, including serum withdrawal [16] and overexpression of the apoptosis regulator p53 [17]. A recent report suggests that A20 inhibits apoptosis in co-operation with a novel anti-apoptotic protein called TXBP151 (Tax 1-binding protein 151) [18]. The precise molecular mechanism of A20's cytoprotective effects remains elusive.

We have previously described two novel proteins, Cezanne and TRABID, which contain a region with sequence similarity to the TRAF-binding domain of A20 [19]. This region encompasses a novel protease domain which belongs to the OTU (ovarian tumour) superfamily. The catalytic domain of Cezanne was recently shown to cleave mono-ubiquitin units from branched polyubiquitin chains [20].

Here we provide direct evidence that the TRAF-binding region of A20 has similar de-ubiquitinating activity. This suggests that de-ubiquitination may be a function conserved throughout the A20 family. The ubiquitin hydrolase activity of A20 appears not to have a global effect on polyubiquitinated proteins and therefore is likely to target specific molecules.

MATERIALS AND METHODS

Mammalian cells

Cos7, HeLa and HEK293 cells were cultured using Dulbecco's modified Eagle's medium/10% (v/v) foetal-calf serum, supplemented with antibiotics.

Escherichia coli expression vectors

pDEST15 (Invitrogen) was used for expression of GST (glutathione S-transferase)–Cezanne fusion proteins in *E. coli*. Construction of pDEST15-Cezanne 126–455 has been described previously [20]. QuikChange[®] technology (Stratagene) was used to introduce a point mutation at codon 209 (cysteine $\rightarrow$ serine; C209S) in pDEST15-Cezanne 126–455 using complimentary primers (sense 5'-GGAGATGGGAACAGCCTCCTGCATGCAG-3'). Manipulations were checked by PCR and DNA sequencing.

Mammalian expression vectors

pHM6 was used to express haemagglutinin (HA) epitope-tagged molecules in mammalian cells. pHM6-Cezanne and pHM6-A20 have been described [19]. Expression vectors for FLAG [Asp-Tyr-

Lys-Asp-Asp-Asp-Asp-Lys (DYKDDDDK)]-tagged versions of A20 comprised pCAGGS-FLAGmA20.1–366, pCAGGS-FLAGmA20.1–468 and pCAGGS-FLAGmA20.1–775 (full length). Expression of E-tagged, full-length A20 was achieved using pCAGGS-E-A20 as described in [18]. Vectors containing FLAG or GAPVYPDPLEPR-epitope-tagged (E-tagged A20) were generously supplied by Professor Rudi Beyaert [Unit of Molecular Signal Transduction in Inflammation, Department of Molecular Biomedical Research, University of Ghent–VIB (Flanders Interuniversity Institute for Biotechnology), Ghent, Belgium]. Quik-Change[®] technology (Stratagene) was used to introduce a point mutation at codon 103 of A20 [TGC (cysteine) $\rightarrow$ to AGC (serine)] in pHM6-A20, pCAGGS-mA20.1–468 or pCAGGS-E-A20 using complementary primers (5'-GAACGGTGACGGCAATAGCCTCATGCATGCCACTTC-TC-3'). Constructs were checked by DNA sequencing. An expression vector containing HA-tagged IKK γ (pM-HA-IKK γ) was also kindly supplied by Professor Beyaert.

Expression of FLAG-tagged ubiquitin was achieved using pcDNA3.1-FLAG-ubiquitin as described in [20]. Plasmids containing HA-tagged versions of ubiquitin mutated at specific codons for lysine residues were kindly provided by Professor Kei-ichi Nakayama (Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan). The mutations (lysine $\rightarrow$ arginine) were designed to generate a ubiquitin protein containing single lysine residues (Lys⁴⁸ or Lys⁶³) or to replace a single lysine residue (Lys⁴⁸ $\rightarrow$ Arg or Lys⁶³ $\rightarrow$ Arg) and have been described previously [21]. cDNA sequences were cloned into pCAGGS, which allowed them to be expressed in mammalian cells. Resulting constructs were denoted as pCAGGS3.1-HA-Ub, pCAGGS3.1-HA-Ub K48, pCAGGS3.1-HA-Ub K63, pCAGGS3.1-HA-Ub K48R and pCAGGS3.1-HA-Ub K63R.

Production of GST-tagged Cezanne proteins in *E. coli*

E. coli (Rosetta[™] strain; Novagen, Madison, WI, U.S.A.) transformed with pDEST15-Cezanne 126–455 or pDEST15-Cezanne 126–455 [C209S (Cys²⁰⁹ $\rightarrow$ Ser)] were cultured in the presence of 0.4 mM IPTG (isopropyl β -D-thiogalactoside) to induce expression of GST-fusion proteins. Bacteria from each 50 ml culture were harvested and lysed in 1 ml of BugBuster[®] (Novagen), 0.04% β -mercaptoethanol, 25 units of benzonase (Novagen), 1 mM AEBSEF [4-(2-aminoethyl)benzenesulphonyl fluoride] and 1 mM EDTA and clarified by centrifugation (20 000 g for 10 min at 4 °C). Hydrolysis reactions or probe binding assays were performed using 2 μ l of lysate. Alternatively, GST-Cezanne 126–455 protein was used following purification (above 99%) using published methods [20].

Preparation of cell lysates and purification of A20 from transfected mammalian cells

Cultures of Cos7 cells in 60 mm-diameter dishes were transfected with various pCAGGS constructs using DEAE-dextran according to standard protocols. After 48 h, cells were washed using PBS before application of 200 μ l of lysis buffer [50 mM Tris (pH 7.6)/0.2% Nonidet P40/150 mM NaCl/0.5 mM EDTA] plus 0.5 mM AEBSEF, a reversible protease inhibitor. Lysates were cleared by centrifugation. Probe binding assays required the use of 45 μ l of lysate. FLAG-tagged A20 was purified from the remaining lysate using 10 μ l of anti-FLAG antibody bound to matrix (M2; Sigma). Following overnight incubation at 4 °C, beads were washed three times using lysis buffer then twice using

50 mM Hepes (pH 7.8)/0.5 mM EDTA. A third of the beads were used in each hydrolysis reaction or probe binding assay.

Synthetic polyubiquitin substrates

Polyubiquitin chains conjugated through Lys⁴⁸ were obtained commercially (Affiniti Research Products, Mamhead, Exeter, Devon, U.K.). Polyubiquitin chains linked through Lys⁶³ were assembled using a mutated version of ubiquitin containing a single lysine residue at position 63. Ligase reaction products containing [Lys⁶³]polyubiquitin were obtained commercially (Boston Biochem Inc., Cambridge, MA, U.S.A.). Polyubiquitin species were separated from monomer by gel filtration using Superdex 75 PC 3.2/30 (Amersham Biotech) with a Pharmacia Biotech SMART system. Fractions containing chains lengths of 4 and above were used as substrate.

In vitro isopeptidase assay

This was performed by incubating potential proteases with substrate in hydrolysis reaction buffer [50 mM Hepes (pH 7.8)/0.5 mM EDTA/0.01 % Brij/3 mM DTT (dithiothreitol)] at 37 °C for various lengths of time. Synthetic branched polyubiquitin chains were used as substrate at a final concentration of 1 μ M. Preparation of test material was carried out as follows: (i) GST-Cezanne 126–455 or GST-Cezanne 126–455 (C209S) were harvested from *E. coli* as described above; (ii) alternatively, FLAG-tagged A20 sequences were tested after immunoprecipitation from Cos7 cells as described above. For inhibition of de-ubiquitinating activity, A20 was pre-incubated in the presence of 2 μ M ubiquitin aldehyde (Affiniti Research Products) for 30 min at 37 °C. Following hydrolysis, ubiquitin was revealed by Western blotting using anti-ubiquitin antibody (1:1000; Zymed, South San Francisco, CA, U.S.A.), horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma) and chemiluminescent detection (Pierce).

Probe binding assay

Synthesis and purification of the ubiquitin-derived probe used in this study (HAUbBr2) has been described previously [22]. It is tagged with HA epitope to facilitate detection. Probe (0.2 μ g) was applied either to Cos7-cell or *E. coli* lysates in the presence of 3 mM DTT and 0.01 % Brij. Reaction mixtures were incubated at 37 °C for 1–3 h. Probe sequences were detected by Western blotting using anti-HA epitope antibodies (1:1000; Roche), HRP-conjugated secondary antibodies (Chemicon, Temecula, CA, U.S.A.) and chemiluminescent detection.

Assay of de-ubiquitination in cultured cells

The effect of A20 on ubiquitinated proteins was assessed using HeLa cells cultured in 60 mm-diameter dishes. They were co-transfected with 0.5 μ g of mammalian expression vector containing E-tagged ubiquitin plus 1.5 μ g of various mammalian expression constructs containing A20 or Cezanne sequences. Transfection was performed using GeneJuice™ (Novagen), following manufacturer's recommendations. Note that empty vector was used to standardize the total amounts of vector used for transfection. After 48 h, cells were treated with 20 μ M MG132 for 1 h (or remained untreated). Cell lysates were then analysed for polyubiquitin and A20 by Western blotting using anti-FLAG (1:1000; M2; Sigma), anti-HA (1:1000; Roche) or anti-(E tag) (1:1000; Amersham Biosciences) primary antibodies,

HRP-conjugated secondary antibodies and chemiluminescence detection.

The effect of A20 on IKK γ ubiquitination was also measured. Cos7 cells cultured in six-well plates were transfected with various combinations of pM-HA-IKK γ (1 μ g), pFLAG-ubiquitin (0.5 μ g), pCAGGS-E-A20 (1 μ g) and pCAGGS-E-A20 C103S (1 μ g). Transfection was performed using DEAE-dextran according to the standard protocol. After 48 h, cells were lysed using 50 mM Tris (pH 7.6)/250 mM NaCl/1 % Triton-X100/0.5 % Nonidet P40/3 mM EGTA/3 mM EDTA/10 % (v/v) glycerol/0.1 mM Na₃VO₄/10 μ M N-ethylmaleimide plus a cocktail of protease inhibitors (Roche). Lysates were clarified by low-speed centrifugation and pre-cleared using Protein G–Sepharose before immunoprecipitation of HA-tagged IKK γ using anti-HA–Sepharose beads (Roche). Beads were then washed extensively using lysis buffer. Immunoprecipitated material or lysates were analysed by Western blotting using anti-FLAG (1:1000; M2; Sigma), anti-HA (1:1000; Roche) or anti-(E tag) (1:1000; Amersham Biosciences) primary antibodies, HRP-conjugated secondary antibodies and chemiluminescence detection.

NF- κ B reporter gene assays

The NF- κ B reporter (kindly provided by Dr Patrick Baeuerle, Micromet AG, Munich, Germany) consisted of an NF- κ B response element upstream of firefly luciferase. To normalize transfection efficiency, cells were co-transfected with pRL-TK (encoding *Renilla* luciferase; Promega). HEK-293 cells were transfected by electroporation and incubated for 16 h. Cells were then treated with interleukin-1 (1 ng/ml), TNF- α (50 ng/ml) or pam3Cys {(S)-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys(4)-OH trihydrochloride; 1 μ g/ml} for 16 h before measurement of NF- κ B activity. Firefly and *Renilla* luciferase activities were assessed using the Dual Luciferase Reporter Assay Kit (Promega) and luminescence counter. Results for NF- κ B activity reflect normalized luciferase values.

RESULTS

A20 binds covalently to a ubiquitin-derived probe

To examine whether A20 has enzymic activity towards ubiquitin, we tested two properties: (i) the ability to bind covalently to a ubiquitin-derived probe, and (ii) hydrolysis of synthetic substrates of branched polyubiquitin chains (Figures 2 and 3). The Cezanne protein, an A20-family member with known de-ubiquitinating activity, served as a positive control in these experiments. The ubiquitin-derived probe used in this study (HAUbBr2) contained a thiol-group-reactive bromoethylamine group at the C-terminus. It was chosen because of its ability to bind covalently to the catalytic cysteine of a ubiquitin hydrolase belonging to the OTU superfamily [18]. The probe is tagged with HA epitope to facilitate detection and is approximately 10 kDa in size.

The ubiquitin-derived probe bound covalently to a fusion protein between GST and the protease domain of our positive control, Cezanne (residues 126–455), as indicated by the substantially altered migration of probe sequences during SDS/PAGE (Figure 2A). The GST-Cezanne sequences identified at 60 kDa by Coomassie Blue staining migrated at 70 kDa following attachment of the probe (compare upper and centre panels, lane 3). Reactivity was not observed with the form mutated at residue 209 (centre panel, lane 2), confirming the involvement of the catalytic cysteine residue in probe binding. As expected, GST-Cezanne

Figure 2 A thiol-reactive, ubiquitin-derived probe binds covalently to A20 sequences

(A) Native or catalytically inactive (C209S) versions of the protease domain of Cezanne (Cez.) were expressed in *E. coli* as GST-tagged proteins. Crude *E. coli* lysates were tested. GST-Cezanne proteins were examined by SDS/PAGE, followed by Coomassie Blue staining (upper panel). Test material containing equivalent quantities of protein was incubated at 37 °C for 2.5 h with a thiol-reactive probe (HAUbBr2). Probe incubated alone was a negative control (no lysate, lane 1). Samples were subjected to SDS/PAGE, and probe sequences were revealed by Western blotting using anti-(HA epitope) antibodies (centre panel). Note that the free probe, which appeared at 10 kDa, is not shown. Test material was also incubated at 37 °C with an excess of branched polyubiquitin chains (Ub). Substrate incubated alone was a negative control (no lysate, lane 1). After hydrolysis, ubiquitin was revealed by Western blotting using anti-ubiquitin antibodies (lower panel). (B) Two FLAG-tagged versions of A20 (residues indicated) were produced in transfected Cos7 cells and tested for reactivity with HAUbBr2. Lysate from untransfected cells served as a negative control (lane 2). Levels of FLAG-tagged proteins were assessed by Western blotting using an anti-FLAG antibody (upper panel). Cell lysates were incubated with HAUbBr2 probe at 37 °C for 2 h. Probe sequences were then revealed by Western blotting using an anti-HA antibody (lower panel). Probe incubated alone was a second negative control (no lysate, lane 1). Abbreviation: UBP, ubiquitin-specific processing protease.

sequences cleaved branched polyubiquitin chains (lower panel, lane 3), whereas the form mutated at the catalytic site was inactive (lower panel, lane 2).

We then assessed the reactivity of HAUbBr2 towards two FLAG-tagged versions of A20 produced in transfected Cos7 cells (Figure 2B). We tested A20 residues 1–366, a stretch that contains

Figure 3 A20 purified from Cos7 cells hydrolyses Lys⁴⁸- and Lys⁶³-conjugated polyubiquitin chains

(A) Various FLAG-tagged versions of A20 were immunoprecipitated from transfected Cos7 cells (two separate clones of mutated A20 1–468 were tested; lanes 7 and 8). Their capacity to hydrolyse polyubiquitin or bind to a thiol-reactive ubiquitin-derived probe was tested in parallel. Precipitated material from untransfected cells served as a negative control (lane 2). The efficiency of each precipitation was assessed by Western blotting using anti-FLAG antibody (upper panel). Immunoprecipitated material was tested in hydrolysis reactions using an excess of polyubiquitin chains linked through Lys⁴⁸ (Ub). Polyubiquitin chains incubated alone were a second negative control (no enzyme, lanes 1 and 6). After hydrolysis, ubiquitin was revealed by Western blotting using anti-ubiquitin antibodies (centre panel). The purified material was also tested for reactivity with HAUbBr2. Probe incubated alone served as an additional negative control (no enzyme, lanes 1 and 6). After incubation, probe sequences were revealed by Western blotting using anti-(HA epitope) antibodies (lower panel). (B) GST-tagged Cezanne protein and FLAG-tagged A20 sequences were purified from *E. coli* or Cos7 cells respectively. These proteins were examined either by Coomassie Blue staining or by Western blotting using anti-FLAG antibodies (upper panel). Purified material was tested in hydrolysis reactions against Lys⁶³-conjugated polyubiquitin chains (Ub). Note that the amount of GST-Cezanne tested corresponded to 0.5 % of the material analysed by Coomassie Blue staining. After hydrolysis, ubiquitin was revealed by Western blotting with anti-ubiquitin antibodies (lower panel).

the putative protease domain alone, and A20 residues 1–468, which also includes a single zinc finger (Figure 1). Incubation of probe with lysate from untransfected cells revealed several minor bands that are probably derived from endogenous ubiquitin hydrolases (lower panel, lane 2). Probing of lysate from cells transfected with either of the A20 constructs revealed in each case a prominent additional band corresponding to ubiquitin-modified A20 (lower panel, lanes 3 and 4). Attachment of the probe to A20 1–366 and A20 1–468 increased their molecular size to about

50 kDa and 60 kDa respectively (compare upper and lower panels, lanes 3 and 4).

A20 hydrolyses distinct structural forms of polyubiquitin

FLAG-tagged versions of A20 purified by immunoprecipitation from transfected Cos7 cells were used to determine ubiquitin hydrolase activity (Figure 3A). Assessment of enzymic function *in vitro* demonstrated that truncated forms of A20, containing the catalytic domain, were highly effective in cleaving polyubiquitin chains linked through Lys⁴⁸ into monomers (centre panel, lanes 4 and 5). The same purified preparations of A20 bound covalently to HAUBr2 (lower panel, lanes 4 and 5). It is highly likely that both the hydrolysis activity and probe binding observed in this experiment were due to A20 sequences, because they were not observed using immunoprecipitates from untransfected cells (lane 2, centre and lower panels). The mutation Cys¹⁰³ → Ser abolished enzymic activity against both polyubiquitin substrate and ubiquitin probe (compare lanes 7, 8 and 9). This confirms that Cys¹⁰³, which is highly conserved among members of the OTU superfamily, is a catalytic residue. The de-ubiquitinating activity of A20 was suppressed completely by ubiquitin aldehyde, a highly specific inhibitor of de-ubiquitinating enzymes (results not shown). Full-length A20 possessed weak ubiquitin hydrolase activity (lane 3) and had little or no reactivity with a ubiquitin probe. Repeated attempts to compare the activities of full-length A20 and shorter versions were hindered because the former was consistently expressed at far lower levels (upper panel, compare lanes 3, 4 and 5). At this stage we cannot make firm conclusions regarding the relative activities of the full-length molecule compared with the protease domain in isolation.

We then examined whether OTU-family proteases have enzymic activity towards an alternative structural form of polyubiquitin linked through Lys⁶³ (Figure 3B). Sequences containing the catalytic domain of Cezanne cleaved dimeric and monomeric ubiquitin from Lys⁶³ polyubiquitin chains (lane 2). De-ubiquitinating activity must be attributed to GST-Cezanne and not to trace amounts of contaminating proteins, because *E. coli* does not contain endogenous ubiquitin hydrolases. A20 1–468 purified from Cos7 cells also cleaved Lys⁶³-linked polyubiquitin to generate trimeric, dimeric and monomeric forms (lane 4). In contrast, the preparation containing catalytically inactive A20 (Cys¹⁰³) produced only a small quantity of trimeric ubiquitin, probably as a result of trace amounts of contaminating endogenous ubiquitin hydrolases (lane 5). We observed less activity using A20 protein purified from Cos7 cells compared with that seen using a preparation of GST-tagged Cezanne 126–455 purified from *E. coli*. This difference is likely to be attributed to the greater concentration of protease used in the latter experiment.

A20 does not have a global de-ubiquitinating activity in cultured cells

We compared the effects of A20 and Cezanne on ubiquitinated cellular proteins in cultured Hela cells (Figure 4A). Full-length Cezanne prevented the build up of ubiquitinated cellular proteins in response to a proteasome inhibitor MG132 (compare lanes 1 and 3, lanes 4 and 5). In contrast, overexpression of wild-type or mutant forms of A20 had no effect on the overall level of ubiquitinated cellular proteins (compare lanes 4, 6 and 7).

We also examined the effect of A20 overexpression on cellular proteins modified with defined structural forms of polyubiquitin (Figure 4B). A20 had no effect on the overall levels of proteins

Figure 4 A20 does not have a global de-ubiquitinating effect in cultured cells

(A) HeLa cells were co-transfected with pcDNA3.1-FLAG-ubiquitin (0.3 μg) plus pHM6 constructs containing HA-tagged versions of Cezanne, A20 or a catalytically inactive form of A20 (C103S). Alternatively, empty pHM6 vector was used as a control (lanes 1 and 4). Cultures received 1.5 μg of expression construct, except one (lane 5), which received 0.1 μg plus a compensatory amount of empty vector (lane 5). Here, inclusion of empty pHM6 ensured that total levels of transfected vector were identical for each well. After 48 h, cells were treated with a proteasome inhibitor (20 μM MG132) for 1 h or remained untreated, as indicated. Cell lysates were tested by Western blotting using anti-FLAG antibodies to detect residual ubiquitinated proteins (upper panel) or with anti-(HA epitope) antibodies to detect A20 or Cezanne (centre panel). Blots were stripped and re-probed using anti-α-tubulin to assess loading (lower panel). (B) Cos7 cells were co-transfected with expression vectors containing various HA-tagged versions of ubiquitin and E-tagged versions of A20. Forms of ubiquitin mutated at specific lysine residues [Lys⁴⁸ (K48), Lys⁶³ (K63), Lys⁴⁸ → Arg (K48R) or Lys⁶³ → Arg (K63R)] were co-expressed with either active or catalytically inactive versions of A20. After 48 h, cell lysates were tested by Western blotting using anti-HA antibodies to detect ubiquitinated proteins (upper panel) or with anti-E tag antibodies to detect A20 (lower panel). Abbreviation: WT, wild-type.

conjugated to Lys⁶³ or Lys⁴⁸ polyubiquitin chains (compare lanes 3 and 4, lanes 5 and 6, lanes 7 and 8, lanes 9 and 10).

De-ubiquitinating activity by A20 may not contribute to its anti-inflammatory effects

IKKγ and TRAF6 proteins are key signal adaptors in pro-inflammatory signalling that are activated by Lys⁶³ polyubiquitin chains. Both molecules are known to interact with A20. We therefore examined whether A20 could target either molecule for de-ubiquitination. Wild-type or catalytically inactive A20 was overexpressed in cultured cells and their effects on the level of ubiquitinated IKKγ were compared (Figure 5A). We observed no

Figure 5 Contribution of ubiquitin hydrolase function to A20's anti-inflammatory effects

(A) Cos7 cells were co-transfected with expression vectors containing HA-tagged IKK γ and FLAG-tagged ubiquitin. The cultures also received expression vectors containing either native or catalytically inactive A20 sequences which were tagged with E-epitope [A20 or mutated A20 (C103S)]. After 48 h, IKK γ protein was purified by immunoprecipitation using anti-HA antibodies. Precipitated material was tested by Western blotting using anti-HA antibodies to detect IKK γ (upper panel) or with anti-FLAG antibodies to detect ubiquitin (centre panel). Expression of A20 was measured in cell lysates by Western blotting with anti-E tag antibodies (lower panel). Abbreviation: i.p., immunoprecipitation. (B) Reporter gene assays were performed using expression vectors encoding native or catalytically inactive versions of A20 (pCAGGS-E-A20 or pCAGGS-E-A20 C103S). HEK-293 cells were co-transfected with 100 ng of pGL2 (NF- κ B reporter), 100 ng of pRL-TK (*Renilla* luciferase control) and various amounts of expression vector (0, 0.2, 1 or 5 μ g, as indicated). After 16 h, cells were stimulated with TNF α , interleukin-1 (IL-1) or pam3Cys for 16 h. Cell lysates were analysed and the ratio firefly/*Renilla* luciferase activity was calculated, which is a measure of NF- κ B activity normalized for transfection efficiency. Mean values calculated from duplicate wells are shown with S.E.M. values. The data are representative of those from three closely similar experiments.

difference, which suggests that the ubiquitinated form of IKK γ is not a physiological substrate for A20. We also tested whether A20 de-ubiquitinates polyubiquitinated TRAF6. Our preliminary results showed a weak or marginal effect (results not shown).

Reporter-gene experiments were performed to examine whether the ubiquitin hydrolase activity of A20 is required for its inhibitory effects on NF- κ B (Figure 5B). As expected, A20 suppressed NF- κ B activation in response to TNF α or interleukin-1 in a dose-dependent manner. Our study also revealed a novel inhibitory effect of A20 on NF- κ B activation in response to pam3Cys, an agonist of TLR-2. Active and inactive (Cys¹⁰³ $\rightarrow$ Ser) forms of A20 had similar inhibitory effects on NF- κ B in response to stimulation with TNF α , interleukin-1 or pam-3-Cys for 16 h. In a series of reporter-gene experiments, we observed similar activities of active and catalytically inactive A20 during 8, 24, 48 and 72 h of stimulation with pro-inflammatory mediators (results not shown). This indicates that the catalytic properties of A20 are not required for NF- κ B suppression under conditions of overexpression.

DISCUSSION

The recently described A20 family contains several molecules that show sequence similarity to the N-terminal TRAF-binding region of A20 [19]. The A20 family is a subgroup of the OTU superfamily, which contains more than 100 proteins that are predicted to function as cysteine proteases [23]. Molecules belonging to the OTU group are divergent, except for two short conserved sequences that contain putative catalytic cysteine and

histidine residues. Here, we have demonstrated that the A20 zinc finger protein contains a functional OTU domain that hydrolyses polyubiquitin chains. We and others have recently found ubiquitin hydrolase activity for three other OTU proteins [20,22,24], and we suggest that de-ubiquitination may be a function conserved throughout the OTU superfamily.

We have demonstrated that A20 and Cezanne can hydrolyse distinct structural forms of polyubiquitin containing either Lys⁴⁸ or Lys⁶³ linkages. These forms are known to have different effects on the proteins to which they are attached. Proteins modified with Lys⁴⁸ polyubiquitin chains are targeted to the proteasome, where they are degraded. In contrast, Lys⁶³ polyubiquitin has the capacity to activate certain signalling molecules and to trigger internalization of transmembrane proteins. Thus OTU-type de-ubiquitinating enzymes may be capable of regulating a diverse range of physiological processes that involve either Lys⁴⁸ or Lys⁶³ polyubiquitination.

The OTU domain of A20 is contained within the N-terminal half of the molecule. Structure-prediction modelling suggests close similarity to papain-like cysteine proteases in which the catalytic residues are present in a deep cleft. Yeast two-hybrid experiments have revealed that the protease domain is also responsible for binding to TRAF molecules [15]. The spatial relationship between the TRAF-binding motif and the active site has not been established. The active-site targets polyubiquitin chains for cleavage and is unlikely to recognize TRAF sequences, which are entirely unrelated to ubiquitin, so the TRAF-binding motif is probably situated outside the catalytic cleft.

Our experiments revealed that A20 does not influence the overall level of cellular proteins modified with either Lys⁴⁸ or Lys⁶³

polyubiquitin chains. We cannot discount the possibility that A20 can cleave free polyubiquitin chains but does not have the capacity to remove ubiquitin from cellular proteins. This mode of action has been described for isopeptidase-T, which generates free ubiquitin monomers from branched chains for recycling [25]. Alternatively, A20 may de-ubiquitinate a restricted subset of cellular protein(s). Regions outside the catalytic domain may target A20's enzymic activity to proteins with which it interacts. This concept has been established for herpesvirus-associated ubiquitin specific protease, which specifically de-ubiquitinates the apoptosis regulator p53 [26,27].

A20 is recruited to the TNFR1 complex following engagement with TNF α [12]. This association is co-ordinated by dual interactions between A20 zinc fingers and IKKs [12,13] and between A20 and TRAF molecules [15]. A number of proteins within this complex are known to be ubiquitinated including IKK γ , TRAF2, RIP and TNFR1 [7–9]. Signal transduction through IKK γ and TRAF molecules relies on their modification with Lys⁶³-linked polyubiquitin chains. Recent studies on CYLD, a tumour suppressor mutated in familial cylindromatosis and a member of the ubiquitin C-terminal hydrolase family (Type I), suggest that NF- κ B activation can be blocked by de-ubiquitination of IKK γ and/or TRAF2. CYLD removes Lys⁶³-linked polyubiquitin chains from both of these molecules and is a potent suppressor of NF- κ B activation [28–30].

To investigate whether the catalytic domain contributes to A20's known anti-inflammatory effects, we examined whether A20 could modulate polyubiquitination of IKK γ or TRAF6. Interestingly, our studies revealed that A20 does not target IKK γ for de-ubiquitination. Similarly, preliminary experiments suggested that A20 has little effect on TRAF6 polyubiquitination. These data indicate that A20 and CYLD use distinct mechanisms to down-regulate NF- κ B. It would have been attractive to speculate that A20 de-ubiquitinates I κ B α . However, persuasive evidence from studies of A20-deficient mice argue against this [10]. Reporter-gene experiments comparing active and catalytically inactive forms of A20 did not reveal a role for the ubiquitin hydrolase domain in NF- κ B inhibition. Our findings are in full agreement with previous data demonstrating that overexpression of the zinc-finger domain of A20 in isolation is sufficient to inhibit NF- κ B [15]. Another laboratory has also found that an intact A20 catalytic domain is not required for NF- κ B inhibition [24]. Their data is not entirely consistent with our results, however, because it suggests that catalytic inactivation of A20 leads to a modest reduction in its ability to suppress NF- κ B. This relatively minor discrepancy could be due to small differences in experimental design. Collectively, both sets of data seem to indicate that A20's de-ubiquitinating activity may not be required for its anti-inflammatory effects. These reporter-gene experiments must be interpreted with a certain degree of caution, however, because overexpression of A20 sequences may not reflect their true physiological roles.

Alternatively, it is possible that the ubiquitin hydrolase domain contributes to A20's cytoprotective functions or its role in regulating skin differentiation [10]. It is also conceivable that the stability of A20 itself could be regulated by self-association leading to self-de-ubiquitination. It has been suggested that A20 protein levels are reduced following phosphorylation by IKK β , presumably through the ubiquitin/proteasome pathway [13]. De-ubiquitination of self could constitute a mechanism to oppose this. Further experiments are required to identify the targets of A20 de-ubiquitination and its biological significance.

Sequencing of the human genome has led to the identification of over 100 putative de-ubiquitinating enzymes. Emerging data has revealed a role for ubiquitin hydrolases in a number of settings,

including proteasome function [2,3], tumour suppression [28–31], apoptosis regulation [26,27] and neural function [32]. Our demonstration of de-ubiquitinating activity for A20, an important regulator of inflammation and cell survival, provides further evidence that ubiquitin hydrolases modulate fundamental and diverse physiological processes.

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