Title: METHOD OF DEUBQUITINATION

Abstract: The invention concerns a new class of deubiquitinating enzymes and methods of deubiquitination using these enzymes or functional fragments or derivatives thereof. In one aspect, the invention covers a method for enzymatic deubiquitination of a molecule which has been ubiquitinated to form a ubiquitinated molecule, comprising the step of contacting the ubiquitinated molecule with an enzyme, wherein the enzyme or a domain thereof has at least 20% amino acid sequence identity with the TRAF2 domain defined by Cezanne amino acid residues 160-416 (SEQ ID NO:1).
Method of Deubiquitination

The present invention relates to deubiquitinating enzymes and methods of deubiquitination.

Recent studies have highlighted the ubiquitination system as a pivotal regulator of multiple cellular processes. Ubiquitin is covalently attached to substrate lysine residues through the concerted action of E1, E2 and E3 ubiquitin ligases which, together, determine substrate specificity. Successive rounds of ubiquitination, where ubiquitin is itself ubiquitinated, generate an isopeptide-linked chain which typically targets the substrate protein to the proteasome where it is degraded. Ubiquitin plays an essential role at several levels during NF-κB signalling, the best characterised being ubiquitination and degradation of IκB. Ubiquitination also provides the signal for partial degradation of NF-κB precursors into active forms. In addition, activation of TNF receptor associated factor (TRAF) and IL-1 receptor associated kinase (IRAK) signalling intermediaries is mediated by 'non-classical' ubiquitin modifications that do not target these proteins to the proteasome.

Ubiquitination can be reversed by deubiquitinating enzymes that cleave isopeptide-linked polyubiquitin chains (reviewed by Wilkinson, KD [2000] Semin. Cell. Dev. Biol. 11: 141-148). Two protease families with deubiquitinating activity have been recognised: ubiquitin C-terminal hydrolases (UCH, type I) and ubiquitin-specific processing proteases (UBP, type II). Family members vary greatly in size (60-300kD) and overall sequence composition but contain very conserved active-site domains (two short conserved sequences that surround the catalytic cysteine and histidine residues). UBP enzymes are able to hydrolyse branched polyubiquitin chains. They play an important role in proteasome function and in generating free ubiquitin monomers from branched chains for recycling (Wilkinson, K.D. et al., 1995, Biochemistry 34: 14535-14546). In addition, some UBP molecules can regulate the fate of specific cellular proteins. Thus removal of ubiquitin chains will
extend the half-life of these molecules and therefore modulate their particular activities in the cell. For example, genetic experiments in Drosophila have revealed that liquid facets protein is the specific target of Fat facets, a UBP molecule involved in eye development. Fat facets stabilises liquid facets through deubiquitination. For the great majority of UBP molecules, however, substrate specificity has not been determined. A family of metalloproteases which cleave ubiquitin or ubiquitin-like molecules has also been identified recently. Deubiquitinating activity by one of these enzymes, Rpn11, is essential for degradation of substrate proteins by the 26S proteasome.

The fate of many proteins depends on a balance between the opposing activities of E3 ligases and deubiquitinating enzymes. Until now, ubiquitin hydrolases that specifically regulate signalling pathways have received little attention. Neither the structure of proteins displaying this activity nor the mechanisms by which they achieve substrate specificity are known in the prior art.

The present inventors disclose novel and inventive deubiquitinating activity in a family of proteins.

According to the present invention there is provided a method for enzymatic deubiquitination of a molecule which has been ubiquitinated to form a ubiquitinated molecule, comprising the step of contacting the ubiquitinated molecule with an enzyme, wherein the enzyme or a domain thereof has at least 20% amino acid sequence identity (for example, at least 80%, 70%, 60%, 50%, 40%, 35% or 30% amino acid sequence identity) with: (1) the TRAFB domain defined by Cezanne amino acid residues 160-416 (SEQ ID NO: 1); and/or (2) the A20 homology domain defined by Cezanne amino acid residues 126-455 (SEQ ID NO: 4); and/or (3) the Cezanne core catalytic domain defined by Cezanne amino acid residues 182-455 (SEQ ID NO: 6).

The TRAFB (TRAF binding) domain has been identified as a conserved domain between the proteins Cezanne (Genbank Accession No. AJ293573), TRABID
(Genbank Accession No. AJ252060) and A20 (Genbank Accession No. M59465), as described below and in Evans, P.C. et al. (2001), Biochem J. 357: 617-623. The protein Cezanne-2 (Genbank Accession No. CAD23047 or AJ430383l; SEQ ID NO: 3) is a further member of this protein family.

The TRAFB domain has structural similarity to the protease papain and contains putative active-site cysteine and histidine residues. Experiments performed by the present inventors have defined the active-site cysteine and histidine residues for Cezanne as residues 209 and 373 respectively (see AJ293573). By homology with the amino acid sequence of Cezanne, active-site cysteine and histidine residues have been identified for Cezanne-2 (Cys210, His367), TRABID (Cys443, His585) and A20 (Cys103, His256). It has further been demonstrated (see Experimental section below) that mutation of Cys103 in A20 abolishes catalytic activity.

The present inventors show that members of the TRAFB-domain family function as deubiquitinating enzymes. The TRAFB domain of Cezanne contains an active catalytic site, which is responsible for the deubiquitinating activity of this molecule. The deubiquitinating activity of this family of proteins is surprising since the TRAFB domain bears no consensus to either the type I or II family consensus of known deubiquitinating enzymes. The invention will allow regulation of ubiquitinated proteins and cellular pathways involving ubiquitination.

Sequence identity refers to identical matches between aligned polypeptide sequences. Alignment of polypeptides can be performed using default parameters of BLASTP version 2.2.3 (see website at ncbi.nlm.nih.gov/blast).

The A20 homology domain is a further domain defined by aligning the sequences of Cezanne (Genbank Accession No. AJ293573), TRABID (Genbank Accession No. AJ252060) and A20 (Genbank Accession No. M59465), as described below.
The Cezanne core catalytic domain defined by Cezanne amino acid residues 182-455 (SEQ ID NO: 6) also displays structural similarity to papain and contains putative active-site cysteine and histidine residues.

The term "enzyme" as used herein refers to a protein or polypeptide that acts as a catalyst in a chemical or biochemical reaction. The term "molecule" encompasses ubiquitin per se, so that "a ubiquitinated molecule" includes ubiquitinated ubiquitin and polyubiquitin.

In one embodiment of the invention, the enzyme or a domain thereof has at least 80% sequence identity with the TRAFB domain defined by SEQ ID NO: 1. For example, the enzyme may be Cezanne (Genbank Accession No. AJ293573) or Cezanne-2 (Genbank Accession No. AJ430383; SEQ ID NO: 3). Alternatively, the enzyme may be or may comprise a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) of Cezanne or Cezanne-2.

In a further embodiment of the invention, the enzyme or a domain thereof has at least 35% sequence identity with the TRAFB domain defined by SEQ ID NO: 1. For example, the enzyme may be A20 (Genbank Accession No. M59465). Alternatively, the enzyme may or may comprise be a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) of A20.

In yet another embodiment of the invention, the enzyme or a domain thereof has at least 30% sequence identity with the TRAFB domain defined by SEQ ID NO: 1. For example, the enzyme may be TRABID (Genbank Accession No. AJ252060). Alternatively, the enzyme may be or may comprise a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) of TRABID.
In a further aspect of the invention, there is provided a method for enzymatic deubiquitination of a molecule which has been ubiquitinated to form a ubiquitinated molecule, comprising the step of contacting the ubiquitinated molecule with an enzyme, wherein the enzyme or a domain thereof comprises a Cys box domain defined by Cezanne amino acid residues 200-220 (SEQ ID NO: 7 or a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) of the Cys box domain. The Cys box, particularly conserved residues therein, is described below with reference to Fig. 14. In the experimental section below, it is shown that mutation of a conserved cysteine residue in the Cys box, for example C209 of Cezanne or residue C103 of A20, abolishes deubiquitination activity. By homology, the Cys box domain active site cysteine is found at residue 210 of Cezanne-2 and 443 of TRABID.

In another aspect of the invention, there is provided a method for enzymatic deubiquitination of a molecule which has been ubiquitinated to form a ubiquitinated molecule, comprising the step of contacting the ubiquitinated molecule with an enzyme, wherein the enzyme or a domain thereof comprises a His box domain defined by Cezanne amino acid residues 365-379 (SEQ ID NO: 8) or a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) of the His box domain. The His box domain, particularly conserved residues therein, is described below with reference to Fig. 14. The active site histidine residues in the His box domain have been identified residue 373 in Cezanne (see Experimental section below), residue 367 in Cezanne-2, residue 585 in TRABID and residue 256 in A20. In an alternative embodiment, an enlarged His box domain comprises a larger domain encompassing Cezanne amino acid residues 365-406 (SEQ ID NO: 9).

In a further aspect of the invention, the enzyme or a domain thereof may be used as a fusion partner. As a fusion partner, the enzyme or a domain thereof is fused with another polypeptide to form a fusion protein (or “fusion”). In one embodiment, the enzyme or a domain thereof is fused to a binding site for a
target molecule. In this way, deubiquitination activity could be targeted to the
target molecule when binding or bound to the binding site. Alternatively, the
enzyme or a domain thereof may be fused with a polypeptide to function in cis
to stabilise or modulate the activity of the fusion by deubiquitination. This
approach may have application, for example, in circumstances where exogenous
DNA is introduced into an organism (such as DNA vaccines or gene therapy)
and it is desirable to control activity of the exogenous DNA or its products.

In another embodiment of the invention, the molecule or target molecule is a
regulator of a cellular process. The importance of deubiquitination of molecules
which regulate cellular processes is now recognised. The present invention
allows modification of such processes by enzymatic removal of ubiquitin from
ubiquitinated molecules and reversing the normal fate (for example, degradation
in the proteasome) of the molecule.

In a preferred embodiment of the invention, the molecule or target molecule
may be a regulator of the inflammatory process. The inflammatory process may,
for example, be regulated by NF-κB. Experimental evidence described below
suggests that Cezanne and A20 are involved in the regulation of NF-κB which
is known to be involved in co-ordinating the inflammatory response. In one
aspect, the present invention allows for suppression of NF-κB, and hence
suppression of the inflammatory response, by enzymatic deubiquitination of
negative regulatory molecules such as IκB which have been ubiquitinatated.

Further experimental evidence described below indicates that NF-κB is
activated by TRABID. An alternative application of the present invention is
activation of NF-κB, and hence activation of the inflammatory response, by
enzymatic deubiquitination of positive regulatory molecules which have been
ubiquitinatated.

In another embodiment, the molecule or target molecule is a regulator of
apoptosis. For example, the inventors show that TRABID is able to protect cells
from TNFα/cycloheximide-induced apoptosis.

In a further embodiment, the molecule or target molecule is involved in B-cell development. Cezanne, for example, has been shown herein to be differentially expressed among B-cells at distinct development stages and may be involved in deubiquitinating a molecule involved in B-cell development.

The method according to the present invention may be used for stabilising the molecule or the target molecule. For example, if the molecule or target molecule is susceptible to degradation following ubiquitination, then the method of enzymatic deubiquitination may be used to remove ubiquitin from the ubiquitin-ated molecule or target molecule, or prevent accumulation of ubiquitin on the molecule or target molecule, thus preventing or minimising ubiquitin-dependent degradation.

Alternatively, the method may be used for targeting or maintaining the molecule or target molecule to or at a subcellular location. For example, ubiquitination has been shown to be involved in targeting proteins to specific locations. Manipulation of ubiquitination levels can allow for the molecule or target molecule to be targeted to a destination location (for example, a subcellular organelle) or maintained at a specific site (for example, the cytoplasm).

The method may also be used for modulating the activity of the molecule or target molecule. Ubiquitination of a molecule or target molecule may increase or decrease its activity. Manipulation of the ubiquitination process, for example by increasing or decreasing the level of ubiquitination, may therefore allow modulation of ubiquitination-dependent activity. For example, if ubiquitination decreases the activity of a molecule, then the method for enzymatic deubiquitination can be used to remove ubiquitin from the ubiquitinated molecule, or prevent accumulation of ubiquitin on the molecule, and thus increase activity of the molecule relative to its ubiquitinated form.
The method may further be used to modify signalling of the molecule or target molecule.

Further provided according to the invention is a method for regulating the method of deubiquitination described herein, comprising the step of contacting (i) a molecule which has been ubiquitinated or (ii) a deubiquitination enzyme with a regulatory polypeptide having at least 20% amino acid sequence identity (for example, at least 80%, 70%, 60%, 50%, 40%, 35% or 30% amino acid sequence identity) with the C-terminal domain of Cezanne defined by Cezanne amino acid residues 444-858 (SEQ ID NO: 5) or a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) thereof.

As shown the experimental section below, the C-terminus of Cezanne, as defined by amino acid residues 444-858 (SEQ ID NO: 5), includes a ubiquitin binding site and has regulatory properties. The regulatory peptide may therefore be used to self-regulate the activity of the deubiquitination enzyme. The regulatory peptide may be part of the deubiquitination enzyme, for example as a fusion partner with the deubiquitination enzyme, and thus act in cis. Alternatively, the regulatory peptide may be discrete from the deubiquitination enzyme and thus act in trans.

In another aspect of the invention, there is provided a method for modulating the activity of a molecule which is capable of being ubiquitinated to form a ubiquitinated molecule, or stabilising the molecule or targeting or maintaining the molecule to or at a subcellular location, comprising the step of contacting the molecule or the ubiquitinated molecule with a polypeptide having at least 20% amino acid sequence identity (for example, at least 80%, 70%, 60%, 50%, 40%, 35% or 30% amino acid sequence identity) with: (1) the TRAFB domain defined by Cezanne amino acid residues 160-416 (SEQ ID NO: 1); and/or (2)
the A20 homology domain defined by Cezanne amino acid residues 126-455 (SEQ ID NO: 4); and/or (3) the Cezanne core catalytic domain defined by Cezanne amino acid residues 182-455 (SEQ ID NO: 6), wherein the polypeptide has deubiquitination activity.

Also provided is a method for modulating the activity of a molecule which is capable of being ubiquitinated to form a ubiquitinated molecule, or stabilising the molecule or targeting or maintaining the molecule to or at a subcellular location, comprising the step of contacting the molecule or the ubiquitinated molecule with a polypeptide comprising a Cys box domain defined by Cezanne amino acid residues 200-220 (SEQ ID NO: 7) or a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) of the Cys box domain, wherein the polypeptide has deubiquitination activity.

Further provided is a method for modulating the activity of a molecule which is capable of being ubiquitinated to form a ubiquitinated molecule, or stabilising the molecule or targeting or maintaining the molecule to or at a subcellular location, comprising the step of contacting the molecule or the ubiquitinated molecule with a polypeptide comprising a His box domain defined by Cezanne amino acid residues 365-379 (SEQ ID NO: 8) or a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) of the His box domain, wherein the polypeptide has deubiquitination activity.

The methods of deubiquitination and/or regulation may be useful in therapy. For example, in a formulated composition, the polypeptide, enzyme or a functional fragment, homologue, variant or mutant (for example, a conservative substituted mutant) of the enzyme may be useful in the treatment of a condition or disease. The condition or disease may, for example, be cancer. The condition or disease may involve inflammation or apoptosis.
In another aspect of the invention there is a protein having an amino acid sequence of Cezanne-2 (SEQ ID NO: 3), or a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) thereof. The novel protein Cezanne-2 belongs to the TRAFB-domain family of deubiquitinating enzymes, with high sequence identity to Cezanne 1.

Also provided is an isolated nucleotide encoding Cezanne-2, functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) thereof. The isolated nucleotide may have the sequence of SEQ ID NO: 2.

A detailed description of the invention, including various embodiments, is provided below with reference to the following figures, of which:

**Fig. 1** shows domain composition of A20, Cezanne and TRABID;

**Fig. 2** shows sequence alignments of Cezanne and other A20-like proteins;

**Fig. 3** shows distribution of Cezanne and TRABID mRNA in various tissues and cells;

**Fig. 4** shows regulation of NF-κB by Cezanne, TRABID and A20 in cultured cells;

**Fig. 5** shows co-immunoprecipitation studies of A20, Cezanne and TRABID with TRAF6;

**Fig. 6** shows intra-cellular localisation of GFP-tagged A20, Cezanne and TRABID with TRAF6;

**Fig. 7** shows domain composition and sequence alignment of Cezanne, Cezanne-2, A20 and TRABID;
Fig. 8 shows further domain composition of Cezanne;

Fig. 9 shows specific interaction of Cezanne with polyubiquitin;

Fig. 10 shows Cezanne binding and cleaving the polyubiquitin gene product;

Fig. 11 shows western blots indicating that Cezanne cleaves free, isopeptide-linked polyubiquitin chains;

Fig. 12 shows western blots indicating that the C-terminal half of Cezanne regulates polyubiquitin hydrolysis;

Fig. 13 shows western blots indicating that Cezanne deubiquitinates cellular proteins in cultured cells;

Fig. 14 shows sequence alignments showing Cys and His regions of Cezanne are distinct from catalytic regions of UCH and UBP molecules;

Fig. 15 shows TRABID downregulation of TNFα/ cycloheximide-induced cell death;

Fig. 16 shows covalent binding of thiol-reactive, ubiquitin-derived probes to Cezanne;

Fig. 17 shows covalent binding of a thiol-reactive ubiquitin-derived probe to A20;

Fig. 18 shows that purified A20 hydrolys polyubiquitin chains and binds covalently to a ubiquitin-derived probe; and

Fig. 19 shows that A20 does not have a generalised deubiquitinating effect in
cultured cells.

The figures are described in more detail below:

In Fig. 1, amino acid sequences were analysed using BLAST and Profilescan (see website at isrec.isb-sib.ch/software/PFSCAN) programmes. The position of recognised domains is represented alongside amino acid number (0-800). Depicted domains are TRAFB (TRAF binding domain), ZF A20 (A20-like zinc finger), NUC (putative nuclear localisation sequence) and ZF NUP (nucleoporin-like zinc finger).

Fig. 2a shows sequence alignment of the TRAFB domain of human Cezanne (EMBL:AJ293573), A20 (EMBL: M59465) and TRABID (EMBL: AJ252060) compiled using Pileup. Hydrophobic residues are displayed as black type on grey background and basic residues as white type on dark grey background. Secondary structure was predicted using the Jpred2 server (see website at barton.ebi.ac.uk/servers/jpred.html) and is depicted. H, alpha-helix; E, extended (beta) sheet. Fig. 2b shows alignment of Cezanne and A20 zinc fingers using Pileup. Text was highlighted for conserved cysteine (white type on black background), hydrophobic (black type on grey) and basic residues (white type on dark grey background). Fig. 2c shows alignment of TRABID and Nup358 (EMBL: L41840) zinc fingers using Pileup. Text was highlighted for conserved cysteine (white type on black background), hydrophobic (black type on grey) and asparagine residues (white type on dark grey background). The sequence identity number ("NO:") of each sequence is given in parentheses after the sequence.

In Fig. 3a, RT-PCR was used to analyse β-actin and Cezanne transcripts. A panel of cDNA from various human tissues obtained commercially (Origene Technologies). This comprised mammary gland (MG), fat (F), foetal liver (FL), foetal brain (FB), lymphocytes (Ly), prostrate (Pr), uterus (Ut), ovary (Ov), pancreas (Pa), adrenal gland (Ad), pituitary gland (Pi), placenta (Pl), testis (Te),
stomach (St), muscle (M) and small intestine (SI). In Fig. 3b, transcripts for β-actin, TRABID and Cezanne were amplified by RT-PCR in a panel of murine tissues that comprised lymph node (LN), lung (Lu), brain (Br), heart (He) and kidney (Ki). In Fig. 3c, mRNA from HMEC-1 (HM), HepG2 (Hep) and MRC5 (MRC) cells were Northern blotted and probed in parallel using 32p-labelled full-length cDNA for TRABID and Cezanne. Membranes were washed before autoradiography was performed. Transcript sizes are indicated.

In Fig 4, cells were cultured in 24-well plates and transfected using calcium precipitation. In Fig. 4a, HEK293 cells were transfected with HA-tagged A20 (A), TRABID (T) or Cezanne (Ce) and incubated for 16 hours. mRNA was then extracted from these cells and untransfected cells (-) before RT-PCR analysis of Cezanne, TRABID and A20 transcripts. Reporter gene assays were performed using pHM6 expression vectors (exp.) encoding full-length TRABID, A20, Cezanne or truncated regions of Cezanne (Cez. 1-443 or Cez. 444-858). In Fig. 4b, HEK293 cells were co-transfected with 100ng pGL2 (NF-κB reporter), 100ng pUT651 (β-galactosidase control) and various amounts of expression vector (exp.). The total amount of DNA transfected was standardised with empty pHM6 vector (empty). Cells were stimulated with TNFα for six hours. Cell lysates were analysed and the ratio Fire-fly luciferase/ β-galactosidase activity was calculated which is a measure of NF-kB activity normalised for transfection efficiency. Similar experiments in HEK293 (Fig. 4c) and EaHy cells (Fig. 4d) used pRL-TK (Renilla luciferase control) instead of pUT651. In these assays, NF-κB activity was represented by the ratio Fire-fly/ renilla luciferase activity. Mean values calculated from duplicate or triplicate wells are shown with standard errors. The data shown is representative of 12 closely similar experiments.

In Fig. 5, HEK293 cells were transfected transiently with pHM6 expression vectors which encoded HA-tagged versions of A20 (A20-ha), Cezanne (Cez-ha) or TRABID (Trabid-ha). These were transfected alone or were co-transfected with FLAG-tagged TRAF6. Cell lysates (15μl) were tested by Western blotting
using anti-flag or anti-ha antibodies. Anti-flag antibody was used to immunoprecipitate TRAF6-FLAG from 1ml lysate. Immunoprecipitating proteins (i.p.) were tested by Western blotting using anti-flag or anti-ha antibodies.

In Fig. 6, HEK293, EaHy and NIH3T3 cells were transfected transiently with EGFP-tagged molecules. Full-length A20, TRABID and Cezanne were analysed alongside TRABID residues 1-345 and Cezanne residues 444-858. Confocal microscopy was performed 16 hours later.

In Fig. 7, sequences were analysed using BLAST and Profilescan. The position of recognised domains is represented. “Protease” domain is synonymous with TRAFB domain.

In Fig. 8, 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 together with amino acid number. The region of A20 homology, core catalytic domain, nuclear localization sequence (NLS) and zinc (Zn) finger are depicted.

In Fig. 9, yeast strain YRG-2 was transfected with either: (i) pGBK7-Cezanne plus pGAD424-polyubiquitin or (ii) empty pGBK7 plus pGAD424-polyubiquitin, before plating onto minimal media (-Trp, -Leu, -His) and incubation at 30°C. After 3 days, colonies were transferred to nitrocellulose membrane, briefly immersed in liquid nitrogen and then incubated with X-gal substrate (overnight at room temperature). This procedure identifies β-gal containing colonies, which become blue (dark colonies in figure).

In Fig. 10 A, B & C, yeast strain YRG-2 was co-transformed with a pGBK7 construct containing full-length, truncated or mutated versions of Cezanne (residues indicated) plus pGAD424-polyubiquitinB. β-galactosidase reporter
gene expression was measured when yeast colony growth on each plate was at maximal rate (day 4 to 6), as assessed by daily monitoring of colony numbers. Yeast colonies expressing reporter genes were identified (A, B, C) and counted (B, C). Transformation efficiency was identical within a single experiment but varied between experiments. The results are representative of three closely similar experiments. (D) *E. coli* were transformed with an IPTG-inducible expression vector containing two lengths of sequence encompassing the putative catalytic domain of Cezanne (residues 126-455 or 182-455, as indicated) and then cultured in the presence or absence of IPTG for 6h. *E. coli* lysates were tested *in vitro* against Arg-Leu-Arg-Gly-Gly-AMC (SEQ ID NO: 10; a fluorescent analogue representing the polyubiquitin cleavage site) and release of AMC was measured using a fluorometer. Lysates from transformed *E. coli* that were not treated with IPTG did not yield fluorescence (data not shown).

In Fig. 11A, two versions of the catalytic domain of Cezanne (182-455 or 126-455) or an irrelevant molecule (control) were expressed in *E. coli* as GST-tagged proteins. Crude *E. coli* lysate, purified protein or purified/dialysed protein were tested (see Experimental procedures). The relative amount of protein in stocks of purified or purified/dialysed material was assessed by Coomassie staining (lower panel). Test material containing equivalent molar quantities of GST-tagged protein were incubated at 37°C with an excess of branched polyubiquitin chains (Ub). Purified isopeptidase-T served as a positive control (Iso.T), while polyubiquitin chains incubated alone were a negative control (no enzyme). Following hydrolysis, ubiquitin was revealed by western blotting using anti-ubiquitin antibody. Cleavage of polyubiquitin into the monomeric form was observed for Cezanne 126-455. This data is representative of six closely similar experiments. In Fig. 11B, equivalent molar quantities of purified Cezanne 126-455 or isopeptidase T (Iso.T) were pre-incubated in the presence or absence of ubiquitin-aldehyde as indicated. An excess of branched polyubiquitin chains (Ub) was then added before further incubation for 30m. Polyubiquitin chains incubated alone were a negative control (no enzyme).
Following hydrolysis, ubiquitin was revealed by western blotting using anti-ubiquitin antibody. Note that monomeric ubiquitin migrated out of this gel.

In Fig. 12, various HA-tagged versions of Cezanne were immunoprecipitated from transfected Cos7 cells and tested in hydrolysis reactions using an excess of branched polyubiquitin chains (Ub). Precipitated material from cells transfected with empty expression vector served as a negative control (vector). Purified isopeptidase-T was a positive control (Iso.T), while polyubiquitin chains incubated alone were a second negative control (no enzyme). Following hydrolysis, ubiquitin was revealed by western blotting using anti-ubiquitin antibody (lower panel). It is possible that the substrate contained trace amounts of a circularised form of polyubiquitin (*), which is known to be resistant to cleavage by isopeptidase T. The efficiency of each precipitation was assessed by western blotting using anti-HA antibody (upper panel).

In Fig. 13A, HeLa cells were co-transfected with pCDNA3.1-FLAG-ubiquitin (0.5µg) plus a pHM6 construct (2.5µg) containing a HA-tagged version of Cezanne (residues as indicated) or empty pHM6 (no Cezanne). Untransfected cells served as a control (untransfected). After 48h, cells were treated with 20µM MG132 for 1h or remained untreated, as indicated. Cell lysates were tested by western blotting using anti-FLAG to detect residual ubiquitinated proteins or with anti-HA antibodies to detect Cezanne. Two independent experiments are shown (lanes 1-10 and lanes 11-16 respectively). A protein that reacted non-specifically with anti-HA antibody served as an internal control for the total amount of cellular proteins loaded (n.s.). In Fig. 13B, Cos7 cells were transfected with a pHM6 construct containing an HA-tagged version of Cezanne (residues as indicated) or with empty pHM6 (vector). After 24h, cells were treated with 20µM MG132 for 1h or remained untreated, as indicated. HA-tagged molecules were immunoprecipitated from Cos7 cell lysates using anti-HA antibody coupled to beads. Co-immunoprecipitating polyubiquitinated proteins were detected by western blotting using anti-ubiquitin antibody. Levels of immunoprecipitated Cezanne were assessed by silver staining. The position
of Cezanne is indicated (*).

In Fig. 14, three multiple sequence alignments were made of putative catalytic regions of representative members of the OTU superfamily (this includes the A20 group), the UCH family or the UBP family. Accession codes are given and residue numbers are indicated. All sequences are human except for AAD31534 (mouse UCH37). Hin-1 was compiled using several Genbank entries (unpublished). Text is highlighted for conserved putative catalytic residues (white on black background), identical residues (dark grey background) and residues with similar properties (light grey background). The sequence identity number ("NO:" for each sequence is given in parentheses after the sequence. In Fig. 15, HEK293 cells expressing GFP-TRABID or GFP alone were exposed to TNFα/ cycloheximide for 3, 6 or 10 hours before staining with propidium iodide and analysis by flow cytometry. The proportion of necrotic cells (propidium iodide-positive) were calculated for cells gated on GFP and for untransfected cells (no GFP). Note that close correlation between cell death in untransfected cells and GFP expressing cells indicates that: 1. GFP itself did not influence survival and 2. GFP did not ‘leak’ from necrotic cells. Results were closely similar for two independent experiments.

In Fig. 16, native or catalytically inactive (C209S) forms of Cezanne were purified from transfected insect cells. (A) The relative amounts and size distribution of protein(s) in stocks of purified material were assessed by SDS-PAGE followed by silver staining. (B) Test material containing equivalent quantities of protein were incubated at 37°C for 1h with an excess of branched polyubiquitin chains (Ub). Substrate incubated alone was a negative control (no enzyme). Following hydrolysis, ubiquitin was revealed by western blotting using anti-ubiquitin antibody. (C) Test material was incubated at 37°C with a thiol-reactive probe (HUAUbVME or HUAUbBr2, as indicated). Probe incubated alone was a negative control (no enzyme). Probe sequences were then revealed by western blotting using an anti-HA epitope antibodies.
In Fig. 17, two FLAG-tagged versions of A20 (residues indicated) were produced in transfected Cos7 cells and tested for reactivity with a ubiquitin-derived probe (HAUbBr2). Lysate from untransfected cells served as a negative control. Levels of FLAG-tagged proteins were assessed by western blotting using an anti-FLAG antibody (upper panel). Cell lysates from transfected or untransfected cultures were incubated with HAUbBr2 probe at 37°C for 2h. 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).

In Fig. 18, 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. The efficiency of each precipitation was assessed by western blotting using anti-FLAG antibody (upper panel). A proportion of immunoprecipitated material was tested in hydrolysis reactions using an excess of branched polyubiquitin chains (Ub). Polyubiquitin chains incubated alone were a second negative control (no enzyme). Following hydrolysis, ubiquitin was revealed by western blotting using anti-ubiquitin antibody (centre panel). The purified material was also tested for reactivity with HAUbBr2. Probe incubated alone served as an additional negative control (no enzyme). Following incubation, probe sequences were revealed by western blotting using an anti-HA epitope antibodies (lower panel). Two experiments are shown (lanes 1 to 5 and lanes 6 to 9).

In Fig. 19, HeLa cells were co-transfected with pcDNA3.1-FLAG-ubiquitin (0.3µg) 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 (vector). Unless otherwise indicated, each well received 1.5µg pHM6 construct. One well received 0.1µg pHM6-Cezanne plus
1.4µg empty pHM6 vector (lane 5). Here, inclusion of empty pHM6 ensured that total levels of transfected vector were identical for each well. After 48h, cells were treated with 20µM MG132 for 1h or remained untreated, as indicated. Cell lysates were tested by western blotting using anti-FLAG antibody to detect residual ubiquitinated proteins or with anti-HA epitope antibody to detect A20 or Cezanne. Blots were stripped and re-probed using anti-α-tubulin to assess loading.
**Example 1: Characterisation of Cezanne and TRABID, A20-like proteins**

**Introduction (Prior Art)**

The endothelium provides a critical barrier to immune cells and exerts control over the inflammatory process. Pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α and interleukin (IL)-1, initiate signalling pathways in endothelial cells leading to activation of NF-κB. This transcription factor elevates the production of several adhesion molecules and chemokines which control docking and transendothelial migration of leukocytes.

NF-κB is sequestered in the cytoplasm of unstimulated cells through binding to inhibitory IκB molecules. Signalling cascades triggered by cytokine stimulation lead to phosphorylation of IκB, which is subsequently ubiquitinated and degraded. NF-κB is released, translocates to the nucleus and engages target nucleotide sequences. Control of IκB phosphorylation is therefore a key element in NF-κB regulation. Engagement of the TNF receptor 1 (TNFR1/p55) leads to recruitment of several signal adaptor molecules including RIP and TRAF2. It has recently been shown that IκB kinases (IKK1 and IKK2), which are responsible for phosphorylation of IκB, are recruited to the TNF receptor signalling complex through association with the adaptor molecule NEMO (IKKγ) which in turn binds to RIP. Localisation of IκB kinases to the signalling complex may facilitate their activation. Similarly, NF-κB activation through IL-1 receptor 1 requires several adaptor molecules including TRAF6. IL-1 and TNFα signalling pathways converge during activation of the IκB kinase signalosome.

The zinc finger molecule A20 is a crucial negative regulator of NF-κB activity. A20 deficient mice cannot regulate NF-κB and develop severe inflammation. Conversely, a strong association exists between endothelial expression of A20 and long-term survival of transplanted organs. Studies in cultured cells have demonstrated that NF-κB activity is down-regulated by over-expression of A20.

Furthermore, over-expression of the C-terminal portion of A20, which contains seven zinc fingers, was sufficient for this activity. It has been shown recently
however, that the A20 zinc finger domain interacts with NEMO and it is possible that this association provides an opportunity for A20 to exert an inhibitory effect on IKK function. The N-terminal region of A20 (residues 1-386) binds to TRAF signal adaptor molecules which are critical components of TNFα and IL-1 signalling pathways. The function of A20 (1-386) has not hitherto been described.

MATERIALS AND METHODS

Cell culture

Human epithelial cells (HEK293) and mouse fibroblasts (NIH3T3) were cultured using DMEM/ 10% foetal calf serum (FCS), supplemented with antibiotics. An endothelial cell line (BaHy926) supplied by M.Rose (Harefield Hospital, Harefield, UK), was cultured using DMEM/ 20% FCS, supplemented with HAT (Gibco BRL, Paisley, UK) and antibiotics.

Isolation of cDNA and cloning into expression vectors

Sequence information for full-length cDNA was generated using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, USA) according to the manufacturer's instructions. This information was used to design primers for amplification of full-length cDNA. Pfu DNA polymerase (Stratagene, Cambridge, USA) was used to amplify full-length TRABID (sense, ATGTCAGAACGTGGAATAAGTGG - SEQ ID NO: 11; antisense, TCATTCACTTTCACTCCTCTTC - SEQ ID NO: 12) and Cezanne (sense, ATGTTCTACCATACTCCAGCTGGC - SEQ ID NO: 13; antisense, TCAGAACCTGTGCACCAGGAGC - SEQ ID NO: 14).

Cloning into expression vectors was achieved through recombinant PCR. pHM6 (Roche, Mannheim, Germany) facilitated production of molecules tagged at the N-terminus with haemagglutinin (HA). Expression was driven by the cytomegalovirus (CMV) immediate-early gene promoter. Full-length Cezanne was cloned into pHM6. An N-terminal region of Cezanne encompassing the putative TRAF binding domain (residues 1-443) was generated using sense (ATGTTCTACCACACATCCAGCTGGC - SEQ ID NO: 13) and antisense
(GTAGCTATGCAGCAGATGC - SEQ ID NO: 15) primers and cloned into pHM6, creating pHM6-Cez1-443. A C-terminal region of Cezanne encompassing the putative nuclear localisation sequence and zinc finger (residues 444-858) was also generated using sense (AGATGAATGTGAAGTGATCCC - SEQ ID NO: 16) and antisense (TCAGAACCTGTGCACCAGGAGC - SEQ ID NO: 14) primers, and cloned to create pHM6-Cez444-858. Full-length TRABID was also cloned into pHM6. pEGFP-C3 (Clontech) utilised the CMV promoter to drive expression of molecules tagged at the N-terminus with green fluorescent protein (GFP). cDNA's encoding full-length Cezanne and residues 444-858 were cloned into pEGFP. cDNA's encoding full-length TRABID and residues 1-345 were also cloned. Primers for TRABID 1-345 comprised sense (ATGTCAGAACGTGGAATTAAGTG - SEQ ID NO: 11) and antisense (TGCTGGAAATACACTTTG - SEQ ID NO: 17). It was ensured that the coding sequence of all molecules would be in-frame with the HA or GFP tags and this was verified by sequencing. Stop codons were positioned immediately after the coding sequence. The expression vector encoding FLAG-tagged TRAF6 has been described previously (Heyninck, K. & Beyaert, R., 1999, FEBS Lett. 442: 147-150).

RT-PCR and Northern blotting
Reverse transcription and PCR was performed as described previously (Evans, P.C. & Kilshaw, P.J., 2000, Transplantation 70: 928-934). Sense (TGGCAGACACCATGCTGAGG - SEQ ID NO: 18) and antisense primers (CGCTTTGACTTCTCCTCGGC - SEQ ID NO: 19) were used to amplify Cezanne transcripts. For TRABID, sense (GAAGATTTGCCCCAACAGTCC - SEQ ID NO: 20) and antisense (AGCTTGCTCCAGGCTGACTAGC - SEQ ID NO: 21) primers were used. For Northern blotting, total RNA was extracted using Trizol as described previously (Evans & Kilshaw, 2000, supra). 20μg RNA was separated using a 1% agarose/formaldehyde gel, blotted onto charged nylon membrane and exposed to ultra-violet light. Prehybridisation was performed at 60°C for 1h using Quickhyb (Stratagene). Full-length cDNA was
labelled with $\alpha-^{32}$P dCTP using the random priming method (Promega; Madison, USA), purified, mixed with 100$\mu$g/ml sonicated salmon sperm DNA and denatured before application to the membrane. After overnight incubation at 60°C, the membrane was subjected to stringent washes (0.1X SSC, 0.1% SDS at 55°C) before autoradiography.

**Transfection of cultured cells**

(i) CaCl$_2$ precipitation. For cells cultured in 24-well plates, DNA was combined with 50$\mu$l 0.25M CaCl$_2$ before addition of 50$\mu$l of 2x BBS (280mM NaCl, 1.5mM Na$_2$HPO$_4$, 2H$_2$O, 50mM BES (pH7; Sigma, Poole, UK). This solution was incubated at room temperature for 30min and added to 1ml growth medium which was then applied to the cells. Cells were incubated overnight in 3% CO$_2$ and then restored to normal culture conditions. (ii) Lipofectamine. 17$\mu$g expression construct and 70$\mu$l lipofectamine (Gibco BRL) were each combined with 1.75ml Optimem-1 (Gibco BRL) and incubated at room temperature for 5min. These solutions were combined, incubated at room temperature for 20min. Cells grown in a 75cm$^2$ flask were washed with Optimem-1 and 3.5ml lipofectamine/DNA/Optimem-1 was applied for 5h before normal culture conditions were restored.

**Reporter gene assays**

The NF-κB reporter (pGL2-NFLUC) comprised NF-κB response elements upstream of firefly luciferase (supplied by M.Turner, The Babraham Institute, Cambridge, UK. The vector is described in Williams D.H. et al. (1995; Eur. J. Imm. 25: 42-47). To normalise transfection efficiency, cells were co-transfected with control constructs which comprised either pUT651 (encoding β-galactosidase; Cayla, Toulouse, France) or pRL-TK (encoding Renilla luciferase; Promega). Cells were transfected by calcium precipitation and tested after 48-72h. β-galactosidase activity was measured as described previously (Heyninck & Beyaert, 1999, *supra*). Fire-fly and renilla luciferase activity was assessed using the Dual Luciferase Reporter Assay Kit (Promega) and luminescence counter (Topcount Microplate Scintillation; Packard, Meriden,
USA).

**Immunoprecipitation**

Confluent cultures of HEK293 cells in 75cm² flasks were transfected transiently using lipofectamine. Cells were lysed after 72h using 2ml Tris-HCl (20mM, pH7.5), Triton X-100 (1%), NaCl (137mM), MgCl₂ (1.5mM) and EGTA (1mM). Debris was removed from lysates by high-speed centrifugation. A total of 1ml lysate was pre-cleared three times using 5μg mouse IgG (Sigma I5381) bound to protein G sephrose beads (Amersham Pharmacia; Uppsala, Sweden).

FLAG-tagged TRAF6 was then immunoprecipitated using M5 anti-FLAG monoclonal antibody (Sigma F4042) which was bound to protein G-sephrose beads (Amersham Pharmacia). Beads were then washed four times with lysis buffer. Lysates (15μl) and immunoprecipitates were analysed by Western blotting. Detection of HA tag was achieved using rat anti-HA monoclonal antibody conjugated directly to HRP (Roche 2013819). FLAG-tagged proteins were identified using M5 anti-FLAG followed by goat anti-mouse HRP (Dako, Cambridge, UK). HRP was developed using chemiluminescent substrate (Pierce, Chester, UK).

**Confocal Microscopy**

Cells cultured on glass coverslips were transfected transiently with GFP-tagged expression vectors using lipofectamine. Live cells were analysed after 24h using a confocal laser scanning microscope with an excitation wavelength of 488nm.

**RESULTS**

Analysis of the predicted amino acid sequences of Cezanne and TRABID using BLAST and Profilescan (see website at isrec.isb-sib.ch/software/PFSCAN) programmes revealed several features (Fig. 1). Cezanne residues 160 to 416 displayed striking identity (39%) to the N-terminal, TRAF binding region of A20 (Fig 2a). This defines a novel domain with conserved structural features which we have named TRAFB (TRAF binding). Cezanne also contains a putative nuclear localisation signal (RRKEKSKRDREKD KKR [SEQ ID NO:
22]; residues 497-513) which conforms to a consensus sequence that has a reported predictive accuracy of 88% (see website at cubic.bioc.columbia.edu/predictNLS). A single A20-like zinc finger is located at the extreme C-terminus of Cezanne (residues 816-838) which conforms to the consensus Cys-X4-Cys-X11-Cys-X2-Cys (SEQ ID NO: 23). This has greatest similarity to A20 zinc fingers 7 (residues 747-767) and 4 (residues 592-612) (Fig 2b).

Similar analysis demonstrated that a TRAFB domain was located in the C-terminal portion of TRABID (residues 392 to 641; Fig 1). This domain has 32% identity with the corresponding domain of A20 (Fig. 2a). Three zinc finger motifs were identified at the N-terminal region of TRABID (Fig. 1). These were unrelated to A20 and Cezanne but showed similarity to zinc fingers found in several nucleoporin molecules (Fig. 2b).

Alignment of the TRAFB domains of Cezanne, TRABID and A20 revealed considerable conservation of hydrophobic and basic residues which suggested structural similarities (Fig 2a). The alignment was analysed using several computer algorithms to create a consensus prediction of secondary structure (JPred; see website at barton.ebi.ac.uk/servers/jpred.html). In general, α-helices and β-sheets were predicted for N-terminal and C-terminal TRAFB regions respectively.

Expression and regulation of transcripts

RT-PCR analysis revealed widespread expression of Cezanne and TRABID transcripts among human and murine tissues (Figs 3a and 3b). Cezanne transcripts were particularly abundant in kidney, heart and foetal liver. RNA from endothelial, hepatocyte and fibroblast cell lines was tested by Northern blotting with cDNA encoding full-length Cezanne and TRABID (Fig. 3c). A dominant, 6kb Cezanne transcript was detected in each cell line. A larger 9.5kb transcript was also detected in endothelial and fibroblast cells, which may be a splice variant. Probing of a multiple-tissue Northern blot (Clontech) also revealed 9.5kb and 6kb Cezanne transcripts and confirmed high level expression in heart tissue (data not shown). TRABID transcripts were also identified in
each cell line and comprised a 3kb species and a minor 1kb variant. It has been shown previously that transcription of A20 is upregulated after NF-κB activation and provides a negative feedback loop in pro-inflammatory responses. We performed RT-PCR analysis of phorbol ester stimulated T-cells (Jurkat line) and TNFα treated HUVEC and demonstrated rapid induction of A20 transcripts. In contrast, activation of NF-κB through either treatment did not alter levels of Cezanne or TRABID (data not shown).

**Cezanne is a negative regulator of NF-κB**

The effects of Cezanne on NF-κB activity and comparison with A20 and TRABID were investigated. Reporter gene experiments were performed in HEK293 cells which were co-transfected with expression plasmids for A20, Cezanne or TRABID. Comparison of the relative contribution of endogenous and transfected molecules was an important pre-requisite to these studies (Fig 4a). HEK293 cells did not contain endogenous levels of A20, Cezanne or TRABID but transcripts were detected in transfected cells indicating that transfection and subsequent transcription were successful. For each expression construct, translation was also verified by Western blotting (see Fig. 5).

Cezanne reduced NF-κB activity in HEK293 cells stimulated with TNFα in a dose dependent manner (Fig 4b). In contrast, TRABID expression was associated with a modest elevation of NF-κB. Comparison of the abilities of A20 and Cezanne to regulate NF-κB demonstrated that A20 had greater potency, i.e. 50ng A20 and 400ng Cezanne expression constructs had similar effects (Fig 4c). This difference could not be attributed to divergent transcriptional (Fig 4a) or translational activity (see Fig.5) and therefore reflects a genuine difference in the efficiency of over-expressed A20 and Cezanne. We predicted that, like A20, the zinc finger-containing region of Cezanne would be sufficient to regulate NF-κB. It was demonstrated however, that expression of this region (Cezanne 444-858) had no effect on NF-κB reporter gene activity whereas expression of the TRAFB domain (Cezanne 1-443) led to marginal suppression (Fig. 4c). Significant reductions in NF-κB activation were only observed after over-expression of full-length Cezanne.
The importance of endothelium in the inflammatory response prompted examination of A20 and Cezanne function in an endothelial cell line (EaHy). In contrast to HEK293 cells, these cells express low constitutive levels of A20 and Cezanne (data not shown). Despite this difference, the effects of Cezanne and A20 on NF-κB activity in EaHy mimicked those observed in HEK293 cells (Fig 4d).

A similar series of experiments demonstrated that activation of NF-κB by IL-1α treatment was also regulated by Cezanne but with reduced efficiency compared to A20 (data not shown).

**Interaction with the signal adaptor TRAF6**

We have defined the TRAFB domain through alignment of A20, Cezanne and TRABID. A20 TRAFB has been shown in the prior art to interact with a TRAF1/TRAF2 heterocomplex and with TRAF6 which are signal adaptor molecules for the TNFR1 and IL-1R1, respectively. We expected that the TRAFB domain of Cezanne and TRABID would facilitate interaction of these molecules with TRAFs.

Studies of over-expressed molecules in HEK293 cells demonstrated that A20, Cezanne and TRABID were co-immunoprecipitated with TRAF6 (Fig. 5). These molecules were not immunoprecipitated when TRAF6 was omitted, demonstrating that interactions were mediated through specific binding to TRAF6. The level of A20, Cezanne and TRABID molecules expressed in transfected cells was similar, therefore the degree of co-immunoprecipitation reflected the relative efficiency of binding to TRAF6, i.e. A20>TRABID>Cezanne. Overall, a relatively small proportion of each TRAFB-containing molecule co-immunoprecipitated with TRAF6 which raises the possibility that these interactions were limited by an endogenous intermediate.

**Intra-cellular localisation**

The intra-cellular distribution of GFP-fusion proteins was assessed in live cells using confocal microscopy (Fig. 6). In epithelial (HEK293) and fibroblast
(NIH3T3) cell lines, GFP-A20 was localised to punctate bodies within the cytoplasm. A similar distribution was observed in an endothelial line (EaHy) but in these cells, GFP-A20 also demonstrated nuclear localisation. These patterns contrasted with GFP-TRABID which was distributed diffusely within the cytoplasm and nucleus of all lines tested and accumulated in the nucleus of most fibroblasts. The zinc finger domain of TRABID (residues 1-345) localised to large punctate bodies within the cytoplasm.

Despite the presence of a putative nuclear localisation sequence, GFP-Cezanne did not localise to the nucleus of epithelial and fibroblast lines and was instead, distributed diffusely within the cytoplasm. Only a proportion of endothelial cells displayed GFP-Cezanne within the nucleus, which suggests that nuclear entry of this molecule is subject to tight control.

Localisation of full-length and the zinc finger-containing region of Cezanne were compared because this may have influenced their differential effects on NF-κB activity (see Fig. 4). Cezanne 444-858 was located exclusively at the plasma membrane of epithelial cells. Staining of transfected cells with a lipid dye (FM 4-64; Molecular Probes Inc., Eugene, USA) revealed that Cezanne 444-858 was adjacent to the plasma membrane and did not insert (data not shown). Punctate distribution within the cytoplasm was observed in endothelial and fibroblast cells with a degree of plasma membrane localisation in the latter.

**DISCUSSION**

It has been demonstrated that over-expression of the C-terminal half of A20, which contains seven zinc fingers is sufficient for this suppression of NF-κB. Relatively little was known in the prior art about the function of the N-terminal portion of A20. This region is responsible for the interaction of A20 with TRAF molecules. Sequence alignment between A20, Cezanne and TRABID identified a novel domain with conserved structural features (TRAFB).

Over-expression studies revealed that Cezanne has the ability to regulate NF-κB in cells of epithelial or endothelial origin. Sequence similarity between Cezanne
and A20 zinc fingers suggests that these molecules may employ similar mechanisms. The relative efficiency of Cezanne and A20 in regulating NF-κB may therefore, be governed by the number of zinc fingers present within these molecules, i.e. one and seven. This is consistent with a previous study demonstrating that at least four A20 zinc fingers are required for efficient modulation of TNFα-induced NF-κB.

**Example 2: Isolation and characterisation of Cezanne-2**

The sequence of Cezanne-2 was derived from expressed sequence tags and genomic sequences available on public databases. The primary amino acid sequences of Cezanne and Cezanne-2 are 90% similar.

A domain composition and sequence alignment of Cezanne, Cezanne-2, A20 and TRABID is shown in Fig. 7.

**Example 3: Characterisation of Cezanne as a deubiquitinating enzyme**

The functional activity of Cezanne has been further tested in the experiments outlined and discussed below.

**MATERIALS AND METHODS**

**Mammalian cells**
Cos7 and HeLa cells were cultured using Dulbecco’s modified Eagle’s medium/10% foetal-calf serum, supplemented with antibiotics.

**Yeast expression vectors**
Cloning was performed by recombinant PCR using Pfu turbo polymerase (Stratagene). The yeast expression vector pGBK7 (Clontech Laboratories) was used to express fusions between the DNA binding-domain (BD) of the Gal4
transcription factor, a myc epitope tag and Cezanne (driven by the ADH1 promoter). The primers used to amplify regions corresponding to Cezanne residues 17-858 (5'-GCTGGAATTCCACCTGGGACATGGATGCTGTTC-3' [SEQ ID NO: 24] and 5'-GGGTGTTGTCGACTCAGAACCTGTGCGACCAGGAG-3' [SEQ ID NO: 25]), residues 17-443 (5'-GCTGGAATTCCACCTGGGACATGGATGCTGTTC-3' [SEQ ID NO: 24] and 5'-GGGTGTTGTCGACCATTGTAGCTATGCGACAGGCACATGC-3' [SEQ ID NO: 26]) or residues 444-858 (5'-GCTGGAATTCCAATGTGAAGTGGATGCACCCACTG-3' [SEQ ID NO: 27] and 5'-GGGTGTTGTCGACTCAGAACCTGTGCGACCAGGAG-3' [SEQ ID NO: 25]) incorporated SalI or EcoRI restriction sites, which facilitated directional cloning of cDNA in-frame with GAL4-BD.

**Bacterial expression vectors**

pDEST15 (Invitrogen) was used for expression of glutathione-S-transferase (GST)-Cezanne fusion proteins in *E. coli* in response to isopropyl-beta-D-thiogalactosidase (IPTG). PCR primers amplified cDNA corresponding to residues 126-455 (sense, 5'-CACCGAGAACCCTGTACTTTCCAGGCTGGTCCCATGTCTCTCCAA-3' [SEQ ID NO: 28]; antisense, 5'-TTACTAGAACCCTCTCAGGATCCAGGGACAGTGCCATCCCTTT-3' [SEQ ID NO: 29]) or residues 182-455 (sense, 5'-CACCGAGAACCCTGTACTTTCCAGGCTGGTCCACATAGG-3' [SEQ ID NO: 30]; antisense as above). Sense primers incorporated nucleotides encoding the tobacco etch virus (TEV) protease recognition site and nucleotides required for topoisomerase cloning. Antisense primers incorporated stop codons. Amplified sequences were cloned initially into pENTR/D-TOPO vectors (Invitrogen) and then into pDEST15, using the GATEWAY system (Invitrogen).
Mammalian expression vectors

pHM6 was used to express molecules tagged at the N-terminus with haemagglutinin (HA) in mammalian cells (driven by a CMV immediate-early promoter). Construction of pHM6-Cezanne1-858 and pHM6-Cezanne1-443 is described above in Example 1 (and see Evans et al., 2001, supra). Expression of FLAG-tagged ubiquitin driven by the CMV immediate-early promoter was achieved using pcDNA3.1-FLAG-ubiquitin, supplied by Dr. I. Dikic (Ludwig Institute for Cancer Research, Uppsala, Sweden).

Mutagenesis

Quickchange technology (Stratagene) was used to change two nucleotides at codon 209 (Cys to Ala) in pGBK7-Cezanne17-858, pHM6-Cezanne1-858 and pHM6-Cezanne1-443 using complimentary primers (sense: 5\'-CTACTGGAGATGGGAACGCCCTCCTGCATGCAGCCTC-3' [SEQ ID NO: 31]). This method was also used to introduce a point mutation at codon 209 (Cys to Ser) in pHM6-Cezanne1-858 (sense 5\'-GGAGATGGGAACGCCTCCTGCATGCAG-3' [SEQ ID NO: 32]). Quickchange PCR using this primer set was unsuccessful when pGBK7-Cezanne 17-443 was used as template. An alternative strategy was employed which involved amplification of two overlapping products (using primers 5\'-GCTGGAAATTCAACCTGGACATGGATGCTGTTC-3' [SEQ ID NO: 33] and 5\'-CTGAAATCTAGTGGCTGTCTCCTGCAGTCC-3' [SEQ ID NO: 34] or 5\'-GGACTCGAGGAGACAGCCTACTTGATCC-3' [SEQ ID NO: 35] and 5\'-GAGTTTGTCGACCTGACCTGTGCACCAGGAG-3' [SEQ ID NO: 36], respectively). The two products were then denatured, annealed and 3' ends were extended using polymerase. cDNA corresponding to residues 17-443 was then amplified by PCR and inserted into pGBK7, following restriction with EcoR1 and SalI. DNA sequencing was used to ensure that each construct was inserted in-frame with N-terminal residues and to verify changes at codon 209.

Yeast 2-hybrid

The yeast two hybrid system was performed according to manufacturers
recommendations (Clontech Laboratories). In the first instance, it was ensured that co-transformation of yeast strain YRG-2 with various pGBK7-Cezanne constructs (17-858, 17-443, 444-858, 17-443 C209S, 17-858 C209A or 17-443 C209A) plus empty pGAD424 would not generate colony growth on minimal media lacking Trp, Leu and His in the presence of 5mM 3-amino-1,2,4-triazole (-Trp, -Leu, -His, +3AT). The cDNA library used was derived from whole mouse embryos and cloned into pGAD424 in-frame with the activation domain of GAL4 (AD) using methods described previously (De Valck, D. et al., 1996, FEBS Lett. 384, 61-64). This library was screened using pGBK7-Cezanne 17-858 and clones containing putative interacting proteins were identified by growth on minimal media lacking essential amino acids (-Trp, -Leu, -His, +3AT) and by expression of β-galactosidase. pGAD424 plasmid DNA was harvested from positive clones and amplified using E.coli as host. Each plasmid was then used to re-transform yeast together with either pGBK7-Cezanne17-858 or empty pGBK7. Colony growth on minimal media lacking Trp and Leu was assessed to ensure that the transformation efficiency using either construct was identical. Clones that reacted exclusively with pGBK7-Cezanne 17-858 were sequenced.

**In vitro peptidase and isopeptidase assays**

*In vitro* hydrolysis assays were performed by incubating potential proteases with substrate in reaction buffer (50mM Hepes (pH7.8), 0.5mM EDTA, 0.01% Brij, 3mM DTT; 40μl total volume) at 37ºC for varying lengths of time.

For peptidase assays, a fluorescent substrate was used Arg-Leu-Arg-Gly-Gly-AMC (Bachem; SEQ ID NO: 10) at a final concentration of 10μM. Hydrolysis of the peptide bond between Gly and AMC was assessed over time using a fluorometer (Cytoflour II, PerSeptive Biosystems). Test material was prepared as follows: *E.coli* (Rosetta, Novagen) transformed with pDEST(15)126-455 or pDEST(15)182-455 were cultured in the presence of 0.4mM IPTG for 6h to induce expression of GST-fusion proteins (or remained untreated as a control). Bacteria from each 50 ml-culture were harvested and lysed in 1ml of BugBuster
(Novagen), 0.04% β-mercaptoethanol, 25u benzonase (Novagen), 1mM AEBSF, 1mM EDTA and clarified by centrifugation (20,000 g for 10m at 4°C). Hydrolysis reactions were performed using 5μl lysate, which gave a final concentration of each GST fusion protein of 15nM (estimated by Coomassie staining of lysates alongside standards of known concentration, following gel electrophoresis).

For isopeptidase assays, a mixed population of branched polyubiquitin chains polymerised through Lys48 (Affiniti Research Products) was used as substrate at a final concentration of 1μM. Preparation of test material was carried out as follows:

(i) GST-Cezanne126-455, GST-Cezanne182-455 and a GST-tagged irrelevant molecule (Vav2, supplied by Dr. M. Turner, Babraham Institute, UK) were harvested from E. coli as described earlier. They were tested in either crude form (denoted as lysate) or were purified using a glutathione column (Pharmacia). Purified material was eluted from the column using 20mM glutathione, 0.1M Tris (pH8), NaCl, 1mM DTT. Material was then used either directly in isopeptidase assays (denoted as purified) or used after dialysis against PBS (denoted as purified/ dialysed). Analysis of the purified material by gel electrophoresis/ silver staining identified only trace amounts of impurities (<1%) which were present at identical levels in each preparation (data not shown). All forms of the GST fusion protein or isopeptidase T (Affiniti Research Products), which served as a positive control, were tested at a final concentration of 15nM. In some experiments, the GST tag was removed from GST-Cezanne 126-455 protein by digestion with TEV protease (Invitrogen). Material was then loaded onto a glutathione column to remove undigested fusion protein and released GST, before final purification of Cezanne 126-455 by gel filtration on a Superose75 column (Pharmacia).

(ii) Alternatively, HA-tagged molecules were tested after immunoprecipitation from Cos7 cells. Cultures in 25cm² flasks were transfected with various pHM6
constructs using DEAE dextran, according to standard protocol. After 40h, cells were washed using PBS before application of lysis buffer (20mM Tris (pH7.5), 1% Triton X-100, 150mM NaCl, 1mM EGTA, 1.5mM MgCl₂) plus reversible protease inhibitors (10μM EDTA, 10μM Leupeptin, 1μM pepstatin and 0.2mM AEBSF). Lysates were cleared by centrifugation and pre-cleared using 150μl Sepharose4B. HA-tagged molecules were then immunoprecipitated using 12μl anti-HA antibody bound covalently to matrix (Roche). Beads were then washed twice using lysis buffer/ 150mM NaCl, once using lysis buffer/ 500mM NaCl and then three times using reaction buffer (see earlier) before 50% were used in hydrolysis reactions. For inhibition of deubiquitinating activity, Cezanne was pre-incubated in the presence of 2mM ubiquitin aldehyde (Affiniti Research Products) for 30m at 37°C. Following hydrolysis, ubiquitin was revealed by western blotting using anti-ubiquitin antibody (1:1000; Zymed), horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma) and chemiluminescent detection (Pierce).

Assay of deubiquitination in cultured cells
HeLa cells cultured in 60mm dishes were co-transfected with 0.5μg pcDNA3.1-FLAG-ubiquitin plus 2.5μg of various pHM6-Cezanne constructs (or with empty pHM6) using Genejuice, following manufacturers recommendations (Novagen). A relatively low amount of FLAG-ubiquitin expression vector was used to ensure that proteins modified with FLAG-ubiquitin were derived from cells that also expressed the HA-tagged molecule. After 48h, cells were treated with 20μM MG132 for 1h (or remained untreated). Cell lysates were then analysed by western blotting using anti-FLAG (1:500; Sigma) or anti-HA (1:1000; Roche) primary antibodies, HRP-conjugated secondary antibodies and chemiluminescent detection.

Co-immunoprecipitation of ubiquitinated cellular proteins
Cos7 cells cultured in 75cm² flasks were transfected with a pHM6 expression vector containing a HA-tagged version of Cezanne (as indicated) or with empty pHM6 (vector), using DEAE dextran. After 24h, cells were treated with 20μM
MG132 for 1h (or remained untreated, as indicated) before addition of lysis buffer (see earlier) plus irreversible protease inhibitors (Roche). Lysates were cleared by centrifugation and pre-cleared twice using 50μl Sepharose4B. HA-tagged molecules were immunoprecipitated using 30μl anti-HA matrix (Roche). Beads were then washed four times using lysis buffer/150mM NaCl. The levels of immunoprecipitated Cezanne protein were revealed by silver staining following gel electrophoresis, according to the manufacturers recommendations (BioRad). Co-immunoprecipitating material was detected by western blotting using anti-ubiquitin antibody (Zymed), HRP-conjugated secondary antibody (Sigma) and chemiluminescent detection (Pierce).

RESULTS

Cezanne cleaves polyubiquitin gene products
Yeast two-hybrid screening of a murine whole-embryo cDNA library with full-length Cezanne showed that 29 clones out of 93 analysed contained polyubiquitin genes. Start-points of polyubiquitin cDNA varied between clones and were in-frame with the GAL4 activation domain (AD). Neither polyubiquitin nor Cezanne bound components of the GAL4 transcription factor, thus demonstrating that the interaction between Cezanne and polyubiquitin was genuine (data not shown).

Database searches have previously identified weak sequence similarity between the N-terminal region of Cezanne and molecules related to Drosophila ovarian tumour protein (OTU). OTU is the founder member of a super-family of over 100 proteins predicted to be cysteine proteases. They contain conserved cysteine and histidine residues in a putative catalytic site. For Cezanne, the residues are C209 and H373 (Fig. 8). A proteolytic function for OTU-like molecules has not been previously demonstrated.

Further experiments were performed to confirm that polyubiquitin was interacting specifically with Cezanne. Here, yeast YRG-2 were transfected with either: (i) pGBK7-Cezanne plus pGAD424-polyubiquitin or (ii) empty
pGBK7T plus pGAD424-polyubiquitin (Fig. 9), before plating onto minimal media (-Trp, -Leu, -His) and assessing expression of β-gal. Colonies producing β-gal were only generated when Cezanne was expressed, indicating that polyubiquitin interacted specifically with Cezanne residues and not with the GAL4 domain fusion partner.

(Note: the yeast two hybrid assays in our earlier US application serial No. 60/399,439 filed on 31 July 2002 used the yeast strain YRG-2 and not HF7c as described. Yeast strain HF7c can be used as an alternative to YRG-2 in yeast two hybrid assays.)

It is thought that the first methionine of human Cezanne is likely to correspond to residue 16 of the GenBank deposited sequence (AJ293573). This is based on multiple alignment of Cezanne sequences from several species, including mouse, pig and zebrafish (not shown). In the yeast 2-hybrid constructs (pGBK7T), Cezanne sequences were cloned down-stream of a GAL4 domain. The protein produced was a GAL4-Cezanne fusion. In several of these constructs, Cezanne sequences started at residue 17. This was chosen rather than residue 16 to avoid creating a second initiation point for translation.

(Note: the experiments depicted in Fig. 9 of our earlier US application serial No. 60/399,439 filed on 31 July 2002 used Cezanne 17-443 and Cezanne 17-443 (C209S) inserts, not Cezanne 1-443 and 1-443 (C209S) as shown.)

The yeast 2-hybrid system was used to investigate whether the putative active site (a catalytic cysteine) of the proposed protease domain (see Fig. 8) may effect the interaction with polyubiquitin which we observed in our initial screen. The ability of this region of Cezanne (residues 17-443) to interact with polyubiquitin was enhanced considerably by mutating cysteine 209 to serine (Fig. 10A, 10B) or to alanine (Fig. 10C). Surprisingly, colonies expressing β-gal were only generated when the mutant form of Cezanne 1-443 was expressed. The present inventors concluded that the catalytic domain of
Cezanne was cleaving polyubiquitin and therefore, undergoing a transient interaction with this molecule that was insufficient to activate the yeast 2-hybrid reporter genes. In contrast, the C209S mutant was behaving as a substrate-trapping mutant and undergoing prolonged interaction with polyubiquitin which was sufficient to activate the histidine and β-gal reporters. These yeast 2-hybrid experiments provided the first experimental evidence that the Cezanne protein has the ability to cleave polyubiquitin. Put another way, the result is consistent with the interpretation that the mutation prevented proteolytic activity and trapped the substrate, thus enhancing activation of yeast 2-hybrid reporter genes. Interestingly, the full-length version of wild-type Cezanne (residues 17-858) yielded a higher colony count when co-transformed with polyubiquitin than the putative protease domain alone (residues 17-443) (Fig. 10B).

A separate yeast 2-hybrid screening experiment was performed using the C-terminal half of Cezanne as bait (residues 444-858). A total of 90 clones out of 92 analysed contained polyubiquitin genes, which were in-frame with the GAL4 AD (data not shown). Analysis of the amino acid sequence of Cezanne 444-858 revealed several structural features (see Fig. 8) but sequence similarity to known ubiquitin-binding domains was not identified. We conclude, therefore, that Cezanne 444-858 contains a novel type of binding element for ubiquitin. Further experiments demonstrated that the C-terminal region generated relatively few colonies compared to the inactive protease domain (residues 17-443 Cys209Ser) suggesting that, in relative terms, it may have a lower affinity for ubiquitin (Fig. 10B).

The greater number of colonies seen with the full-length version compared to the N-terminal region in isolation (residues 17-443) could be explained by a synergistic relationship between binding sites in the C-terminal and N-terminal halves. Alternatively, the C-terminal region may inhibit proteolytic activity and this would have the effect of increasing colony number. When mutated versions of full-length and truncated sequences were compared, colony counts were closely similar (Fig. 10C). This suggests that inactivation of the catalytic
cysteine was sufficient to allow maximal binding to polyubiquitin and full activation of yeast 2-hybrid reporter genes.

Although UCH and UBP molecules have different substrate preferences, both types target the peptide bond at G76, which links ubiquitin units in tandem. Both have the ability to cleave short model substrates. Therefore, to assess directly whether Cezanne has proteolytic activity we tested its ability to cleave a fluorophore from a short peptide corresponding to the C-terminus of ubiquitin (Fig. 10D). Lysates from E.coli expressing a version of the putative protease domain (residues 126-455), cleaved the fluorophore AMC from Arg-Leu-Arg-Gly-Gly-AMC (SEQ ID NO: 10). A shorter version of the catalytic domain, Cezanne 182-455, had little or no activity. Cezanne 126-455 corresponds to the region that displays similarity to the TRAF-binding domain of A20, whereas Cezanne 182-455 corresponds to the predicted core-catalytic domain that displays structural similarity to papain (Fig. 8). Although it is plausible that residues 126-181 are critical for substrate binding or regulation of hydrolysis, it is equally possible that the conformation of the active-site of Cezanne 182-455 may have been compromised by aberrant folding. The lack of reactivity of endogenous E.coli proteins towards this substrate was not surprising because prokaryotes lack ubiquitin hydrolase genes.

Cezanne hydrolyses branched polyubiquitin chains
The potential ability of the papain-like fold of Cezanne to hydrolyse (cleave) branched polyubiquitin chains was examined (Fig. 11A). Hydrolysis experiments in vitro demonstrated that a GST-Cezanne 126-455 fusion protein cleaved ε-amino linked polyubiquitin chains into monomers (lanes 4, 7, 10). This activity was present in crude E.coli lysates containing Cezanne 126-455 (lane 4) and was elevated in purified material (lanes 7, 10). The deubiquitinating activity observed in this experiment must be attributed to Cezanne sequences because it was not observed using an irrelevant GST-fusion protein either purified (lanes 5, 8) or in the presence of endogenous E.coli proteins in the crude lysate (lane 2). The shortened version of the protease
domain (Cezanne 182-455) had little isopeptidase activity against branched polyubiquitin chains (lanes 3, 6, 9). Cezanne’s activity was suppressed by ubiquitin aldehyde, a highly specific inhibitor of deubiquitinating enzymes (Fig. 11B).

The C-terminal half of Cezanne regulates deubiquitination of branched polyubiquitin chains

Full-length Cezanne had the ability to undergo prolonged interaction with polyubiquitin, whereas the catalytic domain of Cezanne (residues 1-443), expressed in isolation, did not (compare Figs 9 and 10A). The present inventors conclude, therefore, that cleavage may be regulated in cis by elements present in the C-terminal half of the Cezanne molecule.

Additionally, hydrolysis experiments were performed to examine the influence of the C-terminal half of Cezanne on deubiquitination of branched polyubiquitin chains and to examine our prediction that C209 is a catalytic residue.

We compared full-length Cezanne and the isolated protease domain alone for the ability to cleave a synthetic substrate of branched polyubiquitin chains using HA-tagged versions of Cezanne immunoprecipitated from transfected Cos7 cells. The full-length molecule was considerably more effective than the protease domain alone (Fig. 12, compare lanes 1 and 3). The mutation C209S abolished activity in the full-length molecule confirming that C209, which is highly conserved between members of the OTU superfamily, is a catalytic residue (compare lanes 1 and 2). We observed very little activity using isolated protease domain immunoprecipitated from Cos7 cells compared with that seen using preparations purified from E.coli (Fig. 10). We attribute this difference to the much greater concentration of protease domain used in the latter experiments.

Cezanne deubiquitinates cellular proteins in cultured cells

To investigate the effect of Cezanne on ubiquitinated cellular proteins, we co-
transfected HeLa cells with expression vectors containing HA-tagged versions of Cezanne and FLAG-tagged ubiquitin (at a ratio of 5:1; Fig. 13A). After 48h, cells were either treated with a proteasome inhibitor (MG132) for 1h or remained untreated. Levels of ubiquitinated cellular proteins in experimental and control cultures were then compared by western blotting for FLAG.

Full-length Cezanne prevented the build up of ubiquitinated cellular proteins in response to MG132 whereas a catalytically inactive version had no effect (Fig. 13A, compare lanes 1 and 3 or lanes 13 and 15). This implies that Cezanne has the capacity to deubiquitinate at least part of the cellular pool of ubiquitinated proteins. Expression of the catalytic domain alone had only a marginal effect on ubiquitination levels (compare lanes 1 and 5). Thus, the C-terminal part of Cezanne is required for efficient hydrolysis of both free and conjugated forms of branched ubiquitin chains (Fig. 12 and 13).

We next examined the capacity of the mutated full-length molecule to bind to polyubiquitinated proteins in cultured cells (Figure 13B). Ubiquitinated cellular proteins could be co-immunoprecipitated with a catalytically inactive version of full-length Cezanne (lane 6) but not with the wild-type molecule (lane 5). It is likely that the catalytically inactive version of Cezanne functions as a substrate-trapping mutant whereas the active version would not be expected to retain polyubiquitin after hydrolysis.

**Cezanne is a novel type of deubiquitinating enzyme**

Database searching and sequence analysis was carried out to examine the relationship between members of the A20 group, the OTU-superfamily, Type I and Type II deubiquitinating enzymes and other cysteine proteases (Fig. 14). Multiple sequence alignment of the putative protease regions of the A20 family, however, revealed that Cezanne shares 39% and 25% sequence identity with A20 and TRABID respectively. The similarity is particularly evident in the areas containing putative catalytic residues (Cys and His regions; Fig. 13). Makarova, K.S. *et al.* (2000, Trends Biochem. Sci. 25: 50-52) suggest that the
catalytic site of OTU proteins may comprise a triad of conserved Cys, His and Asp residues (209, 373 and 206; Cezanne numbering). It is notable that TRABID does not contain the conserved Asp. The Cys and His regions of the A20-family display a significant level of sequence identity with OTU-like molecules but not to the catalytic regions of Type I or Type II deubiquitinating enzymes, apart from conservation of active-site cysteine and histidine residues. We conclude that Cezanne is a novel type of deubiquitinating enzyme.

DISCUSSION

We have demonstrated that Cezanne has deubiquitinating activity against both linear and branched forms of polyubiquitin. We compared the activity of Cezanne to isopeptidase T, a well characterised UBP molecule that is known to hydrolyse the branched form of polyubiquitin chains in preference to linear forms (Wilkinson, K.D. et al.; 1995, supra). In the present Example, we have noted from several experiments that equimolar quantities of Cezanne and Isopeptidase T hydrolysed free, branched polyubiquitin chains at similar rates. The activities of both molecules were suppressed by ubiquitin aldehyde, a highly specific inhibitor of deubiquitinating enzymes.

A catalytic domain of Cezanne is present in the N-terminal half. We provide evidence for an additional ubiquitin binding site in the C-terminal part of the molecule. This region contains a single zinc finger at the extreme C-terminus but is devoid of other known structures, including consensus sequences for known ubiquitin-interacting motifs. Full-length Cezanne hydrolysed free and conjugated forms of branched polyubiquitin chains more efficiently than the catalytic domain alone. These observations are consistent with the idea that the C-terminal ubiquitin binding element may contribute to the overall affinity of Cezanne for ubiquitin. Alternatively, this motif may be required for optimal orientation of polyubiquitin chains with the catalytic site. Other proteases are known to contain substrate-binding sites outside the catalytic region, otherwise known as exosites. Interestingly, prothrombinase contains an exosite that is responsible for the initial interaction of this protease with its substrate. The
anchored substrate is then cleaved at two spatially separated sites. By analogy, it is conceivable that binding of the C-terminus of Cezanne to a polyubiquitin chain may enable cleavage of multiple ubiquitin units in processive fashion.

Our experiments demonstrate that Cezanne can cleave branched polyubiquitin chains from a substantial proportion of cellular proteins. These studies have relied on overexpression of Cezanne in cell lines and it should be noted that is possible that in such circumstances substrate specificity will be broadened well beyond the normal physiological range. The domain composition among deubiquitinating enzymes is diverse and it has been proposed that regions outside the catalytic domain may target specific cellular proteins for deubiquitination. This concept has been established for several UBP molecules including HAUSP, which targets the apoptosis regulator p53. Substrate specificity remains poorly defined, however, for the great majority of ubiquitin hydrolases.

Our previous study revealed that transcripts for Cezanne are expressed widely and are particularly abundant in foetal liver and kidney (see Example 1 and Evans et al., 2001, supra). We have also recently found that Cezanne is expressed differentially among B-cells at distinct developmental stages (data not shown). Primary immature B-cells (IgM+, IgD-) contained 100-fold more Cezanne mRNA than their mature counterparts (IgM-, IgD+) as assessed by real-time PCR using β-actin transcripts as a standard (data not shown). It is possible that the capacity of Cezanne to suppress NF-κB, a powerful survival factor, may influence the fate of immature B-cells.

The amino acid sequence of the protease domain of Cezanne is similar to corresponding regions found in A20 and TRABID (see Example 1 and Evans et al., 2001, supra). In addition, we have recently discovered a close homologue of Cezanne which we have named Cezanne-2 (GenBank accession NM_130901; SEQ ID NO: 3). The catalytic cysteine and histidine residues of Cezanne are conserved throughout the group. Our discovery of enzymatic activity for
Cezanne indicates that proteolysis may be a common function throughout this family.

This study provides the first demonstration of proteolytic activity in the OTU super-family. Cezanne and related enzymes should be classified as founder members of a type III or OTU-type family of deubiquitinating enzymes.

**Example 4: Regulation of cell death by TRABID**

TRABID contains a papain-like fold located over the C-terminal half of the molecule and three zinc finger motifs that resemble those found in Ran binding proteins (RanBP; Fig. 7). Over-expression studies using cultured cells have demonstrated that TRABID protects cells from TNFα/ cycloheximide-induced cell death (Fig. 15). In these experiments, TRABID cDNA was introduced into pEGFP (Clontech), in-frame with green fluorescent protein (GFP). HEK293 cells were transfected with pEGFP-TRABID or pEGFP alone using lipofectamine (Gibco BRL). Cells expressing GFP-TRABID or GFP alone were exposed to TNFα/ cycloheximide before the proportion of necrotic cells (propidium iodide-positive) was calculated for cells gated on GFP and for untransfected cells (no GFP). The present inventors conclude that TRABID has the capacity to prevent cell death after initiation of the TNF receptor signalling pathway.

**Example 5: Characterisation of A20 as a ubiquitin hydrolase**

**INTRODUCTION**

In this example, we demonstrate that the TRAF-binding region of A20 has deubiquitinating activity.

**METHODS**

Mammalian cells

Cos7 and HeLa cells were cultured as described in Example 3 above.
Insect cell expression vectors

pDEST10 (Invitrogen) was used for expression of polyHis-Cezanne fusion proteins in insect cells. PCR primers for amplification of full-length Cezanne cDNA incorporated nucleotides required for topoisomerase cloning. Wild-type and inactive forms of Cezanne were amplified using pHM6-Cezanne and pHM6-CezanneC209S as templates, respectively (Evans et al., 2003). Products were cloned into pENTR/D-TOPO and then into pDEST10, using GATEWAY systems (Invitrogen). Bacmids were prepared by transforming DH10Bac E. coli (Invitrogen) with recombinant pDEST10. Manipulations were checked by PCR and DNA sequencing.

Mammalian expression vectors

pHM6 was used to express haemagglutinin epitope (HA)-tagged molecules in mammalian cells. pHM6-Cezanne and pHM6-A20 are described in Example 1 and Evans et al. (2001, supra). Expression of FLAG-tagged ubiquitin was achieved using pcDNA3.1-FLAG-ubiquitin. Expression vectors for FLAG-tagged versions of A20 comprised pCAGGS-FLAGmA20.1-366, pCAGGS-FLAGmA20.1-468 and pCAGGS-FLAGmA20.1-775 (full-length), which were generously supplied by Prof. R. Beyaert (University of Gent- VIB, Ghent, Belgium). Quickchange technology (Stratagene) was used to introduce a point mutation at codon 209 (TGC (Cys) to AGC (Ser)) in pHM6-A20 or pCAGGS-mA20.1-468 using complementary primers. Constructs were checked by DNA sequencing.

Production of His-tagged Cezanne proteins in insect cells

Sf9 insect cell monolayers were transfected with 2µg/ml bacmid DNA following the manufacturer’s recommendations (Invitrogen). Recombinant baculovirus underwent 4 rounds of amplification. Cultures containing 10^8 cells were infected with virus at a MOI of 5. After 3 days, cells were harvested and lysed. His-tagged proteins were purified using nickel-NTA-agarose beads (15µl of beads/ml of extract; Qiagen) according to the manufacturer’s
recommendations.

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

Cultures of Cos7 cells in 60mm dishes were transfected with various pCAGGS constructs using DEAE dextran, according to standard protocol. After 48h, cells were washed using PBS before application of 200µl lysis buffer (50mM Tris (pH7.6), 0.2% NP40, 150mM NaCl, 0.5mM EDTA) plus 0.5mM AEBSF, a reversible protease inhibitor. Lysates were cleared by centrifugation. Probe binding assays required the use of 45µl lysate. FLAG-tagged A20 was purified from the remaining lysate using 10µl 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 reaction buffer (see Example 3 above). A third of the beads was used in hydrolysis reactions or probe binding assays.

**In vitro isopeptidase assays**

These were performed as described in Example 3 above.

**Probe binding assay**

Synthesis and purification of the ubiquitin-derived probes used in this study (HAUbVME and HAUbBr2) have been described (Borodovsky, A. *et al.*, 2002, Chem. Biol. 9: 1149-159). They are tagged with HA epitope to facilitate detection. Probe (0.2µg) was applied either to cell lysates or purified material in the presence of 3mM DTT and 0.01% Brij. Reactions were incubated at 37°C for 1-3h. Probe sequences were detected by western blotting using anti-HA epitope antibodies, as described in Example 3 above.

**Assay of deubiquitination in cultured cells**

This was performed as described in Example 3 above. Note that empty pHM6 vector was used to standardize the total amounts of vector used for transfection.
RESULTS

Covalent modification of Cezanne using thiol-reactive ubiquitin-derived probes

The ubiquitin-derived probes used in this study contain thiol-reactive groups at the C-terminus, which promote covalent binding to the catalytic cysteine of ubiquitin hydrolases. They are tagged with HA epitope to facilitate detection.

In an attempt to identify reagents that will recognize A20-family ubiquitin hydrolases, we examined the potential ability of two ubiquitin-derived probes to modify Cezanne (Fig. 16). These probes contained vinylmethyl ester (HAUbVME) and bromoethylamine (HAUbBr2) reactive groups, respectively. We have previously identified Cezanne residue 209 as a catalytic cysteine (see Example 3 above).

We expressed active and catalytically inactive (C209S) forms of full-length Cezanne in insect cells. The expected size of full-length Cezanne is approximately 100kD. Analysis of the purified active form, however, revealed fragments at 50kD and 60kD (Fig. 16A). These species were not present in the preparation of catalytically inactive Cezanne and are, therefore, likely to be products of self-cleavage. (We have previously observed self-cleavage of Cezanne when it is produced in high quantities in mammalian cells.) As expected, wild-type Cezanne cleaved branched polyubiquitin chains effectively whereas the mutant form was inactive (Fig. 16B).

Both probes bound covalently to Cezanne (Fig. 16C), as indicated by the substantially altered migration of probe sequences during SDS-PAGE. The size of the probe is approximately 10kD. The Cezanne fragments identified at 50kD and 60kD by silver staining migrated at 60kD and 70kD, respectively, following attachment of probe. Reactivity was not observed with the inactive form, confirming the involvement of the catalytic cysteine. We conclude that HAUbVME and HAUbBr2 are useful reagents for identification of A20-family ubiquitin hydrolases.
A20 hydrolyses branched polyubiquitin chains and binds covalently to a ubiquitin-derived probe

To examine whether A20 has enzymatic activity towards ubiquitin we tested two properties: (i) the ability to bind covalently to a ubiquitin-derived probe (HAUbBr2) and (ii) hydrolysis of a synthetic substrate of branched polyubiquitin chains (Figs 17 and 18).

In the first instance, we assessed the reactivity of HAUbBr2 towards two FLAG-tagged versions of A20 produced in transfected Cos7 cells (Fig. 17). We tested A20 1-366 which contains the putative protease domain alone and A20 1-468 which also includes a single zinc finger (see Fig. 1). Incubation of probe with lysate from untransfected cells revealed several bands that probably derive from endogenous ubiquitin hydrolases (Fig. 17, lane 2). Probing of lysate from either culture of transfected cells revealed an additional band corresponding to ubiquitin-modified A20 (Fig. 17, lanes 3 and 4). A20 1-366 and A20 1-468 migrated at 50kD and 60kD, respectively, following attachment of probe.

A series of experiments employed FLAG-tagged versions of A20 purified by immunoprecipitation from transfected Cos7 cells (Fig. 18). Assessment of enzymatic function in vitro demonstrated that truncated forms of A20, containing the catalytic domain, were highly effective in cleaving branched polyubiquitin chains into monomers (centre panel, lanes 4 and 5). In addition, the catalytic region of A20 bound covalently to HAUbBr2 (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 deubiquitinating activity of A20 was suppressed completely by ubiquitin aldehyde, a highly specific inhibitor of deubiquitinating enzymes (data not shown).

The mutation C103S abolished enzymatic activity against both polyubiquitin
substrate and ubiquitin probe (compare Fig. 18, lanes 7, 8 and 9). This confirms that C103, which is highly conserved between members of the OTU superfamily, is a catalytic residue.

We observed that full-length A20 possessed ubiquitin hydrolase activity (lane 3) but 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).

**A20 does not have a generalized deubiquitinating function in cultured cells**

We compared the effects of A20 and Cezanne on ubiquitinated cellular proteins in cultured cells (Fig. 19). Full-length Cezanne prevented the build up of ubiquitinated cellular proteins in response to MG132 as observed previously (compare lanes 1 and 3, lanes 4 and 5). Over-expression 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).

**DISCUSSION**

The OTU superfamily contains more than 100 molecules that are predicted to function as cysteine proteases (Makarova *et al.*, 2000, *supra*). This group now includes the A20 zinc finger protein and its three homologues, namely Cezanne, TRABID and Cezanne-2. We and subsequently others have shown that hydrolysis of branched polyubiquitin chains is a function conserved among several human OTU proteins (see above and Borodovsky *et al.*, 2002, *supra*). In this Example, we have demonstrated that the A20 zinc finger protein also has deubiquitinating activity.

Yeast 2-hybrid experiments have previously revealed that TRAF-molecules bind directly to the N-terminal half of A20. The interaction has not been mapped at the level of individual residues, however. This region of A20 contains the OTU domain. It has structural similarity to papain-like cysteine
proteases and includes a deep cleft containing catalytic residues. The active-site targets polyubiquitin chains for cleavage and it is unlikely to recognize TRAF sequences. It is conceivable, however, that it may contribute to the binding of ubiquitinated forms of TRAF molecules. The precise spatial relationship between the TRAF binding motif and the active-site remains to be established.

Our experiments demonstrate that A20 does not effect the overall level of ubiquitin-conjugated cellular proteins. It is possible that A20 can cleave free polyubiquitin chains but does not have the capacity to remove ubiquitin from cellular proteins.

Alternatively, it is conceivable that A20 may deubiquitinate a restricted subset of cellular protein(s). It has been proposed that regions outside the catalytic domain may regulate the specificity of deubiquitinating enzymes.

A20 is recruited to the TNFR1 complex following engagement with TNFα. This association is probably co-ordinated by dual interactions between A20 zinc fingers and IKK's and between A20 and TRAF molecules. A number of proteins within this complex are known to be ubiquitinated including TRAF2, RIP and TNFR1. It is possible that A20 may modulate the activity of one or more of these molecules through deubiquitination. Thus ubiquitin hydrolase activity by A20 could play an important role in NF-κB regulation.

It is also conceivable that the stability of A20 itself could be regulated by self-association leading to self-deubiquitination. It has been suggested that A20 protein levels are reduced following phosphorylation by IKKβ, presumably through the ubiquitin/proteasome pathway. Deubiquitination of self could constitute a mechanism to oppose this.

All references cited herein are incorporated in their entirety.
Sequences of SEQ ID NOs 1-9:

SEQ ID NO: 1 – Human Cezanne amino acid residues 160-416.

SEQ ID NO: 2 – Human Cezanne-2 cDNA (GenBank Accession No. AJ430383).

gtcttcctgc tcaaggtcct gctagtgatt gtcactttgc gtcaccatcc cacaaagttg 60
taaagcgcct tcttctctct tctccgcaaat ggaggtgttct caagttgtct ccaacccc 120
acctcgggct actgttgtgg ccagcacttc catgactctac tgcatccct 180

gtcctgtaac acttttctgc gtcaccacgg gcaagaactcg gtcctggcag agacctgtg 240
gacggaactaa actgggacct gacagccgct ctccagcgact atgacagct aacccgaggt 300
ccacacacac aacgacaccc tcggccagt cacagcatacgt ctccactgccc 480

gcgtccacag tgcagctgtg actcgactac gagccgttct cccctgctctg ccaactctac 540
acatttaccct ggcagctgact aatggtgggtct tggccagggc cccctgctgca ccaactctac 600
gacttcatctg agcgacagcct cagctgactc atgatcagact ccgctggtcc ccgctggtcc 660
tccactgactg ccaggtcagc ccaacccgct acaggctgct gcacacgct gacacagctt 720
tgcctctttac atgctgtgcaacttggaagatg tgggttccttc aagccgaggg ctggtgtgt 780
gcagagctgg tttttacagc atagcagccttg gaggcagcagc ggcagggcag 840
tagtccgtgca tggcaggtgaa ccaacagccg ccaggtgtgc ccgctggtcc ccgctggtcc 900
tcgtctcgcc cagcgtggtgc ccagtctgact gcagccgctg ccaacagccg ccgctggtcc 960
gggtgcaggc caacctctgca gacagcgccg ccaacagccg ccgctggtcc ccgctggtcc 1020
tgctctcaggc atatattaga aacggcactag gggttggtgg ccgctggtcc ccgctggtcc 1080
tcaggtgagaag ccccttccttg tccagtcccaattcggaagggg tctcttggcttccggtggtg 1140
tccctcccttg ggacgctcttg ccggctggcccc tgcacctggcttg gcagttgactgctg 1200
gccctggtgtg ctcctgggaat cccggtggaat cccggtggaat 1260
gggttggtgg ccagtctgact gcagccgctg ccaacagccg ccgctggtcc ccgctggtcc 1320
tgctctcaggc atatattaga aacggcactag gggttggtgg ccgctggtcc ccgctggtcc 1380
tcaggtgagaag ccccttccttg tccagtcccaattcggaagggg tctcttggcttccggtggtg 1440
tcggacgccc tggcagcggc gggcgcgcgg cccggtggaat cccggtggaat 1500
tcggacgccc cccggtggaat cccggtggaat 1560
tcggacgccc cccggtggaat cccggtggaat 1620
tcggacgccc cccggtggaat cccggtggaat 1680

aaaacactagg gcgggtccttg gcggctgtgg ccggccagaa ccaactctgcaagc ccaactctgcaagc 1740
aattgcaaga gcgggaactgc gggcagcggc gggcagcggc gggcagcggc gggcagcggc 1800
SEQ_ID_NO: 3 – Human Cezanne-2 protein (Genbank Accession No. CAD23047; see also AJ430383), encoded by nucleotides 94 to 2871 of SEQ ID NO: 2.

MVSSVLVPNPTSAECAWALLHDPMTLDMDAVALSLDFVIRSTGAEPL
ARDLLEKGNWLDTALASDYQLQVRQHINTLPHVFNKGRPGPKPEREPQGPKVERPCL
QRQDDIAEQKEKLRSRGHASSAIVSLARSHVASECNQFPLPMEIYTFQPLDLSVYS
EDFRRPSKNGTGGVDNSEDPAVESLEEFHVFLAHIRLRPRPVVADTMRLDSGEEA
FWIFHDLRLVLRKALYTMRTGAESEALKRRWRRWQQTQONKEEBWIREBETELKLASS
EPRTHSKXIIMPYGIYQSHGKVYDGKQDKNGLLPLDKAPQLLPLGTVVDLQGAMQ
FAPIFPGGILYVSLEVPNPNNPCHCSPLVLAYDOHFLSASVSMBQDQQRESAQVITPLTDS
EKLPLLIFHVDPGVKDVNGKDNLDNARLHIHLLSHLAVMIWTVIRSETR
APLAPFPSTASPAGEDVQLSMDLSLRSDLDSRDSVSNSNSNSNGKNGKDEKKQKEKD
RASDVANKLQGPSFSTLGKLNQMGGLGLVHKMGRANSANGKNGDSAESKEEKKA
SRKGSKEBGEASASTSPKTTTPDLSAQAGASPAEKGGGPRGDQTYSTDVKLSLNI
LRAAMQGERKPIFAGLIIULSTSRHQQHEMIGYFLATSQERFSASBEQRRDARATAAA
AAAATATARKPARRPETEGVPVERASPGPGPPTQLVKLKERRPSPGPAAGRAAARAAG
GTASPPGARRASASQVPGGRSPPPAPARQSVIHVQASARDCEAPAVGALRFCPCTYP
QONRSLSSQSYSPARAAALRVTNTVSLARAVPGALPGAGTACAGAAEHKSQTYYNGFG
SEQ ID NO: 4 – Human Cezanne amino acid residues 126-455.

ALRDGLEFADADAPTARSGNGCGRGGPVGQRQCQRENCAFAYGRAETEHYCSYCREELRREARGARP

SEQ ID NO: 5 – Human Cezanne amino acid residues 444-858.

MNVKWIPPLSS DAQAPLAPQPS SPTASAGDEP RSTPEGSDSD KSSVGSSSTS
NEGGRRKEKS KRDERKDQKR ADSVANLGS FGKTLGSLKLK KNMGGLMHSK
GSPGPGVGTG LGSSGSTETL EKKKNSLKS WKGKKEEAAG DGPVSKPPA
EVGNGGSKY SQEVMQSLSI LRTAMQGEGK PIFVGLKMKG HRHQYQEEMI
QYRLSDAER PLAEIQKKEA ERKIMGIG GGPPPAKKE PDAREQOTPQG
PPAESRAMAF STGYPSGDFTI PRPSGGGHEC QEPFRQLAGG PCVGLPYPYA
TFPRQCPPGR PYPHQSIPS LEPGSHSKDG LRHGAELPPPP YRVADESYNG
YRREPPEPDGW AGGLRGLLPT QTKCKQPNC5 FYGHPPETNRP CSCCYYREBLR
RRREPQGEL LVHRF

SEQ ID NO: 6 – Human Cezanne amino acid residues 182-455.

AGRLLMNWWSV DPTQRRLLPL ATGDDNCM LL HAASLGMWGF HRRDLMLRKA
LYALEMKEV5 KEALKRRRNRN QTQQNKESG LVYTEDWQK EWNELIKLAS
SEPRHMTGNN GANCQGEVSS EPPVYESLSE FHVFVLAVHL RRPQVVADT
MLRDSGGBAP APIPPGIGYL PL8VPSASCH RSPLVLAYDQ AHPSALVSME
QKENTKEQAV IPLITDSEYKL LPLHFADVDP KGWEWGGDDS

SEQ ID NO: 7 – Human Cezanne amino acid residues 200-220.

PLATTGDNC LLHAASLGMW G

SEQ ID NO: 8 – Human Cezanne amino acid residues 365-379.

LVLAYDQAHF SALVS
SEQ ID NO: 9 – Human Cezanne amino acid residues 365-406.
LVLAYDQAHF SALVSMEQKE NTKRQAVIPL TDSEYKLLPL HF
Claims

1. A method for enzymatic deubiquitination of a molecule which has been ubiquitinated to form a ubiquitinated molecule, comprising the step of contacting the ubiquitinated molecule with an enzyme, wherein the enzyme or a domain thereof has at least 20% amino acid sequence identity with: (1) the TRAFB domain defined by Cezanne amino acid residues 160-416 (SEQ ID NO: 1); and/or (2) the A20 homology domain defined by Cezanne amino acid residues 126-455 (SEQ ID NO: 4); and/or (3) the Cezanne core catalytic domain defined by Cezanne amino acid residues 182-455 (SEQ ID NO: 6).

2. The method according to claim 1, wherein the enzyme or a domain thereof has at least 80% sequence identity with the TRAFB domain defined by SEQ ID NO: 1.

3. The method according to claim 2, wherein the enzyme is Cezanne (Genbank Accession No. AJ293573) or Cezanne-2 (Genbank Accession No. AJ430383; SEQ ID NO: 3).

4. The method according to claim 3, wherein the enzyme is or comprises a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) of Cezanne or Cezanne-2.

5. The method according to claim 1, wherein the enzyme or a domain thereof has at least 35% sequence identity with the TRAFB domain defined by SEQ ID NO: 1.

6. The method according to claim 5, wherein the enzyme is A20 (Genbank Accession No. M59465).

7. The method according to claim 6, wherein the enzyme is or comprises a functional fragment, homologue, variant or mutant (for example, a
conservatively substituted mutant) of A20.

8. The method according to claim 1, wherein the enzyme or a domain thereof has at least 30% sequence identity with the TRAFB domain defined by SEQ ID NO: 1.

9. The method according to claim 8, wherein the enzyme is TRABID (Genbank Accession No. AJ252060).

10. The method according to claim 9, wherein the enzyme is or comprises a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) of TRABID.

11. A method for enzymatic deubiquitination of a molecule which has been ubiquitinated to form a ubiquitinated molecule, comprising the step of contacting the ubiquitinated molecule with an enzyme, wherein the enzyme or a domain thereof comprises a Cys box domain defined by Cezanne amino acid residues 200-220 (SEQ ID NO: 7) or a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) of the Cys box domain.

12. A method for enzymatic deubiquitination of a molecule which has been ubiquitinated to form a ubiquitinated molecule, comprising the step of contacting the ubiquitinated molecule with an enzyme, wherein the enzyme or a domain thereof comprises a His box domain defined by Cezanne amino acid residues 365-379 (SEQ ID NO: 8) or a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) of the His box domain.

13. The method according to any preceding claim, wherein the enzyme or a domain thereof is used as a fusion partner.
14. The method according to claim 13, wherein the enzyme or a domain thereof is fused to a binding site for a target molecule.

15. The method according to any preceding claim, wherein the molecule or target molecule is a regulator of a cellular process.

16. The method according claim 15, wherein the molecule or target molecule is a regulator of the inflammatory process.

17. The method according to claim 16, wherein the inflammatory process is regulated by NF-κB.

18. The method according to claim 15, wherein the molecule or target molecule is a regulator of apoptosis.

19. The method according to any preceding claim, for use in stabilising the molecule or target molecule.

20. The method according to any of claims 1 to 18, for use in targeting or maintaining the molecule or target molecule to or at a subcellular location.

21. The method according to any of claims 1 to 18, for use in modulating the activity of the molecule or target molecule.

22. A method for regulating the method of any of claims 1 to 21, comprising the step of contacting

   (i) a molecule which has been ubiquitinated or

   (ii) a deubiquitination enzyme

   with a regulatory polypeptide having at least 20% amino acid sequence identity with the C-terminal domain of Cezanne defined by Cezanne amino acid residues 444-858 (SEQ ID NO: 5) or functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) thereof.
23. A method for modulating the activity of a molecule which is capable of being ubiquitinated to form a ubiquitinated molecule, comprising the step of contacting the molecule or the ubiquitinated molecule with a polypeptide having at least 20% amino acid sequence identity with: (1) the TRAFB domain defined by Cezanne amino acid residues 160-416 (SEQ ID NO: 1); and/or (2) the A20 homology domain defined by Cezanne amino acid residues 126-455 (SEQ ID NO: 4); and/or (3) the Cezanne core catalytic domain defined by Cezanne amino acid residues 182-455 (SEQ ID NO: 6), wherein the polypeptide has deubiquitination activity.

24. A method for modulating the activity of a molecule which is capable of being ubiquitinated to form a ubiquitinated molecule, comprising the step of contacting the molecule or the ubiquitinated molecule with a polypeptide comprising a Cys box domain defined by Cezanne amino acid residues 200-220 (SEQ ID NO: 7) or a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) of the Cys box domain, wherein the polypeptide has deubiquitination activity.

25. A method for modulating the activity of a molecule which is capable of being ubiquitinated to form a ubiquitinated molecule, comprising the step of contacting the molecule or the ubiquitinated molecule with a polypeptide comprising a His box domain defined by Cezanne amino acid residues 365-379 (SEQ ID NO: 8) or a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) of the His box domain, wherein the polypeptide has deubiquitination activity.

26. A protein having an amino acid sequence of Cezanne-2 (SEQ ID NO: 3), or a functional fragment, homologue, variant or mutant (for example, a conservatively substituted mutant) thereof.

27. An isolated nucleotide encoding the protein of claim 26.
28. The isolated nucleotide according to claim 27, having the sequence of SEQ ID NO: 2.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 7
Cezanne

Zn finger

816 838

NLS

407 513

Cys

455

His

443

A20 homology

Core catalytic domain

182

209

373

455

Fig. 8
Fig. 11B
Fig. 12
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<th>Family</th>
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<th>HIS BOX</th>
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<td>(434) ALWRTNGQLGDSVLOATWG (NO: 58)</td>
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<td>cezanne (NP_064590)</td>
<td>(200) FLATTGCNCGLLHAASLGMWG (NO: 7)</td>
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<td></td>
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<td>(201) FLATTGCNCGLLHAASLGMWG (NO: 59)</td>
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<td>(149) IKQIPQGCHQYKTDQDLKES (NO: 63)</td>
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<td>HSFC263 (NP_060140)</td>
<td>(82) IRKTRPQGCGCPRFGFSHLE (NO: 64)</td>
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| UCH        | UCH-L3 (P15374)              | (96) SMKCDNGGCGCIGLQHLLA (NO: 72) | (162) IDEKVDDHSLALVHDLHESGRKPRFPHNHP (NO: 76) |
|            | Uch37 (AAD31534)             | (79) RAEVVBNNQCATLQVSTLV (NO: 73) | (157) AKEBDFSHYSVYPCHFCPEGYLDGLREGTDLG (NO: 77) |
|            | bap1 (AAC15970)              | (82) CAROLIINSQCATLQVSTLV (NO: 74) | (162) VRTMEAVHSVYVPTTCTPFLQCLKVYIDHG (NO: 78) |
|            | uch-11 (P09936)              | (81) SMKCDNGGCGCIGLQHLLA (NO: 75) | (154) VDDKVNPHELFLNNVDQGPRFPGFVPNH (NO: 79) |

| UBP        | TRE2 (P35125)                | (216) HSHASTNLIGNNSIQC (NO: 80) | (995) HNYFAISCHE-DLGFHVTYAKKP-NCKYCV (NO: 84) |
|            | ISO. T (AAA78934)            | (327) TIRALNGCGLYNGVUVNL (NO: 81) | (.778) FOSFAIPEMTTMCSVVCYHIEIK-EGRGVIY (NO: 85) |
|            | UBP-Y (P40818)               | (778) GFRNLINCGLYNGVUVNL (NO: 82) | (1051) KNFGRGIVG-DLGGYTA/VCKNAQQR-FK (NO: 86) |
|            | HAUSP (CAA96580)             | (215) ELKKQPGCNGLQHYLTH (NO: 83) | (448) HILLAVLWHRES-NHGSFYPVFKLMPKGDGKCK (NO: 87) |

Fig. 14
Fig. 15
Fig. 17
Fig. 18
Fig. 19