SUMMARY

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 structure, folding, stability or function (Gavilanes et al., 1982; Watson et al., 1978; Schlesinger et al., 1975; Schlesinger and Goldsteiner, 1975).

The structure of ubiquitin displays some unusual features. One of the nonconforming secondary structural features found in ubiquitin is a parallel G1β-bulge (Vijay-Kumar et al., 1987). The residues participating in the formation of the bulge are Glu64, Ser65 and Gln2.

One of the conserved residues in ubiquitin, 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 (Vijay-Kumar et al., 1987; Chan et al., 1993). 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 UbE64G 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.

Ser65 is the second residue in the parallel G1β-bulge. The % frequency of occurrence of these amino acids in a bulge in these positions is as low as 1.5 (Chan et al., 1993). It was replaced by Asp with a higher % frequency of occurrence. Asp is also the residue that is found in the homologs of ubiquitin in this position (Jentsch et al., 2000). There was not much change in the secondary and tertiary structure of the protein. On the other hand, the content of hydrated hydrophobic residues decreased suggesting compaction of overall structure.

Gln2 forms the X residue in the parallel G1β-bulge. The % frequency of occurrence of Gln2 in this position is as low as 0.34 (Chan et al., 1993). It was replaced

by Asn, a residue most commonly found among ubiquitin homologs in this position and also an amino acid with higher % frequency of occurrence in G1β-bulge. There is not much difference in secondary and tertiary structures when compared to wild type protein except that the environment around Trp residue is slightly altered.

In order to test if the residues in parallel G1β-bulge have any functional significance, the residues have been mutated in ubiquitin to obtain the following substitutions: E64G, S65D and Q2N, where the substituting residues have greater preference for the same secondary structural feature. The variants of ubiquitin UbE64G, UbS65D and UbQ2N have been characterized for their function. Our results establish that *Sacchromyces cervisiae* expressing the mutated proteins UbE64G, UbS65D and UbQ2N, do not complement UBI4 ubiquitin deletion mutants and fail to confer resistance to the antibiotic cycloheximide. They show altered preference for N-end rule. Even though, they can be employed in polyubiquitin chain formation, proteins with destabilizing N-terminal residues show longer half-lives, an indication that the chain is not recognized by the degradation machinery of the proteasome. However, heat sensitivity of the organism and thermal stability of the protein remain unaltered.

Intracellularly, ubiquitin is used as a tag to mark proteins for degradation by ubiquitin proteasome system (UPS) (Gavialnes et al., 1975; Watson et al., 1978). The substrate proteins of UPS include cell cyclins (Goebl et al., 1988), antitumor proteins and transcription factors (Hochstrasser and Varshavsky, 1990). 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 *S.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 (42°C). Most of the mutations in ubiquitin gene failed to complement UBI4 phenotype under heat stress,

unlike the wild type gene. Only one of the mutants caused cell lysis, even at permissive temperature. Interestingly, expression of the same protein (UbEP42) in wild type *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 wild type, 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.

UbEP42 is thermolabile. It does not complement SUB60 cells under stress conditions like heat treatment and exposure to antibiotics. Though UbEP42 can form polyubiquitin chains, substrate protein degradation may not be effective with this tag. UbEP42 altered the half-life of substrate proteins with destabilizing N-terminal 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.

In summary, the studies demonstrate that in spite of their low % frequency of occurrence the residues in parallel G1β-bulge show complete conservation through evolution because of their functional significance, establishing relationship between the primary structure of the parallel G1β-bulge and the functions of ubiquitin. Additionally, the mutant UbEP42 isolated by error prone PCR confers several interesting phenotypic characters to the host cell, the most important feature among them is dosage dependent lethality. Further characterization of this mutant may reveal important details on the mechanisms of ubiquitin functions and its interaction with other proteins. The mutations may have some biomedical relevance.