Construction and functional characterization of double and triple mutants of parallel β-bulge of ubiquitin

Mrinal Sharma & C Ratna Prabha*
Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara 390 002, India

Received 20 May 2011; revised 13 July 2011

Ubiquitin, a small eukaryotic protein serving as a post-translational modification on many important proteins, plays central role in cellular homeostasis and cell cycle regulation. Ubiquitin features two β-bulges, the second β-bulge, located at the C-terminal region of the protein along with type II turn, holds 3 residues Glu64(1), Ser65(2) and Gln2(X). Percent frequency of occurrence of such a sequence in parallel β-bulge is very low. However, the sequence and structure have been conserved in ubiquitin throughout the evolution. Present study involves replacement of residues in unusual β-bulge of ubiquitin by introducing mutations in combination through site directed mutagenesis, generating double and triple mutants and their functional characterization. Mutant ubiquitins cloned in yeast expression vector YEp96 tested for growth profile, viability assay and heat stress complementation study have revealed significant decrease in growth rate, loss of viability and non-complementation of heat sensitive phenotype with UbE64G-S65D and UbQ2N-E64G-S65D mutations. However, UbQ2N-S65D did not show any negative effects in the above assays. Present results show that, replacement of residues in β-bulge of ubiquitin exerts severe effects on growth and viability in Saccharomyces cerevisiae due to functional failure of the mutant ubiquitins UbE64G-S65D and UbQ2N-E64G-S65D.

Keywords: β-Bulge, Cancer, Ubiquitin, Ubiquitin proteasome system (UPS), Virus

Maintenance of equilibrium between protein synthesis and degradation is integral to cellular metabolic regulation. Degradation of cellular proteins which are misfolded, damaged or proteins which have completed their role is of utmost significance for striking this balance. Controlled and selective removal of proteins plays major role in a variety of basic cellular pathways during both cell life and death.

Most of the proteins, destined for degradation are marked off by conjugating to ubiquitin through successive processes of ubiquitin activation, substrate protein recognition and ubiquitination of substrate protein\(^{1,2}\). Ubiquitinated substrate proteins are dragged towards proteasome for degradation. Ubiquitin tags wide variety of key regulators of cell cycle such as cyclins and CDK inhibitor. These regulatory proteins are chief targets for mutation in human cancer. It has been established that a functioning ubiquitin-proteasome system is important for successful infection by members of the Orthopoxvirus family\(^{3,4}\). Individual interactions between poxviral proteins and the ubiquitin-proteasome system have been characterized\(^{5}\). Viruses influence and manipulate the cellular ubiquitin-proteasome pathway for their replication and propagation.

Functional failure of ubiquitin proteasome system (UPS) leads to accumulation of ubiquitin conjugates in neurodegenerative diseases such as neurofibrillary tangles of Alzheimer’s disease and brain stem Lewy bodies in Parkinson’s disease. Recent findings demonstrate that soluble aggregated proteins can inhibit the UPS\(^6\).

Apart from its role in cell cycle and neurodegenerative diseases, ubiquitin proteasome system also regulates apoptosis, signal transduction, induction of the inflammatory response, DNA repair, endocytosis and antigen presentation. Deregulation of this pathway has been implicated as a causative factor in several inherited diseases. An assortment of proteins encoded by oncogenes and suppressors are targets of ubiquitination. UPS can be a probable target for pharmacological intervention in many viral and other detrimental diseases\(^7\). Apart from proteolysis, ubiquitination as a post-translational modification regulates ribosomal functioning, post-replication...
DNA repair and the function of certain transcription factors. Non-degradative activities of ubiquitin are responsible for survival of cells under antibiotic, nutritive, UV and other forms of stress.

Ubiquitin is present in all eukaryotic cells play an indispensable role in selective protein degradation. It is a small and highly conserved protein with 76 amino acid residues. It has a tightly packed globular structure with a hydrophobic core of mixed parallel and anti-parallel β-strands packed against an α-helix and a flexible C-terminal tail. Existence of tight hydrophobic core and extensive hydrogen bonding, make the structure of ubiquitin extremely stable in wideranges of pH and temperature. Compact structure of ubiquitin shows 9 reverse turns and interacts with many proteins through ubiquitin binding domains. Most of the residues of ubiquitin which are known to participate in proteasome recognition and endocytosis occur in 2 hydrophobic clusters. The others reside near the tail region, which is important for ubiquitin conjugation and deubiquitination. Residues surrounding Phe4 are required for endocytosis, whereas residues surrounding Ile44 are required for both endocytosis and degradation by proteasomes.

Highly conserved sequence of ubiquitin hints at importance of residues in its structure and/or function. However, knowledge on structural and functional significance of individual residues of ubiquitin is largely missing. Importance of some of the key residues and structural features in ubiquitin biology have been generated and characterized using mutant forms of ubiquitin.

Ubiquitin features 2 β-bulges. The first one, which is situated at N-terminal region in the type I turn of the β hairpin, has been shown to be in directing the formation and stabilization of transition state and acts as nucleation centre for folding of ubiquitin.

The second β-bulge located at the C-terminal region is formed by 3 residues Glu64 (1), Ser65 (2) and Gln2 (X). Percent frequency of occurrence of these residues in a parallel β-bulge is very low as revealed by amino acid preference table on the basis of analysis of a set of 182 proteins and a total of 362 bulges. However, this structure has been conserved in ubiquitin all the way through the evolution. In the present study, role of amino acids in second β bulge, by replacing the residues and generating double and triple mutants of ubiquitin. Amino acids used for replacement have higher preference for the same secondary structure. Functional characterization of mutants UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D was carried out. The results highlight the functional importance of these residues.

Materials and Methods

Strains, media, reagents—S. cerevisiae strains used in the study were as follows: SUB62 (MATa lys2-801 leu2-3,112 ura3-52 his3-A200 trl-1) is a wild type strain for all four ubiquitin genes and SUB60 (MATa ubi4-A2::LEU2 lys2-801 leu2-3,112 ura3-52 his 3-A200 trp1-1) is a deletion mutant lacking UB14 polyubiquitin gene. S. cerevisiae cultures were grown in synthetic dextrose (SD) medium containing Hi-media yeast nitrogen base (0.67%; without amino acids) and glucose (2%) as carbon source, histidine (20 mg/L), lysine (30 mg/L), uracil (20 mg/L), leucine (100 mg/L) and tryptophan (20 mg/L). Cultures were grown at 30°C at 200 rpm. Escherichia coli DH5α culture was chosen for carrying out all plasmid manipulations and grown at 37°C at 200 rpm in nutrient rich Luria broth (Hi-media). Plasmids were maintained by supplementing ampicillin (50 µg/ml) in the medium.

Construction of plasmid with site directed mutations—UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D—Episomal plasmid YEp96 (YEp96Wt) carrying copper inducible promoter CUP1, synthetic ubiquitin gene having nine restriction enzyme sites, CYC1 terminator cassette and a variant of ubiquitin gene with F45W mutation (YEp96F45W) were gifts from Prof. Daniel Finley.

Recombinant PCR—Recombinant PCR was performed using pfu DNA polymerase using the YEp96 as a template. The pair of mutagenic complementary primers and their non-mutagenic counterparts were designed to bring mutations in ubiquitin gene. In each complementary primer the SalI restriction site was damaged to facilitate the screening process. Forward primer 5’CATTCAGAAGGAGATACCTACCTTACCTTG3’ and the reverse primer 5’CAAGATGTAAGGTATCTCCCTTCTGAATG 3’ were used to get 62bp and 217bp PCR products, which were recombined by gene specific primers to generate 238 bp full length mutant ubiquitin.

Construction of ubiquitin gene derivatives—All ubiquitin gene mutations were constructed in plasmids derived from YEp96, which expresses a synthetic yeast ubiquitin gene from CUP1 promoter.
Mutant ubiquitin gene UbE64G-S65D developed by recombinant PCR, was cloned into BglII and KpnI sites of YEp96 to yield YEp96UbE64G-S65D. To get triple mutant the above PCR product was cloned into YEp96 UbQ2N backbone with sites for XhoI and KpnI restriction enzymes. In order to construct UbQ2N-S65D, single mutant S65D gene was PCR amplified and digested by XhoI and KpnI restriction enzymes to further clone into UbQ2N backbone.

Phenotype analysis

Growth effects—Cultures of SUB60 cells expressing wild type and mutant forms of ubiquitin were grown and their growth was compared with wild type strain for UBI4, SUB62 strain.8 Freshly grown stationary phase cells were used as inoculum for the growth curve. Optical density was recorded at intervals of 2 h at 600 nm. Generation times of the mutants have been calculated from mid-log phase of cultures.

Viability assay—SUB60 yeast transformants were grown to log phase with optical density of cultures reaching a value in between 0.5 to 0.6. The culture was serially diluted 4-fold and plated on SD selection media. Plates were incubated at 30°C and the colonies were counted.

Heat stress complementation—S. cerevisiae SUB60 strain lacking UBI4 gene is hypersensitive to various stresses. SUB60 transformed with YEp96 plasmid carrying mutated ubiquitin gene under CUP1 promoter and was tested for complementation under stress. SUB60 yeast cells can be complemented and rescued from stress by the expression of wild type ubiquitin gene from YEp96 plasmid, hence it was used as a positive control. Heat sensitivity test was done to confirm the functional integrity of the mutant ubiquitins.8 SUB60 yeast transformants were grown to log phase with optical density of the cultures reaching a value of 0.5 to 0.6 and serially diluted four fold and plated on SD selection plates. The plates were incubated at 40°C for variable time periods of 0, 4, 8, 12 and 16 h and shifted back to 30°C and the colonies were counted.

Results

Three mutants namely UbE64G-S65D, UbQ2N-S65D and UbQ2N-E64G-S65D have been constructed by recombinant PCR. YEp96 plasmids carrying the genes for 3 mutants along with wild type ubiquitin were transformed in SUB60 strain which lacks the UBI4 gene to understand the effect of mutant ubiquitin on various functions of S. cerevisiae.

Growth profile—Growth profiles of S. cerevisiae transformed with mutants UbE64G-S65D and UbQ2N-E64G-S65D show slow growth with increased lag phase, while mutant UbQ2N-S65D shows no change in growth when compared with SUB60 transformed by plasmid carrying wild type ubiquitin gene (Fig. 1).

Generation time—SUB60 cells lacking UBI4, SUB62 cells wild type for UBI4 and SUB60 transformants expressing wild type ubiquitin gene from plasmid have a generation time of 2.5 h. Generation time increased for mutants UbE64G-S65D, UbQ2N-E64G-S65D, while it remained unaltered for mutant UbQ2N-S65D. Mutant UbE64G-S65D and UbQ2N-E64G-S65D showed 3.37 h and 4.63 h generation time (Fig. 2).

Viability of cells—Viability assay revealed that mutants UbE64G-S65D and UbQ2N-E64G-S65D gave rise to less colony forming units in comparison...
to wild type ubiquitin gene transformed in SUB60 strain of *S. cerevisiae*, whereas viability of UbQ2N-S65D remained unaffected (Fig. 3).

**Heat stress phenotype**—Heat stress hypersensitivity of the mutants has been analyzed by applying chronic heat stress to SUB60 strain of *S. cerevisiae*, transformed with mutant and wild type ubiquitin genes. SUB60 strain is UBI4 deletion mutant and cannot withstand heat and other forms of stress, while SUB60 transformed with wild type ubiquitin gene can. If the mutant forms of ubiquitin can complement SUB60 cells, the cells may be able to survive under heat stress. However, results show that mutant ubiquitin UbE64G-S65D and UbQ2N-E64G-S65D are hypersensitive to chronic heat and on the other hand UbQ2N-S65D shows moderate sensitivity to heat stress. Mutant UbE64G-S65D and UbQ2N-E64G-S65D show ~10 and ~ 5% survival at 12 and 8 h of heat stress respectively, while UbQ2N-S65D shows 20% survival at 16 h (Figs 4, 5). Non-complementation seen with ubiquitin variants can be interpreted as functional loss due to the incorporation of mutations.

**Discussion**

Several viruses have developed refined mechanisms to manipulate the host ubiquitin-proteasome system (UPS) for their own needs. UPS has been suggested to play an indispensable role in viral maturation, viral progeny release, efficient viral replication, reactivation of virus from latency. Viruses control UPS by averting host immune surveillance by inhibiting antigen processing through down-regulation of MHC molecules. The Epstein-Barr virus encoded nuclear antigen 1 (EBNA1) contains Gly-Ala repeats that interfere with antigen processing and MHC class I-restricted responses by preventing viral protein degradation by the proteasome. Further, the virus down-regulates the host defences against itself by interfering with interferon signaling, in a process involving UPS.

Tumor suppressor protein p53 is a well known substrate for UPS, E3 ligase Mdm2 (murine double minute 2) ubiquitinates the tumour suppressor protein p53 (ref. 21). Protein p53 being the guardian of genome prevents carcinogenesis by inducing the regulation of MHC molecules. The Epstein-Barr virus encoded nuclear antigen 1 (EBNA1) contains Gly-Ala repeats that interfere with antigen processing and MHC class I-restricted responses by preventing viral protein degradation by the proteasome. Further, the virus down-regulates the host defences against itself by interfering with interferon signaling, in a process involving UPS.

![Image](image_url)

**Fig. 3**—Viability (colony forming units) of *S. cerevisiae* strain SUB60, SUB62 and SUB60 transformed with YEp96 plasmid carrying genes for wild type ubiquitin (UbWt), genes for double and triple mutated variants of ubiquitin UbE64G-S65D, UbQ2N-S65D and UbQ2N-E64G-S65D. [**P < 0.01; ***P < 0.001 vs wt. Values are mean ± SD of 3 replications].

**Fig. 4**—Effect of heat stress on SUB60 strain of *S. cerevisiae* expressing variants of ubiquitin. SUB60, SUB62 and SUB60 cells of *S. cerevisiae* transformed with ubiquitin genes for wild type (UbWt) and genes for mutant ubiquitins UbE64G-S65D (I), UbQ2N-S65D (II) and UbQ2N-E64G-S65D (III) were grown on two sets of Petri plates. The first set was incubated at 30°C (optimum temperature) and the second set was incubated at 40°C (heat stress). SUB60 strain lacking UBI4 and SUB60 transformants expressing UbWt from plasmid were used as negative and positive controls, respectively.
expression of genes involved in regulating cell-cycle arrest, DNA repair and apoptosis after DNA damage. A reduced levels of p53 associated with over-expression of Hdm2 (human homologue of Mdm2) have been found in many tumors such as neuroblastoma, melanomas, lung and breast carcinomas.22,23

Considering the central role of ubiquitin proteasome system in cancer, viral and neurodegenerative diseases, several proteasome inhibitor drugs have been formulated to target UPS system e.g. indanone substituted peptides, cyclic tripeptide TMC-95, 2-aminobenzyl-satatine derivative24,25. Several protein-based chimeric molecules have been designated to destroy cancer-promoting proteins i.e. β-catenin, Rb protein, cyclin A, cdk 2. However, the latter class of molecules requires a special delivery system26. Even though, many molecules have been shown to inhibit UPS effectively, there has been no clinical application due to their toxicity. At this juncture, unraveling of finer details of structure and molecular interactions of UPS is the most important step in the direction of development of UPS targeted drugs.

Under optimal conditions of growth SUB62 cells, with all 4 copies of ubiquitin gene intact, SUB60 cells lacking UBI4 and SUB60 cells transformed with wild type ubiquitin gene have generation times in the range of 2.3 to 2.5 h. Increased generation times of SUB60 cells expressing UbE64G-S65D and UbQ2N-E64G-S65D imply that the mutant ubiquitins are not only failing to complement but are also inhibitory to normal ubiquitin functioning. This observation is in concurrence with decreased viability and decreased tolerance towards heat stress observed with the above two mutants, which can be concluded as a consequence of the inhibitory effect of the mutations on the functions of ubiquitin. The structural changes produced due to incorporation of above mutations may be turning the ubiquitin variants into potential competitive inhibitors, which interfere in the functionally important interactions of wild type ubiquitin with other proteins. Such inhibitory analogues of ubiquitin may find clinical applications along with other inhibitors of UPS.

In the present study replacement of residues in β-bulge of ubiquitin generating double and triple mutants resulted in retarded growth and reduced viability. Furthermore, they are not complementing the heat sensitive phenotype. This inactive variants of ubiquitins may be taken as antagonists of ubiquitin in UPS and may influence several vital functions of the cell.

Acknowledgement

Thanks are due to University Grants Commission, India, for the award of major research grant to C.R.P and Prof. Daniel Finley for providing the plasmids and strains.

References

Schlesinger D H, Goldstein G & Nail H D, Complete amino acid sequence of ubiquitin, an adenylate cyclase stimulating polypeptide probably universal in living cells, Biochemistry, 14 (1975) 2214.


Sun Y, E3 ubiquitin ligases as cancer targets and biomarkers, Neoplasia, 8 (2006) 645.

