Chapter 4

Effect of 'trans-acting elements' on substrate half-life

4.1. INTRODUCTION

Depending on their function, the half-life of proteins in a living cell ranges from few seconds to several days. This controlled turnover of a protein is tightly regulated by the proteasome machinery. However, the mechanism by which the half-life of a protein is decided is still not very clear. Some of the short lived proteins carry a signature known as degradation signals or degrons. In bacterial and archebacterial system, a degron called SsrA is popular. SsrA is an 11 residue (AANDENYALAA) sequence, that when fused to proteins with very long half-life in trans, shortens their half- life (Benaroudj and Goldberg, 2000). For example green fluorescence protein (GFP) is very stable in the cell while, GFP SsrA is relatively unstable (Benaroudj and Goldberg, 2000; Benaroudj et al., 2003). In eukaryotic system SsrA like sequences are not well characterized. C-terminus of ornithine decarboxylase (ODC) may be considered as the eukaryotic equivalent. ODC gets degraded by proteasome in an Ub-independent manner. The C-terminal sequences of ODC and antizyme has been shown to be important for this process (Chattopadhyay et al., 2001; Li and Coffino, 1993). The C-terminal sequence of ODC has been shown to compete with Ub for proteasomal interaction (Zhang et al., 2003). This C-terminal sequence was found to be rich in proline (P), glutamic acid (E), serine (S), and threonine (T) called PEST. Apart from ODC, other proteins with similar PEST rich sequences were found to exhibit less than 2 h of intracellular half-lives (Rogers et al., 1986). In addition to such signals, N-terminal residues have also been shown to dictate half-life of proteins and these signals are termed as ‘N-end rule’ (Bachmair et al., 1986; Varshavsky, 1996). However, how exactly these sequence help in shortening the half-life is still unanswered. There are several hypothesis, for instance, the ‘degrons’ might help in substrate recognition and/or act as initiators of degradation. The length, sequence, complexity and location of fusion

of these ‘degrons’ are other important factors. Most probably these ‘degrons’ are context dependent, it is likely it may or may not help in degradation of all the test proteins.

ApoMb was found self-sufficient to interact tightly with proteasome but degradation was a long process. We therefore asked whether fusion of ‘degrons’ in ‘trans’ will affect the half-life of apoMb. Some of the known PEST sequences as well as sequences known to interact with proteasome were tested to address this.

4.2 MATERIALS and METHODS

4.2.1 PEST fusion to the C-terminus of Mb: The PEST sequences from ABCA1 (ATP dependent cassette transporter1), GCN4 (Yeast transactivator) and Hac1 (transcription factor) were fused to the C terminus of Mb cDNA, purified by ion-exchange chromatography and tested for proteasomal degradation assay.

4.2.1A Insertion of PEST sequences in Mb: Synthetic sperm whale Mb cDNA was cloned between PstI and KpnI site of pUC19. PEST sequences from GCN4 (apoMb PEST 1), Hac1 (apoMb PEST 2) and ABCA1 (apoMb PEST 3) were converted to nucleotide and codon preferred by bacterial system were used for the same. This was then annealed to form a cassette. The cassette was prepared in a such way that it contained nucleotide corresponding to PEST along with two stop codons and for directional cloning, sticky end of BsteII (in Mb cDNA) and KnpI (vector) at the ends.

Material: *Restriction enzymes (Fermentas) PstI, KpnI and BsteII, T4 DNA ligation kit (Fermentas).*

Single stranded oligonucleotides containing PEST sequence were synthesized in such a way which when annealed to each other would generate a double stranded

cassette comprising of sticky ends corresponding to restriction enzymes BsteII and KpnI (**Table 4.1**). 10 μM of both the single stranded oligonucleotides were diluted in Tris EDTA buffer (10mM Tris pH 8, 1mM EDTA), denatured at 95ºC for 5 min and incubated at 72ºC for 10 min in a water bath. Following this, the water bath was turned off to allow slow reduction of temperature to room temperature, ensuring proper annealing of two strands. This double stranded cassette was annealed to BsteII and KpnI digested pUC19 plasmid using T4 DNA ligase. The annealed product was transformed in DH5α and colonies were screened for fusion product by restriction digestion using PstI and KpnI enzymes. Positive clones were confirmed by gel shift as compared to wild type (wt Mb =500 bp). The fusion of all the PEST sequences in Mb cDNA was further verified by Sanger sequencing.

Table 4.1: Oligonucleotides used for PEST fusion in Mb

Name	**Primer sequence**
PEST 1Mb F	GTTACCAGGGTAGCAGCAGCACCGATAGCACCCCGTAATGAGGTAC
PEST 1Mb R	CTCATTACGGGGTGCTATCGGTGCTGCTGCTACCCTG
PEST 2Mb F	GTTACCAGGGTCATAGCAGCAGCGATACCTTCACCCCGAGCCCGCTGAA CTGCACCATGGAACCGGCGACCCTGAGCCCGTAATGAGGTAC
PEST 2Mb R	CTCATTACGGGCTCAGGGTCGCCGGTTCCATGGTGCAGTTCAGCGGGCT CGGGGTGAAGGTATCGCTGCTGCTATGACCCTG
PEST 3Mb F	GTTACCAGGGTCGCCCGTTTACCGAAGATGATGCGGCGGATCCGAACGA TAGCG ATATTGATCCGGAAAGCCGCGAAACCGATTAATGAGGTAC
PEST 3Mb R	CTCATTAATCGGTTTCGCGGCTTTCCGGATCAATATCGCTATCGTTCGGA TCCGCCGCATCATCTTCGGTAAACGGGCGACCCTG

4.2.1B Expression and purification of PEST fused Mb: PEST fusion proteins were expressed in DH5α as discussed above. Due to the fusion of PEST sequences, the pI of the fusion protein might change. Therefore, depending on the calculated pI, the purification strategy was designed. The calculated pI of PEST1 Mb was similar to wt. Hence, it was purified by cation exchange chromatography. Since, the expression level of PEST 2 Mb was suboptimal, several attempts to purify PEST2 Mb in optimum

amount failed. PEST3 Mb was purified by anion-exchange followed by gel-filtration chromatography.

Material: *DEAE cellulose (Sigma), 25 ml column (Bio-rad), Amicon ultra centrifugal filter units (Millipore).*

Essential buffer and reagents:

For DEAE cellulose regeneration: 0.1N NaOH containing 0.5 M NaCl, 0.5M NaCl, 0.1 M HCl containing 0.5 M NaCl and 1M NaCl pH 7-8 (with NaOH).

Purification buffer: 10 mM sodium phosphate buffer pH 6.8

Elution buffer: 30 mM sodium phosphate buffer pH 6.8 supplemented with 25 mM or 50 mM NaCl.

- **Regeneration of DEAE resin:** DEAE resin was suspended in MQ water (5 ml/g bead) and poured in column. Resin was washed twice with 2 column volume (CV) 0.1 M NaOH containing 0.5 M NaCl followed by 0.5 M NaCl and 0.1 M HCl containing 0.5 M NaCl. Resin was washed with MQ until the effluent pH was 5 or greater. At this stage resin can be stored in 1M NaCl pH 7-8 at 4°C (with preservative).

Note: A. If in the final stage, pH of effluent is not ~5, wash several times with MQ and repeat the regeneration all over again.

B. Resin should not be left in NaOH or HCl for more than 30 min.

- **Partial purification PEST3 Mb by DEAE cellulose:** DEAE cellulose resin was equilibrated with 2 CV of 10X purification buffer followed by 5 CV of 1X purification buffer. PEST3 Mb expressing cells were lysed by sonication, cleared lysate was loaded on DEAE cellulose column, bound protein was washed with 10-15 CV of purification

buffer. PEST3 Mb was eluted in holo form (reddish brown in color) with the help of 30 mM sodium phosphate buffer supplemented with 25 or 50 mM NaCl.

- **PSET3 Mb purification by gel-filtration chromatography:** Purity of Mb PEST3 eluted from DEAE cellulose column was analyzed on 15% SDS PAGE. To get rid of contaminating proteins, eluted proteins were concentrated using Amicon filter units (3.5 kDa cut-off, Millipore) and further purified by gel-filtration chromatography. Gel-filtration fractions were resolved on 15% SDS PAGE and pure protein fractions were pooled together.

Mb PEST1 and Mb PEST3 were converted to apo form; heme removal was confirmed by UV-Visible spectrometry and proteasomal degradation of PEST1 and PEST3 Mb was done as described previously.

4.2.2 Fusion of probable 'degron' to C-terminus of Mb: We chose to test the effect of the following trans-acting elements on apoMb half-life.

- The C-terminus of ODC was shown to be essential for its ubiquitin independent degradation. It was able to shorten the intracellular half-life of GFP when fused to its C-terminus (Corish and Tyler-Smith, 1999).
- Short sequences derived from N and C-terminus of proteins found to interact with 26S proteasome by indigenous screening (Mb P8 and Mb P13).

Due to the fusion of sequences of varying length and character, the physicochemical properties of Mb might change and interfere with purification. Hence, we decided to switch to affinity purification of Mb using 6 X- histidine tags. The cloning and purification strategy of Mb fusion is summarized in the following schematic (**Fig. 4.1**).

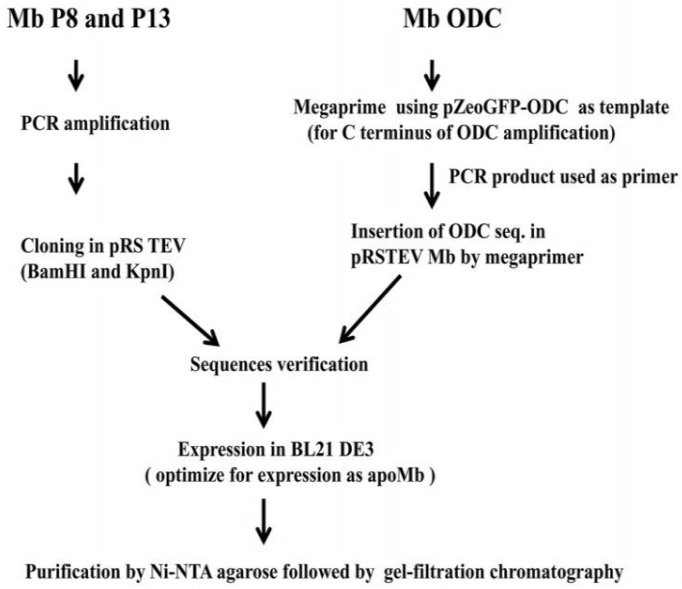

Figure 4.1: Overview of cloning, expression and purification of Mb C-terminal fusion proteins.

4.2.2A. Generating pRSTEV Vector: The pRSETA vector (Invitrogen) has 6X-histidin tag the T7 gene, 10 leader sequences for high level of protein expression, Xpress epitope and enterokinase (EK) recognition and cleavage sequence. All together these components contribute to ~3 kDa extra sequences. In order to get tag free protein (if needed) we replaced Xpress epitope, ER recognition and cleavage site with available protease site (TEV or thrombin) using single step PCR without any cloning in single shot, deletion of Xpress epitope and EK as well as insertion of TEV site was achieved without affecting the MCS.

Material: *pfu PCR kit (Fermentas), 25 mM dNTPs, DMSO, DpnI (Fermentas).*

Bacterial strain: *XL1 blue*

A 50 µl PCR reaction containing 50-60 ng template plasmid and control reaction (without pfu) was set-up. The proofreading activity of pfu enzyme provided error free amplification. The following PCR condition was used: initial denaturation- 95ºC for 5 min, denaturation- 95ºC for 1 min, annealing- 50ºC for 1 min, extension- 72ºC for

1min/kbp and number of cycle 19. The 10 µl PCR product and control reaction (every component except pfu enzyme) were resolved on 0.8% agarose gel. After confirming amplification, DpnI digestion was setup in a 25 µl reaction using 20 µl of PCR product, 2 µl of 10X Tango buffer and 10 units of DpnI for at least 8 h at 37ºC. DpnI would digest the parental plasmid (cleave adenomethylated dam sites). The digested product was then transformed in XL1 blue cells. The colonies were screened, plasmids isolated and finally sequence verified for insertion of TEV recognition sequence and deletion of EK site.

4.2.2B. Cloning wtMb in pRSTEV: The cDNA of Wt Mb was amplified from pUC 19 Mb and cloned in pRSTEV vector via PCR cloning vector pJET.

Material: *pfu PCR kit, pJET 1.2/blunt PCR cloning kit, BamHI and KpnI from Fermentas. Gene gel elute kit (Sigma).*

Bacterial strain: *DH5α*

Mb was amplified using pfu enzyme and Mb cDNA cloned in pUC19 as template. The following PCR condition was used: initial denaturation - 95ºC for 5 min, denaturation- 95ºC for 1 min, annealing- 55ºC for 1 min, extension- 72ºC for 1 min/kbp and number of cycle 29. Pfu polymerase does not have terminal transferase activity, hence the blunt end PCR product was gel extracted using gene gel elute kit, ligated with 50 ng of linearized pJET vector and transformed in DH5α cells. pJET PCR cloning kit utilizes insertional inactivation of the lethal eco47IR gene to obtain a positive clones. Clones were screened for insert using BamHI and KnpI digestion and the insert was gel extracted. The pRSTEV vector was serially digested with BamHI and KpnI, resolved on 0.8% agarose gel and gel extracted. The insert and vector were ligated with the help of T4 DNA ligase. The ligated product was transformed in DH5α, colonies were screened

using BamHI and KpnI digestion and one of them was sequence verified for wtMb sequence.

4.2.2C. Cloning Mb p8 and p13 in pRSTEV: The cloning steps were similar to the above described method. The forward primer was common for the amplification of wt or fusion proteins (P8 and P13). The reverse primers comprised of nucleotides corresponding to C-terminus of Mb, tri-glycine bridge, fusion sequence followed by two stop codons and KnpI site (Table 4.2). Forward primer pRSTEV Mb F and respective reverse primes were used in PCR.

Table 4.2: Oligonucleotides used for fusion of 'probable degrons' to Mb

Name	**Primer sequence**
PRTEV F	CAGCAAATGGGTGAAAACCTGTATTTCCAGGGTGGATCCGAGCTC
PRTFV R	GAGCTCGGATCCACCCTGGAAATACAGGTTTTCACCCATTTGCTG
PRTEV MB F	GGATTCATGGTTCTGTCTGAAGGT
PRTEV MB R	GGTACCTCATTAACCCTGGTACC
PRTEV MB P8 R	GGTACCTCATTACGGTTTTAGGTTCTGGAAGATTTGGTCCAGGTGTTTCATTGAGTCACCCTGGTAAC
PRTEV MB 13 R	GGTACCTCATTAGATTTCTGGGTATGACGGGAATGATGCGATTCCGCAGTGGTTTCCACCCTGGTAAC
PMB ODC F	GGGTTACCAGGGTCATGGCTTCCCGCCGG
PMB ODC R	CCGGTACCTCATTAACGGTCCATCCCGCTCTC

4.2.2D. Cloning Mb ODC in pRSTEV: ODC C-terminal sequences were taken from reported pZeoGFP-ODC construct *(kind gift from Chris Tyler Smith, University of Oxford,UK)* (Corish and Tyler-Smith, 1999).

Material: *pfu PCR kit and DpnI from fermentas.*

In order to clone the C-terminal sequences of ODC, an altogether different approach was used. PCR reaction was carried out with unique primer pair. The forward primer was designed such that the 5' end would contain the C-terminal encoding sequence of Mb and 3' end, the ODC sequences.

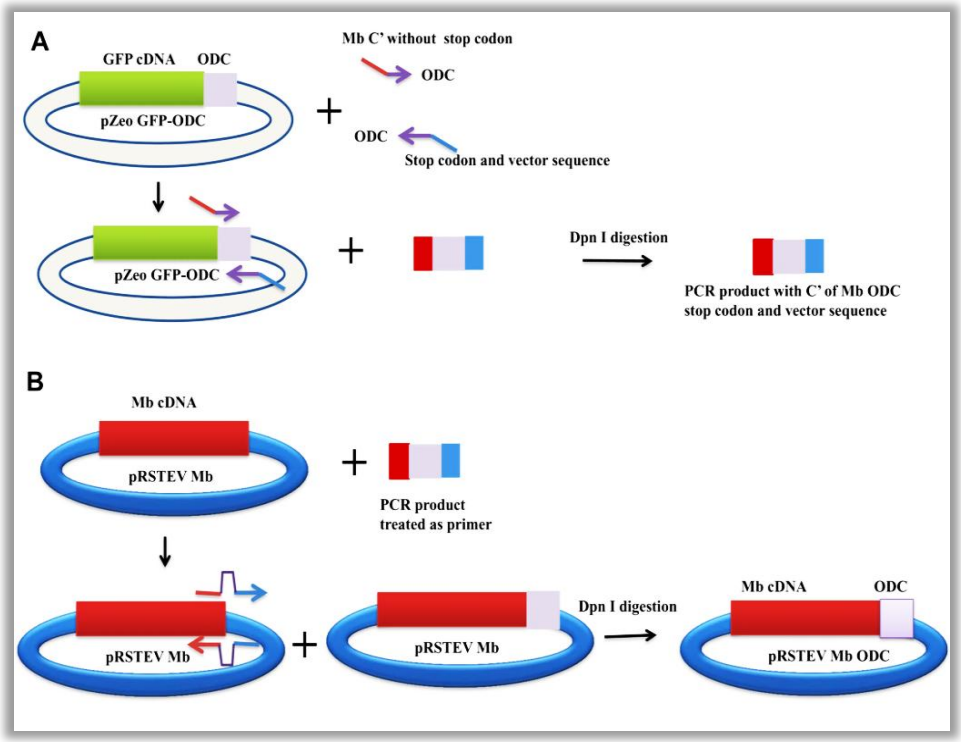

Figure 4.2: Cloning strategy of MbODC. *With the help of specially designed primers set comprising of ODC, C-terminal Mb (forward) and ODC, stop codon and vector sequences (reverse), ODC sequence was amplified from pZeoGFP-ODC (A). This PCR product was treated with DpnI and used as a primer for second step of PCR using pRSTEV MB as template; this PCR step will allow the ODC sequence to fuse to Mb. After DpnI digestion pRSTEV Mb ODC was used for fusion protein production (B).*

The 3'end of the reverse primer on the other hand comprised of ODC sequences, two stop codons and pRSTEV complimentary sequences (**Fig. 4.2**). Using this primer set and pZeoGEP-ODC as template, PCR was done using following conditions: initial denaturation - 95ºC for 5 min, denaturation- 95ºC for 1 min, annealing - 55ºC for 30 sec, extension- 72ºC for 1 min/kbp and number of cycle 29. The template (pZEO GFP-ODC) was next digested by DpnI, leaving the unmethylated amplified product intact. The amplified product harbored the ODC C-terminal sequence in between Mb (last few residues) and pRSTEV vector sequences (few nucleotides after KpnI site). For second round of PCR, 2µl of this product was used as mega primers and pRSTEV Mb as template. Following condition was used for PCR initial denaturation - 95ºC for 5 min,

denaturation- 95ºC for 1 min, annealing - 55ºC for 1 min, extension- 72ºC for 1 min/kbp and number of cycle 19. After confirming amplification on 0.8% agarose gel, as described above unmodified parental plasmid was digested using DpnI and transformed in XL1 blue cells. The colonies were picked, plasmid DNA was extracted and digested with BamHI and KpnI. One of the positive clones was verified by Sanger sequencing.

4.2.3 Expression and purification of wt and fusion proteins: Wt and mutant Mb were transformed in BL21 DE3 cells. Growth condition and IPTG induction was optimized for apoMb production.

Material: *IPTG (Sigma), Ni-NTA-agarose (Invitrogen), 25 ml column (Bio-Rad), 10X protease inhibitor cocktail (Sigma) and lysozyme (Sigma).*

Bacterial strain: *BL21 DE3.*

Essential buffers and reagents:

5X Native purification buffer (100 ml): 250 mM Sodium phosphate monobasic (NaH_2PO4), 2.5 M NaCl, Add 90 ml of Milli Q water, adjust pH to 8 with NaOH. Make volume to 100 ml.

1X Lysis buffer (20ml): 5X Native binding buffer (4 ml), 2 mM β-Mercaptoethanol, 0.1% Triton X -100, 10% Glycerol, and 1 X protease inhibitor (pH 8.0).

Binding buffer (50 ml): 1X Native binding buffer, 2 mM β-Mercaptoethanol, 0.1% Triton X -100, 10% Glycerol and 10 mM imidazole, pH 8.0.

Wash buffer (50 ml): 1X Native binding buffer, 2 mM β-Mercaptoethanol, 0.1% Triton X -100, 10% Glycerol and 20 mM imidazole, pH 8.0.

Elution buffer (50 ml): 1X Native binding buffer, 2mM β-Mercaptoethanol, 0.1% Triton X -100, 10% Glycerol and 250 mM imidazole, pH 8.0.

Instrument: Ultrasonic homogenizer (300VT, BioLogics, Inc), Sorval RC 5C (for centrifugation), Rotospin (Tarson).

The BL21 DE3 cells were transformed with pRSTEV Mb or mutant plasmid. Single colony was inoculated in 10 ml of LB broth and incubated at 37ºC for 5 h, 2 ml of this starter culture was inoculated in 1 l LB broth. Cells were grown up to A_{600nm} 0.4-0.6 at 37ºC then 100 μM IPTG was added to induce the recombinant protein expression. The cells were further incubated for 16 h at 18°C and harvested by centrifugation. Pellets were either stored at -80ºC freezer or used for purification, whereby the pellet was suspended in 1X lysis buffer and incubated on ice for 30 min followed by cell lysis using 5 mm sonication probe for 6 to 8 cycle of 1 min each (70% pulsar). Cell debris was removed by centrifugation for 1 h at 18K and cleared lysate was stored on ice. The Ni-NTA beads (2 to 3ml) were taken in 25 ml column washed several times with MQ water and equilibrated with 15 ml of native binding buffer. The cleared cells lysate was incubated with Ni-NTA column on rotospin at 15 rpm for 1 h at ~4ºC. The flow through was collected to test the protein binding. The column was washed with 100 ml of 1X wash buffer to remove impurity and protein was then eluted using imidazole. The purification was checked on 15% SDS PAGE.

Note: A. Incubation on ice up to 2h after addition of lysis buffer require less sonication cycle.

B. Used Ni-NTA beads can be regenerated by incubating it with 0.5 N NaOH for 30 min and washing thoroughly with MQ water.

Protein purified by Ni-NTA affinity column were concentrated to about 2 mg/ml and further purified by gel filtration chromatography as described earlier. The protein fractions were collected and analyzed on 15% SDS PAGE. Pure protein fractions were pooled together, concentrated and dialyzed overnight with 50 mM Tris pH 7.5.

4.2.4 Proteasome degradation assay: Apo form of wt and different Mb fusion protein were tested for proteasomal degradation as described earlier.

4.3 RESULT and DISCUSSION

ApoMb was self-sufficient for proteasomal degradation. We wanted to test whether trans-acting elements would affect the half-life of apoMb. The sequence, length, complexity and location of fusion are not defined. Effect of most of the trans-acting property is context dependent. To check the effect of some of the trans-acting elements on Mb half-life, we chose to test PEST sequences from a few reported proteins. These included known 'degrons' like C-terminal sequences of ODC, few sequences from N or C-terminal peptides of proteins found to interact with proteasome obtained by in house screening.

4.3.1. Cloning and purification of PEST sequence fused apoMb: To test the effect of some of PEST sequences on apoMb half-life, we selected the reported PEST sequences (8 to 24 residues) from ABCA1, GCN4, and Hac1 with intracellular half-life of less than 1 h. Deletion of PEST sequences of these proteins resulted in intracellular stabilization of these proteins (Kornitzer et al., 1994; Pal et al., 2007; Wang et al., 2003). To fuse these sequences to the C-terminus of Mb cDNA, a cassette with PEST sequence and sticky ends corresponding to BsteII and KpnI was designed and ligated to linearized pUC 19 Mb (with same enzyme sets). The PEST sequence and physicochemical property of PEST fused Mb is summarized in table 4.3. The calculated pI of PEST 1 (GCN4) was similar to wt Mb. PEST1 was purified by cation-exchange chromatography. Due to the fusion of PEST sequences, rich in acidic residues, the calculated pI of Mb PEST3 (ABCA1) dropped to 6. PEST 3 was partially purified on anion exchange resin followed by gel-filtration chromatography. PEST2 (Hac1) was probably unstable in bacterial cells, several attempts to purify PEST2 Mb in optimum amount failed. Hence, PEST 2 was not persuaded further.

Table 4.3: Effect of PEST fusion on physicochemical property of Mb

Protein	Name of 'PEST' containing protein	Calculated pI	MW (kDa)
Wt Mb		9	17.3
Mb PEST1	GCN4 (Yeast trans activator)- 8 residue PEST (SSSTDSTP)	8.7	18
Mb PEST2	Hac1 (transcription factor)- 23 residue PEST (HSSSDTFTPSPLNCTM EPA TLSP)	7.9	19.7
Mb PEST3	ABCA1 (ATP dependent cassette transporter1) - 24 residue PEST (RPFTEDDAADPNDSDIDPE SRETD)	6	20

4.3.2. Cloning and purification of 'probable degrons' fused apoMb: Ornithine decarboxylase has been shown to be degraded with the help of antizyme by proteasome in Ub independent manner. The intracellular half-life of C-terminal ODC sequences fused GFP (to C terminus) has also been shown to be shorter than GFP alone (Corish and Tyler-Smith, 1999). We wanted to test whether the C-terminus of ODC (with critical Cys) would help Mb to be degraded faster by 26S proteasome.

In the quest of finding SsrA like sequences in eukaryotic system, short sequences (15 residues long) from N and C-terminus of proteins were selected. Considering that in most of the known proteasomal substrate, degradation starts from N/ C-termini and generally termini are more flexible in a protein, we designed an indigenous screening method to identify sequences that may interact with immobilized proteasome. Two peptides interacted tightly with proteasome (P8 and P13) and were selected to test their ability to affect Mb half-life. The sequence and calculated pI of Mb fusion protein is summarized in table 4.4. Fusion of these sequences will affect the pI of the fusion protein. In order to maintain uniformity, we switched to affinity purification of these fusion proteins using pRSET A 6X his tag purification vector (Invitrogen). It contains IPTG inducible promoter for recombinant gene expression and apart from other essential component of expression vector it has Xpress epitope and EK recognition and cleavage site to get Tag free recombinant protein. The TEV protease

expression construct was available in lab. We first generated pRSTEV vector by replacing Xpress epitope and EK site by TEV protease cleavage site without affecting the multiple cloning site of pRSET A (**Fig. 4.3A**). This vector was used for expression and purification of wt and C-terminal fusion Mb proteins.

Table 4.4: Sequence, calculated pI and M.W. of Mb fusion proteins

Name	**Fusion sequence**	**MW (kDa)**	**Calculated pI**
Wt	NA	17.3	9
Mb ODC	HGFPPEVEEQDDGTLPMSCAQESGMDR	21.9	6.39
Mb P8	DSMKHLDQIFQNLKP	19.1	8.96
Mb P13	GNHCGIASFPSYPEI	18.9	8.56

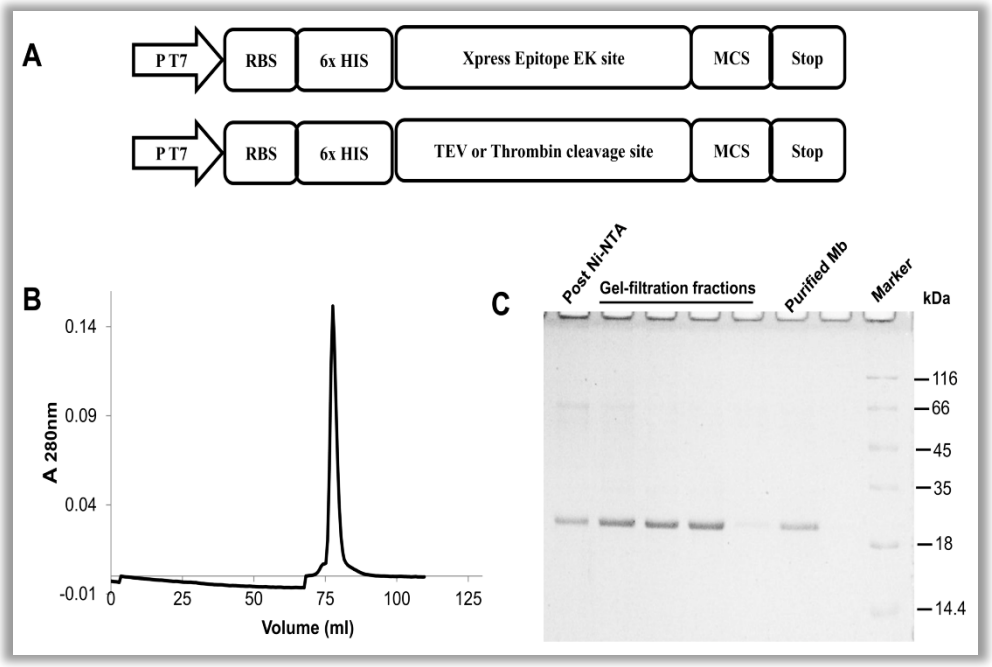

Figure 4.3: Generation of pRSTEV vector and Purification of Mb fusion protein: *The original pRSET A vector was modified to replace Xpress epitope and EK site with TEV or thrombin cleavage site (A). Mb ODC was expressed in BL21 DE3 and purified by Ni-NTA resin followed by gel-filtration chromatography (B). Mb ODC eluted from Ni-NTA column, gel-filtration fractions and protein after dialysis was analyzed on 15% SDS PAGE (C). The purified protein was found to be >95% pure.*

These 'probable degrons' were cloned at the C-terminus of Mb in the modified pRSTEV vector. Proteins were expressed in BL21 DE3. When IPTG was added at higher cell density (A600 nm > 0.8) the Ni-NTA purified wt protein was in holo form

while at low cell density (A600 nm =0.4 to 0.6) it was in apo form. For all the fusion proteins, expression was done at A600 nm~04. Wt and the fusion proteins were purified by Ni-NTA chromatography followed by gel-filtration chromatography (**Fig. 4.3B**). Wt as well as fusion proteins were >95% pure on SDS PAGE. MbODC Ni-NTA purification and gel-filtration chromatogram has been shown as a representative of fusion protein (**Fig. 4.3C**).

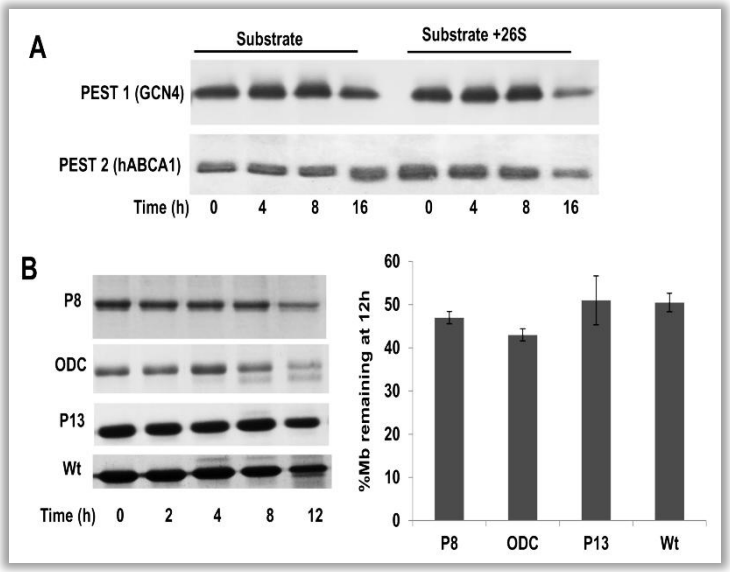

Figure 4.4: Effect of PEST sequences and 'degrons' on Mb half-life. *The proteasomal degradation of apo PEST 1 Mb or PEST 3 Mb was done and substrate remaining was monitored, these PEST sequences were not able to shorten the half-life of apoMb (A). Mb fusion proteins were subjected to proteasomal degradation assay and substrate remaining at 12 h was monitored, none of the fusion protein tested helped in shorting the half-life of Mb (B). In case of Mb ODC at 8 h and 12 h cleaved fragment was observed.*

4.3.3 Effect of PEST or 'probable degron' on apoMb half-life: The apo form of Mb PEST 1 or 3 along with wtMb was incubated with proteasome. The half-life of wt and both the PEST fused proteins were comparable. Similarly none of the probable degrons tested were able to shorten the half-life of apoMb. In case of Mb ODC, truncated product was observed at lower time scale, which might be due to cleavage or degradation of ODC sequences.

4.4 SUMMARY

Although the role of 'degron' in the degradation step is not clear, few recent observations suggest that long unstructured regions (90 to 120 residues) fused (in trans) to proteins, render them susceptible for degradation (Prakash et al., 2009; Prakash et al., 2004). These unstructured regions may help in initiation of degradation most likely by helping the polypeptide to reach the active site. However, generation of such long unstructured regions in a protein is rare other than the intrinsically disordered regions. In contrast to these long unstructured regions short sequence of 11 residues like SsrA has been shown to destabilize proteins like GFP rendering it susceptible to degradation by homologous ATP dependent proteases like 20S-PAN complex from archaebacteria and CLPX protease systems (Benaroudj and Goldberg, 2000; Benaroudj et al., 2003). The exact role of such degrons is still unclear.

We tested the ability of short sequences which have the potential to act as 'degrons' by fusing them to the C-terminus of Mb. Fusion of short, trans acting elements tested did not facilitate Mb degradation. Some of the possible explanations could be -

a) Due to very high affinity of apoMb (nM) for proteasome, additional interactions due to fusion of these elements may not facilitate degradation.

b) In apoMb, degradation might not be starting from C-terminus or else these would work better when attached to the N terminus of Mb.

c) Local structural changes might be more important than fusing unstructured sequences to the termini.