Glomerular disorders are the leading cause of chronic kidney disease like diabetes, hypertension, ischemia *etc*. Recent studies have emphasized the critical role of glomerular SD of podocytes as size selective filtration barrier of kidney and revealed a noval aspect of mechanism that leads to filtration. Disruption of glomerular filtration barrier resulted in loss of permselectivity and appearance of macromolecule in urine (proteinuria). Loss of glomerular protein into urine led to clinical picture of nephritic syndrome, which eventually resulted in end-stage kidney failure. Detail analyses in this direction led to identification of several genes underlying these diseases, which translate to critical structural protein components and mutation/deletion of encoding gene resulted into massive proteinuria.

First molecular component identified at podocyte SD was nephrin in 1998 by Tryggvanson's group, which was followed by others which includes Neph1, ZO-1 *etc*. Neph1 (homologous to Nephrin, 40 % identity) and Nephrin together form heterophilic interaction across the foot process intercellular junction, forming a size selective pore of ~40 nm wide. In addition, Neph1 cross talk with intracellular partner ZO-1 (a tight junction protein), which in turn connected to actin cytoskeleton. Biochemical data concluded that under ischemic condition there is a loss of Neph1/ZO-1 interaction and subsequently led to disorganization of actin cytoskeleton and reperfusion helps in reassociation of the complex. Consequently, Neph1/ZO-1 interaction act as an intermediator, which passes outside extracellular signal to cytoskeleton of podocytes and thus regulate actin assembly.

Later, experimental demonstration by Hubar *et al* 2003, concluded that ZO-1 interacts with cytoplasmic domain of Neph1 *via* its first PDZ domain (Huber *et al.*, 2003b). Recent investigation showed that Neph1 interacts with other PDZ containing proteins like PICK, which regulate various function in podocytes. It is well known fact that function of a protein is chiefly regulated by its structure and subtle change in tertiary structure may impart change in its function. Although, SD components like Neph1 and ZO-1 are of critical importance but three dimensional structure of proteins are not yet elucidated. Therefore, keeping these point in our mind we attempted to solve the structure of cytoplasmic domain of Neph1 (Neph1-CD) and PDZ1 domain of ZO-1 (ZO-1-PDZ1) in solution using biophysical techniques like SWAXS, CD, gel-filtration and molecular modeling.

In Chapter 3, unliganded proteins His-ZO-1-PDZ1 and Neph1-CD were extensively characterized biophysically. Since, crystal structure of ZO-1-PDZ1 is available (Protein Data Bank code 2H3M), but cytoplasmic domain of Neph1 never been characterized using any biophysical tehniques. This is the first report of solution structure of cytoplasmic domain of

Nephl (Nephl-CD). To gain insight into the structure of cytoplasmic domain of Nephl we used three construct of Nephl-CD: His-Nephl-CD, GST-Nephl-CD and tagless-Nephl-CD. Detailed characterization revealed that all the constructs of Nephl-CD *i.e.*, tagged and tagless-Nephl-CD, adopted globular shape in solution (Table 3.1). Structural parameters deduced from SWAXS and secondary structural contents obtained from deconvolution of CD data were used to construct the structural model of Nephl-CD. Analyses of structural model of Nephl-CD exhibited that C-terminal THV motif is surface exposed in all the three constructs. Additionally, biochemical data confirmed binding of ZO-1-PDZ1 with His-Nephl-CD, GST-Nephl-CD and tagless-Nephl-CD in solution (Fig. 3.9). An investigation of stability of tagged and tagless-Nephl-CD as a function of time and temperature using DLS revealed that His-Nephl-CD is most stable in solution as compared to GST-Nephl-CD and tagless-Nephl-CD (Fig. 3.15). In summary, these results establish a 3D model of Nephl-CD in solution and providing a framework for understanding their interaction with ZO-1-PDZ1.

Chapter 4 details the interaction of His-Nephl-CD with ZO-1-PDZ1 in solution. This Chapter reported a complete biochemical and biophysical characterization of His-Nephl-CD•ZO-1-PDZ1 complex. Biophysical data concluded that almost 90-95 % of proteins His-Nephl-CD and His-ZO-1-PDZ1 interacted at 1:1 molar ratio in solution. Resulted model of His-Nephl-CD•ZO-1-PDZ1 complex in solution using SASREF was mapped for identification of novel regulators of intermolecular interactions. One of the interesting finding of this study was the fact that in addition to C-terminal peptide motif (THV), Lys-761 and Tyr-762 of Nephl-CD are also critical. Point mutants (His-Nephl-CD-K761A, His-Nephl-CD-Y762A and His-Nephl-CD-R750A) and deletion mutants (His-Nephl-CD-PDZ) were generated using site-directed mutagenesis. His-Nephl-CD-R750A and His-Nephl-CD-PDZ were used as a positive and negative control, respectively. Mutation of Lys-761 and Tyr-762 to alanine resulted into complete loss of Nephl-CD/ZO-1-PDZ1 interaction *in vitro*, indicating pivotal role played by these residues in interaction. For further validation of the interaction *in vivo*, point mutants K761A, Y762A, R750A and deletion mutant Nephl-PDZ were co-transfected with Myc-ZO-1 in COS-7 cells (Fig. 4.14A). In consistent with *in vitro* results *in vivo* data also showed loss of interaction in mutants K761A and Y762A. Overall, biochemical and biophysical data concluded that residues Lys-761 and Tyr-762 are critical for Nephl/ZO-1 interaction and mutation led to abrogation of the complex even presence of THV was uneffective.

By possibly providing the first evidence of role of residues Lys-761 and Tyr-762 in Neph1/ZO-1 interaction (which present upstream of THV), we hypothesized that residues upstream of THV of Neph1-CD might have role in maintaining specificity of His-Neph1-CD•ZO-1-PDZ1 interaction. As outlined in Chapter 5, competitive inhibition assays between His-Neph1-CD and ZO-1-PDZ1 in presence of peptide at different molar ratio concluded that interaction between His-Neph1-CD and ZO-1-PDZ1 was greatly reduced in presence of peptides. Moreover, pronounced effect was observed in case of longest peptides, interaction decreases linearly as the length of peptide was decreased. In conclusion, upstream residues of Neph1-CD help in rendering stability to the protein-peptide complex. Last part of the Chapter 5 includes, understanding the role of environmental factors in stabilization of His-Neph1-CD•ZO-1-PDZ1 complex. Data suggested loss of interaction under higher concentration of salt and acidic/basic pH, while it remains stable at low ionic strength and neutral pH. Moreover, presence of osmolytes promote interaction and show buffering capacity under thermal stress (specicially 10% sucrose is most effective). Data implied significant role of pH, salt and temperature in His-Neph1-CD/ZO-1-PDZ1 interaction and osmolytes provide extra stability to the complex in solution.

Overall, unliganded proteins, His-Neph1-CD and His-ZO-1-PDZ1, and their 1:1 complex are globular in solution. Present work identified two additional critical residues of cytoplasmic domain of Neph1 and mutation to alanine resulted into loss of Neph1/ZO-1 interaction *in vitro* and *in vivo*. Additionally, data pinpointed critical role of upstream residues of Neph1-CD in His-Neph1-CD•ZO-1-PDZ1 interation. Furthermore, data is suggestive of complex is chiefly regulated by environmental factors like pH, salt, temperature and osmolytes.