Cells communicate with each other and respond to changes in the environment through highly regulated signaling cascades. Signals perceived at the cell surface receptors reach their targets through a number of modular domains like SH3, WW, PTB, PDZ, etc. These domains confer catalytic/structural functions or mediate protein-protein interactions. SH3 domain is one such modular domain that facilitates protein-protein interactions by binding to polyproline motifs on their complementary proteins. They are small 60-80 amino acid long monomers that have a five-stranded, antiparallel closed β-barrel with greek key topology. SH3 domains are known to bind to a consensus XPXXP motif as monomers.

SH3 domain from Eps8 (EGFR protein substrate #8) is peculiar, as it exists in dynamic equilibrium between monomer and dimer states. Three-dimensional structure of Eps8 SH3 has been solved as a novel intertwined, strand exchanged, domain swapped dimer at an alkaline pH of 7.4 and as usual monomer at an acidic pH of 4.0. It has an Ile at 57th amino acid position instead of an aromatic residue (Phe or Tyr), a conserved feature in other SH3 domains. Phage display studies have shown that it binds to a novel PXXDY motif, instead of the consensus XPXXP motif. The PXXDY motif has been mapped to E3B1 (Eps8 SH3 binding protein 1) and RN-tre. E3B1 is a 480 amino acid long protein and is ubiquitously present in all human cells. It has an SH3 domain that binds to Sos-1. Eps8 in complex with E3B1 and Sos-1 activates Rac-GEF activity, thus leading to Rac activation and actin remodeling. In complex with RN-tre, it controls the activity of Rab5 by catalyzing the hydrolysis of guanine nucleotide on activated Rab5, thereby inhibiting receptor internalization and prolonging receptor signaling at the plasma membrane. The unusual structural and binding property of Eps8 SH3 and its importance in cell growth makes it an interesting system for structural studies. As a step in this direction, a part of the work embodied in this thesis intends to elucidate the mechanism of interaction of Eps8 SH3 with E3B1.

The clone of Eps8 SH3 in pGEX-KG was transformed and expressed in *Escherichia coli* strain BL21(DE3). Eps8 SH3 was purified on glutathione agarose affinity column. Glutathione-S-transferase (GST) tag was removed by thrombin digestion and the protein was further FPLC-purified on Superdex-75 column. Initially,

the presence of Ile at the 57th amino acid position was attributed to be one of the reasons for the unusual binding property of Eps8 SH3. Later studies have shown that the mutation of Ile^{57} to Tyr results in the higher binding affinity of Eps8 SH3 for its ligand peptide. In the present work, Ile^{57} was mutated to Tyr (I57Y) through site-directed mutagenesis. The I57Y mutant was also expressed as GST fusion protein and purified on glutathione agarose column by affinity chromatography in a manner similar to Eps8 SH3. Crystallizations were set up for Eps8 SH3 and I57Y along with ligand peptides containing PXXDY motif (peptides 1 and 2) using hanging drop and sitting drop vapor diffusion methods. Crystals obtained were very small in size (20-30 μm) and of irregular shape. Grid of pH, salt and precipitating agent concentrations was set up to improve the size of crystals. Fifty-four different additives were used in the optimized conditions to grow bigger crystals. Techniques like macroseeding and microseeding were also attempted to improve their quality. Unfortunately, all these efforts failed to yield crystals of appropriate size. The small sized crystals obtained were thus tried for X-ray diffraction on mar345 image plate detector on a Rigaku ultraX X-ray generator. Crystals of Eps8 SH3 and I57Y in complex with peptides when mounted on 0.5 mm capillary and exposed to X-rays, failed to diffract. Even cryo temperatures of liquid nitrogen did not result in X-ray diffraction. The small size of crystals itself posed difficulties in mounting them on the smallest loop of 100 μm diameter. Fluorescence spectroscopy and gel filtration techniques were used as an indirect method to elucidate peptide binding property of Eps8 SH3 and I57Y. Fluorescence emission spectra were collected for the proteins alone as well as in the presence of ligand peptides 1 and 2 or a non-specific Sos-1 peptide containing XPXXP motif. There was a blue shift in the emission spectrum when the proteins were mixed with ligand peptides 1 and 2. As expected, there was no shift in the emission spectrum when the proteins were mixed with Sos-1 peptide. This shows that Eps8 SH3 binds to PXXDY motif and not to XPXXP motif. In gel filtration assays, when the proteins were mixed with peptides 1 and 2, they eluted earlier, indicating that Eps8 SH3 and I57Y interact with their ligands.

E3B1 was expressed in fusion with C-terminal His tag using pET-23a expression vector. Whole of the protein was observed in the post-sonication pellet

fraction as inclusion bodies. Thus the protein was purified on Ni-NTA agarose under denaturing condition of 8 M urea. Refolding of E3B1 was tried by dialyzing urea under different pH conditions. The protein precipitated at pH 4.0 and pH 8.0, but remained soluble at pH 9.2. However, E3B1 formed soluble aggregates as revealed by the gel filtration elution profile on Superdex-200 FPLC column. Eps8 SH3 is known to bind to E3B1. Thus, the refolding of E3B1 from sarkosyl solublized inclusion bodies was carried out in the presence of Eps8 SH3 and was later purified on Ni-NTA agarose column. SDS-PAGE gel electrophoresis revealed the presence of Eps8 SH3 along with E3B1. This observation indicated that E3B1 was in fact able to bind to its complementary protein, Eps8 SH3, thus confirming that E3B1 refolded correctly and was purified in its native form. Furthermore, CD spectroscopy also showed the presence of secondary structures in E3B1. The aggregation status of E3B1 when examined by gel filtration chromatography, revealed it to be a dimer. Crystallization of E3B1 in the presence of Eps8 SH3 was set up by hanging and sitting drop vapor diffusion methods. However, the crystals obtained were small in size (10 μm) and the attempts to obtain bigger crystals were unsuccessful.

The biophysical studies presented here thus further prove the unusual ligand binding nature of Eps8 SH3. As the crystal structure of Eps8 SH3 could not be solved due to the small size of crystals, the exact mechanism of its interaction with the complementary protein, E3B1 could not be elucidated.

The binding function of SH3 domains is present in many other proteins of unrelated sequence, but similar structure. According to an estimate, the number of folding spaces available for a protein sequence is approximately 1000. Thus, proteins with different sequence tend to have similar fold. Two such folds, which have similar topology like that of SH3 domains, SH3-like (SH3-fold) and OB-fold were analyzed for fold similarity and sequence dissimilarity.

Twenty different non-redundant proteins having SH3-like fold were structurally superimposed on the reference SH3 domain from chicken spectrin (Chspec) with RMSD values less than 2.00 Å. SH3-fold proteins perform a variety of functions like oligopeptide/DNA/RNA binding, binding to small ligands like biotin, form a part of electron transport system or motor domain, or part of enzymes. The

binding pocket of SH3-fold proteins is conserved. They bind between RT- and n-src loops. All the proteins superposed had no sequence homology between them, but when structure based sequence alignment was carried out, they showed some homology in the β-strand region with respect to the property of amino acids. This is obvious as the core of the barrel is hydrophobic and every alternate residue in the β-strand points towards the same side. The analysis of SH3-fold revealed that β-strands provide the basic framework on which various loops modulate to impart different functions to the fold. Sso7d and the C-terminal domain of HIV-1 integrase (HID) have SH3-fold, which binds to dsDNA. However, HID binds to dsDNA as a dimer, whereas Sso7d binds as a monomer. HID has an open RT-loop. The functional residues are present on the RT-loop, n-src loop and the face of the barrel. The specific residues on HID were mutated (mHID) *in silico* and the structure was energy minimized. The structure of mHID resembled Sso7d and its functional regions were also comparable. This suggests that the newly designed mHID can bind to dsDNA in a manner similar to Sso7d.

OB-folds have fold architecture similar to SH3-folds. However, the difference between these folds is that in SH3-fold, second β-strand kinks in the middle to participate in the formation of both sheets, while in OB-fold, the first β-strand kinks for sheet formation. Twenty-seven non-redundant, different proteins having OB-fold were analyzed for their functional variability, fold similarity and sequence dissimilarity. OB-fold proteins bind to oligonucleotides/oligosaccharides/ oligopeptides. The barrel is formed by five strands over which the loops modulate differently in conformation, sequence and length to facilitate diverse ligand binding. OB-fold proteins were structurally superimposed on the OB-fold domain of cold shock protein B (CspB) with RMSD values less than 2.00 Å. Sequence alignment program Clustal W failed to show any sequence homology between these proteins, however, structure based sequence alignment revealed some sequence homology in the β-strand region owing to the hydrophobic nature of the core. The analysis of SH3- and OB-folds suggests that they provide a good template for protein design to impart new functions to an already existing fold.

There are many features, which are similar in both SH3- and OB-folds. They are five-stranded, closed β-barrel. These folds have a fold related binding face, as the binding pocket is situated between RT- and n-src loops and on the face of sheet 1. The fact that the tertiary structures of proteins have been more conserved during evolution than their primary amino acid sequence, suggests that both the folds might have evolved from a common ancestor. If the folds are examined as one-dimensional chain, it becomes apparent that the major difference between them lies in the insertion/deletion of the first β-strand. It seems that the first β-strand of SH3-fold is inserted between the fourth and fifth strands of OB-fold. This points towards the evolution of folds through gene duplication and shuffling. Since OB-fold is an ancient nucleic acid binding fold, it seems quite probable that SH3-fold has evolved from OB-fold. Both these folds then diverged further to perform different functions by modulation of their loops in conformation, sequence and length.