Isolation of a Dosage Dependent Lethal Mutation in Ubiquitin Gene of *Saccharomyces Cerevisiae*

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Summary: Ubiquitin is a small protein with a highly conserved sequence, playing a pivotal role in ubiquitin proteasome system (UPS). Considering the central role UPS has in cellular homeostasis, several drugs have been developed to target UPS to remove cells responsible for cancer and other neurodegenerative diseases. As an alternative to the above approach, in the present study we have isolated dose dependent lethal form of ubiquitin gene by *in vitro* evolution. *In vitro* evolution is a powerful tool for developing proteins with novel and desirable properties. The ubiquitin gene of *Saccharomyces cerevisiae* was subjected to *in vitro* evolution and lethal mutations were selected. The ubiquitin of *S. cerevisiae* differs only by three residues from human ubiquitin. The mutants were selected by expressing the protein in temperature sensitive *ubi4* deletion mutants of ubiquitin. Most of the mutations in ubiquitin gene failed to complement UBI4 phenotype under heat shock. Only one of the mutants caused cell lysis, even at permissive temperature. Interestingly, expression of the same protein in wild type *S. cerevisiae* cells left them unaffected, establishing the mutant protein as a competitive inhibitor for UPS. Sequencing of the mutant gene showed four completely novel amino acid substitutions. They are namely, Ser20 to Phe, Ala46 to Ser, Leu50 to Pro and Ile61 to Thr. Construction of the mutant ubiquitin gene and characterization of the mutant phenotype along with the nature and location of the mutations are presented.

Keywords: directed evolution; dosage dependent lethal mutation; error prone PCR; *in vitro* evolution; Ubiquitin

Introduction

Designing and engineering macromolecules with desirable characteristics and functions has been a challenge and if the macromolecules happen to be proteins the problem is compounded by the complex reactions *in vivo*. However, random mutagenesis with effective selection strategies made *in vitro* evolution of proteins feasible turning the seemingly impossible task attainable. The technique has already been successfully employed to develop several proteins.[1–5] Here we have exploited *in vitro* evolution to develop a dosage dependent lethal variant of ubiquitin, which can act as an antagonist to UPS.

Ubiquitin is a small, compact globular protein with a highly conserved sequence. It is present in all eukaryotes. Ubiquitin has been isolated and sequenced from a variety of sources and has been found to be identical in all organisms from insects to humans.[6–9] In yeast[10] and Oat[11] replacement of amino acids is seen only in three positions in ubiquitin. Ubiquitin plays key role in protein degradation[12,13] and various other cellular phenomena[14–16] explaining why mutations are not permitted

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in it naturally. The remarkable conservation of ubiquitin along with its smaller size, points to constraints imposed on its sequence by folding, stability and functional interaction with other proteins. A natural variant of ubiquitin from *Autografa californica* displays only 75% identity with ubiquitin protein sequence. This isoform of ubiquitin attenuated the formation of polyubiquitin chain.[17] This observation suggests the potential of ubiquitin variants to interfere with essential cellular phenomena leading to cell death. Though there are many studies with mutated ubiquitins using site directed mutagenesis, including a few from our own laboratory, the mutant ubiquitins do not give rise to the desired phenotype.

Random mutagenesis is a powerful technique for directed or *in vitro* evolution of proteins with novel and desirable properties. In the present study random mutagenesis of ubiquitin gene was carried out by using error prone PCR[5] (polynucleotide chain reaction). Subsequently the mutants were selected for absence of complementation in *UBI4* mutants under stress conditions. Ubiquitin is encoded by four different genes in *S.* cerevisiae. Among them the gene *UBI4* codes for a polyubiquitin natural fusion protein, which is processed post-translationally into free ubiquitin molecules.[18] The *ubi4* mutants are deletion mutants of *UBI4* gene and are sensitive to temperature stress.[19] Among the many mutants generated, one of the mutants UbEP42 turned out to be unique. UbEP42 caused the lysis of ubi4 mutant cells even when the cells were not under any kind of stress. The present work deals with directed evolution of the gene, its sequencing and characterization of the mutant phenotype.

Experimental Part

Yeast Strains, Media, and Plasmids

The *S. cerevisiae* strains used in the study are as follows. SUB62 (*MAT***a** *lys2-801 leu2-3,112 ura3-52 his3-Δ200 tr1-1*) is a wild type strain for ubiquitin genes and SUB60 (*MAT***a** *ubi4-Δ2::LEU2 lys2-801 leu2-3,112 ura3-52 his3-A200 tr1-1*) is a deletion mutant lacking *UBI4* polyubiquitin gene.[19]

S. cerevisiae cultures were grown in synthetic dextrose (SD) medium containing 0.67% Hi-media yeast nitrogen base and 2% glucose as carbon source. Uracil, leucine, tryptophan, lysine and histidine supplements were added as and when required depending on the strain. The cultures were grown at 30 °C at 200 rpm (except where indicated).

Bacterial Strains and Media

Escherichia coli DH5α culture was grown at 37 °C at 200 rpm in nutrient rich Luria broth from Hi-media. Selection pressure of 100 μg/ml of ampicillin was used with the strains transformed by plasmids.

Plasmid Construction and Expression of Ubiquitin

All ubiquitin gene mutations were carried in plasmids derived fromYep96, which expresses a synthetic yeast ubiquitin gene from the *CUP1* promoter. Ubiquitin genes developed by error prone PCR were cloned into the *Bgl* II and *Kpn* I sites of Yep96. Ubiquitin overproduction from the *CUP1* promoter was induced by the addition of 100 μM copper sulphate.

Error Prone PCR

Error prone PCR was performed using the Taq DNA polymerase and a standard reaction system containing 10 mM Tris–HCl buffer (pH 9 at 25 °C), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 250 μM dNTPs each. The error condition were modified by adding the following chemicals in separate reactions: 10 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM dATP, 1 mM dGTP and 1 mM dATP + 1mM dGTP, to reduce the fidelity of Taq polymerase and increase the rate of incorporation of mutations during PCR reaction. The forward primer 5'ATGCA-GATCTTCGTCAAGACGTTAACCGG3' and reverse primer 5'TCCGGTACCC GCTCAACCACCTCTTAG3 were used to generate 240bp amplicon in different error prone conditions and cloned in copper inducible Ub expression plasmid Yep96 at *Bgl*II and *Kpn*I sites.

Mutant Screening

Yep96 plasmid was originally constructed to express the wild type ubiquitin under *CUP1* promoter. The wild type ubiquitin gene in Yep96 was replaced by the mutant forms. SUB60 was transformed with above chimeric constructs and tested for failure of complementation of the heat sensitive phenotype of SUB60 *ubi4* mutant.

Yeast transformants were grown and streaked on SD agar selection media with and without induction by copper sulphate. Plates were incubated at 40 °C for 16 hours and shifted to 30 °C.

Sequence Analysis

The plasmid with mutant gene was sequenced using Sanger's dideoxy sequencing method for the detection of the mutation incorporation in the DNA.

Results and Discussion

Error prone PCR was carried out according to the conditions mentioned in the section of experimental part. The PCR products were cloned in Yep96 and transformed into SUB60 strain of *S. cerevisiae*. In order to give heat stress plates were incubated at 40 °C for 16 hours and then again incubated back at 30 °C which is a favorable temperature for the growth of yeast cells. SUB60 fails to grow, while the wild type strain SUB62 grows normally. SUB60 transformed with Yep 96/Wt (Yep96 carrying wild type gene for ubiquitin) shows normal growth due to complementation under heat stress.

The SUB60 cells transformed by error prone PCR products were selected by incubating at 40 °C for 16 hrs. Several variants were generated in different reaction conditions listed under Experimental part (Figure 1).

The colonies which failed to show complemetation were picked up from master plate and cultured in SD media containing copper sulphate at 30 °C. Interestingly, one of the colonies UbEP42, obtained from the PCR reaction carried out in 0.5 mM $MnCl_2$, showed lysis even at permissive temperature, under copper sulphate induction. Further, the lysis showed concentration dependence on copper sulphate, showing complete lysis at 200 μM copper sulphate (Figure 2c).

The plasmid from EP42 strain, Yep96/UbEP42 was introduced into SUB62 cells with wild type UBI4. In contrast to SUB60, the SUB62 strain did not undergo lysis (Figure 2d). The result suggests that the mutant form of ubiquitin UbEP42 acts as a competitive inhibitor to wild type ubiquitin.

The plasmid from UbEP42 was isolated and the gene was sequenced. Sequencing of the mutant gene showed four completely novel mutations (Table 1).

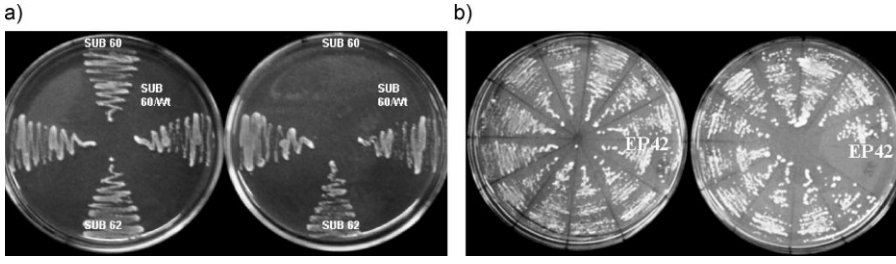

Figure 1.
Isolation of UbEP42 by failure of complementation under temperature stress in SUB60 strain of *S. cerevisiae*. (a) The Petri plate on the left shows growth of SUB60, SUB60/Wt (SUB60 transformed with Yep96 carrying wild type ubiquitin gene) and SUB62 strains at 30 °C (permissive temperature and the Petri plate on the right shows failure of growth of SUB60 cells after incubation at 42 °C, (b) SUB60 strain transformed with products of error prone PCR grown at 30 °C without copper sulphate (petri plate on the left) and with copper sulphate (Petri plate on the right).

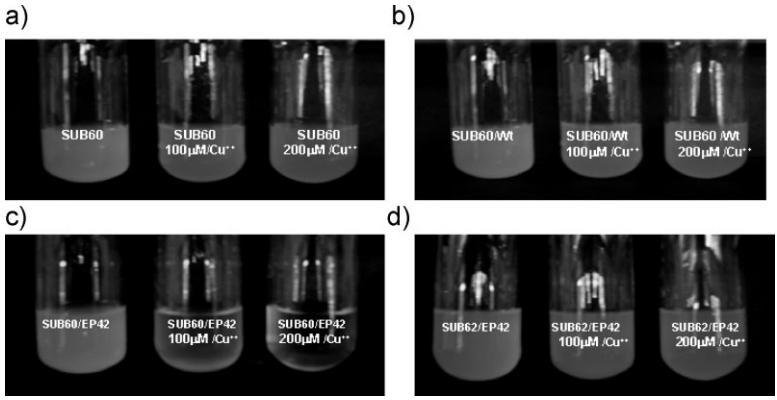

Figure 2.
Effect of expression of UbEP42 on *S. cerevisiae* grown at 30 °C in 0, 100 and 200 μM copper sulphate respectively. (a) The *ubi4* deletion mutant SUB60 strain and (b) SUB60 strain transformed by Yep96 plasmid carrying wild type ubiquitin gene, both showing normal growth at all concentrations of copper sulphate, (c) SUB60 transformed with Yep96/UbEP42 show reduced growth at 100 μM copper sulphate and undergo complete lysis at 200 μM copper sulphate, (d) SUB62 transformed with Yep96/UbEP42 show normal growth irrespective of copper sulphate concentration.

There are seven bases in the original sequence where substitution mutations have occurred. Out of these three are silent or neutral mutations resulting in replacement by synonymous codons and the other four mutations led to replacement of amino acid residues. The mutations Ser20 to Phe and Ala46 to Ser have occurred in type I and type III turns respectively.[20] These two are surface residues and hence the substitution of a hydrophilic residue Ser by a hydrophobic residue Phe may have a drastic effect on the structure of the molecule. Formation of a hydrogen bond between the N of ε-amino group of Lys48 and Ala46 was reported in the wild type ubiquitin earlier, which may be affected with the substitution in the mutant. The third mutation Leu50 to Pro occurring in the β-sheet may be significant as well since the side chain of Leu is buried in the interior of the protein. Moreover, Pro being restricted in geometry due to its torsion angleϕ, introduces kink in the protein backbone. Even though, substitution of Ile61 by Thr is not a drastic change, Ile61 is one of the first residues to be protected from H-D exchange during refolding of ubiquitin.[21] Further, the side chain of Ile is also buried in a hydrophobic pocket Ala46 and Lue67 of the wild type native molecule. Thr being polar may not show same preference (Figure 3). Indeed our results indicate that the thermal stability of

Table 1.
The codon and amino acid residue substitutions observed with the sequence of *UbEP42* mutant and the secondary structures where the mutations are located is given below.

Codon	Residue position	Amino acid	Secondary structure involved
TCC/TTC	20	Ser/Phe	3rd residue of a type I turn
ATC/ATA	23	Ile/Ile	—
CCA/CCG	37	Pro/Pro	—
TTG/TTA	43	Leu/Leu	—
GCC/TCC	46	Ala/Ser	2nd residue of type III turn
CTC/CCT	50	Leu/Pro	β-sheet
ATT/ACT	61	Ile/Thr	Between two turns in the turn rich region

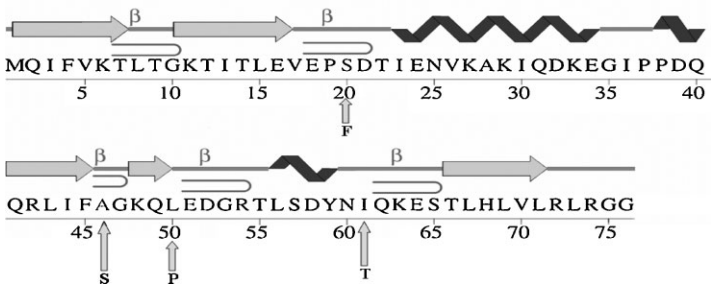

Figure 3.
Sequence of ubiquitin along with its secondary structure is presented above. The amino acid residue substitutions in UbEP42 are indicated with arrows.

UbEP42 is much reduced compared to wild type ubiquitin (unpublished observations, Pradeep Mishra and C. Ratna Prabha). Structural characterization of the mutant protein will give a better picture in this regard.

Ubiquitin of *S.cerevisiae* is almost identical to human ubiquitin in its sequence and structure. Our results indicate possibilities of either the same combination of mutations in ubiquitin or some other mutations generated by *in vitro* evolution of human ubiquitin gene are likely to give rise to dosage dependent lethal effects in human cells as well. Fine tuning the expression of such a dosage dependent mutation in tissue specific manner can have profound medical implications.

Conclusion

In vitro evolution of ubiquitin gene gave rise to a dosage dependent lethal variant of the gene with substitution mutations in four positions, resulting in replacement of four amino acid residues. The following conclusions were drawn from the sequence of the mutated gene: (i) These mutations do not include any of the lysines, which are important for the biological role ubiquitin, (ii) Two of the mutations are present in turns, one is in the (-sheet and one more is in the turn rich region, and (iii) Two of them convert nonpolar residues to polar, one is polar to nonpolar and the other one is nonpolar to nonpolar residue.

Acknowledgements

CRP is grateful to University Grants Commission for the research grant. Plasmid Yep96 and *S. cerevisiae* strains are generous gifts from Prof. Daniel Finley, Department of Cell Biology, Harvard Medical School, Boston, Massachusettes, U.S.A.

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