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# Identification of human YVH1 substrates and binding partners using biological mass spectrometry.

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# IDENTIFICATION OF HUMAN YVH1 SUBSTRATES AND BINDING PARTNERS USING BIOLOGICAL MASS SPECTROMETRY

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## ABSTRACT


### IDENTIFICATION OF HUMAN YVH1 SUBSTRATES AND BINDING PARTNERS BY BIOLOGICAL MASS SPECTROMETRY

Dual specificity phosphatases belong to the protein tyrosine phosphatase family of enzymes. These members have the ability to dephosphorylate both phosphotyrosine and phosphoserine/-phosphothreonine residues on the substrate proteins and have been found to be the regulators of critical cellular functions such as cell growth and cell cycle progression. The first dual specificity phosphatase was identified from *vaccina* virus. Later studies found homologues of VH1 in other organisms including yeast and humans. The human homologue, hYVH1, is a 36kDa enzyme containing a novel zinc finger domain. This research project was carried out as a first step towards the physiological characterization of this enzyme. In this project, we have identified the first associating protein of hYVH1 using affinity chromatography and mass spectrometry. The protein identified is Hsp70, a member of the heat shock family of proteins, known to get induced in response to cellular insults and to prevent apoptosis. Using substrate trapping mutants and two-dimensional gel electrophoresis we have identified unique spots which qualify to be the potential substrate of hYVH1. The identification and functional characterization of these potential substrates and interacting proteins will greatly enhance the elucidation of the physiological relevance of this evolutionary conserved phosphatase.

Dedicated to my family

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| | |
|---|---|
| AB | ammonium bicarbonate |
| Alpha-matrix | alpha-cyano-4-hydroxycinnamic acid |
| ATP | adenosine triphosphate |
| BSA | bovine serum albumin |
| CHAPS | 3-(3-cholamidopropyl)diethyl-ammonio -1propanesulfonate |
| CID | collision induced fragmentation |
| 2-DE | two-dimensional electrophoresis |
| DMEM | Dulbeco's Modified Eagle Medium |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| DSPs | dual specificity phosphatases |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetra-acetic acid disodium salt |
| ESI | electrospray ionization |
| FBS | fetal bovine serum |
| HRP | horse reddish peroxidase |
| IgG | immunoglobin G |
| IEF | isoelectric focusing |
| IP | immunoprecipitation |
| IPTG | isopropyl-beta-D-thiogalactopyranoside |
| MALDI-TOF | Matrix-assisted laser desorption ionization-Time of flight |
| MAPK | mitogen-activated protein kinases |
| MKP | MAPK phosphatases |
| mRNA | messenger RNA |
| MW | molecular weight |
| m/z | mass to charge ratio |
| PBS | phosphate buffer saline |
| pI | isoelectric point |
| PCR | polymerase chain reaction |
| PMSF | phenylmethyl sulfonyl fluoride |
| PSD | post source decay |
| PTPs | protein tyrosine phosphatase |
| PVDF | polyvinylidene fluoride |
| RNA | ribonucleic acid |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| TBS | Tris buffer saline |

# INTRODUCTION

## 1.1 Protein tyrosine phosphatase (PTP) superfamily

In cellular systems, environmental signals are communicated via regulatory post-translational modifications. Phosphorylation is a reversible form of post-translational modification, in which the gamma ($\gamma$) phosphate group of ATP is added to a serine, threonine or tyrosine residue of a target protein by the action of a protein kinase. The event of phosphorylation is reversed by another group of enzymes called phosphatases.

Protein kinases and phosphatases work in a coordinated manner to regulate the activities of proteins and control a wide range of cellular functions (Hunter, 1995). These include communication between cells, proliferation and differentiation, cell cycle control, transcription, mRNA processing, molecular transport into and out of cells, metabolic processes, cytoskeleton organization and cell adhesion (Alonso *et al.,* 2004). Since phosphorylation is reversible, the protein phosphatases play a crucial role in the completion of the process by reversing the phosphorylation state of the protein and bringing it back to its original form whether it had initially been activated or inactivated by phosphorylation **(Figure 1).** The disruption of phosphatase activities puts a cell under a malfunctioning state leading to the development of many diseases including abnormal cell growth, oncogenesis, neuromuscular illness, diabetes, and immune disorders (Alonso *et al.,* 2004; Ducret *et al.,* 2005).

Protein phosphatases are mainly divided into two families. These include

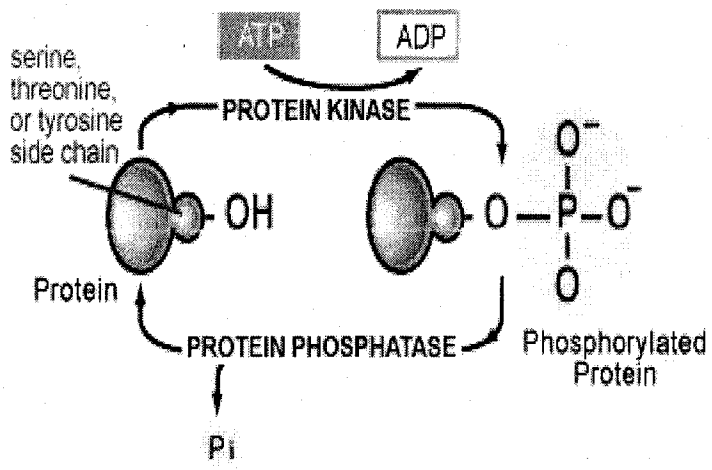



**Figure 1. Post-translational modification of proteins via phosphorylation**. The figure above shows the modification of proteins which can take place at the serine/-threonine or tyrosine residues of the target protein to be phosphorylated by a protein kinase in an ATP-dependent manner. Once phosphorylated the proteins are taken back to their original state by the action of protein phosphatases.

serine/-threonine phosphatases and protein tyrosine phosphatases (PTPs). Serine/-threonine phosphatases are metalloenzymes and they require a metal ion to perform catalysis (Barford *et al.,* 1998). On the other hand, PTPs are classified by the presence of the signature motif $C(X)_5R$ and they mediate catalysis via the formation of a phosphoenzyme reaction intermediate (Stone and Dixon, 1994).

There are approximately 107 PTPs encoding genes in the human genome (Alonso *et al.,* 2004).The PTP family is divided into receptor-like and nonreceptor-like or intracellular enzymes based on the presence of extracellular and transmembrane regions (Barford *et al.,* 1998; Fischer *et al.,* 1991). Furthermore, the intracellular PTPs are subdivided into four subfamilies which include tyrosine specific phosphatases, dual-specificity phosphatases (DSPs), CDC25-phosphatases and low molecular weight phosphatases **(Figure 2)** (Barford *et al.,* 1998; Fauman and Saper, 1996). In addition to the presence of the very essential catalytic domain in these enzymes, other domains also play critical roles and are responsible for the regulation of their catalytic activity, substrate specificity, and targeting to different subcellular locations (Fauman and Saper, 1996; Tonks and Neel, 1996).

**1.2 Catalytic mechanism of PTPs**

The catalytic domains of PTPs are highly conserved among all members of this family (Stone and Dixon, 1994). The first step in catalysis involves a nucleophilic attack of the thiol group of the catalytic cysteine on the phosphoamino acid of the substrate. This results in the formation of a covalent-

bonded thiol-phosphate intermediate between the enzyme and the substrate. The next step includes the release of the substrate from the enzyme pocket. This happens when the catalytic aspartic acid donates a proton to the oxygen on the substrate breaking the bond between the substrate and the phosphate group. In the last step, the catalytic aspartic acid acts as a base and abstracts the hydrogen from a water molecule. This creates a nucleophilic hydroxyl group that attacks the phosphate group breaking the phospho-thiol bond. As a result, the enzyme is regenerated and is free to undergo another round of dephosphorylation **(Figure 3)** (Barford *et al.,* 1998).

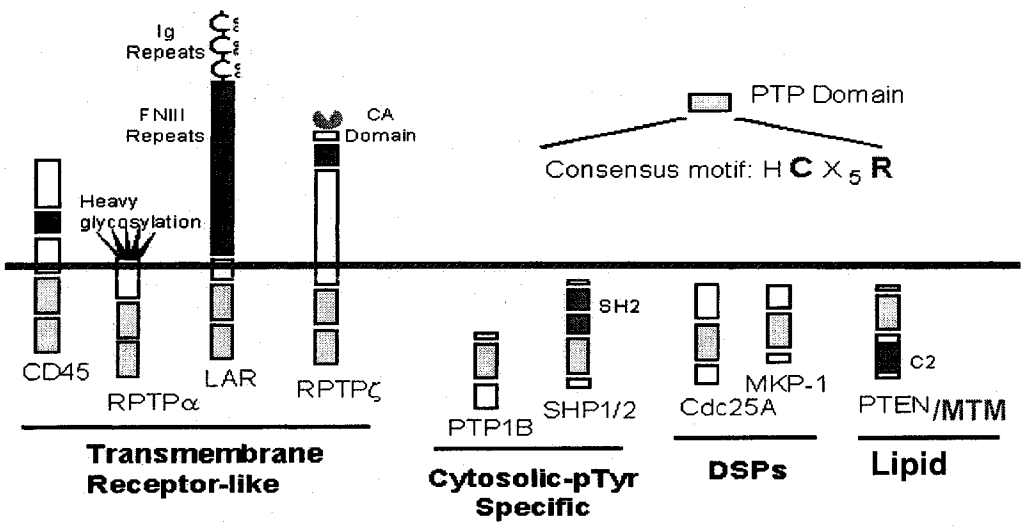



**Figure 2. Schematic of the classification of PTPs.** PTPs are recognized by the presence of the signature motif $C(X)_5R$. The classification scheme above shows receptor PTPs, which contain extracellular and transmembrane regions, and intracellular (cytosolic) PTPs. The intracellular PTPs are further subdivided into three main classes which include tyrosine specific PTPs, dual specificity PTPs (DSPs), and lipid PTPs that dephosphorylate lipid phosphoinositides as their physiological substrates.

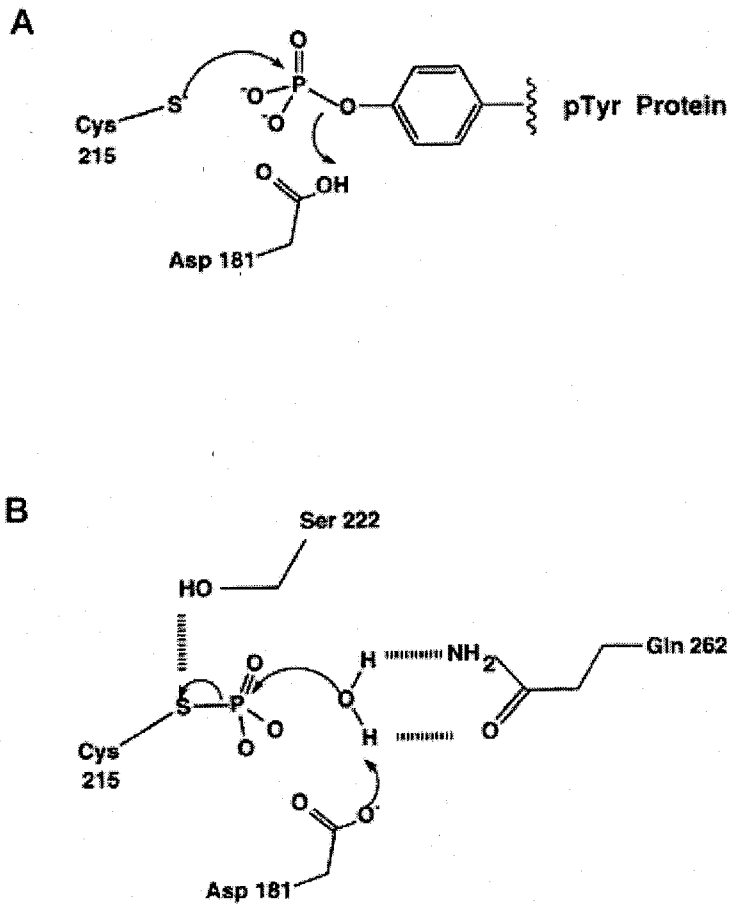



**Figure 3. Catalytic mechanism of PTPs. A)** Formation of the phospho-thiol intermediate between the enzyme and the substrate and the release of the substrate from the enzyme pocket. **B)** Regeneration of the PTP by the break down of the covalent bond between the enzyme and the phosphate group.

### 1.3 Dual specificity phosphatases

Dual specificity phosphatases (DSPs) make a subclass of the PTP family. These enzymes possess the signature catalytic motif $C(X)_5R$, and follow the same catalytic mechanism as other PTPs (Wang *et al.,* 2003). However, as suggested by their name, these enzymes are distinguished from other subclasses of PTPs due to their dual specificity for phosphotyrosine and phosphoserine-/threonine containing substrates (Stone and Dixon, 1994). Structurally speaking, these enzymes contain one serine in their catalytic motif (CXX**G**X**S**R) (Stewart *et al.,* 1999), instead of the two glycines in case of tyrosine specific phosphatases (CXX**G**X**G**R) (Groves *et al.,* 1998). As a result, their enzyme pocket is shallow and can accommodate residues like serine and threonine for hydrolysis (Barford *et al.,* 1998).

To date, there are 38 DSPs found in the human genome. These include MAPK phosphatases (11), Cdc25 phosphatases (3), Cdc14 phosphatases (4), PRL phosphatases (4), and atypical DSPs (17) (Ducret *et al.,* 2005). Among the DSPs, the ones that have been most extensively studied include the Cdc25 phosphatases and MAPK phosphatases (MKPs).

Cdc25 phosphatases regulate the activity of an important group of enzymes called cyclin dependent kinases (CDKs) which are regulators of the mammalian cell cycle (Ferguson *et al.,* 2005). In cases where the phosphorylation of CDKs results in the inactivation of a CDK/cyclin complex (Honda *et al.,* 1992; Liu *et al.,* 1997), Cdc25 phosphatases dephosphorylate and activate the cyclin bound enzyme in response to extracellular signals due to mitogen and growth factors,

resulting in cell cycle progression (Ducret *et al.,* 2005). Hence, the activation of CDKs via dephosphorylation by Cdc25 phosphatases provides an example of how dephosphorylation is not merely restricted to inactivation but also activation of enzymes.

In contrast to Cdc25 phosphatases, MKPs are associated with the activation of a group of enzymes called MAPKs (Wu and Bennett, 2005). MAPKs are important enzymes that play vital roles in signal transduction due to mitogenic signals, apoptosis, cellular survival and stress response (Ducret *et al.,* 2005). They are activated by phosphorylation and in their activated form they, in turn, phosphorylate many proteins including transcription factors to activate gene expression in order to allow a cell to respond to the original signal (Yang *et al.,* 2003). The inactivation of MAPKs by MKPs is necessary to closely regulate cellular responses to signals and preventing the cell from going into an overwhelmed state. In brief, DSPs play important roles in cell cycle regulation and cellular signaling processes and any disturbance in their activity can result in the development of pathological states such as cancer. Therefore, this group of enzymes makes attractive candidates as therapeutic targets to treat human disease (Ducret *et al.,* 2005).

### 1.4 Studies of hYVH1 orthologues

In 1991, the open reading frame H1 in the *HINDIII* restriction fragment of the *vaccina* virus genome was found to have amino acid sequence similarity to protein tyrosine phosphatases. On expression and purification of the protein in the bacterial systems, it showed activity against both phosphoserine-/threonine

and phosphotyrosine residues (Guan *et al.,* 1991). On site directed mutagenesis of the catalytic cysteine residue, the enzyme showed lack of hydrolyses of phosphorylated serine/-threonine and tyrosine residues. Therefore, it was confirmed that the newly found enzyme from the *vaccina* virus shares the catalytic mechanism of PTPs but possess dual activity against phosphoserine/-threonine and phosphotyrosine substrates. Thus, the enzyme was named as VH1 dual-specificity phosphatase (Guan *et al.,* 1991; Guan and Dixon 1991; Pot and Dixon, 1992; Streuli *et al.,* 1990).

One year later, the eukaryotic homologue of the VH1 dual specificity phosphatase was found in yeast *Saccharomyces cerevisiae* and was called yeast VH1 or YVH1. The gene for YVH1 was expressed in *Escherichia Coli* and was shown to possess phosphatase activity. In addition, it was found that inactivation of YVH1 gene decreased the growth rate in yeast increasing the doubling time in yeast to two-folds (Guan *et al.,* 1992) and disrupted sporulation (Park *et al.,* 1996). Moreover, it was observed that the mRNA of this enzyme was dramatically induced by nitrogen starvation conditions (Guan *et al.,* 1992).

In addition to the yeast VH1-like phosphatases, there are only 17 open-reading frames in yeast corresponding to PTPs and dual specificity phosphatases (Muda *et al.,* 1999). Those phosphatases that have been conserved from yeast to humans are of particular interest of study because they are likely to be the regulators of conserved functions in eukaryotic organisms. One such enzyme is the human orthologue of the dual-specificity phosphatase YVH1 called human YVH1 (hYVH1). The mRNA of this enzyme is widely found in

human tissues, with the highest levels found in spleen, testis and ovary. Moreover, the hYVH1 gene is located on chromosome 1q21-q22 which falls in a region amplified in liposarcomas (Muda *et al.,* 1999).

Human YVH1 is a 340 amino acid protein with 31% sequence identity with the yeast enzyme. Interestingly, the human orthologue can restore the yeast phenotypical defects when YVH1 is deleted. The YVH1 orthologues are the only PTPs known to contain a novel zinc finger domain which employs two moles of zinc per mol of protein. It has been shown that this COOH zinc finger is essential for the complementation of YVH1 slow growth phenotype (Muda *et al.,* 1999). However, the physiological function of hYVH1 is unknown and requires knowledge about its interacting proteins and substrates as a first step towards its physiological characterization.

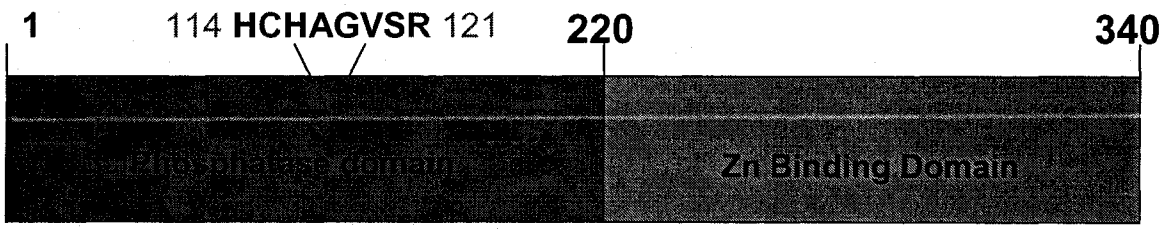



**Figure 4. Schematic of the dual specificity phosphatase hYVH1.** The enzyme contains the signature motif $C(X)_5R$ of PTPs, and the amino acids found in this motif are shown. In addition, the enzyme contains a novel C-terminal zinc finger domain which binds 2 moles of zinc per mol of protein.

### 1.5 Regulation of proteins via protein-protein interactions

The study of interacting proteins is a major resource for understanding the physiological function of an uncharacterized protein. Protein-protein interactions can regulate the activity of enzymes, the stability of proteins, the subcellular distribution of proteins, and the formation of functional multiprotein complexes. For example, specific binding partners of protein kinase C enzymes regulate the activity and subcellular location of different isoforms to gain functional diversity (Poole *et al.,* 2004).

There are many techniques available to study protein-protein interactions. These include yeast two-hybrid system (Miller and Stagljar, 2004), Glutathione-S-transferase fusion based assays (Vikis and Guan, 2004) and blot overlay or far western blot (Hall, 2004), just to name a few. Recently, the use of proteomic based approaches to rapidly identify interacting proteins has become widely utilized. This approach consists of the use of affinity chromatography to capture the multiprotein complex followed by mass spectrometry to sequence and identify the unknown proteins. However, the identification of a novel protein is coupled to its functional characterization in order to elucidate its physiological relevance.

Interacting proteins play an important role in the regulation of many proteins. For example, interacting proteins regulate the localization of Cdc25C in and out of nucleus and effect cell cycle progression (Hutchins and Clarke, 2004). Furthermore, the 14-3-3 interacting proteins bind to phosphorylated serine residues of proteins in the signaling networks and mediate a variety of effects in eukaryotes including conformational changes, inhibition of enzyme activity,

bridging between two molecules and change in localization (Thomas *et al.,* 2005). In addition, the interacting partners of leukocyte common antigen-related (LAR) receptor PTPs (LAR-RPTP) have indicated their role in actin cytoskeleton remodeling (Chagnon *et al.,* 2004). Thus, the knowledge about novel interacting proteins can open unanticipated doors into the understanding of a particular protein.

**1.6 Identification of PTP substrates**

PTPs regulate a broad range of cellular activities. The function of a particular PTP can be understood by knowing the substrate of the enzyme. However, phosphoproteins are low abundance signalling proteins, due to a low stoichiometry of cellular protein phosphorylation, (Shu *et al.,* 2004) with a high turnover (Szeszak, 1976). These factors make the capture of phosphoproteins from the cell a challenging task. An approach to identify the substrates for PTPs relies on the formation of substrate-trapping mutants of the enzyme. Based on the knowledge of the catalytic mechanism of PTPs two substrate-trap mutants can be generated; one by substituting the catalytic cysteine (C) to a serine (S) residue and the other by substituting the catalytic aspartic acid (D) residue to an alanine (A). On expression in cells, these mutants would compete with the wild type enzyme for the substrate. These mutants are designed as such that they would be able to bind the substrate but would disable its release from the enzyme pocket making the substrate amenable to be captured as an enzyme-substrate complex using affinity purification.

The idea of the formation of substrate trap-mutants came from studies on the

prototypical PTP called PTP1B. The crystal structure of the substrate bound (Jia *et al.,* 1995) and unbound enzyme (Barford *et al.,* 1994) was determined. The invariant residues were found by aligning the sequences of the PTP catalytic domains and these residues were found to be clustered around the region of the active site (Flint *et al.,* 1997). Two such invariant residues are the catalytically active cysteine (Barford *et al.,* 1994; Stuckey *et al.,* 1994) and catalytic aspartic acid (Denu and Dixon, 1995; Jia *et al.,* 1995; Zhang *et al.,* 1994). The substitution of the catalytically active cysteine to a serine or alanine residue has been used in the past by several researchers to successfully target PTP substrates (Furukawa *et al.,* 1994; Jia *et al.,* 1995; Shiozaki and Russell, 1995; Sun *et al.,* 1993). However, for other PTPs, the interaction between the enzyme and the substrate is inadequate to allow the isolation of their complex in the absence of the catalytic cysteine residue (Flint *et al.,* 1997). In such cases, the catalytic aspartic acid can be mutated to an alanine to stabilize the interaction between the enzyme-substrate complex. This will allow the substrate to be trapped in the enzyme pocket by hindering the cleavage of the P—O bond in the substrate. The validity of this mutant was tested by expressing GST-tagged (C215S) and (D181A) mutants in COS cells. It was followed by immunoprecipiation with GST-sepharose resin and immunoblotting with anti-tyrosine antibody. Several pTyr proteins were captured, however, the (D181A) mutant proved to be a better mutant (Flint *et al.,* 1997). It also indicates its capacity as a better substrate trap due to a more stabilized interaction between the enzyme and the substrate.

## MASS SPECTROMETRY

Mass spectrometry is a highly sensitive analytical tool (picomole to femtomole sensitivity) that is used to study biomolecules (such as proteins and nucleic acids) (Siuzdak, 1994). The technique is based on determining the mass to charge (m/z) ratio of the ionized particles in their gaseous state. The two most commonly used types of mass spectrometry techniques include MALDI-TOF (Matrix-Assisted Laser Desorption Ionization-Time of flight), and ESI-MS (Electrospray Ionization-mass spectrometry). In general, a mass spectrometer consists of three parts: an ion source which ionizes the sample into gaseous charged particles, an analyzer which determines the mass to charge ratio of the ions and a detector which records the number of ions with the same mass to charge ratio (Aebersold and Mann, 2003).

The two most common types of ionization methods include MALDI (Matrix-Assisted Laser Desorption Ionization) and ESI (Electrospray inonization). ESI is the preferred choice for ionization from solutions (e.g., aqueous or aqueous/organic solvent systems) and is employed to identify proteins from a more complex sample of peptides (Siuzdak, 1994). In order to achieve ionization via ESI, the source of separation of protein mixture (e.g., a chromatographic column) is directly attached to the mass spectrometer. Once separated, the charged particles are directed to the mass analyzer where they are separated according to their m/z ratio and travel to the detector resulting in the generation of a mass spectrum.

ESI is usually coupled with a quadropole analyzer. In this analyzer, ions of a particular m/z ratio are passed through a four rod mass filter and make their way to a collision cell. Once fragmented in the collision cell, the resultant fragments are read by the analyzer and are passed onto the detector to generate a CID (collision induced dissociation) spectrum. The data from the spectrum are matched against protein databases for the identification of the protein (Aebersold & Mann, 2003).

The mass spectrometer used in this research project is a MALDI-TOF instrument. MALDI is the method of choice for the ionization of charged gaseous particles from relatively simpler mixture of peptides. MALDI refers to an ionization procedure in which the sample is crystallized with a matrix such as alpha matrix (alpha-cyano-4-hydroxycinnamic acid). The matrix is desorbed into gaseous ion particles using a nitrogen laser and the ions travel through a time of flight tube to the analyzer (Siuzdak, 1994). Prior to MALDI analysis, the proteins are separated by SDS-PAGE or chromatography. In case of SDS-PAGE, the gels are stained either with coomassie or silver stain. The protein bands are carefully excised from the gel and are in-gel digested using a proteolytic enzyme such as trypsin. The resultant sample consists of a mixture of peptides of different masses (Aebersold and Mann, 2003). The samples can be further processed by a process called zip-tipping to remove salt contamination. The refined sample is applied to the MALDI plate along with the matrix and the plate is inserted into a chamber for the sample to be bombarded and ionized by a nitrogen laser. The data from the mass spectrometer is collected in the form of a mass spectrum

which is a plot of the relative abundance of various ion fragments plotted against their mass-to-charge (m/z) ratio. This mass spectrum is also referred to as a peptide mass fingerprint (PMF) (Aebersold and Mann, 2003). The peptide mass finger print generated by a particular proteolytic enzyme is a signature spectrum of a protein.

MALDI is normally attached to a time of flight (TOF) analyzer which separates the ions based on the difference in their m/z values. The charged particles are accelerated and given their maximum kinetic energies before entering the time of flight tube. The velocity of these particles can be calculated by rearranging the equation for kinetic energy, where $KE=mv^2/2$ or $v= [(2E/m)]^{1/2}$ i.e., the velocity of the particles during their time of flight is inversely proportional to their masses. Therefore, ions of smaller masses will travel faster and reach the detector earlier than the larger ions. (http://www.abrf.org/ABRFNews/1995/December1995/dec95maldi.html).

The TOF analyzer can be used either in a linear mode or a reflector mode. When used in the linear mode, particles of the same molecular weight that slightly differ in their velocities generate a broad peak on the spectrum. The resolution of this spectrum can be improved by using the analyzer in the reflector mode. In this mode, ions that slightly differ in their velocities are first brought to a stop. They are reaccelerated and reflected by a mirror before reaching the detector which results in the generation of narrower and well resolved peaks enhancing the resolution of the spectrum (http://www.abrf.org/ABRFNews/1995/December1995/dec95maldi.html).

Tandem mass spectrometry (MS/MS) refers to successive rounds of mass spectrometry where the fragment ions are further analyzed by a second or a third round of mass spectrometry. Using MALDI, MS/MS analysis can be performed to get partial sequencing information on a peptide through a process called post-source decay (PSD). The result of PSD creates Y-ions, in which the charge of the fragment ion is retained on the C-terminal, and also b-ions, in which the charge is retained on the N-terminal. In MALDI-PSD, the parent ion is fragmented through metastable fragmentation in the time of flight tube. The ions are deflected through a reflector mirror on the end of the tube at different voltage settings (Vacratsis *et al.,* 2003). This generates a spectrum that provides information about the fragmentation pattern and m/z values of the Y and b ions, and allows us to perform *de novo* sequencing of the peptide. As a result, the unknown protein is identified unambiguously.

## Objectives

Dual specificity phosphatases are PTPs that dephosphorylate proteins at serine-/threonine residues in addition to the tyrosine residues. The enzyme to be studied in this research project is also a dual specificity phosphatase, called human YVH1. It is a 36kDa protein and contains a zinc finger domain. Interestingly, hYVH1 has been shown to complement growth defect in yeast under stress conditions such as nitrogen starvation and low temperature, however, the physiological function of this enzyme is still unknown. The purpose of this research project was to take the first step towards the characterization of hYVH1 by using affinity chromatography and mass spectrometry to capture/identify the substrates and interacting proteins of this enzyme.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Bioinformatics and DNA constructs

The amino acid sequence of the dual specificity phosphatase human YVH1 was aligned with the well characterized MAP kinases dual specificity phosphatases (MKPs) using ClustalW software (http://www.ebi.ac.uk/clustalw/) in order to identify the probable catalytic hYVH1 residues. The two expression vectors used in the study were the mammalian expression vector (pCMV) and bacterial expression vector (pGEX-6TI) which contained the FLAG-hYVH1 and GST-hYVH1 recombinant cDNAs, respectively. These vectors were subjected to polymerase chain reaction (PCR) to mutate the catalytic aspartic acid residue (D84) to an alanine (A), using the site directed mutagenesis protocol provided by Stratagene. The sense and anti-sense PCR primers were obtained from Invitrogen and the sequence of the primers was as follows:

5' GTG CCA GCG CTG **GCC** AAA CCC GAG AC 3' (sense)
5' GT CTC GGG TTT **GGC** CAG CGC TGG CAC 3' (antisense)

These primers were resuspended in autoclaved millipore water ($ddH_2O$) at a concentration of 0.1$\mu$g/$\mu$l. The template cDNAs, hYVH1 (pCMV) and hYVH1 (pGEX) were used at a concentration of 0.1$\mu$g/$\mu$l. Other components of the PCR reaction included 10x Pfx amplification buffer, 50mM $MgSO_4$, 10mM dNTP mixture, autoclaved $ddH_2O$ and DNA polymerase. The reaction was performed in autoclaved PCR tubes using a Techgene thermal cycler. The denaturation, annealing and extension temperatures for the PCR reaction were set to 95°C, 55 °C and 68°C, respectively. The PCR product was subjected to Dpn I digestion at

37°C for one hour to digest the methylated parent DNA and was stored at -20°C until ready to be transformed.

**2.2 DNA transformation**

Competent cells (DH5$\alpha$) from *Escherichia Coli* (*E.Coli*) were transformed with the PCR DNA products and the transformed bacterial cells were grown on ampicillin-agar plates. Colonies were picked from each sample plate hYVH1 (pCMV) and hYVH1 (pGEX) into 10ml small cultures which were incubated overnight at 37°C. This was followed by plasmid DNA purification using Promega miniprep kit. Automated DNA sequence analysis for the mutant constructs was done at the great lake institute for environmental research (GLIER), Windsor, ON. The DNA with confirmed sequence for the hYVH1(D84A) mutant for the pCMV and pGEX vectors was amplified in *E.Coli* using large 500ml cultures and was purified using Sigma maxiprep kit.

**2.3 Protein purification**

*E.Coli* competent cells (BLR) were transformed with the bacterial vector pGEX containing hYVH1 (D84A) DNA and the transformed bacterial cells were grown grown on ampicillin-agar plates. Bacterial colonies from agar-ampicillin plates were picked into 10ml cultures and were grown overnight in the shaker at 37°C. These small cultures were transferred into large 500ml cultures and the cultures were grown until the absorbance 600nm was read to be between 0.5-0.6 (approximately 2 hours). At this point, protein expression was induced with 0.4 mM IPTG and the bacterial cultures were grown overnight at room temperature with gentle agitation. The cultures were spun down at 5000rpm for 15min and the

pellet was resuspended in 20ml of STET buffer (10mM Tris-base pH 8.0, 150mM NaCl, 1mM EDTA and 1% Triton) + 0.1% $\beta$-mercarptoethanol + protease inhibitors (aprotinin and PMSF) and the cells were lysed by sonication. The sonicated cells were centrifuged at 12,000rpm for 15min and the clear supernatant was bound to 2.5ml of GST-sepharose resin at 4°C for 2hrs. The beads were washed twice with 20ml of STET buffer + 0.1 % $\beta$-mercaptoethanol. The GST-hYVH1 (D84A) protein was eluted off the beads using 4ml of elution buffer (50mM Tris base pH 8.0, 150mM NaCl, and 0.1 % $\beta$-mercaptoethanol) containing 20mM glutathione. The protein induction and purification efficiency was analyzed by SDS-PAGE. The protein was concentrated and elution buffer was exchanged for storage buffer (50mM Tris pH 7.5, 100mM NaCl, 5mM DTT) using centricons (Amicon).

**2.4 GST pulldown assays**

GST-sepharose resin (30$\mu$l) was washed twice with and resuspended in STET + 0.1 % $\beta$-mercaptoethanol. 200$\mu$g of GST protein was bound to the resin for two hours at 4°C. After two hours, the beads were spun down and the supernatant was removed. The beads were washed once with lysis buffer containing protease inhibitors (PMSF and aprotinin) and 20ml of lysates from HEK293 cells were passed over the beads to preclear the lysates for GST affinity proteins. These precleared lysates were passed over the GST sepharose resin bound GST control, GST-hYVH1 and GST-hYVH1 (D84A) proteins for 4 hours at 4°C. The beads were washed twice with IP wash buffer and were collected by centrifugation at 3,000g for 2min. These beads were resuspended in loading

buffer (2x), analyzed by 10% SDS/PAGE and protein bands were visualized by staining (coomassie or silver staining).

**2.5 Cell culture and transfection**

Human embryonic kidney cells were grown in DMEM/F-12 growth medium supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin in 10cm plates. Cells were subcultured every two to three days once they reached 80-90% confluency. 1.5 x $10^6$ cells were split one day before transfection. These cells were transfected with pCMV containing wild type hYVH1 or hYVH1 (D84A) cDNA. Transfection was done using Fugene reagent (Roche). For all transfections, the mixture was made by mixing Fugene and DNA in a 3:1 ratio into 100 $\mu$l of serum free media (Invitrogen).

**2.6 Cell Lysis**

HEK 293 cells were lysed 24hours post-transfection. The cells were washed with cold (4°C) PBS in the tissue culture hood. Lysing was performed outside the hood on the lab bench by resting the culture plates on ice and adding 1ml of lysis buffer (50mM Tris-base pH 7.4, 1% Triton x-100, 150mM NaCl, 0.1% SDS) for the GST-pull down assays and 500$\mu$l of lysis buffer for the co-immunoprecipitation experiments. The lysates were collected using a 1ml pipette into clean falcon tubes. These fresh lysates were used to carry out further experiments.

**2.7 Co-immunoprecipitation and FLAG elution**

Immunoprecipitations were performed with 20$\mu$l of M**2** agarose resin/ plate of cells. The resin was washed twice with 1ml of IP wash buffer, and it was

resuspended in 500$\mu$l of IP wash buffer. The lysates were incubated with the resin in a 15ml falcon tube in the cold room on a shaker for 4hrs. At the end of the incubation period, the tubes were spun down at 3,000g for 2min in a swinging bucket rotor. The supernatant was decanted and the beads were washed twice with IP wash buffer (50mM Tris base pH 7.4, 0.1% Triton x-100, 150mM NaCl, 0.1% SDS). For two-dimensional electrophoresis experiments without FLAG elution, the second wash was done with 10mM Tris buffer (pH 8.0). These beads were incubated in BIO-RAD sample rehydration buffer (8M urea, 2% CHAPS, 50mM DTT, 0.2% Bio-Lyte® 3/10 ampholyte, 0.001% Bromophenol blue) at room temperature for 30min in 15ml falcon tubes. The tubes were spun down at 4,000g for 2min and the collected supernatant was loaded on the IEF strips (pI range 3-10).

In case of FLAG elution employment, the immunoprecipitants were washed twice with IP wash buffer and hYVH1-substrate complex was eluted with 50$\mu$l of FLAG peptide (100$\mu$g/ml) dissolved in 1xTBS (20mM Tris base pH 7.6, 137mM NaCl). The FLAG elution was repeated, and the supernatants were combined and precipitated in 3 volumes of acetone overnight at -20°C. The precipitates were collected by centrifugation at 14,000g at 4°C for 15min. These precipitates were dissolved in sample rehydration buffer and were loaded on the IEF strips.

**2.8 Gel electrophoresis and western blotting**

Proteins were resolved on 10% or 12% polyacrylamide gels. For western blot analysis, the proteins were transferred to Polyvinylidene Fluoride (PVDF) membrane. The membrane was blocked for 1hr at room temperature in blocking

buffer, 0.3% gelatin ($\alpha$-FLAG primary antibody) and 5% dry milk ($\alpha$-hYVH1 primary antibody) dissolved in 1xTBS-Tween. This was followed by an overnight incubation of the membrane with the primary antibody $\alpha$-FLAG (1:1000) or $\alpha$-hYVH1 (1:3000) at 4°C with gentle agitation. The membrane was washed for 15 min with 1xTBS-Tween for three times. It was incubated with the secondary antibody, goat anti-mouse (1:5000) for $\alpha$-FLAG antibody, and goat anti-rabbit (1:3000) for $\alpha$-hYVH1 antibody. The secondary antibody was conjugated with the enzyme horse reddish peroxidase (HRP) and proteins were visualized by a chemiluminescence machine.

**2.9 Two dimensional electrophoresis**

The protein sample was loaded on the IEF strips and was incubated overnight (passive rehydration). The strips were transferred to an electrophoresis chamber and proteins were resolved in the first dimension at 500V using the preset method. The strips were taken out of the IEF chamber and were equilibrated with BIO-RAD equilibration buffer I (6M urea, 0.375M Tris, pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT) and equilibration buffer II (6M urea, 0.375M Tris, pH 8.8, 2% SDS, 20% glycerol, 2.5% (w/v) iodoacetamide) with slight agitation. The proteins were resolved in the second dimension on 12% polyacrylamide gels. The gels were stained with mass spectrometry compatible silver staining kit (Invitrogen) according to the protocol provided by the manufacturer.

**2.10 Mass Spectrometry**

The unique spots/bands on the gel were carefully excised manually in a vacuum chamber. The protein bands were destained using the reagent provided

in the Invitrogen kit. The proteins were in-gel digested using trypsin digestion buffer (50mM ammonium bicarbonate (AB) containing 13ng/$\mu$l trypsin). The tubes were first incubated on ice for 30min to allow trypsin to enter through the gel around the protein band followed by an overnight trypsin digestion at 37°C on a shaker. The sample was spun down briefly and the peptides were extracted with a mixture of 60% acetonitrile and 1% formic acid solution at 37°C for 45min. The supernatant containing the pool of peptides was collected and was concentrated in the Speed Vac. The samples were spotted on the MALDI plate along with the $\alpha$-matrix for a first round of mass spectrometry. The sample was then desalted with C18 ziptips to improve resolution and reloaded for analysis by the MALDI-TOF instrument (Voyager-DE, Applied Biosystems) equipped with a nitrogen laser of 337nm. This new spectrum was taken as the mass finger print of the p70 band and preliminary database search was done using the peptides from this spectrum against the NCBI database using the prospector software (http://prospector.ucsf.edu/). For MS/MS sequencing MALDI-Post Source Decay (PSD) was performed to obtain partial sequence information. Selected parent ions were analyzed in reflectron mode for sequence determination. PSD spectra was acquired in 8-10 segments with mirror ratios ranging from 1.0-0.25, and "stitched" together using the Data Explorer software. In silico fragmentation using the Prospector software and BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) were used to compare the determined MS/MS sequences with sequences in the NCBI database.

# CHAPTER 3

# RESULTS

## 3.1 Identification of hYVH1 interacting proteins by affinity chromatography and mass spectrometry

### 3.1.1 FLAG immunoprecipitation

Human embryonic kidney (HEK293) cells were used to find the interacting proteins of hYVH1 *in vivo*. Cells were transfected with mammalian pCMV empty vector or pCMV vector containing FLAG-tagged wild type hYVH1. Cellular lysates were collected 24hrs post-transfection and were immunoprecipitated with M2-agarose anti-FLAG resin as described in Materials and Methods. The immunoprecipitants were resolved by SDS-PAGE and were visualized by coomassie staining. On comparison of the control and wild type hYVH1 lanes, a 70kDa protein band was observed in the wild type lane but not in the control lane which was suggestive of an interaction between hYVH1 and p70 **(Figure 5).**

### 3.1.2 Identification of p70 by mass spectrometry

The p70 band was excised out of the gel and was in-gel digested with trypsin which cleaves proteins after basic residues, lysine (K) and arginine (R). The digested sample containing the pool of peptides from the p70 band was loaded onto the MALDI plate to be analyzed by the mass spectrometer. The peptide mass finger print was obtained for p70 showing many peptides in the spectrum **(Figure 6).** However, due to a poor resolution of the spectrum, a second mass finger print was generated following desalting with C18 ziptips **(Figure 7).** This spectrum had an increased signal to noise ratio and better mass accuracy. The

m/z values of selected peptides from this spectrum were used to do a preliminary database search for candidate proteins against the NCBI protein database using the software program MS-FIT. A number of matching hits were obtained **(Table 1)**. However, in order to unambiguously identify the p70 band, the peptide ion with m/z value = 1487 was analyzed by MALDI-PSD to obtain partial sequence information **(Figure 8).** In silico digestion and the resulting fragment ion series (y and b ions) of the precursor ion (m/z = 1487) provided the sequence of the peptide ion as **'TTPSYVAFTDTER'** corresponding to the protein Hsp70**.** Blast search for the obtained peptide sequence corresponded to amino acids $Thr^{37}$-$Arg^{49}$ of Hsp70 sequence as well as of its constitutive active isoform Hsc71. Sequence alignment was done between Hsp70 and Hsc71 using ClustalW and the two proteins were found be ~80% identical **(Figure 9).** Hence, two more peptide fragments were selected from the p70 PMF for MALDI-PSD analysis. The results from PSD analysis (y and b ions) deduced the peptide sequences **'ATAGDTHLGGEDFDNR'** and **'LLQDFFNGR'** for the precursor ions (m/z = 1675) and (m/z = 1109), respectively **(Figure 10 & 11).** Blast search analysis identified these two peptides specific for Hsp70. In addition, from the results of sequence alignment between Hsp70 and Hsc71, the two peptides were found only in Hsp70. Therefore, it was concluded that the unknown p70 protein which was found to interact with hYVH1 by FLAG-IP was the heat shock protein Hsp 70.

### 3.1.3 Interaction specificity of hYVH1 to Hsp70

In order to test the interaction specificity between hYVH1 and Hsp70, co-

immunoprecipitation experiments were carried out *in vivo.* FLAG-Hsp70 and FLAG-Hsc71 were co-transfected with wild type myc.hYVH1 in HEK293 cells. After 24hrs, cells were lysed and the cellular extracts were immunoprecipitated with M2-agarose anti-FLAG resin. Both lysates and immunoprecipitants were resolved by SDS-PAGE and were visualized by western blot analyses. FLAG-Hsp70 and FLAG-Hsc71 were visualized using $\alpha$-FLAG 1° antibody and goat $\alpha$-mouse 2° antibody whereas myc-hYVH1 was visualized by $\alpha$-hYVH1 1° primary antibody and goat $\alpha$-rabbit 2° antibody. The co-immunoprecipitation experiment demonstrated that Hsp70 is able to copurify hYVH1 confirming the mass spectrometry results **(Figure 12A &12B).**

The interaction between hYVH1 and Hsc71 was studied using a set of replicate experiments with FLAG.Hsc71 instead of FLAG.Hsp70 **(Figure 13A &13B).** However, while a large amount of hYVH1 coimmunoprecipitated with FLAG-Hsp70 (Figure 12A; lane 6), very low amounts of hYVH1 coimmunoprecipitated with FLAG-Hsc71 **(Figure 13B; lane 6).** These results suggest that under these conditions the interaction of hYVH1 is much stronger and thus more specific to Hsp70 than Hsc71 supporting the mass spectrometry results identifying Hsp70 as a hYVH1 interacting partner.

### 3.1.4 Endogenous hYVH1 and Hsp70 complex formation.

The purpose of these experiments was to determine if hYVH1 interacts with Hsp70 endogenously and, hence, physiologically. Protein agarose A resin was bound separately to anti-hYVH1 and anti-Hsp70 antibodies. HEK293 cellular lysates from 10cm plates were passed over the resin and the resin bound protein

complexes were isolated and separated by SDS-PAGE. The resolved proteins were visualized by immunoblotting with both anti-hYVH1 and anti-Hsp70 antibodies. The results of hYVH1 IP showed that Hsp70 came down with hYVH1 **(Figure 14A)** and the results of Hsp70 IP showed that hYVH1 came down with Hsp70 **(Figure 14B).** The results showed that endogenous Hsp70 and hYVH1 interact, suggesting the interaction is physiologically relevant.

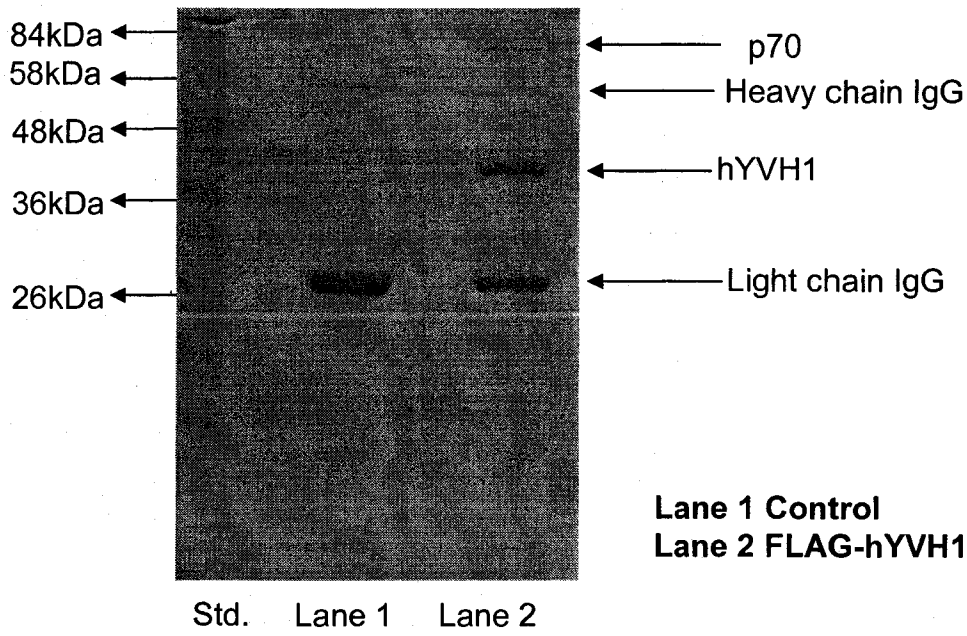



**Figure 5. Detection of p70 using FLAG immunoprecipitation.** The coomassie stained gel above shows the results from a FLAG-IP experiment. Untransfected and FLAG-hYVH1 transfected lysates from HEK293 cells were immunoprecipitated with M2-agarose anti-FLAG resin. The figure above shows the presence of light chain (~26kDa) and heavy chain (~56kDa) bands from the anti-FLAG antibody in both lanes. The wild type FLAG-hYVH1 lane shows the 38kDa hYVH1 band and a novel p70 band which was found to be repeatedly absent in the control lane.

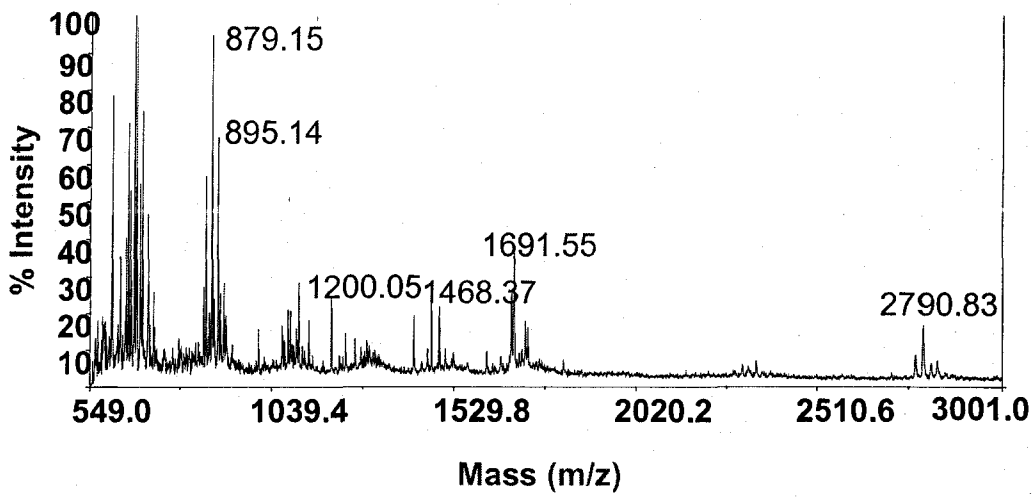



**Figure 6. Peptide mass finger print of p70.** The spectrum above represents the first peptide mass finger print of the p70 band as generated by the MALDI-TOF instrument. The peaks observed in the spectrum are representative of some of the peptides found in the peptide pool of the p70 band which was obtained after in-gel trypsin digestion. However, sample decontamination is required to generate a better quality of mass finger print for p70 with a higher signal to noise ratio.

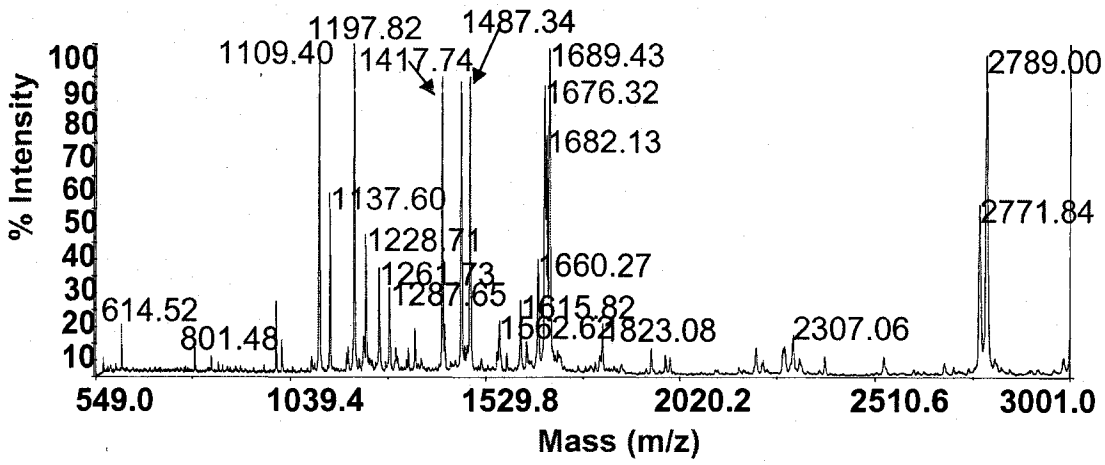



**Figure 7. Peptide mass fingerprint after C18 zip tipping.** The mass spectrum was obtained after C18 ziptipping of the p70 peptide sample. It shows well resolved peaks and the signal to noise ratio of the spectrum has increased after ziptipping. Peptides from this spectrum were used to perform a preliminary database search to obtain a list of candidate proteins for p70.

| Ranking | Accession# | Name of protein |
|---|---|---|
| 1 | 5123454 | Heat shock 70kDa |
| 2 | 14714528 | PMPCB protein |
| 3 | 51094701 | Nexin 8 |
| 4 | 26252129 | ZNF1 protein |
| 5 | 17028348 | MTHFDIL1 Protein |

**Table 1. A number of reported hits from preliminary NCBI database search.**

The table above shows the list of proteins obtained, with the m/z values of the peptides from the p70 mass finger, as a result of a preliminary database search against NCBI using the prospector software.

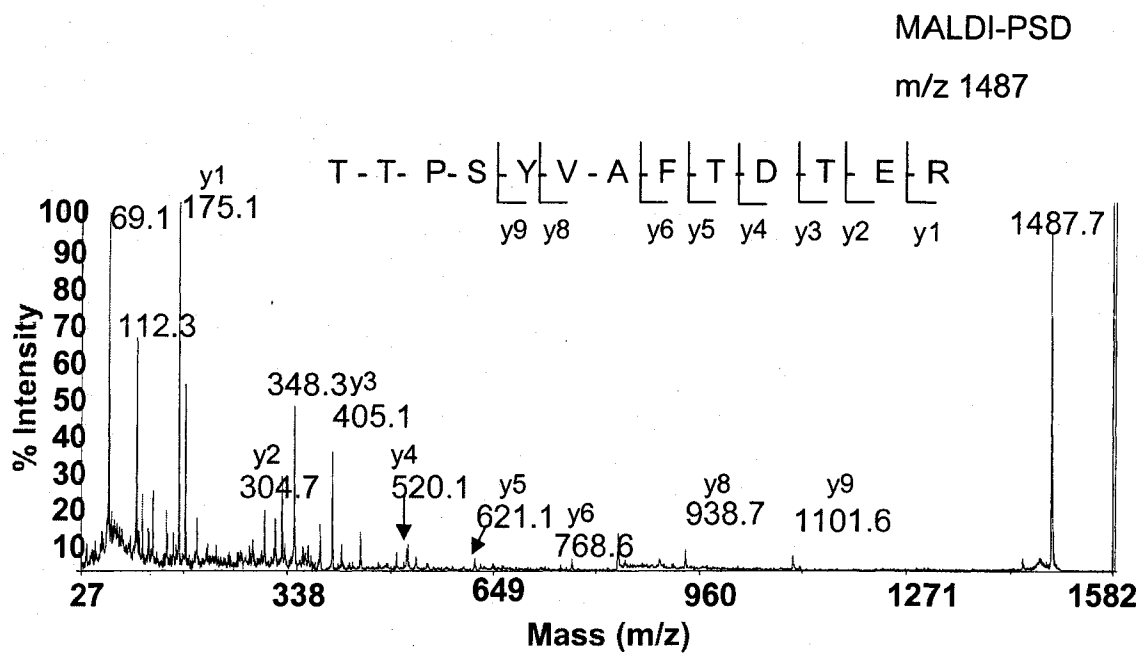



**Figure 8. MS/MS spectrum for 1487 m/z parent ion.** The spectrum above shows results of the ion fragmentation for the parent ion with m/z value of 1487, selected from the p70 mass finger print. The sequence of the peptide along with the y ion fragmentation is shown in the figure. The spectrum represents the peptide which contains amino acids 37- 49 and is found in both Hsp70 and Hsc71 proteins.

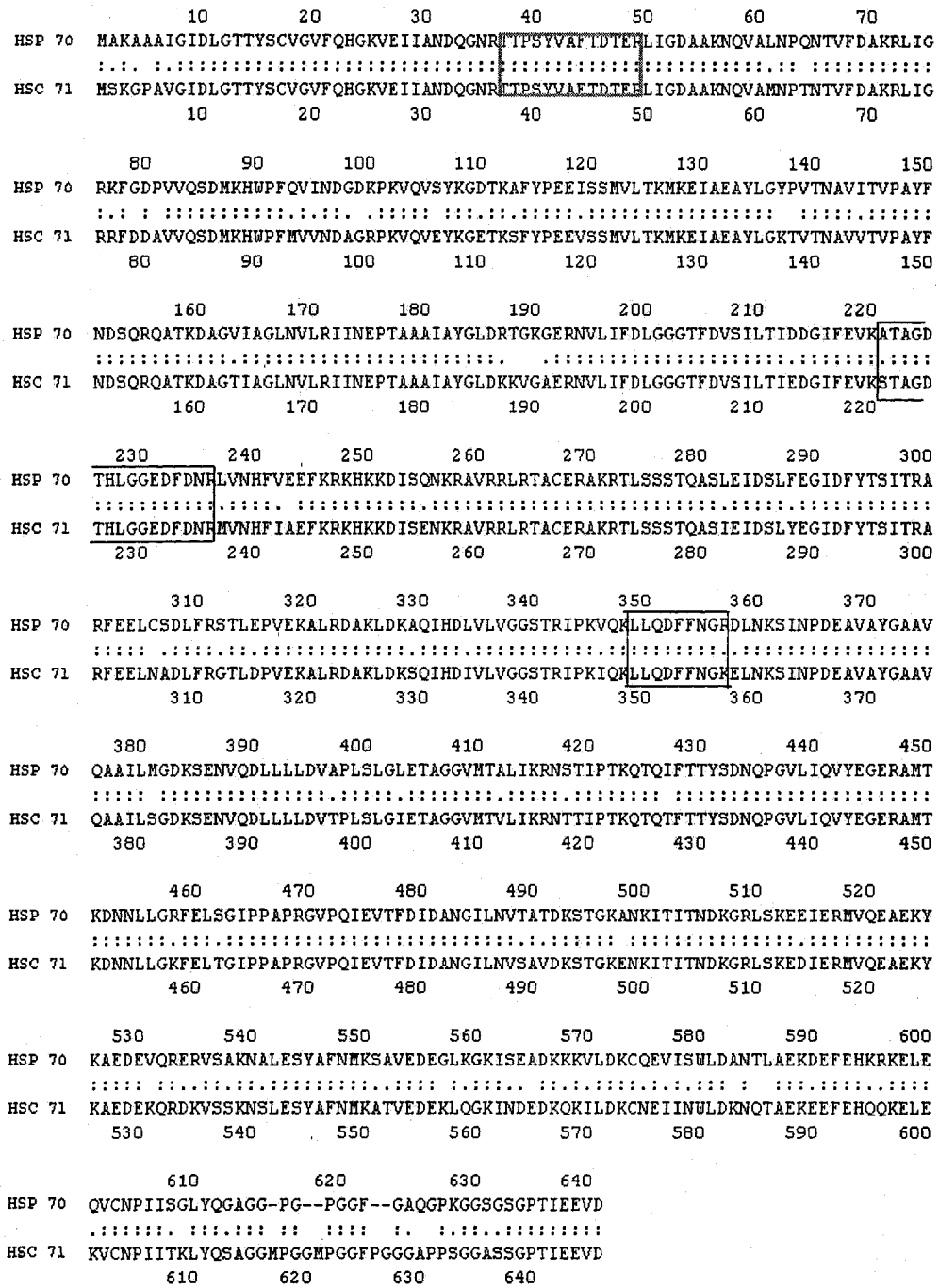



**Figure 9. Alignment of Hsp70 and Hsc71 using ClustalW.** Grey box represents the peptide common in both Hsp70 and Hsc71 (m/z 1487). Two black boxes represent the peptides specific to Hsp70 (m/z 1109 & 1675).

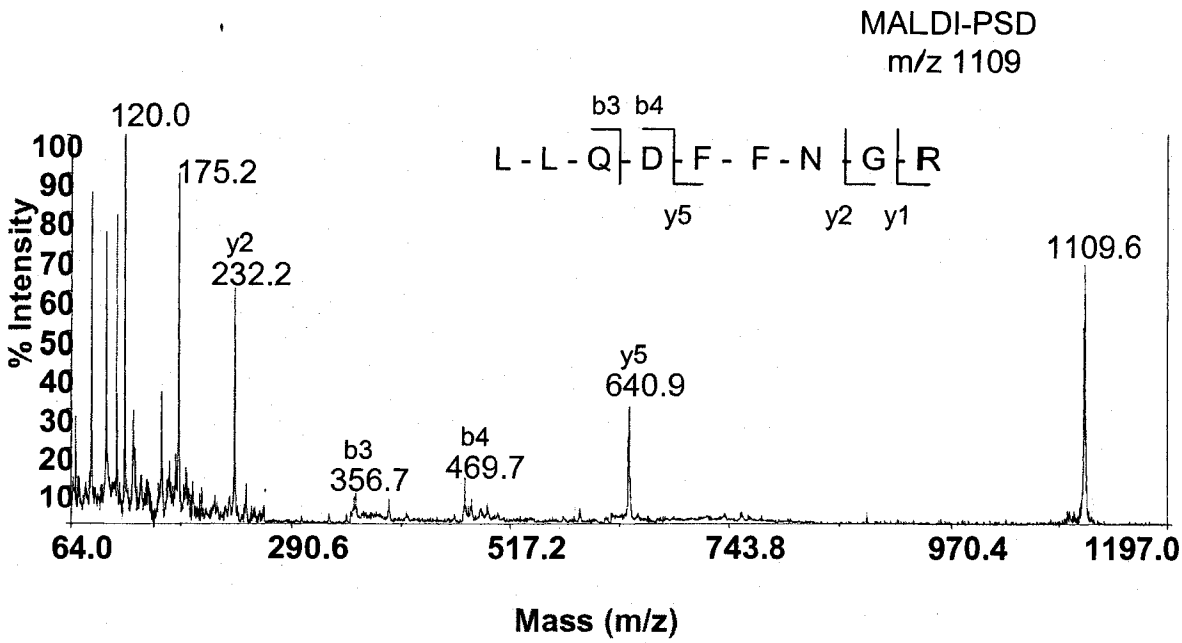



**Figure 10. MS/MS spectrum of the parent ion m/z 1109.** The MS/MS spectrum above shows the ion fragmentation of the peptide with m/z value of 1109. The peptide includes amino acid 349-357 in the Hsp70 amino acid sequence and is not found in Hsc71.

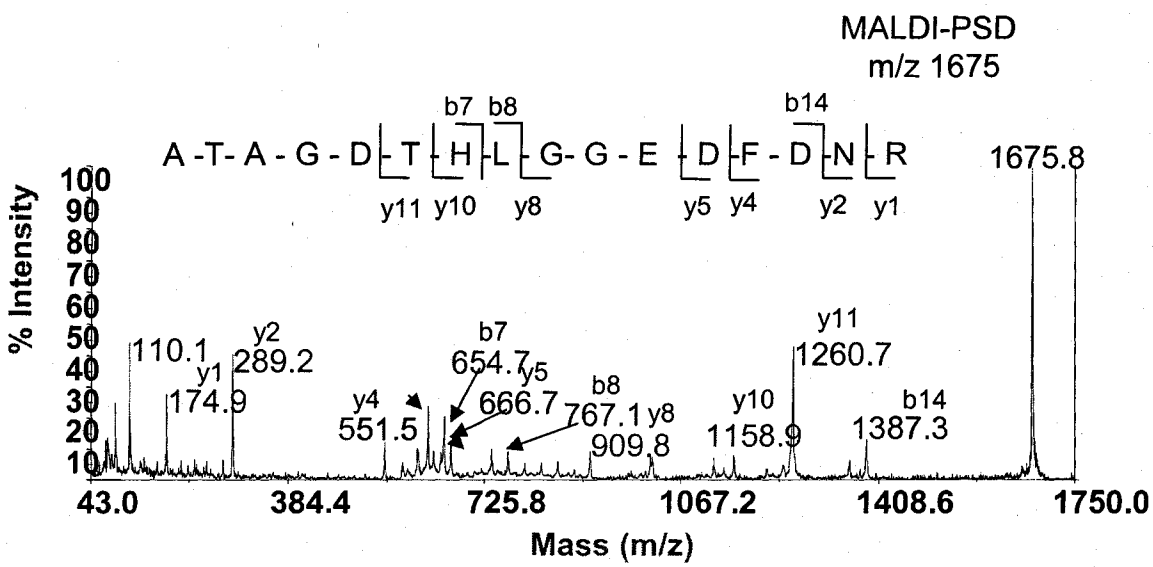



**Figure 11. MS/MS spectrum for the parent ion m/z 1675.** The spectrum above shows the ion fragmentation of the peptide, with m/z value 1675, taken from the peptide mass finger print of p70. The fragment ions include a mixture of y and b ions. The spectrum represents the peptide, with amino acid residues 221-236, found only in the Hsp70 isoform.

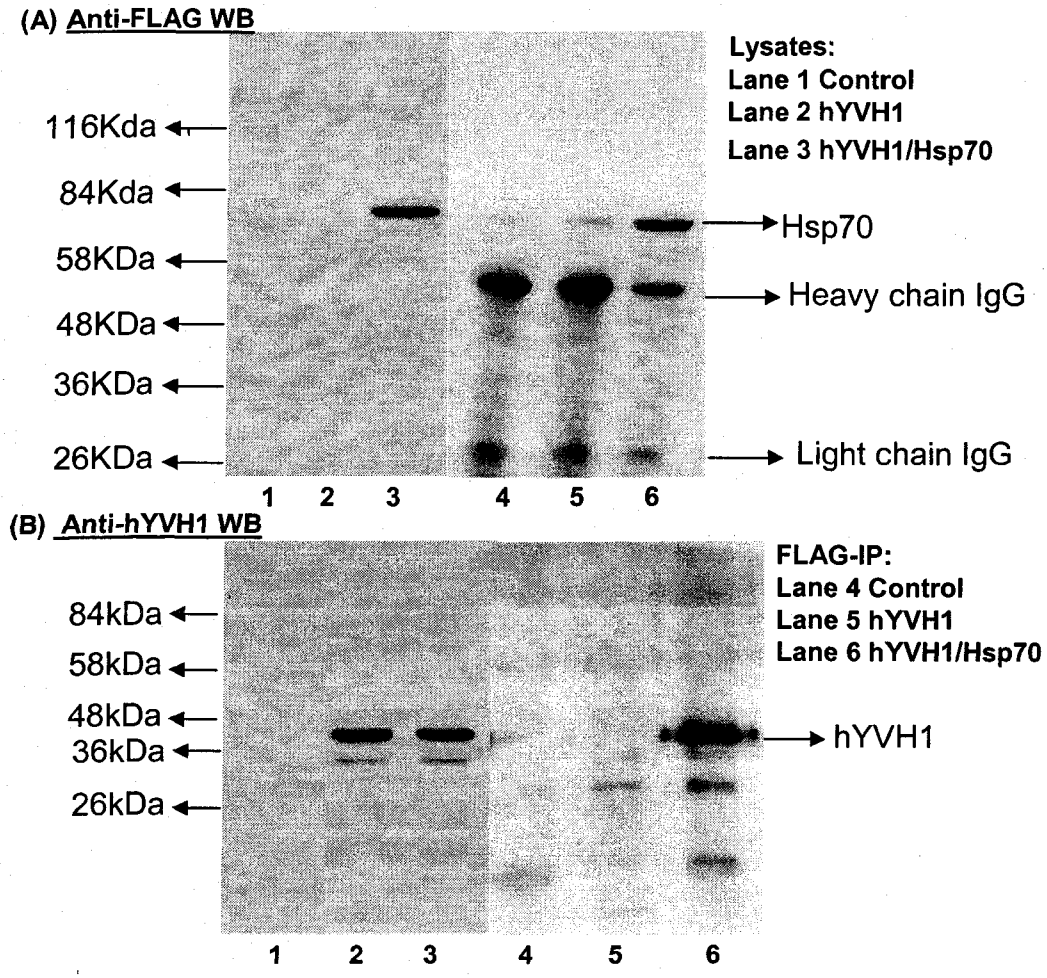



**Figure 12. Overexpression of hYVH1 and Hsp70 in HEK293 cells.** c-myc hYVH1 was transfected alone or co-transfected with FLAG-Hsp70 in HEK293 cells. Lysates were immunoprecipitated with $\alpha$-FLAG resin. Both lysates and immunoprecipitants were visualized with $\alpha$-FLAG and $\alpha$-hYVH1 antibodies. The control lanes represent results for the untransfected lysates. **A)** $\alpha$-FLAG western blot analysis shows the presence of the FLAG-Hsp70 band in the lysates (3) and also in the immunoprecipitants (6). **B)** $\alpha$-hYVH1 western blot shows that the c-myc-hYVH1 cotransfected with FLAG-Hsp70 was found in the lysates (3) and was also co-immunoprecipitated with Hsp70 in the FLAG IP (6).

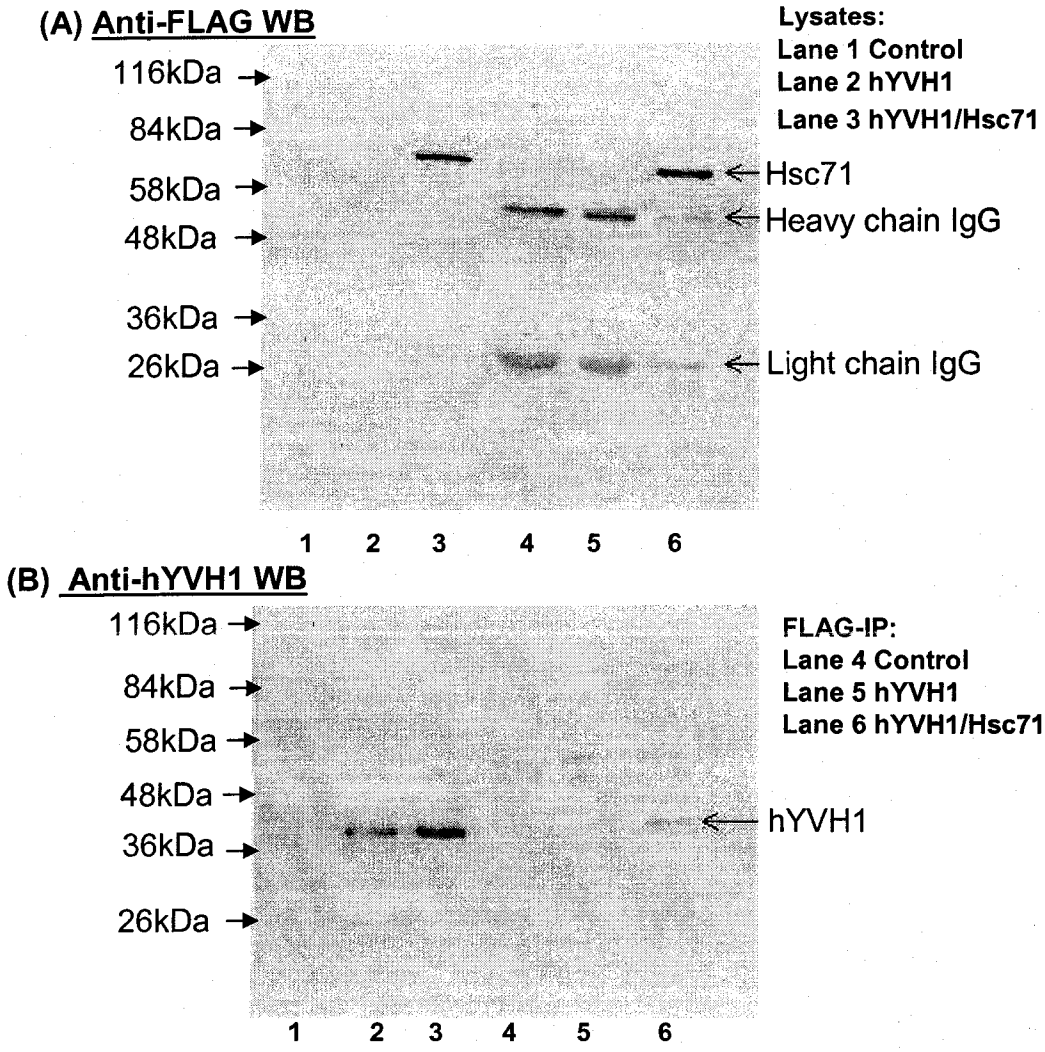



**Figure 13. Co-immunoprecipitation of hYVH1 and Hsc71.** c-myc-hYVH1 was co-transfected with FLAG-Hsc71 in HEK293 cells. Lysates were immunoprecipitated with *α*-FLAG resin. The proteins in the lysates and the immunoprecipitants were visualized by western blot analyses with *α*-FLAG and *α*-hYVH1 antibodies. The control lanes show results for the untransfected lysates. **A)** *α*-FLAG western blot analysis shows that Hsc71 was found in the lysates lane (3) and in the IP lane (6) **B)** *α*-hYVH1 western blot shows normal levels of hYVH1 in the lysates (3) but a very little amount of hYVH1 in the co-IP lane (6).

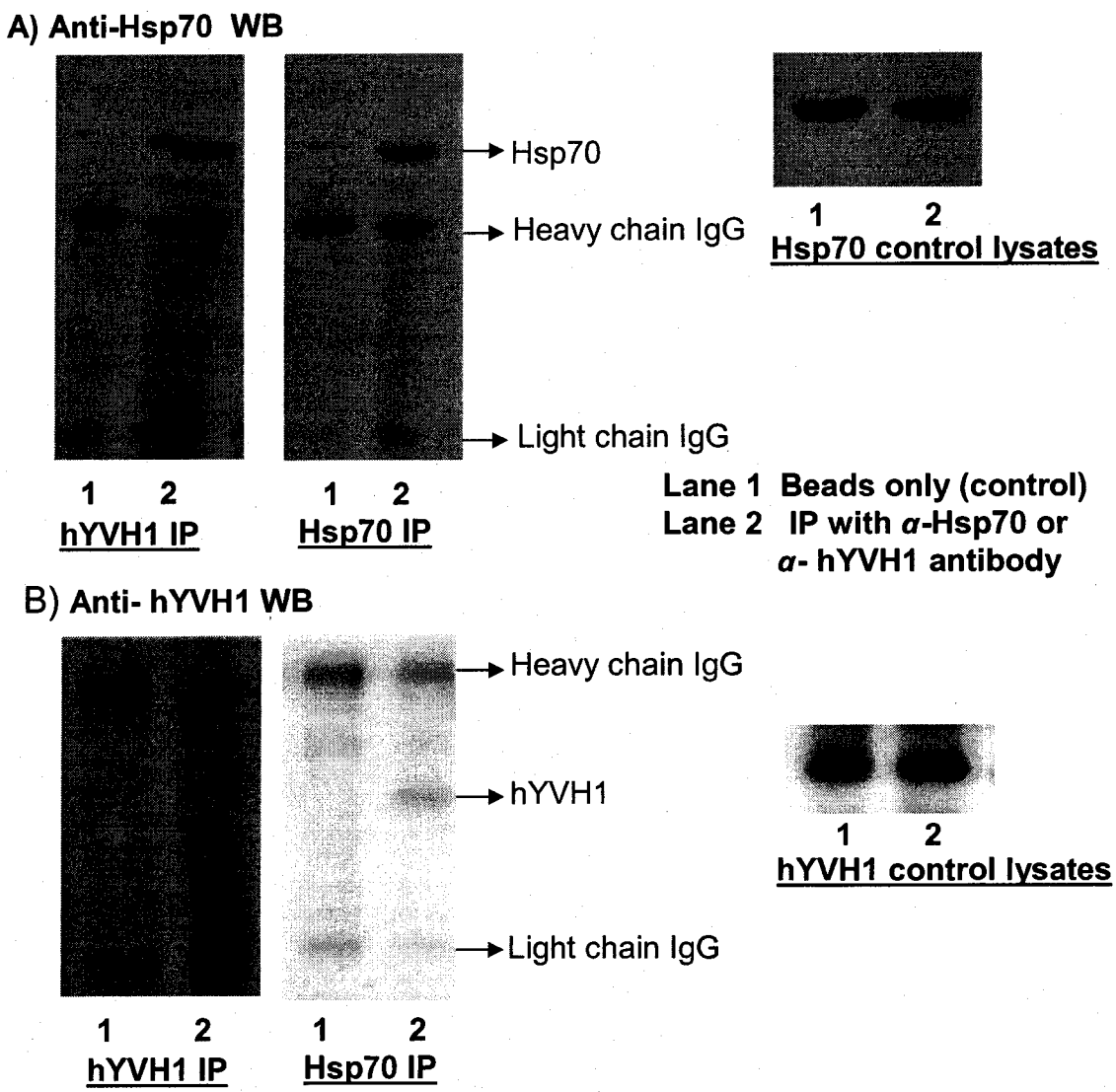



**Figure 14. Endogenous interaction of hYVH1 with Hsp70.** Lysates from two HEK293 plates were bound separately with protein agarose A resin. Two separate immunoprecipitations were performed, one with α-hYVH1 antibody and another with α-Hsp70 antibody. **A)** The immunoprecipitants of the two IPs were visualized with α-Hsp70 antibody. The presence of Hsp70, in case of hYVH1 IP, (2) shows that hYVH1 pulled down Hsp70. **B)** Western blot analysis of the two IPs with α-hYVH1 antibody shows that Hsp70 was also able to successfully pull down hYVH1(2).

### 3.2 Identification of hYVH1 substrates using substrate trap mutants

#### 3.2.1 Construction of substrate trap mutants

It has been demonstrated that PTPs can form substrate traps when certain catalytic residues are mutated (Flint *et al.,* 1997; Furukawa *et al.,* 1994; Jia *et al.,* 1995; Milarski *et al.,* 1993; Shiozaki and Russell, 1995; Sun *et al.,* 1993). Therefore, substrate trap variants of hYVH1 were desired to be used to capture potential substrates. The catalytically active cysteine to serine mutant had been previously constructed and represents one type of substrate trap. The catalytic aspartic acid to alanine mutant was created by first identifying the invariant catalytic aspartic acid residue in hYVH1 sequence. Sequence alignments were done between well characterized MKPs with experimentally determined catalytic residues (PDB codes: 1MKP and 1M3G). In addition, the aspartic acid residue in hYVH1 was also aligned with other orthologues of YVH1 including *S. cerevisiae* and *P. falciparum.* As a result, the position of the catalytic aspartic aicd in hYVH1 was marked at residue number 84 in the hYVH1 amino acid sequence **(Figure 15).**

Using PCR and site-directed mutagenesis, the catalytic aspartic acid residue of hYVH1 was mutated to an alanine and a hYVH1 (D84A) mutant was constructed in a mammalian pCMV vector and a bacterial pGEX vector. The PCR products were analyzed by DNA gel electrophoresis. An expected size of 6Kb band was found for both pCMV.hyvh1 and pGEX.hyvh1 vectors containing the D84A mutation while no plasmid DNA was observed in the control lane **(Figure 16).**

### 3.2.2 Does the interaction between hYVH1 and Hsp70 require catalytic activity?

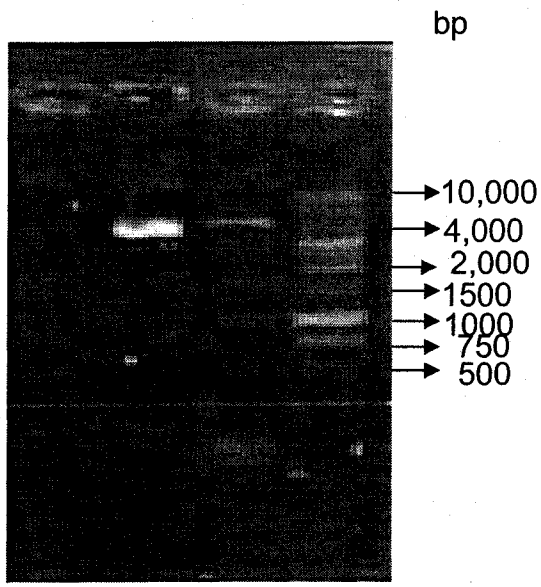
The interaction between hYVH1 and Hsp70 was further studied to determine the status of Hsp70 as a substrate or an associating protein to hYVH1. HEK293 cells were transfected with empty FLAG-pCMV mammalian vector or pCMV vector containing FLAG-hYVH1, FLAG-hYVH1 (C115S) and FLAG-hYVH1 (D84A). Cell lysis was done 24hrs post-transfection. Coimmunoprecipitation experiments were performed with the HEK293 lysates. The immunoprecipitates were resolved by SDS-PAGE and were visualized by coomassie staining. The results showed the Hsp70 band in the wild-type and mutant lanes but not in the control lane. In addition, the intensity of the Hsp70 band was found to be the same for wild-type and mutant forms of hYVH1 **(Figure 17).** The equal binding affinity of Hsp70 with wild-type and mutant hYVH1 indicated that the interaction between the two proteins is independent of the catalytic activity of hYVH1. Thus, the results from this experiment are consistent with the fact that Hsp70 is an interacting partner and not a substrate to hYVH1.

### 3.2.3 GST pull down assays

In an attempt to find hYVH1 substrates, recombinant GST-tagged hYVH1 was used as a bait to fish out the hYVH1-substrate complex. HEK293 lysates from twenty large sized plates (20cm in diameter) were precleared for GST-affinity proteins using GST protein bound to GST-sepharose resin. The pre-cleared

lysates were then used to capture the target enzyme-substrate complex. GST-protein, GST-tagged wild type hYVH1 and GST-tagged hYVH1 (D84A) mutant were bound to GST-sepharose resin. The precleared lysates were passed over the resin bound proteins for 4hrs. The GST-resin was washed to get rid of the non-specific protein binding and was loaded on SDS-PAGE to resolve the proteins bound to the resin. A unique 90kDa band was observed only in the GST-hYVH1 (D84A) lane and not in the control and wild-type hYVH1 lanes **(Figure 18).** The band was excised out of the gel and was analyzed by the MALDI-TOF mass spectrometer. Unfortunately, the p90 turned out to be a bacterial protein which was purified and isolated during the purification of GST-hYVH1 proteins from *E.Coli.* To counteract this problem, we loaded the purified GST-hYVH1 proteins in the absence of 293 lysates as negative controls for our next set of GST-pull down experiments. In addition, to increase the resolution of proteins on the gel we resorted to silver staining. However, no promising unique bands were detected using the in vitro GST-pull down method **(Figure 19).**


**Figure 15. Alignment of hYVH1 with MKP dual specificity phosphatases and YVH1 orthologues.** The sequence alignment of amino acids was done using ClustalW algorithm. The two human MKPs chosen are Psyt MKP-3 and MKP Pac-1 with PDB codes 1MKP and 1M3G, respectively. In addition, hYVH1 is aligned with *P. falciparum* and *S. cerevisiae* YVH1 phosphatases. The catalytic aspartic acid/base residue (D84) and the catalytic cysteine (C115) of hYVH1 are represented in two separate boxes.




Lane 1 Control
Lane 2 pGEX-hyvh1
Lane 3 pCMV-hyvh1
Lane 4 DNA ladder

**Figure 16. Confirmation of PCR products using DNA electrophoresis.** The PCR products were run on a DNA gel to verify for the size and presence of the two types of vectors (pCMV & pGEX) containing mutant hYVH1 cDNAs. No plasmid DNA was found in the control lane (lane 1), however, lane 2 and lane 3 show approximately 6Kb band for pGEX.hYVh1 and pCMV.hYVH1 cDNA, respectively.

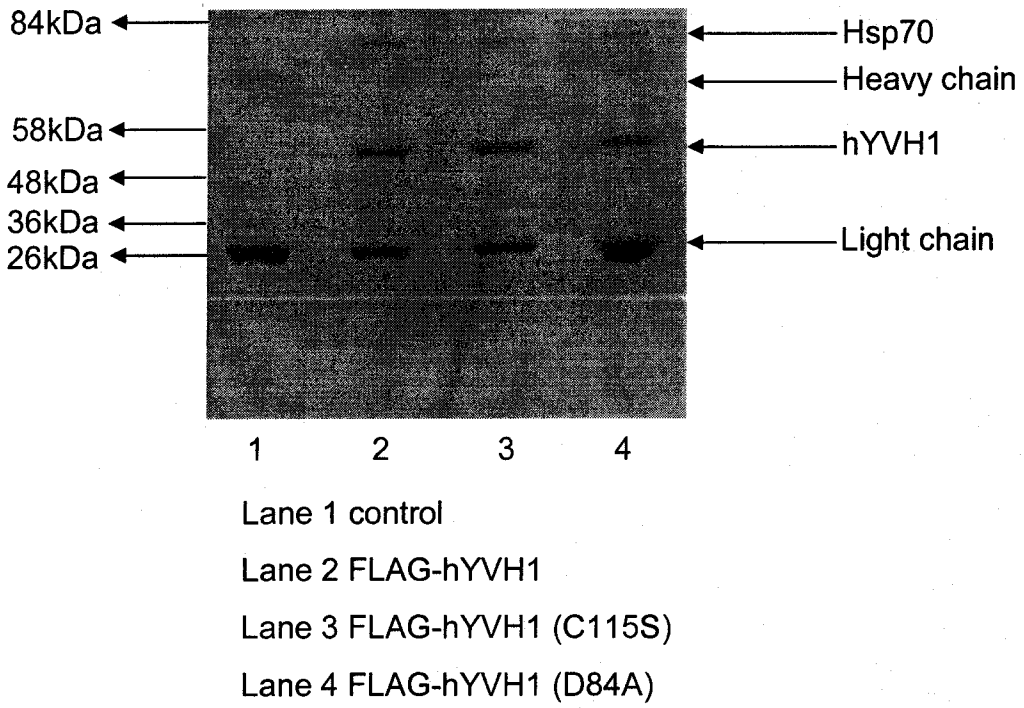



**Figure 17. Hsp70 interacts with wild type and catalytic hYVH1 mutants with equal affinity.** HEK293 cells were transfected with FLAG-tagged wild type and hYVH1 mutants. The lysates were immunoprecipitated with *α*-FLAG resin and the immunoprecipitants were resolved by SDS-PAGE. The coomassie stained gel above shows the presence of an Hsp70 band in lanes 2, 3 & 4 but not in the control lane. The band has the same intensity in the three lanes indicating that Hsp70 interacts with wild type hYVH1 (lane 2), hYVH1-C115S (lane 3), and hYVH1-D84A (lane 4) with equal affinity.

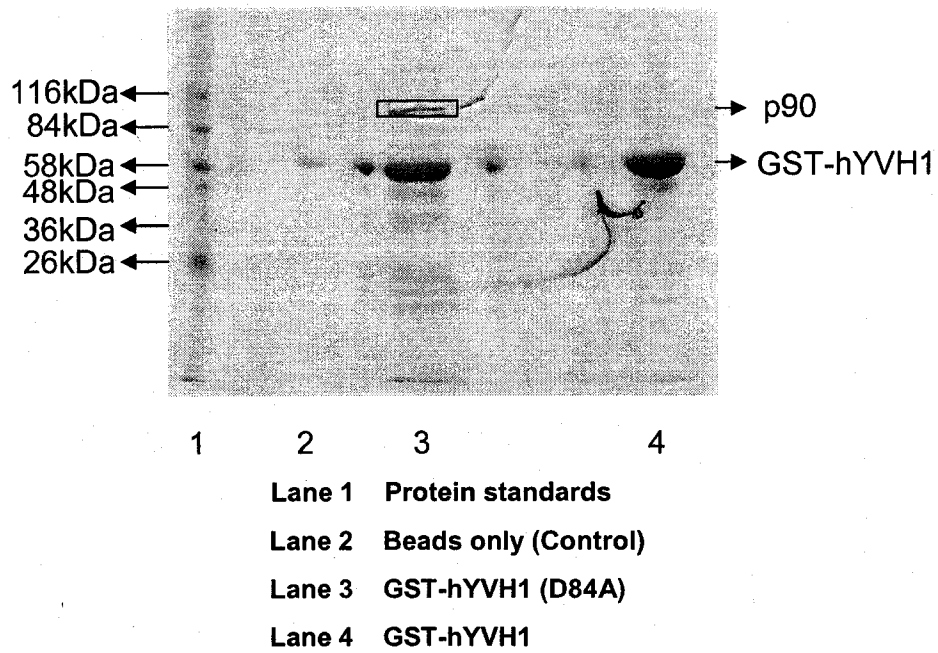
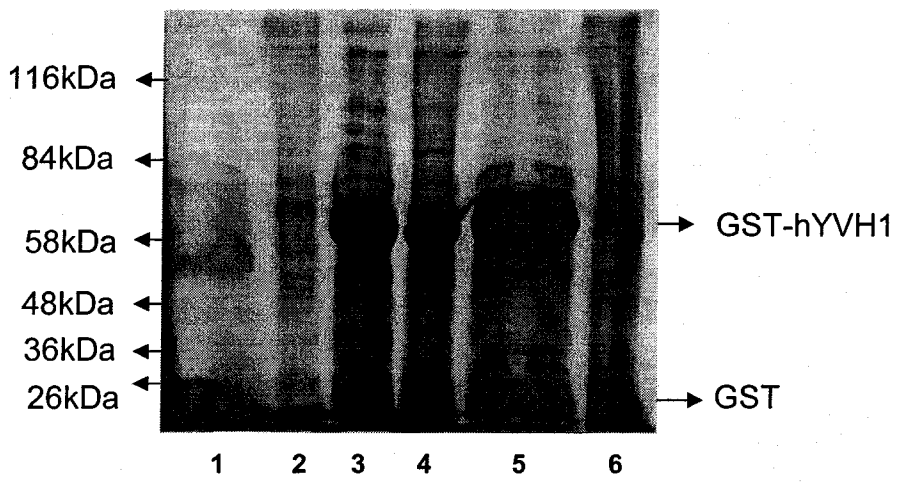



**Figure 18. Coomassie stained gel showing results from a GST pull down assay.** Purified GST-hYVH1 and GST-hYVH1 (D84A) proteins were bound to GST-Sepharose resin. Lysates from HEK293 cells were passed over the resin bound proteins to capture the hYVH1-substrate complex. The resin bound protein complexes were resolved by gel electrophoresis. The coomassie stained gel above showed a unique band (~90kDa) in the mutant lane (3) which was found to be absent in GST-hYVH1 lane (4) and the control lane (2). The band had been excised for mass spectrometry analysis.




**Lane 1 GST protein**
**Lane 2 GST+resin**
**Lane 3 GST-hYVH1(D84A) protein**
**Lane 4 GST-hYVH1(D84A)+ resin**
**Lane 5 GST-hYVH1 protein**
**Lane 6 GST-hYVH1+resin**

**Figure 19. Silver stained gel showing results from a GST pull down assay.** Purified GST, GST-hYVH1, GST-hYVH1 (D84A) were bound to GST-Sepharose resin. HEK293 lysates were precleared for GST proteins and the precleared lysates were passed over the resin bound proteins, GST, GST-hYVH1 & GST-hYVH1 (D84A). The resin and the purified proteins were loaded on SDS-PAGE gels and the gels were visualized by silver staining. The position of GST-hYVH1 protein (wild type and mutant) are marked at ~60 kDa. Some of the GST cleaved off from hYVH1 and is marked at ~26 kDa. The experiment did not show any unique bands on comparison of the control, wild type and mutant lanes.

### 3.3 Identification of hYVH1 substrates using the *in vivo* approach

#### 3.3.1 One dimensional gel electrophoresis and silver staining

Previous attempts using the substrate trapping mutants and coomassie staining revealed few detectable bands besides Hsp70. Therefore, it was decided to use mass spectrometry-compatible silver staining for the FLAG-affinity chromatography experiments to increase sensitivity for capturing the substrates of hYVH1 *in vivo* in HEK293 cells. Cells were transfected with empty pCMV vector or pCMV vector containing wild-type or mutant hYVH1 (hYVH1 - C115S & hYVH1-D84A). After 24hrs, cells were lysed and the lysates were immunoprecipitated using M2-agarose anti-FLAG resin. The immunoprecipitants were resolved by one-dimensional electrophoresis and proteins were visualized by silver staining. The results showed a large number of protein bands on the silver stained gel. However, the heavy and light chain IgGs, hYVH1 (wt & mutant) and Hsp70 bands overlapped some of the potential substrate bands on the gel **(Figure 20)**. Therefore, the protein band visualization needed to be optimized in order to enhance their resolution on the protein gel.

#### 3.3.2 Two dimensional gel electrophoresis

In order to overcome the problem of antibody overlapping as observed in one dimensional electrophoresis, we competitively eluted the hYVH1 complexes using the FLAG peptide and concentrated the eluant by acetone precipitation overnight at -20°C. In addition, proteins were separated by two-dimensional electrophoresis (2-DE) for increased resolution. This technique separates proteins according to their isoelectric point (pI) first, in a process called isoelectric

focusing (IEF) on pH gradient strips. This is followed by the separation of proteins based on their molecular weight (MW) in the second dimension.

HEK293 cells were transfected with pCMV empty vector and hYVH1 (D84A). Cells were lysed 24hrs post-transfection and the control and hYVH1 (D84A) lysates were immunoprecipitated with M2-agarose anti-FLAG resin. The proteins were eluted from the resin using FLAG peptide and were acetone precipitated. The proteins were loaded onto the IEF strips using the passive rehydration method and were resolved in the first dimension according to their pI and in the second dimension according to their MWs.

The results from preliminary efforts using this strategy showed Hsp70, hYVH1 (D84A) and a possible unique spot on the gel around 30kDa (p30) whereas none of these proteins were observed on the control gel **(Figure 21A & 21B).** However, while collecting the eluting peptides some of the resin entered into the sample and hence a slight amount of light and heavy chain IgGs from the anti-FLAG antibody were also observed. Also, it was concluded that the acetone precipitation step was not compatible with IEF.

To confirm that the spot around 30kDa was real, and in the attempt to optimize IEF, we scaled up the number of cells from 5 big plates (20cm in diameter) to 10 big plates. Also, to eliminate sample loss and optimize IEF, 2-DE experiments were performed without FLAG elution. With scaling up the number of cells, we were better able to detect the presence of the p30 spot on the gel with enhanced resolution and confidence using the optimized procedures. Also, reference protein spots (RF1, RF2 & RF3) were found to be helpful in defining

the position of p30 during reproducible trials. The pI of p30 was approximated to be between pI 4.5 to 5.5 using the calculated pI of Hsp70 and hYVH1 **(Figure 22.1 & 22.2).**

Since the expression of Hsp70 increases in response to heat shock, we tested if under these conditions hYVH1 substrates were elevated (i.e. more phosphorylated) **(Figure 23)**. The same reference points and p30 were observed, however, a p30 spot with an altered pI was observed. This may represent a more heavily phosphorylated version of p30 or a different protein. The level of Hsp70 coimmunoprecipitating with hYVH1 also seemed to be enhanced under the heat shock conditions **(Figure 23.1).**

To test if the p30 spot coimmunoprecipitated with the substrate trapping mutant of hYVH1 (D84A) was a substrate or an interacting protein, the experiment was performed comparing wild-type hYVH1 with hYVH1 (D84A). The results showed coimmunoprecipitation of p30 only with hYVH1 (D84A) mutant but not with wild-type hYVH1. This suggests that the p30 protein trapped by the hYVH1 (D84A) mutant represents a potential physiological hYVH1 substrate **(Figure 24).**

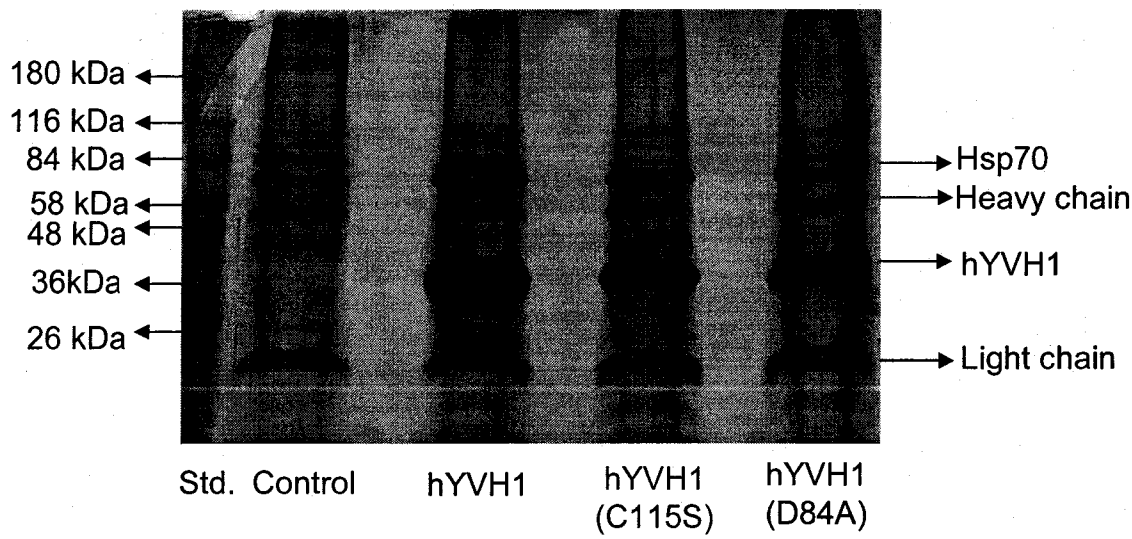
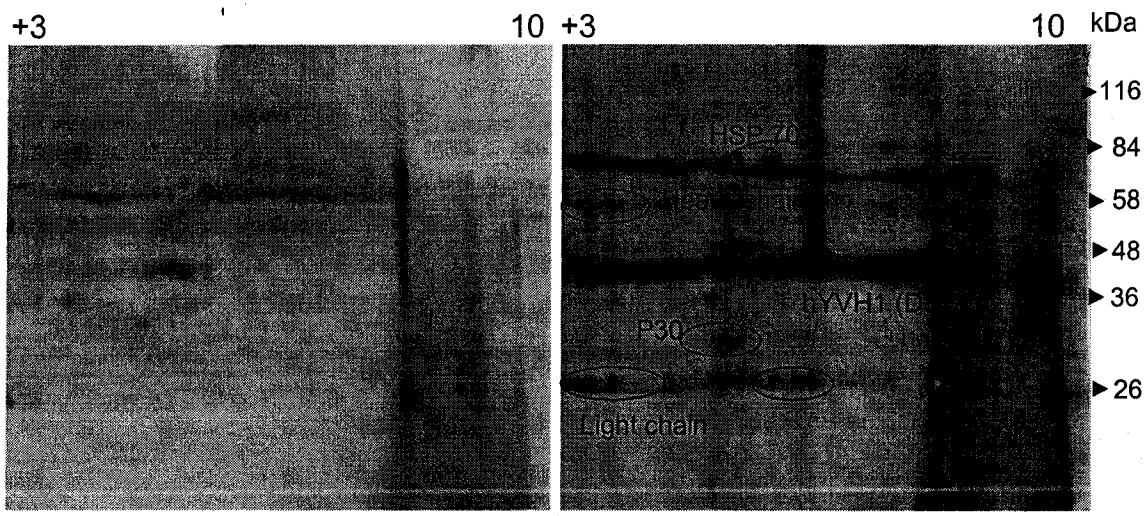



**Figure 20. Identification of hYVH1 substrates using one-dimensional electrophoresis and silver staining.** HEK293 cells were transfected with pCMV vector containing hYVH1 or hYVH1 (C115S) or hYVH1 (D84A).The cellular lysates were immunoprecipitated with *α*-FLAG resin. The immunoprecipitants from the FLAG-IP were resolved by SDS-PAGE and were visualized by silver staining. Hsp70 and hYVH1 bands were observed in hYVH1 and mutant lanes but not in the control lane. The gel also contains numerous other bands, however, due to overlapping of protein bands in one dimensional electrophoresis it was difficult to spot a unique band on the gel in the mutant lanes.




**A) Control** **B) hYVH1 (D84A)**

**Figure 21. Use of two-dimensional electrophoresis and FLAG-elution to capture hYVH1 substrates.** Lysates from HEK293 untransfected cells **(A)** and cells transfected with hYVH1 (D84A) **(B)** were immunoprecipitated with anti-FLAG resin. The hYVH1-protein complexes were eluted with FLAG peptide and were resolved in the first dimension by isoelectric focusing and in the second dimension by SDS/PAGE. The gels were visualized by silver staining. A faint p30 spot was observed in the hYVH1 (D84A) mutant gel which was found to be absent in the control gel.

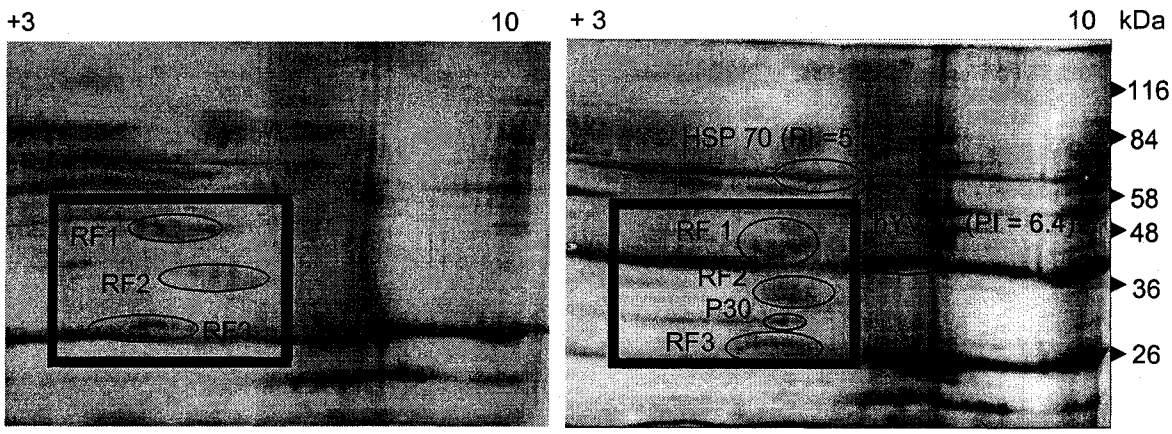
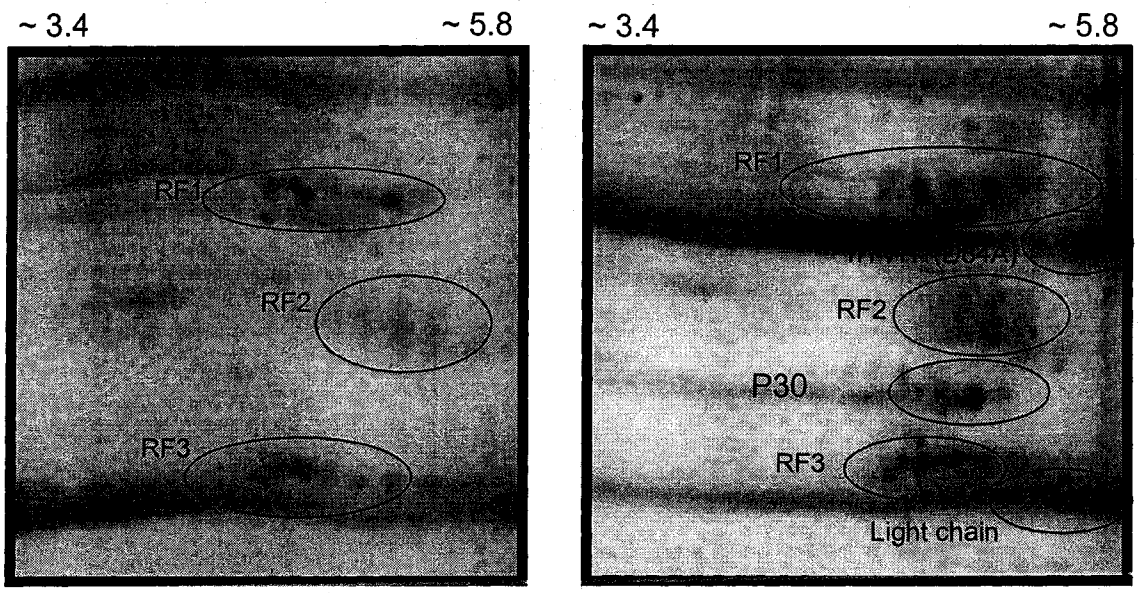
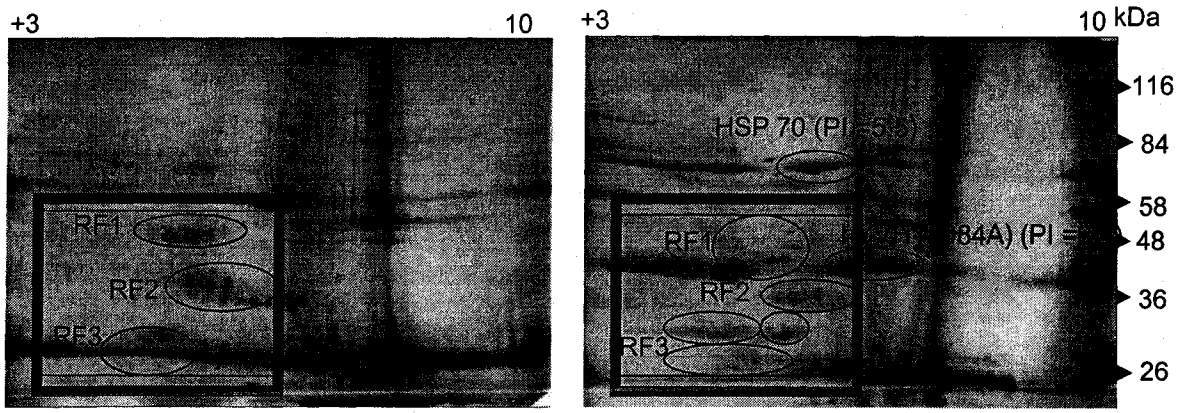



**Figure 22.1 Use of two-dimensional gel electrophoresis and FLAG-immunoprecipitation to capture the substrates of hYVH1.** HEK293 cells were used to perfom FLAG-IP with untransfected lysates **(A)** and lysates from cells transfected with hYVH1 (D84A) DNA **(B)** The enzyme-substrate complex was resolved by two dimensional electrophoresis and gels were visualized by silver staining.



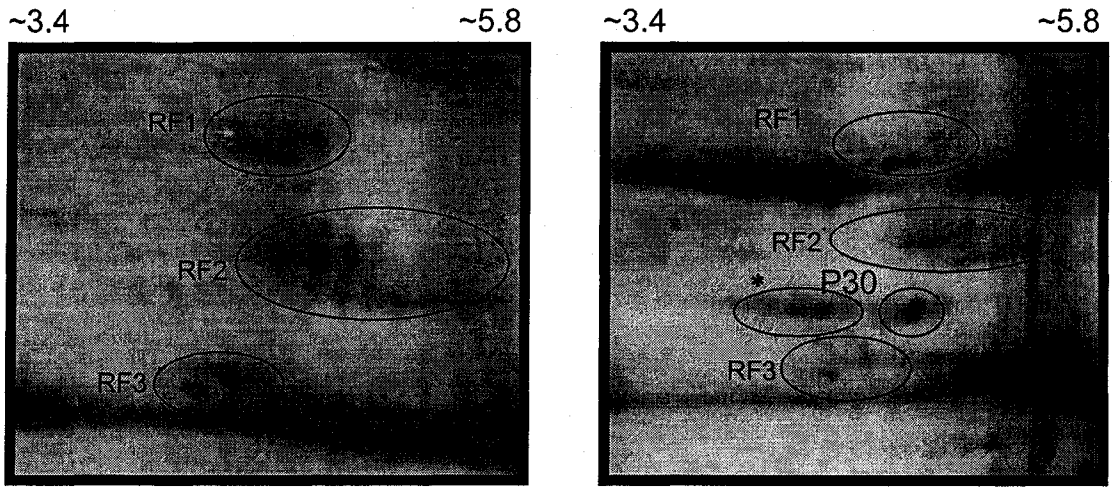

**A) Control** **B) hYVH1 (D84A)**

**Figure 22.2 Enlarged view of p30 with the hYVH1 (D84A) mutant.** The 2D gels above represent a zoomed in view of the p30 area from the FLAG IP experiment (figure 22.1). The p30 spot is marked in reference to three other areas on the gels. The figure above shows the control **(A)** and hYVH1 (D84A) mutant **(B)** results where p30 is only observed on the hYVH1 (D84A) gel.



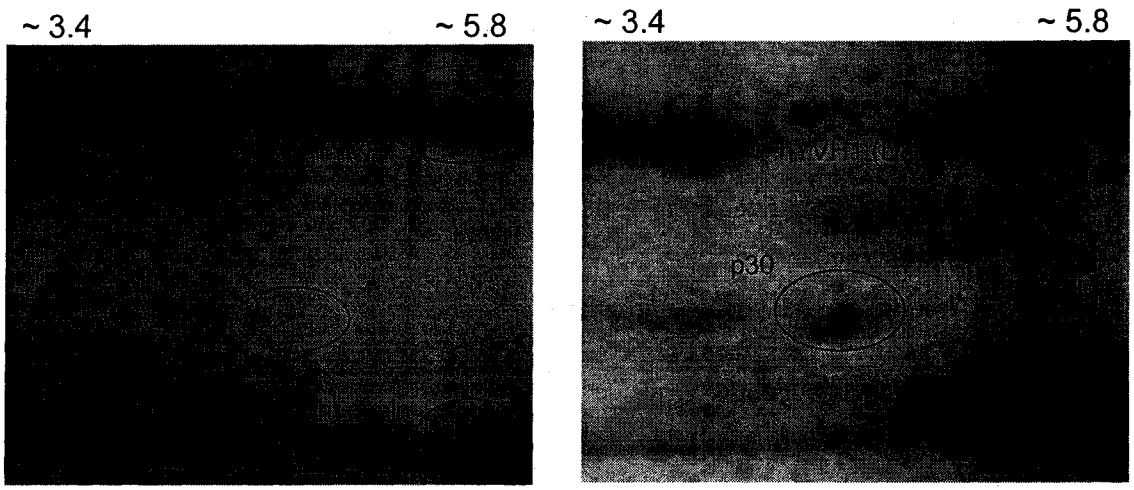

**A) Control** **B) hYVH1 (D84A)**

**Figure 23.1 Use of two-dimensional electrophoresis and FLAG-immunoprecipitation to capture hYVH1 substrates under heat shock conditions.** Both untransfected **(A)** and hYVH1 (D84A) transfected **(B)** HEK293 cells were heat shocked to 42°C for one hour. Cellular lysates were immunoprecipitated with anti-FLAG resin and hYVH1-substrate complex was resolved by 2-DE and visualized by silver staining.




**A) Control** **B) hYVH1-D84A**

**Figure 23.2 Enlarged view of p30 with the hYVH1 (D84A) mutant under heat shock conditions.** The figure above shows a zoomed in view of the FLAG-IP experiment performed under heat shock conditions (figure 23.1). Heat shock conditions resulted in the presence of an additional spot next to p30 as marked by an asterisk which was found to be absent in the control.




**A) hYVH1** **B) hYVH1-D84A**

**Figure 24. Comparison of p30 with wild type hYVH1 and hYVH1 (D84A) mutant using two dimensional electrophoresis.** HEK293 cells were transfected with pCMV vector containing hYVH1 or hYVH1 (D84A). Lysates were immunoprecipitated with $\alpha$-FLAG antibody and proteins were resolved by two-dimensional electrophoresis. The gels were silver stained and the p30 protein was observed with mutant hYVH1 and not wildtype hYVH1 validating its status as a potential substrate of hYVH1.

# CHAPTER 4

# DISCUSSION

The dual specificity phosphatase human YVH1 is a 36kDa protein which contains a novel zinc finger domain. Past studies have shown that hYVH1 can complement the slow growth phenotype in yeast (Muda *et al.,* 1999). However, the exact physiological function of this enzyme is unknown. In this study, we aimed to identify the interacting partners and substrates of hYVH1 as a first step towards the characterization of this evolutionary conserved enzyme.

## 4.1 Identification of Hsp70 as an interacting protein of hYVH1

In this study, we have identified the first novel binding partner of hYVH1 using affinity chromatography and mass spectrometry. The identified protein is Hsp70 which belongs to the heat shock family of proteins. Two mass fingerprints were obtained for Hsp70 in order to have a spectrum with a high signal to noise ratio. To enhance the resolution of the original spectrum, the peptide samples were desalted using the C18 ziptips which are made of hydrophobic reverse phase chromatography resin packed into a pipette tip for rapid desalting. The C18 zip tips decontaminated the sample for any salt impurities that might have retained in the original sample. The result of this step was increased number of peptide peaks detected, higher mass accuracy, and improved resolution. The increase in the number of peptides detected results from improved co-crystal formation and thus ionization of the peptide molecules in the absence of salt. The higher mass accuracy and resolution occurs because eliminating the salt molecules allows for the peptide ions to keep a tighter flight path in the TOF tube.

The peptide masses from this improved ziptipped spectrum were used for preliminary database searching and to select precursor ions for MS/MS analyses. In the process of identification of Hsp70 by mass spectrometry, it was found to be similar with its constitutive isoform Hsc71 and the MS/MS spectrum for the precursor ion (m/z = 1487) with the '**TTPSYVAFTDTER**' was common to both Hsp70 and Hsc71. Our sequence alignment results demonstrated that the two proteins are highly identical to each other. However, the MALDI-PSD analysis for the partial sequencing of two more precursor ions (m/z = 1109 & m/z = 1657) with amino acid sequence **'LLQDFFNGR'** and **'ATAGDTHLGGEDFDNR'**, respectively were only specific to Hsp70 which clearly demonstrated that the interacting protein was indeed Hsp70 and not Hsc71. The interaction between hYVH1 and Hsp70 was also confirmed from our overexpresssion studies via reciprocal western blot analyses. Importantly, Hsp70 and hYVH1 were found to coimmunoprecipitate under endogenous conditions addressing the possibility that the interaction was an overexpression artifact. The endogenous experiment strongly suggests the interaction between hYVH1 and Hsp70 in cells is physiologically relevant. In addition, our comparison for the interaction of hYVH1 with Hsp70 and not Hsc71 demonstrates Hsp70 isoform specificity for hYVH1. The specificity is intriguing since Hsc71 and Hsp70 share such high sequence identity and it would be expected on a biochemical basis that hYVH1 could interact with both equally in vitro. This suggests that in vivo, other factors such as specific subcellular localization, or additional proteins are needed to be present for them to be captured as an *in vivo* protein complex.

Heat stress is a major source of cellular apoptosis. Hsp70 is inducible in response to cellular insults such as heat and other cellular insults playing an anti-apoptotic role under these conditions (Gabai and Sherman, 2002; Pirkkala *et al.,* 2001). Interestingly, hYVH1 is also implicated to counteract stress conditions such as low temperature and nitrogen starvation in yeast (Muda *et al.,* 1999). Hence, the interaction between the two proteins seems to be functionally relevant and requires to be characterized by functional studies.

**4.2 Identification of hYVH1 substrates using the substrate trap mutants**

In this study, two types of substrate trap mutants were used to capture the hYVH1-substrate complex. These two mutants (C115S) and (D84A) were created by mutating the two invariant catalytic residues of hYVH1, the catalytic cysteine and the catalytic aspartic acid, using site-directed mutagenesis. The substrate trap mutants were designed as such that the enzyme was kept enabled to bind the substrate but would not allow its release from the enzyme pocket. The substrate-trapping strategy of the two mutants differed from each other due to their individual roles in the enzyme catalysis. Mechanistically, the C115S mutant will hold the substrate due to hydrophobic and electrostatic interactions between the substrate and the enzyme pocket and is incapable of making a covalent interaction between the enzyme and the substrate. In case of D84A mutant, the enzyme is still able to make a covalent thiol-phosphate intermediate between the enzyme and the substrate due to the nucleophilic attack by the catalytic cysteine. However, a lack of the catalytic aspartic acid residue would not allow the breakage of the phospho-substrate bond resulting in a more stabilized substrate

trap in the enzyme pocket. Therefore, due to the reason of a stronger covalent interaction between the enzyme and the substrate, which occurs with the D84A mutant, it was the first choice to be used for trapping the hYVH1 substrate.

Recombinant GST-tagged proteins are widely used as bait proteins to fish protein complexes (Lee, 2005; Singh et al., 2005; Craig et al., 2004). Our first attempts to capture hYVH1 substrates also used the purified GST-hYVH1 proteins. Both GST-wild-type and GST-hYVH1 (D84A) mutant proteins were immobilized on GST-sepharose resin and fresh lysates from HEK293 cells were passed over the resin bound proteins to capture the substrate. The results identified a unique protein on the gel as an *E.Coli* protein. Experiments were repeated with controls for the bacterial proteins, yet no promising results were obtained using this approach. This was mainly attributed to problems with the instability and unfolding of the enzyme which hinders it from binding its substrate. It was also reasoned to be due to the lack of an intracellular regulation of the enzyme since hYVH1 has been shown to be phosphorylated *in vivo* (Vacratsis, unpublished data).

As an alternative to the GST pull down assays, we used an *in vivo* approach to capture the substrate-phosphatase complex from HEK293 cells. The results of a previous experiment, to check the requirement of the catalytic activity for the interaction between hYVH1 variants and Hsp70, showed no additional proteins bands with coomassie staining. Hence, we decided to use silver staining for increased sensitivity and resolution of proteins for the *in vivo* set of experiments. Although a large number of protein bands were observed by silver staining, we

faced the problem of overlapping of protein bands due to overcrowding of proteins in one dimensional electrophoresis. Moreover, some of the protein bands seemed to be covered by the light and heavy chain IgGs of the anti-FLAG antibody. Therefore, we eluted the proteins using FLAG peptide and precipitated them with acetone. In addition, the problem of overlapping was circumvented by resolving proteins via 2-DE which produced promising results (Figure 21). Final optimization of the IEF conditions involved increasing the cell number used and not performing FLAG elution of the proteins to avoid any possible interference from acetone.

A faint p30 band was observed in our initial attempts to resolve the substrate in the 2-DE experiments. To increase the intensity of the p30 band we scaled up on the number of cells in our experiments by 5-fold. This led to a more pronounced detection of the p30 protein spot on the 2D gels and its location was marked by reference points. The absence of the p30 protein spot in the control gels and wild type hYVH1 gels confirmed it to be a potential substrate of hYVH1. The same set of results was obtained under heat shock conditions at 42° C. Heat shock increased the level of Hsp70 present and increased the intensity of the p30 spot on the gel. In addition a new p30 spot appeared at a region more acidic than the previous p30 protein spot that may represent a heat shock induced hYVH1 substrate. However, since adding phosphate groups to a protein would make the pI shift to the acidic region, the new spot may represent a more phosphorylated version of p30.

Using the substrate trap approach and two-dimensional electrophoresis we

revealed a potential substrate of hYVH1. However, the identification of this protein by in gel trypsin digestion and mass spectrometry faced a technical challenge. The amount of tryptic peptides obtained from the gel was below the detection limit when analyzed by mass spectrometry. This may be due to low level of protein or possibly the acidic p30 may resist in gel trypsin digestion and extraction.

However, using bioinformatics we have narrowed the candidate list to less than 100 human protein sequences that share the pI ~ (4.5-5.2) and MW ~ (27-35kDa) of the p30 protein spot. From this list we searched for feasible candidates. One is an Hsp70 co-chaperone called Bag-1 which is a regulator of the activity of Hsp70 and also is an anti-apoptitic protein. The fact that Bag-1 is a regulator of Hsp70, and Hsp70 is an interacting protein to hYVH1, strikingly increases the relativity of Bag-1 being a potential hYVH1 substrate. Furthermore, Bag family members such as Bag-2 have been found to be phosphorylated (Ueda *et al.,* 2004). More interestingly, one of the Bag-1 isoforms (Bag-1M) has also been found to be phosphorylated (Townsend *et al.,* 2005). It is very promising that future studies testing the possible interaction between hYVH1 and Bag-1 as well as functional studies on Hsp70 and hYVH1 will delineate the the physiological function of the evolutionary conserved dual specificity phosphatase hYVH1.

## FUTURE PROSPECTIVES

This study reported the first interacting protein of the dual specificity phosphatase hYVH1 called Hsp70. Although much analytical work was done to lay the foundation for the characterization of this enzyme, future biochemical and functional studies will further elucidate the nature of the interaction between hYVH1 and Hsp70. The interaction between the two proteins will be studied under various stress conditions such as heat shock, apoptotic inducers and hydrogen peroxide ($H_2O_2$) treatment to see if stress weakens or strengthens the Hsp70 interaction with hYVH1. In addition, the interaction domains of the two proteins will also be studied by making zinc-finger truncated mutants of hYVH1 and substrate binding or ATPase-binding truncated mutants of Hsp70. To determine the biological settings and the domains mediating the interaction, the coimmunoprecipitation experiments will be repeated in other cell lines under various conditions and variants of the two proteins.

Recent preliminary data from our lab shows that hYVH1 may be partially located in the mitochondria. Since Hsp70 has been shown to translocate proteins to the mitochondria, it will be intriguing to study if Hsp70 plays a role in the translocation of hYVH1 to the mitochondria by performing localization studies on the two proteins using immunofluorescence assays. Moreover, enzymatic assays will be done to study the effect of the Hsp70-hYVH1 interaction on the enzymatic activity of hYVH1 and the anti-apoptotic activity of Hsp70. These studies will determine whether Hsp70 increases or decreases the phosphatase activity of hYVH1 or if hYVH1 modulates the anti-apoptotic effects of Hsp70.

Further attempts will be made to determine the identity of the p30 protein. Biochemical fractionation may be used once the data on the localization of hYVH1 is confirmed and can be used to target that particular fraction of cellular extracts to enrich the p30 protein.

Moreover, cell cycle assays will be done to get an insight about the cell cycle stage where levels of the hYVH1-p30 complex are greatest. In addition, co-immunoprecipitation experiments and Western blot studies are currently being done by others in the lab to test if Bag-1 is a hYVH1 substrate using hYVH1 substrate trapping variants and detecting endogenous Bag-1 using an anti-Bag antibody.

In summary, our proteomic approaches using affinity chromatography and mass spectrometry have opened exciting new doors to be explored concerning hYVH1. It is promising that future work based on these studies will elucidate breakthrough details about the physiological function of the hYVH1 phosphatase.

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# VITA AUCTORIS

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## Education

**2003-2005** **University of Windsor**
Masters in Biochemistry

**1999-2003** **University of Toronto**
Bachelors of Science: specialization in toxicology
Took courses in the area of Chemistry, Biochemistry, Molecular Biology, statistics, pharmacology, and toxicology.

## Research Experience

**2003-2005** **University of Windsor**
Research assistant under the guidance of Dr. P.O.Vacratsis
Project title: "**Identification of substrates and interacting proteins using biological mass spectrometry".** We identified the first associating protein of hYVH1. The protein belongs to the heat shock family and is called Hsp70. In addition, we also found a potential (30kDa) substrate protein of this enzyme.

**Summer 2003** **University of Toronto**
Research assistant under the guidance of Dr. J.S. Yeomans
Project title: **"In vivo electroporation of M5 muscarinic receptors in the rat ventral tegmental area."**

**Summer 2001** **Hospital for Sick Children**
Research assistant under the guidance of Dr. B. Alman
Project title: **"Pathogenesis of Ewing's sarcoma."**

## Publications

**Abstract published at the 34th annual meeting for the society for neuroscience (SFN).**
Citation: H. Wang, Z. Butt, G. Chui, A. Madden, S. Steidl, J. Yeomans. In vivo electroporation of M5 muscarinic receptor genes in the rat ventral tegmental area with IRES-hrGFP vectors. Society for Neuroscience, 2004. Online.

## Interests

Sports, Scientific and literature reading, Playing piano.