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Protein-protein interactions involved in the signal transduction pathway of hPTP1E

par

Kristopher Clark

Programme de biologie moléculaire

Faculté des études supérieures

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Ce mémoire intitulé:

Protein-protein interactions involved in the signal transduction pathway of hPTP1E

présenté par:

Kristopher Clark

a été évalué par un jury composé des personnes suivantes:

Dr Martine Raymond, président-rapporteur

Dr Denis Banville, directeur

Dr Alaka Mullick, membre du jury

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ABSTRACT

Protein-protein interactions are an integral component of signal transduction pathways. The interactions are mediated by modular domains which are present within the structure of the signalling molecule. These domains include PDZ, SH2, SH3, WW, PTB and LIM domains.

hPTP1E is a protein-tyrosine phosphatase which contains within its primary structure a region with homology to Band 4.1 proteins, five PDZ domains and a catalytic domain. While the function of this PTPase remains unknown, the structure of hPTP1E suggest it may recruit several proteins into a multiprotein complex. In order to understand the role of hPTP1E, the protein interactions within its signalling cascade were examined.

hPTP1E interacts with ZRP-1 and GEF-5.1 via its second PDZ domain and tuberin via its fourth PDZ domain in the yeast two-hybrid system. In order to characterize these proteins and their interactions, antibodies were generated against ZRP-1 and GEF-5.1. The antibodies which detected the antigen expressed in bacteria were purified by affinity chromatography. The antibodies raised against ZRP-1 and hPTP1E detected proteins of appropriate molecular weights in total cell extracts. HPTP1E is ubiquitously expressed whereas ZRP-1 is restricted to HeLa and MCF-7 cells among the cells tested. Unfortunately, antibodies against GEF-5.1 did not detect a protein of the predicted molecular weight in any of the cell extracts.

Immunoprecipitation of hPTP1E from cells overexpressing regions of ZRP-1 and tuberin with a hemaglutinin tag demonstrated the presence of an interaction between the phosphatase and tuberin *in vivo*. However, ZRP-1 and hPTP1E did not interact under these experimental conditions. Confirmation of the yeast two-hybrid results provides further support for a possible role of hPTP1E in the regulation of endocytosis.

Additional molecules involved in the signalling pathways involving hPTP1E were identified by interaction trap. In one study, the proline rich amino terminus of ZRP-1 interacted with several clones encoding a segment of hCDC47

and NtZRP-p33, a clone containing an SH3 domain. The significance of these findings is unknown. HCDC47 is a minichromosome maintenance protein wich regulate DNA replication. Further, a clone called KIAA0769 containing the sequence of NtZRP-p33 depicts the typical structure for a scaffolding protein.

Another yeast-two hybrid cDNA library screening using the CDC25 homology domain of GEF-5.1 did not detect an interaction with any GTPase but with 14-3-3ε. 14-3-3 proteins are regulatory molecules which interact with various types of proteins by means of a phosphorylated serine residue. Mutational analysis demonstrated that the interaction is dependent on the second serine residue within the consensus sequence R S L S Q G found in GEF-5.1.

The primary structure of the open reading frame of GEF-5.1 was analyzed using profilescan. The software predicted the presence of several domains including a cNMP binding domain, a LTE domain, a PDZ domain, a ras-associated domain and a CDC25 homology domain. A family of guanidine nucleotide exchange factors may exist as clones KIAA0313 and T14G10 have the same structure. These results indicate a role for GEF-5.1 in Ras signalling pathways. Further, its activity may be regulated by the binding of cNMP molecules and 14-3-3ε.

The identification of ZRP-1 and GEF-5.1 interacting proteins as well as the analysis of the primary structure of GEF-5.1 have provided additional information about the function of hPTP1E. This cytoplasmic phosphatase may be involved in the regulation of processes such as transcription, DNA replication and. Further, an interaction between tuberin and hPTP1E suggests a role for this PTPase in the regulation of endocytosis.

RÉSUMÉ

La phosphorylation des protéines est une modification post-traductionelle fréquemment employée pour moduler la transmission des signaux intracellulaires. Il est nécessaire qu'un équilibre du niveau de phosphorylation soit maintenu pour le fonctionnement normal de la cellule sinon des maladies comme le cancer peuvent apparaître.

Les enzymes responsables de la phosphorylation des protéines sont les protéines kinases tandis que les protéines phosphatases enlèvent les groupements phosphate. Les résidus phosphorylés dans les protéines sont certains résidus sérines, thréonines et/ou tyrosines. Les différentes enzymes sont classées en deux familles selon leur spécificité.

Les protéine-tyrosine phosphatases (PTPase) sont elles-même regroupées dans deux familles selon leur localisation intracellulaire: les PTPases de type récepteur et les phosphatases cytoplasmiques. La structure des phosphatases de type récepteur inclus un domaine extracellulaire, un domaine transmembranaire et un (ou deux) domaine(s) catalytique(s). Les PTPases cytoplasmiques contiennent un domaine catalytique unique et généralement un/ ou des domaine(s) responsable(s) de leur localisation intracellulaire ou impliqué(s) dans des interactions protéine-protéine.

Dans notre laboratoire, une phosphatase cytoplasmique dénommée hPTP1E par nous (et PTPL1, PTPBAS, FAP par d'autres) a été isolée. En plus de son domaine catalytique, cette protéine-tyrosine phosphatase contient 1 domaine de type "Band 4.1" qui est impliqué dans la localisation de la protéine à la membrane cellulaire via une interaction avec le cytoskelette, et 5 domaines PDZ. Ces domaines PDZ sont en général impliqués dans les interactions protéine-protéine.

Plusieurs études récentes ont tenté de définir la fonction de hPTP1E. Sato et ses collègues ont isolé hPTP1E lors d'un criblage d'une librairie d'ADNc en utilisant le système des deux-hybrides dans la levure avec la partie cytoplasmique

du récepteur Fas, comme appât. Ils ont aussi démontré que hPTP1E peut inhiber l'effet apoptotique de Fas. L'apoptose des cellules cibles qui est induit par les lymphocytes T cytotoxiques utiliserait le système Fas. De plus, Fas pourrait être associé à des maladies auto-immunes. En plus, hPTP1E pourrait jouer un rôle dans l'apparition de cellules resistantes aux effets de Fas tel que retrouvées dans les sarcomes de Kaposi chez les sidéens. Malgré des données convaincantes, il reste quand même des doutes quant à l'importance de hPTP1E dans ces maladies. Ainsi une étude publiée n'a pu démontrer une interaction entre les homologues de Fas et hPTP1E chez la souris.

Depuis d'autres groupes étudiant les interactions de hPTP1E ont découvert plusieurs protéines qui interagissent avec celle-ci. La première, PARG, est membre de la famille des Rho-GAP, des protéines impliquées dans l'activation des GTPases de type Rho. L'interaction aurait lieu avec le 4ième domaine PDZ de hPTP1E. De plus, le domaine LIM de RIL interagirait avec hPTP1E via ses 2ième et 4ième domaines PDZ. La fonction biologique de ces interactions n'a toutefois pas été déterminée à ce jour.

Pour caractériser la fonction biologique de hPTP1E, nous avons utilisé le système des deux-hybrides de la levure pour identifier des protéines qui interagiraient avec les domaines PDZ de hPTP1E. J'ai ainsi identifié deux protéines nommés ZRP-1 et GEF-5.1, qui se lient à hPTP1E. ZRP-1 possède une structure semblable à celle de zyxin.Ces deux dernières protéines contiennent une région amino-terminale riche en résidus proline et 3 domaines de type LIM à l'extrémité carboxyl terminale. GEF-5.1, d'autre part démontre une homologie marquée aux GEFs de la famille CDC25 impliquées dans l'activation des GTPases de la famille Ras.

Des anticorps ont été générés contre ZRP-1, GEF-5.1 et hPTP1E afin de fournir les outils nécessaires pour mieux caractériser ces différentes protéines. Ainsi, j'ai exprimé et purifié le troisième domaine LIM de ZRP-1 ainsi que le domaine PDZ de GEF-5.1, sous forme de protéines de fusion avec la glutathione-S-transferase (GST). Ces protéines ont servis d'antigène pour générer des

anticorps chez le lapin. Des anticorps dirigés contre le deuxième domaine PDZ de hPTP1E étaient déja disponibles dans le laboratoire. Ces anticorps ont été purifiés sur une colonne d'affinité GST. Les anticorps anti-ZRP-1 et anti-hPTP1E détectent tous les deux des protéines du poids moléculaire attendu. HPTP1E est exprimé d'une facon ubiquitaire tandis que l'expression de ZRP-1 est plus restrainte parmi les cellules testées. Toutefois, les immunoglobulines dirigées contre GEF-5.1 ne détectent aucune protéine du poids moléculaire attendu dans un extrait cellulaire brut.

Parallèlement, d'autres membres du laboratoire ont démontré une interaction entre la tuberine, le produit du gène TSC2, un oncogène impliqué dans la sclérose tubéreuse, et le quatrième domaine PDZ de hPTP1E. Afin de caractériser ces interactions *in vivo*, des immunoprécipitations de hPTP1E à partir de cellules dans lesquelles une région de ZRP-1 et/ou de la tuberine étaient sur-exprimé ont été conduites. Sous les conditions expérimentales utilisées, ZRP-1 n'a pas co-immunoprécipité avec hPTP1E. Cependant une interaction avec la tuberine a été détectée utilisant cette stratégie suggérant que HPTP1E pourrait jouer un rôle dans la modulation de l'endocytose.

La structure de ZRP-1 inclus un domaine riche en proline qui n'est pas nécessaire pour son interaction avec hPTP1E mais qui pourrait interagir avec d'autres protéines en particulier avec des protéines contenant un/ ou des domaine(s) SH3. La moitié amino-terminale de ce domaine a été utilisé pour cribler une librairie d'ADNc par le système des deux-hybrides. Un clone appelé NtZRP-p33 contenant un domaine SH3 a été identifié. La conséquence biologique de cette interaction reste toutefois a être déterminée. Cependant, NtZRP-p33 possède une structure suggérant son implication dans la signalisation intracellulaire.

Un deuxième criblage de la librairie d'ADNc a été initié pour caractériser les protéines impliquées dans le mécanisme de signalisation de hPTP1E. En utilisant le domaine de GEF-5.1 homologue à CDC25, des clones correspondants à la protéine 14-3-3 ont été isolés. Les protéines 14-3-3 forment une famille de

protéines qui régularisent la fonction de plusieurs protéines. Leurs interactions se font via un residu sérine qui est phosphorylé. Des mutations du domaine catalytique ont démontré que l'interaction entre 14-3-3ε et GEF-5.1 est dépendante du deuxième sérine de la séquence R S L S Q G qui se retrouve immédiatement du coté carboxyl terminale du domaine GEF de la protéine GEF-5.1. Ces résultats suggèrent que l'activité de GEF-5.1 pourrait être modulée par la 14-3-3ε.

En conclusion, les résultats expérimentaux présentés dans ce mémoire indique un rôle potentiel de hPTP1E dans plusieurs fonctions cellulaires. En s'associant à la tuberine, hPTP1E pourrait régulariser l'endocytose. Aussi, cette PTPase pourrait être impliquer dans le cycle cellulaire. Ras étant un activateur de la mitose, HPTP1E pourrait moduler l'activité de Ras par voie de GEF-5.1. Ainsi, hPTP1E pourrait agir comme proto-oncogène ou un gène suppresseur des tumeurs. Zyxin est une protéine qui se retrouve près des sites membranaires en association avec le cytoskelette. Puisque la structure de ZRP-1 et zyxin est semblable, ce dernier sert de modèle pour la fonction de ZRP-1. En collaboration avec hPTP1E, ces deux protéines pourrait régulariser la structure du cytoskelette.

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ABBREVIATIONS

AD	Activation domain
AICD	Activation-induced cell death
ALP	Actinin-associated LIM protein
DBD	DNA-binding domain
DMSO	Dimethylsulfoxide
EDTA	Ethylenediamine tetraacetic acid
GAP	GTPase-activating protein
GEF	Guanidine nucleotide exchange factor
GST	Glutathione-S-transferase
HA	Hemaglutinin
IPTG	Isopropyl-β-D-thiogalactoside
LPP	Lipoma preferred partner
MAGUK	Membrane-associated guanylate kinase
MBP	Maltose-binding protein
MCM	Minichromosome maintenance
Mr	Molecular weight
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PH	Pleckstrin homology
PMSF	Phenylmethylsufonyl fluoride
PTB	Phosphotyrosine-binding
PTK	Protein-tyrosine kinase
PTPase	Protein-tyrosine phosphatase
SH2	Src homology 2
SH3	Src homology 3
TSC	Tuberous sclerosis
X-gal	5-bromo-4-chloro-3-indolyl-β-G-galactopyranoside

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Chapter 1: Introduction

1.1 Gene Function

The human genome project was undertaken to provide a physical map of all human genes. The map will be used for identifying the function of a gene and those genes which are responsible for inherited diseases. The nucleotide sequence of a gene provides information about the mechanism of transcriptional regulation and the gene product. Using computational analyses, the open reading frame may be predicted for each gene. Further, the presence of functional domains and homologues may be identified by sequence alignment. At present, the genomic sequence of several organisms have been determined such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Blattner *et al.*, 1997; Cherry *et al.*, 1997; Wilson, 1999). The information derived from studies using less complex organisms as models for specific functions has been invaluable in understanding processes such as cell-cycle regulation and apoptosis within mammalian cells (Bartlett and Nurse, 1990; Hale *et al.*, 1996; Horvitz, 1999; MacNeill *et al.*, 1991). The identification of a homologue for a human gene within one of these organisms will provide information about the function of the gene. However, these organisms are evolutionary distant from *Homo sapiens*. Our genome is larger and will encode proteins with novel activities and biological functions. Although the sequence of a gene will provide important information about its function, it may not necessarily define the function for all genes thus requiring other means for its identification.

Numerous cellular activities require the formation of multiprotein complexes in the presence or absence of nucleic acids. These functions include transcription, translation, cytoskeletal rearrangements and signal transduction. Interest in studies of protein-protein interactions was renewed with the discovery of protein modules regulating protein-protein interactions and the development of methods for their study (Pawson, 1995; Phizicky and Fields, 1995). The

methodology presently used involves both biochemical approaches such as co-immunoprecipitation and co-purification experiments and genetic procedures such as the yeast two-hybrid system. In accordance with this interest, I have studied the protein-protein interactions involved in the signal transduction pathway of hPTP1E, a human protein-tyrosine phosphatase (PTPase), in order to obtain additional information about the function of this protein (Banville *et al.*, 1994).

1.2 Signal Transduction Pathways

Cellular activities are regulated by stimuli which are interpreted via a signal transduction pathway. Upon stimulation of a receptor by its ligand, a series of biochemical events is initiated to activate or inhibit effector molecules resulting in a cellular response such as cell division, cell differentiation, transcription of target genes and regulation of the cytoskeletal configuration.

There are two main principles by which signal transduction pathways function. Protein-protein interactions are involved in the targeting of proteins to specific subcellular locations and the formation of "transducisomes" via scaffold, anchoring and adaptor proteins. On the other hand, enzymatic reactions involving GTPases and their effector molecules, phosphorylation reactions, phospholipases and phosphoinositol kinases regulate the activation state of proteins by their direct modification or indirectly by the production of organic molecules (Pawson and Scott, 1997; Tsunoda *et al.*, 1997).

The proteins involved in signal transduction pathways are composed of an array of protein modules which are assembled in different combinations to provide the protein with the appropriate function (figure 1). The domains involve catalytic domains such as phosphatase or kinase domains while other domains are responsible for mediating protein-protein interactions (Pawson, 1995). The presence of multiple domains required for protein-protein interactions within a single polypeptide chain allows for the assembly of signalling complexes (Pawson and Scott, 1997). The principal role of the transducisomes is to increase the speed of signal transmission and the reduction of cross-talk. Many pathways employ

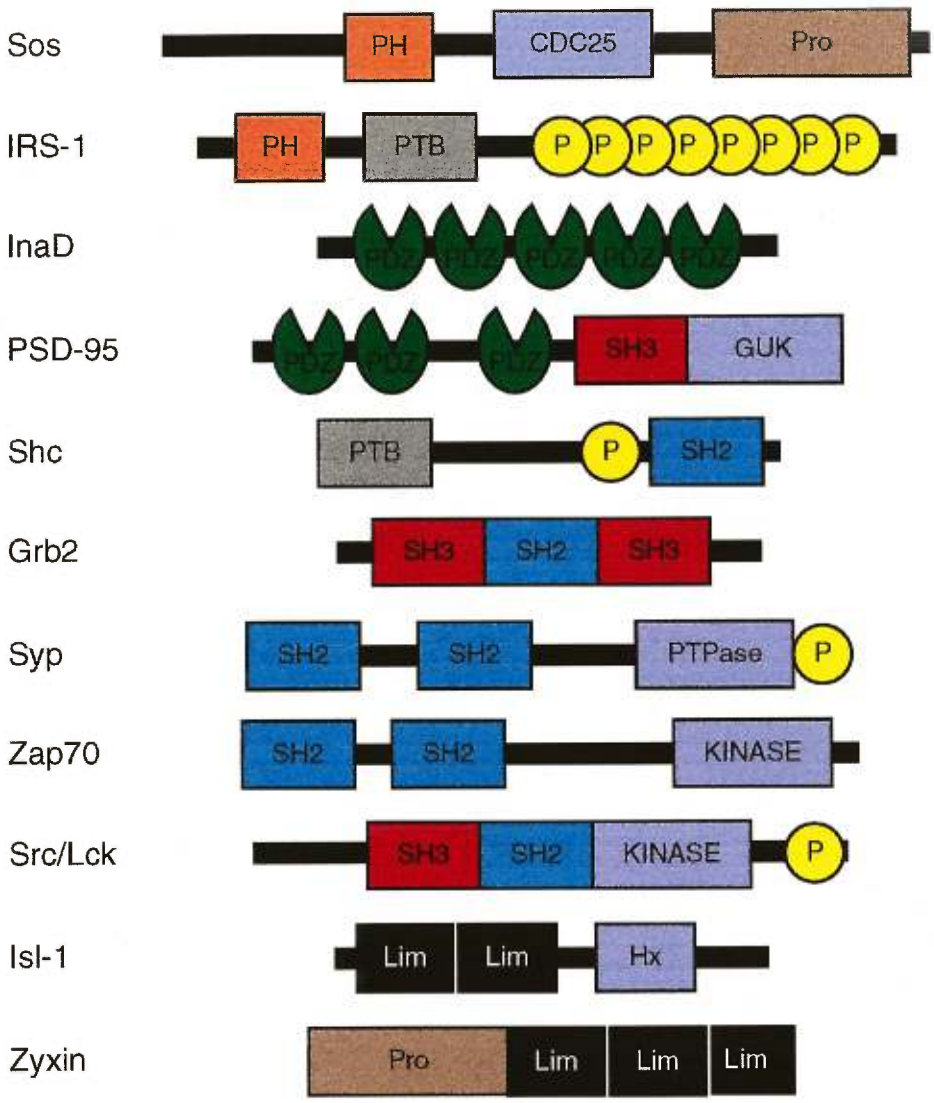

Figure 1- Domains involved in protein-protein interactions. The names of the domains are provided within the representative boxes. P: SH2 binding sites, PH: Pleckstrin homology domain, PTB: Phosphotyrosine binding domain, GUK: Guanylate kinase domain, Pro: Proline-rich region, Hx: Homeobox.

these proteins such as InaD which is involved in the regulation of phototransduction in *Drosophila melanogaster* photoreceptors, the STE5 complex regulating the *S. cerevisae* pheromone mating pathway and the induction of channel aggregation by PSD-95 (Craven and Bredt, 1998; Faux and Scott, 1996; Gomperts, 1996; Tsunoda *et al.*, 1997).

1.3 Protein Modules

Protein-protein interactions are the focal point of signal transduction pathways. The general principles governing these interactions is the presence of a domain which mediates protein-protein interactions in one protein and a short amino acid sequence in the counterpart. The protein modules characterized in recent years include the Src homology domains 2 and 3 (SH2 and SH3), PDZ, LIM, pleckstrin homology (PH), WW and phosphotyrosine-binding (PTB) domains. Numerous proteins which have different functions contain these domains. Examples include Src, Sos, PSD-95, Grb2, zyxin and hPTP1E.

1.3.1 SH2 and PTB Domains

The SH2 domain was identified among kinases due to its effect on both catalytic activity and substrate phosphorylation (Koch *et al.*, 1991). Since its discovery, SH2 domains have been found in hundreds of proteins involved in intracellular signalling. These proteins include enzymes such as kinases, PTPases, phospholipases, GTPase-activating proteins (GAPs) and adaptor proteins such as Crk, Grb2 and Nck which have no catalytic activity but serve to form multiprotein complexes (Koch *et al.*, 1991; Pawson and Gish, 1992). The domain is composed of approximately 100 amino acids and binds to specific phosphotyrosine-containing peptide motifs (Songyang *et al.*, 1993). An important function of this domain is to localize downstream signalling molecules to the membrane by recognizing phosphorylated receptors (Mayer and Gupta, 1998).

Another protein module which interacts with phosphotyrosine containing peptides is the phosphotyrosine binding domain. It has been found primarily in

docking proteins such as IRS-1, Numb, FE65 and Shc (Borg and Margolis, 1998). The specificity of the interactions is maintained by the different sequences flanking the phosphorylated tyrosyl residue. SH2 domains rely on three to six residues at the carboxyl terminus of the phosphotyrosine whereas PTB domains bind to a consensus sequence of NPXpY (Kavanaugh *et al.*, 1995; Songyang *et al.*, 1993).

1.3.2 SH3 and WW Domains

The common feature of SH3 and WW domains is their binding to proline rich regions found in the interacting protein. The unique structure of proline results in an unusual three dimensional configuration when numerous proline residues are found over a short sequence. SH3 and WW domains usually interact constitutively with their counterparts whereas the binding of SH2 and PTB domains to their ligands can be regulated by tyrosine phosphorylation which is maintained by a balance between protein-tyrosine kinases (PTKs) and PTPases in the cell (Mayer and Grupta, 1998).

Both SH3 domains and WW domains are relatively small, each composed of 60 amino acids and 40 amino acids respectively (Mayer and Gupta, 1998; Rotin, 1998). The WW domain has derived its name from 2 conserved tryptophan residues found approximately 20-22 amino acids apart (Bork and Sudol, 1994). Although both domains bind to proline-rich ligands, there exists a difference between the consensus sequences and thus the overall structure of the ligand. WW domains have the ability to distinguish between two different consensus binding sites namely the PY and PPLP motifs whereas SH3 ligands adopt a left-handed polyproline-2 helix conformation with a PxxP core motif (Bradford *et al.*, 1997; Chen and Sudol, 1995; Yu *et al.*, 1994).

1.3.3 PH Domains

The pleckstrin homology domain is composed of approximately 100 amino acids originally identified in pleckstrin, a protein kinase C substrate in activated blood platelets (Haslam *et al.*, 1993; Mayer *et al.*, 1993). The domain is present in numerous proteins involved in intracellular signalling and like the other modular domains described in this chapter, occurs in combination with other domains important for signal transduction. The main function of the PH domain is the regulation of protein localization to the membrane via protein-protein interactions or interactions with phosphoinositols (Lemmon and Ferguson, 1998; Shaw, 1996).

1.3.4 LIM Domains

LIM domains were first recognized in three transcriptional factors involved in developmental processes. The proteins are **L**in-11 from *C. elegans*, **I**sl-1 from rat and **M**ec-3 from *C. elegans* (Freyd *et al.*, 1990; Karlsson *et al.*, 1990; Way and Chalfie, 1988). The acronym was derived from the initial of each of these factors. The LIM domain is composed of approximately 55 amino acids with a precise pattern of cysteine and histidine residues. The consensus sequence has been refined to $CX_2CX_{16-23}HX_2CX_2CX_{16-23}CX_2C$ (where X is any amino acid) (Jurata and Gill, 1998). Each LIM domain coordinates two zinc ions which are instrumental for the maintenance of the appropriate tertiary structure (Kosa *et al.*, 1994; Perez-Alvarado *et al.*, 1994). The formation of two zinc finger structures in every LIM domain similar to zinc fingers present in GATA-1 has led to the speculation about a role for LIM domains in the regulation of transcription or DNA replication due to possible protein-nucleic acid interactions. This hypothesis is still unproven. However, Schmeichel and Beckerle (1994) were able to demonstrate a role for LIM domains in the regulation of protein-protein interactions.

LIM domains have been identified among numerous proteins. The structure of these proteins has provided for classification guidelines. The proteins within this family have been subdivided into 4 groups (Dawid *et al.*, 1995). The initial group contains all the proteins which have 1 or 2 LIM domains at the N-terminus and a homeodomain at the C-terminus. Proteins within this group are transcription factors regulating developmental processes (Curtiss and Heilig, 1998). Group 2 is composed of proteins consisting solely of LIM domains. Proteins within this class include CRIP, CRP1 and PINCH. Proteins containing LIM domains localized at the C-terminus form the third class. This group includes among other proteins zyxin and paxillin which are found at adhesion plaques. Finally, the unclassified proteins are regrouped together. These proteins often contain catalytic domains such as the LIMKs which are kinases and LRG1 being a GTPase-activating protein (GAP).

1.3.5 PDZ domains

The PDZ domain was originally identified among the sequences of three proteins within the membrane-associated guanylate kinase (MAGUK) protein family. The acronym was derived from these proteins namely the post-synaptic density protein **P**SD-95, the *Drosophila* tumor suppressor **D**lg and the tight junction protein, **Z**O-1 (Kennedy, 1995). PDZ domains are not restricted to MAGUKs and have been found in signalling molecules such as kinases and PTPases, syntrophins, nitric oxide synthase, enigma and CASK (Fanning and Anderson, 1998). Most proteins containing PDZ domains have a sub-membranous localization which is important for function (Ponting *et al.*, 1997).

PDZ domains appear as multiples in a wide range of proteins. Certain proteins contain only one PDZ domain while others contain five or nine in the presence or absence of other functional domains. The function of PDZ domains is principally for localization of proteins via protein-protein interactions. The MAGUKs by means of their multiple PDZ domains cluster receptors and channels required for proper synaptic signalling (Craven and Bredt, 1998; Gomperts,

1996). PSD-95 interacts with NMDA receptors, Shaker-type potassium channels and neuroligins (Irie *et al.*, 1997; Kim *et al.*, 1995; Kornau *et al.*, 1995). β-Neurexins and neuroligins mediate neuronal cell-cell interactions. Hence, PSD-95 may colocalize receptors and channels at neuronal cell-cell junctions by means of its numerous interactions (Irie *et al.*, 1997). InaD which possesses five PDZ domains is another example of a scaffolding protein. InaD is involved in photoreceptor signalling. It increases the speed of signalling by co-localizing PLC-β, eye-PKC and a Trp Ca^{2+} channel within vicinity of the rhodopsin receptor which triggers the signalling cascade (Ranganthan and Ross, 1997). Although much information about the functions of PSD-95 and InaD remains unknown, they serve as models for other proteins containing multiple PDZ domains.

PDZ domains recognize carboxyl terminus peptide sequences in many target proteins. PDZ domains can be divided into two classes according to both their structure and recognition motif. In the first group, PDZ domains from proteins Dlg and hPTP1E bind to the consensus S/T-X-V/I-COOH (Songyang *et al.*, 1997). PDZ II domains from proteins LIN-2, p55 and Tiam-1 interacted with peptides containing hydrophobic or aromatic side chains at the carboxyl terminus (Songyang *et al.*, 1997). The selectivity of the interaction can be explained by structural data. The structure of PDZ domains between the two groups is very similar with the exception that class II domains contain a second hydrophobic binding pocket for accommodating bulky hydrophobic side chains (Daniels *et al.*, 1998). In contrast, class I domains contain a hydrophilic pocket for binding of the serine or threonine residue (Doyle *et al.*, 1996). The binding specificity for the C-terminus is achieved via the carboxylate binding loop (Doyle *et al.*, 1996). While the amino acid sequence of the loop differ between the two classes of PDZ domains, the overall structure and binding properties to the COOH group is similar in both cases (Daniel *et al.*, 1998). Two different types of PDZ domains have evolved over the years which interact with the C-terminus of proteins possessing a consensus sequence of either S/T-X-V or aromatic-aromatic-V/A.

PDZ domains not only interact with the C-terminus of target proteins but may also recognize internal STV motifs (Shieh and Zhu, 1996). PDZ domains may also form heterotypic dimers as described for the interaction between neuronal nitric oxide synthase and both PSD-95 and syntrophin (Brenman *et al.*, 1996). Finally, the PDZ domain of actinin-associated LIM protein (ALP) interacts with spectrin-like motifs of α-actinin-2 (Xia et al., 1997).

1.4 Yeast Two-Hybrid System

Several methods have been developed to study protein-protein interactions. The procedures employ biochemical analyses such as co-purification and co-immunoprecipitation or genetic techniques such as the yeast two-hybrid system (Phizicky and Fields, 1995). The main advantage of the yeast two-hybrid system is the ability to rapidly identify and clone genes encoding a novel interacting protein with a protein of interest. Since its initial development in 1989 by Fields and Song, the system has been widely used to understand various cellular functions at the molecular level. The yeast two-hybrid system may be used to characterize a known protein-protein interaction or to screen a library to identify a novel interacting protein (Fields and Song, 1989; Chien *et al.*, 1991).

Many improvements have been incorporated since the initial conception and the system has been modified for each individual need (Brachmann and Boeke, 1997; Brent and Finley, 1997). A yeast one-hybrid system has been designed to study protein-DNA interactions, a reverse two-hybrid system was described for the identification of proteins which can dissociate a protein-protein interaction and a three-hybrid system has been used to identify proteins involved in multiprotein complexes (Li and Herskowitz, 1993; Shih *et al.*, 1996; Vidal *et al.*, 1996; Zhang and Lautar, 1996). Finally, a mammalian two-hybrid system has also been developed with the advantage of expressing proteins containing the appropriate post-translational modifications (Fearon *et al.*, 1992). All these genetic systems for studying macromolecule interactions have been and will

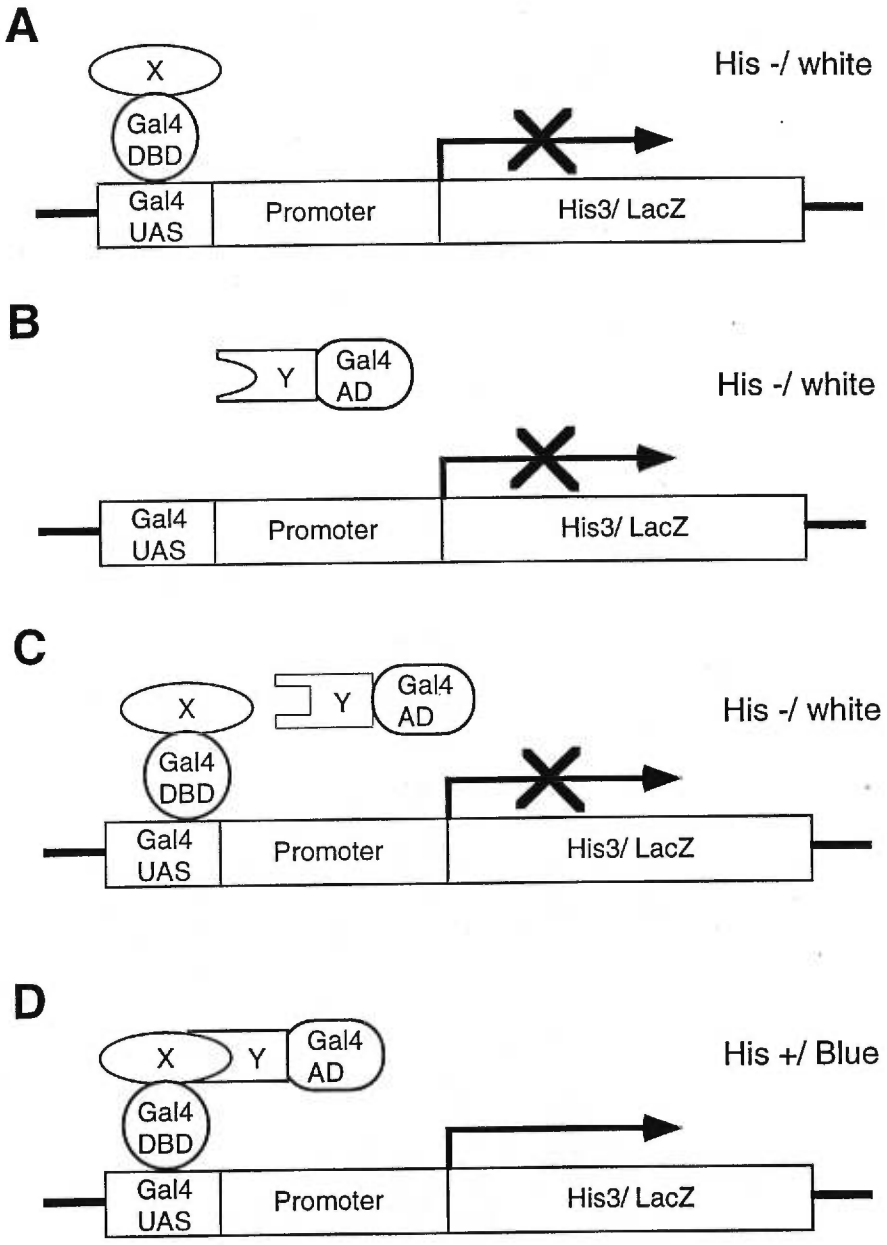

Figure 2- Yeast two-hybrid system. Within this system, a protein of interest X is expressed as a fusion protein with the DNA-binding domain (DBD) of Gal4. A cDNA library is produced such that the proteins are expressed as a fusion protein with the activation domain (AD) of Gal4. If proteins X and Y interact, a functional Gal4 is produced which induces the expression of the reporter genes. When the fusion proteins are expressed independently or the proteins do not interact no expression of the reporter genes is observed.

continue to be useful in deciphering the cellular activities at a molecular level.

The yeast two-hybrid system involves a genetic selection for a protein-protein interaction (figure 2). Gal4 is a transcription factor composed of two essential but separable domains which are the DNA-binding domain (DBD) and the activation domain (AD) (Keegan *et al.*, 1986; Ma and Ptashne, 1987). Further, an irrelevant polypeptide may be placed between the two domains without loss of activity. In the yeast two-hybrid system, a fusion protein is expressed in cells whereby the DNA-binding domain is fused to the protein of interest X (Brachmann and Boeke, 1997; Brent and Finley, 1997; Fields and Song, 1989). A cDNA library is cloned into another vector such that a library of fusion proteins is formed with the activation domain. If a protein Y among the library interacts with protein X, a functional Gal4 transcription activator will be reconstituted. Gal4 will induce the expression of the reporter genes which have been engineered to contain Gal4 DNA-binding sites upstream from the start of transcription. In most systems, two reporter genes are used which include one gene for nutritional selection such as His3 or Leu2 and LacZ with its expression being monitored using an assay relying on the formation of a colored product.

1.5 Protein-Tyrosine Phosphatases

Phosphorylation is an important post-translational modification regulating protein function and cellular activities. The transfer of a phosphate group from ATP to a protein is performed by kinases whereas phosphatases dephosphorylate proteins. These enzymes have been divided into two classes according to their residue specificity: 1) the serine/ threonine specific kinases and phosphatases and 2) the tyrosine specific kinases and phosphatases. The great majority (>99%) of phosphorylation of proteins occurs on serine and threonine residues however, the greatest change (20-200 fold increase) in protein phosphorylation during cell stimulation by growth factors occurs on tyrosine residues (Zhang, 1998). The large increase in tyrosine phosphorylation in proteins in response to specific stimuli indicated a significant role for PTK and PTPases. The experimental data

suggests a role for these enzymes in a range of cellular activities including the cell-cycle, pathogenesis, cell-cell interactions, cell adhesion, B and T-cell activation, apoptosis, proliferation, differentiation and stress responses in the plant (Charbonneau and Tonks, 1992; Dixon, 1996; Hunter, 1995; Klingmüller, 1997; Sato *et al.*, 1995; Tonks and Neel, 1996; Van Vactor *et al.*, 1998; Walton and Dixon, 1993; Xu *et al.*, 1998).

The phosphorylation of tyrosyl residues is a dynamic process *in vivo* requiring a balance between the actions of PTKs and PTPases. In the early stages of the research, PTPases were thought to be merely house-keeping genes which reversed the actions of the kinases. However, the cloning of over a hundred PTPases in the last decade with the identification of specific functions for several of these enzymes has lead to the notion that simultaneous regulation of the kinases and phosphatases is required for proper biological function (Zhang, 1998).

The importance of PTPases could not be further implied than by providing examples of their role in human diseases. PTPases have been associated with viral and bacterial pathogenesis, tumorigenesis, diabetes, Lafora's disease and immunological dysfunction (Minassian *et al.*, 1998; Parsons, 1998; Shultz *et al.*, 1997; Zhang, 1998). Several potential mechanisms exist for the development of a tumor: 1) the constitutive activation of an oncogene, 2) inhibition of a tumor suppressor gene and 3) the deregulation of apoptosis. An example can be provided for each mechanism. Cdc25a which regulates cell-cycle progression is overexpressed in a number of breast tumors implying an oncogenic behavior (Galaktionov *et al.*, 1995). The deregulation of PTKs with mitogenic activity may lead to the development of different cancers. Since PTKs serve as protooncogenes, it was postulated that PTPases may serve as tumour suppressor genes. In 1997, two laboratories independently reported the identification of the first PTPase with tumor suppressor activity namely PTEN or MMAC1 (Li *et al.*, 1997; Steck *et al.*, 1997). The gene encoding the PTPase is mutated in a number of cancers affecting different organs including the brain, breast and the prostate. Mutations within the PTEN/MMAC1 gene have been also associated with bladder

cancers, ovarian cancer, glioblastomas and predisposes people to Cowden's disease (Cairns *et al.*, 1998; Liaw *et al.*,1997; Wang *et al.*, 1997; Yokomizo *et al.*, 1998). The identification of hPTP1E as an inhibitor of Fas-mediated apoptosis has led to studies on the role of this PTPase in disease (Sato *et al.*, 1995). Two independent studies showed a correlation between hPTP1E expression levels and a resistance of cancerous cells to Fas-induced apoptosis (Mori *et al.*, 1996; Ungefroren *et al.*, 1998). Mutations within the PTPase gene designated EPM2A has been associated with the development of Lafora's disease (Minassian *et al.*, 1998). Finally, certain bacteria and viruses depend on PTPases for pathogenecity. The bacteria *Yersinia* and *Salmonella typhimurium* encode PTPases essential for virulence while the *vaccinia* virus encodes the dual specificity PTPase VH1 which is required for viral transcription and infectivity (Bliska *et al.*, 1991; Kaniga *et al.*, 1996; Liu *et al.*, 1995).

The structure of proteins within the PTPase family display a large diversity which has necessitated a classification system (figure 3). The PTPases have been categorized into four groups: 1) the receptor-like PTPases, 2) cytoplasmic PTPases, 3) low Mw PTPases and 4) the VH1 dual specificity phosphatases. The common feature of all these proteins is the catalytic domain which encompasses a signature motif characterized by the sequence (H/V)C(X)$_5$R(S/T) (Zhang *et al.*, 1994). Other than the active site cysteine and arginine, an aspartic acid residue is required for activity while serving as a general acid in the reaction's mechanism (Zhang, 1998). The sequences which differentiate the proteins from each class are those flanking the catalytic domain at the carboxyl and amino termini. The additional sequences serve a regulatory role for the appropriate localization and formation of multiprotein complexes.

1.5.1 Receptor-Like PTPases

The receptor-like PTPases are characterized by an extracellular domain, one transmembrane region followed by one or two catalytic domains. If two catalytic domains are present, usually the carboxyl terminal domain is inactive.

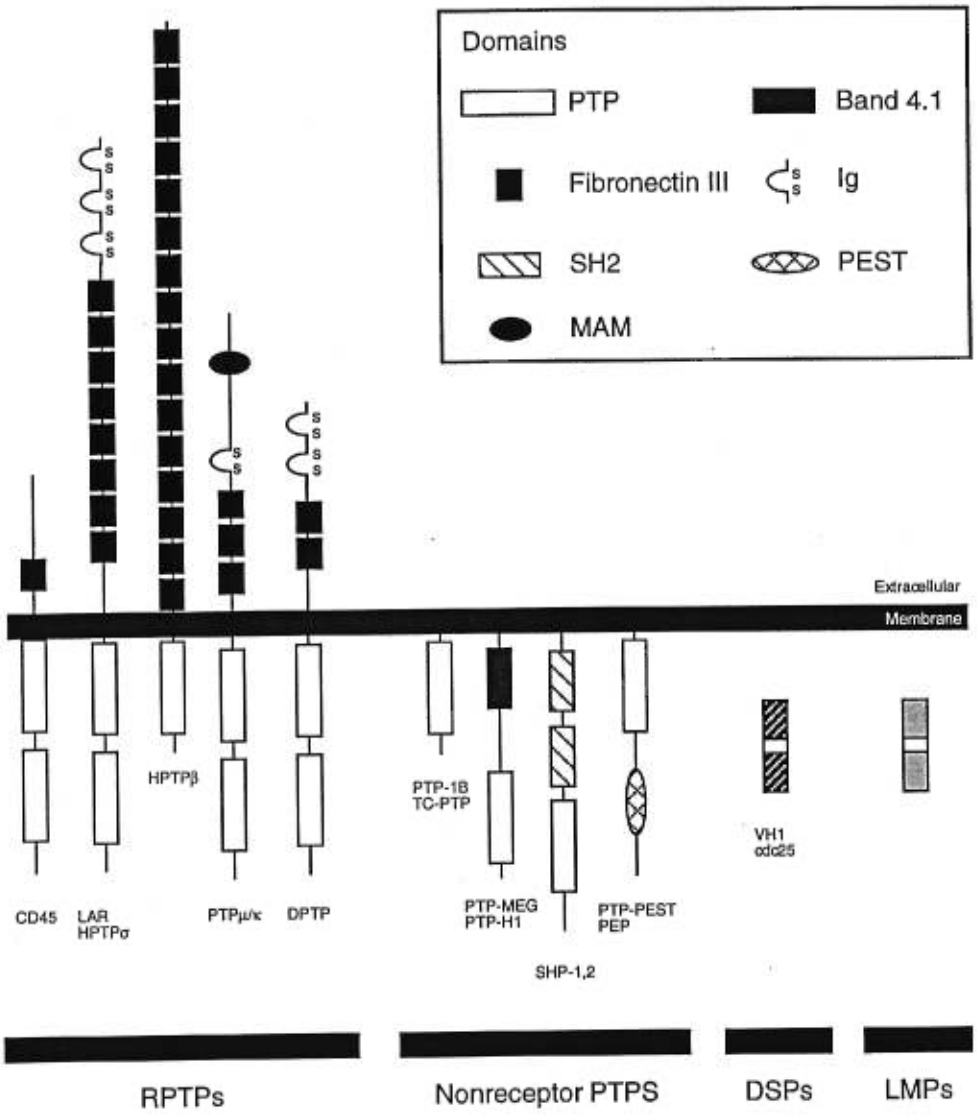

Figure 3- Classification of protein-tyrosine phosphatases. Only selected representatives of each class are shown.

The role of this catalytically inert domain may be to recruit substrate and/or localization (Van Vector *et al.*, 1998). The PTPases within this family are distinguished from one another by the structural diversity of the extracellular domain which can be of varying lengths and contain numerous motifs including fibronectin III-like domains, immunoglobulin domains and MAM domains (Zondag and Moolenaar, 1997). A wealth of information about the function of RPTPs has been provided by Drosophila genetics (Van Vactor *et al.* 1998). The results of these experiments provided insights into the role of RPTPs in the control of axon guidance decisions and suggest a prominent role for this class of PTPases in the nervous system. Other studies have described a role for RPTPs in the homotypic and heterotypic aggregation of cells. The insights into these functions were provided by the similarity of the extracellular domain to the structure of cell adhesion molecules. RPTPμ, RPTPκ and RPTPΨ all participate in homophilic aggregation of cells *in vitro* (Banville, unpublished; Brady-Kalnay *et al.*, 1993; Sap *et al.*, 1994).

1.5.2 Dual Specificity Phosphatases

The dual specificity phosphatases have the unique ability to dephosphorylate phosphoserine, phosphothreonine as well as phosphotyrosyl residues within a protein. The majority of dsPTPases are localized in the nucleus where they inactivate MAP kinases and form complexes with cdk molecules involved in cell cycle regulation (Ramponi and Stefani, 1997).

1.5.3 Low Molecular Weight PTPases

The low molecular weight PTPases have no structural similarities with the other PTPases with exception of the signature motif. The most notable feature is their low molecular weight (Mr) which is only 18 kDa on average. Only a few mammalian low Mr PTPases have been described. The studies involving IF2, a

phosphatase purified from the bovine liver, have indicated a role for this family of PTPases in the regulation of mitogenic signalling (Ramponi and Stefani, 1997).

1.5.4 Cytoplasmic PTPases

Finally, the cytoplasmic phosphatases have a number of regulatory motifs which flank the catalytic domain at the amino and carboxyl termini. These include SH2 domains present in PTPases such as SHP-1 and SHP-2, PDZ domains which are found in hPTP1E and the ERM domain which targets proteins to the cytoskeleton is found in PTPMEG, PTPH1 and hPTP1E (Ahmad *et al.*, 1993; Arpin *et al.*, 1994; Banville *et al.*, 1994; Shen *et al.*, 1991). The role of these regulatory domains has been informative in identifying the function of the PTPases.

Cytoplasmic phosphatases may regulate numerous signal transduction pathways in different cells. They are involved in the regulation of growth factor, cytokine and oligomeric receptor signalling (Tonks and Neel, 1996). Further, these PTPases regulate adhesion processes required for cell spreading, migration and focal adhesion (Oh *et al.*, 1999; Tamura *et al.*, 1998; Yu *et al.*, 1998). The most notorious members of this family are SHP-1 and SHP-2. SHP-1 is predominantly expressed in hematopoietic cells. Genetic disruption of this gene in mice has led to a panoply of hematopoietic abnormalities including immunological dysfunction (Shultz *et al.*, 1997). In contrast, SHP-2 is expressed ubiquitously. The proper function of this PTPase is required during development. A mouse knockout of this gene results in embryonic death (Van Vector *et al.*, 1998). While numerous PTPases will negatively regulate signalling, others demonstrate a positive role. The association of SHP-1 with the erythropoeitin receptor terminates signalling whereas SHP-2 couples the Ras signalling cascade to the PDGFR receptor exerting a positive effect on mitogenic signals within the cell (Klingmüller, 1997).

In recent years, research has focused on the protein-protein interactions mediated by the regulatory domains rather than the identification of *in vivo*

substrates of the PTPases. However, a novel strategy called substrate trapping will steer some of our efforts towards the identification of these elusive substrates. The methodology involves the mutation of either the catalytic cysteine residue to a serine or alanine or the mutation of the conserved aspartic acid residue to an alanine (Flint *et al.*, 1997). Although these mutants are catalytically inert, some phosphatases retain the ability to interact with their substrate allowing for their detection and identification.

1.6 hPTP1E

hPTP1E, a cytoplasmic protein-tyrosine phosphatase, has sustained interest due to its protein structure as well as its inhibitory effect on Fas-mediated apoptosis (Sato *et al.*, 1995). hPTP1E was cloned independently in several laboratories and has numerous names including PTPL1, PTP-BAS and FAP-1 (Banville *et al.*, 1994; Maekawa *et al.*, 1994; Saras *et al.*, 1994; Sato *et al.*, 1995). hPTP1E is composed of 2490 amino acids with a predicted mass of 277kDa. It may also be present in the cell as different alternatively spliced variants with deletions and insertions throughout the protein (Banville *et al.*, 1994; Maekawa *et al.*, 1994). Besides the catalytic domain at the C-terminus, hPTP1E contains an N-terminal domain demonstrating homology to the band 4.1 protein family, five PDZ domains and five PEST sequences. The band 4.1 protein family includes ezrin, radixin, moesin and several protein-tyrosine phosphatases such as PTPH1 and PTPMEG1 (Arpin *et al.*, 1994; Tsukita *et al.*, 1997). Members of the band 4.1 superfamily interact with actin filaments and membrane proteins to organize the cortical actin cytoskeleton. The function of proteins in the band 4.1 family is regulated by the level of phosphorylation. It has been proposed that hPTP1E may regulate the association of band 4.1 proteins with the cytoskeleton as certain members of the family may serve as substrates for the PTPase (Banville *et al.*, 1994).

In order to define the function of hPTP1E, the protein-protein interactions involving the different PDZ domains of the protein-tyrosine phosphatase were

studied. Using the yeast two-hybrid system, two potential candidates were identified which interact with the second PDZ domain of hPTP1E. The full-length cDNA of the two genes were isolated and sequenced. Analysis of the cDNA sequence revealed that one of the proteins displays homology to a guanine nucleotide exchange factor (GEF) and the other to zyxin (Banville *et al.*, unpublished; Murthy *et al.*, 1999). The proteins were called GEF-5.1 for its speculative catalytic function and its clone number and ZRP-1 for Zyxin-Related Protein-1. In contrast, a screening using the fourth PDZ domain of hPTP1E revealed an interaction with tuberin (Banville *et al.*, unpublished).

GEF-5.1 encodes a protein of 1601 amino acids with a predicted mass of 175 kDa (Banville *et al.*, unpublished). Comparison of the protein sequence against the Blast database revealed the presence of a PDZ domain from amino acid 530 to amino acid 600 and a CDC25 domain from amino acid 859 to amino acid 1059. No other information about this clone was available at this time.

ZRP-1 was identified as a protein which interacted with the second PDZ domain of hPTP1E using the yeast two-hybrid system (Murthy *et al.*, 1999). The full-length cDNA encodes a protein of 476 amino acids with a predicted mass of 52 kDa. Analysis of the primary structure of ZRP-1 revealed three distinct functional regions which are homologous to zyxin (Murthy *et al.*, 1999; Yi and Beckerle, 1998). A proline rich region is located at the N-terminus. A number of motifs could serve as ligands for SH3 or WW domains found in other proteins. At the C-terminus, three consecutive LIM domains separated by only 3 or 4 amino acids are found. As previously discussed, LIM domains mediate protein-protein interactions (Schmeichel and Beckerle, 1994). While the carboxyl terminus of ZRP-1 is required for an interaction with hPTP1E, the LIM domains appear to stabilize the interaction. Finally, a putative nuclear exclusion sequence (NES) is found between these two regions of ZRP-1. The NES was identified by sequence alignment with motifs from related proteins. Whether this signal peptide is functional has yet to be determined experimentally.

Tuberous sclerosis is an autosomal dominant trait characterized by the development of benign tumors in many tissues. The hamartomas can develop within the brain, eyes, skin, kidneys, heart, lung and skeleton leading to a broad spectrum of symptoms among patients (Gomez, 1988). Loss of heterozygosity within chromosomal regions 9q34 and 16p13.3 coincide with the development of the disease (Sampson and Harris, 1994). The genes responsible for tuberous sclerosis (TSC) have been called TSC1 and TSC2, respectively.

TSC2 was identified by positional cloning (European Chromosome 16 Tuberous Sclerosis Consortium, 1993). The gene encodes a protein called tuberin of 1784 amino acids with a predicted mass of 198kDa. The most notable feature of the primary structure is a stretch of 58 amino acids at the C-terminus displaying homology to the GTPase-activating protein rap1GAP. Finally, the gene is widely expressed among the different tissues in agreement with the widespread development of hamartomas (European Chromosome 16 Tuberous Sclerosis Consortium, 1993).

1.7 Aims

During two co-op work terms, I was involved in setting up the yeast two-hybrid system and the isolation of several clones which interacted with hPTP1E. These clones were latter named ZRP-1 and GEF-5.1. This initial work on ZRP-1 was included in a recent publication (appendix 1). The aims of the thesis project was to further characterize the protein-protein interactions involved in the signal transduction pathway of hPTP1E. It has been demonstrated in our laboratory using the yeast two-hybrid system that both ZRP-1 and GEF-5.1 can interact with the second PDZ domain while tuberin can interact with the fourth PDZ domain of hPTP1E. In order to obtain further evidence that all these interactions occurred *in vivo*, antibodies against each of the proteins would be required. For this project, I expressed the PDZ domain of GEF-5.1 and the C-terminal region of ZRP-1 as a fusion protein with GST. The purified fusion proteins were used to immunize rabbits for the generation of polyclonal antibodies. Antibodies recognizing the

second PDZ domain of hPTP1E were available in the laboratory. All the antibodies were purified using affinity chromatography. These antibodies were used to characterize the expresssion of proteins in a range of cell lines. Further, the validity of the yeast two-hybrid results was assessed by co-immunoprecipitation studies. The yeast two-hybrid system was used to screen a HeLa cDNA library to identify proteins which could interact with the catalytic domain of GEF-5.1 and the proline-rich N-terminus of ZRP-1. Finally, in order to obtain further information about the biological function of GEF-5.1, computational analyses of this gene along with KIAA0313 and TG14.10 were undertaken.

Chapter 2: Materials and Methods

2.1 GST-Fusion Protein Purification

The third LIM domain of ZRP-1 (aa 398-476, LIM3-GST) and the PDZ domain of GEF-5.1 (aa 511-629, PDZ-GST) were amplified by PCR and cloned into the pGEX-5X-3 expression vector (Pharmacia Biotech) in frame with the glutathione S-transferase (GST) gene. Protein expression and purification was performed according to the manufacturers' protocol. Briefly, a saturated bacterial culture was diluted 40-fold in DYT (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) supplemented with 50 μg/mL of ampicilin. The culture was incubated at 37°C with vigorous agitation until the OD_{600} reached approximately 0.500. The expression of the recombinant protein was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 200 μM and incubating the culture at 37°C for another 3 hours. The culture was centrifuged and the pellet was resuspended in lysis buffer (1% Triton, 10 μg/mL leupeptin in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4 pH 7.3) for LIM3-GST and 0.25% sarkosyl, 10 μg/mL leupeptin in PBS for PDZ-GST). The solution was sonicated to lyse the bacteria. Glutathione sepharose 4B (Pharmacia Biotech) was added to the soluble fraction and incubated at room temperature for 1 hour (LIM3-GST) or overnight (PDZ-GST). The glutathione sepharose beads were washed with lysis buffer. The recombinant proteins were eluted using 10 mM reduced glutathione dissolved in lysis buffer (LIM3-GST) or 20 mM reduced glutathione dissolved in 0.25% sarkosyl, 100 mM Tris pH 8.0 (PDZ-GST). The concentration of the protein was measured using a Bradford assay (Bio-Rad laboratories) and the purity of the proteins was confirmed by SDS-PAGE gel electrophoresis.

2.2 Antibody Production

Rabbits were immunized with 100 μg of the GST recombinant proteins in a solution of Freund's complete adjuvant (Sigma). The immunization was followed by two booster injections every third week using identical quantities of protein but mixed in Freund's incomplete adjuvant (Sigma). The rabbits were bled 10 days after each booster injection. The titer of the antibodies was estimated by Western blots of total bacteria lysates containing the antigen.

2.3 Preparation of GST Affinity Column

2.3.1 Purification of GST

GST was purified under the same conditions as for LIM3-GST described above except the protein was eluted in 10 mM reduced glutathione, 100 mM NaCl in 50 mM Tris pH 8.0 buffer. The protein solution was dialyzed overnight at 4°C against 100 mM NaCl in 100 mM $NaHCO_3$ pH 8.3 buffer. Subsequent dialysis steps were performed against 250 mM NaCl in 100 mM $NaHCO_3$ pH 8.3 buffer and 500 mM NaCl in 100 mM $NaHCO_3$ pH 8.3 buffer.

2.3.2 Activation of Sepharose CL4B and Coupling Reaction

Sepharose CL4B (Pharmacia Biotech) was activated with CNBr for the coupling of GST. 10 g of sepharose CL4B was washed with distilled water on a filter funnel. The sepharose was resuspended in 10 mL of 2M Na_2CO_3. The reaction was initiated by adding 1 g/mL CNBr dissolved in acetonitrile and allowed to proceed for 2 minutes with vigorous agitation. The activated sepharose was immediately washed under suction with ice-cold distilled water followed by coupling buffer (0.5 M NaCl, 0.1M $NaHCO_3$ pH 8.3). The activated sepharose was used immediately for coupling to GST. Approximately 8.5 mg of GST at 0.85 mg/mL in coupling buffer was added to 5 g of activated sepharose. The coupling reaction was performed for 24 hours at 4°C. The GST-sepharose was

washed with coupling buffer and 1 M ethanolamine pH 8.0. The GST-sepharose was blocked with 1 M ethanolamine pH 8.0 for 24 hours at 4°C.

2.4 Antibody Purification

Antibodies were purified using a sequential procedure involving Protein A and GST columns (Harlow and Lane, 1988). The Protein A sepharose (Pharmacia) column was equilibrated in 100 mM Tris pH 8.0 buffer. The serum was buffered with 100 mM Tris pH 8.0 as a final concentration. The buffered serum was incubated on the column for 1 hour with a continuous flow. The column was washed with 10 volumes of 100 mM Tris pH 8.0 and 10 mM Tris pH 8.0. The immunoglobulins were eluted with 100 mM glycine pH 3.0 and the acidic pH neutralized with one-tenth volume of 1M Tris pH 8.0. The samples containing the immunoglobulins were identified using a microtiter Bradford assay (Bio-Rad laboratories). The fractions containing protein were pooled for purification on the GST column. The GST-sepharose column was equilibrated with binding buffer (25 mM K_2HPO_4, 500 mM NaCl, 1% glycerol, 0.1% triton X-100, 0.2% SDS at pH 7.5) (Fisher and Smith, 1988). The antibody solution was incubated on the column for 90 minutes with a continuous flow. The unbound immunoglobulins were collected for analysis. The column was washed with 10 volumes of binding buffer. The anti-GST antibodies were eluted with ½ volume of regeneration buffer (50 mM glycine, 500 mM NaCl, 0.1% triton X-100, 0.02% SDS at pH 2.3) followed by ½ volume of binding buffer (Fisher and Smith, 1988). The acidic pH was neutralized by adding 1/10 volume of 1 M Tris pH 8.0. The column was equilibrated with binding buffer for further purification of sera.

2.5 Bacterial Extracts

For the analysis of antibody purity, bacterial lysates containing the GST-fusion proteins and maltose binding protein (MBP)-fusion proteins were produced. Besides the constructs described above (section 2.1), the second PDZ domain of hPTP1E (amino acid 1361-1461) was cloned in frame with the GST

gene in the pGEX-5X-3 plasmid. Also, the C-terminus of ZRP-1 containing the 3 LIM domains (amino acid 278 to 476) was cloned in frame with MBP in the pMAl-C2 plasmid. Bacteria cultures were induced for protein expression as previously described (section 2.1). After the induction period, the cultures were centrifuged and the bacterial pellet washed with PBS. Proteins were extracted with SDS sample buffer (20% v/v glycerol, 4% w/v SDS, 280 mM 2-mercaptoethanol, 14 mM bromophenol blue, 125 mM Tris pH 6.8). The samples were boiled for 5 minutes prior to loading of SDS-polyacrylamide gels.

2.6 Mammalian Cell Extracts

Total mammalian cell extracts were prepared using a confluent layer of cells growing in a 100 mm dish. The cells were washed with PBS and the proteins were extracted by adding 500 µl of SDS sample buffer. Only 1.5% of the cell extracts were loaded onto the gel.

2.7 Western Blots

Protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane using standard protocols (Ausubel *et al.,* 1998). The membrane was incubated overnight in blocking buffer [5% milk in TBST (150 mM NaCl, 0.1% Tween 20, 100 mM Tris pH 7.5)] at 4°C. The membrane was blotted with the primary antibody for 2 hours while the secondary antibody was added for 1 hour at room temperature. The purified antibodies were used at a concentration of 1:1000 to 1:5000 whereas the horse-radish peroxidase conjugated anti-rabbit immunoglobulin antibody was used at a dilution of 1 in 5000. All antibodies were diluted in blocking buffer. The proteins were revealed using an ECL kit from Amersham and autoradiography.

2.8 Co-Immunoprecipitation

2.8.1 Transfection of Mammalian Cells

The cell line 293S was transfected with expression vectors according to established protocols (Ausubel *et al.*, 1998). 293S cells were maintained in DMEM medium (Gibco BRL) supplemented with 10% fetal calf serum (Wysent). Cells growing exponentially were used for transfection. A total of 20 μg of DNA was used in each transfection of which 2 μg was pFRED25, an expression vector encoding GFP, used as an internal control. The DNA was precipitated at room temperature using a solution of 0.125 M $CaCl_2$ in a BBS buffer (50 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na_2HPO_4 pH 6.95). The precipitate was added to the cells dropwise and the cells were incubated at 37°C, 3% CO_2 overnight. The cells were washed with PBS and maintained in fresh medium at 37°C, 5% CO_2. The cells were harvested 48 hours post-transfection for immunoprecipitation studies.

2.8.2 Immunoprecipitation

Cells were transfected with pcDNA3 vectors expressing the LIM domains of ZRP-1 (aa 272-476) and the C-terminus of TSC2 (aa 1591-1784) with a hemaglutinin (HA)-tag at the N-terminus of the protein. The transfected cells were chilled on ice, washed with PBS and subsequently, lysed in PBS containing 1% triton X-100, 10 mM sodium pyrophosphate, 10% glycerol, 100 μM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/mL aprotinin. hPTP1E was immunoprecipitated using the purified antibody (1-2 μg of protein) and incubating the solution overnight at 4°C on an end-over-end rocker. The immunocomplexes were precipitated with protein A sepharose. The beads were washed 4 times with lysis buffer. The immunoprecipitated proteins were dissolved in SDS sample buffer and separated by SDS-polyacrylamide gel electrophoresis. The coimmunoprecipitated proteins were revealed by Western blotting with an anti-HA antibody (section 2.7). The ascites fluid from clone 9E10 was diluted 1:5.

2.9 Yeast Strains

The detection of protein-protein interactions was performed using two yeast strains, HF7c and SFY526. HF7c [*MAT***a,** *ura*3-52, *his*3-200, *ade*2-101, *lys*2-801, *trp*1-901, *leu*2-3, 112, *gal*4-542, *gal*80-538, *URA3::Gal*$4_{17\text{-mers(x3)}}$-*CYC*1-lacZ, *LYS2::GAL*1_{UAS}-*GAL*1_{TATA}-*HIS*3] was used to screen the cDNA library whereas SFY526 [*MAT***a,** *ura*3-52, *his*3-200, *ade*2-101, *lys*2-801, *trp*1-901, *leu*2-3, 112, *can*^{r}, *gal*4-542, *gal*80-538, *URA3::Gal*1_{UAS}-*GAL*1_{TATA}-lacZ] was employed for the control experiments.

2.10 Yeast Two-Hybrid Library Screening

For separate library screenings, the catalytic domain of GEF-5.1 (aa 722-1145) and the proline-rich region of ZRP-1 either the entire region (aa 1-274) or the N-terminal half (aa 1-156) were cloned into the pGBT9 plasmid in frame with the Gal4-DBD. The cDNA library from HeLa cells was prepared into pGADGH, a yeast GAL4 activation domain cloning vector containing a LEU2 gene marker (ClonTech Laboratories).

The library screening was performed according to the manufacturers' protocols. The plasmids were transformed sequentially into yeast using the lithium acetate method developed by Gietz and coworkers (1992). The bait was initially transformed into the yeast strain HF7c. A positive selection of the transformants was performed on W^- medium (1.7 g/L Difco yeast nitrogen base lacking amino acids and ammonium sulfate, 5.0 g/L ammonium sulfate, 1.3 g/L of dropout mix composed of 3% Ade, 3% Arg, 3% His, 4% Ile, 14% Leu, 4% Lys, 3% Met, 7% Phe, 29% Thr, 3% Trp, 4% Tyr, 3% Ura, 21% Val minus the amino acids under selection and 2% glucose). Clones negative for β-galactosidase expression were selected for transformation with the cDNA library. A saturated culture of the clone containing the bait plasmid was diluted to an OD_{600} of approximately 0.200 in 1L of YPD (20 g/L peptone, 10 g/L yeast extract, 2% glucose). The culture was incubated at 30°C with vigorous shaking until the OD_{600}

was approximately 0.500. The culture was centrifuged and the yeast pellet was washed sequentially with water and 1X TE/LiAc buffer (1 mM ethylenediamine tetraacetic acid (EDTA), 10 mM Tris-HCl, 100 mM lithium acetate pH 7.5). The yeast pellet was finally resuspended in 10 mL of 1X TE/LiAc which was added to a mixture of DNA containing 300 μg of cDNA library and 20 mg salmon sperm DNA diluted in 60 ml of polyethylene glycol (PEG)/ LiAc buffer (40% PEG 4000 in 1X TE/LiAc). The yeast were incubated at 30°C for 30 minutes with vigorous shaking. To the yeast-DNA mixture, dimethylsulfoxide (DMSO) was added to a final concentration of 10%. A heat shock of the yeast was performed for 15 minutes at 42°C. The solution was chilled on ice. The transformants were centrifuged and resuspended in 10 mL of TE (1 mM EDTA, 10 mM Tris-HCl pH 7.5). The transformants were plated on $L^-W^-H^-$ medium. An aliquot of the yeast was also plated on L^-W^- plates to determine the efficiency of transformation. After an incubation of 3 to 7 days at 30°C, the His^+ clones were tested for expression of β-galactosidase. The His^+/β-gal^+ clones were streaked on selective medium to isolate a single colony for characterization of the insert by polymerase chain reaction (PCR) and sequencing.

2.11 β-Galactosidase Assay

Yeast colonies were lifted onto nitrocellulose filters. The yeast cells were lysed by a rapid freeze-thaw cycle in liquid nitrogen. The filter was placed onto a filter presoaked with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as substrate in Z buffer (38 μM 2-mercaptoethanol, 0.8 mM X-gal, 10 mM KCl, 1 mM $MgSO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 60 mM $Na_2HPO_4.7H_2O$ pH 7.0). The filter was incubated at 30°C until the blue color developed.

2.12 Isolation of cDNA Library Plasmid, pGADGH

A single colony was used to segregate the two plasmids by a curing method. The clone was grown in YPD and an aliquot of the culture was plated on

L^- plates. The colonies were replica plated sequentially onto L^-W^- and L^- plates. Clones which could not sustain growth on L^-W^- plates were selected for plasmid purification. The DNA was purified according to a protocol described by Nasmyth and Reed (1980).

2.13 Amplification of pGADGH Plasmid in DH5α

An aliquot of the DNA was used to transform electrocompetent DH5α. A protocol devised by Dower and colleagues (1988) was optimized for plasmids greater than 5 kb. Instead of washing the bacteria pellet with water, 2 consecutive washes with 5% and 10% glycerol were performed. The electroporation conditions were as follows: 25 μF, 2.5 kV and 400 Ω. The transformants were incubated in SOC (20 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 8.6 mM NaCl, 2.5 μM KCl, 10 μM $MgCl_2$, 20 mM glucose) for 30 minutes at 37°C and plated on ampicillin selective medium. Colony PCR was performed on the amp^r colonies to identify clones containing a cDNA insert. Clones with a cDNA insert were grown in 5 mL of DYT containing 50 μg/mL ampicilin. Plasmid DNA was purified using an alkaline lysis method (Ausubel *et al.*, 1998). This DNA was used for transformation of SFY526 cells and DNA sequencing.

2.14 Yeast Two-Hybrid Control Experiments

The protein-protein interaction observed during the cDNA library screening was tested for specificity and potential autoactivation of the β-galactosidase gene by the library protein. Control experiments were performed in SFY526 whereby the following combinations of plasmids were transformed into the yeast: 1) pGADGH-prey alone, 2) pGADGH-prey +pGBT9-bait and 3) pGADGH+ pLAM (a vector encoding for the fusion of the Gal4-DBD with lamin C). The transformation protocol was down-scaled according to requirements. The clones were tested for β-galactosidase expression.

2.15 PCR

The polymerase chain reaction was performed according to standard protocols (Ausubel *et al.*, 1998). The template was either a purified plasmid or a bacteria colony. To the template, GAD2F (5`-CTATTCGATGATGAAGAT-ACCCCAC-3`) and GAD2R (5`-ACGACGTTGTAAAACGACGGCCAG-3`) primers were added at a concentration of 1 pmol/μL, dNTPs at a concentration of 200 μM, a PCR buffer from Pharmacia Biotech was diluted 10-fold and 2 units of Taq DNA polymerase were used. The PCR conditions were as follows: 5 minutes at 94°C followed by 25 cycles of 45s at 94°C, 45s at 52°C and 90s at 72°C, a final extension period was performed at 72°C for 7minutes. The PCR products were analyzed on agarose gel.

2.16 DNA Sequencing

DNA was purified from bacteria by alkaline lysis. The DNA was further purified by PEG precipitation. A 32 μL aliquot of DNA was precipitated by adding 8 μL of 4 M NaCl and 40 μL of 13% PEG 8000 and incubating on ice for 1 hour. The DNA pellet was washed with 70% ethanol and air dried. The sequencing reaction was performed using a dRhodamine kit (Perkin-Elmer). Briefly, approximately 500 ng of DNA was mixed with 8% DMSO, 1.6 pmoles of primer and 4 μL of the mix provided by the manufacturer (includes dNTPs, ddNTPs and enzyme) for a total volume of 10 μL. The reaction conditions were performed following the manufacturer's protocol. The resulting mix of cDNA fragments were separated by denaturing gel electrophoresis and the DNA sequence recorded using an automated ABI PRISM A377 sequencer.

2.17 Mutation Analysis

The S1070I point mutation within the GEF-5.1 clone used as bait for the yeast two-hybrid system screening was performed using sequential PCR reactions. The initial PCR reactions involved primers 5.1GEF.F (5'-

GGATCCCTGGAACACTCTC-3') and S1070I.R (5'-TGAATTTGTGCTTC-CTTGAATCAGTG-3') while the other reaction used primers 5.1GEF.R (5'-CTATAAATTCTTGGTCAA-3') and S1070I.F (5'-CGACAGAGGTCACTGAT-TCAAGGA-3'). The wild-type construct served as template for the reaction. The PCR conditions were as outlined in section 2.15. The PCR products from these two reactions were purified using the Geneclean II kit (BIO 101 Inc.). The full-length fragment was produced by using the PCR products from the initial reactions and the primers 5.1GEF.F and 5.1GEF.R. Slight modifications to the PCR reactions were made due to the small number of complementary bases near the site of mutation. The first three cycles were performed as follows: 30s at 94°C, 45s at 40°C and 90s at 72°C. The mutated product was further amplified by 25 cycles using the above conditions but with an annealing temperature of 55°C. The PCR product was purified using the Geneclean II kit and subsequently, introduced into pGEMT. The fragment containing the point mutation was introduced into the GEF5.1-pGBT9 construct by digesting both the latter pGEMT construct and the wild-type GEF-5.1-pGBT9 construct with *Nde*I and *Bgl*II for 2 hours at 37°C. The cDNA fragments from the enzymatic digestion were separated by agarose gel electrophoresis. The bands of the appropriate size were excised and the DNA purified from the agarose using the Geneclean II kit. The cDNA fragments were ligated and transformed into DH5α by heat shock as described by Inoue *et al.*(1990). The clones containing the point mutation were identified by restriction analysis using *Dde*I and verified by sequencing. The presence of a protein-protein interaction with the clone encoding 14-3-3ε was determined using the yeast two-hybrid system (see above for protocols).

2.18 Primary Sequence Analysis

The primary sequences of clone NtZRP-p33 and GEF-5.1 were compared to all the sequences within the GenBank database. Further, analyses for the identification of domains and motifs was performed using Profilescan software provided online at ulrec3.unil.ch/software/PRFSCAN_form.html.

Chapter 3: Results

3.1 Antibody Production

Antibodies have many applications from detection of proteins to their localization and interaction studies. While ZRP-1 and GEF-5.1 were isolated using the yeast two-hybrid system as proteins which interacted with the second PDZ domain of hPTP1E, little information about the function of these proteins is provided in the literature since both encode novel proteins. Antibodies were raised against the third LIM domain of ZRP-1 and the PDZ domain of GEF-5.1 to increase our repertoire of research tools for the study of these proteins (Figure 4). Besides antibodies against ZRP-1 and GEF-5.1, antibodies against the second PDZ domain of hPTP1E were previously produced in the laboratory.

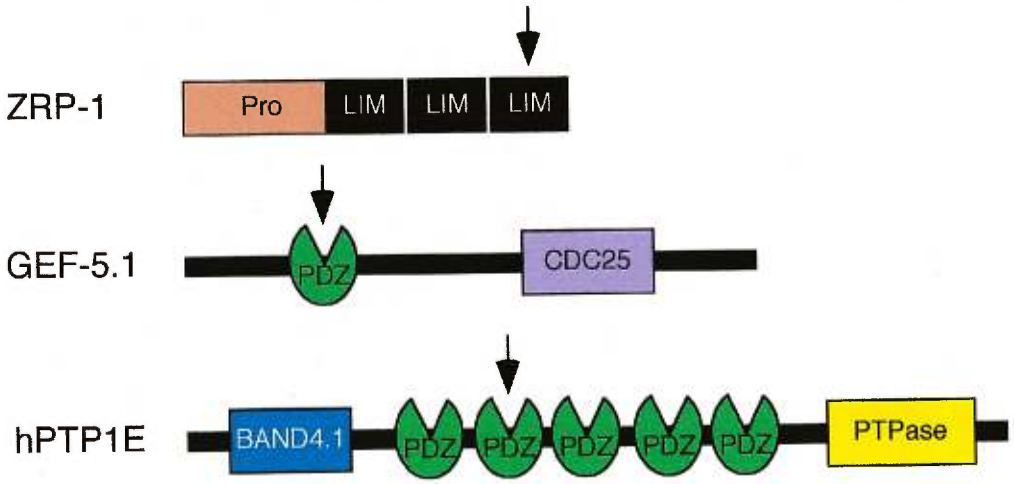

Figure 4- Schematic representation of the regions of ZRP-1, GEF-5.1 and hPTP1E used for antibody production. The domains are indicated by the arrow. Pro: Proline-rich region, CDC25: CDC25 homology domain, Band4.1: Region homologous to Band4.1 proteins.

3.1.1 Purification of GST Recombinant Proteins

The third LIM domain of ZRP-1 and the PDZ domain of GEF-5.1 were expressed as GST fusion proteins to facilitate the purification procedure. In both cases, proteins of the predicted molecular weight were expressed and purified to near homogeneity (Figure 5).

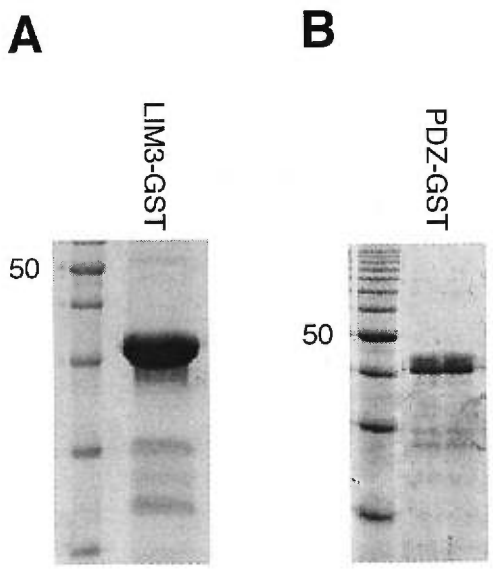

Figure 5- Purification of GST recombinant proteins. A) The LIM3 domain of ZRP-1 and B) the PDZ domain of GEF-5.1 were expressed in *E.coli* as a fusion with GST. The proteins were purified by affinity chromatography using glutathione beads. The purity of the proteins was verified by SDS-PAGE and coomassie stain.

Unfortunately, several contaminating proteins were observed by SDS-PAGE. These proteins may represent degradation products as the contaminants had a lower molecular mass than the protein expressed. The purified sample of LIM3-GST displayed a protein of approximately 33 kDa on SDS-PAGE. Gel separation of proteins co-purifying with the PDZ-GST fusion revealed a doublet at approximately 40 kDa. It is unlikely that the difference in migration may be reasoned by post-translational modifications of the proteins. The yield of each

purified recombinant protein as determined by the Bradford assay was 13 mg of LIM3-GST and 4.5 mg of PDZ-GST per liter of bacteria culture.

3.1.2 Antibody Titer in Sera

The purified proteins were used to immunize 2 rabbits for the production of polyclonal antibodies. The sera collected from each rabbit was tested for its ability to recognize the recombinant protein in a total bacterial extract. After the second bleeding, the antibody titer in the sera of each animal was high enough to detect the antigen (Figure 6). Further, a proportion of the antibodies recognized specifically the GST moiety.

3.1.3 Antibody Purification

In order to determine the contribution of antibodies specifically recognizing the portion of the fusion protein belonging to ZRP-1 and GEF-5.1 and to decrease the background created by the GST antibodies, the sera was fractionated into its two main components. Two sequential purification steps involving a protein A sepharose column and a GST affinity column were used. The separation of the antibodies was followed by Western blot analysis after each purification step. The results for the sera obtained from one rabbit immunized with LIM3-GST are provided in figure 7. Similar results were obtained for the purification of the antibodies against the PDZ domains of GEF-5.1 and hPTP1E. The results show that both immunoglobulins bind to protein A sepharose but only the antibodies recognizing GST bind to the GST affinity column. Using both columns, the anti-GST immunoglobulins could be separated from the anti-ZRP-1 immunoglobulins very efficiently. There was no indication of any significant antibody loss during the purification procedure.

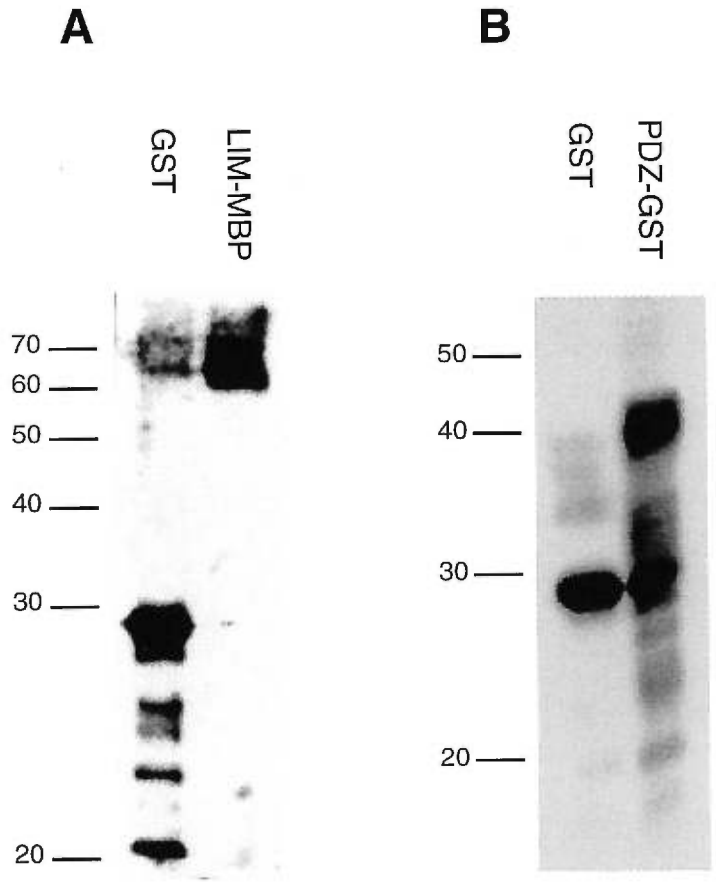

Figure 6- The antibodies recognize the fusion proteins injected in the rabbits. Total cell extracts of *E. coli* expressing GST, or the PDZ-GST, or LIM1-2-3-MBP fusion proteins were separated by SDS-PAGE and transferred onto nitrocellulose. The proteins were detected by chemiluminescence and autoradiography. A= anti-ZRP-1 antibody, B= anti-GEF-5.1 antibody.

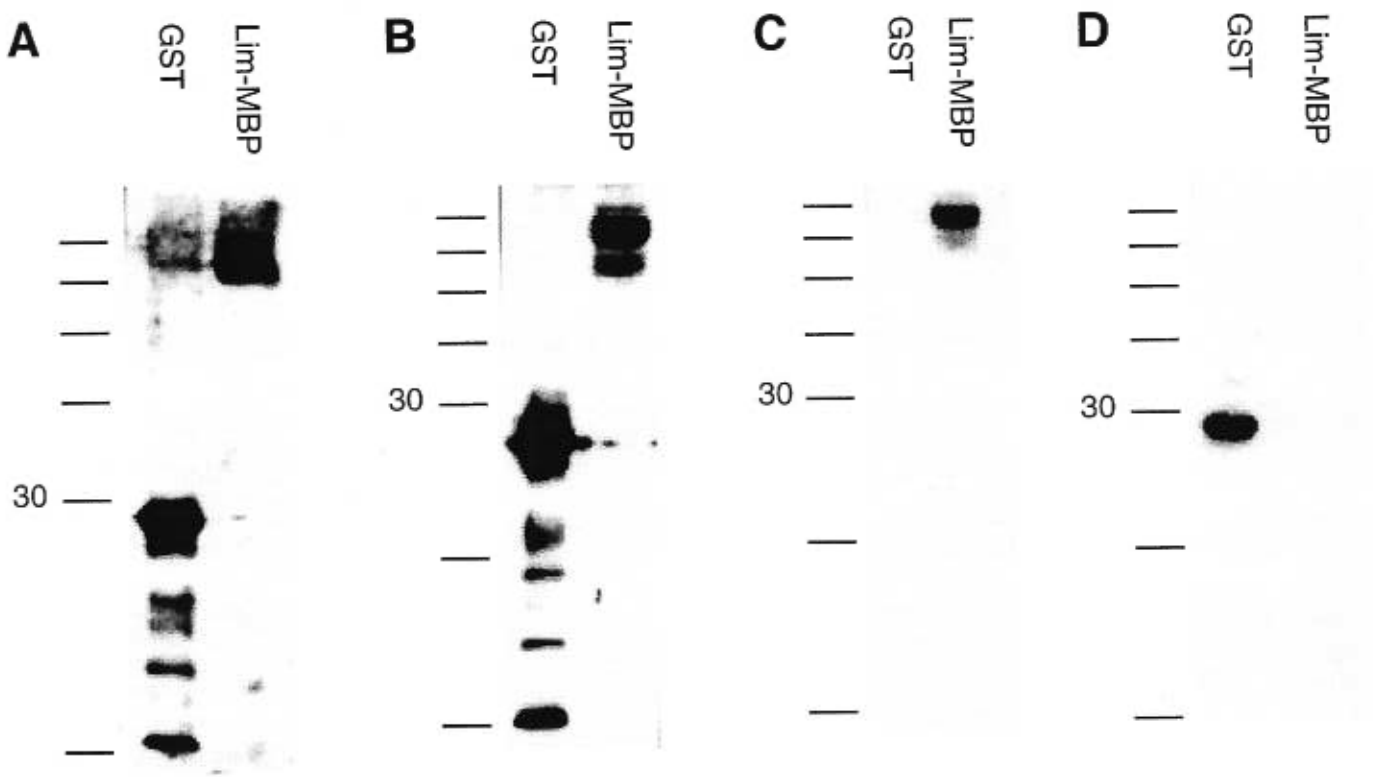

Figure 7- Purification of antibodies against the LIM3 domain of ZRP-1. The antibodies were purified using a two-step process. A proteinA-sepharose column was used in conjunction with a GST affinity column. The segregation of the anti-ZRP-1 and anti-GST antibodies was followed by Western blot. In lane 1, a bacterial extract containing GST and in lane 2, a bacterial extract containing the LIM3-MBP fusion protein were loaded on gel. The antibody fractions from each purification step were used to blot. A= crude serum, B= an aliquot from the immunoglobulins eluted from the proteinA-sepharose column, C= flow through from GST column, D= fraction eluted from GST column.

3.1.4 Proteins Detected in Mammalian Cell Extracts by Antibodies

Although the antibodies recognized the recombinant proteins used for the immunization of the rabbits, it was not clear whether they could recognize mammalian proteins. Further, the main disadvantage of polyclonal antibodies is a high background due to the mixture of immunoglobulins capable of recognizing a large range of epitopes. It is possible that other proteins besides the protein of interest may be detected during different experiments making the results difficult to interpret.

These questions were answered by analysing the proteins detected by the antibodies in different cell lines. A number of cell lines from a wide range of origins were used to screen the expression of these proteins including CHO, COS1, 293S, HeLa and MCF7 cells derived from Chinese hamster ovaries, monkey kidneys, human fetal kidney, human cervival cancer tissue and a lung carcinoma, respectively. A ubiquitous expression pattern is observed for proteins detected by the antibodies raised against GEF-5.1 and hPTP1E. However, the proteins recognized by antibodies against ZRP-1 are restricted to a few cell lines. The proteins are not expressed in CHO, COS1 or 293S cells but were detected in HeLa and MCF7 cells (Figure 8A).

The molecular weight of the proteins detected by each antibody was approximated by means of standards. The anti-ZRP antibodies recognized two proteins of approximately 50k Da. The relative size of the slower migrating protein corresponds to the predicted molecular weight of ZRP-1. It is unlikely that the proteins detected are both ZRP-1 with a differential phosphorylation state because the mobility shift is greater than normally contributed by phosphate groups. The identity of the smaller protein is unknown but may possess a domain with homology to the third LIM domain of ZRP-1.

The anti-GEF-5.1 antibody also detects two proteins with a large difference in molecular weight (Figure 8B). The largest protein has a mass of approximately 90 kDa while the smaller protein has a mass of 38 kDa. Unfortunately, neither of these proteins has a mass corresponding to the one

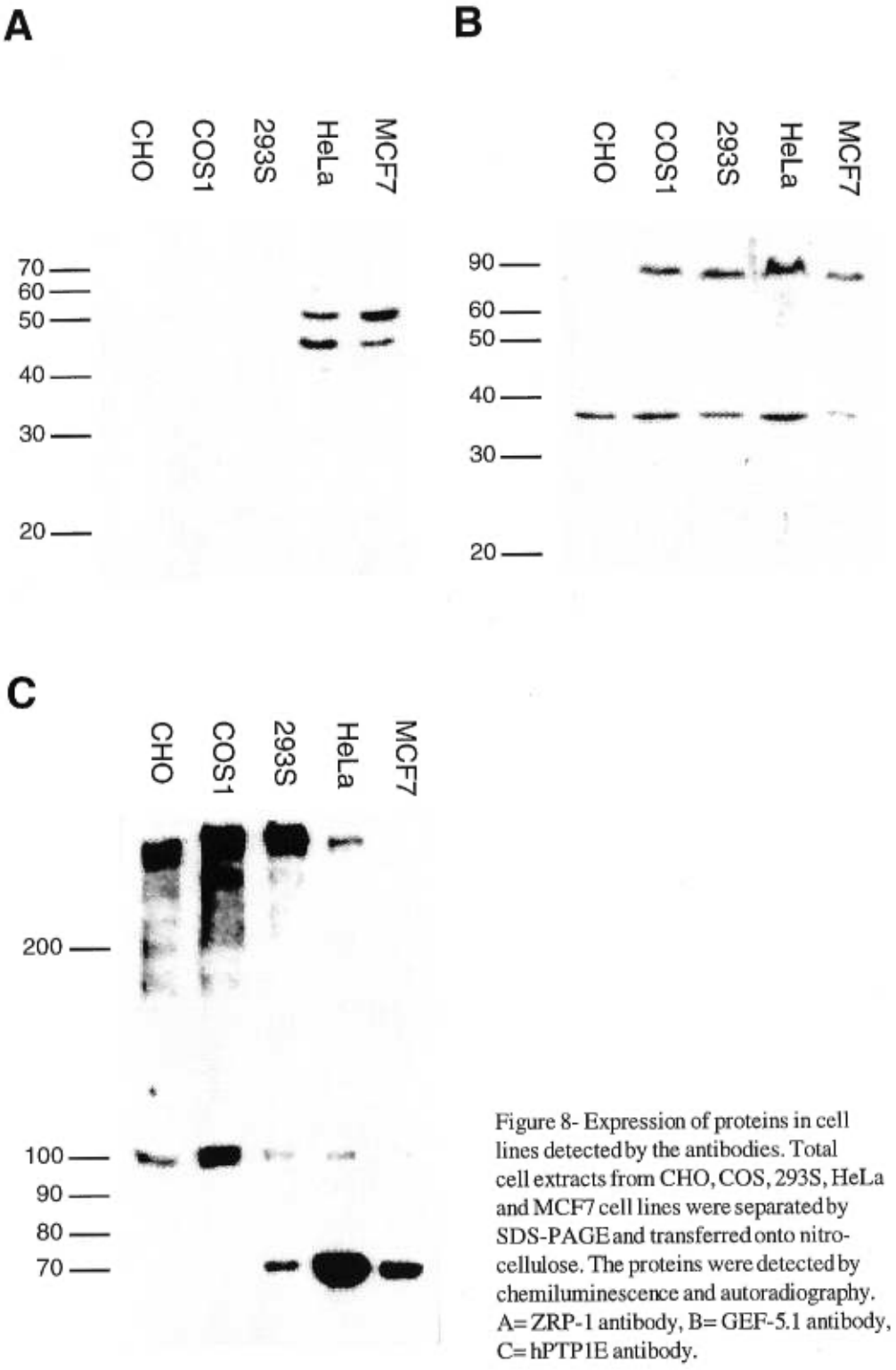

Figure 8- Expression of proteins in cell lines detected by the antibodies. Total cell extracts from CHO, COS, 293S, HeLa and MCF7 cell lines were separated by SDS-PAGE and transferred onto nitrocellulose. The proteins were detected by chemiluminescence and autoradiography. A= ZRP-1 antibody, B= GEF-5.1 antibody, C= hPTP1E antibody.

predicted for GEF-5.1 which is 175 kDa.

The expression of proteins recognized by anti-hPTP1E is ubiquitous within the cell lines tested (Figure 8C). Three different proteins were detected with a mass of approximately 70 kDa, 100 kDa and 275 kDa. The predicted mass of the full-length hPTP1E protein is 275 kDa which corresponds to the slower migrating protein. The observation of alternatively spliced variants of the hPTP1E protein has been reported previously (Banville *et al.*, 1994; Maekawa *et al.*, 1994). The hypothesis remains as to whether the lower molecular weight proteins are a product of alternative splicing. Interestingly, the ratio of the concentration of each protein changes between cell lines. The 293S and HeLa cell lines express all three proteins. The 275 kDa protein has the highest level of expression in 293S cells whereas HeLa cells express the 70 kDa protein in greatest yield. All other cell lines tested express only a subset of these proteins. MCF7 cells express only the 70 kDa protein whereas the CHO and COS1 cells express both the 275 kDa and 100 kDa protein. It will be interesting to determine if the change in ratio of the relative expression levels of the different proteins has any functional significance.

3.2 Co-Immunoprecipitation Studies

Yeast two-hybrid studies revealed interactions between the second PDZ domain of hPTP1E and both, ZRP-1 and GEF-5.1 while the fourth PDZ domain of hPTP1E interacts with tuberin (Banville, unpublished; Murthy *et al.*, 1999). To confirm that the interactions also occur *in vivo,* the relevant regions of ZRP-1, GEF-5.1 and TSC2 were cloned into an expression vector such that a fusion with a HA-tag was formed. The co-immunoprecipitation of these proteins with hPTP1E was monitored by Western blot analysis. Expression of the fusion protein within cells transfected with the GEF-5.1 construct was undetectable. The results of the immunoprecipitation studies with cells transfected with ZRP-1 and TSC2 are provided in figure 9. In panel A, it is shown that hPTP1E is immunoprecipitated only in the presence of antibody. No protein is detected in the lanes where only protein A sepharose was added to the sample. Another blot

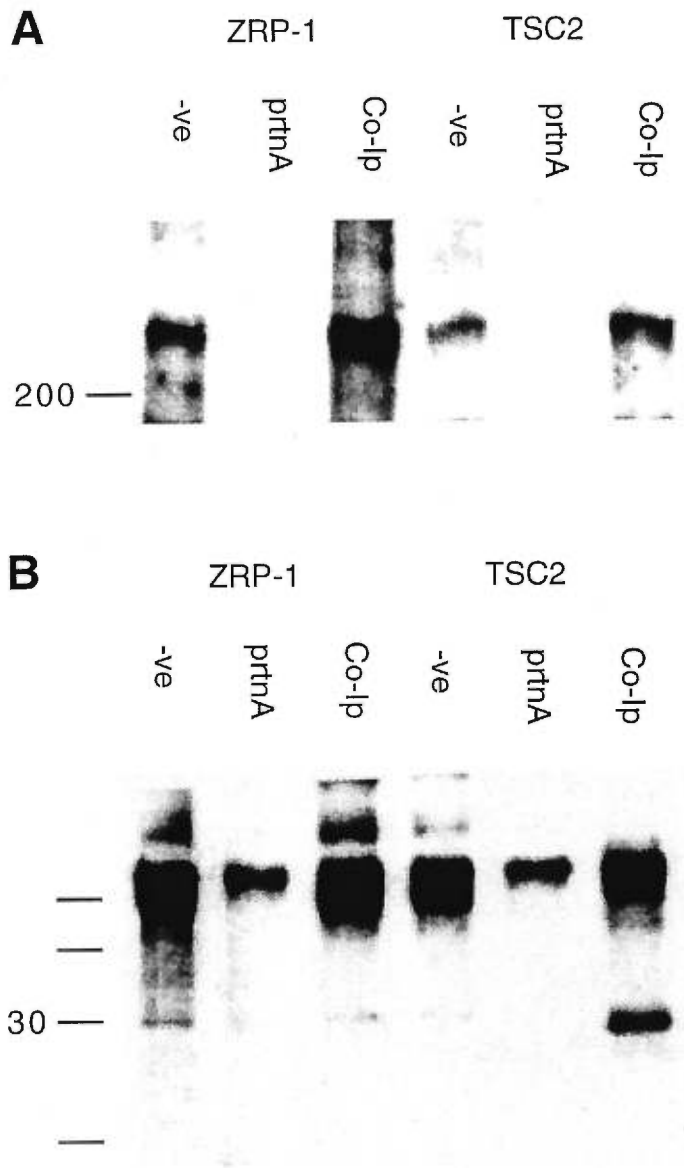

Figure 9- Co-immunoprecipitation of hPTP1E with ZRP-1 and TSC2. The three LIM domains of ZRP-1 and the C-terminus of TSC2 were expressed in 293S cells as a fusion protein with a HA-tag. The endogenous hPTP1E protein was precipitated and the co-precipitating proteins were detected by Western blot. The co-immunoprecipitation of the proteins is depicted in the lane called Co-Ip. Control experiments include the co-immunoprecipitation of proteins from non-transfected cells (lane -ve) and an experiment where the precipitating antibody was omitted (lane prtnA). In panel A, the detection antibody recognizes hPTP1E whereas the one used for panel B detects the HA-tag.

containing the same samples was probed with an antibody raised against the HA-tag to identify co-immunoprecipitating proteins. The fusion proteins have a predicted mass of approximately 30 kDa. It is evident that the C-terminal region of ZRP-1 did not interact with hPTP1E under these experimental conditions. However, a specific band at 30 kDa was observed for the experiment involving TSC2. The C-terminus of tuberin associates *in vivo* with hPTP1E. The fusion protein did not interact with protein A resulting in its precipitation nor does hPTP1E directly interact with the hemaglutinin moeity of the fusion protein.

3.3. Yeast Two-Hybrid System

3.3.1 Proline-Rich Region of ZRP-1

The main application of the yeast two-hybrid system is to isolate cDNA fragments encoding a protein which interacts with a protein of interest (Chien *et al.*, 1991). A library screening using the interaction trap with the second PDZ domain of hPTP1E as bait identified an interaction with the C-terminal half of ZRP-1 which contains three LIM domains (Murthy *et al.*, 1999). The structure of ZRP-1 also includes a proline rich region at the N-terminus. These regions serve as ligands for SH3 and WW domains. To identify potential interacting partners of ZRP-1 via this region, the yeast two-hybrid system was used to screen a HeLa cDNA library. When the entire amino half (aa 1-274) of ZRP-1 was expressed in yeast cells, it was lethal and no transformants could be isolated after numerous attempts. A positive control was performed to ensure that the yeast cells were competent and the plates contained the appropriate nutritional selection.

A truncated form of the proline rich region was subsequently used as bait for the yeast two-hybrid system. Transformants were obtained at a frequency similar to other clones. However, the doubling time of the yeast cells had increased. Finally, the proline rich region did not serve as an activation domain or aggregate host transcription factors to the LacZ promoter as the transformants were negative for the expression of β-galactosidase. This bait fulfilled all the requirements to be successfully utilized in the yeast two-hybrid system.

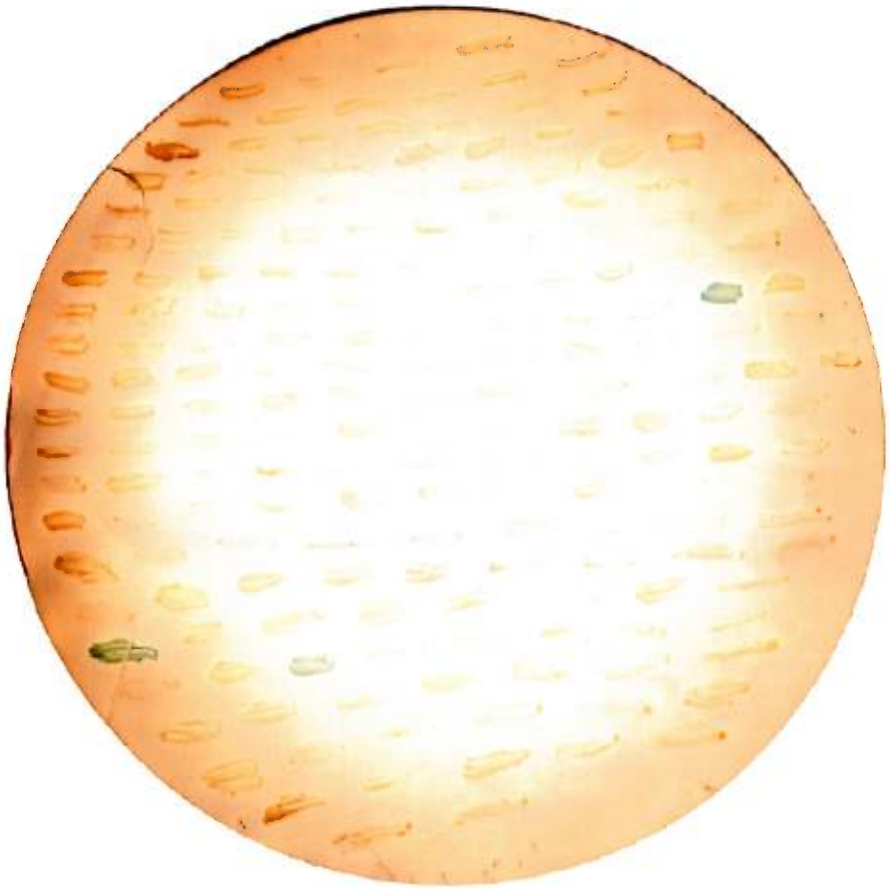

Figure 10- A representative β−galactosidase filter assay performed during a yeast two-hybrid cDNA library screening. The yeast colonies were transferred to a nitrocellulose filter. After a freeze-thaw cycle, the filter was overlayed onto a filter pre-soaked with X-gal. The blue colonies represent potential candidates.

A HeLa cell cDNA library was transformed into yeast expressing the amino half of the proline-rich region (aa 1-156) of ZRP-1 as a fusion with the GAL4-DBD. A total of 12 million clones were screened for their ability to activate the reporter genes. Several hundred clones grew on His^- plates but only 16 were also capable of activating the expression of β-galactosidase. A representative β-galactosidase filter assay is provided in figure 10. Only the blue colonies were subsequently analyzed as the white colonies are false positives.

Table 1- Identity of clones demonstrating an interaction with the proline rich region of ZRP-1

Representative clone	No. of Times Isolated	Homology to known protein
NtZRP-207	9	MCM7
NtZRP-3018	2	human quinone oxidoreductase
NtZRP-3094	2	human deocystidine kinase
NtZRP-3276	1	human succinate dehydrogenase
NtZRP-p33	1	proteins with SH3 domains

Initial characterization of the clones involved the determination of the cDNA sequence of the insert. On the basis of their sequence, the clones were classified into 5 groups. The results are presented in table 1. Several of the clones were isolated on multiple occasions whereas others were obtained only once. In addition, different cDNA extension products were isolated which originated from the same gene. The origin of the inserts was determined by aligning the cDNA sequence against those present in the GenBank database. All the clones except for NtZRP-p33 encode proteins which lack an SH3 or WW domain. The group with the greatest variation in sequence encoded segments of MCM7 which is a member of the minichromosome maintenance gene family (figure 11). A total of 9 clones were isolated independently. While certain clones were identical, others contained additional sequence towards the 5' end of the gene. The longest clone has an open reading frame of 275 amino acids while the shortest clone has an open reading frame of 122 amino acids. No structural motifs were identified within this region.

Other clones lacking an SH3 domain encoded segments of enzymes either required for DNA replication such as deoxycystidine kinase or involved in metabolism such as succinate dehydrogenase.

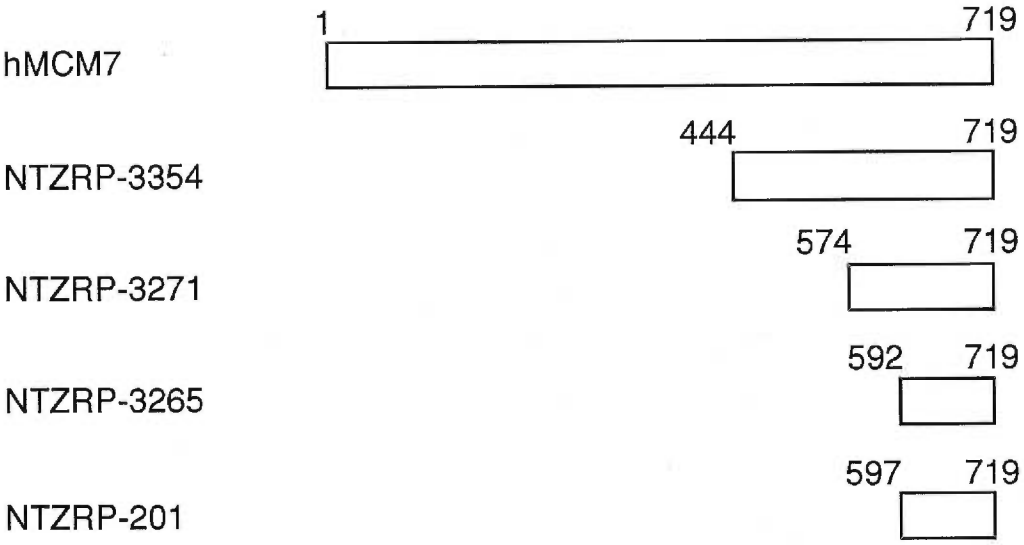

Figure 11- Schematic representation of the MCM7 clones isolated using the yeast two-hybrid system with the N-terminal region of ZRP-1 serving as bait. The numbers refer to the corresponding amino acids within the primary sequence of MCM7.

Finally, the clone NtZRP-p33 was derived from a novel gene. The clone has an open reading frame of 185 amino acids without an initiation codon indicating the absence of coding sequence at the N-terminus. The sequence of this clone is identical to a portion of the clone KIAA0769 which contains additional sequence at both the 5' and 3' ends (Nagase *et al.*, 1998). The predicted primary structure of NtZRP-p33 demonstrates homology towards numerous signal transduction molecules which contain an SH3 domain. The analysis of the primary structure of the protein using software such as Profilescan predicted the presence of a SH3 domain within the sequence of NtZRP-p33. It is encoded between amino acid 83 and 144 as depicted in figure 12. Interestingly, a similar analysis of the primary structure of KIAA0769 revealed the presence of a second SH3 domain at the carboxyl terminus. It is important to note that the NtZRP-p33 clone contains

A

```
1     TG AAA GCT GAA GCC CGG TTG GAC CTG CTA AAG CAG ATT GGT GTT TCT
        K   A   E   A   R   L   D   L   L   K   Q   I   G   V   S   15

48    GTG GAC ACA TGG CTA AAG AGT GCC ATG AAC CAA GTA ATG GAA GAA CTG
        V   D   T   W   L   K   S   A   M   N   Q   V   M   E   E   L   31

96    GAA AAT GAG CGA TGG GCC CGC CCT CCT GCA GTG ACC AGT AAT GGC ACT
        E   N   E   R   W   A   R   P   P   A   V   T   S   N   G   T   47

144   TTA CAC TCG CTT AAT GCA GAT ACC GAA AGA GAA GAA GGC GAA GAG TTT
        L   H   S   L   N   A   D   T   E   R   E   E   G   E   E   F   63

192   GAA GAT AAC ATG GAT GTT TTC GAT GAC AGC AGT TCC AGC CCT TCT GGC
        E   D   N   M   D   V   F   D   D   S   S   S   S   P   S   G   79

240   ACC TTA AGA AAT TAT CCA CTC ACC TGC AAA GTT GTT TAT TCC TAC AAG
        T   L   R   N   Y   P   L   T   C   K   V   V   Y   S   Y   K   95

288   GCT TCT CAA CCA GAT GAG TTG ACC ATT GAG GAA CAT GAG GTG TTA GAA
        A   S   Q   P   D   E   L   T   I   E   E   H   E   V   L   E   111

336   GTG ATT GAA GAT GGA GAT ATG GAA GAC TGG GTA AAG GCT CGA AAT AAA
        V   I   E   D   G   D   M   E   D   W   V   K   A   R   N   K   127

384   GTT GGC CAA GTG GGT TAT GTG CCA GAA AAG TAC CTA CAG TTT CCC ACC
        V   G   Q   V   G   Y   V   P   E   K   Y   L   Q   F   P   T   143

432   TCG AAC AGC CTC CTG AGC ATG CTG CAG TCC CTG GCC GCT TTG GAC AGT
        S   N   S   L   L   S   M   L   Q   S   L   A   A   L   D   S   160

480   CGG TCA CAC ACG TCC AGC AAT TCC ACG GAA GCA GAA CTC GTT TCA GGC
        R   S   H   T   S   S   N   S   T   E   A   E   L   V   S   G   177

528   AGC CTC AAC GGA GAT GCC AGT GGT AAA GAC TGA AAC AAG GCA CTC TTT
        S   L   N   G   D   A   S   G   K   D   -                       194

576   ATT TGG TCA CGT TTG ACC TAA ACT GTA TTG CCC TGG AGT AAG CCT TCA

624   GTA GGA TAA AAA GCA CAA CTT TCC TCC TGT CCT ATA ATA TGC TTT TCT

672   TTA TAT GGT CAC GTT TGT CCT GCA CAG TAG AAC CCT AAA GTT AAT CAT

720   TCA TCA GTG AGT AAA AAA ATT ACA ACT TTA ATC CT
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B

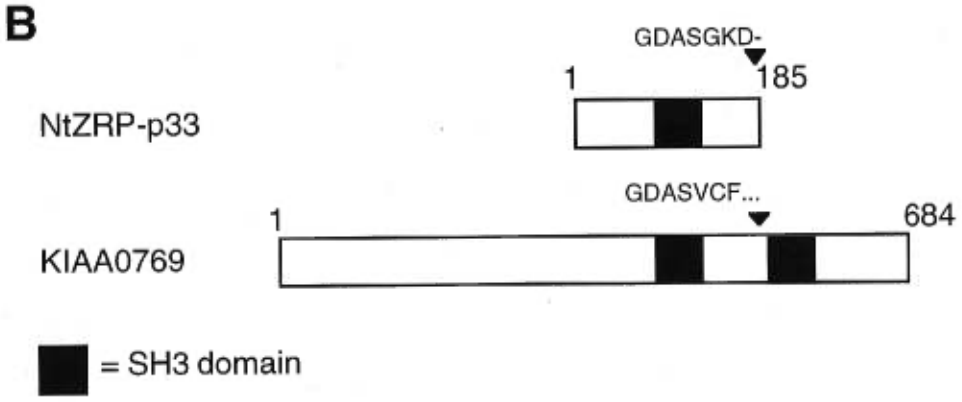

Figure 12- Analysis of clone Nt-ZRP-p33. A) The cDNA sequence of the clone along with the predicted primary structure of the protein are presented.The SH3 domain is underlined. B) A schematic representation of the sequence overlap between clone NtZRP-p33 and KIAA0769. The arrow indicates the location where the sequences of the two proteins differ.

```
1 LYN          -EQGDIVVALYPYDG IHPDDLSFKKGEKMK VL---EEHGEWWKAK SLLTKKEGFIPSNYV AKLNT--
2 SRC          -GGVTTFVALYDYES RTETDLSFKKGERLQ IVN--NTEGDWWLAH SLSTGQTGYIPSNYV APSDS--
3 NCK1         -AEEVVVVAKFDYVA QQEQELDIKKNERLW LL---DDSKSWWRVR NSM-NKTGFVPSNYV ERKNS--
4 GRB2.1       ----MEAIAKYDFKA TADDELSFKRGDILK VLN--EECDQNWYKA ELN-GKDGFIPKNYI EMKPH--
5 KIAA0769     DASVCFVKALYDYEG QTDDELSFPEGAIIR ILNKENQDDDGFWEG EFN-GRIGVFPSVLV EELS---
6 GRB2.2       -QQPTYVQALFDFDP QEDGELGFRRGDFIH VMD---NSDPNWWKG ACH-GQTGMFPRNYV TPVNRNV
7 NCK2         ----------FNYMA EREDELSLIKGTKVI VM---EKCSDGWWRG SYN-GQVGWFPSNYV TEEGDS-
8 NCK3         -QVLHVVQALYPFSS SNDEELNFEKGDVMD VIEKPENDPEWWKCR KIN-GMVGLVPKNYV TVMQN--
9 ABL          -NDPNLFVALYDFVA SGDNTLSITKGEKLR VLG--YNHNGEWCEA QTKNGQ-GWVPS-YI TPVNS--
10 NtZRP-p33   -NYPLTCKVVYSYKA SQPDELTIEEHEVLE VIE--DGDMEDWVKA RNKVGQVGYVPEKYL QFPTSNS
                    *** * *   * ***  *** *  **         *        ** **** ***
```

Figure 13- Sequence alignment of SH3 domains from signal transduction molecules. The residues conserved within 70% of the sequences are indicated with an asterisk.

numerous stop codons at the 3' end strongly suggesting the end of the open reading frame. The presence of additional sequence at the 3' end of KIAA0769 may be a result of differential splicing.

The sequence of SH3 domains from different genes were aligned and presented in Figure 13. The level of identity between any two SH3 domains is within the range of 20 to 40%. However, a number of residues with similar physical and chemical characteristics are often substituted. All the sequences present were derived from human genes but a similar degree of homology is noticed upon alignment of SH3 domains originating from different species such as *Drosophila melanogaster, dictyostelium discoideum* and *Caenorhabditis elegans.* The important criteria for proper SH3 function is the overall secondary structure described as a β-barrel.

Table 2 - Yeast two-hybrid system control experiments for the clones isolated using the N-terminus of ZRP-1.

Clone #	Alone	+Bait	+pLam
NtZRP-207	-	+	-
NtZRP-3018	-	+	-
NtZRP-3094	-	+	-
NtZRP-3276	-	+	-
NtZRP-p33	-	+	-

In the yeast two-hybrid system, the fusion proteins are over-expressed and targeted to the nucleus. Therefore, there is a potential for the detection of non-specific interactions. The validity of the interactions was determined using the yeast strain SFY526 under controlled conditions. The results are presented in Table 2 for representative clones classified according to their sequence. The expression of β-galactosidase is reported as a (+) for blue colonies and (-) for white colonies. None of the clones served as DNA-binding domains capable of associating with Gal4 binding sites nor aggregate host transcription factors as no β-galactosidase expression was observed when the library plasmid encoding the prey was transformed into yeast alone. The specificity of the interactions was

monitored by cotransforming SFY526 with the prey plasmids and pLAM which encodes Lamin C as a fusion with the gal4 DNA-binding domain. No interaction was observed between Lamin C and any of the clones isolated during the library screening. The cDNA library clones interacted solely with the proline rich region of ZRP-1.

3.3.2 CDC25 domain of GEF-5.1

The sequence of GEF-5.1 contains a putative catalytic domain with homology to CDC25 which is a guanidine nucleotide exchange factor (GEF). In an attempt to isolate GTPases which may serve as substrates for GEF-5.1 or regulatory molecules of GEF-5.1 activity, a HeLa cell cDNA library was screened using the yeast two-hybrid system with the CDC25 homology domain (aa 722-1145) of GEF-5.1 as bait. After screening 4.6 million clones, a total of 22 His^+/ β-gal^+ clones were identified.

Table 3- Identity of clones isolated during a yeast two-hybrid screening of a Hela cDNA library using the CDC25 homology domain of GEF-5.1.

Representative clone	No. of Times Isolated	Homology to known protein
GEF-1.08	5	14-3-3ε
GEF-3.29	1	KIAA0269

The clones were categorized according to their cDNA sequence. Only two different sequences were isolated during the screening (Table 3). After alignment of the sequences against the DNA sequences present in the GenBank database, the two sequences were identified as 14-3-3ε and KIAA0269. The cDNA library clone corresponding to 14-3-3ε was isolated 5 times while the one for KIAA0269 was isolated only once. The members of the 14-3-3 family regulate the function of signalling molecules (Burbelo and Hall, 1995; Morrison, 1994; Reuther and Pendergast, 1996). KIAA0269 is a cDNA sequenced by Nagase *et al.* (1996) during a bulk sequencing project. To date, it is the only documented information about this clone. The protein encoded by this gene is rich in proline residues

which constitute about 18% of the primary structure. The biological function of this protein remains unknown.

Table 4 - Yeast two-hybrid system control experiments for the clones isolated using the CDC25 homology domain of GEF-5.1.

Clone #	Alone	+Bait	+pLam
GEF-1.08	-	+	-
GEF-3.29	-	+	-

Control experiments were performed to determine whether the cDNA library clone could serve as a DNA-binding domain and whether the interaction between the CDC25 homology domain and the prey were specific. In both cases, the cDNA library clones did not interact with Lamin C and could not activate the expression of β-galactosidase independently of the bait (Table 4). Blue colonies were observed only when the yeast was cotransformed with the bait and prey plasmids.

Table 5- Mutation analysis of the protein-protein interaction between the CDC25 homology domain of GEF-5.1 and 14-3-3ε using the yeast two-hybrid system.

		β-Gal
Consensus:	R S X pS X P	
GEF-5.1:	R S L S Q G	+
Mutation:	R S L I Q G	-

The residues required for an interaction between GEF-5.1 and 14-3-3ε were determined using the yeast two-hybrid system. Interactions involving members of the 14-3-3 family is usually mediated by a consensus sequence described by the sequence R S X pS X P where the second serine residue is phosphorylated (Muslin *et al.*, 1996). The phosphorylation event is crucial for an interaction to occur and serves as a regulatory mechanism. A similar sequence

was noticed within the primary structure of GEF-5.1. The sequence was R S L S Q G which deviates slightly from the consensus sequence. It has been reported that the proline residue may be substituted in certain cases for other amino acids without significant effects on the binding affinity (Muslin *et al.*, 1996). In order to confirm that this motif was recognized by the 14-3-3ε clone, the second serine residue was mutated to an isoleucine. This mutation prevented 14-3-3ε from interacting with GEF-5.1 as no β-galactosidase was expressed (Table 5).

3.4 ProfileScan

At the time, a partial cDNA clone of GEF-5.1 was isolated during a yeast two-hybrid library screening. The full-length sequence was subsequently cloned in the laboratory. The predicted open reading frame is 1601 amino acid with a calculated mass of 175kDa. During database searches, it was evident that a putative catalytic domain with GEF activity was present. However, no other information was available about the protein structure. A computer program available on the world wide web called ProfileScan predicts the presence of putative functional motifs and domains within the primary structure of a protein.

After analysis of the amino acid sequence of GEF-5.1, numerous domains were identified. These regions have been highlighted in figure 14. The structure of GEF-5.1 includes a cNMP binding domain from amino acid 280 to 380, a LTE domain from amino acid 419 to 453, a PDZ domain from amino acid 662 to 669, a ras-associated domain from amino acid 749 to 835 and a CDC25 homology domain from amino acid 859 to 1059. In addition to the domains predicted by computer software, a consensus binding motif for 14-3-3 proteins from amino acid 1067 to 1072 and finally, a consensus binding site for PDZ domains at the C-terminus were defined experimentally. This elaborate primary structure has revealed speculations about the potential function and regulation of GEF-5.1.

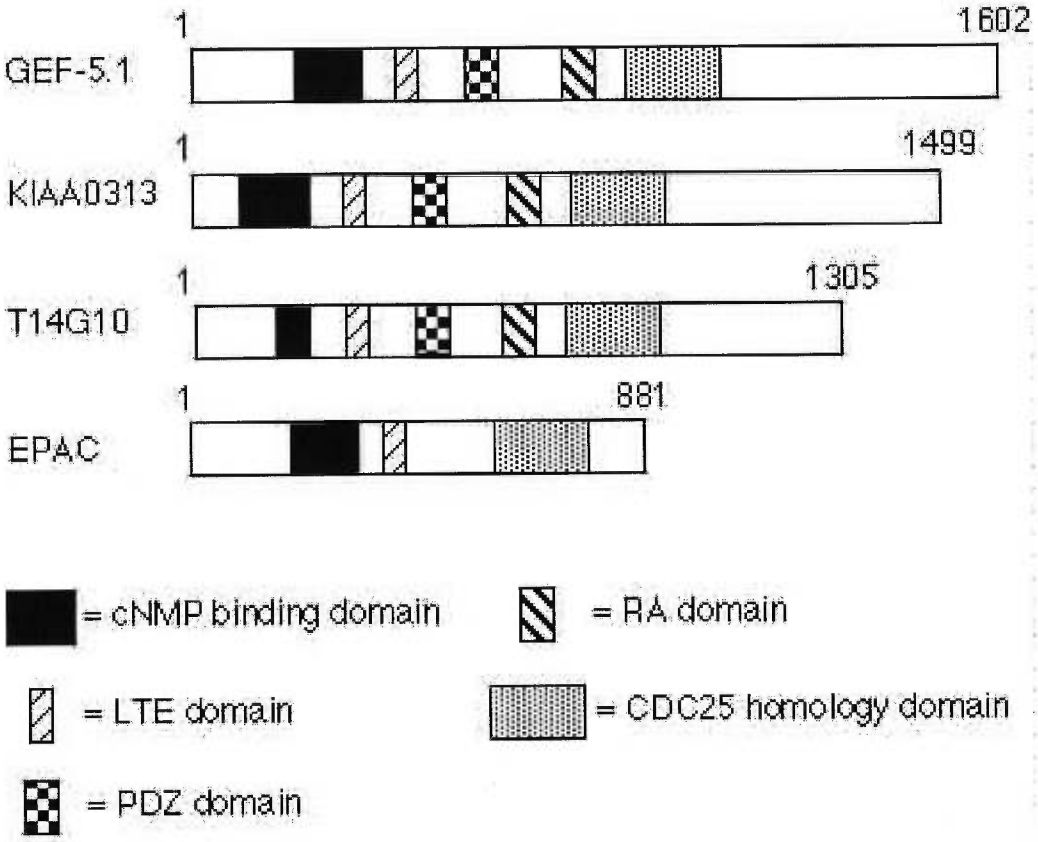

Figure 14- Comparison of the protein structure of GEF-5.1 with *the Caenorhabditis elegans* clone T14G10.2, a predicted human open reading frame KIAA0313 and EPAC, a guanidine nucleotide exchange factor for Rap1.

During a comparison of the primary sequence of GEF-5.1 with sequences present in the GenBank database, a number of genes displayed homology to this novel protein. The most notable were KIAA0313, a cDNA sequence defined during bulk sequencing, T14G10.2, a *Caenorhabditis elegans* clone and EPAC, a guanidine nucleotide exchange factor for Rap1 (de Rooij *et al.*, 1998; Nagase *et al.*, 1997; Wilson *et al.*, 1994). These sequences were also analyzed using ProfileScan. Interestingly, the structure of KIAA0313 and T14G10.2 were similar to that of GEF-5.1 whereas the structure of EPAC is significantly different. KIAA0313 and T14G10.2 have all the domains arranged in the same manner as GEF-5.1 but no consensus sequence for the binding of 14-3-3 proteins was

noticed. The structure of EPAC lacks primarily a ras-associated domain and the CDC25 homology domain but includes a cAMP-binding domain.

Chapter 4: Discussion

4.1 Antibody Production

Antibodies have become an indispensable research tool for studying specific proteins. The primary use of antibodies is the detection of proteins within protein extracts, blood, cells or tissues. The different applications provide a wealth of information about the protein (Harlow and Lane, 1988). The concentration of specific proteins is quantified using an enzyme-linked immunosorbent assay, their identity is confirmed by Western blot, their localization within cells is detected by immunocytochemistry and the expression of proteins in tissue can be monitored by immunohistochemistry. Immunoprecipitation is a method for purifying proteins and the multiprotein complexes present within the cell. Finally, the activity of specific signal transduction pathways may be regulated by antibodies. This polyvalent usage of antibodies and the simple methods for their production have made the development of specific immunoglobulins a routine process.

4.1.1 Recombinant Protein Purification

Since ZRP-1 and GEF-5.1 were novel genes, polyclonal antibodies were raised in rabbits. The antigens were expressed in *Escherichia coli* as GST fusion proteins. Upon purification of the proteins, contaminants were present within the extract. The contaminating proteins should have had little effect on the production of antibodies of the appropriate specificity for they are thought to be degradation products of the fusion protein.

The expression of the PDZ-GST fusion protein led to the purification of two proteins. The proteins were detected as a doublet after separation by SDS-PAGE. The reason for the production of two GST-fusion proteins is that the initial stop codon is inefficient in arresting translation due to the presence of nonsense suppressors. Mutations within tRNA molecules can result in an anticodon which recognizes a specific stop codon. In lieu of arresting translation, an amino acid is

transferred to the polypeptide chain. The ribosomes will continue translation until the second stop codon. These tRNA molecules are called nonsense suppressors (Lehninger *et al.*, 1993). A method to circumvent this problem is to identify a bacteria strain which does not contain a nonsense suppressor capable of recognizing the initial stop codon.

The yield of fusion proteins obtained after purification corresponded to previously reported results (Ausubel *et al.*, 1998). The difference in yield between the two proteins is principally due to the efficiency of the purification step rather than the expression levels. The PDZ-GST protein was very insoluble. The addition of N-laurylsarkosyl was necessary to solubilize the protein. Even under these stringent conditions, analysis of the samples revealed that only a fraction of the protein was solubilized. Further, sarkosyl may reduce the affinity of GST for glutathione decreasing the yield of purified protein. It was not necessary to optimize conditions for protein purification because each injection required only 100μg of protein which is a small quantity compared to the yield using bacterial expression systems.

4.1.2 Immunization Protocol

Two possibilities were at my disposition as to the choice of antigen used for immunization of the rabbits: 1) the entire GST fusion protein could be injected or 2) the GST moiety could be cleaved, allowing for the injection of the protein of interest. The former was chosen as the GST moiety was believed to increase the antigenicity of the protein. This would result in a larger titer of antibody present in the serum. However, a disadvantage is associated with this immunization protocol. The serum will not only contain antibodies recognizing the protein of interest but also GST. The ratio of the titer of each antibody is affected by the immunization protocol. The injection of soluble GST fusion proteins results in a 3:1 ratio in favour of the anti-GST antibodies (Rothel *et al.*, 1997). This ratio may be reversed by injecting the antigen after denaturation with a urea/dithiotreitol solution (Rothel *et al.*, 1997). However, this methodology was not used for ethical

reasons. The presence of antibodies against GST could increase the background of future experiments.

The presence of antibodies detecting GST within the serum required the separation of the two principal antibodies. As outlined in the methods, a protein A sepharose column followed by a GST affinity column were used. This methodology was used rather than the classical antigen affinity columns because the antigen was unstable and precipitated from solution at 4°C. The main difference between the two protocols is that in the former, the host antibodies remain in the fraction containing the antibodies of choice. The presence of the host antibodies is not detrimental as their contribution to the experiment may be substracted by performing a negative control using the pre-immune serum. Further, it may actually be an advantage. The host antibodies may serve to block non-specific sites.

4.1.3 Antibody Specificity

4.1.3.1 Characterization of α-ZRP-1 Antibody

The antibodies against ZRP-1, GEF-5.1 and hPTP1E were capable of detecting proteins by Western blot. However, doubts have been forwarded about the actual identity of the mammalian proteins recognized by each antibody. The anti-ZRP-1 antibody detected two proteins with an estimated mass of 48 and 52 kDa. The slower migrating protein is believed to be ZRP-1 as its estimated molecular mass corresponds to the calculated mass of ZRP-1.

Another explanation for this observation is that the smaller protein has a LIM domain with a high degree of identity to the third LIM domain of ZRP-1. The protein sequences within the GenBank database which demonstrate the greatest level of identity to the third LIM domain of ZRP-1 are those of lipoma preferred partner (LPP) and zyxin (Macalma *et al.*, 1996; Petit *et al.*, 1996). The percent identity is 77% for LPP and 64% for zyxin. However, the calculated mass of these proteins is 67 kDa for LPP and 63 kDa for zyxin. These values are much

greater than the observed value of approximately 48 kDa. This protein may be a novel LIM-containing protein.

Finally, the expression pattern of ZRP-1 contradicts the results observed by Northern blot. The tissue distribution of the ZRP-1 mRNA was ubiquitous with slight variations in expression levels (Murthy *et al.*, 1999). ZRP-1 was not detected by Western blot in 293S nor COS1 cells which have been derived from kidney cells. The protein expression of ZRP-1 may be regulated at the translation step instead of transcription. Further, the cells have been immortalized. This process may have affected the expression of numerous genes which no longer represents their expression *in vivo*. A simple experiment to answer all of these questions would be to detect the protein in human tissue by immunohistochemistry using ZRP-1 specific antibodies. In addition, the mRNA expression profile of ZRP-1 should be examined and correlated with the amount of ZRP-1 protein present.

4.1.3.2 Characterization of α-GEF-5.1 Antibody

The anti-GEF-5.1 antibody also detected two proteins but failed to recognize a protein of 175 kDa, the calculated mass of GEF-5.1 (Banville *et al.*, unpublished). Several reasons may explain this result. Total cell extracts were loaded onto gel. The concentration of GEF-5.1 within the gel may be below detectable limits under these experimental conditions. An approach to circumvent these problems would be to perform an immunoprecipitation of the proteins. The protein may not be expressed under these conditions but may be induced in response to specific stimuli. The results obtained by Northern blot also suggest a low level expression of this protein under homeostasis. No signal was detected when a blot with mRNA from human tissues was hybridized with a GEF-5.1 probe (Banville *et al.*, unpublished). Again the identity of the two proteins is unknown but may be proteins which possess a PDZ domain displaying homology to the one from GEF-5.1.

4.1.3.3 Characterization of α-hPTP1E Antibody

The antibody raised against the second PDZ domain of hPTP1E recognized three proteins with mass of approximately 70 kDa, 100 kDa and 275 kDa. The larger protein has a molecular weight equivalent to the one predicted for the full-length sequence of the phosphatase (Banville *et al.*, 1994). The other two proteins may be the result of alternative splicing. Two independent studies have revealed the existence of alternatively spliced transcripts (Banville *et al.*, 1994; Maekawa *et al.*, 1994). Each study describes the sequence of the full-length cDNA along with the sequence of two transcripts being the product of alternative splicing. However, the latter sequences differ between the two studies. There exists at least 4 alternatively spliced products but many more may be present. The mass predicted for the translated protein from the transcripts produced by alternative splicing do not correlate with the 70 kDa and 100 kDa proteins detected by the anti-hPTP1E antibody. It will be necessary to conduct an exhaustive study on this issue to determine the number and sequence of each transcript. The results would have great implications about the regulation of hPTP1E function. Further, the expression level of each protein in the different cell types varied significantly. The implications of this observation on the function of hPTP1E and the regulation of the phosphatase are still unknown.

4.1.4 Conclusions

The methodology employed for the production of antibodies was an efficient means for the generation of large quantities of specific immunoglobulins. In addition, a simple two-step method was devised for the purification of the antibodies. Unfortunately, characterization of the antibodies suggest that they may have a limited utility. The antibodies against ZRP-1, GEF-5.1 and hPTP1E can all detect proteins with the expected specificity by Western blot. However, these antibodies recognize various proteins within mammalian cells. More concrete

evidence to the identity of these proteins is required. Further, experiments requiring these antibodies will have to be designed carefully to allow for proper interpretation of the results.

4.2 Co-Immunoprecipitation Studies

4.2.1 Co-Precipitation of hPTP1E and ZRP-1

The function of ZRP-1 is still undetermined. However, studies of related proteins have provided many insights. Proteins related to ZRP-1 include zyxin, the LPP and enigma. Zyxin is involved in organizing the cytoskeleton at adhesion plaques (Beckerle, 1997). The LPP gene is involved in chromosome rearrangements resulting in tumorigenesis (Petit *et al.*, 1996). Finally, enigma is a downstream signalling molecule for the Ret receptor (Durick *et al.*, 1998). Zyxin has served as a model for ZRP-1 function due to its identical structural motifs. Zyxin is a phosphoprotein localized at focal adhesion plaques where it interacts with a number of different proteins to regulate the cytoskeletal configuration (Beckerle, 1997). The proline rich domain of zyxin binds α-actinin which crosslinks actin filaments, vav, a guanine nucleotide exchange factor for the Rho family of GTPases which regulate cytoskeletal rearrangements and VASP, a signalling molecule of the Abl signalling pathway localized at integrin-rich adhesion plaques (Crawford *et al.*, 1992; Hobert *et al.*, 1996; Reinhard *et al.*, 1995). Finally, the cysteine rich protein 1 (CRP1) interacts with the first LIM domain of zyxin (Crawford *et al.*, 1994, Schmeichel and Beckerle, 1994). While numerous proteins which bind to zyxin have been identified, other proteins potentially interact with zyxin as no interacting proteins for the LIM domains 2 and 3 have been identified. Since zyxin is capable of interacting with numerous proteins, investigations to identify proteins which interact with ZRP-1 may provide helpful information about its function.

ZRP-1 interacts with the second PDZ domain of hPTP1E in the yeast two-hybrid system. In order to confirm the interaction in a cellular environment, co-

immunoprecipitation studies were undertaken. Under the experimental conditions attempted in this study, no interaction was detected. Analysis of the cells transfected with the vector expressing a region of ZRP-1 demonstrated that the recombinant protein was expressed. Additional refinement of the immunoprecipitation conditions allowed for the detection of an interaction between ZRP-1 and hPTP1E *in vivo* (Murthy *et al.*, 1999) (see appendix 1).

4.2.2 Co-Precipitation of hPTP1E and TSC2

An interaction between the TSC2 gene product, tuberin and hPTP1E was observed by co-immunoprecipitation experiments. The combination of the results from the yeast two-hybrid system, *in vitro* protein-protein binding studies and co-immunoprecipitation studies strongly suggest a role for hPTP1E in the regulation of functions dependent on TSC2.

Since the identification of the TSC2 gene, studies have focused on its biological function. It has been demonstrated that tuberin can activate the GTPase function of rap1a *in vitro* (Wienecke *et al.*, 1995). Rap1a is a member of the Ras superfamily of GTPases which are involved in the control of cell proliferation and have oncogenic properties (Bos, 1998). Hence, cells lacking tuberin would express rap1a with an inappropriate activation state leading to deregulated mitogenic signalling (Wienecke *et al.*, 1995).

Rab GTPases regulate endocytosis (Schimmöller *et al.*, 1998). A macromolecular complex is formed at the docking site whereby rabaptin-5 recruits the key molecules involved in endocytosis. It binds to rab5, Rabex-5 which serves as a GEF for rab5 activation, and tuberin (Horiuchi *et al.*, 1997; Stenmark *et al.*, 1995; Xiao *et al.*, 1997). The association of rabaptin-5 with both rab5 and tuberin has led to the demonstration of GAP activity of tuberin towards rab5 (Xiao *et al.*, 1997). It is unknown which molecules are present in the complex at which stages of the endocytic process. The complex may be formed at all times with the regulation of the activity of rabex-5 and tuberin allowing for the appropriate activation state of rab5 or the recruitment of the rab5 effectors is

temporally regulated and the nature of the complex dictates the activation state of rab5 (Xiao *et al.*, 1997).

A functional assay for endocytosis revealed that tuberin negatively regulates fluid-phase endocytosis by modulating the activity of rab5 (Xiao *et al.*, 1997). A model has been proposed to explain the role of tuberin in the pathogenesis of tuberous sclerosis in light of its regulatory role of endocytosis. Tuberin may regulate the internalization of growth factor receptors or other membrane-bound signalling molecules resulting in the turnover of these proteins. Cells lacking tuberin could lead to the missorting and slow turnover of mitogenic effectors resulting in hamartomas (Xiao *et al.*, 1997).

The demonstration of an interaction between hPTP1E and tuberin implies a role for hPTP1E in endocytosis. HPTP1E may also be recruited to the multi-protein complex as the region of tuberin mediating its interaction with rabaptin-5 is distinct from the C-terminal tail required for the interaction with hPTP1E (Banville *et al.*, unpublished; Xiao *et al.*, 1997). Within the mechanism of endocytosis, hPTP1E may regulate the phosphorylation state of tuberin which may determine its activity, it may regulate the phosphorylation state of other proteins within the complex or it may assist in the recruitment of proteins required for the proper regulation of the endocytosis process by acting as a scaffold protein. These hypotheses have not been investigated to date. It will be necessary to characterize the role of hPTP1E in the regulation of endocytosis.

4.3 Yeast Two-Hybrid System

The yeast two-hybrid system is a genetic method for studying protein-protein interactions (Fields and Song, 1989). The main application of this technology is the screening of a cDNA library to identify cDNA inserts encoding a protein which interacts with a protein of interest (Chien *et al.*, 1991). Although many studies have been successful in identifying protein-protein interactions, numerous false negatives and false positives may arise. The system depends on the formation of a hybrid protein. The fusion of the protein of interest with the

DBD or the AD may have adverse effects on the conformation of the protein. The deviation of the protein conformation away from its native form will inevitably change the range of protein interactions. Fortunately, this problem should not occur at a high frequency for the modular domains involved in signal transduction pathways such as SH2, SH3, PTB and PDZ domains.

The orientation of the fusion protein may also contribute to artefactual results. Usually the fusion is formed at the C-terminus of the DBD as is the case in these studies. This strategy is ideal for investigating interactions involving PDZ domains as the ligand for these domains is the C-terminal tail (Fanning and Anderson, 1998). In contrast, interactions dependent on the N-terminus will not be detected under these circumstances. Beranger and colleagues (1997) have constructed plasmids for use in the yeast two-hybrid system with opposing fusion polarity. A comparison of the two methods showed that the orientation of the fusion protein may affect the strength of the interaction from 4 to 7-fold. It is important to design the fusion protein for use as bait carefully to increase the chances of an effective library screening.

Finally, interactions with meaningless biological significance are observed because the fusion proteins are directed to the same cellular compartment and are present at very high concentrations. In the classical two-hybrid system, the proteins are targeted to the nucleus (Fields and Song, 1989). Other methods detect an interaction within the cytoplasm at submembranous locations (Aronheim *et al.*, 1997). Thus, it is necessary to assess each interaction carefully to determine its biological significance. Further, the screening of a cDNA library using the yeast two-hybrid system by no means reveals all the possible interactions due to these inherent problems.

The shortcomings of the yeast two-hybrid system mentioned are present within all the systems devised to date. Other problems may be associated specifically with one type of method. For my yeast two-hybrid studies, the classical method was utilized whereby the detection of a protein-protein interaction is dependent on a transcriptional output. This strategy has several

limitations. The overexpression of proteins in the nucleus which normally reside in other cellular compartments may be lethal or the protein may be nonfunctional. On the other hand, nuclear proteins which are themselves transcriptional activators or repressors cannot be used as bait (Brackmann and Boeke, 1997).

During a cDNA library screening, false negatives and false positives may arise. Unfortunately, the detection of false negatives is impossible unless one is investigating known interactions. Before dismissing the yeast two-hybrid system, it is important to study a number of baits from the same gene including the orientation of the fusion proteins. If the yeast two-hybrid system fails, it may be necessary to use classical biochemical techniques. In contrast, the false positives are easily tested using a couple of yeast strains and constructs (Bartell *et al.*, 1993). In my studies, the yeast strains HF7c and SFY526 were used for the screening process and the control experiments, respectively. The advantage of using these two strains is the difference in genotype. In HF7c, the LacZ gene is under the control of a hybrid promoter composed of CYC1 with Gal4 binding sites found upstream while the reporter gene in SFY526 is regulated by the GAL1 promoter (Bartell *et al.*, 1993; Feilotter *et al.*, 1994). While both promoters respond to GAL4, it is improbable that the same artefacts will affect the different promoters. Hence, an interaction observed in both yeast strains should require a direct interaction between the two proteins. The specificity of the interaction must be determined. All this requires is to test a panel of irrelevant proteins which should not interact with the prey. In these studies, Lamin C was used as an irrelevant bait. All these problems are present during each screening and must be kept in mind.

4.3.1 Identification of ZRP-1 Interacting Proteins

Although the yeast two-hybrid system has inherent problems, it has been exhaustively employed for the identification of protein-protein interactions. Using the N-terminal half of the proline rich region of ZRP-1, I identified an interaction with hCDC47 and NtZRP-p33, a clone encoding an SH3 domain. It is difficult to

assess the significance of these interactions as very little or no information about the clones is discussed in the literature. Other interactions with enzymes such as succinate dehydrogenase were observed. However, these interactions do not appear to be biologically significant.

DNA replication is an essential process during the cell cycle which must be highly regulated to ensure that each daughter cell obtains only 1 copy of each chromosome. Several members of different protein families play crucial roles in modulating DNA replication. The minichromosome maintenance (MCM) protein family which may function as licensing factors is one such family (Kearsey and Labib, 1998). There are six members denoted MCM2 to MCM7 within eukaryotes. The primary structure similarities are confined to a 200 amino acid core sequence. However, several MCM proteins including MCM7 contain a zinc-finger motif with a consensus corresponding to $CX_2CX_{18-19}CX_{2-4}C$ (Schulte *et al.*, 1996). The function of this domain is thought to be the mediation of protein-protein interactions as MCM proteins do not interact directly with DNA (Kersey and Labib, 1998; MacKay and Crossley, 1998). Other characteristics of MCM proteins include nuclear localization either bound to chromatin or free within the nuclear matrix (Fujita *et al.*, 1996; Kimura *et al.*, 1994). The interaction with chromatin is necessary for proper function but is dependent on the cell cycle phase (Fujita *et al.*, 1996; Kubota *et al.*, 1995; Madine *et al.*, 1995). Further, phosphorylation of MCM proteins regulates their function throughout the cell-cycle (Kimura *et al.*, 1994; Schulte *et al.*, 1995).

A model for the initiation of DNA replication as controlled by the MCM proteins has been reviewed by Kearsy and Labib (1998). Initially, MCM proteins bind to chromatin in a multiprotein complex which includes the origin recognition complex. At the beginning of the S phase, MCM proteins are phosphorylated by cyclin dependent kinases. DNA replication is initiated at sites marked by MCMs. As elongation of the replication fork proceeds, the MCM proteins dissociate from the DNA. A mechanism to ensure that the DNA is replicated only once per cell cycle has been devised. The phosphorylated MCM proteins cannot reassociate

with the chromatin possibly because of the hyperphosphorylated state but also because the expression of other proteins required for their association with chromatin such as Cdc6 has been down regulated.

The detection of an interaction between ZRP-1 and hCDC47/MCM7 implies a possible role for ZRP-1 in the regulation of DNA replication. The data which contradicts this hypothesis are: 1) proline rich regions are ligands for SH3 and WW domains. The primary structure of hCDC47 does not contain such a domain. 2) the only domain possibly involved in the recruitment of proteins is the zinc-finger-like motif (Schulte *et al.*, 1996). However, this motif is present at the N-terminus of hCDC47 and is excluded from all the clones isolated during the yeast two-hybrid system. The results suggest that the interaction is mediated by the C-terminus. 3) Finally, hCDC47 has a pI of 5.05 (Kearsey and Labib, 1998). The negative charge may contribute to non-specific interactions.

While there exists many indications that the interaction is an artefact, other results suggest that the interaction is biologically significant. Among all the clones isolated during the yeast two-hybrid cDNA library screening, the clones encoding a fragment of the hCDC47 gene demonstrated the strongest interaction with ZRP-1. Also a large number of clones were isolated with different but overlapping sequences.

Finally, the hypothesized function of ZRP-1 is modeled on the findings of zyxin as it is the closest related protein. Zyxin, a phosphoprotein located at adhesion plaques, possesses a nuclear exclusion sequence which allows the protein to shuttle from the membrane to the nucleus (Nix and Beckerle, 1997). The functional implications of this observation has yet to be deciphered but Nix and Beckerle (1997) have contributed a number of hypothesis about the functional relevance of this behaviour. Zyxin may be necessary for the transmission of the signal from adhesion plaques to the nucleus. Zyxin may transport transcription factors to the nucleus or inhibit other processes while present in the nucleus. ZRP-1 is thought to be a phosphoprotein localized to sub-membranous regions. Sequence alignments defined a putative NES within the sequence of ZRP-1.

Although ZRP-1 may be primarily located at the membrane, it may shuttle to the nucleus delivering a signal for DNA replication by regulating the function of hCDC47. A thorough investigation is required to assess the significance of the interaction due to the ambiguity of the results.

Interestingly, another nuclear protein interacts with ZRP-1 in the yeast two-hybrid system. The initial isolation of a cDNA fragment of the ZRP-1 gene was reported by Lee and colleagues (1995). Using the yeast two-hybrid system, the C-terminal half of ZRP-1 interacted in a ligand dependent manner with both the thyroid hormone receptor and the retinoic acid receptor, RXR. These two receptors regulate the transcription of certain genes involved in the response to stimulation by thyroid hormone and retinoic acid. It will be interesting to determine whether ZRP-1 can modulate the function of these two receptors after translocation from the cytoplasm to the nucleus.

The interaction between the clone NtZRP-p33 and ZRP-1 has the greatest potential of being biologically relevant. The sequence of NtZRP-p33 encodes an SH3 domain. This correlates with the expected result for an interaction with a proline rich region. This clone is derived from a novel gene. The sequence of a clone called KIAA0769 within the GenBank database includes the sequence of clone NtZRP-p33 (Nagase *et al.*, 1998). However, KIAA0769 encodes a second SH3 domain present at the C-terminus which is not present within the sequence of NtZRP-p33. At the N-terminus of KIAA0769 are three consensus sequences for tyrosine phosphorylation which may serve as ligands for SH2 or PTB domains. It will be necessary to obtain the full-length sequence of NtZRP-p33 to assess its relationship to the clone KIAA0769. It is possible that the mRNA corresponding to the sequence of KIAA0769 be the only form expressed or two different mRNA molecules may result from the same gene due to alternative splicing.

The structure of the protein suggest a role as a signal transduction molecule. KIAA0769 may serve as a scaffolding protein recruiting proteins via interactions with the two SH3 domains and the phosphorylated tyrosine residues. It will be interesting to determine if KIAA0769 can also interact with ZRP-1. A

comparison in the binding properties of the two proteins may suggest a plausible mechanism for regulating their function. Further, hPTP1E once recruited to the multiprotein complex may dephosphorylate the putative phosphotyrosine residues at the N-terminus.

Unfortunately, only the N-terminal half of the proline rich region of ZRP-1 could be used for the cDNA library screening because the expression of the fusion protein containing the entire region was lethal. It will be necessary to determine if the C-terminal half of the proline rich region could be used as bait in the yeast two-hybrid system. Other proteins may be able to interact with this region with further implications as to the biological function of ZRP-1.

The studies of the protein-protein interactions involving ZRP-1 suggest a role for this protein in regulating the cell-cycle and transcription. Further, ZRP-1 may be involved in the pathogenesis of certain diseases. Chromosomal mapping located the ZRP-1 gene to the chromosome segment 7q22 (Yi and Beckerle, 1998). Deletions within this region of the genome cause cytogenetic abnormalities associated with malignant myeloid diseases and uterine leiomyoma (Johansson *et al.*, 1993; Xing *et al.*, 1997). Unfortunately, the identity of the gene responsible for the diseases is unknown. ZRP-1 may also be required for the pathogenecity of *Neisseria gonorrhoeae* which infects human cervical epithelial cells. The bacteria possess outer membrane proteins called opacity-associated (Opa) proteins. During the intracellular phase of *N. gonorrhoeae* pathogenesis, the bacteria probably interact with host proteins. During a yeast two-hybrid screen to identify host proteins which interact with Opa proteins, a clone encoding a fragment of ZRP-1 was isolated (Williams *et al.*, 1998). Unfortunately, the authors did not attempt any experiments to understand the significance of this interaction.

4.3.2 Characterization of GEF-5.1 and Identification of GEF-5.1 Interacting Proteins

GEF-5.1 contains a CDC25 homology domain. CDC25 is a guanine nucleotide exchange factor specific for Ras (Overbeck *et al.*, 1995). This suggests a plausible role for GEF-5.1 in the regulation of members of the Ras GTPase superfamily which control proliferation of cells. In an attempt to identify potential substrates of GEF-5.1, the CDC25 homology domain of GEF-5.1 was used as bait for the screening of a cDNA library in the yeast two-hybrid system. Unfortunately, no GTPase was identified among the six clones sequenced. At this stage, it may be more efficient to test the activity of GEF-5.1 towards representative GTPases of each family using an *in vitro* assay. This assay could also be used to investigate the mode of regulation of GEF-5.1.

Although no GTPase was isolated during the screening, 14-3-3ε which may regulate the activity of GEF-5.1 was identified. 14-3-3 proteins form a family of 7 proteins (Reuther and Pendergast, 1996). Each isoform has been implicated in numerous cellular functions such as apoptosis, cell cycle check points, ADP ribosylation, hydroxylation and secretion (Reuther and Pendergast, 1996; Zha *et al.*, 1996). The function of 14-3-3 proteins depends on their interaction with target proteins. The interactions are mediated by phosphoserine residues within the consensus sequence RSXpSXP (Muslin *et al.*, 1996). 14-3-3 proteins can bind to protein kinase C, Raf, the Cdc25 phosphatase, Bad, PTPH1, IRS-1 and tryptophan hydroxylase (Banik *et al.*, 1997; Conklin *et al.*, 1995; Craparo *et al.*, 1997; Freed *et al.*, 1994; Toker *et al.*, 1992; Zha *et al.*, 1996; Zhang *et al.*, 1997). In certain cases, 14-3-3 has a positive effect on the target protein while in others, it plays an inhibitory role. The association of 14-3-3 proteins with tryptophan hydroxylase enhances the activity of the enzyme while it inhibits the kinase activity of PKC (Banik *et al.*, 1997; Toker *et al.*, 1992).

14-3-3 proteins bind to their target proteins via a phosphoserine residue (Muslin *et al.*, 1996). The sequence RSLSQG which deviates slightly from the consensus sequence was identified among the region of GEF-5.1 used as bait. To test whether this motif was responsible for the interaction between GEF-5.1 and 14-3-3ε, the second serine residue was mutated to an isoleucine. This abolished

the interaction observed in the yeast two-hybrid system. The second serine residue within the above motif is critical for the interaction. However, it remains to be determined whether the serine residue is phosphorylated. It is important to note that all the clones isolated during the cDNA library screening encoded the 14-3-3ε family member. Although the interactions of all seven 14-3-3 isoforms is mediated by the consensus motif, other regions of the protein determine the specificity of the interaction (Muslin *et al.*, 1996). The specificity observed in these experiments suggest that the interaction is not an artefact but is biologically relevant.

The function of this interaction has yet to be investigated. Along with the structural information, the interaction provides support for a role of GEF-5.1 in Ras signalling. 14-3-3 proteins have been involved in the regulation of Raf activity, a downstream effector of the Ras signalling pathway (Reuther and Pendergast, 1996). Genetic studies using *Drosophila melanogaster* has demonstrated a positive regulatory role for 14-3-3ε in Ras signalling required for development of the eye (Chang and Rubin, 1997). Interestingly in this pathway, 14-3-3ε acts downstream from Raf. Hence, the same 14-3-3 isoform can regulate the Ras signalling pathway by means of different mechanisms. Numerous hypothesis as to the role of 14-3-3ε in regulating GEF-5.1 may be postulated. 14-3-3ε could activate or inhibit the enzymatic activity of GEF-5.1. The interaction may be necessary for proper cellular localization of the protein. GEF-5.1 may be relocated to a region rich in substrate after interaction with 14-3-3ε.

The primary structure of GEF-5.1 was analyzed using software for predicting the presence of functional domains. A number of domains have been identified which provide significant information about the potential function and mode of regulation of GEF-5.1. The most notable domain is the CDC25 homology domain. Proteins containing this domain form a family of proteins with guanidine nucleotide exchange activity (Overbeck *et al.*, 1995; Quilliam *et al.*, 1995). The other family of GEFs possess a Dbl homology domain. These proteins activate GTPases within the Rho family (Overbeck *et al.*, 1995; Quilliam *et al.*,

1995). Although the substrate of GEF-5.1 remains unknown, certain clues are provided by the presence of an LTE domain. This domain is present in GEFs specific for Ras GTPases (Lai *et al.*, 1993). Members of the protein family include CDC25, SOS, Ras-GRF and Sdc25. Another link to the Ras GTPase signalling pathways is via the Ras-associated (RA) domain (Ponting and Benjamin, 1996). This domain is present not only in GEFs but proteins with a wide range of functions. RA domains mediate interactions with GTP-bound Ras. Proteins containing RA domains are believed to be downstream effector molecules of Ras signalling. The implications of this domain among the structure of GEFs has been postulated to link the activation of Ras to the activation of other small GTPases (Ponting and Benjamin, 1996). Considering this possibility plus the idea that GEF-5.1 is a Ras-specific GEF, a positive feedback loop could be created whereby the activated Ras molecules bind to GEF-5.1 allowing for the activation of additional Ras molecules.

The activity of GEF-5.1 may also be regulated by either the levels of cGMP or cAMP within the cell. A cNMP binding domain was predicted between residues 280 to 380. This mechanism has support from a recent report which describes the activation of EPAC by cAMP (de Rooij *et al.*, 1998). The first report on this mode of regulation of GEFs which mimics that of cAMP-dependent kinases leads to the hypothesis that different families of GEFs may be defined according to the mechanism of regulation.

GEF-5.1 may also recruit other components of the signalling pathways. It has been demonstrated using the yeast two-hybrid system that the C-terminus tail of GEF-5.1 interacts with the second PDZ domain of hPTP1E (Banville *et al.*, unpublished). GEF-5.1 also possesses a PDZ domain which can interact with another signalling molecule. This multiprotein complex could allow for a rapid transmission of the signal from the phosphatases to another component of the pathway or vice versa.

Comparison of the sequence of GEF-5.1 with those present in the GeneBank database has identified two other proteins with similar structures. The

human clone KIAA0313 and the *C. elegans* clone T14G10.2 both possess LTE, RA, CDC25 homology, cNMP-binding and PDZ domains (Nagase *et al.*, 1997, Wilson *et al.*, 1994). These proteins may have similar functions. The genetics of the nematode have been well characterized with the recent completion of the genome project (Wilson, 1999). Functional studies of the clone T14G10.2 by genetic manipulation of *C. elegans* would be helpful to elucidate the function of GEF-5.1.

4.4 Biological Function of hPTP1E

The simultaneous emergence of the yeast two-hybrid system along with the discovery of numerous domains which mediate protein-protein interactions has resulted in the publication of large amounts of information about the mechanism of the interactions as well as the identification of binding partners. Several groups have identified proteins which interact with hPTP1E via its PDZ domains using the yeast two-hybrid system. Sato and coworkers (1995) demonstrated an inhibitory effect of hPTP1E on Fas-mediated apoptosis via an interaction between the second PDZ domain of hPTP1E and the C-terminus of Fas. Further, this inhibitory effect of hPTP1E is reduced upon microinjection of a tripeptide with the C-terminal sequence of Fas, SLV (Yanagisawa *et al.*, 1997). The C-terminal tail of Fas can interact with the second and fourth PDZ domains of hPTP1E (Saras *et al.*, 1997a).

The physiologic importance of these findings is still a debatable issue. Sato *et al.* (1995) found a correlation between the level of hPTP1E expression and inhibition of Fas-mediated apoptosis. Other studies involving Fas and hPTP1E expression in human leukaemia/lymphoma cell lines revealed a discrepancy with the previous results, neither the presence nor absence of hPTP1E could be correlated with Fas-mediated apoptosis (Hedlund *et al.*, 1998; Komada *et al.*, 1997). Further, the role of hPTP1E in Fas signalling is not evolutionary conserved. Cuppen and colleagues (1997) presented strong evidence that the mouse homologue of hPTP1E namely PTP-BL does not interact with mouse Fas.

These investigations were initiated due to the divergence in the C-terminal sequence of Fas which is responsible for the interaction with hPTP1E. PDZ domains interact with the C-terminal peptides of target proteins with a consensus sequence of T/SXV. Only human Fas fulfills this requirement as the sequence for mouse Fas is CLE, rat Fas is SLE and NLV for bovine Fas. The studies involved interaction trap experiments, apoptosis functional assays and differential mRNA expression of PTP-BL and Fas. The results from these studies suggest the presence of different mechanisms for the regulation of Fas-mediated apoptosis between species and cell type. It will be necessary to further define the role of hPTP1E in Fas-mediated apoptosis in the future.

Additional protein-protein interaction studies have identified two novel proteins which interact with hPTP1E and its mouse homologue, PTP-BL. PARG1 which is a protein with GAP activity for the Rho family of GTPases interacts with the fourth PDZ domain of hPTP1E (Saras *et al.*, 1997b). The Rho family of GTPases which include Rho, Rac and Cdc42 are implicated in the regulation of cytoskeletal rearrangements (Zigmond, 1996).

RIL was identified as a protein which can interact with both the second and fourth PDZ domains of PTP-BL via a LIM domain (Cuppen *et al.*, 1998). The primary structure of RIL includes an N-terminal PDZ domain and a C-terminal LIM domain. Further, several tyrosine residues could be phosphorylated by PTKs. Cuppen *et al.* (1998) demonstrated that RIL can serve as an *in vitro* substrate of PTP-BL. Since RIL can interact with both the second and fourth PDZ domain of PTP-BL and potentially form homodimers via a PDZ-LIM interaction between 2 RIL molecules, a mechanism may exist for the formation of a multiprotein complex which could regulate cytoskeletal structures. The presence of hPTP1E may be required for the regulation of the phosphorylation state of the proteins. The significance of these interactions are still not understood. However, they strongly suggest an imminent role for hPTP1E in the regulation of the cytoskeleton.

In parallel to these studies, we have investigated the protein-protein interactions involving hPTP1E. Using the yeast two-hybrid system, several novel clones were identified and isolated which interacted with this PTPase. ZRP-1 and GEF-5.1 interact with the second PDZ domain while tuberin interacts with the fourth PDZ domain of hPTP1E. Other potential molecules involved in the signalling cascade of hPTP1E were identified using the yeast two-hybrid system to isolate clones which interact with ZRP-1 and GEF-5.1. While these molecules do not interact directly with hPTP1E, they provide additional insight into the biological function of this PTPase. The demonstration of an interaction between TSC2 and hPTP1E *in vivo* provides further evidence for a regulatory role of hPTP1E during endocytosis.The work on ZRP-1 provides for a plausible role for hPTP1E in DNA replication and an unknown function associated with the clone NtZRP-p33. Likewise the results from experiments involving the function of GEF-5.1 suggest a role for hPTP1E in the Ras signalling pathway. Since the downstream effectors of Ras signalling involve primarily kinases such as Raf, the MAP kinases and ERK, hPTP1E would probably have a role in down regulating Ras signalling (Denhardt, 1996). Further, the Ras pathway is used by growth factors to transduce mitogenic signals to the nucleus. HPTP1E could have an inhibitory role in mitogenic signalling. If this is the case, hPTP1E may be another example of a tumour suppressor gene. The conclusions from the ZRP-1 and GEF-5.1 merge to emphasize a role for hPTP1E in the regulation of DNA replication.

While many studies have focused on molecular events, other investigations have attempted to define a function for hPTP1E. The data strongly suggests a role for hPTP1E in modulating immune responses. Fas-mediated apoptosis is a mechanism used by cytotoxic T lymphocytes to destroy target cells. HPTP1E controls the deletion of target cells as it is a negative regulator of Fas. A decrease in expression of hPTP1E has been associated with an increase in susceptibility to Fas-mediated apoptosis by peripheral T cells exposed to IL-2 (Zhou *et al.*, 1998). Activation-induced cell death (AICD) in CD4+ Th cells is a mechanism used especially by viruses to deplete a population of lymphocytes.

The mechanism involves the triggering of apoptosis by Fas when the cells are restimulated with antigen. It was observed that Th2 cells which are more resistant to AICD express higher levels of hPTP1E than Th1 lymphocytes (Zhang *et al.*, 1997).

Finally, hPTP1E may contribute to the pathology of several diseases including cancer, human T cell leukemia virus type 1-associated myelopathy/tropical spastic paraparesis and AIDS-associated Kaposi's Sarcoma. Tumor cells may avoid deletion by the host immune system via a resistance to apoptosis. Human pancreatic adenocarcinomas express both Fas and Fas ligand yet, are resistant to apoptosis. This resistance was correlated with high expression levels of hPTP1E (Ungefroren *et al.*, 1998). Other human cancers characterized by resistance to Fas-mediated apoptosis do not express hPTP1E. In these cells, other mechanisms regulating apoptosis have evolved. HTLV-I encodes a protein called tax which can inhibit Fas-mediated apoptosis. Although infected cells normally express tax, some infected cell lines were negative for tax but were still resistant to Fas-mediated apoptosis. In these cases, a correlation with the level of expression of hPTP1E and resistance to apoptosis was observed (Arai *et al.*, 1998). Further, hPTP1E was selectively overexpressed in cell lines derived from HAM/TSP patients (Arai *et al.*, 1998). Kaposi's sarcoma cells which are resistant to Fas-mediated apoptosis express high levels of hPTP1E mRNA (Mori *et al.*, 1996). While the overexpression of hPTP1E in cells may have detrimental effects leading to disease, it may also help cure other diseases. Steroidal and nonsteroidal hydroxytamoxifen antiestrogens inhibit cell growth of breast cancer cells induced by EGF or IGF-1 via upregulation of hPTP1E (Freiss *et al.*, 1998).

These studies have provided, in most part, a correlation between in the expression of hPTP1E with a disease. The molecular mechanism has yet to be deciphered. It would be interesting to determine whether any of the molecules stated in this study including GEF-5.1, tuberin, and ZRP-1 along with their interacting partners are required for the development of these diseases.

In conclusion, hPTP1E is a large cytoplasmic phosphatase which possesses five PDZ domains. HPTP1E may serve as a scaffolding protein whereby it recruits numerous proteins via its PDZ domains. The role of the molecules potentially involved in the signalling cascade of this PTPase suggest a function for hPTP1E in the regulation of endocytosis, DNA replication, cell cycle regulation and cytoskeletal rearrangements.

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APPENDIX 1

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ZRP-1, a Zyxin-related Protein, Interacts with the Second PDZ Domain of the Cytosolic Protein Tyrosine Phosphatase hPTP1E*

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Kishore K. Murthy, Kristopher Clark, Yves Fortin, Shi-Hsiang Shen, and Denis Banville‡

From the Pharmaceutical Biotechnology Sector, Biotechnology Research Institute, Montreal, Quebec H4P 2R2, Canada

Protein-protein interactions play an important role in the specificity of cellular signaling cascades. By using the yeast two-hybrid system, a specific interaction was identified between the second PDZ domain of the cytosolic protein tyrosine phosphatase hPTP1E and a novel protein, which was termed ZRP-1 to indicate its sequence similarity to the Zyxin protein family. The mRNA encoding this protein is distributed widely in human tissues and contains an open reading frame of 1428 base pairs, predicting a polypeptide of 476 amino acid residues. The deduced protein displays a proline-rich amino-terminal region and three double zinc finger LIM domains at its carboxyl terminus. The specific interaction of this novel protein with the second PDZ domain of hPTP1E was demonstrated both *in vitro*, using bacterially expressed proteins, and *in vivo*, by co-immunoprecipitation studies. Deletion analysis indicated that an intact carboxyl terminus is required for its interaction with the second PDZ domain of hPTP1E in the yeast two-hybrid system and suggested that other sequences, including the LIM domains, also participate in the interaction. The genomic organization of the ZRP-1 coding sequence is identical to that of the lipoma preferred partner gene, another Zyxin-related protein, suggesting that the two genes have evolved from a recent gene duplication event.

Protein-protein interactions play a crucial role in maintaining the normal function of cells. Such interactions are important in the transmission of signals within the cell. Scaffolding, anchoring, and adaptor proteins, which bring together the various signaling molecules through such interactions, are determinant in fine-tuning these pathways and often contain modular structural domains mediating protein-protein interactions (1–3). A partial list of these domains include Src homology 2 and 3 (SH2 and SH3) domains (4), Tyr(P) binding domains (5), pleckstrin homology domain (6, 7), and PDZ domain (8, 9).

PDZ domains consist of a motif of approximately 90 amino acid residues, found in one or multiple copies in a variety of signaling proteins. This domain derives its name from the three proteins originally shown to contain these sequences as follows: post-synaptic density protein, PSD-95 (10), the *Drosophila* septate junction protein discs-large tumor suppressor, Dlg (11), and the epithelial tight junction protein, ZO-1 (12). Other PDZ domain-containing proteins include nitric oxide synthase (13), the *Drosophila* dishevelled protein, Dsh (14), the channel-interacting PDZ domain protein (15), etc. (for reviews see Refs. 9 and 16).

PDZ domains have also been found in a subfamily of protein tyrosine phosphatases (PTPs),[1] which includes PTPH1 (17), PTPMEG (18), and hPTP1E (19). The latter, also called PTP-BAS, PTPL1, and FAP-1, is a cytosolic PTPase and contains five PDZ domains in addition to a single tyrosine phosphatase catalytic domain. This protein also contains other distinct structural elements including a band 4.1 homology domain and five PEST regions (19–22). A recent study revealed that the second PDZ domain (PDZ2) of hPTP1E interacts with a sequence within the last 15 amino acids at the COOH terminus of Fas, a cell-surface receptor involved in the apoptotic pathway (22). In addition, a GTPase-activating protein (GAP) with activity toward Rho (PTPLI-associated RhoGap 1; PARG-1) interacts with PDZ4 of hPTP1E (23). By using the yeast two-hybrid system and the PDZ2 of hPTP1E as bait, we have screened a HeLa cell cDNA library to identify other binding partners. We have cloned and characterized a novel protein (ZRP-1, zyxin-related protein-1) that interacts strongly with this domain. ZRP-1 is a 476-amino acid protein containing 3 LIM domains at its COOH terminus and a proline-rich NH_2-terminal segment. The region of ZRP-1 involved in the interaction with the PDZ2 domain of hPTP1E has been characterized. The specificity as well as the *in vitro* and *in vivo* interactions of these proteins have also been demonstrated.

MATERIALS AND METHODS

Identification of Interacting Proteins Using the Two-hybrid System

The yeast two-hybrid system was employed to identify novel proteins interacting with the PDZ domains of hPTP1E. The MATCHMAKER (CLONTECH Laboratories) HeLa cell cDNA library was screened essentially following the protocol outlined by the manufacturers. All yeast transformations were performed using the lithium acetate method of Gietz *et al.* (24). Plasmid DNA was prepared by the method of Nasmyth and Reed (25). The inserts were amplified by PCR using the oligos GAD1F (5′-TACCACTACAATGGATGATG-3′) and M13 "Universal" primer flanking the insert in the plasmid pGADGH. The amplified PCR products were sequenced directly using an ABI377 "Prism" automated DNA sequencer.

For β-galactosidase liquid assays, the assays were done essentially as outlined (26). In brief, colonies of SFY526 containing the various constructs of ZRP-1 in pGADGH along with PDZ2 in pGBT-9 were grown overnight in Leu^-, Trp^- medium at 30 °C. The cells were pelleted and the cell wall disrupted with liquid nitrogen. The released β-galactosidase was assayed using *O*-nitrophenyl-β-D-galactopyranoside as substrate. The resultant color was measured at 420 nm and the β-galactosidase activity calculated.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF093834, AF093835, AF093836, AF000974.

‡ To whom correspondence should be addressed: Pharmaceutical Biotechnology Sector, Biotechnology Research Institute, 6100 Royalmount Ave., Montreal, Quebec H4P 2R2 Canada. Tel.: 514-496-6134; Fax: 514-496-6319; E-mail: Denis.Banville@NRC.CA.

[1] The abbreviations used are: PTP, protein tyrosine phosphatases; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; HA, hemagglutinin; bp, base pair; MBP, maltose-binding protein; oligo, oligonucleotide; kb, kilobase pair; GAP, GTPase-activating protein.

Plasmid Constructs and Interaction Studies

Two-hybrid Screening—The PDZ domains of hPTP1E (PDZ1(amino acid 1092–1184), PDZ2 (amino acid 1361–1461), and PDZ4 + 5 (amino acid 1787–1968)) were amplified by PCR from a hPTP1E clone isolated previously and inserted into the *Bam*HI site of pGBT9. A *Bgl*II site was introduced in the oligos to bring the sequence in frame with that of the GAL4 DNA binding domain. Clones with the correct orientation were selected by colony PCR using a forward oligonucleotide (5′-TCATCG-GAAGAGAGTAG-3′) specific to the plasmid and an internal oligonucleotide from the hPTP1E sequence.

In Vitro Interaction Studies—To confirm *in vitro* the protein-protein interaction observed in the yeast two-hybrid system, the carboxyl-terminal portion of ZRP-1 containing the 3 LIM domains was expressed as a fusion protein with the maltose-binding protein (MBP). The carboxyl-terminal portion (amino acids 278–476) was amplified from the cDNA clone isolated from the two-hybrid system using specific oligos containing an *Eco*RI and an *Xba*I site in the forward and reverse oligos, respectively, for cloning purposes. The fragment was inserted in frame into an *Eco*RI/*Xba*I-digested plasmid pMal-C2 (New England Biolabs). The DNA fragments encoding the PDZ domains of hPTP1E as amplified for the two-hybrid constructs were fused in frame with the glutathione *S*-transferase protein in pGEX-5X-3 (Amersham Pharmacia Biotech). The DNA sequences of all plasmid constructs was verified on an Applied Biosystems Inc. DNA sequencer. The GST-PDZ and the maltose-binding protein-LIM (MBP-LIM) fusion proteins were expressed in DH5α and used for *in vitro* interactions. The proteins in the bacterial pellet from 1.5 ml of culture were solubilized in 250 μl of Buffer 1: Tris-buffered saline, 1% Nonidet P-40 containing the protease inhibitors soybean trypsin inhibitor (50 μg/ml), leupeptin (10 μg/ml), phenylmethylsulfonyl fluoride (40 μg/ml), and sonicated. 50 μl of each of the extracts was mixed with 20 μl of glutathione-Sepharose CL4B beads and the volume brought up to 250 μl with Tris-buffered saline, 0.1% Nonidet P-40 containing protease inhibitors (Buffer 2). The reaction was allowed to proceed for 1 h at room temperature on an end-on-end shaker. The glutathione beads were separated by a brief centrifugation, washed 5 times with Buffer 2, and suspended in 25 μl of SDS loading buffer. The bound proteins were analyzed by Western blot.

In Vivo Interaction Studies—For these studies, the carboxyl-terminal portion of ZRP-1 containing all 3 LIM domains (and its own termination codon) was cloned into the mammalian expression vector pAC-TAG2 (27) in such a way as to produce a fusion gene controlled by the cytomegalovirus promoter and encoding three hemagglutinin epitopes fused to the amino terminus of the recombinant protein. As for the construct for *in vitro* interaction studies, the carboxyl-terminal portion (amino acids 278–476) was amplified by PCR using ZRP-1-specific oligos containing *Nhe*I and *Bgl*II sites in the forward and reverse oligos, respectively. This fragment was cloned in frame into pAC-TAG2 vector digested with *Xba*I and dephosphorylated. After a 3-h ligation, a fill-in reaction was carried out using the Klenow fragment of T4 DNA polymerase, and the ligation was continued overnight at 14 °C. Clones containing the insert in the correct orientation were identified by colony PCR, and positive constructs were verified by DNA sequencing. To confirm the *in vivo* interaction of ZRP-1 with PDZ2 of hPTP1E, the hemagglutinin (HA)-tagged ZRP-1 protein was expressed in 293-T cells. The construct containing ZRP-1 sequences in the expression vector pAC-TAG2 was transfected into 293-T cells by the modified calcium phosphate method (28). Transiently expressed HA-ZRP protein was used for immunoprecipitation studies. Two different approaches were assayed as follows: (i) co-precipitation of HA-ZRP protein using an antibody to PDZ2 of hPTP1E, and (ii) co-precipitation of hPTP1E using anti-HA antibody. The protocols used for both these studies were similar and are outlined below.

Cells transfected with the HA-ZRP plasmid were harvested after 48 h. The cells were lysed in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 100 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin) per 10-cm dish. The plates were incubated on ice for 30 min, and the lysed cells were collected by scraping, and the cell debris was removed by centrifugation for 5 min at 4 °C in a microcentrifuge. 500 μl of dilution buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) were added to the cleared supernatant to reduce the concentration of Nonidet P-40. To 250 μl of diluted supernatant, anti-PDZ2 polyclonal antibody (5 μl of antiserum) or anti-HA monoclonal antibody (0.4 μg) (3F10, Roche Molecular Biochemicals) was added and the reaction continued to proceed on an end-on-end shaker overnight at 4 °C. To this mixture, 25 μl of protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) was added and the reaction continued for an additional hour. The gel-bound immunoprecipitates were washed 4 times (20 min each wash) with lysis buffer containing 0.1% Nonidet P-40. After the final wash the gel pellet was taken in 50 μl of SDS-polyacrylamide gel electrophoresis buffer, analyzed on a 10% polyacrylamide gel, and blotted onto a nitrocellulose membrane according to standard protocols. The blots were blocked with 5% non-fat milk in phosphate-buffered saline, 0.1% Tween 20. The immunoprecipitates obtained using anti-PDZ2 antibody were detected using anti-HA antibody (1:5000 dilution), and those precipitated by the anti-HA antibody were detected using anti-PDZ2 antibody (1:3,000 dilution). The bound first antibody was detected using horseradish peroxidase-labeled goat anti-rabbit IgG (1:5000 dilution) or goat anti-mouse IgG (1:5000 dilution) antibody. The bound secondary antibody was detected using the enhanced chemiluminescence (ECL) system from Amersham Pharmacia Biotech.

Isolation of ZRP-1 cDNAs

A human breast cDNA library in λgt-10 (CLONTECH Laboratories) (~500,000 clones) was screened using a 367-bp PCR product containing the first and part of the second LIM domains of ZRP-1 as probe. The probe was labeled with [α-^{32}P]dCTP, using the "Ready-To-Go" random labeling kit from Amersham Pharmacia Biotech. Hybridization and washes were performed according to the manufacturer's instructions.

Isolation of cDNA by Anchored PCR

Anchored PCR was performed using the 5′- rapid amplification of cDNA ends system from Life Technologies, Inc. DNA was prepared from total RNA using random hexamer DNA primers. The reverse transcribed single-stranded cDNA was tailed with deoxycytosine (dCTP) using deoxynucleotide terminal transferase. Anchored PCR was performed essentially as described by Loh *et al.* (29) with a forward primer consisting of a unique sequence (the anchor sequence) followed by a series of 14 d(G) (the anchor poly(G) primer) and a second forward primer containing only the anchor sequence. The reverse primer was a nested primer specific for ZRP-1. The fragments were cloned into the pGEM-T vector and sequenced. Subsequently the entire sequence was rechecked by sequencing directly both strands of PCR-generated products amplified from cDNA without subcloning the DNA fragments.

Western Blot

The proteins bound to the glutathione beads were separated by SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane using standard protocols. The co-precipitation of the MBP-LIM fusion protein with GST-PDZ2 was detected using a polyclonal anti-MBP antibody (New England Biolabs) at a dilution of 1:10,000. The bound antibody was detected using a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Bio-Rad) (dilution 1:5000) and the ECL system from Amersham Pharmacia Biotech.

Northern Blot

The expression of ZRP-1 mRNA was studied with a human multiple tissue Northern blot (CLONTECH Laboratories). The probe used was a 367-bp PCR fragment containing the first and part of the second LIM domains of ZRP-1 labeled with [α-^{32}P]dCTP, using the "Ready-To-Go" random labeling kit from Amersham Pharmacia Biotech. Hybridization and washes were performed according to the manufacturer's instructions.

Isolation and Characterization of Human ZRP-1 Genomic Sequences

The upstream and downstream regions of the human *ZRP-1* gene were amplified from total genomic DNA using the Genomic Walking Kit from CLONTECH. To obtain the 5′-flanking sequence of the gene, the following gene-specific oligonucleotides were used: primer 1, (ZRP-R9) 5′-GCCCCGACATGGCCTGGAAAGG, and primer 2, (ZRP-R10) 5′-CCCGAGCCTCTGGCCTTCACC. The 3′ portion of the gene was obtained using the following primers: 1, (ZRP-F2) 5′-CTGAGCCAGCCTC-CAGAGGAT, and 2, (ZRP-F1) 5′-GACCGGATCCTGCGGGCTATG. The entire coding region of the gene was amplified by long PCR using the Expand DNA polymerase mix from Roche Molecular Biochemicals, with the following oligonucleotides: ZRP-F3, 5′-TCAAGACCGCTGTCTG-GAGTCC, and ZRP-R8, 5′-CTGGAACTGAGAACCCAGCAGGTA. With the exception of two gaps within intron 4 and intron 7, respectively, the sequence of the entire *ZRP-1* gene has been determined and deposited in GenBank™ with the following accession numbers: AF093834, AF093835, and AF093836.

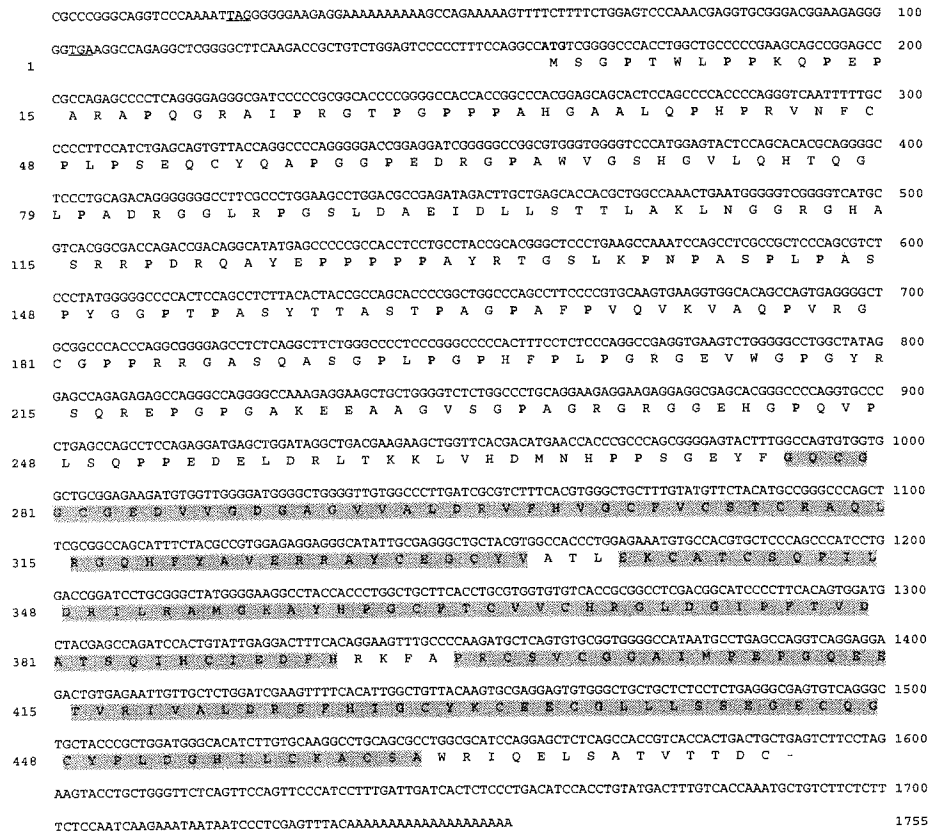

FIG. 1. **Nucleotide and deduced amino acid sequences of the composite ZRP-1 cDNA and protein.** The nucleotide sequence is numbered from the 5′ end of the longest fragment obtained by reverse transcriptase-PCR. The first ATG is at position 160 and is shown in *bold type*. The 3 LIM domains are *shaded*. The proline residues in the amino-terminal half of the protein are shown in *bold letters*.

RESULTS

Identification of a Novel LIM Domain Containing Protein Interacting with the Second PDZ Domain of hPTP1E in a Yeast Two-hybrid Screen—The PDZ2 domain of hPTP1E was fused to the Gal4 DNA binding domain containing plasmid pGBT9 and used as bait to screen a HeLa cell cDNA library using the yeast two-hybrid system (30). Analysis of a total of 1.7×10^7 clones resulted in the identification of approximately 400 candidates as determined by the His+ screen. Most of these were, however, eliminated upon testing for their ability to activate the β-galactosidase gene. Among the 20 positive clones, two overlapping sequences derived from the same gene were found to interact strongly with the PDZ2 domain of hPTP1E as determined by the intensity of the blue color indicator. The longest clone, C90, possessed an open reading frame of 261 amino acids and interacted more strongly than its shorter counterpart (194 amino acids open reading frame). By using the C90 cDNA sequence, a human breast cDNA library was screened, yielding 7 clones, the longest of which was 1.5 kb and contained the sequence encoding the last 432 amino acid residues. The remainder of the sequence was obtained by rapid amplification of cDNA ends-PCR performed on total RNA from HeLa cells.

The composite nucleotide sequence of the ZRP-1 cDNA was established and deposited with GenBank™ (accession number AF000974). The human ZRP-1 cDNA sequence is 1755 base pairs long and displays an open reading frame of 1428 bp with a translational initiation codon at nucleotide positions 160–162 and a stop codon at positions 1588–1590. The sequence around the ATG matches the Kozak consensus initiation sequence (31), and two stop codons are present in this reading frame within the upstream 5′-noncoding region. The open reading frame predicts a 476-amino acid polypeptide with a calculated mass of 50.3 kDa (Fig. 1). The amino-terminal sequence of the predicted protein is enriched in proline residues that account for nearly 20% of all amino acid residues. The second half of the protein contains three cysteine-rich zinc binding domains referred to as LIM domains. These domains are protein motifs of approximately 55 amino acid residues containing the consensus peptide sequence $CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-21}CX_{2-3}$(C,H,D), where X represents any amino acid (32–36). Alignments of the LIM domain sequences of ZRP-1 and those of the related proteins of the Zyxin family of proteins that includes Zyxin (37, 38), LPP (39), and Enigma (40) are shown in Fig. 2.

Despite the fact that all four proteins possess a proline-rich amino-terminal region, their actual amino acid sequences differ significantly in this region. The region of highest homology is that comprising the three LIM domains. Indeed, within this region, ZRP-1 displays 71.9 and 57.5% identity with LPP and Zyxin, respectively. Of the three LIM domain, LIM3 is the most conserved, showing 77% identity between ZRP-1 and the corresponding region of LPP. The similarity between LPP and ZRP-1 is also evident when the genomic organization of the two genes is compared. As shown in Fig. 3, the *ZRP-1* gene coding region consists of 9 exons distributed over approximately 5 kilobases of DNA. As for the LPP protein, the amino-terminal proline-rich region of ZRP-1 is encoded by 5 exons, and the first two LIM domains are encoded by separate exons, whereas the third LIM domain is coded for by the last two exons. Table I summarizes the nucleotide sequences of the splice sites. Sequence comparisons of the *ZPR-1* and the *LPP* genes revealed that the locations of the splice sites are identical suggesting that the two genes result from a recent gene duplication event.

The tissue distribution of the ZRP-1 mRNA was examined by Northern blot analysis (Fig. 4). A strong signal was observed in most tissues including heart, placenta, lung, liver, kidney, and pancreas. A weaker signal was obtained in brain and skeletal muscle. The observed size of the message is approximately 2 kb suggesting that the full-length sequence has been cloned.

ZRP-1 Interacts Specifically with the Second PDZ Domain of hPTP1E—To confirm the specificity of the interaction between

ALIGNMENT OF LIM DOMAINS OF ZRP-1, LPP, ZYXIN AND ENIGMA

LIM-1

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ZRP-1   GQCGGCGEDVVGDGAGVVALDRVFHVGCFVCSTCRAQLRGQHFYAVERRAYCEGCYV
LPP     GRCARCGENVVGEGTGCTAMDQVFHVDCFTCIICNNKLRGQPFYAVEKKAYCEPCYI
ZYXIN   ELCGRCHQPLARAQPAVRALGQLFHIACFTCHQCAQQLQGQQFYSLEGAPYCEGCYT
ENIGMA  PVCHQCHKVI--RGRYLVALGHAYHPEEPVCSQCGKVLEEGGFFEEKGAIFCPPCYD
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LIM-2

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ZRP-1   EKCATCGQPILDRILRAMGKAYHPGCFTCVVCHRGLDGIPFTVDATSQIHCIEDFH
LPP     EQCNVCSKPIMERILRATGKAYHPHCFTCVMCHRSLDGIPFTVDAGGLIHCIEDFH
ZYXIN   EKCNTCGEPITDRMLRATGKAYHPHCFTCVVCARPLEGTSFIVDQANRPHCVPDYH
ENIGMA  PSCAKCKKKITGEIMHALKMTWHVHCFTCAACKTPIRNRAFYME-EGVPYCERDYE
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LIM-3

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ZRP-1   PRCSVCGGAIMPEPGQEETVRIVALDRSFHIGCYKCEECGLLLSSEGECQGCYPLDGHILCKACSA
LPP     PRCSVCKEPIMPAPGQRETVRIVALDRDFHVHCYRCEDCGGLL-SEGDNQGCYPLDGHILCKTCNS
ZYXIN   PRCSVCSEPIMPEPGRDETVRVVALDKNFHMKCYKCEDCGKPLSIEADDNGCFPLDGHVLCRKCHT
ENIGMA  TKCHGCDFKI--DAGDR---FLEALGFSWHDTCFVCAICQINL--EG--KTFYSKKDRPLCKSHAF
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FIG. 2. **Sequence comparison of the LIM domains of ZRP-1 with those of human LPP, Zyxin, and Enigma.** The cysteine and histidine residues characteristic of LIM domains are in *bold letters,* and the conserved amino acids are *shaded.* A conserved alanine at position −6 with respect to the conserved histidine is *underlined.*

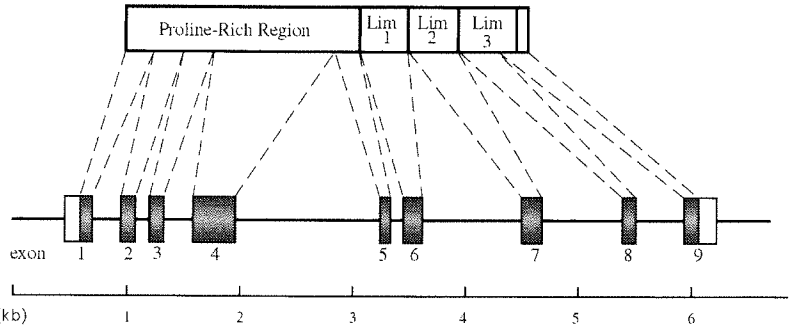

FIG. 3. **Genomic organization of the human *ZRP-1* gene.** The various domains of the ZRP-1 protein are shown *above* a schematic representation of the human *ZRP-1* locus. The *boxes* represent the 9 exons, and the *shaded* portions correspond to the coding sequence.

TABLE I
Intron-exon slice junction sites of the human ZRP-1 gene

Exon	Size	Splice donor	Splice acceptor	Intron Size
	bp			*bp*
1	~240	CC CAC GGA GCA G gtaaggcagcccttgtg. . .	. . .ctcttccctcaacccag CA CTC CAG CCC	254
2	128	CAG CAC ACG CAG gtgagaccgggatcgt. . .	. . .cctggctccatcctcag GGG CTC CCT GCA	119
3	126	CCA GAC CGA CAG gtgactctgcccctcct. . .	. . .cctgcgtttctcctcag GCA TAT GAG CCC	261
4	372	CAC GGG CCC CAG gtgagccctggggaact. . .	. . .tgattcccaccttccag GTG CCC CTG AGC	~1300
5	94	GG GAG TAC TTT G gtgagctgaggctgtgg. . .	. . .tgctcccctgtcctcag GC CAG TGT GGT G	113
6	170	GGC TGC TAC GTG gtgagtggctggggctg. . .	. . .gcattccttcccaacag GCC ACC CTG GAG	884
7	179	G GAC TTT CAC AG gtcaggcctggcctcca. . .	. . .gtttttttcccctgcag G AAG TTT GCC CC	~700
8	121	TAC AAG TGC GAG gtcaggggcccccagca. . .	. . .tgcttctttttcaacag GAG TGT GGG CTG	425
9	278			

ZRP-1 and the PDZ2 of hPTP1E, its interaction was tested with other PDZ domains of hPTP1E. The PDZ domains 1 (amino acids 1092–1184), 4, and 5 (amino acids 1787–1968) of hPTP1E were cloned into pGBT9 in frame with the DNA binding domain of GAL4 and co-transformed with ZRP-1 (in pGADGH) into SFY526. Neither of the domains activated the β-galactosidase gene, suggesting a lack of interaction (data not shown). Additionally, no interaction was observed when the PDZ domains of two related PTPases, PTPH1 (17) and PTPMEG (18), were assayed under the same conditions (results not shown), thus substantiating the specificity of the interaction of ZRP-1 with the PDZ2 of hPTP1E.

To determine the contribution of the various domains of ZRP-1 and that of its carboxyl-terminal residues to its interaction with the PDZ2 domain of hPTP1E, several constructs containing various combinations of LIM domains were prepared in the plasmid pGADGH (Fig. 5*A*). The yeast strain SFY526 was co-transformed with these LIM constructs and the PDZ2-containing plasmid. The yeast colonies obtained after transformation were plated, and the intensity of interaction was assessed by both filter-lift and liquid β-galactosidase assays. The results obtained from filter-lift assays are presented in Fig. 5*A*. An interaction was observed only in constructs containing the carboxyl-terminal fragment of ZRP-1 (constructs 1–4). No interaction was observed with the other constructs suggesting that an intact carboxyl terminus is required for this interaction. The intensity of the interaction was dependent on the number of LIM domains present, being strongest in the presence of all three LIM domains and weakest when only one LIM domain was present (compare constructs 1 and 4). Clone C90 (the longest clone obtained from the two-hybrid screen), which contains 63 extra amino acids in addition to the

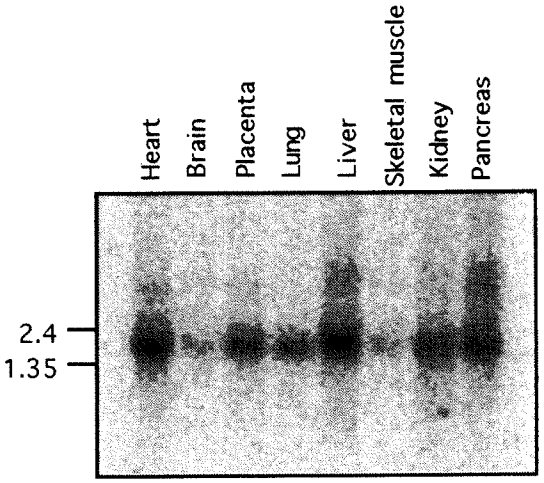

FIG. 4. **Tissue distribution of ZRP-1 mRNA.** A human multiple tissue Northern blot (CLONTECH) was probed with a ^{32}P-labeled 367-bp fragment containing the first and part of the second LIM domains of ZRP-1.

three LIM domains and the carboxyl-terminal tail, gave the strongest signal. The variation in the intensity of interactions was not due to variations in the level of expression of the different constructs as demonstrated by a Western blot of the Gal4-activating domain fusion proteins using a Gal4AD monoclonal antibody (Fig. 5*B*) showing that all constructs were expressed at comparable levels. Liquid assays for β-galactosidase activity were carried out using the various LIM domain constructs, and the results are shown in Fig. 5*C*. The results obtained parallel those obtained with filter-lift assays and show that the β-galactosidase activity decreases with a reduction in the number of LIM domains, and the activity is close to background in the constructs containing the three LIM domains but lacking the last 11 carboxyl-terminal amino acid residues. The data suggest that in addition to the carboxyl-terminal sequence, the rest of the protein molecule may be important in stabilizing the interaction.

In Vitro and in Vivo Demonstration of Interaction between ZRP-1 and hPTP1E—The interaction of the PDZ2 of hPTP1E with ZRP-1 was confirmed by both *in vitro* and *in vivo* studies. For *in vitro* studies the PDZ domain was expressed as a GST fusion protein (GST-PDZ), and the carboxyl-terminal portion containing the LIM domains of ZRP-1 (amino acid residues 278–476) was expressed as a maltose-binding protein fusion (MBP-LIM). Both fusion proteins were induced with isopropyl-1-thio-β-D-galactopyranoside as outlined under "Materials and Methods." The solubilized proteins from bacterial lysates were mixed and allowed to interact at room temperature. The GST fusion protein was pelleted down using glutathione-Sepharose beads. The bound proteins were eluted with SDS buffer, analyzed by SDS-polyacrylamide gel electrophoresis, and probed with a monoclonal antibody to MBP on a Western blot. When both fusion proteins were mixed, the MBP-LIM fusion protein was co-precipitated by glutathione-Sepharose beads along with GST-PDZ indicating that the two proteins interacted with each other *in vitro* (Fig. 6, *lane 1*). The MBP-LIM protein did not bind to glutathione-Sepharose beads alone (Fig. 6, *lane 2*) or to glutathione-Sepharose beads in the presence of GST (Fig. 6, *lane 4*).

For *in vivo* studies, the 3 LIM domains and the carboxyl-terminal tail of ZRP-1 were expressed as a hemagglutinin epitope-tagged protein (HA-ZRP), and its interaction with endogenous hPTP1E was studied. The interaction in the cells was demonstrated by co-precipitation of the transiently expressed HA-ZRP with the endogenous hPTP1E. The results obtained are shown in Fig. 7. In the first set of experiments (Fig. 7*A*), HA-ZRP was expressed in 293-T cells, and the endogenous hPTP1E was precipitated by anti-PDZ2 antibody. The co-precipitation of the expressed HA-ZRP was detected using anti-HA antibody (Fig. 7*A*, *lane 2*). The deletion of the last cysteine residue of ZRP-1 did not prevent the interaction of the two proteins (Fig. 7*A*, *lane 3*). Deletion of the carboxyl-terminal 11 amino acid residues, however, completely abolished the interaction (Fig. 7*A*, *lane 4*). *Lanes 1* and *5* correspond to controls with no HA-ZRP transfected (−) and after transfection of the cells (+) but immunoprecipitated with preimmune serum. To demonstrate the level of transiently expressed HA-ZRP in these cells, an aliquot of each cell lysate was analyzed using an anti-HA antibody (Fig. 7*B*). A distinct band in the 27-kDa region was observed only in the HA-ZRP sample. The other bands observed in the negative control represent the background of the HA antibody used.

The reciprocal experiment, *i.e.* the ability of the transiently expressed HA-ZRP to immunoprecipitate endogenous hPTP1E, was also evaluated (results not shown). HA-ZRP was immunoprecipitated using anti-HA antibody, and the presence of hPTP1E in the precipitate was assayed using anti-PDZ2 antibody. A clear band in the 275-kDa region was detected in the sample prepared from the HA-ZRP-expressing cells with no distinct protein band in the pcDNA3 control. The results of these studies clearly demonstrate that ZRP-1 interacts with hPTP1E *in vivo*.

Finally, since an earlier study (22) had shown that the cytoplasmic tail of the membrane receptor protein Fas interacts with PDZ2 of hPTP1E (FAP-1), we were concerned that none of the clones isolated in our screenings corresponded to the Fas sequence. To verify the presence of the Fas sequence in our HeLa cDNA library, we used PCR. This experiment demonstrated that Fas cDNAs were indeed present in the library (results not shown). We then cloned the carboxyl-terminal part of Fas, amino acid 219–319 (41), as used by Sato *et al.* (22) in pGADGH and studied its interaction with PDZ2 (in pGBT9). Under these conditions, both filter-lift and liquid β-galactosidase assays suggested that this interaction was much weaker in comparison to that elicited by ZRP-1 (Fig. 8 and results not shown). Indeed, under our assay conditions, the strength of the interaction with Fas was roughly one-tenth of the intensity of the interaction observed with ZRP-1.

DISCUSSION

By using the second PDZ domain of hPTP1E as a bait in the yeast two-hybrid system, we have isolated a novel protein (ZRP-1) that interacts strongly with PDZ2 of hPTP1E. ZRP-1 contains three cysteine-rich zinc binding LIM domains. These latter domains are themselves protein interacting modules present in a large number of proteins with diverse functions (32–35). ZRP-1 belongs to a group of LIM proteins which includes Zyxin, a member of the cell adhesion complex (37, 38, 42, 43), LPP, a preferred fusion partner of HMGIC in lipomas (39), and Enigma (40). The LIM domains of ZRP-1 display a high degree of sequence similarity with two proteins belonging to this group, namely LPP (39) and Zyxin (42). These proteins each contain three carboxyl-terminal LIM domains that represent the regions of highest similarity between them. The overall identity between ZRP-1 and LPP in the three LIM domains is 71.9%, with the highest identity between the two proteins being within their last two LIM domains (77%). A similar pattern was observed between ZRP-1 and Zyxin. The overall identity to ZRP-1 in the three LIM domains is 57.5%, with the highest identity being within the last two LIM domains, 61.5% in LIM-2 and 64.5% in LIM-3. These three proteins also possess a proline-rich NH_2-terminal region. However, the identity be-

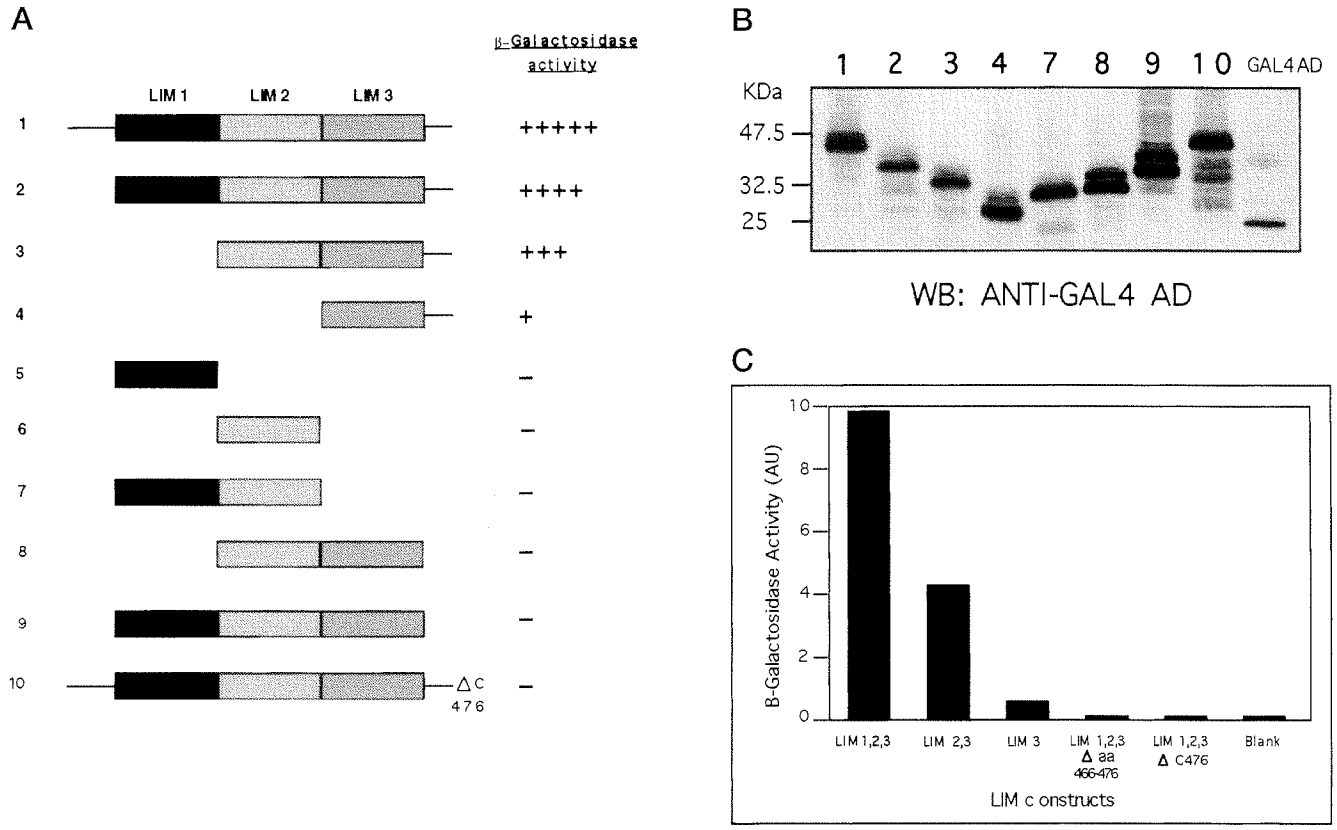

FIG. 5. **Identification of the region involved in the interaction between ZRP-1 and the PDZ2 domain of hPTP1E in the yeast two-hybrid system.** *A*, filter-lift β-galactosidase assays: 10 constructs containing different portions of ZRP-1 were used to study the interaction with PDZ2 domain of hPTP1E. These constructs contain the sequence corresponding to the following portions of the ZRP-1 protein: *1*, amino acids 215–476; *2*, amino acids 278–476; *3*, amino acids 338–476; *4*, amino acids 398–476; *5*, amino acids 278–337; *6*, amino acids 338–397; *7*, amino acids 278–397; *8*, amino acids 338–465; *9*, amino acids 278–465; and *10*, amino acids 215–475. The results have been scored as "+" and "–," with the number of + signs being directly related to the intensity of the interaction. *B*, Western blot (*WB*) analysis of the GAL-4 activating domain fusion proteins using a GAL-4 AD mAb. The yeast strain CG-1945 was used to express the various ZRP constructs. Protein samples equivalent to 0.3–0.5 A_{600} units of cells were electrophoresed on a 12% polyacrylamide-SDS gel. The numbers *above* each lane correspond to the constructs depicted in *A*, *C*, β-galactosidase liquid assays: the constructs used for these assays correspond to constructs 2 (3 LIM domains), 3 (LIM domains 2, 3), 4 (LIM domain 3), 9 (LIM domains 1, 2, 3 minus the last 11 amino acid residues), and 10 (deletion of the last cysteine residue only) represented in *A*. *Blank* represents PDZ2 alone. Yeast colonies containing the desired ZRP-1 construct and the PDZ2 construct were grown overnight in Leu^-, Trp^- medium, the cells disrupted with liquid nitrogen, and the released β-galactosidase activity measured using *O*-nitrophenyl-β-D-galactopyranoside as substrate. The activity is presented as β-galactosidase units (arbitrary units, *AU*).

tween these domains is much lower.

From the crystal structures of the peptide bound and free PDZ3 domains of PSD-95 (44) and the human homologue of *Drosophila* Dlg, DlgA (45), it was determined that the carboxyl-terminal sequence (S/T)*X*V in the PDZ domain binding protein was involved in the interactions. In keeping with the hypothesis that the carboxyl-terminal fragment of ZRP-1 may be involved in the interaction with PDZ2 of hPTP1E, we observed no interaction in the absence of the carboxyl-terminal 11 amino acid residues of ZRP-1 in both yeast or mammalian cells. The carboxyl-terminal sequence of ZRP-1, ..VTTDC-COOH, is significantly different from the consensus sequence of (S/T)*X*V originally suggested to be the required peptide motif at the carboxyl terminus for interaction with PDZ domains (44–45). By using oriented peptide libraries, Songyang *et al.* (46) have suggested that the carboxyl-terminal requirement for interaction with the PDZ2 of hPTP1E is -(E/V)(T/S)*X*(V/I)-COOH. This library was biased since it did not contain any cysteine or tryptophan residues at the COOH terminus. ZRP-1 on the other hand contains a cysteine at the COOH terminus. By using the above approach, this class of proteins would be missed. In another study using neuronal nitric oxide synthase, Stricker *et al.* (47) have identified a peptide sequence D*X*V-COOH to be important in the interaction. This again is different from the sequence (S/T)*X*V-COOH suggested to be the conserved sequence required for interaction with PDZ domains. These observations would further suggest that other carboxyl-terminal sequences could also define the specificity for particular PDZ domains. Deletion of the last cysteine residue abolished the interaction in the yeast two-hybrid system where the specific interaction of hPTP1E PDZ2 domain and the ZRP-1 protein is assayed. This suggests that the carboxyl-terminal amino acid residue is indeed involved in the interaction. However, removal of this last cysteine residue did not affect the interaction of ZRP-1 with endogenous hPTP1E. This apparent inconsistency could result from several factors. It is indeed conceivable that in mammalian cells, the constraints for the interaction of the two proteins are different than those in the yeast cells and that the presence of the complete hPTP1E protein stabilizes the interaction. Finally, by deleting the last cysteine residue from ZRP-1, a novel carboxyl terminus is created whose sequence (. . . VTTD-COOH) may represent a novel PDZ-binding motif capable of interacting with any one of the five hPTP1E PDZ domains. Further studies will be necessary to clarify these observations.

Similar observations have been made recently (48) in the course of a study of the interaction of a LIM domain-containing protein, RIL, with PTP-BL, the murine homologue of the human hPTP1E. The RIL protein interacts with both the PDZ2 and PDZ4 domains. The carboxyl-terminal sequence of RIL

contains the sequence VELV-COOH, which does not match with either the consensus (S/T)*X*V-COOH or the sequences -(E/T)(T/S)*X*(V/I)-COOH (for PDZ2) and -(I/Y/V)YYV-COOH (for PDZ4) identified by Songyang *et al.* (46). For the interaction of RIL with PDZ4 to occur, the carboxyl-terminal sequence is required. In addition, the interaction is much stronger in the presence of the LIM domain, an observation similar to that seen with ZRP-1. These observations are consistent with the concept of the LIM domains also playing an important role in stabilizing the interaction with the PDZ domain of hPTP1E. The importance of upstream peptide sequences in the interactions with PDZ domains has also been suggested in another study with neuronal nitric oxide synthase (47). These authors observed that the preference of Asp at position −2 is determined by tyrosine 77 of neuronal nitric oxide synthase. Mutation of Tyr-77 and Asp-78 to His-77 and Glu-78 results in a change in the specificity from Asp-Xaa-Val to Thr-Xaa-Val. The data suggest that in addition to the carboxyl-terminal sequence, other structural features in the rest of the protein molecule are also important in defining the specificity of the interaction with PDZ domains. In an earlier study of the apoptosis-mediating Fas protein, Sato and co-workers (22) have shown that human FAS interacts with the second of five PDZ domains of hPTP1E (FAP-1) through its carboxyl-terminal 15 amino acid residues. The PDZ2-Fas interaction follows the typical consensus pattern as the carboxyl-terminal sequence of Fas is SLV-COOH. It is surprising to note though that the sequence of the Fas protein is not very conserved between different species with an overall identity between human (49) and mouse (50) sequences being only 49.5% (51). More unexpectedly, this divergence extends to the amino acids at the very carboxyl terminus with the sequence being . . . SLV-COOH in human, . . . CLE-COOH in mouse, . . . SLE-COOH in rat (52), and . . . NLV-COOH in bovine (53). No interaction of the PDZ2 domain of PTPBL (the mouse equivalent of hPTP1E) was observed with the carboxyl terminus of mouse FAS (54). All this suggests that the physical interaction between the Fas protein and the PDZ domain of hPTP1E may not be required for their respective functions in species other than human. Our data demonstrate that the binding of ZRP-1 to the PDZ domain of hPTP1E is much stronger than the binding of Fas to the same PDZ domain via its consensus (S/T)*X*V carboxyl-terminal sequence. The biological significance of this observation remains to be established.

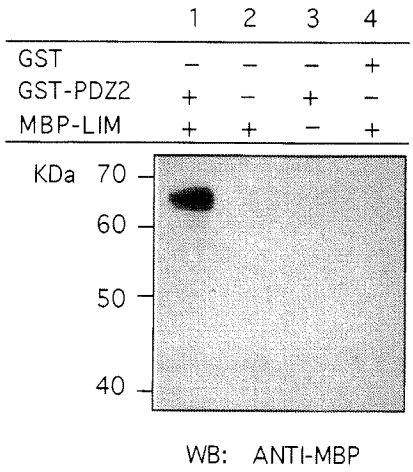

FIG. 6. ***In vitro* interaction of PDZ2 with the carboxyl-terminal LIM domain containing region of ZRP-1.** The PDZ2 domain of hPTP1E and the carboxyl-terminal region of ZRP-1 containing all three LIM domains were expressed as a GST fusion and MBP fusion protein, respectively. The fusion proteins were expressed in bacteria and assayed for interaction as outlined under "Materials and Methods." The protein complex was precipitated using glutathione-Sepharose beads. The proteins bound to the beads were analyzed by Western blot (*WB*) analysis using an anti-maltose binding protein antibody. Lanes are labeled as follows: *1,* GST-PDZ2 + MBP-LIM; *2,* MBP-LIM alone; *3,* GST-PDZ2 alone; and *4,* GST + MBP-LIM.

The broad tissue distribution of ZRP-1, as demonstrated by Northern analysis and reflected by the large number of related sequences in the EST data base (NCBI), suggests a ubiquitous role for this protein in cellular function. A search through the GenBank™ data base revealed that ZRP-1 was identical to a part of the TRIP6 sequence (GenBank™ accession number L40374) (55), identified as a thyroid receptor interacting protein. This sequence contained only two LIM domains and the carboxyl-terminal portion of the protein. However, these authors suggested that it would be unlikely that TRIP6 is involved in thyroid receptor function and that its similarity to Zyxin probably reflects a common subcellular localization. In the course of the preparation of this manuscript, the complete sequence of the human TRIP6 mRNA was reported (GenBank™ accession number AJ001902) (56). The sequence of this gene product is identical to that of ZRP-1. The gene has been assigned to a segment of human chromosome 7q22 between the erythropoietin and the plasminogen activator inhibitor-1 precursor genes (56). This region of chromosome 7 is often deleted in malignant myeloid diseases and uterine leiomyoma. Since the molecular mechanisms of these diseases are not yet clearly understood, the involvement of ZRP-1 in these cancers needs to be evaluated.

Group 3 LIM proteins, to which ZRP-1 belongs, have been shown to be involved in a number of interactions involving both the LIM domains and the non-LIM domain portion of the molecule. In the case of Zyxin, the first LIM domain binds cysteine-

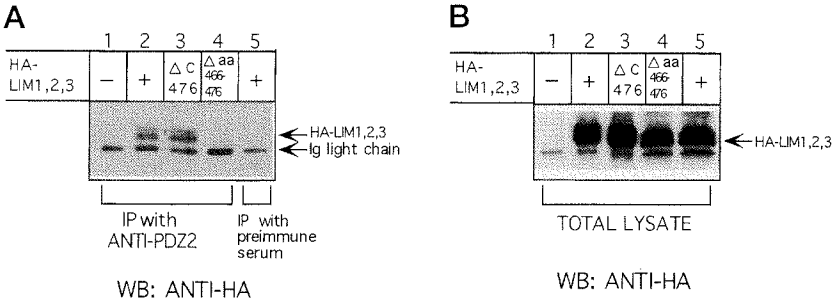

FIG. 7. ***In vivo* interaction of the carboxyl-terminal portion of ZRP-1 with hPTP1E.** ZRP-1 was expressed as a HA-tagged protein in 293-T cells and used for immunoprecipitation studies. The co-precipitation of ZRP-1 with PDZ2 of hPTP1E is shown in *A*. The endogenous hPTP1E was precipitated with PDZ2 antibody, and the co-precipitated HA-ZRP-1 was detected by anti-HA antibody. The co-immunoprecipitation of HA-ZRP-1 with hPTP1E is shown in *lane 2*. Immunoprecipitation experiments using the ΔC476 construct is shown in *lane 3* and that with Δ amino acids (Δ*aa*) 466–476 is presented in *lane 4*. The background of the system is represented by *lane 1* with no HA-ZRP-1 transfected and *lane 5* for which the preimmune serum was used. *B*, the amount of HA-LIM proteins expressed after transfection of the various constructs is shown. *WB,* Western blot.

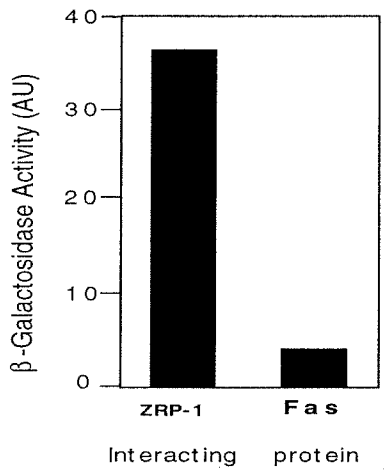

FIG. 8. **Comparison of the intensity of ZRP-1 and Fas interactions with PDZ2 of hPTP1E.** A clone of ZRP-1 containing amino acid residues 215–476 and a construct of Fas containing the entire death domain and the rest of the carboxyl-terminal portion were used in this study. The plasmids were coexpressed with the PDZ2 domain plasmid in SFY526 cells and the interaction quantitated by liquid β-galactosidase assay. The β-galactosidase activity is displayed in arbitrary units (*AU*).

rich protein, and its proline-rich amino-terminal region binds α-actinin (57) as well as the human proto-oncogene product VAV, an SH3 adaptor protein (58). Enigma, another member of this group, recognizes the active endocytic codes of the insulin receptor, a region characterized by two tyrosine-containing tight turn motifs through the third LIM domain (40) and the receptor tyrosine kinase, Ret, through its second LIM domain (59). Furthermore, the mitogenic signaling by Ret/ptc2, a papillary thyroid cancer oncogene product, was shown to require the association with Enigma via the LIM 2 domain (60). In addition to the interactions with heterologous regions of proteins, LIM domains have been shown to function as protein dimerization domains. For example, LIM domains of cysteine-rich protein can efficiently homodimerize (61). The various protein-protein interactions in which Zyxin and Enigma are involved suggest that these molecules act as scaffolding proteins, playing an important role in bringing together many of the signaling molecules (36). ZRP-1, which possesses a similar structure, is also likely to be involved in interactions other than with the PDZ domain of hPTP1E. The three LIM domains of ZRP-1 as well as its proline-rich amino-terminal region are potential protein-protein interaction modules. In a recent study using *Neisseria gonorrhoeae* opacity-associated (Opa) as bait in the yeast two-hybrid system, this protein was shown to interact with the ZRP-1 protein (62). The authors suggest that ZRP-1 may be involved in the mechanism of bacterial pathogenesis.

In addition to the interaction of ZRP-1 and Fas with PDZ2 of hPTP1E, an additional protein has been shown to interact with another structural domain of this enzyme. Thus, a GTPase-activating protein (PARG-1) was shown to interact with PDZ4 of hPTP1E (23). With interacting partners identified for two of five PDZ domains of hPTP1E, it is very likely that this enzyme is part of a larger multiprotein complex involved in signal transduction. In the case of InaD, a PDZ domain containing protein involved in *Drosophila* vision, most of the proteins involved directly in phototransduction bind directly to this protein via its five PDZ domains (63). This protein acts as a scaffolding protein enhancing the speed and efficiency of vision in *Drosophila*. It will be interesting to see if hPTP1E also acts as a scaffolding protein in addition to its function as a phosphatase. Further work is necessary to identify the other members of the hPTP1E complex.

Analysis of the genomic organization of the *ZRP-1* gene and comparison with that of the *LPP* gene revealed a striking similarity indicating a common origin by gene duplication. Interestingly, there are also major differences between the two genes. Whereas the *LPP* gene is located on chromosome 3, between 3q27 and 3q28 (39), the *ZRP-1* gene is located on chromosome 7 (56). The major difference between the two genes resides, however, in their respective size. Whereas *ZRP-1* sequence is contained within a 6-kilobase segment of genomic DNA, the *LPP* gene was estimated to be dispersed over at least 400 kb of genomic DNA (39).

In conclusion we have identified a novel protein that interacts strongly with PDZ2 of hPTP1E. This protein also contains other potential protein interacting modules making it a potential scaffolding protein. Identification of other proteins interacting with the various domains of hPTP1E and of ZRP-1 is required for a better understanding of the role of these proteins in cellular function.

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