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Comparative Structural Analysis of the SCF E3 Ligase Component Hrt1p and its Mutant (C81Y) of *S.cerevisiae* by Homology Modelling

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Abstract: ***The ubiquitin proteasome degradation mechanism (UPS) is one of the finest cellular processes which is essential in driving biological processes in the unidirectional manner such as cell division, DNA replication, and DNA repair. The mechanism ensures that the proteins recycling are achieved efficiently after attaining the crucial function. The UPS involved the marking of the proteins by Ub (71 AA residue chain) ligation and channelizing into the 26S proteasome for degradation. The tagging of target protein is achieved by sequential reaction by the E1, E2, E3 ligases where E3 ligase recruits the target protein for marked destruction. The Hrt1 protein of the S. cerevisiae is the catalytic subunit of the SKP1-Cul-Fbox E3 ligase (SCF) and essential in nature. Here in this study we carried out the 3D structure prediction analysis of the Hrt1p and one of its mutants (C81Y), defective in substrate recruitment, by homology modelling. We report that WT Hrt1p and mutant Hrt1p (C81Y) differs in their structural aspect of alpha helix and β-sheets, which might explain the functional heterogeneity in the WT and mutant.***

Keywords: UPS, Hrt1, homology modelling, F- Box, Cell cycle

1. Introduction

The cell division process in the development of organism is the key event for successful replication of organism. The process of doubling from the single is regulated by various factors and processes. The process of replication of genomic material is unidirectional phenomena. The directionality of this process is ensured by the UPS system by recycling of the proteins product that participates in the process. The ubiquitination of proteins is post-translational modification which required for protein turnover by 26S proteasome [1].The ubiquitination of target protein requires the sequential reaction of E1, E2 and E3 ligase complexes[2][3]. The E3 enzyme reaction gives the specificity of the target protein degradation by recruitment of the target protein by one of its partner. There are various classes of E3 enzymes such as, HECT family, RING finger ligase family, SCF ligase family, and APC family [1]. The APC and SCF family ligases mostly involved in the cell cycle regulation [3], where SCF regulates the G1-S phase transition. The SCF ligases are a subgroup of Cullin- RING ligeses. The Cullin–RING ligase (CRL) forms the largest group of E3 ligases in all eukaryotes. The basic structural components of the CRL are Hrt1/RBx1/Roc1, a cullin, a linker protein and one of many alternative substrate receptors [4] [5]. There are three cullin in the budding yeast (Cdc53, Cul3, and Rtt101). The C terminal cullin portion binds to the small RING domain subunit Hrt1 which recruits and activates the E2, Cdc34 [6] [7]. The N terminal regions of cullins interact with substrate receptor units F box, SOCS box, or DCAF proteins usually linker protein (Skp11, Elc1, and Mms1). Depending on type of cullin different classes of CRls are formed.

The SCF complex component, Hrt1p/Rbx1p/Roc1p is conserved from yeast to mammalian cells and contains ring finger domain and interacts with the various F Box containing proteins[6][8].The Hrt1p interacts with the F box proteins Grr1p and Cdc4 p [6][8] involved in the bud formation. In *S. cerevisiae* Hrt1p is essential and requires for viability. One of the mutants of Hrt1 (Hrt1-C81Y) has been reported which is defective in interactions with the F box proteins but does not affects the viability of the cells [9]. The mutant allele containing cells showed the altered morphology and was defective in degradation of several of SCF targets such as Gic2p, Cdc4p, and Met 30.

The 3D structure of the *S. cerevisiae* Hrt1p and its mutant (C81Y) is not known. The functional studies carried out on the mutant (C81Y) needs explanation about possible difference from the WT protein. Here in this study we carried out the structure prediction and comparison of the Hrt1p and previously reported mutant C81Y allele [9] by homology modelling. The functional characteristics were also discussed in the light of the predicated structure. We looked for the explanations of the functional heterogeneity among the WT and mutant protein in the modelled structure. We proposed that homology modelling generated 3D structure of the Hrt1p and mutant could help to design the inhibitors and drugs to alter the function of this class of proteins in the future ranging from yeast to mammals.

2. Materials and Methods

2.1 Retrieval of Hrt1p Sequence

The primary protein sequence of the Hrt1, (Accession No Q08273, Q06640 resp.) of *Saccharomyces cerevisiae* was retrieved from Uniprot Knowledgebase [10]. The protein sequences were retrieved in FASTA format and used for further analysis. The mutant sequence of Hrt1p was generated by replacing the ‘C’ at position 81 with ‘Y’.

2.2 Primary sequence analysis and alignment

The physiochemical properties of the Hrt1p and its mutant were calculated using the Protparam [11] [12].The proteins sequence alignment of FASTA sequence of Hrt1p was done using the Clustal W program [13][14]. Default parameters were applied and the aligned sequences were inspected for numbers of gaps and insertions. (**Figure 1**)

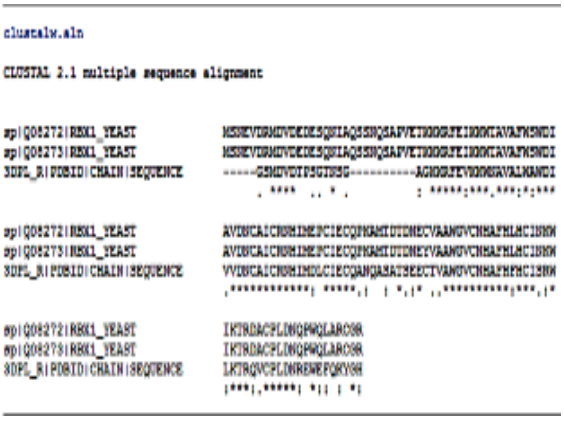


Figure 1 Multiple sequence alignment of the wild type Hrt1p, mutant Hrt1p and the selected template.

2.3 Secondary structure prediction

Secondary structure prediction tools SOPMA (**http://npsa-pbil.ibcp.fr/**) was used for the prediction of secondary structure of the Hrt1p and mutant (C81Y). The self-optimized prediction method with alignment (SOPMA) is a tool to predict the secondary structure of a protein [15]. The methods are based on fact that short homologous sequence of amino acids would form similar secondary structure. It has a database of 126 chains of non-homologous proteins against which the queries were matched.

2.4 Homology modelling

The 3D structure predication of the Hrt1p and mutant (C81Y) was done using the Modeller [16] [17]. The web based modeller is used for homology or comparative modelling of protein three-dimensional structures. The user provides an alignment of a sequence to be modelled with known related structures and modeller automatically calculates a model containing all non-hydrogen atoms. Modeller implements comparative protein structure modelling by satisfaction of spatial restraints, and can perform many additional tasks, including de novo modelling of loops in protein structures, optimization of various models of protein structure with respect to a flexibly defined objective function, multiple alignment of protein sequences and/or structures, clustering, searching of sequence databases, comparison of protein structures etc [18][19].

2.4.1 Structure refinement

The modelled structures were refined using Rosetta server Rosetta is the premier software suite for modelling macromolecular structures [20]. Rosetta design can be used to identify sequences compatible with a given protein backbone. Some of Rosetta design's successes include the design of a novel protein fold, redesign of an existing protein for greater stability, increased binding affinity between two proteins, and the design of novel enzymes. The modelled structure evaluated through ProSA [21]. The web server provides an easy-to-use interface to the program ProSA which is frequently employed in protein structure validation [22]. ProSA calculates an overall quality score for a specific input structure. If this score is outside a range characteristic for native proteins the structure probably contains errors. A plot of local quality scores points to problematic parts of the model which are also highlighted in a 3D molecule viewer to facilitate their detection. To visualize the generated models of proteins Rasmol program was used. RasMol is a molecular graphics program intended for the visualization of proteins, nucleic acids and small molecules the program reads in molecular coordinate files and interactively displays the molecule on the screen in a variety of representations and color schemes[23].

3. Results & Discussion

3.1 Primary sequences analysis and physiochemical properties of Hrt1p and mutant (C81Y)

The 121 amino acid long Hrt1p has 18 negatively charged (Asp+Glu) and 14 (Arg+hys) positively charged residues. The computed pI value was 5.49. This suggested the acidic nature of protein. The mutant Hrt1p (C81Y) showed the same pI value as SH group gets replaced with benzene group. The estimated half life time of Hrt1p and mutant (C81Y) protein was less than 20 hours (yeast, in vivo) and by N-end rule which relates the half-life of a protein to the identity of its N-terminal residue[24].The N-terminal of the sequence considered is M (Met) (Table 1). An instability index value less than 40 is supposed to be stable. As both the values are above 40 it can be said that both wild type and mutant are unstable. GRAVY score with negative values states that the protein is hydrophilic and those with positive values are the one which are hydrophobic. Both proteins are hydrophilic.

Table 1: Physiochemical properties of the Hrt1p and mutant (C81Y) as predicated by Protoparam

Protein	Instability index	Aliphatic index	GRAVY SCORE	Nature
Hrt1p (W.T.)	52.86	62.15	-0.596	hydrophilic
Hrt1p (C81Y)	49.85	62.15	-0.627	hydrophilic

3.2 Secondary structure prediction

Formation of alpha helix and beta sheet are called as secondary structure of protein. The Hrt1p and mutant secondary structure prediction using SOPMA revealed that coils (63.64%) dominated among secondary structure elements followed by helix (19.83%) and sheets (14.88%).

Homology modelling

The three dimensional structure of Hrt1 protein and mutant (C81Y) was generated by Modeller. The BLAST analysis against protein data bank suggested the template '3DPL' which is "**Structural Insights into Nedd8 Activation of Cullin-Ring Ligases: Conformational Control of Conjugatin**". This template had 56% identity and 94%

query cover to the query sequence. The program generated 18 different models for both wild type and mutant out of which one was selected for each category and the results were analyzed on the basis of dope energy. Less the energy, better stability to the structure. The dope energy after minimization for the wild type came out to be -6871.8828 and for mutant type came out to be -8847.7402.Then both the structures were further submitted for refinement which was done through Rosetta server.

3.3 Structural validation

The generated structure by homology modelling was analyzed using the SAVES server "Structural Analysis and Verification Server" (www.nihserver.mbi.ucla.edu)[25]. (**Figure2**). Procheck tool was used. PROCHECK checks the stereo chemical quality of a protein structure by analyzing residue-by-residue geometry and overall structural geometry**.** Errat analyzes the statistics of non-bonded interactions between different atom types and plots the value of the error function versus position of a 9-residue sliding window, calculated by a comparison with statistics from highly refined structures. Also ProSA was used which calculated z scores and the local alignment of the structure (**Figure3**).The green speck in between is the 81st location containing the cysteine residue in the wild type Hrt1p **(Figure4**).

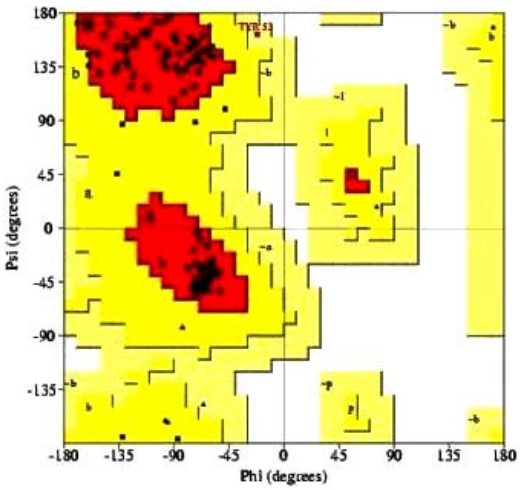


Figure 2(A): Ramachandran plot for wild type Hrt1p showed: - 91.6% core region, 7.5% allow and 0.0% disallowed region.

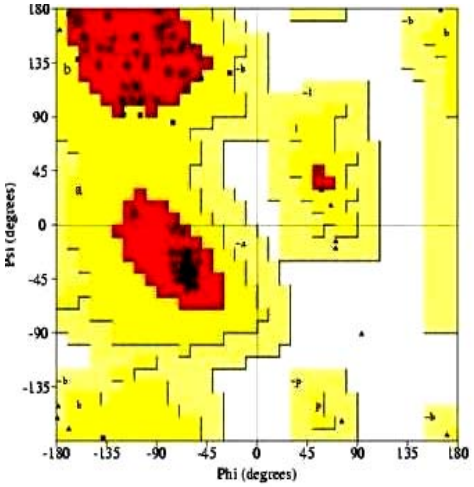


Figure 2(B) Ramachandran plot for mutant Hrt1p 93.1% core region, 6.9% allow and 0.0% disallowed region, Errat value – 91.5.

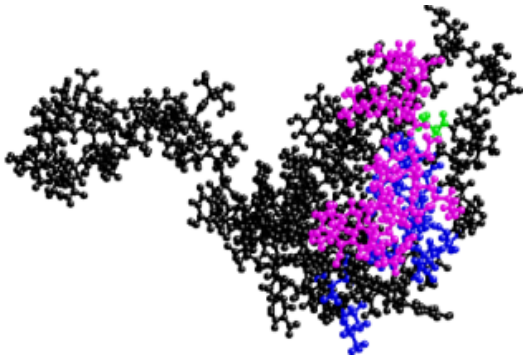
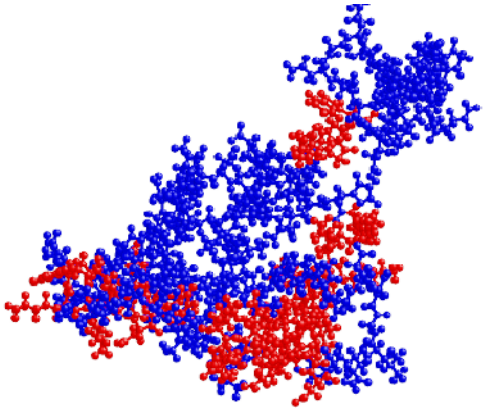


Figure 3: 3D structures of a) wild type Hrt1p. The green spec represents 81st position where C81Y change carried out.



(b) 3D structure of mutant Hrt1p. The red region shows the helical region.

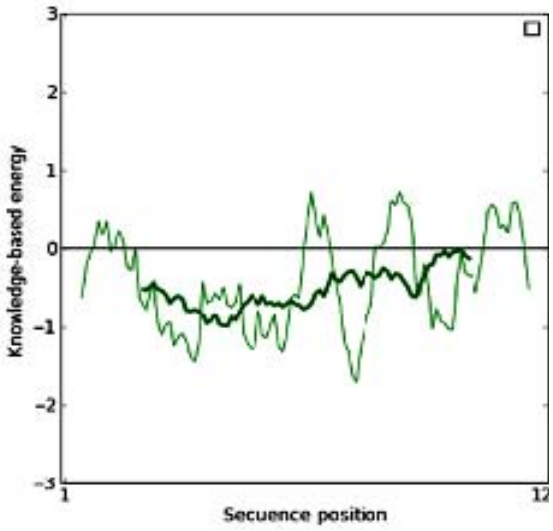
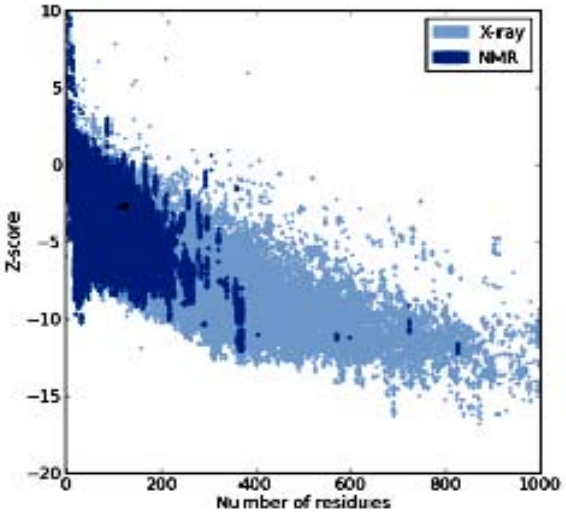


Figure 4 (a) ProSA analysis of mutant Hrt1p, showed graph between knowledge based energy and the energy of our structure. A positive value depicts errors and negative values are relevant.



(b) Z score based on X ray and NMR data. Z score represents the accuracy of the structure of proteins.

4. Conclusion

In this study the 3D structure of both wild type and mutant (C81Y) Hrt1p was generated using modeller. The template taken was same for both the structure queries. The template taken was of Cullin Ring Ligase which is also an important complex of SCF complex. SCF complex consists of 4 Subunits; Skp1, Cullin, F box Protein and Hrt1. Skp1 forms part of the horseshoe-shaped complex, along with Cullin (cul1). Skp1 is essential in the recognition and binding of the F-box. The cullin forms the major structural scaffold of the SCF complex, linking the Skp1 domain with the Rbx1/Hrt1 domain. Hrt1p contains a small zinc-binding domain called the RING Finger, to which the E2-ubiquitin conjugate binds, allowing the transferral of the ubiquitin to a lysine residue on the target protein.

Hrt1p forms an important interactive partner as it contains the ring finger domain (zinc binding), to which the E2 ubiquitin conjugate binds, allowing transfer of Ubiquitin to a lysine residue of target protein. On visualizing the structure of wild type it was found that majority of secondary structure was composed of helix followed by sheets. It was observed that it exhibited an open structure which has active residues from 2-91 position and the mutation was carried out on position 81, replacing C with Y. In case of wild type both helical atoms and sheet were present but in case of mutant, it was observed that the structure got compacted and the number of helical atoms showed an increase while the sheet atoms were reduced to zero.

It was observed that there was a major difference between the 3D structures of the Hrt1p and Hrt1p. The wild type initially consisted of 251 helical atoms and 188 sheet atoms labelling it as 50% helical and other part consisting of sheets and turns. On the other hand, the mutant type was found to have 597 helical atoms, almost double of the previous wild type. Another major change observed was the lack of beta sheets suggesting that during the conversion from wild type to mutant, all the sheets got converted into helical state owing to the compacted structure of the molecule. Also the amino acids involved in the substitution played a major role in the outcome. As cysteine is involved in thioester bond formation it forms a conjugate with cofactor. In case of Hrt1p a zinc binding domain called ring finger was involved in binding with E2. Thus on substitution, this interactions might get affected leading to the loss of ubiquitination activity. It might be said that the structure showed transition from an open to closed state. It can be hypothesized that tyrosine being hydrophobic tends to lie on the inner side, thereby leading to compact nature of the mutant protein.

5. Future Scope of study

The present study with the Hrt1p which is functionally conserved from yeast to human has potential to develop the inhibitors against it for controlling the activity of this molecule.

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