PUBLICATIONS AND PRESENTATIONS

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1) C. Ratna Prabha*, **Pradeep Mishra** and M. Shahukar, (2009) Isolation of a dosage dependent lethal mutation in Ubiquitin gene of *Saccharomyces cerevisiae. Macromolecular Symposia*, (in press).

2) **Pradeep Mishra**, S. Volety, Ch. Mohan Rao and C. Ratna Prabha*, (2009) Glutamate 64 to glycine mutation in ubiquitin makes it structurally more stable and functionally impaired. *Journal of Biochemistry*, published in advanced access July, 2009 (in press).

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PRESENTATIONS

1) **Pradeep Mishra**, S. Volety, D. Trivedi, Harsh Patel, Ch. Mohan Rao and C. Ratna Prabha*, Glutamate 64 to Glycine mutation in ubiquitin makes it more stable than wild type. Presented in 'Advances in Structural Biology and Structure Prediction' 11th ADNAT Convention, Hyderabad Feb. 2007.

2) C. Ratna Prabha*, **Pradeep Mishra**, Mitali Shahukar Isolation of a dosage dependent lethal mutation in ubiquitin gene of *saccharomyces cerevisiae,* Presented in '4th International Symposium on Macro- and Supra-molecular Architectures and Materials (MAM-2008)', Universität Düsseldorf, D-40225 Düsseldorf, Germany 2008.

3) **Pradeep Mishra** and C. Ratna Prabha*, The effects of S65D and Q2N mutations on the multiple functions of ubiquitin in *Saccharomyces cerevisiae.* Presented in 'National Symposium on Cellular and Molecular Biophysics', Hyderabad, Jan 2009.

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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: *in vitro* evolution, Ubiquitin, dosage dependent lethal mutation, directed evolution, error prone PCR.

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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 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 100μg/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), 1.5mM $MgCl_2$, 50mM KCl, 0.01% gelatin, 250μM dNTPs each. The error condition were modified by adding the following chemicals in separate reactions: 10mM $MgCl_2$, 0.5mM $MnCl_2$, 1mM dATP, 1mM dGTP and 1mM 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'ATGCAGATCTTCGTCAAGACGTTAACCGG3' and reverse primer 5'TCCGGTACCC GCTCAACCACCTCTTAG'3 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.

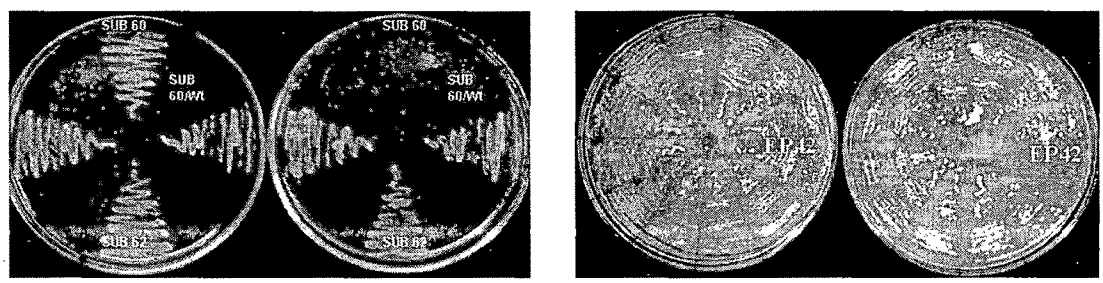
Sequence Analysis

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 40^0C for 16 hours and then again incubated back at 30^0C 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 16hrs. Several variants were generated in different reaction conditions listed under Experimental part (Figure1).



(a) (b)

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).

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 0.5mM $MnCl_2$, showed lysis even at permissive temperature, under copper sulphate induction. Further, the lysis showed concentration dependence on copper sulphate, showing complete lysis at 200μM 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.

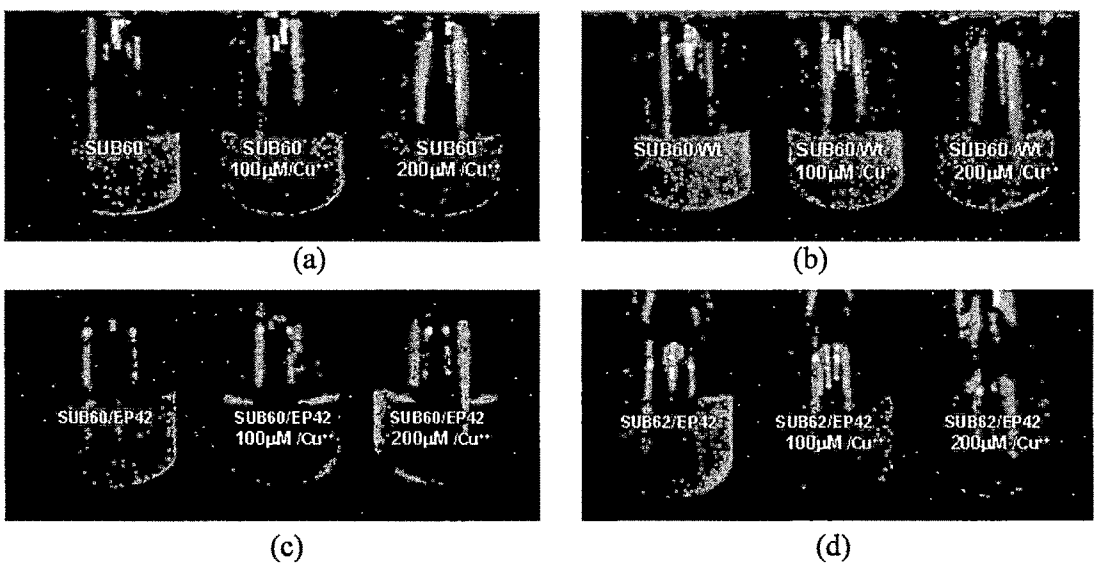


Figure 2. Effect of expression of UbEP42 on *S. cerevisiae* grown at 30°C in 0, 100 and 200μM copper sulphate respectively. (a) The *ubi4* deletion mutant SUB60 strain and (b) SUB60 strain transformed by Yep96 plasmid carrying wild type ubiquitin gene, both showing normal growth at all concentrations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

The plasmid from UbEP42 was isolated and the gene was sequenced. Sequencing of the mutant gene showed four completely novel mutations (Table1).

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

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

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.

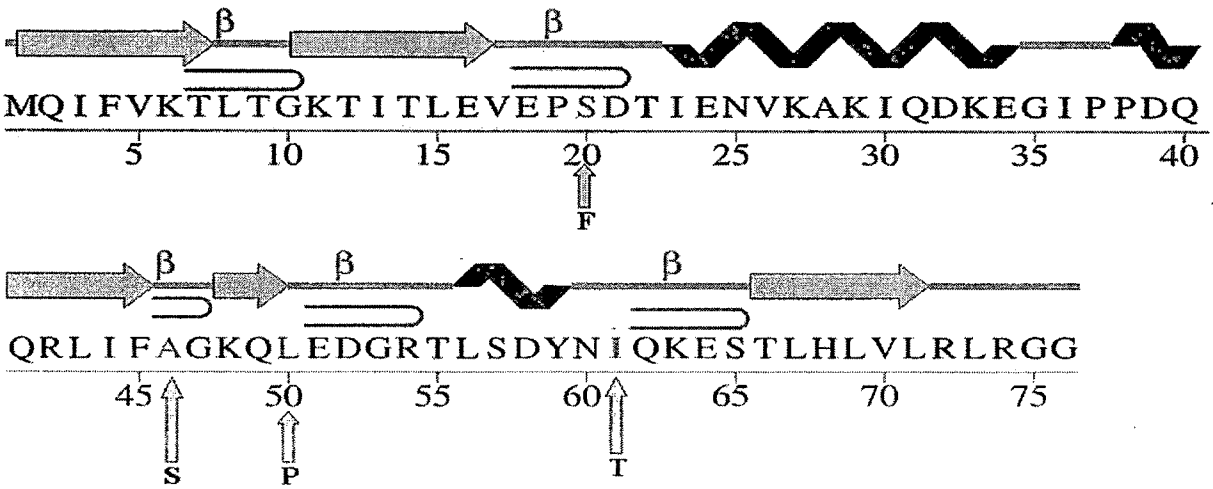


Figure 3. Sequence of ubiquitin along with its secondary structure is presented above. The amino acid residue substitutions in UbEP42 are indicated with arrows.

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 β-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.

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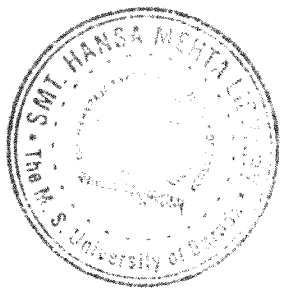

Regular Paper
(Protein Structure in the field of Biochemistry)

Glutamate64 to glycine substitution in G1β-bulge of ubiquitin impairs function and stabilizes structure of the protein

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Short title: Structure and Function of E64G Mutation in Ubiquitin

Key words: Ubiquitin, mutations in ubiquitin, ubiquitin structure, ubiquitin function, G1β-bulge of ubiquitin

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Summary

Ubiquitin is a globular protein with a highly conserved sequence. Sequence conservation and compact structure make it an ideal protein for structure-function studies. One of the atypical secondary structural features found in ubiquitin is a parallel G1β-bulge. Glutamate at 64 is the first residue of this β-bulge and the third residue in a type II turn. However, glycine is seen in these positions in several proteins. To understand the effects of substitution of glutamate64 by glycine on the structure, stability and function of ubiquitin, mutant UbE64G has been constructed and characterized in *Saccharomyces cerevisiae*. The secondary and tertiary structures of UbE64G mutant protein are only marginally different from wild-type protein (UbWt) and fluorescent form of ubiquitin (Ub F45W). The earlier studies have shown that the structure and stability of UbWt and UbF45W were similar. However, UbE64G has less surface hydrophobicity than UbWt. UbE64G is found to be more stable compared to UbF45W towards guanidinium chloride induced denaturation. *In vivo* complementation shows substrate proteins with Pro as the N-terminal residue, which undergo ubiquitination, have extended half lives with UbE64G. This altered preference for Pro as opposed to Met might be related to natural preference of glutamate at 64^{th} position in ubiquitin.

The function of a macromolecule is a consequence of its structure and dynamics. A small, globular protein such as ubiquitin with extensively hydrogen bonded structure and evolutionary conservation presents an excellent model system to understand structure function relationships. Ubiquitin has no cysteines, metal ions or cofactors. Ubiquitin has been isolated and sequenced from a variety of sources and has been found to be identical in all higher eukaryotes from insects to humans [*1-4*]. In yeast [*5*] and oat [*6*] ubiquitin amino acid replacement is seen in only three positions of the entire sequence. Ubiquitin is a remarkable protein from the functional point of view as well. Ubiquitin forms conjugates with histones playing a key role in cellular nucleic acid metabolism [*7*]. Ubiquitination of many important cellular proteins marks them for ATP dependent degradation mediated by proteasomes [*8, 9*] and by lysosomes in case of certain membrane proteins [*10*]. The indispensable role of this protein and its striking conservation in nature reflect the structural constraints imposed on almost every residue in the sequence of the protein and its interaction with enzymes involved in ubiquitin conjugation.

The X- ray crystallographic structure of ubiquitin reveals a globular α/β structure with hydrophobic core surrounded by five strands of β-sheet and four turns of α-helix. The compact structure of ubiquitin has nine reverse turns [*11*]. Pulsed H-D exchange NMR experiments of ubiquitin folding indicated that the backbone amide protons of N-terminal β-sheet and α-helix are protected early. The C-terminal half of the protein exhibits a relatively slow folding kinetics [*12*]. Partially folded state of ubiquitin stabilized in methanol/water mixture revealed conservation of native secondary structural elements in the N-terminal half. But the C-terminal half which is predominantly β strand in character undergoes a transition to helical state [*13-15*]. While folding, the N-terminal portion of ubiquitin serves as an autonomously folding chassis governing the folding of rest of the protein through tertiary interactions [*16*].

Strict conservation of ubiquitin sequence leaves no scope of comparison with its homologs from other species to understand the role of individual residues. However, site directed mutagenesis has been successfully employed to elucidate specific functions of many residues [*17-*

19]. Ubiquitin does not contain any Trp residues. In order to study the structural properties of ubiquitin using intrinsic tryptophan fluorescence, tryptophan was introduced in place of phenylalanine at position 45 in the ubiquitin sequence. The folding kinetics and stability of this protein were investigated using fluorescence spectroscopy and it was found to behave like the wild type protein [*20*]. In most of the earlier studies the structure and folding of mutant ubiquitins were investigated in great detail. However, the functional consequences of mutations in ubiquitin molecule were largely ignored.

Ubiquitin has two β-bulges. The first one is located in N-terminal region in the type I turn of the β-hairpin. This region displays native like structure in early folding intermediates. Occurrence of β-bulge in type I turn, however, is uncommon [*11*]. Hence, it became a topic for extensive studies. Peptides from N-terminal region of ubiquitin have been found to attain β-hairpin conformation autonomously in the presence [*21*] and the absence of organic solvents [*16*], confirming the potential of this peptide to act as an initiation site for protein folding. The replacement of TLTGK sequence, which forms type I turn along with β-bulge, by another type I turn forming NPDG resulted in non-native strand alignment [*22*].

The second β-bulge also displays some unusual features. It is a parallel G1 β-bulge, which is very rare. Glu64 forms the third residue in a type II turn and first residue in the β-bulge [*11*]. Generally first residue in a β bulge is a Gly. Further, the homologs of ubiquitin of Rub1 and NEDD8 display Gly in this position [*23*] (Figure 1a). This unusual feature and its conservation through millions of years of evolution make it an interesting protein for structure function studies. Moreover, this acidic residue is adjacent to a basic residue Lys63, which has been found to be important for UV repair of DNA [*24*], resistance of cells to stress conditions [*25-26*] and endosomal degradation of certain proteins [*10*]. Significance of this structural feature in ubiquitin biology is the main focus of this work. In order to understand the importance of glu64 in the structure, stability and function of ubiquitin, a variant of ubiquitin (UbE64G) has been engineered using site directed mutagenesis and characterized by circular dichroism (CD) and fluorescence

spectroscopy. The position of this mutation in the 3 dimensional structure of the protein is shown in Figure 1b. Its stability was evaluated by guanidine hydrochloride and thermal denaturation studies. Our results show that the mutant protein is structurally similar to wild type protein (UbWt), except that its surface hydrophobicity is reduced. In the present study two forms of ubiquitin, namely the wild type ubiquitin (UbWt) and fluorescent variant of ubiquitin (UbF45W) were used as controls. UbE64G was constructed from UbF45W, thus containing the double mutation.

The gene for UbE64G was introduced into a suitable vector and expressed in UBI4 mutant of *Saccharomyces cerevisiae*, a polyubiquitin deletion mutant, to validate its functional integrity by complementation. The polyubiquitin gene UBI4 is expressed under stress and rescues the organism in extreme conditions [*27*]. The mutant protein UbE64G was found to be functionally complementary and rescued UBI4 mutants of *S. cerevisiae* under thermal stress (results not shown). The effect of mutation on the turnover of proteins was studied with two different constructs of ub-β-galactosidase fusion protein, having Met and Pro as the N-terminal residues of β-galactosidase in UBI4 mutant expressing UbE64G. According to N-end rule proteins with Met as N-terminal have longer half lives, where as proteins with Pro as their N-terminal are extremely short lived [*28*]. Interestingly, our results show that proteins with Pro as the N-terminal residue have extended half lives with UbE64G complementation compared to the UbWt control. Thus, a point mutation leads to altered substrate preference.

Experimental Procedures

Site directed mutagenesis, construction of *UbE64G* plasmid

Plasmid pKK 223-3 carrying ubiquitin gene with F45W mutation was a gift from Prof. Mark Searle's laboratory [*22*]. The ubiquitin gene from pKK 223-3 was subcloned into pUC18. The mutation for UbE64G was introduced following Genei in vitro site directed mutagenesis

protocol. Introduction of mutation was confirmed by sequencing. The mutated fragment from pUC18 vector was cloned back and pKK 223-3-E64G was obtained.

Purification of UbWt, Ub F45W and UbE64G proteins

The proteins UbWt, UbF45W and UbE64G (double mutant) were purified using the methods given in literature [*29-31*]. Since wild type ubiquitin is known to be a heat stable protein the heat step was employed during the purification of the protein. The supernatant that was collected, after cell lysis and precipitation of DNA, was heated at 85-90°C in the presence of 1mM DTT for 15 minutes. The supernatant contained ubiquitin while most of the proteins coagulated and were removed by centrifugation [*29*]. This step was adapted for the purification of UbF45W earlier [*20*]. This purification step based on the thermal stability of ubiquitin could be adapted to the purification of UbE64G as well. The following changes were made in the protocol. After cell lysis, the lysate was fractionated [*31*] up to 85% ammonium sulphate precipitation. After dialysis ubiquitin containing fraction was purified on Sephadex G-50 column. The protein was concentrated by precipitation and dialysis. The protein stocks were stored in 10%glycerol at -20°C. Protein concentration for F45W was determined using its extinction coefficient of 6744 M^{-1} cm^{-1} at 280 nm [*32*]. Mutant ubiquitin UbE64G was purified using the same protocol that was used for the wild type protein.

CD and Fluorescence Spectroscopy of Ub F45W and Ub E64G:

CD spectra were recorded using a Jasco J-715 spectropolarimeter. Far-uv CD spectra were recorded with 1mm path length cells. Spectra were recorded in the range of 200-250 nm with a scan speed of 50nm/ sec. The spectra were accumulated for six times and averaged to improve the signal to noise ratio. Protein solutions were prepared in 10mM Tris HCl, pH7.4 and concentration of protein was 0.25mg/ ml. The near-uv CD spectra of the proteins were recorded between 250 and 320nm. Path length was 1cm and protein concentration was 0.774mg/ ml.

Appropriate blanks were prepared for all samples and the spectra of the samples were blank corrected.

Fluorescence spectra were recorded using Hitachi F-4010 fluorescence spectrophotometer using excitation and emission bandpasses of 5nm respectively. Protein concentration was 0.1mg/ ml. Samples were excited at 295nm to record the intrinsic tryptophan fluorescence in the range of 300-400nm.

ANS was used as an extrinsic fluorophore at a concentration of 50μM. ANS was excited at 390nm and emission was recorded between 450-600nm. Slit width was 5nm. Protein concentration was 0.2mg/ ml. Protein solutions were prepared in Tris HCl buffer, pH 7.4.

Construction of yeast vector carrying *UbE64G* gene:

Yeast-bacteria shuttle vector pUb175-E64G was constructed from pUb175 by replacing Ub gene under CUP-1 promoter with UbE64G gene, which can be induced in yeast by 10-100μM copper sulphate. The mutant ubiquitin gene from pKK 223-3-E64G was PCR amplified. The resultant DNA was subcloned into pUB175 yeast expression vector. The plasmid pUB175 was a kind gift from Prof. Daniel Finley. Similarly the gene for UbF45W was also cloned to have a control for fluorescence studies. The plasmid pUB175-E64G was digested with restriction enzymes and later it was sequenced for confirmation.

The vector was transformed into yeast UBI4 mutant, which lacks the polyubiquitin gene. The gene for UBI4 expresses under stress and mainly responsible for the survival of the organism under stress conditions. In absence of this gene the organism can grow at 30°C (permissive temperature), but fails to grow at 39°C and above. Complementation by functional ubiquitin restores wild type phenotype to the organism. This system was used for complementation tests with UbE64G mutant.

Effect of the ubiquitin mutation on substrate protein turnover

SUB60 (*MAT*a *ubi4-Δ2::LEU2 lys2-801 leu2-3,112 ura3-52 his3-Δ200 trp1-1*) and SUB62 (*MAT*a *lys2-801 leu2-3,112 ura3-52 his3-Δ200 trp1-1*) strains of *S. cerevisiae* [*33*] were used for *in vivo* studies. SUB60 and SUB62 strains of *S. cerevisiae* were transformed with yeast plasmid Yep96 [*24*] carrying the three variants of ubiquitin gene, namely wild type or UbWt, UbF45W and UbE64G and the recipients were referred to as Yep96/wt, Yep96/F45W and Yep96/E64G. SUB60 double transformed with pUB23, a 2μ -based vector expressing ubiquitin-X-βGal (Ub-X-βGal) fusion gene under the control of galactose inducible GAL10 promoter with X position as Met and Pro in independent sets. SUB62 cells were also transformed with pUB23 [*28*] with X position as Met and Pro. *S. cerevisiae* transformants were grown in synthetic galactose media at 30°C, conditions under which Ub-X-βGal is constitutively expressed. The cells were grown with and without 100μM of $CuSO_4$ in two independent sets for the induction of UbWt, UbF45W and UbE64G from Yep 96. β-galactosidase activity assay was used as a measure of protein stability in this set of experiments.

Results

CD and fluorescence spectra of the three variants of ubiquitin

Far-uv CD spectra were recorded to study the changes in secondary structure due to the mutation in the sequence of ubiquitin. The far-uv CD spectrum of UbE64G shows slight changes in secondary structure content, with respect to both UbWt and UbF45W (Figure 2). Percentages of various components of secondary structure in far-uv CD spectra of UbWt, UbF45W and UbE64G were caculated using CD Pro software [*34*] and are shown in Table 1. The values obtained with UbWt are in agreement with X-ray crystallographic data [*34*]. The values observed with the two variants UbF45W and UbE64G appear to be similar to those observed with UbWt. However, UbE64G shows 4-5% decrease in α-helical content and 3% increase in β-sheet. The

changes in secondary structure of UbE64G could be due to enhanced flexibility introduced by the substitution.

The CD spectrum of a protein in the near-uv spectral region (250-320nm) can be sensitive to certain aspects of tertiary structure. At these wavelengths the chromophores are the aromatic amino acids and disulfide bonds, and the CD signals they produce are sensitive to the over all tertiary structure of the protein. Hence, tertiary structure is a qualitative measure of the over all conformation of the molecule [*35-38*]. Based on near-uv CD spectra, the tertiary structure of UbE64G and UbF45W appear to be similar, an indication that the conformation of the protein around the aromatic residue is unaffected by this mutation (Figure 3).

Due to lack of tryptophan, wild type ubiquitin (UbWt) does not display any fluorescence properties. On the other hand, UbE64G and UbF45W display very similar fluorescence properties, indicating that the environment around aromatic amino acid residue Trp45 is almost identical (Figure 4).

UbWt shows greater surface hydrophobicity than UbE64G

Fluorescence spectra of ubiquitin with extrinsic fluorophore 1-anilino 8- naphthalene sulphonic acid (ANS) showed higher intensity with UbWt compared to UbE64G. ANS is known to bind to the exposed hydrophobic surfaces of proteins and show enhanced fluorescence compared to its weak fluorescence in aqueous media. Figure 5 shows that the intensity of ANS fluorescence is higher when bound to UbE64G compared to that in the case of UbF45W and this indicates that UbE64G has more exposed hydrophobic surface than UbF45W. However, higher intensity of ANS fluorescence observed with UbWt is due to greater exposure of hydrophobic residues to the surface in the wild type protein compared to UbE64G (Figure 5). Similar results were obtained with two other extrinsic fluorophores, Bis-ANS and pyrene (results not shown). The resultant differences seen in surface hydrophobicity of UbE64G perhaps can be attributed to minor alterations in structure leading to changes in the extent of exposure of hydrophobic

residues. In addition the mutant replaces E with G which differ in hydropathy index, E is -3.5 while G is -0.4 [*39*].

Thermal denaturation and renaturation studies showed that the two proteins follow the same pathway during folding and unfolding experiments and do not show much change in their conformation. In retrospective, it could be reasoned that it was possible to use the thermal treatment step in the protocol for purification of UbF45W for UbE64G as well without any modification, due to their similar thermal stabilities.

However, the guanidine hydrochloride denaturation curves of UbE64G and UbF45W were not coincident. The $C_{1/2}$ values of guanidine hydrochloride (gdmCl) for UbE64G and UbF45W were 3.6M and 3.24M (figure 6c). The far and near-uv CD spectra recorded in the absence and presence of 4M guanidine hydrochloride suggests that UbE64G retains some amount of its secondary and tertiary structure where as UbF45W loses tertiary structure completely (Figure 6a and 6b) in the presence of 4M guanidine hydrochloride.

In vivo studies:

UbI4 gene cluster has 4-5 copies of ubiquitin expressed as a single polypeptide chain, which is processed into ubiquitin molecules post-translationally. SUB60 mutants lacking UbI4 fail to withstand stress conditions, but grow normally at 30°C. SUB60 mutants lacking UBI4 gene and SUB62 wild type cells were transformed with yeast plasmids (carrying variants of ubiquitin gene as indicated), Yep96/wt, Yep96/F45W and Yep96/E64G. Yep96/wt, Yep96/F45W and Yep96/E64G are plasmids carrying ubiquitin gene under CUP-1 promoter and have tryptophan auxotrophy for selection. These transformants were co-transformed by pUB23, a plasmid with wildtype ubiquitin gene fused to *lacZ.* According to N-end rule, the stability of a protein is determined by its N-terminal residue. β-galactosidase fusion with stabilizing N-terminal residues like Met, is cleaved by ubiquitin hydrolases releasing a free β-galactosidase with longer half life.

Those fusions with destabilizing residues such as Pro are not processed and a polyubiquitin chain is built subsequently on the ubiquitin in the fusion protein, resulting in degradation of the β-galactosidase. The ubiquitin-β-galactosidase fusion is under pGAL10 promoter. β-galactosidase activity assay has been used as a measure of protein stability. Our results show β-galactosidase activity remained more or less unchanged in Yep96/wt, when Met (stabilizing residue) was present as the N-end residue. β-galactosidase activity showed a decline, when Pro was present as the N-end residue. However, the β-galactosidase activity increased for Pro- β-galactosidase in the UbE64G mutant, compared to UbWt and UbF45W in Ubl4 mutant strain SUB60 (Table 2).

Discussion

Proteins showing complete conservation of their sequence through millions of years of evolution are examples of optimal balance of structure and function. Ubiquitin is one such protein, showing no change in sequence from insects to humans and only three substitutions in yeast. A close look at its structure indicates that in the second β-bulge there are certain unusual features. The first residue in the β-bulge has a glutamate in place of glycine, the later being a preferred choice in such structures. Structural homology searches also revealed that close structural homologs of ubiquitin, NEDD8 and Rub1 [*40*] and ubiquitin like protein from baculovirus *Autographa californica* [*41*] have glycine at the same position. Interestingly, those structures are identical.

From our results it is clear that this substitution has been well accommodated locally. However, replacement of glutamate by glycine has affected the overall conformation of the protein, by altering the ionic and hydrophobic interactions, and changing the surface hydrophobicity. In addition, this mutation makes the protein UbE64G less stable in phosphate buffer (pH 7), precipitating it from solution (results not shown). Besides, the protein UbE64G was found to be more stable than UbF45W towards guanidine hydrochloride denaturation under

equilibrium conditions. In 4M guanidine hydrochloride UbF45W loses all its tertiary structure content, whereas UbE64G retains about half of it. Secondary structure of UbE64G remains unchanged even at 4M guanidine hydrochloride (Figure 6a). On the other hand, UbF45W lost most of its secondary structure. It was observed earlier that removal of surface charges stabilized the protein [*42, 43*] and removal of certain charged residues influenced the pKa values of neighbouring residues [*43*].

As described under Experimental Procedures section UbE64G could be purified by adapting the heat treatment, used for purification of UbWt and UbF45W, because of its thermal stability. Since UbE64G and UbF45W are thermostable, it appears that thermal treatment does not lead to complete denaturation (Figure 7). The thermal denaturation profiles appear similar. Thermal unfolding and refolding of UbE64G also did not differ from that of UbF45W.

The changes in structure of the molecule which are evident *in vitro*, appear to have a bearing on the function of the molecule. Interestingly, UbE64G has extended the half life of unstable substrate proteins with N-terminal Pro residue.

In conclusion, replacing the conserved glutamate at position 64 with glycine leads to subtle changes in structure as indicated by 4-5% decrease in helicity, increased stability towards guanidinium chloride denaturation and more importantly altered substrate interaction. The wild type protein leads to slower turnover of protein with N-terminal Met residue, compared to the proteins with N-terminal Pro residue. The mutant has reversed behaviour, it indeed extended the half life of protein with N-terminal Pro, which should have been removed from the system faster.

This study provides an interesting example of a point mutation with subtle structural alteration and with a significantly altered function.

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CONFLICT OF INTEREST
None declared.

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Table 1

Secondary structural analysis of far-uv CD spectra using CD Pro software

Type of secondary structure	UbWt* (X-ray)	UbWt	UbF45W	UbE64G
A-helix	16	17	16	11.5
B-sheet	32	31	32	35
Turns and random coil	52	52	52	53.5

* The values reported by Chyan *et al.*, 2004 [*34*]

Table 2

Effect of UbE64G on the half-life of proteins in the UbI4 background.

Strains of *S.cerevisiae* used in the experiment	Ub-Met-β Gal nmoles of ONPG/ minute/ mg protein		Ub-Pro-β Gal nmoles of ONPG/ minute/ mg protein	
	After induction by copper sulphate	Without copper sulphate	After induction by copper sulphate	Without copper sulphate
SUB60	-8.283±1.161	-11.4667±1.180	-1.653±0.231	-2.283±0.234
SUB62	-11.627±1.871	-14.08±1.624	-2.323±0.372	-2.750±0.361
SUB60 β-Gal	55.303±8.026	49.717±9.027	16.843±1.740	16.613±2.054
SUB62 β-Gal	55.963±8.792	46.873±9.071	2.038±0.901	1.937±0.236
SUB60 β-Gal/UbWt	48.747±2.941	39.480±4.842	4.653±0.738	7.183±0.639
SUB60 β-Gal/F45W	48.373±7.338	41.293±4.905	6.297±0.702	7.983±0.623
SUB60 β-Gal/E64G	48.713±10.745	40.017±6.380	12.417±0.453	12.5±1.241

Table 2. Effect of UbE64G on the half-life of proteins in UbI4 background. *S.cerevisiae* strains SUB60, SUB62, SUB60 transformed by plasmidsYep96/UbWt, Yep96/F45W and Yep96/E64G expressing the three forms of ubiquitin, namely UbWt, F45W and E64G. These cells were also transformed by pUb23 expressing Ub-β-galactosidase fusion with Met and Pro as the N-terminal residues. In SUB62 cells were co-transformed by plasmids Yep96/UbWt, Yep96/F45W, Yep96/E64G and pUb23 expressing Ub-β-galactosidase fusion with Pro as the N-terminal residue. SUB60 and SUB62 were used as controls.

Legends for Figures and Table

Figure 1. a) the sequences of Rub1 and NEDD8 have been aligned with ubiquitin to show the preference for G in the first position of β-bulge (indicated in bold). b) The position of E64 in 3D structure of ubiquitin.

Figure 2. Far uv CD spectrum of three forms of ubiquitin, UbWt (1), UbF45W (2), UbE64G (3)

Figure 3. Near uv CD spectrum UbF45W (1), UbE64G (2)

Figure 4. Fluorescence emission spectra of UbWt (1), UbF45W (2), UbE64G (3) recorded after exciting the protein at 280nm

Figure 5. Fluorescence emission spectra of of the three forms of ubiquitin UbWt (1), UbF45W (2) and UbE64G (3) bound by extrinsic fluorophores ANS recorded after exciting the ANS at 280nm

Figure 6 (a). Far uv CD spectra of UbE64G and UbF45W recorded in 0M guanidine hydrochloride (1 and 2 respectively) and 4M guanidine hydrochloride (3 and 4 respectively). (b) Near uv CD spectra of UbE64G and UbF45W recorded in 0M guanidine hydrochloride (1 and 2 respectively) and 4M guanidine hydrochloride (3 and 4 respectively). (c) Gaunidine hydrochloride denaturation curves of UbE64G (1) and UbF45W (2).

Figure 7. Far-uv CD spectra of UbF45W and UbE64G before thermal denaturation (1 and 2 respectively) and after thermal denaturation (3 and 4 respectively).

Figure 1

UBI - [1]MQIVKTLTGKTITLEVESSDTIDKVKSKIQDKEGIPPDQQRLIFA[45]
RUB1 - MIVKVTLTGKEISVELKESDLVYEIKELLEEKEGIPPSQQRLIFQ
NEDD8 - MLIKVKTLTGKEIEIDIEPTDKVERIKERVEEKEGIPPQQQRLIYS

UBI - [46]GKQLEDGRTLSDYNIQK**E**STLHLVLRLRGG[76]
RUB1 - GKQIDDKLTVTDAHLVE**G**MQLHLVLTLRGG
NEDD8 – GKQNNDEKTAADYKILG**G**SVLHLVLALRGG

Figure 1. a) Sequence alignment of RUB1 and NEDD8, the homologs of ubiquitin with the sequence of ubiquitin UBI. In the 64th position in RUB1 and NEDD8 G is conserved, where as in ubiquitin in all eukaryotes in the same position E is found.

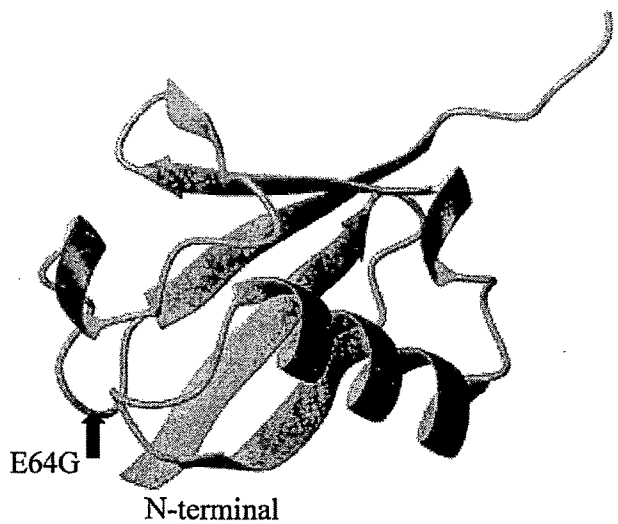

Figure 1. b) The 3D structure of ubiquitin. The arrow indicates the position of E64G substitution.

Figure 2

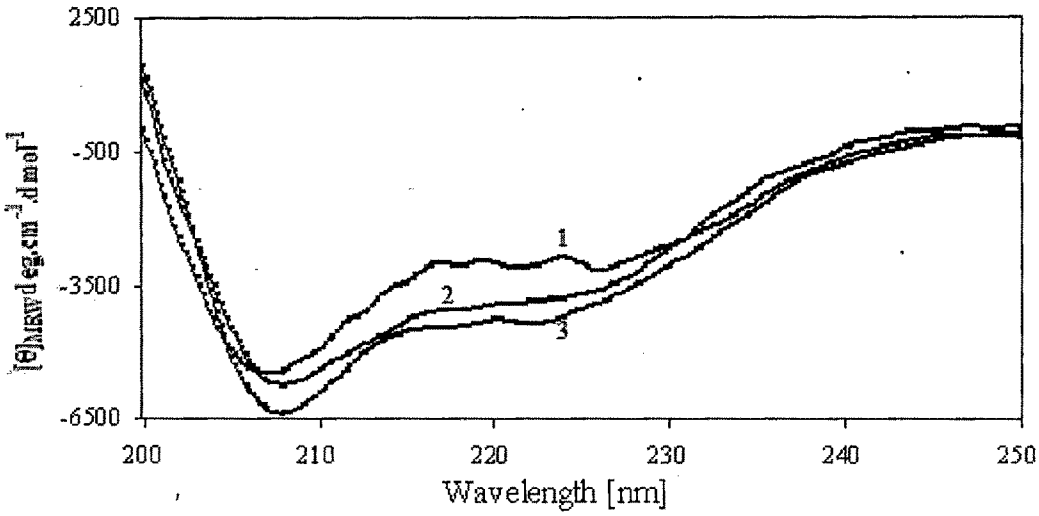

Figure 3

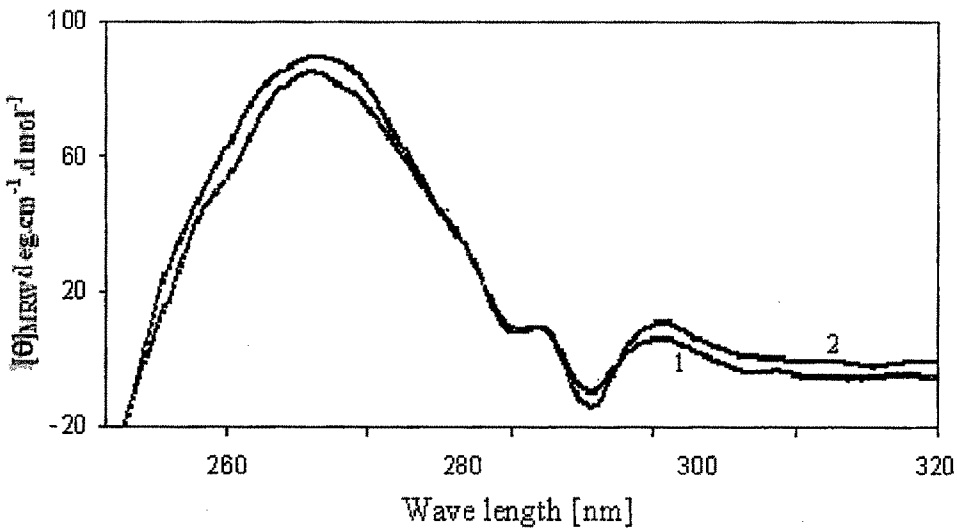

Figure 4

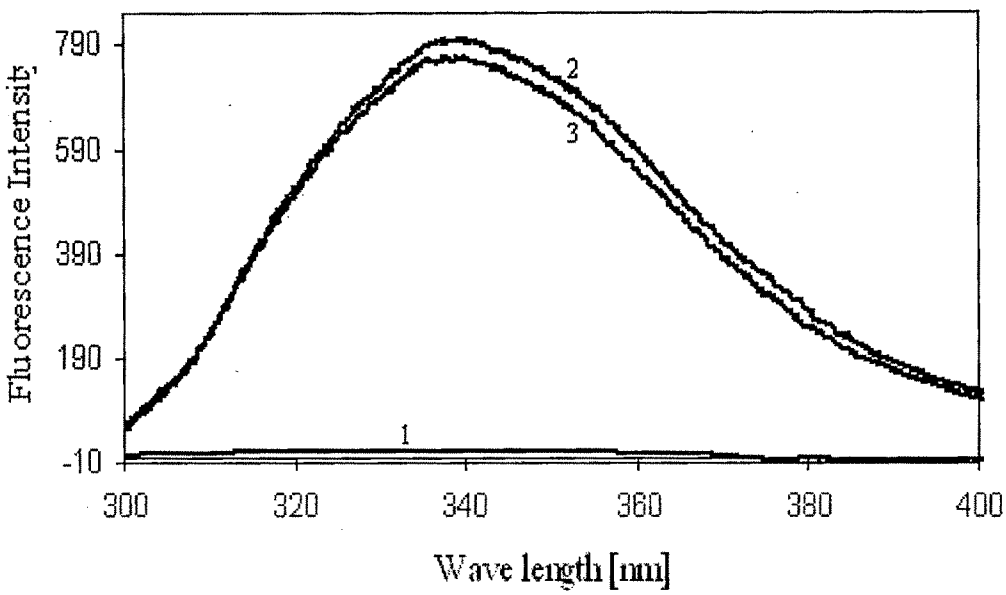

Figure 5

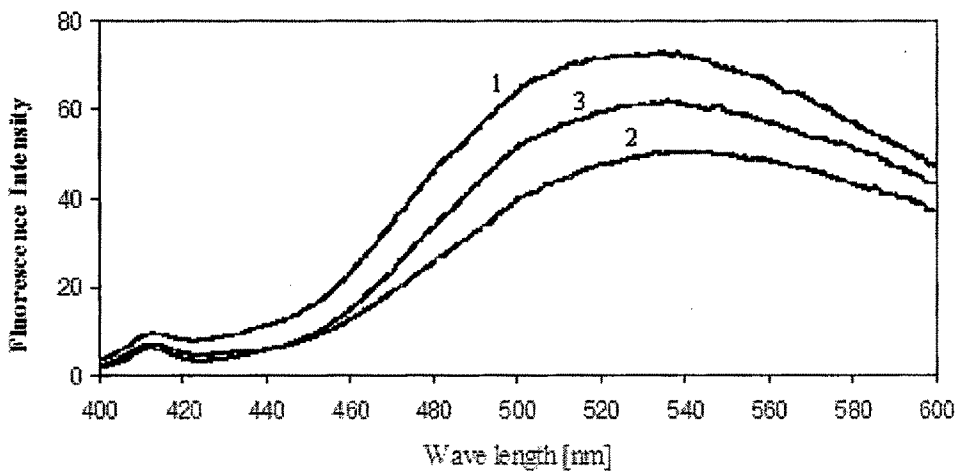

Figure 6

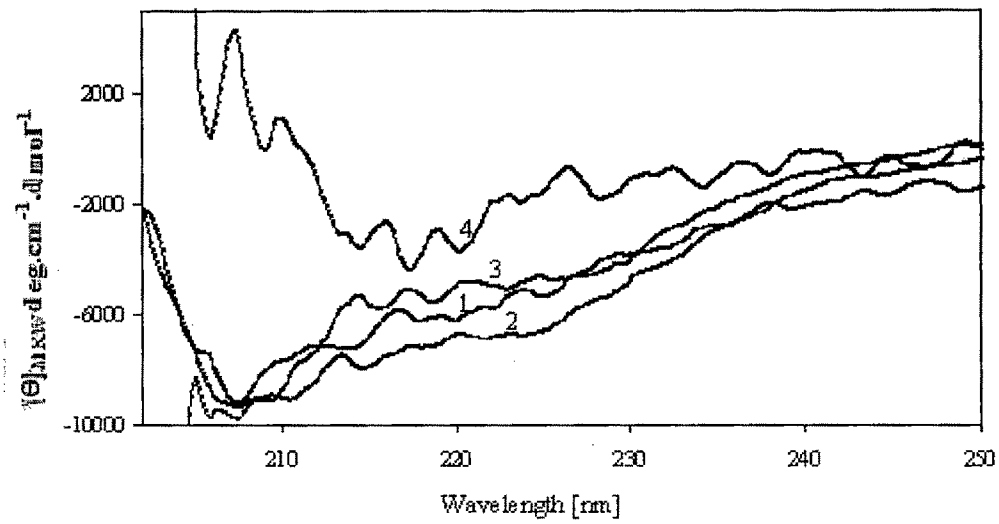

Figure 6 a

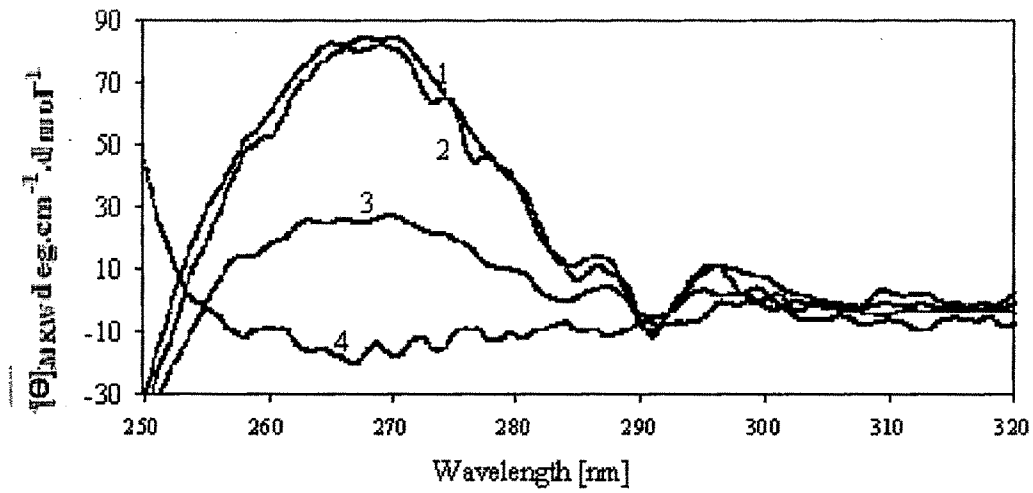

Figure 6 b

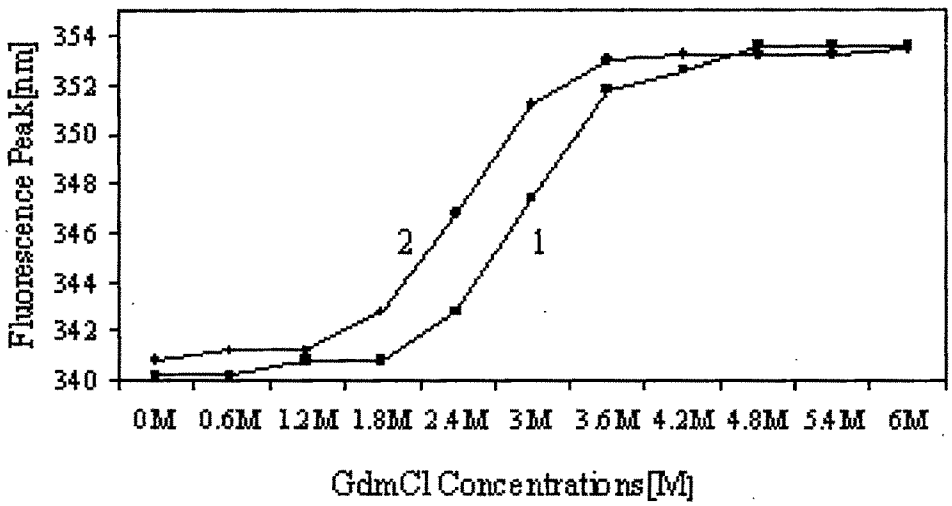

Figure 6 c

Figure 7

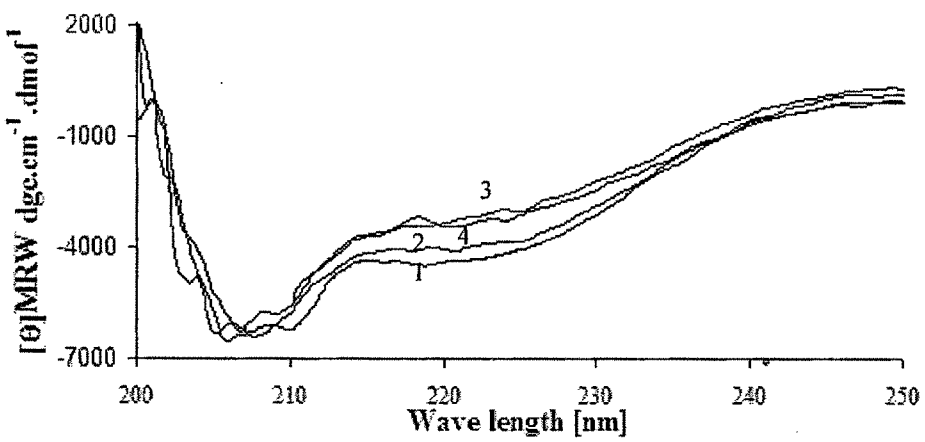

Figure 7. Far-uv CD spectra of UbF45W and UbE64G before thermal denaturation (1 and 2 respectively) and after thermal denaturation (3 and 4 respectively).

GLUTAMATE 64 TO GLYCINE MUTATION IN UBIQUITIN MAKES IT MORE STABLE THAN WILD TYPE

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ABSTRACT:

Ubiquitin is a small, globular protein found in all eukaryotes. Its sequence has been found to be identical in insects to higher eukaryotes such as human. Sequence variations are seen only in three positions in organisms as diverse as yeast and plant. Ubiquitin is also one of the most stable proteins found in eukaryotic cells. Marked conservation of the residues in this protein suggests a role for all residues in protein folding, stability or function. One of the conserved residues, Glu64 is the third residue in a type II turn. It also forms the first residue of a β-bulge. Type II turns and β-bulge usually contain Gly rather than Glu seen in ubiquitin. However, such judgment based on consensus does not take into account the global effects a residue has in specific situations. Thus, we chose to study the effects of substitution of Glu by Gly on folding, stability and function of ubiquitin. Spectroscopic characterization of Ub[F45W][E64G] mutant shows under normal conditions the secondary and tertiary structures of protein are only marginally different from native protein. Despite minimal changes in the conformation the mutant precipitates out at high concentrations of salt compared to wild type, suggesting alterations in the balance of electrostatic and hydrophobic interactions. The mutant shows higher hydrophobicity as measured by ANS fluorescence. Guanidinium chloride induced denaturation of the mutant shows molten globule like intermediate.

INTRODUCTION:

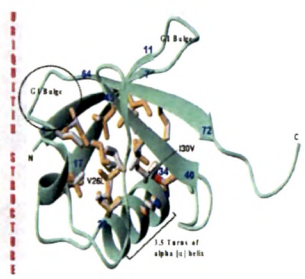
Ubiquitin is a small protein with 76 amino acid residues. It is present ubiquitously in all eukaryotic cells, playing important role in ATP dependent proteasome mediated protein degradation.

Ubiquitin is conjugaed to proteins forming polyubiquitin chains. Polyubiquitination earmarks a protein for degradation.

Ubiquitin protein conjugation is a three step process.

Lys 11& Lys 48 are known to praticipate in ployubiquitin chain formation.

Lys 63 is essential for stress survival in *Saccharomyces cerevisiae*. Mutation in this position leads to impaired DNA repair (Daniel Finley *et al.*, 1995).



SECONDARY STRUCTURAL FEATURES OF UBIQUITIN

Ubiquitin is an α/β protein. Its structure comprises of an α helix, a 3_{10} helix, two parallel and three anti parallel β sheets.

There are eight reverse turns in its structure.

Ubiquitin has two β bulges. The first β bulge is present at the N-terminal region second is located in the C-terminal part of the protein. It involves Glu64(1), Ser65(2) & Gln2(N).

UNUSUAL FEATURES OF SECOND BETA BULGE IN UBIQUITIN

Glu64 is located in the Type II turn in the third position (i+2) and first position (1) of Beta [β] Bulge.

The presence of Glu64 is supported by unusual Φ and Ψ conformation angles.

These conformation angle are commonly observed with Gly and therefore conventionally Gly is favored at (i+2) position of Type II turn and first position (1) of [β] bulge.

Sequence & Structural homology searches also revealed that a close structural homolog of ubiquitin, NEDD8 (also known as Rub1) has glycine in the same position.

Sequence Alignment Structure Alignment

We decided to study the consequences of replacement of glu64 by gly on the structure, function, folding and stability of the protein.

Substitution of charged amino acid (Glu) with neutral amino acid (Gly) might change the pattern of charge charge interaction on the surface, consequently alter the stability.

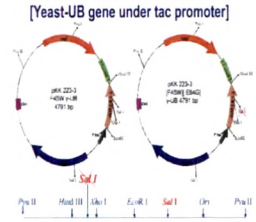
Ubiquitin interacts with number of proteins, change in the surface residue will most likely have a bearing on its function.

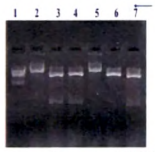
METHODS:

Site directed mutagenesis, construction of Ub[F45W][E64G] plasmid :

E.coli EXPRESSION VECTOR :

[Yeast-UB gene under tac promoter]





Lane 1 : λ *Hind* III Digest Marker

Wild Type:

Lane 2 : pKK-223[F45W] Undigested

Lane 3 : *Sal* I Digest

Lane 4 : *Sal* I *Xba* I Digest

Mutant :

Lane 5 : pKK-223[F45W][E64G] Undigested

Lane 6 : *Sal* I Digest

Lane 7 : *Sal* I *Xba* I Digest

Digestion pattern of pKK223-3 [F45W] & pKK223-3 [F45W][E64G]

Purification of Wt[Ub], Ub[F45W] and Ub[F45W][E64G] proteins:

Wt[Ub] is wild type ubiquitin. Ub[F45W] is a fluorescent variant of ubiquitin (Mark S.Searle,*et.al.*2003), used as a control in far uv CD and fluorescence spectra. Ub[F45W][E64G] is a mutant constructed in which Glu was replaced by Gly in the 64th position.

The proteins Wt[Ub] and Ub[F45W] were purified using the methods given by Ciechanover *et.al*,(1980) and Ecker *et.al*.(1987).

Mutant ubiquitin Ub[F45W][E64G] could be purified using the same protocol without making any major changes in the protocol used for purification of Wt[Ub] and Ub[F45W].

Protein concentration for Ub[F45W] was determined using its extinction coefficient of 6744 $M^{-1}cm^{-1}$ at 280 nm (V. Hippel *et.al.*1989).

CD and Fluorescence Spectroscopy of Ub [F45W] & Ub[F45W][E64G]:

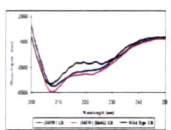
The Far uv CD spectra of protein were recorded between 200 and 250nm with path length of 1mm and protein concentration of 0.25mg/ml prepared in 10mM Tris HCl, pH 7.4.

The near uv CD spectra of the proteins were recorded between 250 and 320nm. path length was 1cm and protein concentration was 0.774mg/ ml prepared in 10mM Tris HCl, pH 7.4.

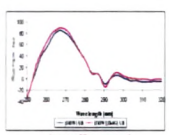
Intrinsic fluorescence spectra of protein were recorded between 320-440nm by exciting at 280nm & 295nm with concentration of 0.2mg/ ml prepared in Tris HCl buffer pH 7.4.

Bis ANS was used as an extrinsic fluorophore at a concentration of 10μM. Bis ANS was excited at 390nm and emission was recorded between 450-600nm. Protein concentration was 0.2mg/ ml. Protein solutions were prepared in Tris HCl buffer pH 7.4. The experiment was repeated with 50 μM ANS. The excitation wavelength was 370nm. Other parameters were not changed.

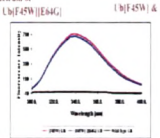
RESULTS:



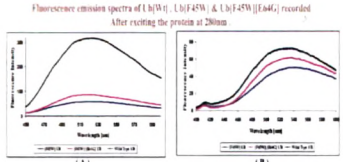
Far uv CD spectrum of Ub[Wt], Ub[F45W] & Ub[F45W][E64G]



Near uv CD spectrum of Ub[F45W] & Ub[F45W][E64G]

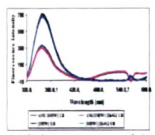


Fluorescence emission spectra of Ub[Wt] , Ub[F45W] & Ub[F45W][E64G] recorded After exciting the protein at 280nm

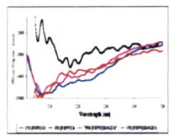


(A) (B)

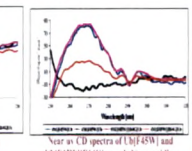
Fluorescence emission spectra of Ub[Wt] , Ub[F45W], Ub[F45W][E64G] with extrinsic fluorophores Bis- ANS (A) and ANS(B).



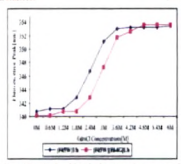
Fluorescence spectrum showing resonance energy transfer between tryptophan of ubiquitin and ANS. The spectra of Ub[F45W][E64G] and Ub[F45W] were recorded in absence and presence of ANS



Far uv CD spectra of Ub[F45W] and Ub[F45W][E64G] recorded in guanidine hydrochloride



Near uv CD spectra of Ub[F45W] and Ub[F45W][E64G] recorded in guanidine hydrochloride



Gaunidine hydrochloride denaturation curves of Ub[F45W] and Ub[F45W][E64G]

DISCUSSION:

Secondary structures of Ub[F45W][E64G] and Ub[F45W] appear to be nearly same and are apparently more than that of wild type Ub.

Tertiary structure of Ub[F45W][E64G] and Ub[F45W] seem to be alike.

Wild type Ub does not show any intrinsic fluorescence peak as it lacks Trp. Ub[F45W][E64G] and Ub[F45W] show almost identical intensities of fluorescence, indicating that the environment around aromatic amino acid residues namely Trp45 and Phe4 is almost identical.

Fluorescence spectra of ubiquitin with extrinsic fluorophore ANS, showed higher intensity with Ub[F45W][E64G] compared to Ub[F45W] & [Wild tpye] Ub. Higher intensity of ANS fluorescence is due to greater exposure of hydrophobic residues on the surface in the mutant protein. Same result was observed with another extrinsic fluorophores Bis-ANS.

Fluorescence resonance energy transfer spectrum confirms the above observation.

Thermal denaturation and renaturation showed that the two proteins Ub[F45W][E64G] and Ub[F45W] follow same path during folding and unfolding(results not shown). Hence, the mutation does not effect the thermal stabilty of Ub[F45W][E64G].

The guanidine hydrochloride denaturation curves of Ub[F45W][E64G] and Ub[F45W] are different. Ub[F45W][E64G] retains its secondary and tertiary structure even after Ub [F45W] loses tertiary structure and most of its secondary structure.

CONCLUSIONS:

From our results it is clear that substitution of glutamate 64 by glycine is well accommodated in ubiquitin. However this replacement has affected the overall conformation of the protein, by disturbing the ionic and hydrophobic interactions, exposing more hydrophobic residues to the surface.

In addition, this mutation makes the protein Ub[F45W][E64G] less stable in phosphate buffer, pH 7, precipitating it from solution (results not shown). Besides, the protein Ub[F45W][E64G] was found to be more stable than Ub[F45W] in guanidine hydrochloride denaturation under equilibrium conditions.

Thermal unfolding and refolding of the protein did not differ from that of UbF45W.

The fact that this mutation was never entertained by the molecule in nature drives us to a foregone conclusion that under certain conditions, the altered structure and stability of the molecule interfere with its function.

ACKNOWLEDGEMENTS:

CRP is grateful to University Grants Commission India for funding the project and making the work possible.

CRP thanks Prof. Mark S.Searle,University of Nottingham,Nottingham UK for the kind gift of Plasmid pKK 223-3 expressing Ub[F45W].

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ISOLATION OF A DOSAGE DEPENDENT LETHAL MUTATION IN UBIQUITIN GENE OF *Saccharomyces cerevisiae*

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[2] *Bhanuben and Ratilal Doshi School of Biosciences, Sardar Patel University, Vallabh Vidyanagar - 388120, India*

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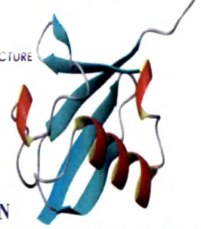
ABSTRACT

Ubiquitin is a small, compact globular protein with a highly conserved sequence. Its sequence has been found to be identical in all higher eukaryotes from insects to humans [1-4]. The ubiquitin of *Saccharomyces cerevisiae* differs only by three residues from the human ubiquitin [5]. Intracellularly, ubiquitin is used as a tag to mark proteins for degradation by ubiquitin proteasome system (UPS) [6,7]. The substrate proteins of UPS include cell cyclins, antitumor proteins and transcription factors. Considering the pivotal role UPS has in cellular homeostasis, several drugs with anticancer potential have been developed to target the system. Since mutations are not naturally permitted in ubiquitin, artificially induced lethal mutations with regulated expression may have a potential to target cancer cells in a similar way. Site directed mutagenesis of ubiquitin has limited scope in giving rise to a lethal gene with desirable phenotype. In contrast random mutagenesis with proper screening strategy is better suited for attaining such a goal. Hence, in the present study artificial mutagenesis was used to generate mutant forms of ubiquitin in *Saccharomyces cerevisiae*. After random mutagenesis the ubiquitin gene was cloned under CUP1 promoter and introduced into temperature sensitive ubiquitin mutant of *S.cerevisiae* by transformation. The mutants were selected by expressing the protein in the UBI4 mutants lacking the UBI4 gene cluster. The UBI4 mutants grow normally at permissive temperature (30°C), but fail to do so under heat stress (40°C). Most of the mutations in ubiquitin gene failed to complement UBI4 phenotype under heat stress, unlike the wildtype gene. Only one of the mutants caused cell lysis, even at permissive temperature. Interestingly, expression of the same protein in wildtype *S.cerevisiae* cells left them unaffected. This result establishes that the mutant protein acts as a competitive inhibitor for UPS and its effect is diluted out in UBI4 wildtype, where ubiquitin is expressed in several copies. Sequencing of the mutant gene showed four completely novel mutations. These mutations do not include any of the lysines. Two of them are present in turns, one is in the β-sheet and one more is in the turn rich region. Two of them are substitutions of nonpolar residues by polar, one is polar residue by nonpolar and the other one is nonpolar by nonpolar residue. This mutant is likely to produce lethality in a dosage dependent manner in higher eukaryotes as well, leaving a possibility for selective targeting of diseased cells by expressing under native tissue specific regulatable promoters with an immediate application in cancer therapy.



INTRODUCTION

Ubiquitin is a small α/β protein with 76 amino acid residues. It is present ubiquitously in all eukaryotic cells, playing important role in ATP dependent proteasome mediated protein degradation.

- Ubiquitin is conjugated to proteins forming polyubiquitin chains.
- Polyubiquitination earmarks a protein for degradation.
- Conjugation of proteins by ubiquitin is a three step process.
- Lys 11 and Lys 48 are known to praticipate in ployubiquitin chain formation.
- Lys 63 is essential for stress survival in *Saccharomyces cerevisiae*. Mutation in this position leads to impaired DNA repair (Finley *et al.*, 1995).
- Primary structure of ubiquitin shows total conservation from insects to humans making interspecies comparison impossible. A comparison of ubiquitins from yeast and human show differences only in three positions. Strong sequence conservation suggests that the vast majority of amino acids that make up ubiquitin are essential as apparently any mutations that have occurred over evolutionary history have been removed by natural selection.

YEAST UBIQUITIN (P04838)

```
MQIFVKTLTG  KTITLEVESS  DTIDNVKSKI  QDKEGIPPDQ  QRLIFAGKQL
1           10          20          30          40          50
EDGRTLSDYN  IQKESTLHLV  LRLRGG
51          60          70      76
```

PLANT UBIQUITIN (Callis & Vierstra *et al.*, 1989)

```
MQIFVKTLTG  KTITLEVESS  DTIDNVKAKI  QDKEGIPPDQ  QRLIFAGKQL
1           10          20          30          40          50
EDGRTLADYN  IQKESTLHLV  LRLRGG
51          60          70      76
```

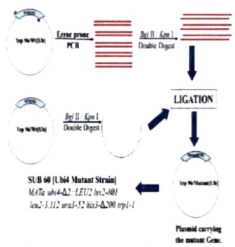
HUMAN UBIQUITIN (P02248)

```
MQIFVKTLTG  KTITLEVEPS  DTIENVKAKI  QDKEGIPPDQ  QRLIFAGKQL
1           10          20          30          40          50
EDGRTLSDYN  IQKESTLHLV  LRLRGG
51          60          70      76
```

Random mutagenesis is a powerful technique for **in vitro evolution of** proteins with novel properties. In the present study random mutagenesis was carried out by using error prone PCR. The resultant amplicons were cloned into shuttle vector, purified and transformed into stress-hypersensitive UBI4 mutant of yeast and looked for complementation, under stress.

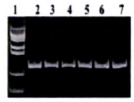
PLAN OF WORK

STEPS FOR ERROR PRONE PCR



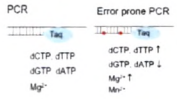
MATERIALS AND METHOD

AMPLICONS GENARATED UNDER VARIOUS ERROR PRONE CONDITIONS



Lane 1:100bp Marker Ladder
Lane 2:Standard condition
Lane 3:10mM $MgCl_2$
Lane 4: 0.5mM $MnCl_2$
Lane 5: 1mM dATP
Lane 6: 1mM dGTP
Lane 7: 1mM dATP/dGTP

Above PCR products were obtained under error prone conditions and were similar to the amplicons obtained under standard condition with respect to Length.

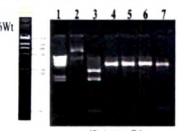


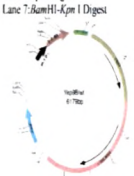
STANDARD CONDITION

Denaturation temp.	:94° C for 5 minutes.
Annealing temp.	:60°C for 30 seconds.
Extension temp.	:72°C for 30 seconds.
Final extension.	:72°C for 10 minutes.
Final hold	:4°C

Yep96/Wt[Ub] DIGESTION PATTERN

Lane 1:*λHind* III Digest Marker
Lane 2:Undigested plasmid Yep96/Wt
Lane 3:*Hind* III Digest
Lane 4:*Bam* HI Digest
Lane 5:*Bgl* II Digest
Lane 6:*Kpn*I Digest
Lane 7:*Bam*HI-*Kpn* I Digest





OLIGONUCLEOTIDE REGION MUTATED BY ERROR PRONE PCR

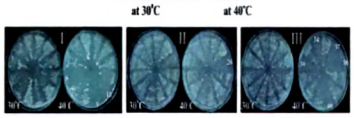
AGATCT ... GGT GGT TGA GGTACC
Bgl II ... Gly Gly Kpn I

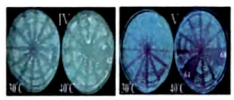
FORWARD PRIMER:
5'ATGCAGATCTTCGTCAAGACGTTAACGG 3'
REVERSE PRIMER:
5'TCCGGTACCCGCTCAACCACCTCTTAG 3'

RESULTS AND DISCUSSION

SCREENING OF MUTANTS BY COMPLEMENTATION UNDER HEAT SHOCK

Without heat sterss at 30°C **Heat stress at 40°C**



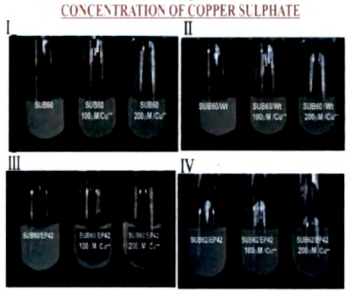


UBIQUITIN GENE AMPLIFICATION OF MUTANT PLASMIDS

PCR of the mutants (colony No. 6,9,11,26,30,34,37,38,40,42,64 & 68) in lane 3 to 14 respectively checked on 2 % agarose, With PCR of Yep 96/Wt[Ub] in lane 2 & 100bp ladder (DNA Marker) lane 1

The mutant plasmid obtained from Colony No. 42 (Yep 96/EP42) transformed into *S.cerevisiae* showed lethal phenotype under induction in UBI4 background even at permissive temperature.

LETHAL EXPESSION OF Yep96/EP42 IN VARYING CONCENTRATION OF COPPER SULPHATE

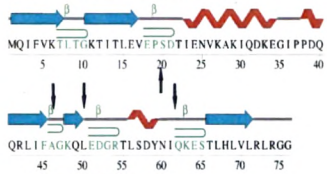


UBI4 mutant of *S.cerevisiae* (I, II and III) with 0 μM, 100 μM and 200μM $CuSO_4$,transformed with plasmid carrying Yep96/Wt [I] and Yep 96/EP42 [II] the lethal form of ubiquitin gene under copper inducible CUP1 promoter at 30°C (permissive temperature).

(IV) SUB 62 strain of *S.cerevisiae* (wild type for ubiquitin) with 0 μM, 100 μM and 200μM $CuSO_4$transformed with plasmid carrying Yep 96/EP42 at 30°C (permissive temperature).

SEQUENCING OF THE MUTANT GENE SHOWED FOUR NOVEL MUTATIONS

- These mutations do not include any of the lysines.
- Two of them are present in turns, one is in the β-sheet and one more is in the turn rich region.
- Two of them convert nonpolar residues to polar and one polar to nonpolar And the other one is nonpolar to nonpolar residue.



ACKNOWLEDGEMENTS

•CRP is grateful to University Grants Commission, India for funding the project Plasmid Yep96/wt and Saccharomyces cerevisiae strains werekind gifts Prof. Daniel Finley Department of Cell Biology, Harvard Medical School, Boston, Massachusetts .

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The effects of S65D and Q2N mutations on the multiple functions of ubiquitin in *Saccharomyces cerevisiae*

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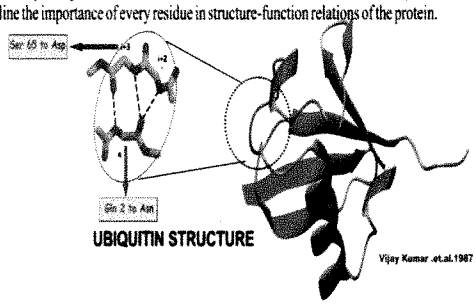
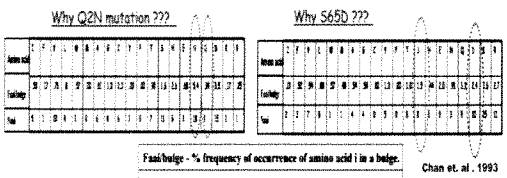
INTRODUCTION

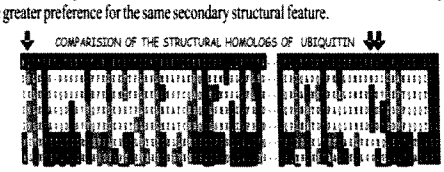
Ubiquitin is a highly conserved eukaryotic protein. It has a compact globular structure which makes it a paradigm for structural studies. Its smaller size and evolutionary conservation underline the importance of every residue in structure-function relations of the protein.



The structure of ubiquitin displays some unusual features. One of the nonconforming secondary structural features found in ubiquitin is a parallel G1β-bulge. In the present study we have focused on two residues Ser65 and Gln2, which are present in the second and X positions of the parallel G1β-bulge.

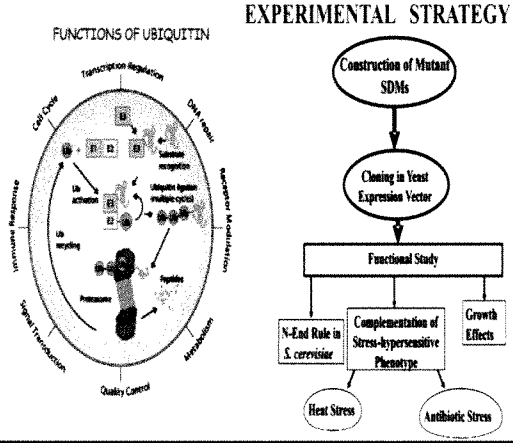


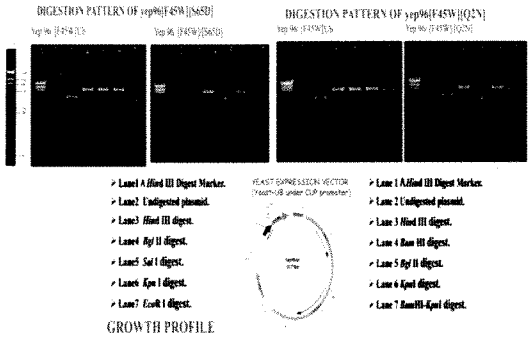
The % frequency of occurrence of these amino acids in a bulge in these positions is as low as 1.5 and 0.34 respectively. In order to test if these residues have any functional significance, we have mutated ubiquitin to obtain S65D and Q2N substitutions, where the substituting residues have greater preference for the same secondary structural feature.

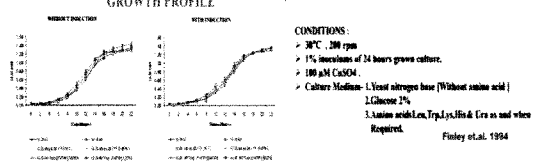


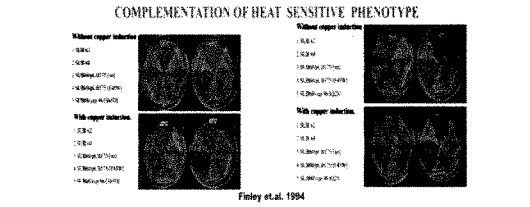
The variants of ubiquitin UbS65D and UbQ2N have been constructed and characterized for their structure and function.

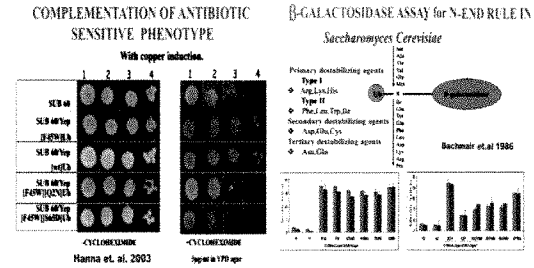
EXPERIMENTAL STRATEGY











Our results establish that *Sacchromyces cervisiae* expressing either of the two mutated proteins UbS65D and UbQ2N, become sensitive to antibiotic cycloheximide and show altered preference for N-end rule. However, heat sensitivity of the organism and thermal stability of the protein remain unaltered.

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