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Deciphering the molecular functionality of Cdc45 in replisomal complex

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ABSTRACT

The members of DHH superfamily have been reported with diverse substrate spectrum and play pivotal roles in replication, repair, and RNA metabolism. This family comprises phosphatases, phosphoesterase and bifunctional enzymes having nanoRNase and phosphatase activities. Cell cycle factor Cdc45, a member of this superfamily, is crucial for movement of the replication fork during DNA replication and an important component of the replisome. The specific protein-protein interactions of Cdc45 with other factors along with helicase moderate the faithful DNA replication process. However, the exact biochemical functions of this factor are still unknown and need further investigation. Here, we studied the biochemical roles of Cdc45 and its molecular interactions within the replisomal complex. The alteration in the level of protein, observed when DNA damage is induced *in-vivo*, suggests its association with DNA replication stress. We analyzed protein Cdc45, providing new insights about the molecular and biochemical functionality of this replisomal factor.

1. Introduction

The genome surveillance system consists of a diverse array of proteins as part of replisome including Cdc45, MCM, GINS, primase, DNA pol, sliding clamp, and single-strand binding protein etc. [1]. The cell cycle factor, Cdc45, coordinates helicase movement and its functioning [2]. Cdc45 is a member of the DHH family of proteins, consisting of RecJ, polyphosphatase, prune, phosphoesterase and bifunctional enzymes. The role of Cdc45 protein is important for the assembly of the active helicase called the CMG complex (Cdc45-MCM-GINS) and is associated with stress tolerance in prokaryotes and eukaryotes [3–6]. However, the biochemical roles of cdc45 are not clearly understood. The orthologs of Cdc45 exist in archaeal and prokaryotic organisms, suggesting universal functional homology in DNA replication process in living organisms [7]. Cdc45 mutations can lead to a distinct phenotype characterized by craniosynostosis and anorectal malformation. The members of this DHH family are mainly phospho-esterase and phosphatases. The structural similarities between Cdc45 and archaeal GAN nuclease might suggest an evolutionary relationship. It is speculated that Cdc45 may have originated from an ancestral exonuclease-like protein [8]. However, the biochemical functionality of Cdc45 still remains ambiguous. Here, we report molecular and biochemical analysis of the cell cycle factor, Cdc45 and its interactions in replisome.

2. Material and methods

2.1. Expression analysis, and assays

The coding region of hcdc45 was PCR amplified using the template hcdc45 clone (a kind gift from Dr. Anna Szambowska) and cloned in the double restricted pET28a (Novagen) vector to form a clone plasmid pRS1, which is used for protein expression of cdc45. The coding region of cdc45 is also cloned in pCMV6 with FLAG tag, as pRS2 for cell culture and replisome analysis. The sequence of the clone is confirmed by DNA sequencing, indicating no mutations in clones. hCdc45 purified by heterologous expression in *E.coli* BL21DE3, contains a full-length Cdc45 polypeptide with a C-terminus comprising the 6X His-tag. Protein purity was assessed on SDS-PAGE. The confirmation of Cdc45 was done using western blot against His-tag mouse monoclonal antibodies (Immunotag). The protein has been purified with homogeneity and then used for activity assay analysis. The phosphatase and phosphodiesterase activity of Cdc45 was estimated as reported previously [7]. The clone pRS2,

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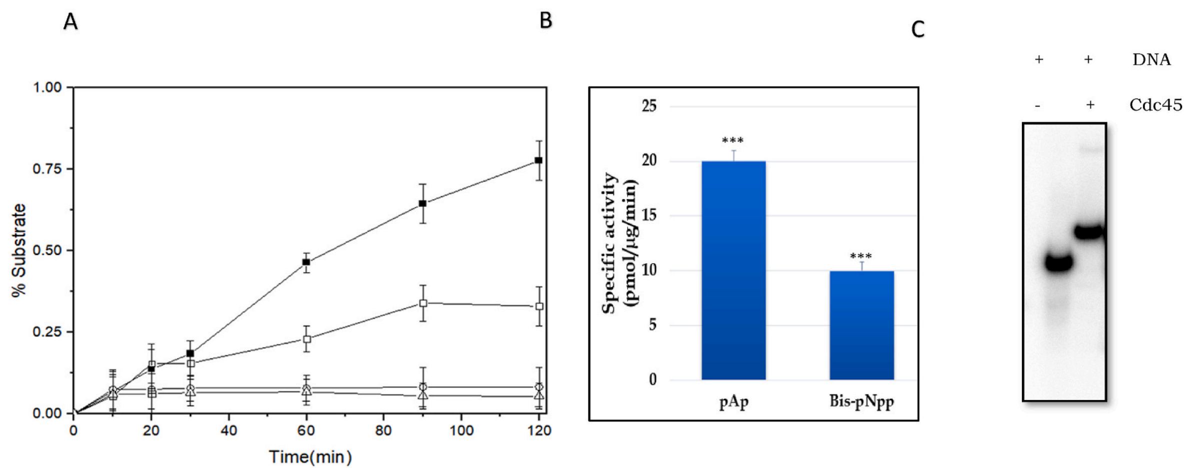

Fig. 1. Biochemical functionality of Cdc45 protein
[A] The hydrolytic activity of purified hCdc45 protein on pAp and Bis-pNpp *in-vitro*. The activity was measured at various time intervals on phosphoadenosine phosphate (pAp) for native (wild-type) WT Cdc45 and mutant (MT) Cdc45 proteins; (WT, ■) (MT, o) and bis-nitrophenyl phosphate (Bis-pNpp) [(WT, □)] (MT, Δ). [B] The specific activity of Cdc45 observed to be higher for pAp. [C] DNA binding and shift (EMSA) analysis for 10mer oligonucleotide.

(having cdc45 gene cloned in pCMV6 Flag), is transfected into HEK293T using Lipofectamine 2000 (Life Technologies, USA) as per the manufacturer's protocol. Subsequently, on the second day, when the plate was confluent, the cells were harvested and the Cdc45 was immunoprecipitated using anti-FLAG beads (Sigma) as per manufacturer's protocol. The presence of FLAG tagged Cdc45 is validated using anti-flag antibodies. The complex of Cdc45 and its associated protein is subjected to MS analysis for identification of its components in an outsourcing facility. Quantification of the blots were done using ImageJ (http://rsb.info.nih.gov/ij/) ImageJ is a Java based (runs on all operating systems) freeware by Wayne Rasband from National Institute of Health (USA).

3. Results

3.1. In vitro phosphoesterase activity analysis

The purified hCdc45 was tested for its phosphoesterase activity including CysQ like phosphatase activity. The purified Cdc45 can hydrolyze phosphoadenosine phosphate (pAp) *in vitro*. CysQ-like phosphatase activity is a unique feature of the proteins of the DHH family. This activity suggests that Cdc45 can interact and bind with smaller ligands including pAp and it can break the terminal ester bond. The specific activity of the Cdc45 protein was observed to be 20.5 pmol/g/min on pAp. In our screening, we also observed that purified Cdc45 can hydrolyze bis-pNpp in vitro, suggesting phosphodiesterase activity potential. The specific activity is observed to be 10 pmol/μg/min. We observed that the rate of hydrolysis of pAp is higher in comparison to Bis-pNpp, which also suggests a higher affinity for pAp (Fig. 1). The putative active site residues interactions to be involved in catalysis are analyzed and represented in Fig. 3 (A-G) for pAp and Bis-pNpp. The analysis suggests that 3′-phosphoadenosine phosphate is preferred over bis-pNpp. The hydrolysis bis-pNpp is similar activity like RecJ and RecJ-like members. There is no exonuclease activity observed in the Cdc45 protein in our analysis (data not shown).

3.2. Interactions within replisome complex

The cell division cycle protein Cdc45 is speculated to be an important factor for replication fork establishment and a key point of movement molecular interaction within the CMG complex. The treatment with mitomycin C may lead to alkylation and retarded DNA replication. The basal expression of Cdc45 changed with the concentration of DNA cross-linker mitomycin C (MMC) in Human Kidney (HK-2) cells. We observed that the Cdc45 level gets altered with mitomycin C, treatment indicating its association with DNA damage tolerance in a dose-dependent manner (data not shown). The expression level of Cdc45 is tightly regulated for proper molecular interactions within replisomal complex during cell cycle process. We identified important factors associated with Cdc45-FLAG followed by immunoprecipitation analysis, represented in Fig. 4. This represents the interacting partners having association with the Cdc45 functioning in replisomal complex.

4. Discussion

There are various biological functions associated with DHH family members. The substrate spectrum of this superfamily of proteins is diverse, ranging from smaller molecules like phosphoadenosine phosphate and dinucleotides to larger DNA molecules. Notably, the smaller RNA (>5mer) can alter nucleic acid metabolism by altering priming for transcription and subsequently leading to changes in global gene expression [10]. Therefore, the concentration of these smaller molecules, by-product of nucleotide degradation, may be regulated by DHH family protein like NrnA. The metabolite, 3′-phosphoadenosine 5′-phosphate (pAp) is generated during nucleotide metabolism and acyl carrier protein synthesis. 3′-phosphoadenosine 5′-phosphate can act as a substrate for NrnA. It is observed that 3′-phosphoadenosine phosphate is preferred over bis-pNpp. Further, the hydrolysis of pAp & bis-pNpp suggests that the target protein has similar functionality like RecJ and NrnA [13].

The analysis of the specific rate of reactions with RecJ, NrnA orthologs of this family suggests that there is comparatively low enzymatic activity *in-vitro*. This could be because of two probable reasons. Either Cdc45 is not processive like RecJ and NrnA or different *in-vitro* experimental conditions than physiological conditions. Further, we also checked the exonuclease function of the Cdc45 using ssDNA as substrate. However, we could not observe exonuclease activity on nucleic acids during the analysis (data not shown). This may be because of the different DHH domain architecture, as similarly observed in previous studies [8,11] (Fig. 1). There are observations which suggest that DHH family proteins and NrnA-like proteins can have pAp-phosphatase activity, but no exonuclease like function [9]. However, these unique aspects of these proteins need to be investigated further to decipher their physiological significance. The *in-vivo* roles of Cdc45 need to be examined.

There are specific interactions of Cdc45 with protein of replisome

Fig. 2. – (A) Multiple sequence alignment: of representative DHH family members. The sequence alignment is built using sequences of Cdc45, RecJ core (60–411) of *Thermus Thermophilus*, NrnA (YtqI) of *Bacillus subtilis* and Mycobacterial NrnA. The conserved motifs are represented as boxes as per Arvind & Koonin, 1998. https://www.ebi.ac.uk/Tools/msa. Madeira F, Park YM, Lee J, et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Research. 47(W1): W636–W641. (B) **Phylogenetic distribution**: of the DHH family proteins including Cdc45 and its others orthologs in across different phyla with minimum query coverage of 50% and presence of DHH motif is depicted as circular dendrogram.

Fig. 3. - (A–G) The putative active site residues interactions to be involved in catalysis:
The three-dimensional structural model for Cdc45 (PDB ID 5DGO) interacting with phosphoadenosine phosphate (pAp) and Bis-p-nitrophenyl phosphate (Bis-pNpp) was modelled (A and E respectively). The substrate pAp binding site is zoomed in the image (B). interacting with the ligand pAp, (C and D); the putative active site residues interacting with the ligand Bis-pNpp (F and G).

Fig. 4. Interactome of hCdc45 in the replisomal complex. The key interactions have been highlighted.

complex. The role of DHH family of protein is associated with diverse functions in nucleic acid metabolism, repair, recombination, and stress tolerance [7,15]. The replisome has specialized proteins to avoid replication stress from the replication fork, including depletion or inhibition of DNA polymerases, and depletion of dNTPs in the nucleotide pool [8,9]. DNA polymerases and helicases along with Cdc45 determine this replisome function during the cell cycle. The main function of Cdc45 seems to be holding MCM and GINS subunits together in functional

helicase. There may be loss of function of this protein during the evolution in the DHH family as suggested by previous observations [8–10]. It is suggested that there is a shift in functionality of the DHH protein Cdc45 from phospho-esterase to the clamp holder of the active CMG complex [11,14]. The phosphatase activity suggests similar functionality of Cdc45 to other members of this family, indicating divergent evolution of the DHH family proteins including bifunctional exonucleases [11–13] (Fig. 2).

The molecular interaction within the CMG complex is the determining force for the proper functioning of the helicase for strand separation during DNA replication. The cell division cycle protein Cdc45 is speculated to be an important factor for replication fork establishment and movement. The treatment with mitomycin C may lead to alkylation and retarded DNA replication [7]. The basal expression of Cdc45 changed with the concentration of DNA cross-linker mitomycin C (MMC) in Human Kidney (HK-2) cells. Cdc45 level gets altered with mitomycin C treatment, confirming its association with DNA damage tolerance. The expression level of Cdc45 is strictly regulated for proper molecular interactions within replisomal complex and cell cycle process. The association of these proteins determines the replisome functionality. The detailed component analysis may provide better understanding of genomic integrity, replisome functioning, and its association with malignancies and tumors as future directions.

5. Conclusion

It is observed that purified Cdc45 protein can hydrolyze smaller ligands like pAp *in-vitro* indicating its unique ability to interact and hydrolyze smaller molecules or ligands, which can fit into its catalytic site. These ligands like pAp can avoid stearic hindrance, which exists for longer oligonucleotides. These interactions of Cdc45 further affirm divergent evolution of the DHH family proteins, suggesting Cdc45 being an important node of the replisomal interactome for accurate functioning of CMG and replisomal complex. Further investigations are required to decipher the precise role of it in DNA replication and its correlation with *in-vivo* phospho-esterase activity in preserving genomic integrity.

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Ethics approval

Not applicable.

Consent to participate

Not applicable.

CRediT authorship contribution statement

Arathi Radhakrishnan: Methodology. **Chandresh Sharma:** Conceptualization, Methodology, Resources. **Viveka Nand Malviya:** Formal analysis, Investigation, Methodology. **Rajpal Srivastav:** Conceptualization, Investigation, Methodology, Project administration, Resources, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

No competing interest to declare.

Data availability

Already provided in the manuscript

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101643.

References

[1] C. Kohler, D. Koalick, A. Fabricius, A.C. Parplys, K. Borgmann, H. Pospiech, F. Grosse, Cdc45 is limiting for replication initiation in humans, Cell Cycle 15 (7) (2016) 974–985, https://doi.org/10.1080/15384101.2016.1152424.

[2] Y. Fu, Z. Lv, D. Kong, Y. Fan, B. Dong, High abundance of CDC45 inhibits cell proliferation through elevation of HSPA6 55 (7) (2022) e13257.

[3] R. Srivastav, R. Sharma, S. Tandon, C. Tandon, Role of DHH superfamily proteins in nucleic acids metabolism and stress tolerance in prokaryotes and eukaryotes, Int. J. Biol. Macromol. 127 (2019) 66–75.

[4] R. Zhang, Z. Liu, G. Zhang, CDC45 modulates MCM7 expression and inhibits cell proliferation by suppressing the PI3K/AKT pathway in acute myeloid leukemia, Am J Transl Res 13 (9) (2021) 10218–10232.

[5] K.S. Makarova, E.V. Koonin, Z. Kelman, The CMG (CDC45/RecJ, MCM, GINS) complex is a conserved component of the DNA replication system in all archaea and eukaryotes, Biol. Direct 7 (2012) 7.

[6] I. Krastanova, V. Sannino, H. Amenitsch, O. Gileadi, F.M. Pisani, S. Onesti, Structural and functional insights into the DNA replication factor Cdc45 reveal an evolutionary relationship to the DHH family of phosphoesterases, J. Biol. Chem. 287 (2012) 4121–4128.

[7] R. Srivastav, D. Kumar, A. Grover, A. Singh, B. Manjasetty, R. Sharma, B. Taneja, Unique subunit packing in mycobacterial nanoRNase leads to alternate substrate recognitions in DHH phosphodiesterases, Nucleic Acids Res. 42 (12) (2014) 7894–7910.

[8] L. Pellegrini, Structural insights into Cdc45 function: was there a nuclease at the heart of the ancestral replisome? Biophys. Chem. 225 (2017) 10–14.

[9] M.E. Turner, K. Huynh, R.K. Carroll, S.J. Ahn, K.C. Rice, Characterization of the Streptococcus mutans SMU.1703c-SMU.1702c Operon Reveals its role in Riboflavin Import and response to acid stress, J. Bacteriol. 203 (2) (2020) e00293, 20.

[10] L. Sanchez-Pulido, C.P. Ponting, Cdc45. The missing RecJ ortholog in eukaryotes? Bioinformatics 27 (2011) 1885–1888.

[11] A. Szambowska, I. Tessmer, P. Kursula, C. Usskilat, P. Prus, H. Pospiech, F. Grosse, DNA binding properties of human Cdc45 suggest a function as molecular wedge for DNA unwinding, Nucleic Acids Res. 42 (4) (2014) 2308–2319, https://doi.org/10.1093/nar/gkt1217.

[12] J. Takaya, S. Kusunoki, Y. Ishimi, Protein interaction and cellular localization of human CDC45, J. Biochem. 153 (2013) 381–388.

[13] Y. Uemura, N. Nakagawa, T. Wakamatsu, K. Kim, G.T. Montelione, J.F. Hunt, S. Kuramitsu, R. Masui, Crystal structure of the ligand-binding form of nanoRNase from *Bacteroides fragilis*, a member of the DHH/DHHA1 phosphoesterase family of proteins, FEBS (Fed. Eur. Biochem. Soc.) Lett. 587 (16) (2013) 2669–2674.

[14] Anna Szambowska, Ingrid Tessmer, Piotr Prus, Bernhard Schlott, Helmut Pospiech, Frank Grosse, Cdc45-induced loading of human RPA onto single-stranded DNA, Nucleic Acids Res. 45 (2017), https://doi.org/10.1093/nar/gkw1364.

[15] H.P. Lu, X.F. Du, J.D. Li, S.N. Huang, R.Q. He, et al., Expression of cell division cycle protein 45 in tissue microarrays and the CDC45 gene by bioinformatics analysis in human hepatocellular carcinoma and patient outcomes, Med. Sci. Mon. Int. Med. J. Exp. Clin. Res. 24 (27) (2021) e928800, https://doi.org/10.12659/MSM.928800.