Abstract

Protein Tyrosine Phosphatases (PTPs) initiate, modulate and terminate key cellular processes by dephosphorylating phosphotyrosine (pY) residues on signaling proteins. The coordinated action of PTPs with their cognate tyrosine kinases is crucial for the maintenance of cellular homeostasis. Five Receptor Tyrosine Phosphatases (RPTPs) DLAR, PTP99A, PTP69D, PTP10D and PTP52F are involved in the axon guidance process of the fruit-fly *Drosophila melanogaster*. The receptors in these RPTPs comprise of Cell Adhesion Molecules (CAMs) while the cytosolic region contains the catalytic PTP domains. Extensive studies on the genetic interactions between these RPTPs reveal that these five RPTPs collaborate, compete or are partially redundant in some developmental contexts. While the genetic interactions between these RPTPs are well characterized, the role of domain-domain interactions and the mechanism(s) of substrate recognition are poorly understood. The aim of this study was to understand the molecular basis for these interactions using a combination of biophysical, biochemical and structural biology tools.

This thesis is organized as follows:

Chapter 1: The introductory chapter of this thesis highlights the mechanistic issues in signal transduction with an emphasis on the role of the RPTPs in the neuro-development of *Drosophila melanogaster*. The first part of this chapter describes the structural features and the catalytic mechanism of the PTP domain. This is followed by a description of the mechanisms that modulate the activity of a PTP domain. The latter part of the chapter summarizes the role of these RPTPs in axon guidance of *Drosophila melanogaster*. The interactions between the RPTPs based on genetic data provide a mechanistic hypothesis that could be examined *in vitro*. The studies described in the subsequent chapters of this thesis were performed to evaluate this hypothesis.

Chapter 2: This chapter reports our observations on the so-called construct dependence on the expression of recombinant PTP domains in *Escherichia coli*. This chapter details the strategies used to obtain recombinant PTP domains in a soluble form suitable for biochemical and structural studies. This study involved substantial optimization in the size of the protein and overexpression strategies to avoid inclusion-body formation. Five strains of *E. coli* as well as three variations in purification tags viz., poly-histidine peptide attachments at the N- and C-termini and a construct with Glutathione-S-transferase at the N-terminus were examined. In this study, we observed that inclusion of a 45 residue stretch at the N-terminus was crucial for the over-expression of the PTP domains, influencing both the solubility and the stability of these
recombinant proteins. While the addition of negatively charged residues in the N-terminal extension could partially rationalize the improvement in the solubility of these constructs, conventional parameters like the proportion of order-promoting residues or the aliphatic index did not correlate with the improved biochemical characteristics. The findings in this chapter suggest that the inclusion of additional parameters like secondary structure propensities apart from rigid domain predictions could play a crucial role in obtaining a soluble recombinant protein upon expression in E. coli.

Chapter 3: This chapter reports the crystal structure of the PTP domain of PTP10D and PTP10D-substrate/inhibitor complexes. These structural studies revealed aromatic ring stacking interactions that mediate substrate recruitment into the PTP active site. In particular, these studies revealed the role of conserved aromatic residue in Motif 1 (Phenylalanine 76 in case of PTP10D). Mutation of Phenylalanine 76 residue to a Leucine (similar to the mutation found in the inactive distal PTP domains in other bi-domain PTPs) resulted in a sixty-fold decrease in the catalytic efficiency of the enzyme. Fluorescence kinetic measurements to monitor ligand binding showed a three fold increase in the half time of enzyme-ligand complex formation. These studies highlight the role of the KNRY loop in substrate recruitment at the active of the PTP domain and the role of this segment in modulating the kinetics of the enzyme-substrate complex formation.

Chapter 4: This chapter describes a strategy to utilize protein-protein interaction data to identify putative peptide substrates for a given protein. This study was performed in collaboration with Shameer Khader and Prof. R. Sowdhamini at the National Center for Biological Sciences (NCBS). This integrated search approach, called ‘PeptideMine’ was developed into a web-server for experimental and computational biologists. The PeptideMine strategy combines sequence searches in the ‘interacting sequence space’ of a protein using sequence patterns or functional motifs. A compilation of indices that describe the chemical and solubility properties of potential peptide substrates to facilitate investigation by in vitro or in silico studies is also obtained from this server. The biological significance of such a design-strategy was examined in the context of protein-peptide interactions in the case of RPTPs of Drosophila melanogaster.

Chapter 5: In this chapter, we report an analysis of the influence of the membrane distal (D2) domain on the catalytic activity and substrate specificity of the membrane proximal (D1) domain using two bi-domain RPTPs as a model system. Biochemical studies reveal contrasting roles for the D2 domain of the Drosophila Leukocyte antigen Related (DLAR) and Protein Tyrosine Phosphatase on Drosophila chromosome band 99A (PTP99A). While D2 lowers the catalytic activity of the D1 domain in DLAR, the D2 domain of PTP99A leads to an increase in the catalytic activity of its D1 domain. Substrate specificity, on the other hand, is cumulative,
whereby the individual specificities of the D1 and D2 domains contribute to the substrate specificity of these two-domain enzymes. Molecular dynamics simulations on structural models of DLAR and PTP99A revealed a conformational rationale for the experimental observations. These studies suggested that concerted structural changes mediate inter-domain communication resulting in either inhibitory or activating effects of the membrane distal PTP domain on the catalytic activity of the membrane proximal PTP domain.

Chapter 6: This chapter describes biochemical studies to understand the role of the D2 domain of PTP99A. While the catalytic activity of PTP99A is localized to its membrane proximal (D1) domain, the inactive membrane distal (D2) domain influences the catalytic activity of the D1 domain. Phosphatase activity, monitored using small molecule as well as peptide substrates, suggested that the D2 domain activates D1. Thermodynamic measurements on the bi-domain (D1-D2 protein) as well as single domain PTP99A protein constructs suggest that the presence of the inactive D2 domain influences the stability of the bi-domain protein. The mechanism by which the D2 domain activates and stabilizes the bi-domain protein is governed by a few interactions at the inter-domain interface. In particular, we note that mutating Lys990 at the interface attenuates inter-domain communication. This residue is located at a structurally equivalent position to the so-called allosteric site of a canonical PTP, PTP1B. These observations suggest functional optimization in bi-domain RPTPs wherein the inactive PTP domain modulates the catalytic activity of the bi-domain enzyme.

Chapter 7: This chapter summarizes the experimental and computational studies on the *Drosophila melanogaster* PTP domains. The salient features of the experimental data that revealed hitherto uncharacterized sequence-structure relationships in the conserved PTP domain are highlighted. The latter part of this chapter briefly suggests the scope of future research in this area based on some of the findings reported in this thesis.

Appendix : This thesis has an appendix section with four parts. These comprise of technical details and auxiliary work that was not included in the main text of the thesis. Appendix I describes cloning strategies, purification protocols and a list of all recombinant proteins used in this study. Appendix II describes the standardization of the ‘Three Phase partitioning’ protocol for refolding and solubilization of protein from inclusion bodies. Appendix III includes the immunochemical work performed to elucidate the localization of PTP10D in *Drosophila* embryos. Appendix IV describes the work on a Quercetin 2,3 Dioxygenase from *Bacillus subtilis* with an emphasis on the role of metal ions in modulating catalytic activity in this class of proteins.