Studies on Mutant forms of the Ubiquitin Conjugating Enzymes UBC1 and UBC4

Synopsis submitted for the degree of Doctor of Philosophy in Biochemistry

By

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Introduction

Proteins undergo continuous turnover in the cell. The continuous degradation of proteins might appear to be a wasteful process, but it is a key to maintaining cellular homeostasis[1]. The rate at which individual proteins are degraded can range from few minutes to several days. Cells encompass multiple proteolytic systems to provide specificity during the degradation process and in turn provide check points to avoid excessive breakdown of proteins. Indeed the rate of protein synthesis and degradation should be maintained by the cell to avoid an excessive loss of mass. Proteolysis can be broadly classified into two main streams, lysosomal degradation and proteasomal degradation. Proteasomal degradation again occurs by two types: ubiquitination independent (via lysosome) and ubiquitination dependent routes.

Lysosomal degradation:

Lysosome is a membrane-bound organelle consisting of an array of non-specific proteases, which cleave any protein delivered to it, into individual amino acids.

Proteasomal degradation

Specific proteins including misfolded or short-lived proteins undergo proteolysis by the **ubiquitin-proteasome system**. This system is the most robust system and can serve as default system to degrade almost any protein when necessary. It coordinates various cellular events—such as cell cycling, it removes proteins that are no longer required by cell and it provides an important quality control check by continuously eliminating proteins which are damaged or non-functional. Under all these conditions, these enzymes act with great specificity by identifying individual proteins among the thousands present in a cell. Furthermore, it also distinguishes different forms of the same protein depending on factors such as subcellular localization, posttranslational modification, or inclusion in multiprotein complexes.

Proteasome is a cylindrical protein complex found in cytosol, which cleaves proteins tagged by ubiquitin for destruction. Ubiquitination generally occurs at lysine residues of substrate proteins, generating an isopeptide bond. To accomplish this task E1 (ubiquitin activating enzyme) activates ubiquitin in presence of an ATP molecule. This ubiquitin is transferred to an E2 (ubiquitin conjugating enzyme) and finally E3 (ubiquitin ligase) covalently attaches ubiquitin to lysine residue of the substrate protein to be degraded. Several rounds of this cycle take place to form a polyubiquitin chain and protein is degraded and released in the form of peptides.

Ubiquitin Conjugating Enzymes

Efficiency of E2s to interact with Ub, E1, E3 and targeted substrate makes them the central players of the ubiquitin cascade. All E2s contain a core domain, also known as the catalytic domain or the UBC domain containing the active site Cysteine which interacts with E1. E2s have been structurally classified based on the extensions they possess surrounding the core domain. UEV (Ubiquitin E2 Variant) proteins also have a UBC domain, but they lack Cys residue. Class I E2s consist of only the core domain (example Ubc4), class II E2s consist of C-terminal extension domain surrounding the core domain (example Ubc1), class III has an N-terminal extension domain (UbcH6) and class IV has both the additional extensions (E2-230K). So far, 13 genes of E2 are known in *Saccharomyces cerevisiae* and at least 38 in human[2]. A high degree of sequential homology is seen between UBC domains of different E2s which adopt similar structures consisting of four α helices, an anti-parallel β sheet made by four strands and a short 3_{10} helix. The active site Cys where Ub binds is present in a shallow groove formed by a short loop connecting α helix 2 with α helix 3 and a long loop proximal to active site. Many studies have proved that E2s are known to contribute in determining the length and type of ubiquitin chains during the chain assembly reaction[2].

In *S. cerevisiae* E2s are encoded by UBC genes. The class II E2 enzyme Ubc1 from *S. cerevisiae* is a flexible two-domain protein comprising of an N-terminal catalytic domain and a C-terminal UBA domain. This C-terminal UBA domain, belonging to all class II proteins consists of three α helices[3]. Unlike the catalytic domain, the UBA domain is able to interact with mono or polyubiquitin chains in a non-covalent manner[4]. Subsequently this may either result in inhibition of the degradation process or may follow the normal route via ubiquitin conjugation. Binding of Ub to UBA domain does not affect the initial thio-ester reaction at catalytic domain. Ubc1 undergoes polyubiquitination via K48 pathway in absence of an E3[5].

Class I E2s consists of entirely the conserved core domain. The genes of UBC4 and UBC5 encode almost identical proteins (92% identical residues) of 16 kDa molecular weight[6]. Overall, UBC4 and UBC5 proteins are highly similar in sequence to proteins UBC1, UBC2 and UBC3. UBC2 (RAD6) is involved in DNA repair, induced mutagenesis and sporulation, UBC3 (CDC34) is required for G1 to S phase transition of cell cycle, UBC1 is important for protein degradation of short-lived proteins specially during G_0-G_1 transition phase accompanying spore germination. UBC4 is expressed at exponential phase of growing cells; while UBC5 at stationary phase and both are heat inducible genes. It is also well known that UBC4 and UBC5 are redundant in functions. However, deletion of both the genes results in slow growth and inviability at elevated temperatures[7]. In addition to the polyubiquitin gene *UBI4*, UBC4 and UBC5 are essential modules of a dynamic pathway of eukaryotic stress response[6].

UBC1, UBC4, UBC5 are such E2s, which in spite of belonging to different groups, constitute a sub-family required for cell growth and viability. Single genes are dispensable, but mutant cells where all three genes are deleted are not viable. This proves the important role played by these enzymes in ubiquitin-dependent protein degradation in eukaryotic cells. Although

these enzymes are partially complementing in function, each one has its specific role. UBC1 is essentially required during early stages of cell growth after germination and is also involved in protein turnover. UBC1 can partially complement functions of UBC4 and UBC5 upon over-expression[7].

E2-25k is a human homolog of yeast Ubc1, known to interact with UBB^{+1} which is a frame shift mutant of ubiquitin and thereby leading to neurotoxic disorders. It is also able to synthesize Lys^{48} linked free polyubiquitin chains in the absence of any E3 ligase[8]. A chimeric protein was constructed in which the UBA domain of E2-25K was fused to the E2 domain of yeast UBC4, showed no polyUb synthesis activity, suggesting that polyubiquitination by E2-25K is dependent on the relative conformations of the E2 and UBA domains and their specific interactions[9].

Ubiquitin conjugating enzymes are also known to be involved in polyglutamine protein aggregation[10]. In yeast, the proteins which are essential for cell cycle progression are assembled by anaphase-promoting complex (APC) in association with E2s. On screening all budding yeast E2s, study revealed that Ubc1 and Ubc4 are the crucial partners for APC. Ubc4 was found to be involved in monoubiquitination of multiple lysines on APC targets and Ubc1 was involved in K48 linked polyubiquitination on pre-attached ubiquitin. E2-25k was also found to be involved in promoting APC dependent chain assembly on pre-attached ubiquitins. Yeast strains lacking both Ubc1 and Ubc4 displayed functional loss towards mitotic APC[11]. Several studies report that a HPN motif is found conserved across the known E2s where histidine within this motif is found oriented to structurally stabilize a tight turn motif in all E2s[12].

Hypothesis proposed

Based on this literature and on performing sequence alignment between Ubc1 and E2-25K protein sequences by clustal omega tool, it was found that the catalytic N and C-terminal domains were found to be homologous, with a major exception lying only at the linker (Figure 1). The Ubc1 linker is composed of 22 amino acids; while E2-25k has 6 amino acids. To understand the importance of linker, we proposed to construct a chimeric protein (c-UBC1) by swapping E2-25k linker with that of Ubc1. This might help to appreciate the functional differences arising out of swapping and may provide insights for better understanding on the role of this protein in protein aggregation diseases.

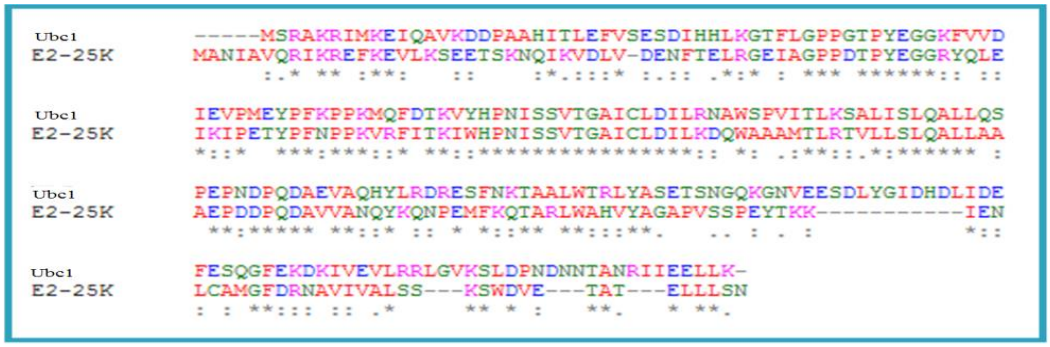

Figure1. Sequence alignment of Ubc1 and E2-25k using clustal omega

A study reports that when a residue between two homologues i.e. residues in UbcH8 (Asp) was substituted with their counterpart from UbcH6 (Glu) it affected the binding profile for E3. Thus, suggesting that even a minimal difference in side chain characteristics could affect the specificity for E3 interaction[13]. It is well-known that UBC4 and UBC5 have 92% sequence similarity with redundancy in functions. Hence, we decided to check stability indices specifically of those amino acid residues which differ in the two proteins and established that if we replace Ubc4 residues for those of Ubc5 one by one, the overall stability of protein was found to be increasing theoretically (Table 1). Thus, we proposed to create single point mutations for UBC5 by site directed mutagenesis and further carry out its functional characterization. A study shows that, minor alterations in side-chain characteristics would affect protein stability *in vivo.* This study would help us understand if UBC4 and UBC5 have any functions that are distinct from each other. Moreover, if these single point mutation can alter the spatial geometry of the molecule?

Residue Position	**Mutation**	**β-turn propensity value***	**Secondary structure involved**
15	E →G	0.87→1.09	i of turn1
20	T → A	1.08→0.81	i
42	A→S	0.81→1.29	i of turn 3
81	A→S	0.81→1.29	i of turn 7
82	N→S	1.02→1.23	(i+1) of turn 7

Table 1(a): β-turn potentials using Hutchinson *et al.*, 1994* stability indices for amino acids

Residue Position	**Mutation**	**Chou Fasman β-strand value** **	**Secondary structure involved**
68	I→V	1.6→1.7	(i+1) of 4th β-strand
69	S→N	0.75→0.89	(i+2) of 4th β-strand

Table 1(b): β-strand potentials using Chou and Fasman stability indices for amino acids (1978) **

Residue Position	Mutation	Chou Fasman α-helix value***	Secondary structure involved
126	H→Q	1→1.1	X of α-helix 3
133	R→K	0.98→1.16	(i+1) of α-helix 4
134	P→A	0.57→1.42	(i+2) of α-helix 4
140	R→K	0.98→1.16	X of α-helix 4

Table 1(c): α-helix potentials using Chou and fasman stability indices for amino acids (1978)***

This led us to two major objectives which are as follows:

Major objectives proposed

1) Swapping of yeast (UBC1) linker for mammalian (E2-25k) followed by functional and structural studies.
2) To construct single mutants for UBC5 followed by its functional analysis.

Results

Objective 1: Swapping of yeast (Ubc1) linker for mammalian (E2-25k) followed by functional and structural studies.

A. Construction of c-UBC1 in shuttle vector for functional characterization.

YEp96, a shuttle vector between yeast and bacteria was used for the study. c-UBC1 and UBC1 were constructed by site-directed mutagenesis in YEp96. The genes are placed under a CUP1 promoter. Expression of the genes is inducible by 100μM copper sulphate. The plasmids were confirmed by sequencing.

B. Various functional assessments for c-UBC1

UBC1 mediated functions are critical for cell growth and viability at a specific point in the yeast life cycle, during the transition period after a state of quiescent. Once the constructs were ready; they were transformed into *S. cerevisiae* for *in vivo* characterization. For this first yeast transformation protocol was standardised first and constructs were successfully transformed into respective strains along with control plasmids. Transformants were confirmed by selection and tested under various conditions.

B. i) Estimation of doubling time

Since UBC1 is specially required in early stages of cell growth we wanted to test c-UBC1 can complement or not? *Δubc1* and UBC1 were taken as controls. The results show that c-UBC1 is functionally active with a doubling time of 1.8 hours which is almost same as UBC1 (1.8 hours).

B. ii) Survival under various stress conditions

UBC1 and E2-25K both are involved in protein turnover of eukaryotic cells. We wanted to test whether c-Ubc1 can fulfill the same function or not?

B. ii. a) Survival under Heat stress

Cells were grown in SD liquid medium containing required nutrients. Appropriate aliquots were spread on supplemented SD plates in duplicates in presence or absence of an inducer and incubated at 37°C for various time intervals. The results show that c-UBC1 exhibits increased resistance compared to UBC1.

B .ii. b) Survival under thermotolerance test

Cells were grown in SD liquid medium containing required nutrients. For pre-treatment cultures were shifted to 37ºC for 1 h. Prior to or after incubation for 5 min at 52ºC appropriate aliquots were spread on SD plates and incubated at 30ºC. The results show that c-UBC1 behaves similar to UBC1.

B. ii. c) Antibiotic complementation test

To evaluate the involvement of c-UBC1 in ubiquitin-mediated protein turnover, we examined its sensitivity towards various translational inhibitors (L-Canavanine, Hygromycin-B, and Cycloheximide). Canavanine (an arginine analog) when incorporated into proteins, is known to generate structurally altered proteins, which are rapidly degraded. Hygromycin-B and cycloheximide inhibit protein synthesis at two different levels of translocation step of the translation process. c-UBC1 exhibited almost similar resistance as UBC1.

C. Sub-cloning of c-UBC1and UBC1 in bacterial expression system for structural characterization.

For this, pET28 (a) was the vector of choice which has 6XHis tag. Sub-cloning was done using site-directed mutagenesis and the constructs were confirmed by insert release. Once confirmed, they were transformed into E.*coli* BL21 (DE3) strain. Ubc1 was used as a control.

D. Structural characterization of c-Ubc1.

D.i. Over expression and purification of the protein in bulk quantity

This was done in two stages. Initial stages required optimization for over-expression of c-Ubc1. The over-expression was obtained at a concentration of 1mM IPTG, upon overnight incubation at RT. Once optimized the protein isolation was scaled up and purified by affinity chromatography using Ni-NTA beads.

D.ii.To study protein folding under denaturing conditions

The denaturation curve was studied using different concentrations of guanidine hydrochloride (GndHCl). The data suggests that, c-UBC1 has a compact structure, which unfolds in presence of a denaturant.

D.iii. Structural characterization of c-Ubc1 using spectroscopy

Circular dichorism spectroscopy studies will be carried out in near and far UV range.

Objective 2: To construct single mutants of UBC5 followed by their functional analysis.

A. Construction of point mutations in shuttle vector for functional characterization.

YEp96 shuttle vector between yeast and bacteria was used for the study. All the respective single point mutations were constructed by site-directed mutagenesis. The genes are placed under a CUP1 promoter. Expression of the genes was induced by 100µM copper sulphate. The plasmids were confirmed by sequencing. WT (UBC4) was used as a control for the studies.

B. Various functional assessments for the constructs.

Since UBC4 and UBC5 are closely related in sequence with redundancy in functions. However it is still puzzling why these two genes exist separately in nature is not yet understood. To gain insights into this we performed various functional tests. Once the constructs were ready, the constructs were successfully transformed into respective strains along with controls. Transformants were confirmed by selection and tested under various conditions. Till now UBC4-T20A, UBC4-S69N, UBC4-N82S and UBC4-R133K have been characterised.

B.i) Estimation of doubling time

To evaluate the functionality of these mutants growth curve was monitored. *Δubc4ubc5,* WT and UBC4 were taken as controls. Our results show that these point mutations are functionally active and did show significant differences in the growth and survival of yeast when compared to UBC4 under normal conditions and on over-expression too.

B.ii.) Survival under various stress conditions

As UBC4/UBC5 is inducible by heat shock; also Ubc4 is required by cells in combating cellular response generated by heat stress we wanted to examine if the point mutations also could perform the same? Moreover do these point mutations behave same as theoretical analysis done for protein stability indices?

B.ii. a) Survival under Heat stress

Cells were grown in YPD liquid medium containing required nutrients. Appropriate aliquots were spread on supplemented SDA plates in duplicates in presence and absence of an inducer and incubated at 37°C for various time intervals. The results show that, the mutants except Ubc4-R133K did not have any negative effect with respect to heat stress in the presence and absence of inducer. Interestingly Ubc4-S69N behaved even better than the WT under both criteria. The mutations except R133K do not seem to affect the polyubiquitin chain formation. Hence, E1 and E3 might not be able to differentiate between wild-type Ubc4 and the mutation, thus not causing any lethality to yeast cell.

B.ii. b) Survival under thermotolerance test

Cells were grown in SD liquid medium containing required nutrients. For pre-treatment cultures were shifted to 37ºC for 1 h. Prior to or after incubation for 5 min at 52ºC appropriate aliquots were spread on SD plates and incubated at 30ºC. Our results show that thermo-sensitivity and induced resistance of Ubc4R133K mutant was very similar to Ubc4 cells. Ubc4N82S mutant however, exhibited constitutive thermotolerance: 100% survived the acute heat shock, in contrast it left < 1 % of wild-type and Δubc5 and 2% of Δubc4 alive.

B.ii. c) Antibiotic complementation test

As reported earlier Ubc4 is required by cells in eliminating cellular response caused by amino acid analogs we examined the sensitivity of point mutants towards various translational inhibitors (Canavanine, Hygromycin B and cycloheximide). Out of four mutants, only Ubc4R133K showed no growth in presence of canavanine and cycloheximide. Thus, this particular mutation might be affecting the E2-E3 pairing; hence unable to degrade misfolded or truncated proteins efficiently.

Construction of all the 11 mutants carrying the single point mutations has been accomplished. Functional studies with four of them (T20A, S69N, N82S and R133K) have been completed. Characterization of rest of them would be carried out.

Conclusion

In-vivo studies with c-UBC1 construct having the E2-25k linker performed equally well in the yeast system as compared to original yeast linker suggesting that it's length does not have any significance in relation to the functions tested. Structural studies of c-UBC1 reveal, it has a compact structure, which unfolds in presence of a denaturant. Further circular dichorism studies would provide more insights to the structure and its relation to function. In conclusion, c-UBC1 was found to be functionally active and comparable with wild type. *In-vivo* studies with Ubc4T20A, Ubc4S69N, Ubc4N82S, and Ubc4R133K suggests that all are functionally active and not detrimental to yeast cell. Out of four mutants, only Ubc4-R133K showed no growth in presence of canavanine and cycloheximide. Thus, this particular mutation might be affecting the E2-E3 pairing; hence unable to degrade misfolded or truncated proteins efficiently. Thus, a point mutation from arginine to lysine is not always neutral. In certain structural or functional contexts, such a mutation can be devastating to function[14].

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Publications

1. Manuscript is under preparation entitled, "T20A, S69N, N82S, R133K substitutions of Ubiquitin Conjugating Enzyme UBC4 for UBC5 deciphers the functions for redundant genes
2. Manuscript is under preparation entitled, "E2 enzymes: Expanding roles from carrier enzymes to central players of ubiquitination"

Presentations/ conferences attended during PhD tenure:

1. **Poster** presentation on "Functional Characterization of Ubiquitin Conjugating Enzyme UBC4 with N82S Mutation in Saccharomyces cerevisiae" at 86th Annual Conference of Society of Biological Chemists (SBC-2017) organized by the School of Life Sciences, Jawaharlal Nehru University, New Delhi during 16th-19th Nov, 2017. **Varsha Raimalani** and C. Ratna Prabha*

2. Abstract got accepted for **poster** presentation entitled, "Structural and Functional Characterization of chimeric Ubiquitin Conjugating Enzyme(c-UBC1) in S.cerevisiae" for The Protein Society's 31st Annual Symposium, held in Montreal, Canada, 2017and will be published by Wiley as a special edition of **The Protein Science. Varsha Raimalani,** C. Ratna Prabha*

   Received International travel grant for the same by DBT-CTEP scheme

3. **Poster** presentation on "Functional studies on Ubiquitin Conjugating Enzyme UBC4 with R133K mutation in *S.cerevisiae"* at Indo-US Conference on Advances in Enzymology: Implications in Health, Diseases and Therapeutics,17-19 jan 2017, ACTREC, Navi Mumbai
   Varsha Raimalani and C. Ratna Prabha*

4. **Oral** presentation on "Functional Characterization Chimeric UBC1 (c-UBC1) Engineered by Swapping of Human Linker in Yeast Ubiquitin Conjugating Enzyme UBC1" at National Symposium on Microbial Biotechnology: Advances and Future Trends, 26/2/14 organized by Dept. Of Microbiology and Biotechnology, Genetics & Bioinformatics, N.V.P.A.S college, Vallabh Vidyanagar. **Varsha Raimalani**, Srivalli Puttagunta, C. Ratna Prabha *

5. Participated in the two day National Symposium on "Omics to Structural Basis of Diseases" organised during Sept 30-Oct 1, 2016 at Department of Biochemistry, M.S.University of Baroda.

6. Participated in National Seminar on “Molecular Basis of Diseases” organised during 1-2Aug, 2014 by Department of Biochemistry, M.S.University of Baroda.

7. Participated in the Open House Science Fair organised during 11-12 March, 2012 as a part of 150th birth anniversary celebration of Maharaja Sayajirao Gaekwad III at Department of Biochemistry, M.S.University of Baroda.
8. Participated in the XXVI Gujarat Science Congress held on Feb 26,2012 at the Maharaja Sayajirao University of Baroda.

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