A yeast model for the study of human DFNA5, a gene mutated in nonsyndromic hearing impairment

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Received 30 January 2003; received in revised form 8 May 2003; accepted 14 May 2003

Abstract

A mutation in human DFNA5 is associated with autosomal dominant nonsyndromic hearing impairment. The function of DFNA5 protein remains unknown and no experimental model has been described so far. Here we describe fission yeast Schizosaccharomyces pombe as a model organism for studying the function of heterologously expressed DFNA5. We have expressed wild-type as well as mutant DFNA5 alleles under control of regulatable nmt1 promoter. Yeast cells tolerated expression of wild-type DFNA5, while expression of the mutant DFNA5 allele, which is responsible for nonsyndromic autosomal dominant hearing impairment, led to cell cycle arrest. We identified new rat and horse DFNA5 homologues and we describe a domain of homology shared between DFNA5 and the Mcm10 family of DNA replication proteins. Genetic interactions between heterologously expressed DFNA5 and a fission yeast cdc23 (mcm10) mutant support a possible link between DFNA5 and Mcm10 proteins.

Keywords: Cell cycle; MCM10; Schizosaccharomyces pombe; Hearing impairment; DFNA5; Yeast

1. Introduction

Hearing impairment is the most frequent sensory handicap. Most of the childhood to early adulthood onset forms of nonsyndromic hereditary hearing impairment exhibit an autosomal dominant inheritance pattern and are indicated by DFNA. To date, more than 90 chromosomal loci for nonsyndromic hearing impairment have been mapped to the human genome (Hereditary Hearing Loss Homepage, Van Camp and Smith, World Wide Web URL: http://www.uia.ac.be/dnalab/hhh).

Human DFNA5 (ICERE-1) is one of the genes associated with nonsyndromic autosomal dominant hearing impairment. An insertion/deletion mutation in intron 7 of this gene causes skipping of exon 8, resulting in premature termination of the open reading frame [1]. In addition to its relevance to hearing impairment, the expression pattern of human DFNA5 indicated that this gene might participate in the tumor biology of breast and skin cancers [2,3]. The physiological function of the DFNA5 protein remains unknown and computational analysis did not give any information on the protein function [1]. In addition, no significant homology with any other protein could be detected. Here we report that DFNA5 shares sequence similarity with the Mcm10 family of DNA replication proteins.

The Mcm10 (dna43) gene was first identified in yeast Saccharomyces cerevisiae in two genetic screens for genes required for chromosomal DNA replication and mini-chromosome maintenance [4,5]. Mcm10 is required for efficient initiation of DNA replication. Mcm10 may also be required for the elongation step of DNA replication [6]. Recent observations showed that Mcm10 performs its function after prereplicative complex assembly and is required for loading of Cdc45 onto chromatin [7] (J. Gregan and S.E. Kearsey, unpublished data). All known members of the Mcm10 family share a conserved zinc-finger-like motif, referred to as the CCCH domain [8], which is essential for the Mcm10
function [9,10]. The hallmarks of the CCCH domain are conserved cysteine residues resembling a zinc-finger motif as well as conserved adjacent glycine and arginine residues (Fig. 1).

2. Materials and methods

2.1. Fission yeast methods

Media, growth conditions and genetic methods were as described by Moreno et al. [11]. Thiamine was used at 5 μg/ml to repress the nmt1 promoter.

Flow cytometry analysis and microscopy were as described in Ref. [12].

2.2. Plasmid constructs

2.2.1. Horse and rat homologues of DFNA5

A horse skin library in λ TriplEx (Clontech) were plated using standard techniques. A 1500-bp mouse DFNA5 hybridization probe was PCR-generated using primers 5'-GTTGATGCTGGAGGAGACC-3' and 5'-GTCTTTGACCTGATGCTCC-3' and the previously cloned mouse Dfna5h as template [1]. The probe was purified with the QiAquick Gel Extraction Kit (Qiagen). Membranes resulting from plaque lifting were hybridized with the 32P-labelled probe using standard techniques. Positive clones were rescreened to purity and phagemid was excised according to the manufacturer’s guidelines. Inserts were sequenced using the Big-Dye Dye Terminator Cycle Sequencing Kit (ABI). Fragments were separated on an ABI 3100 automated DNA sequencer (ABI).

After isolation of the partial rat cDNA, a rat-specific probe was used for PCR primers 5'-GAGGCTTCC-

GTTCCCTTGGC-3' and 5'-CAGCAGTTTGCTGGTGTCGC-3'. In addition, 5'-RACE experiments were performed on the rat library using a rat DFNA5-specific primer 5'-CCACGATTTTGATACGATGCTCC-3' and a TriplEx vector primer 5'-TCGGGAAGACCGCCATTGTGTGTTG-3'.

2.2.2. Cloning of human full-length and mutant DFNA5

To obtain a human full-length DFNA5 clone, a PCR reaction was performed on Human Leukocyte Marathon-Ready cDNA (Clontech) using the Advantage cDNA PCR kit (Clontech) and the following primers: 5'-CCACGATTTTGATACGATGCTCC-3' and 5'-GAGGCTTCC-

GTTCCCTTGGC-3'. In addition, 5'-RACE experiments were performed on the rat library using a rat DFNA5-specific primer 5'-CCACGATTTTGATACGATGCTCC-3' and a TriplEx vector primer 5'-TCGGGAAGACCGCCATTGTGTGTTG-3'.

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GTTCCCTTGGC-3'. In addition, 5'-RACE experiments were performed on the rat library using a rat DFNA5-specific primer 5'-CCACGATTTTGATACGATGCTCC-3' and a TriplEx vector primer 5'-TCGGGAAGACCGCCATTGTGTGTTG-3'.

To obtain a human mutant DFNA5 clone, total RNA was isolated from EBV-transformed lymphoblastoid cell lines derived from DFNA5 patients [1] using Trizol (Life Technologies). cDNA was prepared using random hexamers from the SuperScript Preamplification System (Life Technologies). A similar PCR reaction as described above was performed. The resulting PCR fragments were cloned into the pCR4-TOPO vector using the TOPO TA Cloning kit (Invitrogen), and colonies that contained mutant DFNA5 were selected.

Both the human full-length and the mutant DFNA5 clones were completely bidirectionally sequenced in order to exclude the introduction of cloning errors.

Mouse Dfna5h was PCR amplified from the pBluescript-Dfna5h using oligonucleotide primers 5’-HolmousedFNA5 (ATAGCTCGAGATGCTGGGCGCAAGCAACTC) and 3’-SmalmediumFNA5 (ATAGCCGGGCGCTAGTCTGGGAC-
TGTAG) and cloned into XhoI and SmaI sites of the plasmid pREP3x, resulting in pREP3x-mDFNA5.

Human DFNA5 was excised from the plasmid pT-Adv-hDFNA5 by BamHI and SmaI and cloned into BamHI and SmaI sites of the plasmid pREP3x, resulting in pREP3x-mDFNA5. Mutant DFNA5 was excised from the plasmid pCR4-hDFNA5mut by EcoRI and cloned into the EcoRI site of the plasmid pT-Adv-hDFNA5, replacing the wild-type DFNA5 allele and resulting in pT-Adv-hDFNA5mut plasmid. Mutant DFNA5 was excised from the plasmid pT-Adv-hDFNA5mut by BamHI and HincII and cloned into the BamHI and SmaI sites of the plasmid pREP3x, resulting in pREP3x-hDFNA5mut plasmid. All constructs were verified by sequencing.

2.3. Yeast strains

Degron-cdc23-M36 strain P1146 (cdc23-M36-td) was constructed by BglII linearization of the plasmid pSMRG2+deg+cdc23 (J. Gregan and S.E. Kearsey, unpublished data) and integration at the cdc23 locus of the Schizosaccharomyces pombe strain P155 (cdc23-M36 h−), which contained cdc23-M36 temperature-sensitive allele. A similar strategy was used to construct degron-cdc23-IE2 strain P1145 (cdc23-IE2-td), where pSMRG2+deg+cdc23 plasmid was integrated at the cdc23 locus of the S. pombe strain P893 (cdc23-IE2 leu1-32 h−), which contained the cdc23-IE2 temperature-sensitive allele.

Fig. 2. Multiple alignment of DFNA5 proteins. DFNA5 homologues from human (Swiss-Prot: O60443), mouse (Swiss-Prot: Q9Z2D3), horse (GenBank: AY194290) and partial sequence of the rat (GenBank: AY194291) were aligned using CLUSTAL W [22]. Identical amino acid residues are shaded in black and similar amino acid residues are in gray shades.
DFNA5 proteins were heterologously expressed in a wild-type S. pombe strain P138 (ade6-M210 leu1-32 ura4-D18 18 h)

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) assay

RT-PCR were performed with total cellular RNA, avian myeloblastosis virus reverse transcriptase and Tfl DNA polymerase (Promega) according to the manufacturer’s protocol.

Two oligonucleotide primers, DFNA5 5’ (atggttaccaag-cacagag) and DFNA5 3’ (tcagatcacaactctg), were used to amplify a 515-bp RT-PCR product corresponding to the human DFNA5 mRNA. The identity of the DFNA5 RT-PCR product was confirmed by sequencing.

3. Results

3.1. Sequence analysis

We used PROSITE [14] to search SWISS-PROT, TrEMBL and PDB databases using a consensus sequence of the CCCH domains of Mcm10 proteins as a PROSITE entry. Apart from known Mcm10 homologues, we identified the human DFNA5 protein and its mouse homologue Dfna5h (Fig. 1). Interestingly, the mutation associated with nonsyndromic hearing impairment was a region containing the CCCH domain [1]. Iterative PSI-BLAST searches [15] with the CCCH domains did not detect any significant homologies other than DFNA5, Dfna5h and members of the Mcm10 family.

To further characterize the DFNA5 proteins, we identified additional DFNA5 homologues. Screening one million plaques from a horse skin cDNA library resulted in eight positive clones. Three of these clones were chimeric, four contained partial horse DFNA5 mRNA and one contained the complete horse DFNA5 mRNA (2219 bp; GenBank accession number AY194290). The horse DFNA5 protein showed 75.0% similarity with human and 66.6% similarity with mouse DFNA5.

Only one partial cDNA clone resulted after screening 10^6 clones from a rat brain cDNA library (1515 bp; GenBank accession number AY194291). The rat brain library was rescreened using a rat-specific DFNA5 probe. In addition, 5’-RACE experiments were performed on the rat brain library. However, we did not succeed in generating a full-length rat DFNA5 clone. The partial rat DFNA5 protein showed 90.7% similarity with mouse, 64.0% with human and 60.4% with horse DFNA5 (Fig. 2). Both rat and horse DFNA5 homologues share the domain of homology with the Mcm10 family (Fig. 1). Nuclear localization has been shown experimentally for the human, Xenopus, S. pombe and S. cerevisiae Mcm10 proteins [16]. Subcellular localization of DFNA5 proteins has not been determined yet, but both human DFNA5 and mouse Dfna5h as well as horse DFNA5 proteins show a stretch of positively charged amino acid residues (KKKR) near their N-termini, which resemble a basic core of the monopartite nuclear localization signal K(K/R)X(K/R) [17]. Although PSORT II prediction identified a “pat4” type of nuclear localization signal in all DFNA5 homologues, it predicted the cytoplasmic localization of DFNA5 proteins as the most likely [18]. Therefore, the subcellular localization of DFNA5 proteins will need to be experimentally determined.

3.2. Heterologous expression of DFNA5 genes in yeast

To further characterize DFNA5, we have cloned wild-type alleles of the mouse Dfna5h and human DFNA5 as well as the mutant DFNA5 allele from DFNA5 patients and expressed them in the fission yeast S. pombe under a control of regulatable nmt1 promoter [19]. The nmt1 promoter can be repressed in the presence of thiamine in the growth medium and it can be induced by removing the thiamine. While heterologous expression of the human and mouse DFNA5 proteins did not result in any apparent phenotype, expression of the mutant DFNA5 allele caused loss of viability of the yeast cells as assessed from growth on plates lacking thiamine (Fig. 3A). We also observed a reduction in

Fig. 3. Heterologous expression of the DFNA5 proteins in fission yeast. (A) Wild-type strain (P138) transformed with either empty vector pREP3x (pREP), or construct containing mouse Dfna5—pREP3x-mDFNA5 (DFNA5mouse), or human wild-type DFNA5—pREP3x-hDFNA5 (DFNA5) or mutant allele of human DFNA5—pREP3x-hDFNA5mut (DFNA5mut) were grown on EMM plates with or without added thiamine for 3 days at 32 °C. (B) Wild-type strains (P138) transformed with either empty vector pREP3x (wt-pREP) or construct containing mutant DFNA5 allele—pREP3x-hDFNA5mut (DFNA5mut) were grown in EMM media without thiamine to induce expression of the mutant DFNA5 gene for the indicated time. Aliquots were taken and plated onto YE3S plates to determine viability. Percentage of relative viability was calculated as (number of colonies at time = t since thiamine withdrawal)/100(number of colonies at t = 18 h since thiamine withdrawal). Values represent averages of two experiments. (C) Flow cytometry analysis of the strains described for panel A. Aliquots for flow cytometry were taken at indicated time since thiamine withdrawal. (D) Light microscopy images of ethanol-fixed cells as described for panel A taken at indicated time since thiamine withdrawal. Left-hand panels show phase images and right-hand panels show DAPI staining of DNA. (E) RT-PCR assays detecting expression of wild-type and mutant alleles of the human DFNA5 heterologously expressed in the yeast cells. RT-PCR assays were performed with total RNA isolated from the wild-type yeast strain P138 (wt) (lanes 1 – 2) or the P138 strain transformed with either empty vector pREP3x (pREP) (lanes 3 – 4) or pREP3x-hDFNA5 construct (DFNA5) (lanes 5 – 10) or pREP3x-hDFNA5mut construct (DFNA5mut) (lanes 11 – 16). Strains were grown in EMM medium with or without thiamine for 20 h. RT-PCR products obtained after 25 or 28 cycles of PCR amplification are shown. RT-PCR products are also seen under + thiamine conditions (lanes 5.6, 11,22) as the nmt1 promoter is known to be leaky when repressed with thiamine. Control reactions after 28 cycles of PCR amplification where no reverse transcriptase was added (− RT) to exclude DNA contamination in the samples are shown. DNA size marker (1-kb DNA ladder, Invitrogen) is given in lane 17 (M).
A

[Images of circular patterns with +thiamine and -thiamine labels]

B

[Graph showing % relative viability vs. induction of expression in hours, with markers for pREP and DFNAs^mut]

C

[Images of cell growth patterns over time, labeled as DFNAs^mut-thiamine, DFNAs-thiamine, and DFNAs^mut+thiamine]

D

[Images labeled as DFNAs^mut (24h-thiamine), DFNAs^mut (30h-thiamine), and pREP (30h-thiamine)]

E

[Image of gel electrophoresis with markers for 1018bp and 506bp, with bands labeled as 28, 28, 28, 25, 25, 25, 25, 25, 25]
viability in exponentially growing cells after transfer to medium lacking thiamine (Fig. 3B). Flow cytometry of exponentially growing wild-type haploid fission yeast cells shows only peak corresponding to 2C DNA content. This is because cells enter S phase before cytokinesis is completed. Flow cytometry analysis showed that yeast cells expressing

![Image](image_url)

Fig. 4. Mutant DFNA5 allele heterologously expressed in fission yeast has synthetic phenotype with cdc23 mutant. (A) cdc23-M36-td and cdc23-IE2-td strains containing temperature-sensitive alleles of cdc23 and transformed with either empty vector pREP3x (cdc23-M36-td, cdc23-IE2-td) or construct containing mutant DFNA5 allele—pREP3x-hDFNA5mut (cdc23tstd-DFNA5mut, cdc23-IE2-td-DFNA5mut) were grown on EMM with or without thiamine at permissive temperature, 25 °C, for 24 h and fixed with ethanol. Left-hand panels show phase images and right-hand panels show DAPI staining of DNA. Percentage of elongated cells (longer than 15 μM) is shown. (B) Flow cytometry analysis of the cdc23-M36-td strain transformed with either empty vector pREP3x (cdc23-M36-td) or construct containing mutant DFNA5 allele—pREP3x-hDFNA5mut (cdc23tstd-DFNA5mut). Strains were grown in EMM media without thiamine at permissive temperature (25 °C) for 16 h to induce expression of the mutant DFNA5. The cultures were then split and one half was shifted to nonpermissive temperature (37 °C) to inactivate the Cdc23 protein. Aliquots for flow cytometry were taken at indicated time.
the mutant DFNA5 arrested in cell cycle with approximately 1C DNA content, suggesting that the cells were blocked in G1 or early S phase (Fig. 3C). Interestingly, this is the phenotype associated with mcm10 mutants [5,8]. However, unlike many fission yeast mutants blocked in DNA replication, cells expressing mutant DFNA5 did not elongate signiﬁcantly (less than 5% of cells were elongated) and arrested cells did not show a “cut” phenotype where septation and/or cytokinesis occur in the absence of normal sister chromatid separation (Fig. 3D). We used an RT-PCR assay to conﬁrm that both the wild-type and the mutant alleles of human DFNA5 are expressed in the yeast cells upon induction (Fig. 3E).

3.3. Genetic interactions between DFNA5 and mcm10

Following a possible link between DFNA5 and Mcm10 proteins, we tested the effect of DFNA5 expression in a fission yeast mcm10 homologue, the cdc23 mutant. Neither human nor mouse DFNA5 proteins were able to complement a fission yeast cdc23 mutant (not shown), which is not surprising since the proteins do not share signiﬁcant similarity outside of the CCCH domain. When mutant DFNA5 was expressed in a cdc23 temperature-sensitive degron mutant strains (cdc23-M36-td, cdc23-IE2-td), even at the permissive temperature there was a signiﬁcantly higher number of elongated cells compared to strains not expressing the mutant DFNA5 (Fig. 4A). Elongated cells indicate a cell cycle block, which uncouples growth from cell cycle events (e.g. block in chromosomal DNA replication). Moreover, when mutant DFNA5 expression was induced in a cdc23 temperature-sensitive degron mutant and subsequently shifted to nonpermissive temperature, cells arrested with unreplicated DNA earlier than strains not expressing mutant DFNA5 allele (Fig. 4B). Therefore, in fission yeast, heterologous expression of the mutant DFNA5 allele has a synthetic phenotype with the cdc23.

4. Discussion

Here we describe the first model system for studying molecular effects caused by the mutation in the DFNA5 gene, which was found to be responsible for nonsyndromic hearing impairment. Comparison of the wild-type and the mutant DFNA5 alleles heterologously expressed in yeast allowed us to examine a phenotype associated with a dominant negative or gain of function effect of the DFNA5 mutant. We provide several lines of evidence suggesting a possible link between the DFNA5 and Mcm10 proteins. Firstly, we identiﬁed a domain of homology shared between the Mcm10 family of replication proteins and members of the DFNA5 family, including the newly described horse and rat DFNA5 homologues. The function of the CCCH domain remains obscure, but its importance is underlined by the fact that it is essential for the function of Mcm10 proteins. In addition, this domain is absent in the dominant DFNA5 mutant resulting in hearing impairment. Secondly, we found that heterologous expression of the mutant DFNA5 in fission yeast leads to a block in G1 or early S phase of the cell cycle with approximately 1C DNA content. Furthermore, expression of the mutant DFNA5 in the yeast cdc23 mutant has a synthetic growth defect and the arrest caused by cdc23 inactivation is faster when the mutant DFNA5 is induced. The observed effect could be either direct or indirect and it is possible that there are other currently unidentified cellular processes affected by the mutant DFNA5 allele.

Could DFNA5 have a function related to the DNA replication function of Mcm10? This cannot be strongly predicted since the proteins do not share signiﬁcant similarity outside of the CCCH domain and it is unlikely that DFNA5 is a distant homologue of Mcm10 proteins. However, it is of interest that the reduction in DFNA5 expression can contribute to the etoposide resistance in human melanoma cells. The etoposide-resistant phenotype may be due to an increased cellular susceptibility to drug-induced apoptosis [3]. Etoposide is used as an antineoplastic agent and causes DNA strand breakage via inhibition of DNA topoisomerase II. This could implicate DFNA5 in some aspect of the response to DNA damage. Indeed, alterations in DNA repair proteins have been observed in drug-resistant melanoma lines [20,21]. Alternatively, DFNA5 and Mcm10 proteins may have unrelated functions, but share the CCCH domain to perform an activity unrelated to DNA replication. Further experiments are required to clarify the function of DFNA5. Our fission yeast model now provides a tool to examine the molecular basis underlying the DFNA5 hearing impairment in human.

Acknowledgements

This work was supported by a grant from the Cancer Research UK.

LVL holds a research position with the Flemish FWO (Fonds voor Wetenschappelijk Onderzoek). This research was supported by a grant of the GSKE (Geneeskundige Stichting Koningin Elisabeth) to GVC and LVL, and by a grant of the University of Antwerp to LVL.

The construction of the horse cDNA library was funded in part by The United Professional Horsemen’s Association, The American Saddlebred Horse Association and The Belgian Draft Horse Corporation.

We thank Lynne Larkman for technical assistance.

References


