RESEARCH COMMUNICATION

Isolation and characterization of two novel A20-like proteins

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The transcription factor nuclear factor κB (NF-κB) plays a pivotal role in inflammatory processes through induction of adhesion molecules and chemokines. The zinc finger molecule A20 is an important negative regulator of NF-κB. The mechanism utilized by A20 is not fully understood, but A20 has been shown to bind to tumour-necrosis-factor-receptor-associated factor (TRAF) molecules, which are necessary for pro-inflammatory cytokine signalling. We report two novel genes, *Cezanne* (cellular zinc finger anti-NF-kB) and *TRABID* (TRAF-binding domain), with sequence similarity to A20. Co-immunoprecipitation studies indicated that TRAF6 was able to interact with both Cezanne and TRABID. In contrast, reporter gene experiments revealed a specific ability of Cezanne to down-regulate NF-κB. It is likely, therefore, that Cezanne participates in the regulation of inflammatory processes.

Key words: inflammation, nuclear factor κB, TRAF molecules.

INTRODUCTION

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 nuclear factor κB (NF-κB). This transcription factor elevates the production of several adhesion molecules and chemokines which control docking and transendothelial migration of leucocytes.

NF-κB is sequestered in the cytoplasm of unstimulated cells through binding to inhibitory κB (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 (reviewed in [1]). Engagement of the TNF receptor 1 (TNFR1/p55) leads to recruitment of several signal adaptor molecules, including receptor interacting protein and TNF-receptor-associated factor 2 (TRAF2). It has recently been shown [2] that IkB 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 receptor interacting protein. Localization 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 [3]. 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 [4]. Conversely, a strong association exists between endothelial expression of A20 and long-term survival of transplanted organs [5]. Studies in cultured cells have demonstrated that NF-κB activity is down-regulated by overexpression of A20. Furthermore, overexpression of the C-terminal portion of A20, which contains seven zinc fingers, was sufficient for this activity [6]. The mechanism utilized by A20 has not been fully elucidated. It has been shown recently [2], 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 IkB kinase function. The N-terminal region of A20 (residues 1–386) binds to TRAF signal adaptor molecules [6], which are critical components of TNFα and IL-1 signalling pathways. The function of A20 (1–386) is unclear, but it is possible that TRAF binding is important for recruitment of endogenous A20 to TNFR and IL-1 receptor 1 signalling complexes.

We report the isolation of two novel genes with sequence similarity to A20 and assess their ability to influence NF-κB.

MATERIALS AND METHODS

Cell culture

Human epithelial cells (HEK-293) and mouse fibroblasts (NIH3T3) were cultured using Dulbecco's modified Eagle's medium/10% fetal-calf serum, supplemented with antibiotics. An endothelial cell line (EaHy), supplied by M. Rose (Harefield Hospital, Harefield, Middx., U.K.), was cultured using Dulbecco's modified Eagle's medium/20% fetal-calf serum, supplemented with hypoxanthine/aminopterin/thymidine (Gibco BRL) and antibiotics.

Isolation of cDNA and cloning into expression vectors

Sequence information for full-length cDNA was generated using the SMART rapid amplification of cDNA ends (RACE) cDNA Amplification Kit (ClonTech) according to the manufacturer's instructions. This information was used to design primers for

Abbreviations used: NF-κB, nuclear factor κB; IκB, inhibitory κB; TNF, tumour necrosis factor; TRAF, TNF-receptor-associated factor; TNFR1, TNF receptor 1; IL, interleukin; RACE, rapid amplification of cDNA ends; HRP, horseradish peroxidase; HA, haemagglutinin; GFP, green fluorescent protein; RT, reverse transciption; FLAG epitope, DYKDDDDK; Cezanne, cellular zinc finger anti-NF-kB; TRABID, TRAF-binding domain.

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The nucleotide sequence data reported have been deposited in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AJ293573 and AJ252060.

amplification of full-length cDNA. *Pfu* DNA polymerase (Stratagene) was used to amplify full-length TRABID (TRAF-binding domain) (sense, 5′-ATGTCAGAACGTGGAATTAA-GTGG-3′; antisense, 5′-TCATTCATCTTCATCATCCTCAT-CTTC-3′) and Cezanne (cellular zinc finger anti-NF-kB) (sense, 5′-ATGTTCTACCATACATCCAGCTGGC-3′; antisense, 5′-TCAGAACCTGTGCACCAGGAGCA-3′).

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 immediate-early gene promoter. Full-length Cezanne was cloned into pHM6. An N-terminal region of Cezanne encompassing the putative TRAF-binding domain (TRABID) (residues 1–443) was generated using sense (5′-ATGTTCTACC-ATACATCCAGCTGGC-3′) and antisense (5′-GTAGCTAT-GCAGCAGATGC-3′) primers and cloned into pHM6, creating pHM6-Cez1–443. A C-terminal region of Cezanne encompassing the putative nuclear localization sequence and zinc finger (residues 444–858) was also generated using sense (5′-AGATG-AATGTGAAGTGGATCCC-3′) and antisense (5′-TCAGAA-CCTGTGCACCAGGAGC-3′) primers, and cloned to create pHM6-Cez444–858. Full-length TRABID was also cloned into pHM6.

pEGFP-C3 (ClonTech) utilized the cytomegalovirus promoter to drive expression of molecules tagged at the N-terminus with green fluorescent protein (GFP). cDNAs encoding full-length Cezanne and residues 444–858 were cloned into pEGFP. cDNAs encoding full-length TRABID and residues 1–345 were also cloned. Primers for TRABID 1–345 comprised sense (5′-ATGTCAGAACGTGGAATTAAGTGG-3′) and antisense (5′-TGCTGGAATACACTTTGC-3′). 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 DYKDDDDK (FLAG)-tagged TRAF6 has been described previously [7].

Reverse transcripton (RT)-PCR and Northern blotting

RT-PCR was performed as described previously [8]. Sense (5′-TGGCAGACACCATGCTGAGGG-3′) and antisense (5′-CG-CTTTGACTTCTCCTTCCGC-3′) primers were used to amplify Cezanne transcripts. For TRABID, sense (5′-GAAGAT-TTGCCCCCAACAGTCC-3′) and antisense (5′-AGCTTGCT-CCAGGCTGACTAGC-3′) primers were used. For Northern blotting, total RNA was extracted using Trizol as described previously [8]. RNA (20 μg) was separated using a 1 % agarose/formaldehyde gel, blotted on to a charged nylon membrane, and exposed to UV light. Pre-hybridization was performed at 60 °C for 1 h using Quickhyb (Stratagene). Full-length cDNA was labelled with [α-^{32}P]dCTP using the random priming method (Promega), purified, mixed with 100 μg/ml of sonicated salmon sperm DNA, and denatured before application to the membrane. After overnight incubation at 60 °C, the membrane was subjected to stringent washes [0.1 × SSC (0.015 M NaCl/0.0015 M sodium citrate), 0.1 % SDS at 55 °C] before autoradiography.

Transfection of cultured cells

$CaCl_2$ precipitation

For cells cultured in 24-well plates, DNA was combined with 50 μl of 0.25M $CaCl_2$ before addition of 50 μl of 2 × BBS [280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM BES, pH 7, (Sigma)]. This solution was incubated at room temperature for 30 min and added to 1 ml of growth medium, which was then applied to the cells. Cells were incubated overnight in 3 % CO_2 and then restored to normal culture conditions.

LIPOFECTAMINE™

A 17 μg portion of expression construct and 70 μl of LIPOFECTAMINE™ (Gibco BRL) were each combined with 1.75 ml of Optimem-1 (Gibco BRL) and incubated at room temperature for 5 min. These solutions were combined and incubated at room temperature for 20 min. Cells grown in a 75 cm² flask were washed with Optimem-1 and 3.5 ml of LIPOFECTAMINE™/DNA/Optimem-1 was applied for 5 h before normal culture conditions were restored.

Reporter gene assays

The NF-kB reporter (pGL2) comprised an NF-κB response element upstream of firefly luciferase (supplied by M. Turner, The Babraham Institute, Cambridge, U.K.). To normalize 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 48–72 h. β-Galactosidase activity was measured as described previously [7]. Firefly and *Renilla* luciferase activities were assessed using the Dual Luciferase Reporter Assay Kit (Promega) and luminescence counter (Topcount Microplate Scintillation; Packard, Meriden, CT, U.S.A.).

Immunoprecipitation

Confluent cultures of HEK-293 cells in 75 cm² flasks were transfected transiently using LIPOFECTAMINE™. Cells were lysed after 72 h using 2 ml of Tris/HCl (20 mM, pH 7.5), Triton X-100 (1 %), NaCl (137 mM), $MgCl_2$ (1.5 mM) and EGTA (1 mM). Debris were removed from lysates by high-speed centrifugation. A total of 1 ml of lysate was pre-cleared three times using 5 μg of mouse IgG (Sigma) bound to Protein G–Sepharose beads (Amersham Pharmacia Biotech). FLAG-tagged TRAF6 was then immunoprecipitated using M5 anti-FLAG monoclonal antibody (Sigma) which was bound to Protein G–Sepharose beads (Amersham Pharmacia Biotech). Beads were then washed four times with lysis buffer. Lysates (15 μl) and immunoprecipitates were analysed by Western blotting. Detection of HA tag was achieved using rat anti-HA monoclonal antibody conjugated directly to horseradish peroxidase (HRP; Roche). FLAG-tagged proteins were identified using M5 anti-FLAG followed by goat anti-mouse HRP (Dako). HRP was developed using chemiluminescent substrate (Pierce, Chester, U.K.).

Confocal microscopy

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

RESULTS

Isolation of two novel genes with similarity to A20

Human expressed sequence tags were analysed for the presence of A20 homologues by performing BLAST (http://www.hgmp.mrc.ac.uk) searches with the full-length coding se-

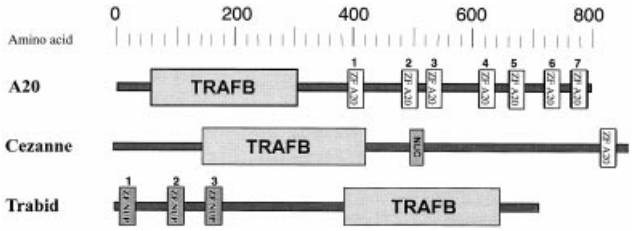

Figure 1 Domain composition of A20, Cezanne and TRABID

Amino acid sequences were analysed using BLAST and Profilescan programs. The position of recognized domains is represented alongside amino acid number (0–800). Depicted domains are TRAFB (TRABID), ZF A20 (A20-like zinc finger), NUC (putative nuclear localization sequence) and ZF NUP (nucleoporin-like zinc finger).

quence of A20 (accession number M59468). Partial coding sequence was found for two novel genes, *Cezanne* and *TRABID*, which share structural features and sequence similarity with A20.

Full-length cDNA for Cezanne was generated by RACE PCR using human peripheral blood lymphocyte cDNA. A PCR product of 3.1 kb was isolated and sequence information was deposited in GenBank® (accession number AJ293573). Two in-frame stop codons were identified upstream of the first initiating codon ATG (nt 155) which precedes an open reading frame of 2574 nt. Full-length cDNA for TRABID was generated by RACE PCR using human umbilical-vein endothelial cell cDNA. A PCR product of 2.7 kb was isolated and sequenced (accession number AJ252060). The predicted initiating codon (nt 407) is preceded by an in-frame stop codon and the subsequent open reading frame is 2126 nt in length.

Analysis of the predicted amino acid sequences of Cezanne and TRABID using BLAST and Profilescan (http://www.isrec.isb-sib.ch/software/) programs revealed several features (Figure 1). Cezanne residues 160–416 displayed striking homology (39%) with the N-terminal, TRAF-binding region of A20 (Figure 2a). This defines a novel domain with conserved structural features which we have named TRAFB (TRAF binding). Cezanne also contains a putative nuclear localization signal (RRKEKSKRDREKDKKR; residues 497–513), which conforms to a consensus sequence that has a reported predictive accuracy of 88% (http://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-Xaa$_4$-Cys-Xaa$_{11}$-Cys-Xaa$_2$-Cys. This has greatest similarity to A20 zinc fingers seven (residues 747–767) and four (residues 592–612) (Figure 2b).

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

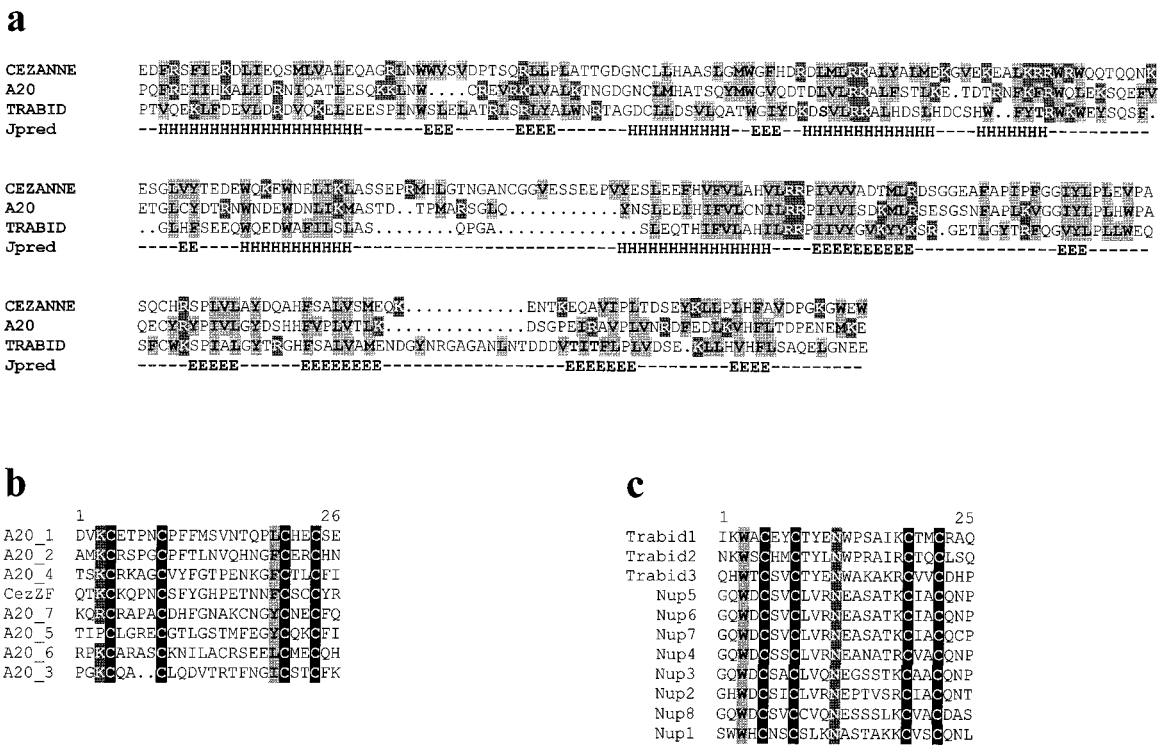

Figure 2 Sequence alignments

(**a**) Sequence alignment of the TRABID human Cezanne (accession number AJ293573), A20 (accession number M59465) and TRABID (accession number AJ252060) compiled using Pileup. Hydrophobic residues are displayed as black letters on grey background and basic residues as white letters on dark grey background. Secondary structure was predicted using the Jpred2 server and is depicted. H, alpha-helix; E, extended (beta) sheet. (**b**) Alignment of Cezanne and A20 zinc fingers using Pileup. Text was highlighted for conserved cysteine (white letters on black background), hydrophobic (black letters on grey background) and basic (white letters on dark-grey background) residues. (**c**) Alignment of TRABID and Nup358 (accession number L41840) zinc fingers using Pileup. Text was highlighted for conserved cysteine (white letters on black background), hydrophobic (black letters on grey background) and asparagine residues (white letters on dark-grey background).

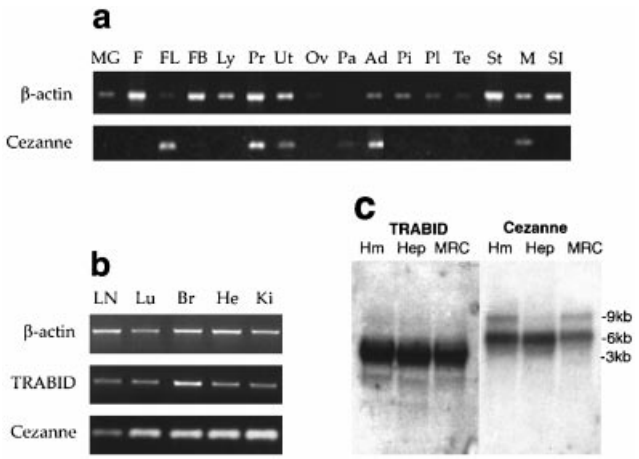

Figure 3 Distribution of Cezanne and TRABID mRNA

(**a**) RT-PCR was used to analyse β-actin and Cezanne transcripts in a panel of cDNA from various human tissues obtained commercially (OriGene Techologies Inc., Rockville, MD, U.S.A.). This comprised mammary gland (MG), fat (F), fetal liver (FL), fetal 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). (**b**) 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). (**c**) mRNA from HMEC-1 (Hm), HepG2 (Hep) and MRC5 (MRC) cells were Northern blotted and probed in parallel using ^{32}P-labelled full-length cDNA for TRABID and Cezanne. Membranes were washed before autoradiography was performed. Transcript sizes are indicated to the right of the blots.

to zinc fingers found in several nucleoporin molecules (Figure 2b).

Alignment of the TRAFB domains of Cezanne, TRABID and A20 revealed considerable conservation of hydrophobic and basic residues, which suggested structural similarities (Figure 2a). The alignment was analysed using several computer algorithms to create a consensus prediction of secondary structure (JPred; http://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 (Figures 3a and 3b). Cezanne transcripts were particularly abundant in kidney, heart and fetal liver. RNA from endothelial, hepatocyte and fibroblast cell lines was tested by Northern blotting with cDNA encoding full-length Cezanne and TRABID (Figure 3c). A dominant, 6 kb Cezanne transcript was detected in each cell line. A larger 9.5 kb 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 9.5 kb and 6 kb Cezanne transcripts, and confirmed high-level expression in heart tissue (results not shown). TRABID transcripts were also identified in each cell line and comprised a 3 kb species and a minor 1 kb 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 [9]. We performed RT-PCR analysis of phorbol ester stimulated T-cells (Jurkat line) and TNFα-treated human umbilical-vein endothelial cells and demonstrated rapid induction of A20 transcripts. In contrast, activation of NF-κB through either treatment did not alter levels of Cezanne or TRABID (results not shown).

Cezanne is a negative regulator of NF-κB

The presence of an A20-like zinc finger and TRAFB domain within Cezanne prompted investigation of the effects of Cezanne on NF-κB activity and comparison with A20 and TRABID. Reporter gene experiments were performed in HEK-293 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 prerequisite of these studies (Figure 4a). HEK-293 cells did not contain endogenous levels of A20, Cezanne or TRABID; however, 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 Figure 5).

Cezanne reduced NF-κB activity in HEK-293 cells stimulated with TNFα in a dose-dependent manner (Figure 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. 50 ng of A20 and 400 ng of Cezanne expression constructs had similar effects (Figure 4c). This difference could not be attributed to divergent transcriptional (Figure 4a) or translational (see Figure 5) activity, and therefore reflects a genuine difference in the efficiency of overexpressed 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 (Figure 4c). Significant reductions in NF-κB activation were only observed after overexpression 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 with HEK-293 cells, these cells express low constitutive levels of A20 and Cezanne (results not shown). Despite this difference, the effects of Cezanne and A20 on NF-κB activity in EaHy mimicked those observed in HEK293 cells (Figure 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 with A20 (results not shown).

Interaction with the signal adaptor TRAF6

We have defined the TRAFB domain through alignment of A20, Cezanne and TRABID. The function of the TRAFB domain is uncertain, but A20 TRAFB has been shown to interact with a TRAF1/TRAF2 [6] heterocomplex and with TRAF6 [7], which are signal adaptor molecules for the TNFR1 and IL-1 receptor 1 respectively. We predicted that the TRAFB domain of Cezanne and TRABID would facilitate interaction of these molecules with TRAF's.

Studies of overexpressed molecules in HEK-293 cells demonstrated that A20, Cezanne and TRABID were co-immunoprecipitated with TRAF6 (Figure 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

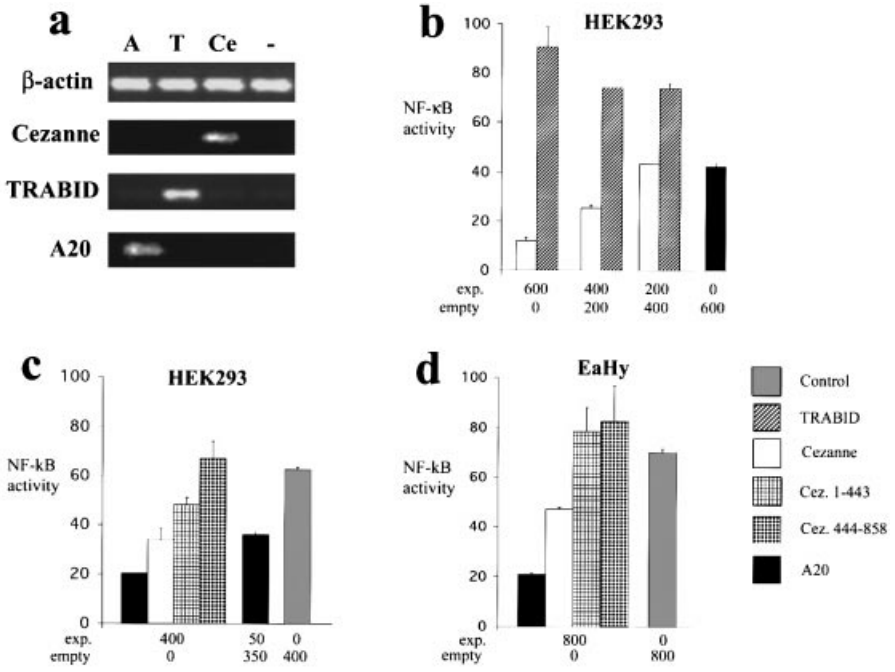

Figure 4 Regulation of NF-κB

Cells were cultured in 24-well plates and transfected using calcium precipitation. (**a**) HEK-293 cells were transfected with HA-tagged A20 (A), TRABID (T) or Cezanne (Ce) and incubated for 16 h. 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 encoding full-length TRABID, A20, Cezanne or truncated regions of Cezanne (1–443 or 444–858). (**b**) HEK-293 cells were co-transfected with 100 ng of pGL2 (NF-κB reporter), 100 ng of pUT651 (β-galactosidase control) and various amounts of expression vector (exp.). The total amount of DNA transfected was standardized with empty pHM6 vector (empty). Cells were stimulated with TNFα for 6 h. Cell lysates were analysed and the ratio of firefly luciferase/β-galactosidase activity was calculated, which is a measure of NF-kB activity normalized for transfection efficiency. Similar experiments in HEK-293 (**c**) and EaHy cells (**d**) used pRL-TK (*Renilla* luciferase control) instead of pUT651. In these assays, NF-κB activity was represented by the ratio of firefly/*Renilla* luciferase activity. Mean values calculated from duplicate or triplicate wells are shown by standard error bars. The data shown are representative of 12 similar experiments. Cez., Cezanne.

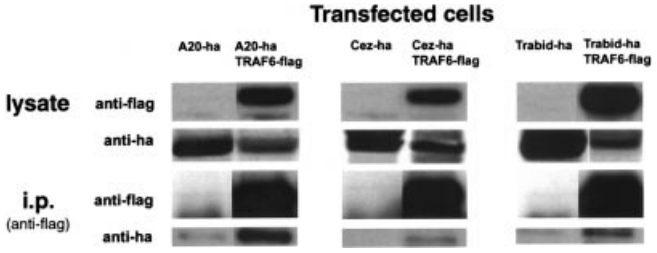

Figure 5 Co-immunoprecipitation studies

HEK-293 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 (15 μl) were tested by Western blotting using anti-FLAG or anti-HA antibodies. Anti-FLAG antibody was used to immunoprecipitate TRAF6-FLAG from 1 ml of lysate. Immunoprecipitating proteins (i.p.) were tested by Western blotting using anti-FLAG or anti-HA antibodies.

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.

Intracellular localization

The intracellular distribution of GFP fusion proteins was assessed in live cells using confocal microscopy (Figure 6). In epithelial (HEK-293) and fibroblast (NIH3T3) cell lines, GFP–A20 was localized to punctate bodies within the cytoplasm, which is consistent with previous reports [10,11]. A similar distribution was observed in an endothelial line (EaHy), but in these cells GFP–A20 also demonstrated nuclear localization. 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) localized to large punctate bodies within the cytoplasm.

Despite the presence of a putative nuclear localization sequence, GFP–Cezanne did not localize 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.

Localization of full-length and the zinc finger-containing region of Cezanne were compared because this may have influenced their differential effects on NF-kB activity (Figure 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, Eugene, OR, U.S.A.) revealed that Cezanne 444–858 was adjacent to the plasma membrane and did not insert (results not shown). Punctate distribution within

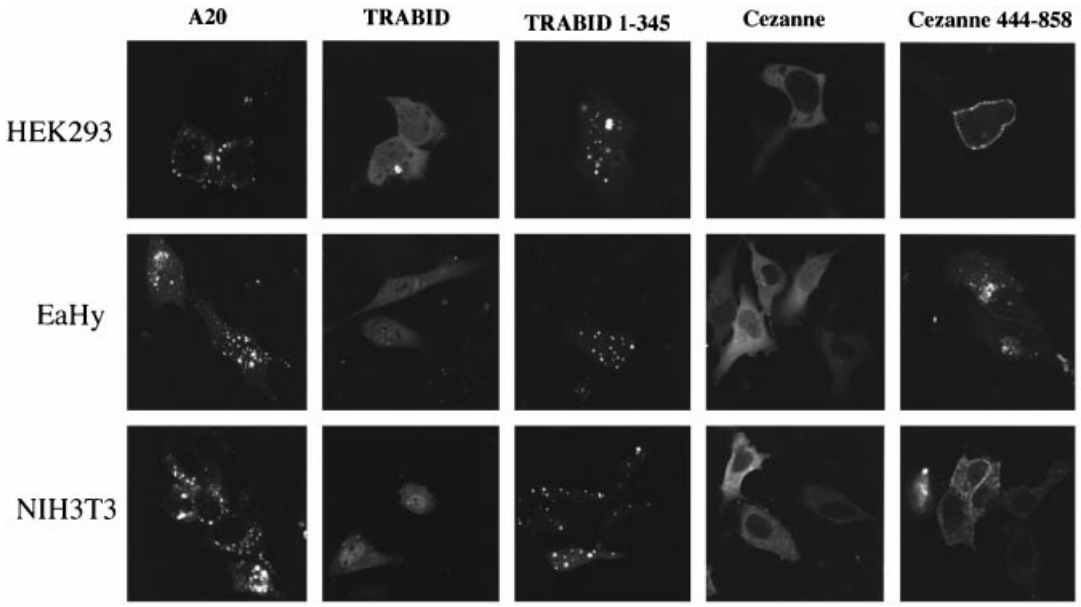

Figure 6 Intracellular localization of GFP-tagged molecules

HEK-293, EaHy and NIH3T3 cells were transfected transiently with enhanced GFP-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 h later.

the cytoplasm was observed in endothelial and fibroblast cells with a degree of plasma membrane localization in the latter.

DISCUSSION

The mechanism underlying the ability of A20 to regulate NF-κB is not completely understood, but it has been demonstrated that overexpression of the C-terminal half, which contains seven zinc fingers, is sufficient for this suppression. Relatively little is known, however, about the function of the N-terminal portion of A20. This region is responsible for the interaction of A20 with TRAF molecules, and it has been tentatively suggested that this interaction may be important in directing endogenous A20 to signalling complexes. We report for the first time, isolation of genes which have sequence similarity with the N-terminal region of A20. Sequence alignment between A20, Cezanne and TRABID identified a novel domain with conserved structural features (TRAFB). Apart from the presence of a TRAFB domain, A20 and TRABID had no other similarities, and presumably have divergent functions. Indeed, TRABID had no significant effect on NF-κB activation. This is consistent with the view that the TRAFB domain of A20 has no direct involvement with regulation of NF-κB, but may provide an essential scaffold.

Overexpression 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 respectively. This is consistent with a previous study [12] which demonstrated that at least four A20 zinc fingers are required for efficient modulation of TNFα-induced NF-κB.

It seems plausible that the zinc finger of Cezanne is involved in NF-κB suppression. Further studies, however, demonstrated that the full-length molecule was required for NF-κB inhibition and that the C-terminal half, which contains the zinc finger, was insufficient. We suggest that the TRAFB is important because it facilitates interaction with TRAF molecules which may chaperone Cezanne to TNFR1 or IL-1 receptor 1 complexes. Three pieces of evidence are consistent with this view: (i) Cezanne and TRAF molecules [13] are both distributed within the cytoplasm, and this co-localization is facilitated by the TRAFB of Cezanne. Conversely, the zinc finger-containing region of Cezanne in isolation will not co-localize with TRAF molecules, and may be sequestered away from signalling apparatus; (ii) Cezanne binds to TRAF molecules; and (iii) TRAF molecules are recruited to signalling complexes in response to cytokine treatment [1]. We observed that Cezanne had relatively weak TRAF-binding ability compared with A20, and note that this could also compromise the ability of Cezanne to regulate NF-κB.

Cezanne and TRABID were expressed at a low constitutive levels in tissues and cultured cells and were not induced by cytokine stimulation. Although overexpression of Cezanne and TRABID revealed a specific effect of the former on NF-κB activation, the role of physiological levels of these novel proteins is unknown. Gene-targeting studies are underway which will address the role of endogenous Cezanne in the control of the inflammatory process.

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