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Dahmcke, Christina Mackeprang; Büchmann-Møller, Stine; Nielsen, Niels Aagaard; Mitchelmore, Cathy

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Altered splicing in exon 8 of the DNA replication factor CIZ1 affects subnuclear distribution and is associated with Alzheimer's disease

Christina Mackeprang Dahncke a, Stine Büchmann-Møller b, Niels A. Jensen c, Cathy Mitchelmore a,⁎

a Eucaryotic Cell Biology Research Group, Department of Science, Roskilde University, Roskilde, Denmark
b Institute of Cell Biology, Zurich University, 8093, Switzerland
c Molecular Neurobiology Laboratory, Medical Biotechnology Center, University of Southern Denmark, J.B. Winslowsvej 25, Odense C, Denmark

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ABSTRACT

In order to understand the gene-mediated processes underlying sporadic Alzheimer’s disease (AD), we carried out a subtractive cloning screen for novel AD candidate genes. We identified the gene encoding the DNA replication factor CIZ1 (CDKN1A interacting zinc finger protein 1) as being more highly expressed in Alzheimer tissue than in healthy brains. We show here that an isoform of CIZ1 which lacks a glutamine-rich region, due to alternative splicing in exon 8, is upregulated in AD brains relative to the full-length CIZ1 protein. We demonstrate for the first time that a minimal 28 amino acid sequence within this region is required for CIZ1 to associate with the nuclear matrix and to form nuclear foci.

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Introduction

Alzheimer’s Disease (AD) is a debilitating disease, characterised by the progressive loss of memory and cognitive abilities. Neuropathologically, the disorder is associated with the extracellular deposition of β-amyloid aggregates in senile plaques and the intracellular accumulation of neurofibrillary tangles. These changes lead to the loss of synaptic function and neuronal cell death in specific brain regions (Terry et al., 1998). The first regions affected by neuronal loss and AD pathology include the entorhinal cortex and the CA1 subregion of the hippocampus (Braak and Braak, 1991; Gomez-Isla et al., 1996; West et al., 1994). Since these regions are essential for the flow and integration of information through the hippocampus, this explains why the early symptoms of AD are related to defective memory (Eichenbaum, 2001; Morris, 1999).

Genetically inherited mutations which lead to early-onset AD (onset before the age of 60) have so far been identified in three genes: amyloid precursor protein, presenilin 1 and presenilin 2 (Price and Sisodia, 1998; Sisodia et al., 1999). However, early-onset AD comprises only about 5% of AD cases. The majority of AD cases are late-onset (after 65 years of age), and occur sporadically. Several genetic risk factors have been described for the late-onset form of AD, notably the ε4 allele of the apolipoprotein E gene (Corder et al., 1993; Farrer et al., 1997; Saunders et al., 1993), but the precise etiology of sporadic AD is poorly understood.

Several reports have indicated that the inappropriate re-entry of post-mitotic neurons into the cell cycle may lead to disruption of cell function in AD (Nagy, 2000; Zhu et al., 2006). In addition to elevated levels of cell cycle proteins, there is evidence that AD-affected neurons have passed the G1/S-checkpoint and initiated DNA replication (Vincent et al., 1997; Yang et al., 2001, 2003). CDKN1A (p21Cip1) is a major factor in blocking the onset of S phase. CIZ1 was first isolated in a yeast two-hybrid screen for cyclin E/CDKN1A interaction partners and shown to bind to CDKN1A (Mitsui et al., 1999). It was proposed that CIZ1 regulates entry into S phase by inducing the cytoplasmic export of CDKN1A (Mitsui et al., 1999). CIZ1 contains two glutamine-rich domains in the N-terminal end, and three zinc fingers in the C-terminal end which are capable of binding to DNA (Mitsui et al., 1999; Warder and Keherly, 2003). Murine Ciz1 has been shown to stimulate DNA replication in NIH3T3 cells. Consistent with a role in DNA replication, Ciz1 forms nuclear speckles, and colocalises with PCNA in replication foci during S phase (Coverley et al., 2005).

There is currently no effective therapy preventing cell loss in AD. Identification of genes whose expression is altered during the beginning stages of the disease should aid in identifying molecules that are potential targets for therapy and lead to a better understanding of the disease process. Using a subtractive cloning approach, we previously reported the identification of NDRG family member 2 (NDRG2) as a protein upregulated in AD and associated with AD pathology (Mitchelmore et al., 2004). Here, we describe additional candidate genes, isolated in the same screen, which are upregulated in AD. Of these, the gene encoding CDKN1A interacting zinc finger protein 1

Abbreviations: CIZ1, CDKN1A interacting zinc finger protein 1; CDKN1A, Cyclin-dependent kinase inhibitor 1A (p21Cip1); AD, Alzheimer's disease; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; NDRG2, NDRG family member 2; GFAP, glial fibrillary acidic protein.
CIZ1 was selected for further study, due to its known role as a DNA replication factor.

Results

Identification of genes upregulated in AD brains

Using a subtractive cloning technique to detect candidate genes upregulated in the hippocampal region of AD brains, we previously isolated a total of 42 cDNAs (Mitchelmore et al., 2004). In order to test the remaining uncharacterised candidate genes for upregulation, we used a slot blot approach where plasmid DNA containing cDNA inserts were separately hybridised with labelled cDNA probe prepared from either control or AD hippocampus mRNA. Changes in gene expression that are related to AD are expected to be highest in the hippocampus, which is one of the brain regions most affected by AD pathology (Braak and Braak, 1991; West et al., 1994). GAPDH mRNA levels are unchanged in AD, allowing its use as a normalisation control (Gutala and Reddy, 2004). After normalisation of the signal to GAPDH, we identified 10 new candidate genes whose expression was upregulated in AD by more than 2-fold compared to control hippocampus. Upregulation of the mRNAs for NDRG2 and GFAP in AD confirms our previous results (Mitchelmore et al., 2004), whereas upregulation of the polyadenylated form of mitochondrial 16S ribosomal RNA (MTRNR2) is probably a result of the increased mitochondrial DNA observed in AD (Hirai et al., 2001). Polyadenylation of the mitochondrial 16S ribosomal RNA and highly related cDNAs have previously been observed (Baserga et al., 1985; Guo et al., 2003). The results of the slot blot should be considered semiquantitative, due to variations inherent in any hybridization reaction, the high complexity of cDNA probes, and to detection limitations for low abundance genes (Wang and Brown, 1991).

In order to confirm the slot blot results, we selected two of the novel AD candidates to determine expression levels in human brain RNA distinct from that used in the subtractive cloning. In contrast to the slot blot, northern blotting was carried out on whole brain RNA. By northern blotting, we showed a modest (25% and 23%, respectively) increase in the mRNA levels for EFNA3 and CIZ1, respectively, in AD compared to normal brain (data not shown). Since inappropriate entry into the cell cycle may play a role in AD (Nagy, 2000) and CIZ1 can act as a DNA replication factor (Coverley et al., 2005), we decided to examine CIZ1 tissue expression and cellular localisation in more detail.

Differential expression of CIZ1 splice forms in AD brain

CIZ1 mRNA expression was examined in normal and AD afflicted tissue as well as in a range of human tissues and cell lines by PCR amplification of the region surrounding exon 8. As shown in Fig. 2, the splice variant CIZ1S, which contains a 168 nt deletion in exon 8, is upregulated in AD compared to normal hippocampi, taking into consideration the different levels of GAPDH, used for normalisation, in the two tissues. Quantification of the data in Fig. 2 shows that levels of the full-length CIZ1 are unaltered in AD, whereas expression of the splice variant CIZ1S is upregulated 2.5-fold in AD compared to normal hippocampus, after normalisation to GAPDH. As expected, GFAP expression is increased in AD compared to normal hippocampus, by 5.6-fold, due to astrogliosis (Diedrich et al., 1987).

In AD hippocampus, CIZ1S expression is comparable to that of full-length CIZ1. In contrast, expression of CIZ1S is distinctly lower than the full-length transcript in all the examined normal tissues and cell lines. Amongst the cell lines, CIZ1 was more highly expressed in the Ad5-transformed HEK293 and MCF7 cell lines, and was undetectable in the U118 glioblastoma cell line. Alternative splicing in exon 8 results in the in-frame removal of 56 amino acids (CIZ1S) or 28 amino acids.
(CIZ1M) in the second glutamine-rich domain (Fig. 3). However, transcripts with the shorter deletion in exon 8 were not detected, probably due to a very low mRNA expression.

**Association of CIZ1 with the nuclear matrix requires a glutamine-rich domain**

In order to examine the importance of alternative splicing in exon 8 for the subcellular localisation of CIZ1 protein, we attempted to amplify the coding region of CIZ1 from hippocampus and cerebellum, where both CIZ1 and CIZ1S are expressed (see Fig. 2). However, we were unable to clone the coding region of CIZ1S, although we could obtain clones for both CIZ1 and CIZ1M. Mouse N2A neuroblastoma cells were therefore transfected with expression plasmids for full-length CIZ1 and the shorter isoform CIZ1M (Fig. 3A).

Cell lysates were separated into four fractions: soluble proteins, two fractions of chromatin-bound proteins, and proteins bound to the nuclear matrix. Both CIZ1 and CIZ1M proteins appeared in the soluble fraction and the DNase-treated chromatin fraction. However, the majority of full-length CIZ1 protein was recovered in the nuclear matrix fraction. Deletion of 28 amino acids in the glutamine-rich Q2-region almost completely abrogated association of CIZ1M with the nuclear matrix (Fig. 3B).

In accordance with the fractionation results, immunofluorescent staining of the transfected cells showed that full-length CIZ1 protein appeared in speckles which resemble replication foci (Figs. 4A–C). However, in some cells CIZ1 appeared to be localized mainly in the cytoplasm, where it might be sequestered when it is not involved in DNA replication (Figs. 4D–F). The smaller splice variant CIZ1M did not form the speckled pattern, but instead it localized solely in the nucleus in a diffuse pattern (Figs. 4G–I). Therefore, the region lacking in CIZ1M seems to be important for the location and appearance of CIZ1 in the cell. Since this region is also lacking in the CIZ1S variant, it is likely that the pattern of CIZ1S resembles that shown here for CIZ1M.

**Discussion**

In this study, we demonstrate the altered processing of CIZ1 mRNA in the hippocampus of AD patients. This results in the upregulation of an alternatively spliced form of CIZ1, in which the encoded protein lacks a glutamine-rich domain. This smaller transcript, CIZ1S, is present as a minor component in a range of normal tissues and cancer cell lines, suggesting that the ratio of CIZ1S to the full-length CIZ1 is normally regulated. Misregulation of alternative splicing has previously been reported for sporadic AD, including exon 5 skipping for the Presenilin-2 transcript and exon 10 inclusion in the Tau transcript (Glitz et al., 2006; Manabe et al., 2003). Misregulation of splicing in AD may be a result of genetic mutations in the gene itself or reflect changes in the regulators of splicing, for example as a result of hypoxic stress (Manabe et al., 2003). In the case of CIZ1S, a 168 nt region located centrally in exon 8 is spilled out. Although the “intron” conforms to the GT–AG rule, there is no polypyrimidine tract, and the mechanism regulating alternative splicing in exon 8 is unclear.

Our results demonstrate that the majority of full-length human CIZ1 protein is bound to the nuclear matrix, following extraction with nuclease and high salt. Accordingly, our immunofluorescent staining revealed the localisation of CIZ1 in nuclear speckles. This is in agreement with results obtained with endogenous mouse Ciz1, in which nuclear-matrix-associated Ciz1 is present in replication foci (Ainscough et al., 2007). Our observation that some CIZ1 is released by nuclease treatment is consistent with the role of CIZ1 as a DNA-binding protein (den Hollander et al., 2006; Warder and Keberly, 2003). Furthermore, we observe a proportion of CIZ1 in the soluble fraction, consistent with reports that mouse Ciz1 is present in the nucleus as both a soluble and a detergent-resistant fraction (Coverley et al., 2005). We also observe formation of CIZ1 aggregates in the cytosol of some transfected cells. This may result from self-aggregation of CIZ1 due to its glutamine-rich domains, or to the cytoplasmic sequestration by CIZ1 of cell cycle proteins such as CDKN1A (Mitsui et al., 1999).

Alternative splicing in CIZ1S results in the removal of amino acids 379–434 in the second glutamine-rich domain. Deletion of a corresponding region by alternative splicing has been reported in the embryonic form of mouse Ciz1, called ECiz1 (Coverley et al., 2005) (Fig. 3). We used a transcript with a shorter deletion within exon 8 in cell transfection studies, in order to delineate the role of this region in CIZ1 localisation, since we were unable to clone CIZ1S by PCR. The 28 amino acid region lacking in CIZ1M is also lacking in CIZ1S, and therefore we postulate that the appearance and localisation of CIZ1S resembles that of CIZ1M. However, we cannot exclude the possibility that the larger deletion in CIZ1S, removing 56 amino acids in the glutamine-rich domain of CIZ1, causes additional changes in protein localisation. The CIZ1M variant, lacking amino acids 407–434, showed a disruption of the speckled pattern, and instead a diffuse localization in the nucleus was observed. Furthermore, the CIZ1M protein was readily extracted into the soluble and chromatin fractions, and only weakly associated with the nuclear matrix in our fractionation studies. Ainscough and others carried out immunofluorescence staining with NIH3T3 cells and GFP-tagged ECiz1 (Ainscough et al., 2007). They demonstrated that ECiz1 is less efficiently concentrated in nuclear foci than full-length Ciz1. However, in contrast to our results with CIZ1M, they showed that GFP-ECiz1 resists extraction with detergent and nuclease/high-salt, indicating attachment to the nuclear matrix. They conclude that sequences in the C-terminal third of Ciz1 are responsible for immobilization on the nuclear matrix. However, these results cannot be directly compared to ours, because they use a GFP tag and a milder 0.5 M NaCl extraction, either of which may affect subnuclear attachment.
Two main species of CIZ1 protein are observed in nontransfected cells: 95 and 120 kDa in human, and 100 and 125 kDa in mouse cells (Coverley et al., 2005; Mitsui et al., 1999). In human U2-OS cells, the 95 kDa species is detergent-soluble whereas the 120 kDa form of CIZ1 fractionates in the NP-40 resistant cell pellet (Mitsui et al., 1999). However, in NIH3T3 cells, the p100 form of Ciz1, but not the p125 form, is resistant to 2 M NaCl extraction, suggesting a stronger association of the p100 form with subnuclear structures (Ainscough et al., 2007). These differences may reflect the different detergent treatments and cell lines used. It may also suggest that there are differences in the behaviour of mouse and human CIZ1, which share only about 70% homology. The relation between the 95 and 120 kDa protein species is currently unclear. However, the larger 120 kDa species behaves like full-length CIZ1 does in our hands, whereas the 95 kDa protein in human cells and CIZ1M are both detergent-soluble. The 28 amino acid region lacking in CIZ1M is also lacking in CIZ1S, and therefore we postulate that CIZ1M and CIZ1S behave similarly. Thus, the 95 kDa form may arise from alternative splicing in exon 8 and thereby generating an isoform lacking part of the second glutamine-rich domain, corresponding to either CIZ1M or CIZ1S.

We demonstrate for the first time that human CIZ1 associates with the nuclear matrix. Furthermore, we report that in human CIZ1, amino acids 407–434 affect the formation of speckles and attachment to the nuclear matrix. Since this exon 8-encoded region is also lacking in the CIZ1S variant, we postulate that the appearance and localisation of CIZ1S resembles that of CIZ1M. Mouse ECIz1 retains the ability to promote DNA replication, suggesting that exon 8 is not required for this basic function (Coverley et al., 2005). There are 4 binding regions for CDKN1A on CIZ1: one between the two glutamine-rich domains, two in the zinc finger region, and one in the C-terminal end (Mitsui et al., 1999). Since these regions are outside of the regions deleted in CIZ1M and CIZ1S, it is likely that CIZ1M and CIZ1S also bind to

![Fig. 4. Subcellular localization of V5-tagged CIZ1 isoforms. Staining for the full-length variant of CIZ1 showed a speckled pattern, localized in varying patterns: in the nucleus (A), cytosol (D) or both (not shown). The CIZ1M variant lacking part of the second polyglutamine domain was diffusely distributed throughout the nucleus (G). (A, D, G) V5 staining; (B, E, H) DAPI staining; (C, F, I) merged images.](image-url)
CDKN1A. Ciz1 is proposed to play a role in replication factory formation, by tethering replication factories to the nuclear matrix (Ainscough et al., 2007). Thus, assembly of CIZ1 into nuclear foci is likely to be important for regulated entry into the S-phase of the cell cycle. The increased expression of CIZ1S compared to CIZ1 in AD may therefore be expected to contribute to disregulation of cell cycle control.

Experimental methods

Slot blot

A 1 kb PCR product for human GAPDH cDNA was cloned into the pCR-TOPO vector (Invitrogen). The other plasmids used in the slot blot, containing cDNA inserts in the pZL1 plasmid (Invitrogen), were isolated in a subtractive screen for genes upregulated in AD (Mitchelmore et al., 2004; Table 1). Plasmid DNA (1 μg) was denatured in 0.3 M NaOH for 10 min at 68 °C, neutralised and bound in duplicate to a Hybond-XL membrane in a slot–blot vacuum apparatus. The membrane was cut in half and the duplicate blots were separately hybridised to radioactively labelled first-strand cDNA (100 ng) which had been prepared from either AD or control hippocampus RNA (Mitchelmore et al., 2004). To block non-specific binding, the hybridisation buffers contained 500 μg denatured salmon sperm DNA and 5 μg poly-dT20, and the overnight hybridisation was carried out at 72 °C. Stringent hybridisation and wash conditions were used to reduce cross-hybridisation with related sequences. Bound label was quantified with a phosphorimager.

RT-PCR

cDNA synthesis on U118 mRNA was performed using SuperScript III Reverse Transcriptase Kit (Invitrogen). First-strand cDNA from liver and colon, Marathon cDNA from cerebellum and hippocampus, and cDNA from a human cell line panel were all purchased from Clontech. The 25 μl PCR reaction contained 2.5 μl cDNA, 10 pmol each primer, 2.5 μl reaction buffer, 0.2 M dNTPs and 0.5 μl Advantage II Polymerase (Clontech). The PCR program was: 1 cycle at 94 °C; 5 cycles at 94 °C for 20 s, 72 °C 2 min; 15–25 cycles at 94 °C for 30 s, 72 °C 2 min; and 1 cycle at 94 °C 5 min.

The following primer pairs were used in PCR: CIZ1 (5′ TATCCGAAA-CACACAGACACACACACAC 3′ and 5′ AACCCACAACTTGTTCCTGCTGAG 3′); GAPDH (5′ TGAAGCTGAGCTACCCGATTGTTGT 3′ and 5′ CATGGGCGGATAGGTCACCC 3′); and GAP (5′ TACCCCTTCACCA-CATAGTGAC 3′ and 5′ CTATCTTCGAGGACTTGCTGCTG 3′). The expected fragment sizes were: GAPDH 983 bp, GAP 424 bp, CIZ1 585 bp, CIZ1M 501 bp and CIZ1S 417 bp. The identity of the PCR bands obtained for CIZ1 were confirmed by sequencing.

Cell transfections

The coding region of CIZ1 was obtained by PCR amplification from cerebellum cDNA and cloned into the pcDNA6/V5HisA vector (Invitrogen), in frame with the C-terminal V5-epitope. The inserts were completely sequenced, and shown to encode full-length CIZ1 as well as the CIZ1M splice variant. Mouse neuroblastoma (N2A) cells were grown in Dulbecco’s modified Eagle’s medium containing 10% FBS with penicillin/streptomycin. 2.5 × 10^5 cells/well were seeded in a 24-well plate, transfected with 4 μg DNA per well using Lipofectamine 2000 (Invitrogen), and harvested after 48 h.

Immunocytochemistry

2 × 10^4 cells were grown on coverslips in 24-well plates for 24 h before transfection, and then grown for 48 h before being fixed in cold methanol for 5 min. Cells were blocked in PBS containing 0.2% Triton X-100 and 3% BSA and washed in PBS with 0.2% Triton X-100. Mouse anti-V5 antibody (Invitrogen) was diluted 1:400, incubated overnight on the coverslips in a humidity chamber at 4 °C, and was detected using Alexa Fluor 488 anti-mouse antibody. Coverslips were mounted onto slides using Vectashield mounting medium with DAPI (Vector). Images were collected using a Leica FW 4000 camera with a 100× oil objective.

Cell fractionation and western blots

The protocol is adapted from (Farley et al., 2006; He et al., 1990). Cell pellets were washed twice in PBS and centrifuged 5 min at 2000 rpm. The cell pellet was resuspended in cold cytoskeletal (CSK) buffer (20 mM PIPES (Sigma), 100 mM NaCl, 300 mM Sucrose, 3 mM MgCl2, 1 mM EGTA (Sigma), protease inhibitor (1:500 v/v) (Sigma), 0.5% Triton X, pH 7), incubated at room temperature for 5 min and centrifuged 5 min at 2000 rpm. The supernatant was collected and labeled “Soluble fraction”. This step was repeated once, and the pellet was resuspended in CSK buffer containing 250 U/ml DNase I (Roche) and incubated 15 min at 20 °C, followed by 20 min at 37 °C. Then (NH4)2SO4 was added to give a final concentration of 0.25 M, and the samples were incubated 5 min on ice. Hereafter the samples were centrifuged at 8000 rpm for 5 min. The supernatant was collected and labeled “Chromatin DNase fraction”. The pellet was then resuspended in CSK buffer containing 2 M NaCl, incubated 5 min on ice, and centrifuged 5 min at 13,000 rpm. The supernatant was collected and labeled “Chromatin NaCl fraction”.

The remaining protein was resuspended in 8 M urea solution (8 M urea pH 8, 100 mM Na2HPO4, 10 mM Tris–HCl), and labeled “Nuclear Matrix fraction”.

Protein concentration was measured using the Bradford assay. Proteins were separated on 4–12% Tris–Glycine SDS gels (Anamed), and transferred to PVDF membranes (GE Healthcare). Immunoblotting was performed using the West Dura kit (Pierce). Primary antibodies were diluted as follows: anti-V5 (1:20,000), mouse anti-α-tubulin and rabbit anti-histone H3 (1:10,000, Sigma); and rabbit anti-lamin A/C (1:4000, Cell Signaling Technology). Protein bands were detected using a Biospectrum AC imaging System (UVP).

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References


