Chapter 2

A potential role for calcium/calmodulin-dependent protein kinase related peptide in neuronal apoptosis: in vivo and in vitro evidence


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Abstract
Previously, we have established that a product of the Doublecortin-like kinase (DCLK) gene, DCLK-short, is cleaved by caspases during serum deprivation. Subsequently, the N-terminal cleavage product of DCLK-short facilitates apoptosis in the neuroblastoma cell line NG108. As this N-terminal cleavage product is highly homologous to CaMK-Related Peptide (CARP), another DCLK gene splice-variant, we aimed to determine possible apoptotic properties of CARP in vivo and in vitro.

We report highly specific CARP expression in apoptotic granule cells in the rat dentate gyrus after adrenalectomy relative to healthy granule cells. CARP is significantly up-regulated in the supra pyramidal blade of the dentate gyrus, with varying levels of up-regulation, depending on the extent of adrenalectomy-induced apoptosis. Similar to the caspase-cleaved N-terminus of DCLK-short, CARP over-expression itself facilitated apoptosis in serum-deprived NG108 cells. Furthermore, CARP facilitated polymerization of tubulin in vitro and was capable of interacting with Grb2, an intracellular protein involved in vesicle trafficking. Together, our data demonstrate a facilitating role for CARP in the apoptotic process in granule cell populations sensitive to adrenalectomy and suggest that this pro-apoptotic effect is mediated by increasing the stability of the microtubule cytoskeleton.

Introduction
Granule cells that are destined to die through apoptosis are known to have, when compared to healthy cells, altered electrophysiological, morphological and cytoskeletal characteristics, which are accompanied by triggering of specific gene expression profiles (Stienstra and Joels 2000; Nair, Karst et al. 2004). A well-established model for the induction of apoptosis in the rat dentate gyrus (DG) is adrenalectomy (ADX). ADX-induced apoptosis typically affects only a small subset of dentate granule neurons, whereas most surrounding cells remain viable (Sloviter, Sollas et al. 1993; Hu, Yuri et al. 1997).

Previously, we have identified the expression of the Doublecortin-Like Kinase (DCLK) gene in the hippocampus of ADX rats (Vreugdenhil, de Jong et al. 1996; Vreugdenhil, de Jong et al. 1996). This gene contains a doublecortin (DCX) domain as well as a Calcium/calmodulin dependent protein kinase (CaMK)-like domain, and is subject of alternative splicing (Vreugdenhil, Engels et al. 2001; Burgess and Reiner 2002). DCLK-long and Doublecortin-Like (DCLK), exhibit high homology with DCX and are both expressed during development where they control neuronal migration and neurogenesis (Figure 1) (Deuel, Liu et al. 2006; Koizumi, Tanaka et al. 2006; Shu, Tseng et al. 2006). DCLK-long and DCX have similar biochemical and biophysical characteristics (Lin, Gleeson et al. 2000). These proteins function as microtubule associated proteins thereby affecting cytoskeleton stability (Gleeson, Lin et al. 1999; Burgess and Reiner 2000).
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Figure 1. The main proteins generated by the DCLK gene. DCLK-long and DCL are mainly expressed during embryonic development (Deuel, Liu et al. 2006; Koizumi, Tanaka et al. 2006; Shu, Tseng et al. 2006), while DCLK-short is mainly expressed in the adult brain (Engels, Lucassen et al. 1999; Engels, Schouten et al. 2004). CARP is expressed at very low levels but induced in the hippocampus by kainate-induced seizures (Vreugdenhil, Datson et al. 1999) and in striatal neurons by D1-agonists and cocaine (Berke, Paletzki et al. 1998). The arrow indicates the location of the caspase cleavage site (Kruidering, Schouten et al. 2001).

DCLK-short contains a CaMK-like catalytic domain and is abundantly expressed in limbic structures of the adult brain (Vreugdenhil, Engels et al. 2001; Burgess and Reiner 2002; Engels, Schouten et al. 2004). The DCLK gene also encodes a transcript that lacks both the DCX and CaMK-like domains. This 55-amino-acid peptide, called CaMK-related peptide (CARP) (Vreugdenhil, Datson et al. 1999); also called Ania-4 (Berke, Paletzki et al. 1998), largely overlaps with the serine/proline (SP)-rich domains of DCLK and DCL (Figure 1). CARP expression is below detection levels under normal conditions. In contrast, CARP mRNA is highly up-regulated by kainate-induced seizures in the hippocampus (Vreugdenhil, Datson et al. 1999). CARP is also induced in striatal neurons by D1-receptor agonists (Berke, Paletzki et al. 1998; Glavan, Sket et al. 2002). Interestingly, it has been shown that the caspase-cleaved SP-rich N-terminal fragment of DCLK-short exacerbates apoptosis in NG108 neuroblastoma cells (Kruidering, Schouten et al. 2001).
Therefore the structural overlap between CARP and the SP-rich N-terminus of DCLK-short may predict involvement of CARP in apoptosis in neuronal cells. Moreover, the homology between DCLK-long/DCL and CARP and the recent observations that the DCX-domain containing products of the DCLK gene (DCLK-long, DCL) are crucial for DCLK gene function (Shu et al., 2006) has lead us to investigate the effect of CARP on DCL-induced microtubule polymerization. Additionally, we have screened for candidates for CARP protein-protein interactions. Since CARP was originally identified in the hippocampus of ADX rats (Vreugdenhil et al., 1999), we used this model to investigate the possible role of CARP in neuronal apoptosis in vivo. We have also studied the effect of CARP over-expression on the fate of NG108 cells in vitro. Together, our results indicate that CARP has pro-apoptotic properties in neuroblastoma cells in vitro and in granule cells in the DG of ADX rats in vivo.

Materials and Methods

Animals and Surgery

26 Male Wistar rats, weighing 150-170 g, were housed two per cage (12h/12h light/dark cycle, lights on 9 AM). Animals had access to food and water ad libitum and were handled and weighed daily (9h-10h AM). After 10 days all animals (then weighing 200-250g) were adrenalectomized between 9 and 12 AM to ensure low circulating corticosterone levels. ADX was performed under isoflurane anaesthesia as described (Meijer and de Kloet 1995). After ADX, all animals had free access to 0.9% saline. Two days after ADX (day 12), a tailcut blood sample was obtained in EDTA-coated capillaries and kept on ice. Samples were centrifuged at 10,000 rpm for 10 min. and plasma was stored at -20 °C until use. Three days after ADX (day 13) animals were decapitated and trunk blood was collected in EDTA-coated tubes and kept on ice. Blood plasma was obtained by centrifuging at 3000 rpm for 15 min. at 4 °C and stored at -20 °C for determination of plasma corticosterone levels by radio immuno assay as described (Karssen, Meijer et al. 2005). Animals were considered properly adrenalectomized if corticosterone values were below 1.00 μg/dl on day 12. Of the 26 rats that were adrenalectomized, 6 had a plasma corticosterone level of 1.00 μg/dl or higher. These animals were excluded from the
experiment (Supplementary Table 1). Brains were quickly taken from the skull and snap-frozen in isopentane on a mixture of ethanol absolute and dry ice. Coronal sections (20 μm) were cut using a cryostat and thaw-mounted on poly-L-lysine coated slides. Sections were stored at -80 °C until use. All animal treatments were approved by the Leiden University Animal Care and Use Committee (UDEC# 01022).

**Constructs**

The DCLK-short construct has been described previously (Kruidering, Schouten et al. 2001). The CARP expression plasmid was constructed using CCAGGATCC ACCATGGGCCCTGGGGAAGAAGAGTC as a sense oligonucleotide and GCAGAATTC TTACACTGAGTCTCCTGAGTCCAAATC as antisense oligonucleotide and Pfu as a proofreading polymerase. After purification on QuiaQuick columns, the fragment was digested with BamHI and EcoRI (underlined in oligonucleotides) and subcloned into the corresponding sites of pcDNA3.1 (Invitrogen, Groningen, The Netherlands).

*In situ* hybridization was performed using oligonucleotides as described (Meijer, Steenbergen et al. 2000). DCLK-short was detected using 45mers recognizing the 3' untranslated region of the DCLK-short transcript and CARP was detected using a 45mer recognizing the 3'-untranslated region of the CARP transcript. Mismatch oligonucleotides with 4-5 substitutions were used as control. The DNA sequences are:

1. TGGTAGTAGTCCAAAGACCTTGATCTCTGGATGGTAAACCCGTGG
2. TGGTAGAGTAGTCCATAGACCGTGATCTCTGCTGATTTACCCGTGG
3. GATGCTTGCTTAGGAAATGGGAAACCTTGATCCCATCACAAACCA
4. GATGCTTGAATAGAAACGGGAACCTCGATCCCATAAACCA

No. 1 is the perfect match recognizing DCLK-short, No. 2 its mismatch control, No. 3 is the perfect match recognizing CARP and No. 4 its mismatch control (substitutions are underlined). Following labelling of the oligonucleotides, slices were exposed to an X-OMAT AR film (Kodak) for approximately 5-7 days. Films were scanned and relative optical densities (RODs) of hippocampal subfields CA1, CA3 and DG and background (area between the cell layers of CA1 and DG) were measured using NIH Image 1.62. The background was subtracted from the RODs of the corresponding areas.

Photographic emulsion in a glass container was liquefied at 42 °C for 30 min. in a water bath and kept at 42 °C during the entire dipping procedure. Hybridized slices were dipped into the emulsion and placed in an upright position in order to dry overnight in the dark and exposed for approximately 3 weeks. The emulsion was developed as follows: developer for 10 min., distilled water for 1 min., 5% acetic acid for 1 min., distilled water for 1 min., fixer for 5 min. and finally distilled water for 1 min. After developing the slices were kept under running water for 1h and air-dried. Sections were counterstained by Nissl staining: cresyl violet solution (0.5 %) for 10 min. and dehydrated in a graded series of ethanol 50, 70, 80, 100, 100% for 30 sec. and 4 times 1 min. respectively. Sections were air-dried, covered with permount and a microscopic coverslip (24x50 mm) and analysed by bright field microscopy using polarising light. Counting both healthy and picnotic nuclei, as observed with Nissl staining, the percentage of apoptotic cells in the suprapyramidal blade of the DG was investigated. Picnosis is a well-known hallmark of neurons that are dying through programmed cell death (Sloviter, Sollas et al. 1993). Picnotic DG granule cells are characterized by small, round, densely stained nuclei that are fragmented (Insert in Figure 2C). Both healthy and apoptotic nuclei of the suprapyramidal blade of the DG were counted in one microscopic field, at a magnification of 400x. The percentage of apoptosis was estimated (% apoptosis = (number of apoptotic cells/total number of cells) x 100) and plotted against the measured RODs of the corresponding *in situ* hybridization. The 20 properly adrenalectomized animals were included in this experiment (see above). Of these adrenalectomized animals, 16 displayed apoptosis, while 4 did not.

**Cell Culture and Microinjection Experiments**

NG108-15 cells were grown as described previously (Kruidering, Schouten et al. 2001). All cell culture chemicals were obtained from Life Technologies, Inc.
Brains were quickly taken from the skull and snap-frozen in isopentane on a mixture of ethanol absolute and dry ice. Coronal sections (20 μm) were cut using a cryostat and thaw-mounted on poly-L-lysine coated slides. Sections were stored at -80 °C until use. All animal treatments were approved by the Leiden University Animal Care and Use Committee (UDEC#01022).

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NG108-15 cells were grown as described previously (Kruidering, Schouten et al. 2001). All cell culture chemicals were obtained from Life Technologies, Inc.
Transient transfection experiments were performed with Superfect (Qiagen, Valencia, CA) according to the protocol of the manufacturer. Cells were exposed to staurosporine 2 h after transfection, and the viability was assessed by microscopy based on cell morphology. Cells were seeded on glass-bottomed coverslip dishes (Matteck Corp., Ashland, OR) 24–48 h prior to injection. Nuclear microinjection was performed using an automated microinjection system (Eppendorf Transjector 5246, micromanipulator 5171). Identical standardized conditions of pressure (150 hectopascals) and time (0.1 s) were used for microinjection in all experiments. DCLK-short and CARP DNA plasmids were mixed 1:5 with EGFP-N1 reporter plasmid (CLONTECH, Palo Alto, CA) in ultrapure water to a final concentration of 100 ng/μl of plasmid. Cells were injected, and the next morning the number of green, EGFP-N1 expressing, viable cells was counted (t=0). Cells were washed three times with serum-free medium to remove all serum and kept in serum-free medium. Cells were counted again 48 h after serum withdrawal. Viability was expressed as EGFP positive cells at a given time after serum withdrawal as percentage of green cells at t=0. For each construct, at least 400 green cells were counted from an average of six independent injection experiments. Injection of pcDNA 3.1 plasmid served as control.

**Tubulin polymerization assay**

To quantitatively analyze microtubule polymerization, 100 μl of pure tubulin at 1 mg/ml in G-PEM buffer (80mM Pipes pH 6.9, 2 mM MgCl₂, 0.5 mM EGTA, 10 mM GTP) plus 5% (v/v) glycerol was added to 10 μl of recombinant wild type DCL protein and/or synthetic CARP peptide at different concentrations (30 μg/μl DCL, 20 μg/ml or 100 μg/ml CARP). Taxol was used as a positive control. According to manufacturer’s instruction, tubulin polymerization was detected by measuring the absorbance of the solution at 340 nm at 37 °C kinetically for 60 minutes (HTS7000 spectrophotometer, BioRad).
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Immunoprecipitation and Western blotting
Because CARP was predicted to interact with Grb2 (see results section), we investigated a possible protein-protein interaction between CARP and Grb2 by incubating increasing concentrations of CARP peptide (0; 0.5; 1; 5; 10 and 15 μg) with COS cell protein lysates. Total protein was extracted by lysing a million cells in lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Triton. Subsequently immunoprecipitation was performed using a Grb2-agarose conjugate (Santa Cruz): Grb2 protein conjugated to agarose beads in PBS containing 0.1% azide, 0.1% BSA and 10% glycerol. For detection of CARP and Grb2, equal amounts of protein were separated by electrophoresis on an SDS-polyacrylamide gel (12%) and semidy electroblotted to a polyvinylidene difluoride membrane, Immobilon-P (Millipore Corp., Bedford, MA). Blots were blocked with blocking buffer (Tris-buffered saline, 0.2% Tween (TBS-T), 10% milk) and incubated with primary antibodies (1:1000) for 1h at room temperature in blocking buffer. The anti-CARP antibody was produced in rabbits by injection of a 55-amino acid-long synthetic peptide corresponding to the N-terminal domain of DCLK, designated CARP. The anti-CARP antibody is capable of recognizing DCLK-long and -short and DCL in addition to the CARP peptide (Vreugdenhil et al., 1999). The anti-Grb2 antibody and horseradish peroxidase (HPA)-conjugated secondary antibodies (used at 1:5000) were from Santa Cruz. Blots were washed three times with blocking buffer, incubated with secondary HPA-conjugated antibodies for 1 h at room temperature, and washed five times. Binding was detected by enhanced chemiluminescence.

Analysis and Statistics
A motif scan of the full length DCLK protein sequence was performed using http://scansite.mit.edu/motifscan_seq.phtml. Corticosterone radioimmunoassay data were analysed using the SECURIA II program. In situ hybridization relative optical densities (RODs) of hippocampal subfields CA1, CA3 and DG and background were measured using NIH Image 1.62. The background signal was subtracted from corresponding measurements for each of the areas studied. Western blot RODs were analyzed in a similar manner. Significant differences were determined with one-way anova, with posthoc Tukey HSD (honest significant
difference) test. Pearson’s Correlation test was used to calculate correlation coefficients and to determine significant correlations. For the output of the tubulin polymerization assay the maximal level of tubulin polymerization for the indicated samples after 60 minutes of incubation was statistically analyzed using one-way anova with Tukey-Kramer multiple comparisons test. For all tests probability level of 5% was used as the minimal criterion of significance.
Results

**CARP expression in the DG of ADX animals**

Adrenalectomy has been widely used to induce apoptosis in adult dentate gyrus granule cells (Sloviter et al, 1989; 1993). To study possible involvement of CARP in neuronal apoptosis, we have studied CARP expression in ADX animals with different levels of apoptosis in the DG. We observed an increase in CARP mRNA expression level specifically in the supra pyramidal blade of the DG in ADX rats (Figure 2A). No changes in CARP expression were observed in the other subfields of the hippocampus (CA1 and CA3). Using the same tissue sections, both the extent of apoptosis (Figures 2B/C and 2E/F) and CARP mRNA expression were investigated (Figures 2A and 2D). The extent of apoptosis ranged from 0% to 30% while ROD measurements for CARP varied from 5 to 110 (arbitrary units). A significant and positive correlation (correlation coefficient 0.66; p<0.01) was found between CARP mRNA expression and the relative number of picnotic nuclei representing apoptotic cells. Specifically, high levels of apoptosis were correlated with high CARP expression and vice versa (Figure 2G). No significant change in DCLK-short expression was found in the DG of ADX animals, regardless of the presence or absence of corticosterone along with no correlation between the level of DCLK-short mRNA expression and the percentage of apoptosis (data not shown). Brightfield microscopy using polarising light revealed that silver grains, representing CARP transcripts, were exclusively colocalized with apoptotic cells while the number of silver grains found in healthy granule cells was equal to background (Figures 2H and 2I), suggesting a role for CARP in the process leading to ADX-induced apoptosis in DG granule cells.
Figure 2. CARP is specifically expressed in apoptotic granule cells in the hippocampus of ADX rats. A-F: in situ hybridization analysis of CARP in ADX animals with high (A-C) and low (D-F) numbers of apoptotic cells. A and D are CARP in situ hybridization autoradiograms. Note the up-regulation of CARP mRNA in the suprapyramidal blade of the dentate gyrus (indicated by arrowhead) in ADX rats with apoptotic cells (A) compared to ADX animals without apoptotic cells in the DG (D). B, C and E, F are the corresponding Nissl-stained sections to visualize apoptotic cells (arrows and insert in F). G: Correlation between the percentage of apoptosis in the suprapyramidal blade and the expression level of CARP (correlation coefficient 0.66; p<0.01). Apoptotic cells were counted in 6 rats with at least 8 sections per animal and the corresponding RODs of the hybridization signal is indicated. See text for further details.

H: Microscopical view visualizing nissl-stained nuclei. Apoptotic cells can clearly be seen by their picnotic appearance (arrows). I: Same section as H but exposed to polarising light to reveal silver grains representing CARP transcripts (arrows). Note that CARP expression is located in, or very near to apoptotic cells although some at low levels (indicated by asterisks) and that the hybridization signal in healthy granule cells is below detection levels.

CARP micro-injection in NG108 cells

Previously, we have shown that DCLK-short is cleaved by activated caspases and that the N-terminal cleavage product facilitates staurosporine-induced apoptosis in NG108 cells. As 38 out of the 55 amino acids of the CARP peptide are identical (63%) within the 60 amino acids-long N-terminal cleavage product of DCLK-short (Figure 1), we decided to study the effect of CARP over-expression on staurosporine-induced apoptosis in NG108 cells. With that aim we have micro-injected CARP, DCLK-short and control constructs in NG108 neuroblastoma cells and monitored their fate. Cells micro-injected with control vector showed 74.5% viability after 24 hours of serum deprivation, whereas only 55.4% of all NG108 cells were viable after micro-injection of the CARP-expressing construct when exposed...
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to 24 hours of serum deprivation. In contrast, injection of the DCLK-short construct did not alter the number of apoptotic cells after serum deprivation (Figure 3). When injected in healthy, non-serum deprived NG108 cells the CARP construct did not decrease viability (data not shown). These observations suggest that CARP acts as a facilitator of apoptosis in neuronal cells, but has no apoptosis-inducing properties of its own.

![Figure 3. Effect of microinjected CARP and DCLK-short constructs on neuronal cell death induced by serum withdrawal. Cells were injected with different constructs mixed with eGFP as described. At t=0, green viable cells were counted and serum was withdrawn. Cells were re-counted 24 hrs after serum withdrawal. Viability is expressed as % of green cells at t=24 relative to t=0. For each construct at least 400 cells have been counted in at least 4 injection series. Injection of empty vector served as control. Cells were injected with CARP and DCLK-short. * Statistically significant relative to control (T-test, p<0.05).](image)

**CARP and DCL-induced tubulin polymerization**

The DCLK gene, which generates CARP by alternative splicing, has been shown to be associated with the stability of microtubules (Kim et al, 2003, Shu et al, 2006, Vreugdenhil et al, 2007), a process that is severely affected by apoptosis. DCL is a microtubule-associated protein with high homology to CARP (Vreugdenhil et al., 2007). Therefore, we have studied *in vitro* the effect of CARP on DCL-induced polymerization of microtubules. Recombinant DCL was incubated with purified tubulin in the presence or absence of different concentrations of synthetic CARP peptide (20 or 100 μg/ml) and the degree of polymerization was measured (Figure 4). As a positive control for this assay Taxol was used. Taxol was able to induce...
polymerization, with a similar level of polymerization as observed with the addition of DCL alone after 60 minutes. Recombinant DCL directly affected tubulin polymerization and an increase in the total amount of polymerized tubulin was observed in all samples containing DCL (Figure 4A). We found that addition of the highest concentration of CARP (100 μg/ml) facilitated DCL-induced polymerization of tubulin, whereas the lowest concentration (20 μg/ml) did not, indicating a dose dependent effect of CARP on DCL-induced tubulin polymerization. In contrast, CARP in the absence of DCL did not positively affect polymerization. This is more clearly illustrated by Figure 4B, which shows the maximal level of tubulin polymerization for the indicated samples after 60 minutes of incubation.

Figure 4. Effect of CARP on DCL-induced microtubule polymerization. Two concentrations (20 μg/ml or 100 μg/ml) of synthetic CARP were incubated with recombinant DCL protein (30 mg/μl) and purified tubulin (1 mg/ml). The turbidity of the DCL/tubulin mixture was monitored at 340 nm for 60 min. Taxol was used as a positive control (different from control (p<0.01)). Addition of synthetic CARP to the DCL/tubulin mixture increased DCL-mediated tubulin polymerization, while addition of CARP in the absence of DCL did not facilitate polymerization (A).
CARP and Grb2 interaction in vitro

The primary amino acid sequence of CARP does not contain obvious protein motifs linking it to specific biological functions e.g. transcription or enzymatic activity. As the CARP peptide is also small in size (55 amino acids) and the S/P-rich C-terminal parts of DCX and DCLK, to which CARP is highly homologous, are implicated in protein interactions (Friocourt et al., 2001; Moores et al., 2004), we speculated that CARP also exerts its effect by interacting with other proteins. To identify potential interacting proteins we conducted an in silico search using a motif scan (http://scansite.mit.edu/motifscan_seq.phtml). This motif scan of the full length DCLK protein sequence revealed a high concentration of protein-phosphorylation motifs within the CARP domain (for details see supplementary figure S1). In addition, the CARP domain is predicted to interact with SH3 domain containing proteins, in particular with Grb2. To study a possible protein-protein interaction

Figure 4. (Continued) The maximal level of tubulin polymerization for the indicated samples after 60 minutes of incubation is shown in B. *, significantly different from DCL (p<0.05). All DCL containing samples were significantly different from control (p<0.001). The graphs are representative of two independent experiments with similar results.
between CARP and Grb2 we incubated increasing concentrations of CARP peptide (0, 0.5, 1, 5, 10 and 15 μg) with COS cell lysates to more closely mimic a cellular context. Immuno precipitation using Grb2 protein coupled to agarose beads was performed to specifically pull-down Grb2-interacting proteins. Western blot analysis using a DCLK/CARP specific antibody of Grb2-captured lysates showed a 10 kD immunoreactive band that co-migrated with the synthetic CARP peptide and a 50 kD band corresponding to the Grb2 protein (Figure 5A). In addition, quantification of the RODs revealed that CARP-Grb2 interaction was dependent on the absolute concentration of added synthetic CARP peptide (Figure 5B), whereas the amount Grb2 protein was equal in all samples (Figure 5C). Thus, in vitro, CARP is able to interact with Grb2 in a dose-dependent manner.

Figure 5. CARP protein-protein interaction with Grb2. A: Western blot showing CARP co-precipitation with Grb2 protein. Increasing concentrations of synthetic CARP peptide (0; 0.5; 1; 5; 10 and 15 μg) and synthetic CARP peptide as a positive control (++) are indicated. The 10 kD band corresponds to CARP and the 50 kD band corresponds to Grb2.
Discussion

We have investigated the role of the DCL K gene in neuronal apoptosis by studying CARP expression in the hippocampus of rats with varying degrees of apoptosis, 3 days after ADX. Under physiological conditions, CARP expression is low or even below detection levels in the adult brain. Previously, induction of CARP mRNA has been associated with kainate-induced seizures in hippocampal neurons (Vreugdenhil et al., 1999) and with administration of D1-agonists in striatal neurons (Berke, Paletzki et al. 1998; Glavan, Sk et et al. 2002). However, during these processes, CARP induction has not been associated with neuronal apoptosis. Here, we show a novel association, i.e. a correlation between CARP mRNA expression and ADX-induced apoptosis in DG granule cells and specific expression of CARP in these apoptotic neurons. Moreover, CARP over-expression in neuronal cells facilitated apoptosis neuronal cells. CARP was also able to simulate DCL-induced tubulin polymerization in vitro. Thus, our data for the first time demonstrate a pro-apoptotic role for this non-DCX domain-containing splice product of the DCLK gene. These findings may be of importance in understanding the functions of members of the DCLK gene family and the molecular basis of apoptosis in specific neuronal populations.

CARP is specifically expressed in apoptotic DG cells following ADX. The extent of changes in CARP expression varied considerably across animals. This may be a consequence of the variability among animals in the number of degenerating cells after ADX (Sloviter, Sollas et al. 1993). This variability allowed us to examine the relation between CARP expression and the presence of apoptotic cells in the DG. We demonstrated a significant and positive correlation between CARP mRNA expression and ADX-induced apoptosis in DG granule cells. This suggests that either cells that have become apoptotic produce large amounts of CARP transcripts or that CARP represents a pro-apoptotic signal, consequently enhancing the rate of apoptosis. To investigate these possibilities we have exposed brain sections to photographic emulsion. Importantly, high levels of CARP transcripts were found in or close to apoptotic cells, but were absent in healthy granule cells. This strongly indicates that the observed increase of CARP in the DG

Figure 5. (Continued) B: Relative quantification (ROD) of CARP peptide levels shows dose dependency. Note that 5 μg of CARP peptide is sufficient to saturate the Grb2-agarose conjugate. 0 μg CARP is used as base line. C: Relative quantification (ROD) of Grb2 protein levels shows equal levels of Grb2 in all samples.
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of ADX rats is a consequence of expression of CARP in apoptotic granule cells specifically. This observation raises the question whether CARP is involved in the initiation of the apoptotic process or whether the apoptotic process induces the expression of CARP. Since most, but not all, picnotic granule cells contained CARP transcripts and non-picnotic granule cell (including pre-apoptotic cells) were completely lacking CARP transcripts, it seems more likely that the apoptotic process induces the expression of CARP. This suggests that CARP exerts its function, albeit presently unknown, in an apoptotic context. This hypothesis is strengthened by our findings in NG108 neuroblastoma cells.

**CARP exacerbates apoptosis in NG108 cells**

Previously, we have shown that the N-terminus of DCLK-short, when cleaved from the full length protein, facilitates serum-deprived apoptosis in neuronal cell lines (Kruidering, Schouten et al. 2001). Since the 38 N-terminal amino acids of CARP are identical to this N-terminus, CARP itself may have similar pro-apoptotic effects. In line with the hypothesis that CARP has a function mostly in an apoptotic context, is the finding that CARP over-expression in non-serum deprived NG108 cells did not alter the number of apoptotic cells. In contrast, CARP over-expression in NG108 cells endangered by serum deprivation exacerbated apoptosis. CARP may therefore have pro-apoptotic properties exclusively in neuronal cells that are programmed to die via an apoptotic process. Given the high expression of DCLK gene products in the hippocampus (Engels et al., 1999; Vreugdenhil et al., 1996b; 1999) CARP may enhance apoptosis in a similar manner in granule cells destined to die through apoptosis after ADX.

**CARP interacts with Grb2 in vitro**

CARP is highly homologous to the S/P rich C-terminal parts of both DCX and DCLK, which are implicated in protein interactions (Friocourt et al., 2001; Moores et al., 2004). In addition, the DCLK gene has recently been implicated in intracellular vesicle trafficking (Duel et al., 2006). In this respect it is important to note that CARP was found to interact with Grb2, most likely through SH-3 domain binding.
Inspection of the primary amino-acid structure of CARP did not reveal any known homology domains that could link a possible biological role of CARP to enzymatic, katalytic, transcriptional or any other activity. The sequence GKSPSPSPTSPGSLR of CARP was predicted to interact with Grb2, an intracellular adapter protein containing a SH3-SH2-SH3 configuration that has been implicated in tyrosine-kinase receptor signalling (for review see (Tari and Lopez-Berestein 2001) and regulation of the actin cytoskeleton (for review see (Buday, Wunderlich et al. 2002). Grb2 is part of the Trk-receptor complex, where it transduces neurotrophin-binding to activation of the Ras GTP-exchange factor Sos (Lowenstein, Daly et al. 1992; Egan, Giddings et al. 1993) and becomes internalized in and transported by signalling vesicles (Howe and Mobley, 2005), ultimately leading to activation of the Ras-ERK kinase cascade (for review see (Katz and McCormick 1997). From this perspective, CARP may influence the viability of granule cells in the DG in concert with a change in the availability of neurotrophic factors and their receptors after ADX (Schaaf, De Kloet et al. 2000; Nichols, Agolley et al. 2005). Interestingly, Neurotrophin-3 is reported to be down-regulated after ADX in the DG (Chao, Sakai et al. 1998; Hansson, Cintra et al. 2000) but not in other hippocampal subfields (Hansson, Cintra et al. 2000). Moreover, injection of Neurotrophin-4/5 grants protection against ADX-induced apoptosis of DG cells (Qiao, Hughes et al. 1996), which suggests a negative correlation between CARP expression and neurotrophin levels. CARP is also highly induced in the rat DG by kainate-induced seizures (Vreugdenhil, Datson et al. 1999), a phenomenon that is accompanied by induction of several neurotrophins (Gall and Lauterborn 1992; Lindvall, Kokaia et al. 1994). The fact that CARP expression is not associated with apoptosis in this seizure model leaves open the possibility that CARP induction is under control of distinct signalling cascades and that its induction, as well as its function, are highly context dependent. In accordance with this is the observed pro-apoptotic effect of CARP during serum deprivation in NG108 cells. Thus, CARP might facilitate apoptosis only in a small subset of the granule cell population in the DG that is deprived of growth factors. Together, our data indicate in vitro interaction of CARP and Grb2 in a dose-dependent fashion. As such, binding of CARP to Grb2 might lead to a downstream shift of neurotrophin signalling cascades and/or availability in vivo.
CARP enhances DCL-induced tubulin polymerization

The mechanism by which CARP affects neuronal viability is presently unknown. The doublecortin family consisting of DCX, DCLK-1 and DCLK-2 are known to induce microtubule polymerization and stabilisation (Francis, Koulakoff et al. 1999; Lin, Gleeson et al. 2000; Edelman, Kim et al. 2005). This, in combination with the structural overlap between DCX, DCLK-1 and DCLK-2 on the one hand and CARP on the other hand, raises the possibility that CARP affects DCLK gene-induced microtubule polymerization and thus cytoskeleton stability, thereby affecting neuronal viability. In addition, Shu et al. propose that the DCX domain containing isoforms of the DCLK gene are responsible for its functions (Shu et al., 2006), suggesting regulation of these isoforms (i.e. DCL) may be of importance. Using a tubulin polymerization assay we found that DCL was capable of tubulin polymerization, an observation that is well in line with previous results (Vreugdenhil et al., 2007). In this assay, CARP increased DCL-induced polymerization of tubulin in a dose dependent fashion. Thus, CARP influences the stability of microtubules in vitro, suggesting that in addition to DCX-domain containing isoforms, DCLK gene products without DCX-domains also have biologically relevant functions. Taxol, a well-known microtubule-polymerizing compound with anti-tumorigenic properties, was used as a positive control. Arrest of the cytoskeleton underlies the tumor-suppressing properties of this agent. Taxol is also able to selectively kill granule cells in the DG in vivo (Kim, Mitsukawa et al. 2002). Similarly, CARP may exert its pro-apoptotic properties through stabilization of the microtubule skeleton. CARP mRNA is induced in hippocampal neurons during kainate-induced seizures (Vreugdenhil, Datson et al. 1999) in striatal neurons by D1-receptor agonists (Berke, Paletzki et al. 1998; Glavan, Sket et al. 2002) and in apoptotic cells in the DG of ADX rats (this study). A common feature of these three challenges is the requirement of cytoskeleton rearrangements underlying the plasticity of specific neuronal circuits (Morimoto, Fahnestock et al. 2004; Everitt and Robbins 2005; Luo and O'Leary 2005). In this respect it is of interest to note that the DCLK gene is also found in a screen for candidate plasticity genes in the hippocampus by Hevroni et al., where it is designated Candidate Plasticity Gene 16 (CPG16) (Hevroni et al., 1998). CARP may function as a modulator of these rearrangements, perhaps by affecting neurotrophin signalling and/or cytoskeleton
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Carp and DCLK-short in apoptosis

Previously, we reported that DCLK-short is a substrate for caspases in vitro and in vivo and that DCLK-short cleavage by caspases is necessary for apoptosis to proceed. The finding that calpain, a protease with a wider variety of substrates than caspases, is capable of breaking down DCLK proteins also suggests a role for the DCLK gene in apoptotic processes or the prevention thereof (Burgess and Reiner 2001). Moreover, a recent study in zebrafish demonstrates that gene knockdown of DCLK (zDCLK) induces a significant increase of apoptotic cells in the central nervous system (Shimomura et al., 2007). Interestingly, DCLK-short cleavage by caspases generates a N-terminal fragment that overlaps largely with CARP in its primary structure and has similar pro-apoptotic properties as CARP when studied during serum-deprived apoptosis (Kruidering, Schouten et al. 2001). This suggests that CARP and the N-terminus of DCLK-short share a common motif that is responsible for the observed pro-apoptotic properties. Apparently, cleavage of this N-terminal motif from DCLK-short is a crucial step in revealing its pro-apoptotic characteristics. CARP mRNA is specifically up-regulated by kainate-induced seizures in the hippocampus, in striatal neurons by D1-receptor agonists and in apoptotic DG cells after ADX. These processes are not accompanied by up-
regulation of DCLK-short, raising the possibility that the extreme induction of CARP (also called ania-4, Berke et al., 1998)) is a consequence of a shift in mRNA splicing. Interestingly, alternative splicing of ania-6 in the striatum is under control of distinct signalling cascades (Berke, Paletzki et al. 1998). A similar mechanism may be responsible for our findings regarding the DCLK gene.

In conclusion, we here demonstrate that CARP mRNA is up-regulated in the suprapyramidal blade of the DG in rats after ADX and that this induction of CARP is specific for apoptotic granule cells. Furthermore, CARP over-expression in serum deprived NG108 cells exacerbated apoptosis. CARP is also able to simulate DCL-induced polymerization of tubulin \textit{in vitro}. CARP may play a role in these \textit{in vivo} and \textit{in vitro} models, perhaps by affecting neurotrophin signalling and/or cytoskeleton stability (Figure 6). Thus, our data for the first time demonstrate a pro-apoptotic role for this non-DCX domain-containing splice product of the DCLK gene. As such, and given its highly specific neuronal expression, CARP may be an important modulator of processes underlying the molecular basis of apoptosis in neuronal populations.
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References


Shimomura S, Nagamine T, Nimura T, Sueyoshi N, Shigeri Y, Kameshita I.


Supplementary Material

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Supplementary table 1. Corticosterone levels of the 26 adrenalectomized rats. 6 animals were excluded from the experiment, as their corticosterone levels were above 1,00 μg/dl (bold text).
Supplementary Figure S1. Predicted interaction sites within the full-length DCLK gene. The two DCX domains are indicated (purple), as well as the C-terminal kinase domain (yellow). Note the extremely high number of potential interaction sites located within the S/P-rich CARP domain (red).