CREB is cleaved by caspases during neural cell apoptosis

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Abstract Programmed cell death, or apoptosis, is a tightly regulated process mediated by selective cleavage of proteins by caspases, resulting in ordered destruction of the cell. In addition to structural proteins, proteins that mediate anti-apoptotic signal transduction are also substrates; their destruction eliminates potential futile attempts to escape execution. We asked whether cAMP response element binding protein (CREB), a transcription factor that mediates nerve growth factor (NGF) survival signals, is a target for caspases during apoptosis. CREB was specifically cleaved by caspases in neuroblastoma extracts, and in cells induced to undergo apoptosis by staurosporine. The destruction of CREB eliminates a key factor that could reverse apoptosis.

Key words: Caspase; Apoptosis; Signal transduction; cAMP response element binding protein; Neuroblastoma; In vitro reconstitution

1. Introduction

Apoptosis, a quick, clean cellular execution, is carried out through the activation of caspases, which proteolytically cleave and activate downstream factors that disassemble the cell, such as the actin-severing protein gelsolin [1] and DNase (cleavage of ICAD releases the DNase CAD) [2]. Caspases also inactivate proteins required for survival, such as poly-ADP-ribose polymerase (PARP) [3] and several anti-apoptotic signal transduction molecules, including Ras-GAP, Raf-1, MEKK1, and Akt [4,5]. Since caspase activity plays a role in the pathogenesis of neurodegenerative disease [6], and caspase inhibition delays progression of neurodegeneration, for example in mouse models of Huntington's disease [7], it is of biological interest to characterise caspase substrates in neural apoptosis.

CREB (cAMP response element binding protein) regulates many aspects of neuronal function such as nerve cell excitation, CNS development, long-term memory formation, and circadian rhythms [8]. CREB plays a key role in regulating neuronal survival and differentiation in response to neurotrophic factors NGF, BDNF, FGF and IGF-1 [9–12]. CREB is activated as a transcription factor by phosphorylation at Ser-133, through both the Ras/Raf/Mek/Erk/Rsk and the PI3K/PDK/Akt pathways [13–16]. Phospho-CREB accumulates in the nucleus in response to retrograde transport of the NGF receptor tyrosine kinase, TrkA [17], which is presumably conveyed to the cell body via 'signalling vesicles' [18]. CREB appears to be a primary transcriptional activator of the anti-apoptotic gene, *bcl-2* [14,16,19,20]. Inhibition of CREB activity induces apoptosis in sympathetic neurones [19], while CREB overexpression inhibits apoptosis induced by okadaic acid [21].

Many metabolic pathways are regulated in both forward and reverse directions, which ensures that futile cycles, reactions in both directions simultaneously, do not occur. The emerging picture is that the regulation of apoptosis follows the same scheme: signalling proteins that turn off the cell death programme are selectively destroyed. We now present evidence that the transcription factor CREB is a target of caspases during neural apoptosis both in vitro and in vivo.

2. Materials and methods

All reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted.

2.1. Cell culture and preparation of cytosolic extracts and nuclei

Human neuroblastoma SY5Y cells and rat phaeochromocytoma PC12 cells were cultured as described [5]. Nuclei and cytosol were prepared exactly as described [5].

2.2. In vitro reconstitution of apoptosis and visualisation of chromatin condensation

Aliquots of 40 μl of SY5Y cytosol (500 μg protein) were incubated with 5×10^6 PC12 nuclei in the absence or presence of 2 μM cytochrome *c*, 1 μM DEVD-CHO (Biomol, Plymouth Meeting, PA, USA), 1 μM IETD-CHO (Biomol), 1 μM YVAD-CHO (Biomol), 100 μM z-VAD-fmk (Enzyme Systems Products, Livermore, CA, USA), 1 mM sodium orthovanadate, 5 μM okadaic acid (Life Technologies, Gaithersburg, MD, USA), an ATP-regenerating system (8 mM creatine phosphate, 1 mM ATP, and 5 μg/ml creatine kinase) or ATP-depleting system (5 mM glucose and 14.2 U/ml hexokinase, Serva, Heidelberg, Germany) and made up to a final volume of 50 μl with cytosol buffer. Reactions were incubated at 37°C for 4 h.

Visualisation of chromatin condensation with Hoechst 33342 (Molecular Probes, Eugene, OR, USA) was carried out as previously described [5].

2.3. Immunoblotting

Protein was measured as described [5] and equivalent amounts of cytosolic and nuclear proteins were resolved on SDS–polyacrylamide gels and transferred to nylon-reinforced nitrocellulose (OptiTran, Schleicher and Schuell, Dassel, Germany). Membranes were probed with rabbit polyclonal antibodies against PARP (Roche Molecular Biochemicals, Auckland, New Zealand), phospho-specific CREB (Ser-133) and CREB (gifts from Michael Comb, New England Biolabs, Beverly, MA, USA), caspase-9 (Pharmingen, San Diego, CA, USA), caspase-6 (Biosource, Nivelles, Belgium), ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and caspase-3 (mouse monoclonal from Transduction Laboratories, Lexington, KY, USA). Proteins were detected using horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies (Amersham, Bucking-

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hamshire, UK) and immunoblots were visualised using enhanced chemiluminescence Western blotting detection reagents (Amersham).

2.4. Staurosporine-induced apoptosis

Six to eight plates of confluent SY5Y cells were washed and harvested in serum-free RPMI 1640 medium prior to reculturing for various times at 37°C in serum-free medium containing 0.5 μM staurosporine. For the caspase activity assays, SY5Y cells recultured for 24 h in medium containing 10% foetal calf serum were used as a control. Protein lysates were prepared from cells according to the method of Wang et al. [22].

2.5. Caspase activity assays

Cleavage of the fluorogenic caspase substrates DEVD-AFC, IETD-AFC, YVAD-AFC (Biomol) and VEID-AFC (Pharmingen) were measured in a Perkin-Elmer LS50B luminescence spectrophotometer (excitation 400 nm; emission 505 nm). Activities are expressed as fold induction over a non-apoptotic control.

3. Results and discussion

We examined whether CREB was affected during cytochrome *c*-mediated apoptosis in extracts from neural cells. Previously, we identified the pro-survival kinase Akt as a caspase substrate during neural cell apoptosis using in vitro reconstitution of apoptosis [5]. Exogenously added cytochrome *c* activates the Apaf-1/caspase-9 signalling cascade [23] in cytosol derived from human neuroblastoma (SY5Y) cells [5]. CREB was cleaved in the reactions where cytochrome *c* was added to initiate the apoptotic mechanism, yielding a cleavage fragment of approximately 30 kDa (Fig. 1A, lanes 2 and 5). Production of this cleavage fragment of CREB correlated with caspase-9 and caspase-3 activation, indicated by the presence of cleavage fragments p37 and p17, respectively (Fig.

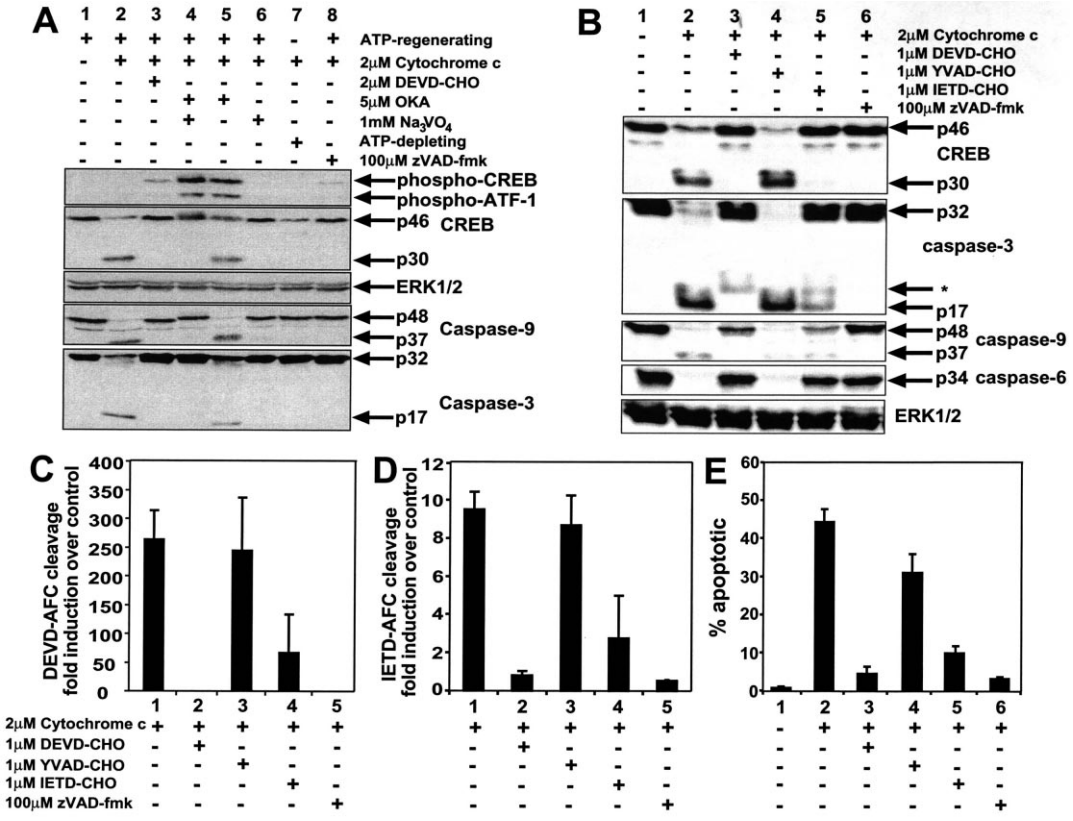

Fig. 1. CREB cleavage during in vitro reconstitution of apoptosis is caspase-dependent. A: CREB cleavage is specific and correlates with caspase activation. Rat phaeochromocytoma (PC12) nuclei were incubated in the presence of cytosol harvested from growing human neuroblastoma (SY5Y) cells, in the presence of an ATP-regenerating system (lanes 1–6, and 8) or an ATP-depleting system (lane 7), with the following additions: cytochrome *c* (lanes 2–8), DEVD-CHO (lane 3), orthovanadate (lanes 4 and 6), okadaic acid (lanes 4 and 5), or z-VAD-FMK (lane 8). Reactions were incubated at 37°C for 4 h, after which the samples were analysed by immunoblotting using an antibody specific for proteins labeled at the right of panels. The phospho-CREB antibody cross-reacts with the transcription factor ATF-1. Cleavage fragment sizes are indicated for caspase-9, caspase-3, and CREB. The blot for ERK1/2, which is not affected in these reactions, shows that equal protein was loaded on the gel and that CREB was specifically cleaved in the reactions where caspase cleavage and activation was detected. Data are representative of four independent experiments. B: Caspase inhibitors inhibit CREB cleavage. SY5Y cytosol and PC12 nuclei were incubated together as in A in the presence of an ATP-regenerating system, with cytochrome *c* (lanes 2–6), DEVD-CHO (lane 3), YVAD-CHO (lane 4), IETD-CHO (lane 5), and z-VAD-fmk (lane 6). After incubation, the samples were analysed by immunoblotting using an antibody specific for proteins labeled at the right of panels. The asterisk denotes an incompletely processed form of caspase-3. Only DEVD-CHO and z-VAD-fmk completely inhibited CREB cleavage. Data are representative of five independent experiments. C and D: The amount of CREB cleavage in B correlates with the amount of activity in the presence of peptide inhibitors. SY5Y cytosol and PC12 nuclei were incubated with an ATP-regenerating system and cytochrome *c* as in A with the peptide inhibitors DEVD-CHO (lane 2), YVAD-CHO (lane 3), IETD-CHO (lane 4) or z-VAD-fmk (lane 5) as in B. Caspase activity was measured by cleavage of substrates DEVD-AFC (C) and IETD-AFC (D). Caspase activities are expressed as fold induction over a control reaction performed in the absence of cytochrome *c*. Results are averages of three experiments for reaction 1 and standard errors are indicated. Results are averages of two experiments for reactions 2–5 and ranges are indicated. E: CREB cleavage correlates with apoptotic chromatin condensation. SY5Y cytosol and PC12 nuclei were incubated together as in A in the presence of an ATP-regenerating system, with cytochrome *c* (lanes 2–6) and the peptide inhibitors shown. Nuclei were fixed and stained with Hoechst 33342 and scored for apoptotic morphology. Results are averages of four independent experiments and standard errors are indicated.

1A). CREB cleavage was specific because there was no change in the amounts of the control protein ERK (Fig. 1A). CREB cleavage was inhibited in the presence of the caspase inhibitors DEVD-CHO and z-VAD-fmk (Fig. 1A, lanes 3 and 8). These results suggest that CREB can be cleaved by caspases during apoptosis.

The activity of caspase-9 has been proposed to be negatively regulated by phosphorylation [24]. We asked if inhibitors of tyrosine or serine/threonine phosphatases affected the apoptotic mechanism and CREB cleavage in our in vitro system. The data suggest that phosphatases affect the apoptotic mechanism and CREB cleavage in our in vitro system. The tyrosine phosphatase inhibitor orthovanadate did inhibit CREB cleavage and caspase-3 and caspase-9 activation (Fig. 1A, lanes 4 and 6). The serine/threonine phosphatase inhibitor okadaic acid by itself had no effect on CREB cleavage or caspase activation (Fig. 1A, lane 5). We detected a slight increase in molecular weight in procaspase-9 in reactions containing both orthovanadate and okadaic acid, but not each individually [5] (Fig. 1A). This increase indicates that caspase-9 is phosphorylated at both tyrosine and serine/threonine residues. However, our data, and those of others, suggest that only tyrosine phosphorylation regulates caspase-9 activity [25,26].

Active CREB, phosphorylated at Ser-133, was detected in reactions containing okadaic acid (Fig. 1A, lanes 4 and 5) but not orthovanadate by itself (Fig. 1A, lane 6). Phospho-CREB was also detected in lower amounts in the reactions containing the caspase inhibitors DEVD-CHO and z-VAD-fmk (Fig. 1A, lanes 3 and 8). The buildup of phospho-CREB in the presence of caspase inhibitors suggests a relationship between the phosphorylation state of CREB and caspase-mediated cleavage. Serine-133-phosphorylated CREB may be a preferred substrate when caspase activity is low; in any case its presence in the pool of total CREB had no effect on CREB cleavage (Fig. 1A, lane 5).

ATP is required for initiation of apoptosis in vitro [27] and for caspase activation in the presence of cytochrome *c* (Fig. 1A). Interestingly, total CREB protein levels were reproducibly decreased without production of the 30 kDa cleavage fragment in reactions where ATP was depleted even though caspase-3 and caspase-9 were not activated (Fig. 1A, lane 7).

To look more closely at the caspase dependence of CREB cleavage, additional reactions were performed in the presence of different caspase inhibitors (non-cleavable peptide substrates, Fig. 1B–E). Only DEVD-CHO, which strongly inhibits caspase-3 and -7, and z-VAD-fmk (a general caspase inhibitor) completely blocked CREB cleavage. These inhibitors blocked cleavage and activation of caspase-3, as well as caspase-6 and caspase-9, which are cleaved by caspase-3 [28] (Fig. 1B, lanes 3 and 6; Fig. 1C,D, reactions 2 and 5). IETD-CHO (a caspase-6, -8 and -10 inhibitor) partially inhibited caspase activation and CREB cleavage (Fig. 1B, lane 5; Fig. 1C,D, reaction 4), while the caspase-1 inhibitor YVAD-CHO had no effect on caspase activation or CREB cleavage (Fig. 1B, lane 4; Fig. 1C,D, reaction 3). The activity induced by cytochrome *c* towards the DEVD substrate was many times greater than towards IETD, but the relative inhibition by non-hydrolysable analogues was similar (compare Fig. 1C and D). The inhibitors that were most effective in preventing CREB cleavage were also the most effective in preventing chromatin condensation in nuclei (Fig. 1E). These data suggest that CREB can be specifically cleaved by caspases and that CREB cleavage correlates with caspase activity and apoptosis in nuclei.

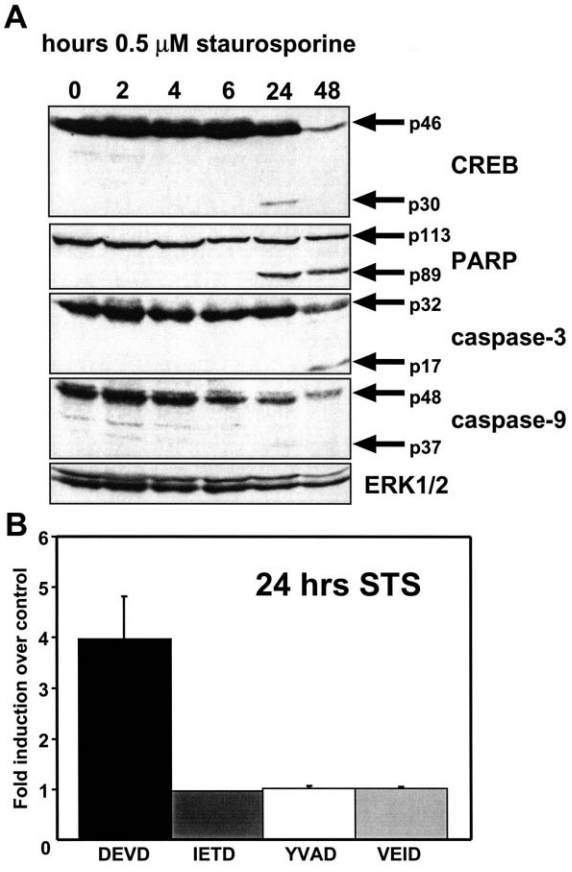

Fig. 2. CREB cleavage during staurosporine-induced apoptosis correlates with DEVD-specific caspase activity. A: SY5Y cells were treated with 0.5 μM staurosporine for the times indicated. Whole cell lysates were then analysed by immunoblotting using antibodies specific for the proteins labeled at the right of the panels. Cleavage fragments for CREB, PARP, caspase-3 and caspase-9 are indicated. Weak signals of cleavage fragments for caspase-3 (p17) and caspase-9 (p37) were seen only after 48 h of staurosporine treatment. Data are representative of four independent experiments. B: SY5Y cells were cultured for 24 h in the absence (control) or presence of 0.5 μM staurosporine and caspase activities were measured by cleavage of peptide substrates DEVD-AFC (black bar), IETD-AFC (dark grey bar), YVAD-AFC (white bar), and VEID-AFC (light grey bar). Results are expressed as fold induction of caspase activity over serum-recultured control. Results are averages from four independent experiments and standard errors are indicated.

We asked whether CREB cleavage occurred in vivo in SY5Y cells induced to undergo apoptosis by staurosporine. CREB cleavage occurred 24 h following staurosporine treatment, concomitant with cleavage of the caspase-3 substrate PARP (Fig. 2A). Caspase activity was detected at 24 h when measured using different fluorogenic substrates (Fig. 2B), even though the processing of caspase-3 and -9 was not detected by immunoblotting until 48 h after staurosporine treatment (Fig. 2A). At 48 h further degradation of CREB protein was apparent.

The CREB sequence ILNDLSSD (137–144) suggests a caspase-6 or -8 cleavage site, however, there was no measurable induction of activity towards the IETD (caspases-6, -10, -8) substrate, YVAD (caspase-1) or VEID (caspase-6; Fig. 2B).

Only DEVD-directed caspase activity was induced in the staurosporine-treated cells (Fig. 2B). These data suggest that CREB is a substrate for caspase-3 or -7 in vivo, which is consistent with the in vitro data.

CREB appears to be a key target for destruction, being downstream of both Raf-1 and Akt, which are also cleaved by caspases, and upstream of the important survival protein, Bcl-2. Why inactivate CREB by proteolysis, rather than by dephosphorylation? Caspase-mediated cleavage of key proteins involved in survival signalling makes their inactivation irreversible and eliminates possible conflicting signals. That this occurs at a late stage of apoptosis enforces no possibility of escape from execution, and avoids a possibly messy 'undead' state somewhere between life and death. In addition, CREB may function without activation by phosphorylation. It has been reported that non-phosphorylated CREB has anti-apoptotic activity and can suppress AP-1 (c-Jun/c-Fos heterodimer) transcriptional activity by competing for the AP-1 binding site on target genes [29].

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