The human homologue of *Caenorhabditis elegans* CED-6 specifically promotes phagocytosis of apoptotic cells

Elke Smits*, Wim Van Criekinge*, Geert Plaetinck and Thierry Bogaert

A key feature of the process of programmed cell death (apoptosis) is the efficiency with which the dying cells are recognized and engulfed by phagocytes [1]. Apoptotic cells are rapidly cleared either by neighbouring cells acting as semi-professional phagocytes or by experts of the macrophage line, so that an inflammatory response is avoided [2]. The *Caenorhabditis elegans* gene *ced-6* is required for efficient engulfment of apoptotic cells [3] and is one of a group of genes that define two partially redundant parallel pathways for the engulfment process [4,5]. These pathways may be conserved across evolution, as two other engulfment genes have human homologues. A CED-5 homologue is part of a human CrkII–DOCK180–Rac signaling pathway proposed to mediate cytoskeletal reorganization [6–8] and a CED-7 homologue is similar to the ABC transporters [9,10]. Here, we report the cloning and characterization of human CED-6, a human homologue of *C. elegans* CED-6. The 34 kDa hCED-6 protein is expressed in most tissues, some human cancer cells, and in primary human macrophages. We developed an assay that quantitates the phagocytic activity of mammalian macrophages: the number of apoptotic cells that have been internalized is measured by the uptake of lacZ-positive apoptotic cells by adherent transgenic macrophages. The results of this assay demonstrate that overexpression of hCED-6 promotes phagocytosis only of apoptotic cells and suggest that hCED-6 is the mammalian orthologue of *C. elegans* CED-6 and is a part of a highly conserved pathway that specifically mediates the phagocytosis of apoptotic cells.

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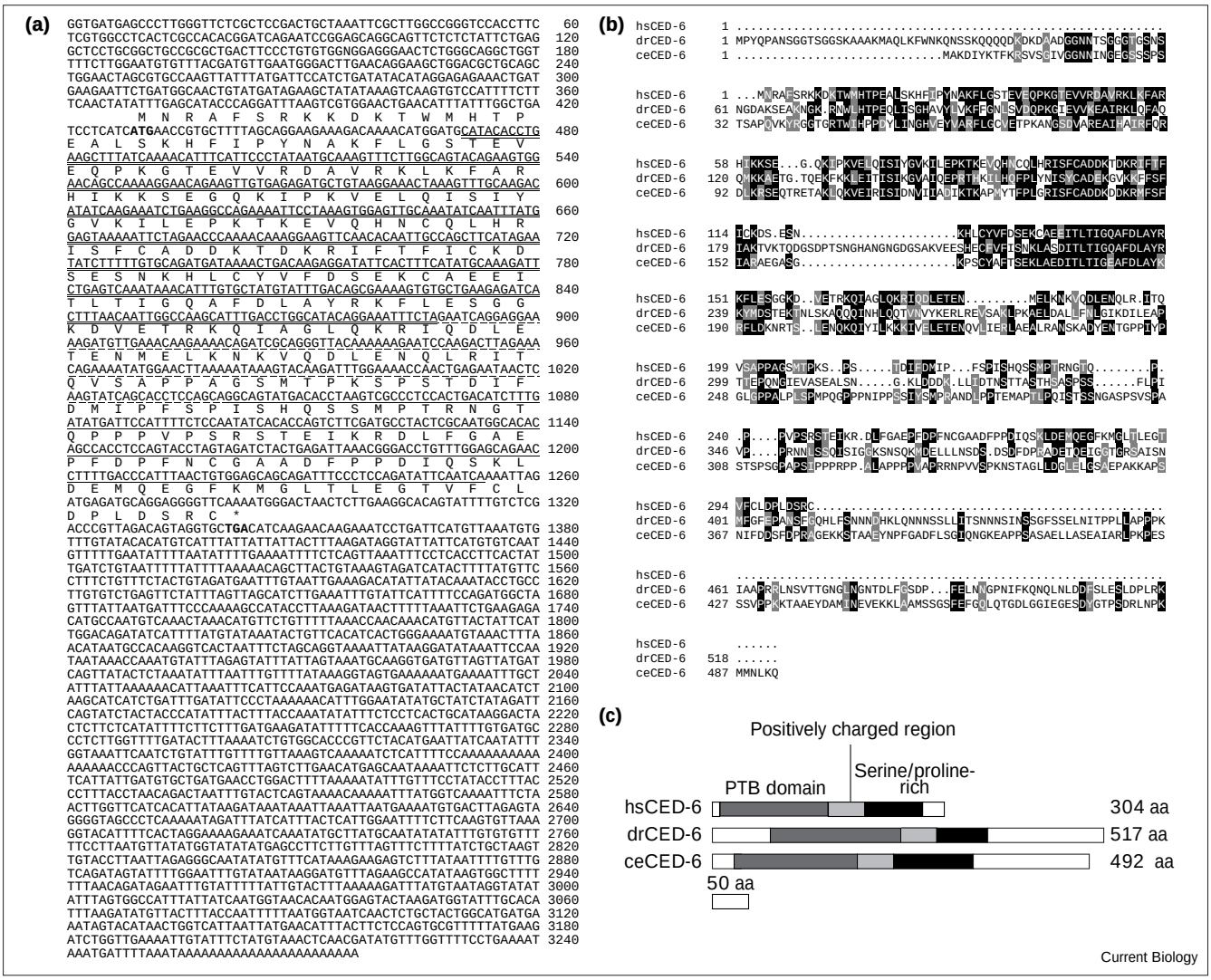
Results and discussion

Human *CED-6*, the human homologue of *C. elegans ced-6*, is located on chromosome 2, region q32.2–q33. The hCED-6 protein (304 amino acids) contains a putative phosphotyrosine-binding (PTB) domain at its amino terminus, a positively charged region and a carboxy-terminal serine/proline-rich region (Figure 1a). It is structurally similar to *C. elegans* CED-6 (492 amino acids) and *Drosophila melanogaster* CED-6 (517 amino acids; Figure 1b). The sequence of the latter was obtained from overlapping expressed sequence tags (ESTs). Interestingly, hCED-6 shows the highest homology to the other two proteins in the amino-terminal region, and the carboxy-terminal part (downstream of the serine/proline-rich region) is absent (Figure 1c). Although the PTB domain was initially thought to end at Asp194, it might extend further, including part of the predicted coiled-coil region, which is clearly conserved among the three species. Several PTB-domain-containing proteins, such as Shc, Sck, Numb, FE65, Disabled, DOC-2, P96 and IRS-1, have been found to act as adaptor molecules in signal transduction pathways [11]. This might be achieved by their ability to bind to phosphorylated tyrosines on activated receptors and/or phospholipids, and by their recruitment of downstream molecules to form a signalling complex [12]. The interaction of hCED-6 with candidate receptors remains to be determined.

The expression of *hCED-6* mRNA was examined by both northern blotting and reverse-transcriptase (RT)–PCR. *CED-6* is widely expressed in human tissues and human cancer cell lines (Figure 2). The transcript size determined by northern blotting was 3.2 kb, confirming the size estimated from cDNA length. Expression of *hCED-6* was also detected by RT–PCR in primary human macrophages (data not shown). Savill *et al.* [13] have previously shown that neighbouring cells such as glomeral mesangial cells also function in clearance of apoptotic neutrophils in the kidney. The CED-6 signal might also be present in most human cells that act as phagocytes and in professional phagocytes of the macrophage line, as well as in tumour cells.

To determine whether the function of hCED-6 in the clearance of apoptotic cells is conserved in mammals, we developed an assay that quantitates the phagocytic activity of mammalian macrophages by automatically measuring the number of apoptotic cells that have been internalized. The presently known technique for quantitating the phagocytosis of apoptotic cells is based on time-consuming microscopic counting of the number of apoptotic cells inside phagocytes [14]. There is thus a need for a reliable and reproducible method that does not require scoring by visual inspection and is therefore more suitable for use in

Figure 1

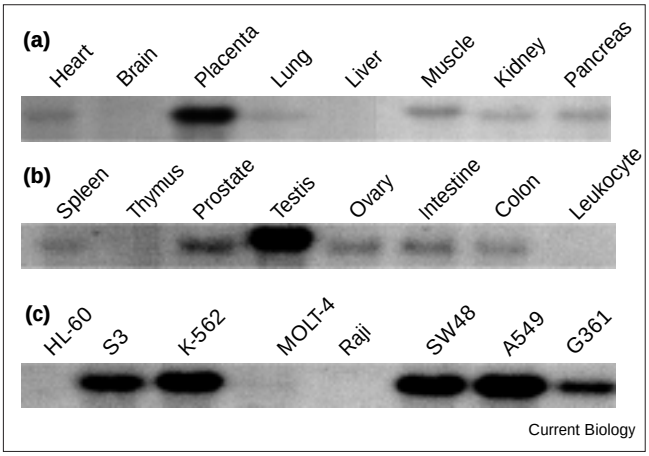


Structural characterization of human CED-6 (GenBank accession number AF191771). (a) Consensus cDNA sequence (3.2 kb) and protein sequence (molecular weight 34.4 kDa) of human CED-6. Protein domains are marked as follows: double underline, PTB domain; dashed underline, positively charged region; single underline, serine/proline-rich region; bold, start and stop codons. (b) A multiple alignment of hCED-6 (hsCED-6) with *D. melanogaster* and *C. elegans* CED-6 [3] (drCED-6 and ceCED-6, respectively). Black and grey boxes indicate identical and similar amino acids, respectively. (c) Schematic structure of human, *D. melanogaster* and *C. elegans* CED-6 [3]. The PTB domain, positively charged region and serine/proline-rich domain are indicated; aa, amino acids.

automated medium- and high-throughput screening. In our assay, mouse J774 macrophages in 96-well plates were challenged with apoptotic mouse Ba/F3 pre-B cells stably transfected with the reporter gene lacZ, which encodes β-galactosidase. Apoptosis of Ba/F3 cells was induced by growth-factor withdrawal 20 hours before the assay [15]. These were optimized conditions, during which 20–30% of the Ba/F3 cells are annexin-V-positive and less than 5% are propidium-iodide-positive, showing that they are in the early stages of apoptosis [16] (data not shown). After washing the non-ingested apoptotic cells away, fluorescein di-β-D-galactopyranoside, a fluorogenic substrate for β-galactosidase, was added. Green fluorescence intensity was measured with an automatic plate reader using 480 nm excitation and 520 nm emission. This phagocytosis assay was validated by showing that exclusively engulfed apoptotic cells were measured (see Supplementary material).

As shown in Figure 3a, the uptake of apoptotic cells by J774 macrophages transfected with *hCED-6* is 40–50% ($p < 0.005$) higher than uptake by J774 cells or J774 cells transfected with the empty vector. CD36 is one of the adhesion molecules on the surface of macrophages that has been implicated in the clearance of apoptotic cells [17,18].

Figure 2



Expression pattern of human *CED-6* in normal human tissues and cancer cell lines. (a) Human multiple tissue MTN-1 blot (Clontech) containing RNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. (b) Human multiple tissue MTN-II blot (Clontech) containing RNA from spleen, thymus, prostate, testis, ovary, small intestine, colon (mucosal lining) and peripheral blood leukocytes. (c) Blot of RNA from human cancer cell lines: HL-60, promyelocytic leukaemia; S3, HeLa cells; K-562, chronic myelogenous leukaemia; MOLT-4, lymphoblastic leukaemia; Raji, Burkitt's lymphoma; SW48, colorectal adenocarcinoma; A549, lung carcinoma; G361, melanoma. All blots were hybridized with a human *CED-6* probe. The transcript size determined by northern blotting was 3.2 kb, confirming that estimated from cDNA length.

J774 macrophages transfected with CD36 were used as a specific positive control in our phagocytosis assay of apoptotic cells and were shown to have an 80% ($p < 0.001$) higher uptake than the control macrophages. We then explored whether overexpression of hCED-6 affected phagocytosis in general or specifically enhanced the engulfment of apoptotic cells. We found that viable cells from the Ba/F3 cell line failed to interact with macrophages in our assay (Figure 3b). Also, there was no difference in phagocytosis of labelled *Escherichia coli* between control J774 cells and J774 cells stably transfected with *hCED-6* and CD36 (Figure 3c). These results suggest that hCED-6 specifically mediates phagocytosis of apoptotic cells.

In conclusion, we have demonstrated that *hCED-6* is an orthologue of *C. elegans ced-6*, as it is structurally and functionally conserved across evolution. The most obvious sequence feature of hCED-6 is a highly conserved PTB domain, which strongly suggests that transduction of the engulfment signal within the macrophage involves tyrosine phosphorylation. Furthermore, we established a functional requirement for hCED-6 during the phagocytosis process in mammals, which appears to be specific to apoptotic cells. We suggest that CED-6 is involved in a pathway for clearance of the apoptotic bodies that is conserved through evolution, and that there is a common molecular mechanism responsible for the engulfment of apoptotic cells in all metazoans.

Supplementary material

Additional methodological details and a figure showing validation of the mammalian assay for phagocytosis of apoptotic cells are available at http://current-biology.com/supmat/supmatin.htm.

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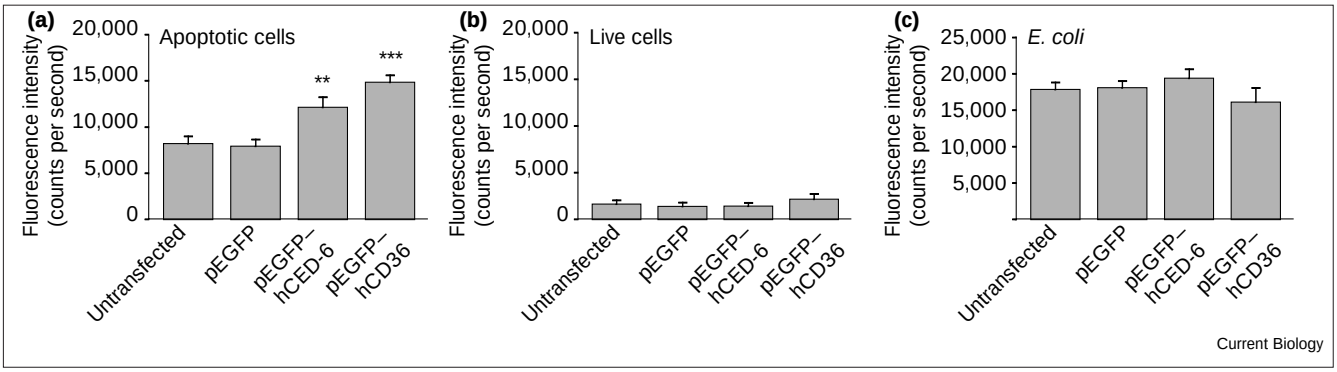
Figure 3



Functional characterization of human CED-6 in a new mammalian phagocytosis assay. Phagocytosis of (a) apoptotic Ba/F3 cells, (b) live Ba/F3 cells, and (c) *E. coli* by J774 macrophages that were left untransfected or stably transfected with plasmids encoding enhanced green fluorescent protein (EGFP), EGFP fused to human CED-6, and EGFP fused to human CD36 (see Supplementary material). EGFP was used to check transfection efficiency; the weak EGFP signal in transfected macrophages did not interfere with the fluorescence readout of the phagocytosis assay (data not shown). Fluorescence intensity is proportional to the number of macrophages that had ingested β-galactosidase-expressing Ba/F3 cells or that had ingested fluorescein-labelled *E. coli*. Data are expressed as the mean ± standard error of the mean ($n = 24$). Comparison with uptake by J774 cells: **, $p < 0.005$, ***, $p < 0.001$.

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Supplementary material

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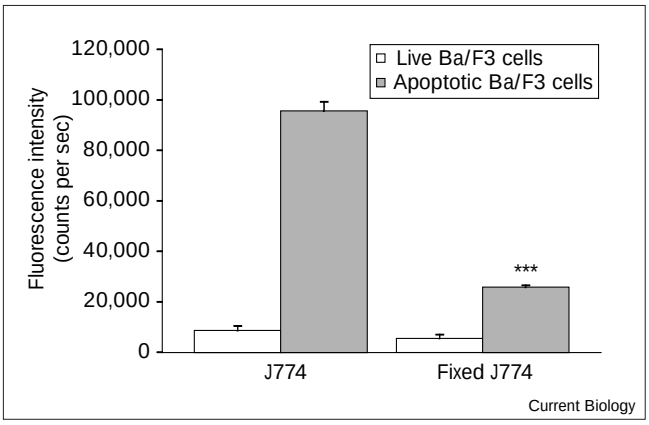
Figure S1



Validation of the phagocytosis assay of apoptotic cells. Phagocytosis of apoptotic Ba/F3 cells and live Ba/F3 cells by J774 macrophages and J774 macrophages fixed with 3.7% formaldehyde to discriminate between mere binding and active engulfment of apoptotic cells. Fluorescence intensity is proportional to the number of macrophages that had ingested β-galactosidase-expressing Ba/F3 cells. Data are expressed as the mean ± standard error of the mean (n = 7). Comparison with uptake by J774 cells: ***, $p < 0.001$.

Supplementary material and methods

Cloning

NCBI database searches using the BLAST algorithm detected several ESTs with statistically significant similarities to *C. elegans CED-6* (GenBank accession numbers AA159394, R76378, THC117484, R65982, AA369714, R65983, D82787, AA443368, AA431995, R33389, R53881 and H03749). By using overlapping PCR with the primers 5′-GCATACACCTGAAGCTTTATCAAAAC-3′ and 5′-GATTTCTTGATATGTCTTGCAAACT-3′ on AA159394 and 5′-AGTTTGCAAGACATATCAAGAAATC-3′ and 5′-CAAATGCTTG-GCCAATTGTTAAAGTGATCTCTTCAGCACACTTTTCGCTGTCAA-ATACATAG-3′ on R76378, we were able to reconstitute a full open reading frame (see Figure 1a). This sequence was further verified by 5′ rapid amplification of cDNA ends and colony hybridisation. The *D. melanogaster* homologue was identified using both *C. elegans CED-6* and human *CED-6* in database searches. Subsequently, two *D. melanogaster* ESTs, LD20509 (GenBank accession number AA540723) and LD09044 (AA390841), were sequenced and aligned. The cDNA fragment containing the full length sequence of *hCED-6* was subcloned into the pEGFP-n3 eukaryotic expression vector (Clontech) cut with *Bgl*II and *Sal*I.

CD36 (GenBank accession number M98399) was amplified from a placenta cDNA library with primer 5′-CGGACTCAGATC-TATGGGCTGTGACCGGAACTGT-3′, which was designed to have a *Bgl*II restriction site, and primer 5′-GCGGTACCGTCGACTTTTAT-TGTTTTCGATCTGCATGCAC-3′, which has a *Sal*I site. The ampli-fied fragment including the full-length sequence of CD36 was subcloned into the pEGFP-n3 eukaryotic expression vector cut with *Bgl*II and *Sal*I.

Cell culture

The J774A.1 mouse monocyte-derived macrophage cell line (American Type Culture Collection code TIB67) was maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% Myoclone super plus fetal bovine serum (Life Technologies). The growth factor interleukin-3 (IL-3)-dependent murine Ba/F3 pre-B-cell line was cultured as described previously [S1].

Northern blotting

Human multiple tissue northern blots MTN-1 and MTN-II (Clontech), which contain in each lane 2 μg poly-A^+ RNA from a different human tissue or cancer cell line, were hybridized with a *hCED-6* probe comprising the open reading frame according to the manufacturer's instructions.

RT–PCR

Human primary macrophages were isolated and cultured as described previously [S2]. RNA was isolated from human primary macrophages using the RNeasy mini kit (Qiagen). Starting from this RNA, first-strand cDNA was generated using the Ready-To-Go T-primed first-strand kit (Pharmacia Biotech). PCR was performed with primers 5′-CGCAAG-GATCCCCATGAACCGTGCTTTTAGCAGGAAG-3′ and 5′-GATC-TACTAGGTACTGGAG-3′ followed by nested PCR with primers 5′-CGCAAGGATCCCCATGAACCGTGCTTTTAGCAGGAAG-3′ and 5′-GCGGATGGTACCGTCGACTGCTGATACTTGAGTTATTCTCAG-3′. PCR products were analyzed on agarose gels and subsequently purified, cloned and sequenced.

Transfection

J774 cells were transfected with the plasmids pEGFP-n3 (Clontech), human full length *CED-6* cloned into the multicloning site of pEGFP-n3, and human full length CD36 cloned into the multicloning site of pEGFP-n3. Double-pulse electroporation of 3×10^6 cells and 30 μg DNA was performed with Easyject Plus electroporator system (Equibio). Electroporation parameters for the first and second pulse were, respectively, a voltage of 750 V and 150 V; a capacitance of 25 and 1500 μF; and a resistance of 99 Ω for both pulses. Clones were selected after 72 h with 400 μg/ml G418 antibiotic (Duchefa), and checked for GFP expression. The weak GFP expression of trans-fected macrophages did not interfere with the fluorescence readout of the phagocytosis assay (data not shown). Ba/F3 cells were transfected by electroporation as described previously [S3].

Phagocytosis assays

Parental or stably transfected J774 cells (1×10^5) were dispensed into 96-well plates (Costar) 36 h before phagocytosis assays. Ba/F3 cells that had been deprived of IL-3 by washing three times in IL-3-free medium 20 h before phagocytosis assays were used as a source of apoptotic cells. Ba/F3 cells cultured in the presence of IL-3 were taken as a control.

Ba/F3 cells (1×10^6) in 100 μl DMEM were overlaid onto wells of macrophages. After 1 h incubation at 37°C in 5% CO_2, wells were washed three times with PBS (Life Technologies). Fluorescein di-β-D-galactopyranoside (Molecular Probes; 10 μM) was added to each well and the plates were incubated in the dark for 1 h at room temperature. Microscopic analysis showed Ba/F3 cells phagocytosed by J774 cells. Green fluorescence intensity was measured with an automatic fluores-cence plate reader Victor 2 (Wallac) using 480 nm excitation and 520 nm emission.

Supplementary references

S1. Palacios R, Henson G, Steinmetz M, Mckearn JP: Interleukin-3 supports growth of mouse pre-B-cell clones in vitro. *Nature* 1984, 309:126-131.

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