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Caspase cleavage of Ets-1 p51 generates fragments with transcriptional dominant-negative function

Souhaila CHOUL-LI*, Catherine LEROY*, Gabriel LEPRIVIER*[†], Clélia LAITEM*[‡], David TULASNE*^{§1} and Marc AUMERCIER*^{§1}

* CNRS UMR 8161, Institut de Biologie de Lille; Institut Pasteur de Lille; Univ Lille Nord de France; IFR 142; B.P. 447, 1 rue Calmette, 59021 Lille Cedex, France

[†] Present address: Department of Molecular Oncology, British Columbia Cancer Research Centre, Vancouver, British Columbia V5Z 1L3, Canada

[‡] Present address: Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, UK

[§] These authors conducted equally this work

¹ Corresponding authors:

Marc Aumercier; Institut de Biologie de Lille, CNRS UMR 8161, 1 rue du Prof. Calmette, BP 447, 59021, Lille Cedex, France; Tel.: +33 320 871 097; Fax: +33 320 871 111; E-mail: marc.aumercier@ibl.fr

David Tulasne; Institut de Biologie de Lille, CNRS UMR 8161, 1 rue du Prof. Calmette, BP 447, 59021, Lille Cedex, France; Tel.: +33 320 871 257; Fax: +33 320 871 111; E-mail: david.tulasne@ibl.fr

Short title: Caspase cleavage of Ets-1 p51

Abbreviations used: DBD, DNA binding domain; DTT, dithiotreitol; EBS, ETS-binding site; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; TAD, transactivation domain; TORU, TPA oncogene response unit; WT, wild-type; zVAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

SYNOPSIS

Ets-1 is a transcription factor that plays an important role in various physiological and pathological processes, such as development, angiogenesis, apoptosis and tumour invasion. Here we demonstrate that Ets-1 p51, but not the spliced variant Ets-1 p42, is processed in a caspase-dependent manner in Jurkat T-leukaemia cells undergoing apoptosis, resulting in three C-terminal fragments Cp20, Cp17 and Cp14 and a N-terminal fragment, Np36. *In vitro* cleavage of Ets-1 p51 by caspase-3 produces fragments consistent with those observed in cells undergoing apoptosis. These fragments are generated by cleavage at three sites located in the exon VII-encoded region of Ets-1 p51. This region is absent in the Ets-1 p42 isoform, which therefore cannot be cleaved by caspases. In Ets-1 p51, cleavage generates C-terminal fragments containing the DNA binding domain but lacking the transactivation domain. The Cp17 fragment, the major cleavage product generated during apoptosis, is devoid of transcriptional activity and inhibits Ets-1 p51-mediated transactivation of target genes by competing with Ets-1 p51 for binding to Ets-binding sites present in the target promoters. In this study, we demonstrate that caspase cleavage of Ets-1 within the exon VII-encoded region leads to specific down-regulation of the Ets-1 p51 isoform during apoptosis. Further, our results establish that caspase cleavage generates a stable C-terminal fragment that acts as a natural dominant-negative of the full-length Ets-1 p51 protein.

Key words: Ets-1 isoforms, apoptosis, caspases, exon VII-encoded region, dominant negative, transcriptional regulation.

INTRODUCTION

Apoptosis is a genetically controlled form of cell death that plays a critical role during development and tissue homeostasis by ensuring the removal of damaged or unnecessary cells [1]. Apoptotic stimulation leads to the cascade activation of cysteine proteases called caspases, which carry out the apoptotic program by cleaving numerous substrates [2]. Disruption in the regulation of apoptosis underlies the pathogenesis of many human diseases, including autoimmune diseases, neurogenerative disorders and cancers [3].

Since caspase-mediated proteolysis is crucial for the apoptotic process [4], identification of the molecular targets of caspases is necessary to better understand apoptotic signal transduction. Indeed, several classes of cellular proteins have been shown to be cleaved by caspases during apoptotic cell death [5]. This usually results in either inactivation or activation of proteins that protect living cells or promote cell death. Among those substrates, several transcription factors are inactivated during apoptosis by caspase-3 including SRF [6], Retinoid Acid Receptor- α (RAR- α) [7] and NF-kappa B p65/RelA subunit [8]. Likewise, in the Ets transcription factor family, Fli-1 has been shown to be cleaved in murine preB leukemic cells undergoing programmed cell death by a caspase-like activity and *in vitro* by caspase-3 [9].

Ets-1 is the founding member of the Ets family characterised by a well-conserved DNA-binding domain (DBD), called the ETS domain. It regulates gene expression by binding to specific DNA elements, called Ets-binding sites (EBS), found in the promoters of its target genes [10, 11]. By binding to these elements, Ets-1 activates the transcription of various genes involved in numerous cellular mechanisms such as development, angiogenesis, proliferation and apoptosis [12]. However, when overexpressed, Ets-1 is involved in the development of

invasive pathologies such as rheumatoid arthritis [13], glomerulonephritis [14] and cancers [12].

In humans, two isoforms of Ets-1 have been described: p51, the predominant, full-length isoform, and p42, a shorter, alternatively spliced isoform that lacks the exon VII-encoded region [15]. As a result of their respective structures, Ets-1 p51 and Ets-1 p42 isoforms have different DNA binding and transcriptional properties [16-18]. Ets-1 p51 is autoinhibited for DNA binding due to the presence of two inhibitory domains that flank and interact with its DBD [19]. To counteract autoinhibition and improve DNA binding, Ets-1 p51 interacts with partners, enabling them to cooperatively bind to adjacent DNA elements [20]. Ets-1 p51 can also counteract its autoinhibition without any interaction partners by binding to a particular palindromic arrangement of two EBS separated by 4 bp, as in the *stromelysin-1* matrix metalloproteinase and the *p53* oncosuppressor promoters [17, 21, 22]. Two molecules of Ets-1 p51 cooperatively bind to the EBS palindrome, thereby forming an Ets-1 p51/DNA/Ets-1 p51 ternary complex that is critical for full transactivation [17]. In contrast, Ets-1 p42 only forms a binary Ets-1 p42/DNA complex, which is much less efficient in promoting transactivation [17, 18]. These differences contribute to the Ets-1 isoform-specific modulation of particular target genes, illustrating that Ets-1 function can be regulated through the exon VII-encoded region [23, 24].

Ets-1 p51 displays pro- and anti-apoptotic functions according to the biological context. Ets-1 p51 is required for the formation of a stable DNA-p53-CBP complex to induce pro-apoptotic genes in the process of UV-induced apoptosis in embryonic stem cells [25]. Furthermore, overexpression of Ets-1 p51 in human umbilical vein endothelial cells induces apoptosis under serum-deprived conditions by up-regulating pro-apoptotic genes [26]. Anti-apoptotic functions have been ascribed to Ets-1 p51 in vascular smooth muscle cells protecting them from undergoing apoptosis by activating the transcription of $p21^{\text{waf1/cip1}}$ [27].

Moreover, Ets-1 p51 inhibits the expression of the pro-apoptotic gene *bax* by interacting with the transcription factor Growth Factor Independent-1 (GFI-1) in the composite site present in the *bax* promoter [28]. In contrast, Ets-1 p42 isoform expression seems to be essentially associated with apoptosis. The expression of Ets-1 p42, unlike that of Ets-1 p51, promotes Fas-mediated apoptosis by directly up-regulating ICE/caspase-1 gene expression in human colon cancer DLD-1 cells [24, 29]. Ets-1 p42 expression can also induce apoptosis of these cells in low-serum conditions [30]. Furthermore, Ets-1 p42-expressing DLD-1 cell xenografts in nude mice inhibit the tumorigenicity of the DLD-1 cancer cells by inducing apoptosis [29].

Previous studies have shown that the basal level of Ets-1 p51 decreases in cells undergoing apoptosis. For example, an apoptotic inducer, curcumin, decreases the amount of Ets-1 p51 protein in human endometrial carcinoma HEC-1-A cells in a time- and dose-dependent manner [31]. Likewise, flow cytometry and confocal microscopy analysis have shown that Ets-1 expression is down-regulated in peripheral blood mononuclear (PBMC) and bursal cells induced to apoptosis [32].

In this study, we demonstrate that the induction of apoptosis in human acute T-cell leukaemia (Jurkat) cells causes a decrease in Ets-1 p51 levels that is associated with the generation of caspase-dependent fragments. Furthermore, we characterise the mechanisms and the functional consequences of Ets-1 p51 cleavage on the transcriptional modulation of Ets-1 target genes.

EXPERIMENTAL

Cell culture and induction of apoptosis

Human Embryonic Kidney (HEK-293) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; InvitrogenTM, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 50 µg/ml gentamycin. To induce apoptosis, HEK-293 cells were incubated

24 h after transfection in medium supplemented with 1 μ M staurosporin (Calbiochem[®], San Diego, CA, USA) for 8 h. Jurkat human T-leukaemia cells were cultured in RPMI 1640 medium (Invitrogen[™]) supplemented with 10% FBS and 50 μ g/ml gentamycin. To induce apoptosis, Jurkat cells (3×10^6) were incubated in medium supplemented with 1 μ M staurosporin for 4 h. Madin-Darby Canine Kidney (MDCK) epithelial cells were cultured in DMEM supplemented with 10% fetal calf serum, 50 μ g/ml gentamycin and 100 μ g/ml penicillin-streptomycin. To induce apoptosis, MDCK cells were incubated 24 h after transfection in medium supplemented with 10 μ M anisomycin (Calbiochem) for 8 h. To inhibit caspase activity, cells were preincubated for 30 min with 20 μ M benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-FMK; Calbiochem) prior to treatment with staurosporin. Rabbit synovial fibroblasts (HIG-82) cells (ATCC, Manassa, VA, CRL-1832) were cultured in F-12 medium (Invitrogen[™]), supplemented with 10% FBS and 50 μ g/ml gentamycin, between passage 7 and 14.

Cell lysates

Jurkat and HEK-293 cells were lysed by sonication (10 s, 16 μ m amplitude) in 100 μ l of buffer (50 mM Tris pH 8; 150 mM NaCl; 0.1 mM EDTA; Complete[™] protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany)). MDCK cells were suspended in 200 μ l of lysis buffer (25 mM HEPES pH 7.5; 100 mM NaCl; 1.5 mM $MgCl_2$; 0.5 mM EGTA; 0.25 mM EDTA; 0.1% Nonidet P-40; 10 mM NaF; 20 mM β -glycerophosphate; 1 mM Na_3VO_4 ; 1 mM PMSF; 10 μ g/ml leupeptin; 10 μ g/ml aprotinin). Total protein concentration was measured by colorimetry (Bio-Rad assay) before Western blot analysis.

Expression vector construction

The construction of a pcDNA3 vector expressing human Ets-1 p51 fused to a Flag tag is described in ref. [33]. The pcDNA3 vector expressing human Ets-1 p42 fused to a Flag tag was obtained by the same strategy as the Flag-Ets-1 p51 pcDNA3 expression vector, using the human Ets-1 p42 pSG5 expression vector [17] as a template. To produce Ets-1 p51 mutants, Asp (D) residues were replaced by Asn (N) residues in position 262 for D1N, 262/287/290 for D3N and 262/287/290/313 for D4N using the QuickChange Site-directed Mutagenesis Kit (Stratagene®, La Jolla, CA, USA). Briefly, the pcDNA3 vector expressing human Ets-1 p51 fused to a Flag tag was used as a template for PCR amplification, using the following primers in which mutated codons are underlined: 5'-TACGATAGTTGTAATCGCCTCACCCAG-3' and 5'-CTGGGTGAGGCGATTACAACCTATCGTA-3' for the D262N mutation; 5'-GACAGCTTCAACTCAGAGAACTATCCGGCTGCC-3' and 5'-GGCAGCCGGATAGTTCTCTGAGTTGAAGCTGTC-3' for the D287/290N mutations and 5'-CGGGACCGTGCTAACCTCAATAAGGACAAG-3' and 5'-CTTGTCCTTATTGAGGTTAGCACGGTCCCG-3' for the D313N mutation.

To produce the Cp17 fragment, the sequence coding amino acids 291-441 of Ets-1 p51 was obtained by PCR amplification, using the following primers: 5'-AATCACTAAGATCTTATCCGGCTGCCCTGCCCAACC-3' and 5'-TATGCGGCCGCTCACTCGTCGGCATCTGGCTTGAC-3'. The PCR product was then cloned between the *Bam*HI and *Not*I restriction sites in a modified pcDNA3 expression vector (kindly provided by Dr D. Monté). This vector contained the coding sequence for a Flag epitope followed by a *Bam*HI site. To produce the Np36 fragment, the sequence coding amino acid 1-262 of Ets-1 p51 was obtained by PCR amplification, using the following primers: 5'-ATTAAAGATCTAAGGCGGCCGTCGATCTC-3' and 5'-AATCACTAAGATCTATCACAACCTATCGTAGCTCTC-3'. The PCR product was then cloned in the *Bam*HI site of the modified pcDNA3 expression vector. For all vectors, correct

insertion was checked by sequencing. The construction of the -478/+4 wild-type and EBS mutant human *stromelysin-1* promoter pGL3 reporter vector (Promega, Madison, WI, USA) and the construction of the -607/+51 TORU mutant human *collagenase-1* promoter pGL2 reporter vector are described in refs. [17, 33]. The -607/+51 wild-type human *collagenase-1* promoter pGL2 reporter vector as well as the c-Jun and the c-Fos encoding pSG5 vectors were kindly provided by Dr M. Duterque-Coquillaud.

The construction of the bacterial expression vector containing the cDNA sequence of human Ets-1 p51 is described in ref. [17].

Transient transfection

HEK-293, HIG-82 or MDCK cells were grown in six-well plates to reach 40-60% confluence at the time of transfection. Transfection of HEK-293 and HIG-82 cells was performed by incubating the ExGen 500 transfection reagent, according to the manufacturer's instructions (Euromedex, Souffelweyersheim, France), with 1 µg of expression vectors. Transfection of MDCK cells was performed using the lipofection method [34].

***In vitro* caspase cleavage reactions**

After 48 h of transfection, HEK-293 cells and Jurkat cells were lysed in 80 µl of caspase buffer (20 mM PIPES pH 7.2; 100 mM NaCl; 1% Chaps; 10% sucrose; 5 mM dithiotreitol (DTT); 0.05 mM EDTA). Cell lysates were incubated 4 h at 37°C with 1 µl of purified caspase. Reactions were stopped by incubation at 70 °C for 1 min in denaturing buffer (125 mM Tris-HCl pH 6.8; 4% SDS; 20% Glycerol; 100 mM DTT). Total cell lysate proteins were then analysed by Western blot. Purified active caspases were generously provided by Dr G.S. Salvesen. Titration of the active recombinant caspases were obtained using *in vitro* cleavage

against synthetic substrates as previously described (caspase-3, 13.9 μ M; caspase-6, 7.5 μ M; caspase-7, 19.7 μ M; caspase-8, 15 μ M; caspase-9, 41.5 μ M) [35].

Transfection and luciferase reporter gene assay

HEK-293 cells were grown in 12-well plates to reach 40-60% confluence at the time of transfection. Transfections were then performed by incubating the ExGen 500 transfection reagent, according to the manufacturer's instructions (Euromedex), with 500 ng of vectors. Transfections were carried out with increasing amounts of the Cp17 or the Np36 expression vector and human *stromelysin-1* or *collagenase-1* promoter reporter vector in the presence or absence of the Ets-1 p51 expression vector. Cells were harvested 48 h after transfection with 200 μ l of passive lysis buffer (Promega) and each supernatant was sequentially tested for firefly and *renilla* luciferase activity (Dual-Luciferase[®] Reporter Assay System, Promega) using a Centro LB 960 (Berthold, Bad Wildbad, Germany) luminometer. For each expression vector combination and concentration, firefly luciferase activity (pGL-2/3 constructs) was normalised to the *renilla* activity (pRL-null normalisation vector (Promega)) to correct for variation in the number of transfected cells. Expression of proteins of interest was tested by Western blot analysis.

Western blot analysis

Total cell lysate proteins were boiled in Laemmli buffer (50 mM Tris pH 6.8; 2% SDS; 5% β -mercaptoethanol; 10% glycerol; 0.1% bromophenol blue) and resolved by SDS/PAGE. Proteins in gels were transferred to a HybondTM-C Extra membrane (Amersham Biosciences, Piscataway, NJ, USA) and blocked for 1 h at room temperature in 5% non-fat milk in PBS solution. The membrane was then incubated with the primary antibody for 1 h at room temperature in blocking buffer. Ets-1 was detected with the C-20 polyclonal antibody directed

against the C-terminal region or the N-276 polyclonal antibody directed against the N-terminal region (Santa Cruz Biotechnology[®], Santa Cruz, CA, USA), Erk-2 with the C-14 polyclonal antibody (Santa Cruz Biotechnology[®]), cleaved caspase-3 with the 5A1 polyclonal antibody (Cell Signaling Technology[®], Beverly, MA), c-Jun with the H-79 polyclonal antibody (Santa Cruz Biotechnology[®]) and c-Fos with the H-125 polyclonal antibody (Santa Cruz Biotechnology[®]). The washed membrane was then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology[®]) in blocking buffer. Bound antibodies were visualized using Western Lightning[™] chemiluminescence detection system (Perkin Elmer Life Sciences Biotechnology[®]). For quantification of Western blot protein expression, luminescence was recorded using a digital camera with a cooled charge-coupled device (LAS 3000; Fuji) and quantified using the Multi Gauge V3.0 image analysis software (Fuji Film). Reprobing was performed using the Re-Blot Plus Strong Antibody Stripping solution according to the manufacturer's instructions (Millipore, Temecula, CA).

Electrophoretic mobility shift assay (EMSA)

Double-stranded synthetic oligonucleotides corresponding to the WT and EBS mutant of the human *stromelysin-1* (-223/-193) promoter region were end-labelled using T4 polynucleotide kinase and [γ -³²P]ATP, and were subsequently purified by electrophoresis on a 20% polyacrylamide non-denaturing gel in TBE buffer (90 mM Tris-Borate; 1 mM EDTA). Cell lysates prepared (30 μ g) from HEK-293 cells transfected with the expression vector, empty or encoding Cp17 fragment, or with recombinant Ets-1 p51 protein (1 μ g) were incubated with 0.5 ng of labelled probe in 40 μ l binding reaction buffer (20 mM Tris pH 7.9; 80 mM NaCl, 1 mM EDTA; 2 mM DTT, 40 μ g/ml poly[dI-dC]poly[dI-dC] (Pharmacia, Piscataway, NJ, USA); 10% glycerol) for 30 min on ice. Complexes formed were resolved on a 5%

polyacrylamide (29:1 acrylamide:bisacrylamide; Euromedex) non-denaturing gel in 0.25X TBE buffer at room temperature. Gels were dried and autoradiographed at -80 °C. For probe competition, 400 ng of non-labelled probe (800X) were added to the reaction mixture. For protein competition, cell lysates (30 µg) prepared from HEK-293 cells transfected with increasing amounts of the Cp17 expression vector was incubated with recombinant Ets-1 p51 protein (0.5 µg) with 0.5 ng of WT-labelled probe in the same conditions.

Expression and purification of Ets-1 p51 protein

Ets-1 p51 was expressed and purified using the T7-Impact™ System (new England Biolabs®) as previously described [17].

RNA extraction and RT-PCR assays

RNA was extracted from HIG-82 cells using TRIzol®, according to the manufacturer's instructions (Invitrogen™). Reverse-transcription was performed with random hexameric oligonucleotides with 1 µg of cDNA using the RevertAid™ First Strand cDNA kit (Fermentas, Burlington, Ontario, Canada). PCR reactions were performed with the High Fidelity PCR Master mixture (Roche, Meylan, France) with 500 ng of reverse-transcribed RNAs and with 125 ng of forward and reverse primers for each of four genes: human *ets-1*, 5'-ATGAAGGCGGCCGTCGATCTC-3' and 5'-TTGGTCCACTGCCTGTGTAG-3'; *gapdh* (glyceraldehyde-3-phosphate dehydrogenase), 5'-ATGAGGTCCACCACCCTGTT-3' and 5'-ATCACTGCCACCCAGAAGAC-3'; *gelatinase-B*, 5'-CTGGGCAAGGGCGTCGTGGTC-3' and 5'-CGTGGTGCAGGCGGTGTAGGAG-3'; and *uPA*, 5'-CCATCCCGGTCCATACAGACT-3' and 5'-TCACAGCTTGTGCCAAAATTG-3'. PCRs were run for 35 cycles with 30 s denaturation at 94°C, 30 s hybridization at 55°C and 30 s

elongation at 72°C. Amplified products were analysed on 2% agarose gels run in 0.5X TBE buffer and stained with ethidium bromide.

RESULTS

Ets-1 is a substrate for caspases

To investigate Ets-1 fate during programmed cell death, Jurkat T-leukaemia cells, which endogenously express Ets-1 p51 and Ets-1 p42 isoforms, were treated with the apoptotic inducer staurosporin (Figure 1A). This stress stimulus was found to activate caspase-3 with detection in Western blot of the specific p19 and p17 caspase-3 fragments (Figure 1A, lane 2). This effect was associated with a decrease in Ets-1 levels and generation of three new Ets-1 fragments of about 14, 17 and 20 kDa in apparent molecular mass, designated as Cp14, Cp17 and Cp20, with Cp17 being the predominant fragment. These fragments were detected using an anti-Ets-1 antibody directed against its DBD located in the C-terminal region. Using an anti-Ets-1 antibody directed against its Pointed domain located in the N-terminal region, the same blot revealed a new Ets-1 fragment of about 36 kDa in apparent molecular mass, designated as Np36 (Figure 1A, lane 2). This suggests that Ets-1 factor was cleaved, leading to generation of three C-terminal and one N-terminal fragments. Furthermore, Ets-1 processing was abrogated by the addition of the potent general caspase inhibitor zVAD-FMK to Jurkat cells medium prior to staurosporin treatment (Figure 1A, lanes 3 and 4), demonstrating that caspases are involved in Ets-1 cleavage.

To confirm that caspases are able to process the Ets-1 transcription factor, Jurkat cell extracts were exposed to recombinant active caspase-3 and analysed by Western blot using the same antibodies as in the previous experiment (Figure 1B). Results showed that recombinant active caspase-3 reduced endogenous Ets-1 levels (Figure 1B, lanes 1 and 2) and produced the same fragments as those generated after staurosporin treatment (compared

Figure 1A, lane 2 and Figure 1B, lane 2), i.e. the Cp14, Cp17 and Cp20 C-terminal and the Np36 N-terminal fragments. These results unequivocally demonstrate that the Ets-1 protein is processed by caspases during apoptosis.

Ets-1 p51, but not Ets-1 p42, is cleaved *in vitro* by recombinant active caspase-3

To determine if one or both Ets-1 isoforms constitute caspase-3 substrates, Ets-1 p51 or Ets-1 p42 were exogenously expressed in HEK-293 cells, which do not encode Ets-1. Cell lysates were then exposed to recombinant active caspase-3 (Figure 2A). Western blot analysis revealed that exogenous Ets-1 p51 levels were drastically reduced after incubation with recombinant active caspase-3 (Figure 2A, lanes 3 and 4), whereas exogenous Ets-1 p42 levels were not affected (Figure 2A, lanes 5 and 6). Furthermore, the decrease in Ets-1 p51 intensities was associated with the production of the Cp14, Cp17, Cp20 and Np36 fragments (Figure 2A, lane 4). Thus, Ets-1 p51, but not Ets-1 p42, is a substrate of recombinant active caspase-3 *in vitro*.

The same experiment was performed using other recombinant active caspases, in particular caspase-3, -6, -7, -8 and -9 (Figure 2B). Results showed that among the tested proteases, the caspase-3 was the only one that cleaved exogenous Ets-1 p51 (Figure 2B, lanes 2-7), while Ets-1 p42 was not cleaved by caspase-3 or any other caspase (Figure 2B, lanes 8-13).

Ets-1 p51, but not Ets-1 p42, is cleaved in cells undergoing apoptosis

We then checked the consequences of apoptotic signals on the cleavage of Ets-1 p51 and Ets-1 p42 by caspases. Ets-1 p51 and Ets-1 p42 were expressed in HEK-293 cells, in the presence or absence of the apoptotic inducer staurosporin (Figure 3A). Western blot analysis of cell lysates showed that the three C-terminal cleavage fragments were generated in cells

expressing Ets-1 p51 (Figure 3A, lanes 5 and 6). Furthermore, the incubation of cells with zVAD-FMK abrogated the production of the cleavage fragments (Figure 3A, lanes 7 and 8). In contrast, the exogenous expression of Ets-1 p42 in HEK-293 cells did not generate any proteolytic fragments (Figure 3A, lanes 9-12). Taken together, these results show that exogenous Ets-1 p51, but not Ets-1 p42, is cleaved by caspases in apoptotic HEK-293 cells.

To determine whether the differential caspase cleavage of the two Ets-1 isoforms could occur in endogenous conditions, apoptosis of Jurkat cells was induced by staurosporin treatment and cell lysates were analysed by Western blot at several time intervals (Figure 3B). Upon induction of apoptosis, the three C-terminal fragments were generated as expected. The level of the Cp20 fragment, detected after 2 h, declined over the 8 h time course, suggesting that it may be degraded. In contrast, the Cp17 and Cp14 fragments were stable over time. Along with the generation of fragments, Ets-1 p51 levels simultaneously greatly decreased in a time-dependent manner, while Ets-1 p42 levels declined only weakly over the same period. The quantification of Ets-1 isoform levels in three independent experiments (Figure 3C) confirmed that the amount of Ets-1 p51 decreased about 60% during the 8 h time course experiment, while Ets-1 p42 level remained unchanged. Furthermore, the quantification of Cp17, the main fragment, showed that it was stable over time (Figure 3C). Incubating cells with zVAD-FMK prior to staurosporin treatment abrogated the reduction of Ets-1 p51 levels (Figure 3B, lane 5 and Figure 3C) as well as the formation of cleavage fragments (Figure 3B, lane 5). Our results demonstrate that in Jurkat cells undergoing apoptosis, Ets-1 p51, unlike Ets-1 p42, is down-regulated. Taken together, this suggests that caspase cleavage of Ets-1 p51 during apoptosis contributes to the efficient degradation of Ets-1 and generates fragments of Ets-1.

Identification of caspase cleavage sites in Ets-1 p51

The cleavage motif for caspases is a sequence of four amino acids, numbered P4-P1, which is processed after the invariant P1 aspartic acid residue. The Asp-X-X-Asp is a cleavage motif present in many caspase-3 substrates [36, 37]. The amino-acid sequence of human Ets-1 p51 has three putative cleavage sites for caspases in the exon VII-encoded region, DSCD²⁶², a double site DSFD²⁸⁷SED²⁹⁰, and DRAD³¹³ (Figure 4Aa). The position of these sites is consistent with the generation of three C-terminal fragments of 20, 17 and 14 kDa (Figure 4Ab). In addition, the location of these sites in the exon VII-encoded region is consistent with the resistance of Ets-1 p42 to caspase cleavage.

To determine whether Ets-1 p51 is cleaved by caspases at these sites, site-directed mutagenesis was performed: (i) on the first putative caspase site in which Asp²⁶² was substituted by Asn (D1N); (ii) on the two first putative caspase sites in which Asp²⁶² and a double sites Asp²⁸⁷/Asp²⁹⁰ were mutated to Asn (D3N) and (iii) on all the caspase sites of the exon VII-encoded region (D4N) (Figure 4Ac).

After the exogenous expression of WT or mutant (D1N, D3N, D4N) Ets-1 p51 in HEK-293 cells, lysates were exposed to recombinant, active caspase-3 and analysed by Western blot (Figure 4B). In contrast to WT Ets-1 p51 (Figure 4B, lane 2), all three mutants were resistant to caspase-3 cleavage (Figure 4B, lanes 4, 6 and 8). Thus, DSCD²⁶² was an essential site for *in vitro* caspase-3 cleavage because its mutation abrogates the processing at the other sites.

Second, we assessed the role of these sites for Ets-1 p51 processing in MDCK cells undergoing apoptosis. MDCK cells exogenously expressing WT or mutant Ets-1 p51 were incubated with or without anisomycin and cell lysates were analysed by Western blot (Figure 4C). As expected, WT Ets-1 p51 generated the C-terminal fragments with Cp17 predominating (Figure 4C, lane 2). It is noteworthy that Ets-1 fragments occurred in the absence of any apoptotic inducer; this may be due to stress caused by transfection conditions

(Figure 4C, lane 3), as previously reported for MDCK cells [38, 39]. The D1N mutant abrogated Cp20 generation, while Cp17 and Cp14 were still detectable (Figure 4C, lane 4), demonstrating that Cp20 was generated through cleavage on Asp²⁶² amino acid. The D3N mutant abrogated generation of all the fragments (Figure 4C, lane 6), demonstrating that cleavage on DSFD²⁸⁷SED²⁹⁰ is necessary for generation of the main Cp17 fragment and the secondary Cp14 fragment. Taken together, these results demonstrate that Ets-1 p51 was cleaved at caspase sites with aspartic acid located at positions 262, 290 and 313, allowing generation of the three C-terminal fragments Cp20, Cp17 and Cp14, respectively. In addition, DSCD²⁶² and DSFDSED²⁹⁰ sites are critical for cleavage *in vitro* and during apoptosis, respectively.

Cp17 binds to the EBS palindrome of the *stromelysin-1* promoter and competes with Ets-1 p51 for binding

Caspase cleavage during apoptosis generated Cp17, the major C-terminal fragment that conserves the DNA-binding domain surrounded by the two inhibitory domains (Figure 5A). This suggests that Cp17 would be able to bind DNA as Ets-1 p51 does. To test this hypothesis, the nucleotide sequence of Cp17, which codes for amino acids 291-441 of Ets-1 p51 (Figure 5A), was cloned into a eukaryotic expression vector and used in an electrophoretic mobility shift assay (EMSA).

Assays were carried out using: (i) lysates from HEK-293 cells transfected with empty vector, (ii) lysates from HEK-293 cells transfected with Cp17 expression vector or (iii) Ets-1 p51 recombinant protein. Proteins were incubated with 30-mer ³²P-labeled DNA probes containing WT or mutant EBS palindromes of the *stromelysin-1* promoter (Figure 5B). Results showed that Ets-1 p51 formed a complex with the WT promoter (arrow 3; Figure 5B, lanes 5). This may correspond to a ternary complex with two protein molecules bound to the

WT EBS palindrome, as previously reported [17]. Two complexes were detected with lysates from cells transfected with Cp17. The slower migrating band was probably a ternary complex (arrow 3'; Figure 5B, lane 9) because we detected a smaller binding species, which may correspond to a binary complex formed by one protein molecule bound to the *stromelysin-1* promoter (arrow 2'; Figure 5B, lane 9). Mutation of the EBS palindrome prevented both Ets-1 p51 and Cp17 forming DNA/protein complexes (Figure 5B, lanes 6 and 10). These complexes were disrupted by an excess of non-labelled WT DNA probe (Figure 5B, lanes 7 and 11), but not by an excess of non-labelled double-mutant DNA probe (Figure 5B, lanes 8 and 12), confirming that these complexes were specific to the EBS palindrome.

Next, we assessed the ability of Cp17 to compete with Ets-1 p51 for DNA binding. EMSA experiments were carried out using a 30-mer ³²P-labelled WT DNA probe and increasing concentrations of Cp17 exogenously expressed in HEK-293 cells, with or without recombinant Ets-1 p51 (Figure 5C). Results showed that the Ets-1 p51/ DNA/Ets-1 p51 ternary complex (Figure 5C, arrow 3, lane 6) was disrupted in the presence of Cp17 (Figure 5C, lanes 7-9). This disruption was accompanied by the formation of intermediate complexes (indicated by arrow i) with lower molecular masses, which may be composed of both Ets-1 p51 and Cp17. Thus, the Cp17 cleavage fragment competes with Ets-1 p51 for binding to the EBS palindrome in the *stromelysin-1* promoter.

Cp17 cleavage fragment is a dominant negative for Ets-1 p51 transactivation

The caspase cleavage within the exon VII-encoded region separates the N-terminal transactivation domain from the C-terminal DNA binding domain. This suggests that Cp17 would be a dominant negative for Ets-1 p51-mediated transcription. To test this hypothesis, luciferase transactivation assays were performed with the WT or EBS mutant *stromelysin-1* promoter using increasing concentrations of Cp17 with or without Ets-1 p51 (Figure 6A).

Cp17 was unable to substantially activate the *stromelysin-1* promoter on its own (Figure 6A, upper part, lanes 1-4). Nevertheless, it repressed in a dose-dependent manner the Ets-1 p51-induced activation of the WT promoter (Figure 6A, upper part, lanes 5-8), with more than 60% suppression when present at a 1:1 ratio with Ets-1 p51 (Figure 6A, lane 7). This was mediated through the EBS palindrome because mutation of both EBS abrogated any Cp17 effects (Figure 6A, middle part). Protein expression was confirmed by Western blot for Ets-1 p51, Cp17, and Erk-2 as a loading control (Figure 6A, lower part). Thus, Cp17 acts as a dominant negative for Ets-1 p51 transactivation of the *stromelysin-1* promoter mediated through the EBS palindrome.

Second, we examined the effect of Cp17 on the *collagenase-1* matrix metalloproteinase promoter, another Ets-1 target gene, which is synergistically activated by Ets-1 p51 and c-Jun/c-Fos (AP-1) complex, through the TPA (12-O-tetradecanoylphorbol-13-acetate) Oncogene Response Unit (TORU), composed of an EBS adjacent to an AP-1 site [40]. Luciferase transactivation assays were performed with *collagenase-1* promoter (WT or EBS/AP-1 mutant) using increasing concentrations of Cp17 with or without Ets-1 p51 and the c-Jun/c-Fos complex (Figure 6B). Cp17 repressed, in a dose-dependent manner, the synergistic activation of the *collagenase-1* promoter induced by Ets-1 p51 and the c-Jun/c-Fos complex (Figure 6B, upper part). This was mediated through the TORU, since its mutation abrogated any Cp17 effects (Figure 6B, middle part). Thus, Cp17 acts also as a dominant negative for Ets-1 p51 transactivation of the *collagenase-1* promoter mediated through the TORU.

We then checked the transcriptional response of the N-terminal Np36 fragment. The nucleotide sequence, which codes for amino acids 1-262 of Ets-1 p51, was cloned in a eukaryotic expression vector and used in luciferase transactivation assays. Results showed that Np36 does not inhibit the Ets-1 p51-induced transcriptional activation of the *stromelysin-*

1 and *collagenase-1* promoters, due to the absence of the DBD domain (Figure 6D). Thus, in contrast to Cp17 fragment, the Np36 fragment is not able to inhibit the transcriptional activity of Ets-1 p51.

Cp17 decreases the Ets-1 p51 target genes expression

Given the dominant-negative function of Cp17, we explored its effect on the expression of endogenous Ets-1 p51 target genes. To this aim, we performed RT-PCR analysis on HIG-82 rabbit synovial fibroblasts, which endogenously express the Ets-1 protein and represent a good model to study the transcriptional regulation of Ets-1 target genes [41]. We transfected these cells with an empty vector, with a human Ets-1 p51 expression vector or with both human Ets-1 p51 and Cp17 expression vectors. Results showed that the overexpression of human Ets-1 p51 alone induced an increase in the mRNA levels of well-known Ets-1 target genes, such as *gelatinase-B* and *uPA* (Figure 7; compare lane 2 and 3), indicating that Ets-1 p51 up-regulated the expression of these genes. However, the overexpression of both Ets-1 p51 and Cp17 decreased the mRNA levels of these target genes (Figure 7; lane 4). This shows that Cp17 inhibits the expression of endogenous Ets-1 target genes, confirming its dominant-negative function on Ets-1 p51.

DISCUSSION

Although previous studies have described the down-regulation of the Ets-1 p51 protein in cells undergoing apoptosis [31, 32], the molecular mechanism underlying this effect was not known. In this study, we demonstrated that the down-regulation of Ets-1 p51 protein in Jurkat T cells undergoing apoptosis is a consequence of its caspase-dependent cleavage. Caspase cleavage generated three C-terminal fragments, Cp14, Cp17 and Cp20, and one detectable N-terminal fragment, Np36. Screening the amino acid sequence of Ets-1 for putative caspase

cleavage sites made possible to identify three consensual sites (DXXD) located in the exon VII-encoded region: DSCD²⁶², a double site DSFD²⁸⁷SED²⁹⁰ and DRAD³¹³. Mutational analysis indicated that Ets-1 p51 processing occurs at these sites, thus generating the Cp20, Cp17 and Cp14 fragments, respectively. Cp17 was the major fragment generated during apoptosis, suggesting that DSFD²⁸⁷SED²⁹⁰ is the main cleavage site. The N-terminal Np36 fragment detected during apoptosis may be the N-terminal counterpart of the C-terminal Cp20 fragment generated through cleavage at the DSCD²⁶² site. Consistent with the location of the caspase sites in the alternate exon VII-encoded region, Ets-1 p42 was not processed by caspases in *in vitro* cleavage experiments, nor during apoptosis.

The exon VII-encoded region of Ets-1 is a regulatory domain described as (i) a regulator of DNA-binding [16, 17, 41], (ii) a mediator of protein-protein interactions [42] and (iii) a target of calcium-mediated phosphorylation [43, 44]. The cleavage of Ets-1 p51 and not Ets-1 p42 allocates a new function to the exon VII-encoded region, thus expanding on the functional differences between the two Ets-1 isoforms.

Cleavage of Ets-1 p51 in the exon VII-encoded region separate the transactivation domain — crucial for transcriptional activity — from the rest of the molecule but leave the DNA-binding domain intact. Therefore, C-terminal Ets-1 fragments containing the DBD should be able to bind to Ets-1 p51 target promoters without activating transcription, suggesting that they have a dominant-negative function. Our results confirmed this hypothesis for the major cleavage product, Cp17. Its DNA-binding and transcriptional properties were assessed on the EBS palindrome of the *stromelysin-1* promoter, which has been shown to be an important target gene for Ets-1 and is involved in the process of tumour invasion and metastasis [45]. Our results demonstrated that Cp17 competes with Ets-1 p51 for binding to the EBS palindrome of the *stromelysin-1* promoter, demonstrating its dominant-negative effect on Ets-1 p51 binding. This competition involves the formation of an intermediate

complex, suggesting that both proteins interact with each other on the EBS palindrome of the *stromelysin-1* promoter. Cp17 forms also a binary complex with the DNA, which may represent a transient complex facilitating the binding of a second Cp17 molecule to form a ternary complex. This is consistent with previous work that showed that deleting the N-terminal region of Ets-1 p51 spanning from amino acids 1-300 does not alter the binding stoichiometry for the EBS palindrome found in the *stromelysin-1* promoter [17, 18]. It is noteworthy that we did not detect any binary complexes involving Ets-1 p51 because full-length Ets-1 p51 more readily forms ternary complexes. Furthermore, Cp17 inhibited Ets-1 p51 transactivation of the *stromelysin-1* promoter. This effect may be the result of Cp17 binding to the palindromic EBS, which then becomes inaccessible to Ets-1 p51. Cp17 also repressed the Ets-1 p51-mediated transactivation of the *collagenase-1* promoter. This may result from the ability of Cp17 to replace Ets-1 p51 in the interaction with the AP-1 complex (c-Fos/c-Jun), required for combinatorial regulation. This is supported by the fact that the interaction of Ets-1 p51 with the AP-1 complex is mediated through the DBD domain, which is conserved in Cp17 [12]. The N-terminal Np36 fragment did not have any effect on the Ets-1 p51-induced transcriptional activation of either the *stromelysin-1* or the *collagenase-1* promoters. This is consistent with the inability of Np36 to bind DNA because it lacks the DBD domain. This confirms that the dominant-negative effect of Cp17 effectively results from its binding to the Ets-1 p51 target promoters. Finally, Cp17 decreased Ets-1-induced expression of *uPA* and *gelatinase-B*, two well-known target genes of Ets-1, confirming the dominant-negative function of Cp17 on Ets-1 p51.

Ets-1 functions have been largely studied through the use of artificial dominant-negative constructs made up of the DBD domain of the molecule [23, 46]. These constructs have helped to demonstrate, for instance, the involvement of Ets-1 in tumorigenesis and angiogenesis [46-48]. The Cp17 fragment represents a natural dominant negative generated

by proteolytic cleavage, able to inhibit the transactivation response induced by the full-length protein. However, in contrast to artificial DBD constructs, Cp17 possesses the two inhibitory domains flanking the DBD. Cp17 is thereby more specific, particularly for promoters that have either palindromic EBS or adjacent DNA elements and that can be activated only by overriding Ets-1 autoinhibition.

Along the same lines, we recently demonstrated that a novel Ets-1 isoform, p27, which lacks the Pointed and the transactivation domains but conserves the DBD flanked by the two inhibitory domains, also acts as a dominant negative of Ets-1 p51 [33]. The overexpression of this novel isoform in invasive mammary carcinoma cells represses proliferation, transformation and invasion, and reduces the growth of tumour xenografts in nude mice. The splice variant Ets-1 p27 and the Ets-1 p51 cleavage product Cp17, both of which possess the DBD and the inhibitory domains, represent natural dominant negatives of the full-length protein generated by two different mechanisms.

The caspase-mediated generation of dominant-negative fragments has also been described for several other transcription factors. For instance, NF- κ B p65/RelA subunit [8] and SRF [6] provide two examples of transcription factors in which caspase cleavage leads to separation of the transactivation domain from the DNA binding domain. These truncated proteins are transcriptionally inactive and also act as dominant-negative inhibitors [6, 8].

Since some studies have shown that Ets-1 plays a role in apoptosis through the control of gene expression critical to this process, we can assume that down-regulation of Ets-1 p51 during apoptosis could prevent this transcriptional program. Following this line of reasoning, the dominant-negative function of Cp17 may provide an additional mechanism to amplify the cell death process by inhibiting the Ets-1 p51-mediated expression of anti-apoptotic proteins. This would be consistent with a previous study demonstrating that artificial Ets-dominant negatives suppress Ets transcriptional activity and induce programmed cell death — mediated

by lower levels of c-myc expression — in thyroid carcinoma cell lines [23]. Although the anti-apoptotic target genes of Ets-1 p51 and their promoters are currently not well characterised, it would be interesting to assess the dominant-negative properties of Cp17 on these target genes. In addition, we demonstrated that caspase cleavage targets Ets-1 p51 without affecting the Ets-1 p42 isoform. Consequently, during apoptosis, the ratio between the two isoforms is modified in favour of the Ets-1 p42 isoform. Interestingly, it has been shown that, compared to Ets-1 p51, Ets-1 p42 is preferentially associated with the induction of apoptosis. Therefore, the modification of the ratio between the two isoforms may be a mechanism favouring cell death through the specific preservation of the pro-apoptotic isoform.

Taken together, our data suggest a novel mechanism of Ets-1 p51 regulation through caspase-mediated cleavage and generation of a dominant-negative fragment, which may play an active role during apoptosis.

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FIGURE LEGENDS

Figure 1 Caspase cleavage of Ets-1

(A) Cleavage of Ets-1 in Jurkat cells undergoing apoptosis. Jurkat cells were treated with staurosporin (1 μ M) for 4 h (lanes 2 and 4) or left untreated (lanes 1 and 3), in the presence (lanes 3 and 4) or in the absence (lanes 1 and 2) of zVAD-FMK (zVAD; 20 μ M). NS, non-specific band. **(B)** *In vitro* cleavage of Ets-1 by recombinant active caspase-3. Total cell lysates were incubated in the absence (lane 1) or in the presence (lane 2) of recombinant active caspase-3 (0.17 μ M) for 4 h at 37°C. **(A and B)** Cell lysates (30 μ g total proteins) were analysed by Western blot using an anti-Ets-1 (C-20) directed against the DBD located in the C-terminal region. The membrane was stripped and reprobed sequentially first with an anti-Ets-1 (N-276) antibody directed against its pointed domain located in the N-terminal region, then with an anti-cleaved caspase-3 antibody and, finally, with an anti-Erk-2 antibody as a loading control. Arrows indicate cleavage fragments. Molecular mass, indicated on the left of each panel, is given in kDa.

Figure 2 Ets-1 p51, but not Ets-1 p42, is cleaved by caspase-3 *in vitro*

(A) *In vitro* cleavage of Ets-1 isoforms by recombinant active caspase-3. pcDNA3 expression vectors without insert (control; lanes 1 and 2), encoding Ets-1 p51 (lanes 3 and 4) or Ets-1 p42 (lanes 5 and 6) were transfected in HEK-293 cells. After 48 h of transfection, total cell

lysates were incubated in the absence (lanes 1, 3 and 5) or in the presence (lanes 2, 4 and 6) of recombinant active caspase-3 (0.17 μ M) for 4 h at 37°C. **(B)** *In vitro* cleavage of Ets-1 isoforms by a set of recombinant active caspases. pcDNA3 expression vectors without insert (lane 1), encoding Ets-1 p51 (lanes 2-7) or Ets-1 p42 (lanes 8-13) were transfected in HEK-293 cells and incubated with recombinant active caspase-3 (lanes 3 and 9), -6 (lanes 4 and 10), -7 (lanes 5 and 11), -8 (lanes 6 and 12) or -9 (lanes 7 and 13). **(A and B)** Cell lysates (30 μ g total proteins) were analysed by Western blot using an anti-Ets-1 (C-20) antibody directed against its C-terminal region and an anti-Ets-1 (N-276) antibody directed against its N-terminal region. Arrows indicate cleavage fragments. Molecular mass, indicated on the left of each panel, is given in kDa.

Figure 3 Ets-1 p51, but not Ets-1 p42, is cleaved in HEK-293 and Jurkat cells undergoing apoptosis

(A) Cleavage of transfected Ets-1 isoforms in HEK-293 cells undergoing apoptosis. pcDNA3 expression vectors without insert (control; lanes 1-4), encoding Ets-1 p51 (lanes 5-8) or Ets-1 p42 (lanes 9-12) were transfected in HEK-293 cells. After 24 h, cells, previously incubated with (lanes 3-4, 7-8 and 11-12) or without (lanes 1-2, 5-6 and 9-10) zVAD-FMK (zVAD; 20 μ M), were treated with staurosporin (10 μ M) (lanes 2, 4, 6, 8, 10 and 12) or left untreated (lanes 1, 3, 5, 7, 9 and 11) for 8 h. Cell lysates (30 μ g total proteins) were then analysed by Western blot using an anti-Ets-1 (C-20) antibody and an anti-Erk-2 antibody as a loading control. **(B)** Cleavage of Ets-1 isoforms in Jurkat cells undergoing apoptosis. Jurkat cells, previously incubated with (lane 5) or without (lanes 1-4) zVAD-FMK (zVAD; 20 μ M), were treated with staurosporin (1 μ M) for the indicated time. Cells were then lysed and analysed by Western blot using an anti-Ets-1 (C-20) antibody. The membrane was stripped and reprobed sequentially using an anti-cleaved caspase-3 antibody and then an anti-Erk-2 antibody as a

loading control. NS, non-specific band. (C) Luminescence from the Western blot was recorded with a CCD camera and the expression levels of the Ets-1 isoforms and the Cp17 fragment were quantified. The percentage of expression was calculated using the signal intensity for Ets-1 p51 at $t = 0$ h as the reference ($n=3$; error bars indicate S.D.).

Figure 4 Identification of the caspase cleavage sites in Ets-1 p51 processing

(A, a) Schematic representation of Ets-1 p51 and Ets-1 p42 proteins, showing the putative caspase cleavage sites (DSCD²⁶², DSFD²⁸⁷SED²⁹⁰ and DRAD³¹³) in the exon VII-encoded region of Ets-1 p51. Pointed domain, transactivation domain (TAD), inhibitory domains (I) and DNA binding domain (DBD) are indicated. The caspase cleavage sites are boxed. (A, b) Schematic representation of Cp20, Cp17 and Cp14 fragments generated by cleavage of Ets-1 p51 at the putative caspase cleavage sites. (A, c) Sequences of wild-type and mutant (D1N, D3N and D4N) Ets-1 p51, in which Asp (D) residues were mutated to Asn (N). (B) Cleavage of wild-type and mutant Ets-1 p51 by recombinant active caspase-3. pcDNA3 expression vectors encoding wild-type (WT, lanes 1-2) or mutant (D1N, lanes 3-4; D3N, lanes 5-6; D4N, lanes 7-8) Ets-1 p51 were transfected in HEK-293 cells. After 48 h of transfection, cell lysates (30 μ g total proteins) were incubated in the absence (lanes 1, 3, 5 and 7) or in the presence (lanes 2, 4, 6 and 8) of recombinant active caspase-3 (0.17 μ M) for 4 h at 37°C. (C) Cleavage of wild-type and mutant Ets-1 p51 in MDCK cells undergoing apoptosis. pcDNA3 expression vectors encoding wild-type (WT, lanes 1-2) or mutant (D1N, lanes 3-4; D3N, lanes 5-6; D4N, lanes 7-8) Ets-1 p51 were transfected in MDCK cells. After 48 h of transfection, cells were treated with anisomycin (10 μ M) (lanes 2, 4, 6 and 8) or left untreated (lanes 1, 3, 5, 7) for 8 h. (B and C) Cell lysates (30 μ g total proteins) were analysed by Western blot using an anti-Ets-1 (C-20) antibody. Longer exposure of the same Ets-1 Western blot is shown in the lower panel.

Figure 5 Cp17 binds to the Ets-binding sites (EBS) palindrome of the *stromelysin-1* promoter and competes with Ets-1 p51 for binding

(A) Structure of the Cp17 fragment compared to the full-length Ets-1 p51. Pointed domain, transactivation domain (TAD), inhibitory domains (I) and DNA binding domain (DBD) are indicated. **(B)** Lysates (30 µg) from HEK-293 cells transiently transfected with the empty vector (1 µg, lanes 1-4), the Cp17 expression vector (1 µg; lanes 9-12) or the Ets-1 p51 recombinant protein (1 µg; lanes 5-8) were incubated with wild-type (WT, lanes 1, 3-5, 7-10, 11 and 12) or EBS mutant (lanes 2, 6 and 10) ³²P-labelled DNA probes (0.5 ng) in the absence (lanes 1-2, 5-6 and 9-10) or in presence of unlabeled competitors (WT 800X, lanes 3, 7 and 11 or EBS mutant 800X, lanes 4, 8 and 12). DNA/protein complexes were separated by electrophoresis in a 5% non-denaturing polyacrylamide gel. Arrow 2' indicates potential binary Cp17/DNA complex. Arrows 3 and 3' indicate potential ternary Ets-1 p51/DNA/Ets-1 p51 and Cp17/DNA/Cp17 complexes, respectively. **(C)** Lysates (30 µg) from HEK-293 cells transiently transfected with the empty vector (1 µg; lanes 1) or increasing concentrations of the Cp17 expression vector (0.45-1 µg; lanes 2-5 and 7-10) in the absence (lanes 1-5 and 10) or in the presence of Ets-1 p51 recombinant protein (0.5 µg; lanes 6-10) were incubated with the WT ³²P-labelled DNA probe (0.5 ng). DNA/protein complexes were separated by electrophoresis in a 5% non-denaturing polyacrylamide gel. Arrows 3, 3' and 2', as above; arrow i indicates the putative Ets-1 p51/DNA/Cp17 intermediate ternary complex.

Figure 6 Cp17, but not Np36, acts as a dominant negative for transactivation of the *stromelysin-1* and *collagenase-1* promoters mediated by Ets-1 p51

(A) Effect of Cp17 on basal and Ets-1 p51-induced *stromelysin-1* promoter activity. pGL3 luciferase reporter constructs driven by the *stromelysin-1* promoter (200 ng; WT, left panel;

EBS mutant, middle panel) were transfected into HEK-293 cells in the absence (-) or in the presence (+) of Ets-1 p51 expression vector (100 ng; lanes 5-8) and with increasing amounts of the Cp17 expression vector (0-200 ng; lanes 2-4 and 6-8). **(B)** Effect of Cp17 on Ets-1 p51-induced *collagenase-1* promoter activity. pGL2 luciferase reporter constructs driven by the *collagenase-1* promoter (100 ng; WT, left panel; TORU mutant, middle panel) were transfected into HEK-293 cells in the absence (-) or in the presence (+) of Ets-1 p51 (100 ng; lanes 2 and 4-7), c-Jun (100 ng; lanes 3-7), c-Fos (100 ng; lanes 3-7) and increasing amounts of the Cp17 expression vector (0-100 ng; lanes 5-7). **(C)** Effect of Np36 on Ets-1 p51-induced *stromelysin-1* and *collagenase-1* promoter activities. WT *stromelysin-1* or *collagenase-1* promoter reporter vector (*stromelysin-1*, 200 ng, left panel; *collagenase-1*, 100 ng, middle panel) were transfected into HEK-293 cells in the absence (-) or in the presence (+) of the Ets-1 p51 expression vector (100 ng; *stromelysin-1*, lanes 2-5; *collagenase-1*, lanes 2 and 4-7), c-Jun (100 ng; *collagenase-1*, lanes 3-7), c-Fos (100 ng; *collagenase-1*, lanes 3-7) and with increasing amounts of the Np36 expression vector (*stromelysin-1*, 0-200 ng, lanes 3-5; *collagenase-1*, 0-100 ng, lanes 5-7). **(A, B and C)** Luciferase activity, measured 48 h after transfection, was normalised to *renilla* activity using a pRLnull vector (1.25 ng) and is expressed as the percentage of WT construct activity induced by Ets-1 p51 (*stromelysin-1*) or by the synergistic effect of Ets-1 p51 and c-Fos/c-Jun (*collagenase-1*). Cell lysates were analysed by Western blot using indicated antibodies to assess protein expression. Results are the average of two experiments performed in triplicate.

Figure 7 Cp17 decreases the Ets-1 p51 target genes expression

Reverse transcription (RT)-PCR analysis of Ets-1 p51 target genes. Empty vector (1 µg; lane 2), human Ets-1 p51 expression vector (500 ng; lane 3) or human Ets-1 p51 (500 ng) and Cp17 (500 ng) expression vectors (lane 4) were transfected into HIG-82 cells. After 48 h,

861 RNAs of cells were retrotranscribed and cDNAs were amplified by PCR using *uPA* and
862 *gelatinase B* primers (see Experimental). Amplified PCR products were resolved on an
863 agarose gel stained with ethidium bromide. Amplification of human *ets-1* was used to assess
864 expression of the transfected Ets-1 p51 expression vector; amplification of *gapdh* with
865 specific primers was used as a loading control and amplification without template as a
866 negative control (NC, lane 1).

Figure 1

Figure 2

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20090877

Figure 3

Figure 4

B

C

Figure 5

Figure 6

Figure 7