# The Insulin Signaling Pathway:

## Evidence that Tax1bp1/Txbp151 is a dimeric human Grb14 interacting protein.

**By**

**Ronald Agatep**

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

**MASTER OF SCIENCE**

Department of Human Genetics
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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**The Insulin Signaling Pathway: Evidence that Tax1bp1/Txbp151 is a dimeric human Grb14 interacting protein**

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## ABSTRACT

The adapter protein Grb14 (Growth Factor Receptor Bound 14) has been shown to directly interact with the cytoplasmic domain of the activated insulin receptor. To date, the function of Grb14 and the purpose of its association with the IR are unclear. To aid in the elucidation of Grb14's function, human Grb14 (hGrb14) interacting proteins were isolated from an adult human liver cDNA library using a modified yeast two-hybrid system. Three interacting proteins were identified: a novel protein named Gip1 (Grb14 Interacting Protein-1), the gene product of *KIAA0093* (a *NEDD4* homologue), and Tax1bp1/Txbp151 (Tax1-binding protein-1/Tax1-binding protein 151). The hGrb14: Tax1bp1 interaction was studied. The Tax1bp1 primary sequence was found to encode an internal coiled-coil domain, a carboxyl-terminal LIM domain and a novel domain shared only with the Ndp52 protein. In the two-hybrid system, the amino acid residues 236 to 593 of the Tax1bp1 coiled-coil domain mediated its association with hGrb14. Furthermore, this portion of Tax1bp1 could also mediate self-association in the two-hybrid system. In a genetically modified CHO (Chinese hamster ovary) cell line constitutively overexpressing the human insulin receptor and human Grb14 and transiently overexpressing Tax1bp1, an interaction between Tax1bp1 and hGrb14 was observed. Structural characterization of the *TAXBP1* gene was also performed. The *TAX1BP1* gene, mapped to 7p14-7p15, contained 16 exons that spanned 70 KB. These results provide evidence that Tax1bp1 is a self-associating human Grb14 interacting protein.

# ACKNOWLEDGMENTS

I remember when I first walked through P306 thinking, "Alright, this guy uses Macs!". Much has happened since that day. This is my chance to give thanks for the time and knowledge you have given to me.

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I am lucky to have met each of you early in my scientific career.

Thanks, Ron

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| 3-AT | 3-amino-1,2,4-triazole |
| aa | amino acid(s) |
| Ab | Antibody |
| AD | GAL4 activating domain (amino acids 768-881) |
| APS | Ammonium persulfate |
| BD | DNA Binding Domain |
| bp | Base Pair |
| dATP | deoxyadenosine triphosphate |
| dCTP | deoxycytidine triphosphate |
| dGTP | deoxyguanosine triphosphate |
| dNTP | deoxynucleotide triphosphate |
| dTTP | deoxythymidine triphosphate |
| DMF | N,N-dimethlyformamide |
| DNA | deoxyribonucleic acid |
| ds DNA | double stranded DNA |
| DTT | dithiothreitol |
| *E.coli* | *Echerichia coli* |
| EDTA | ethylene diaminetetraacidic acid |
| FCS | Fetal Calf Serum |
| *g* | Earth's gravitational constant |
| Grb | Growth Factor Receptor Bound |
| IR | Insulin Receptor |
| Irs | Insulin Receptor Substrate |
| hGrb14 | *Homo sapiens* orthologue of Grb14 |
| HRP | Horseradish peroxidase |
| LB | Luria Bertani |
| LiAc | Lithium Acetate |
| MCS | Multiple Cloning Site |
| ONPG | ortho-nitrophenyl-D-pyranogalactoside |
| ORF | Open Reading Frame |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PEG | Polyethylene Glycol |
| PMSF | phenylmethylsulfonyl flouride |
| rGrb14 | *Rattus norvegicus* orthologue of Grb14 |
| RPM | Rounds per minute |
| *S. cerevisiae* | *Saccharomyces cerevisiae* |
| SC- | Synthetic complete omission media |
| TBS-T | Tris-Cl Buffered Saline-Tween-20 |
| TE | 10 mM Tris-Cl; 1 mM EDTA pH 8.0 |
| UTR | untranslated region |
| w/v | weight/volume |
| X-gal | 5-bromo-4-chloro-3-indolyl-ß-D-galactoside |

" There is no other species on Earth that does science. It is so far, entirely a human invention. It is only a tool. But it is by far the best tool we have, self-correcting, ongoing, applicable to everything. It has two rules. First: there are no sacred truths; all assumptions must be critically examined; arguments from authority are worthless. Second: whatever is inconsistent with the facts must not be confused with how it is and with how we wish it to be."

**Carl Sagan, Cosmos**

# 1. INTRODUCTION

## *1.1 Diabetes mellitus*

Diabetes mellitus, one of the most common metabolic diseases, affects an estimated 5% of the world population (Kahn, 1998). It is characterized by high circulating blood glucose which can lead to several complications including ketoacidosis, hypertension, hypertriglyceridemia, kidney failure, blindness, and stroke (Taylor, 1999). It has no cure. There are two major clinical forms of diabetes: Type I diabetes or Insulin-dependent diabetes mellitus (IDDM) and type II diabetes or Non-insulin dependent diabetes mellitus (NIDDM). The latter, also known as adult-onset diabetes, accounts for the majority of diabetic cases and is therefore a major focus of research.

### *1.1.1 Type I Diabetes or Insulin-Dependent Diabetes Mellitus (IDDM)*

Type I diabetes or insulin-dependent diabetes mellitus (IDDM) is a multifactorial disease controlled by genetic and environmental factors. IDDM, also referred to as juvenile-onset diabetes, is estimated to account for 5% of the diabetic cases in the Caucasoid population (Todd, 1999). Although the cause of IDDM is unknown, it is often associated with an autoimmune response that destroys the insulin producing pancreatic ß–cells resulting in the absence of systemic insulin (Todd, 1999). In humans, 19 susceptibility loci have been linked to IDDM (Tisch and McDevitt, 1996). The HLA genes, which encode proteins that aid in self and non-self recognition, appear to be major genetic contributors to this form of diabetes (Acha-Orbea *et al.*, 1987; Todd *et al.*, 1987; Thorsby *et al.*, 1996; She, 1996). In addition, incomplete concordance is also seen in monozygotic twins suggesting an environmental influence (Barnet *et al.*, 1981).

Environmental factors including viral infection, diet, and climate may be related to the development IDDM (Trevisan *et al.*, 1998).

### *1.1.2 Type II Diabetes or Non-insulin Dependent Diabetes Mellitus (NIDDM)*

Type II diabetes or NIDDM accounts for 90 to 95% of all diabetic cases (Taylor *et al.*, 1999). Affected individuals typically exhibit symptoms at approximately 40 years of age, however, a rare autosomal dominant form that develops before the age of 25, designated maturity onset diabetes of the young (MODY), has also been described (Tattersall, 1974). The prevalent form of NIDDM is characterized by two defects: i) decreased insulin sensitivity in peripheral insulin sensitive tissues and ii) dysregulated insulin secretion (Kahn, 1996). The high concordance rate observed in twins, which ranges from 50 to 100% (Gottlieb and Root, 1968; Barnett *et al.*, 1981; Newman *et al.*, 1987), and the higher incidence of NIDDM in offspring of diabetic parents (Gottlieb, 1980; Knowler, 1990) indicate that genetic factors contribute to the disease. Furthermore, the complex mode of inheritance observed in families affected with NIDDM, and its association with several factors including diet, weight, physical activity, and age suggest that the prevalent form NIDDM is a polygenic, multifactorial disease (Kahn, 1996).

## *1.2 Insulin*

Fredrick Banting, working with Charles Best first discovered and isolated the protein insulin, which was subsequently shown to have a role in glucose metabolism and

diabetes (Banting and Best, 1922). Insulin is a 5.7 kDa protein composed of two polypeptide chains, A and B, which are linked by two disulfide bonds. It is produced by the pancreatic ß-cells from a single genetic locus (Bell, 1980) encoding the pre-proinsulin polypeptide. Pre-proinsulin consists of a signal peptide, and the A, B, and C chains. The signal peptide directs pre-proinsulin to the vesicular transport system where it is processed to its mature form (Lehninger *et al.*, 1992). In response to elevated blood glucose, insulin is released from the ß-cells into the bloodstream, where it migrates to insulin sensitive tissues directing them to activate specific cellular responses.

### *1.3 Insulin induces cellular events in tissues*

In humans, the three major insulin sensitive tissues include skeletal muscle tissue, adipose tissue and liver tissue. These tissues, along with the pancreas, play an important role in glucose storage and utilization. In response to glucose, insulin is released from the pancreas into the bloodstream and subsequently stimulates several tissue dependent cellular responses. Glucose uptake, glycogen synthesis, and lipogenesis are distinct metabolic endpoints of the insulin signal, however, other responses including cell growth, amino acid/ion transport, and cell survival are also regulated by insulin (Yenush and White, 1997: Lamothe *et al.*, 1998). Some tissue specific cellular events regulated by insulin are listed in Table 1.

**Table 1: Cellular events regulated by insulin (Lamothe et al., 1998)**

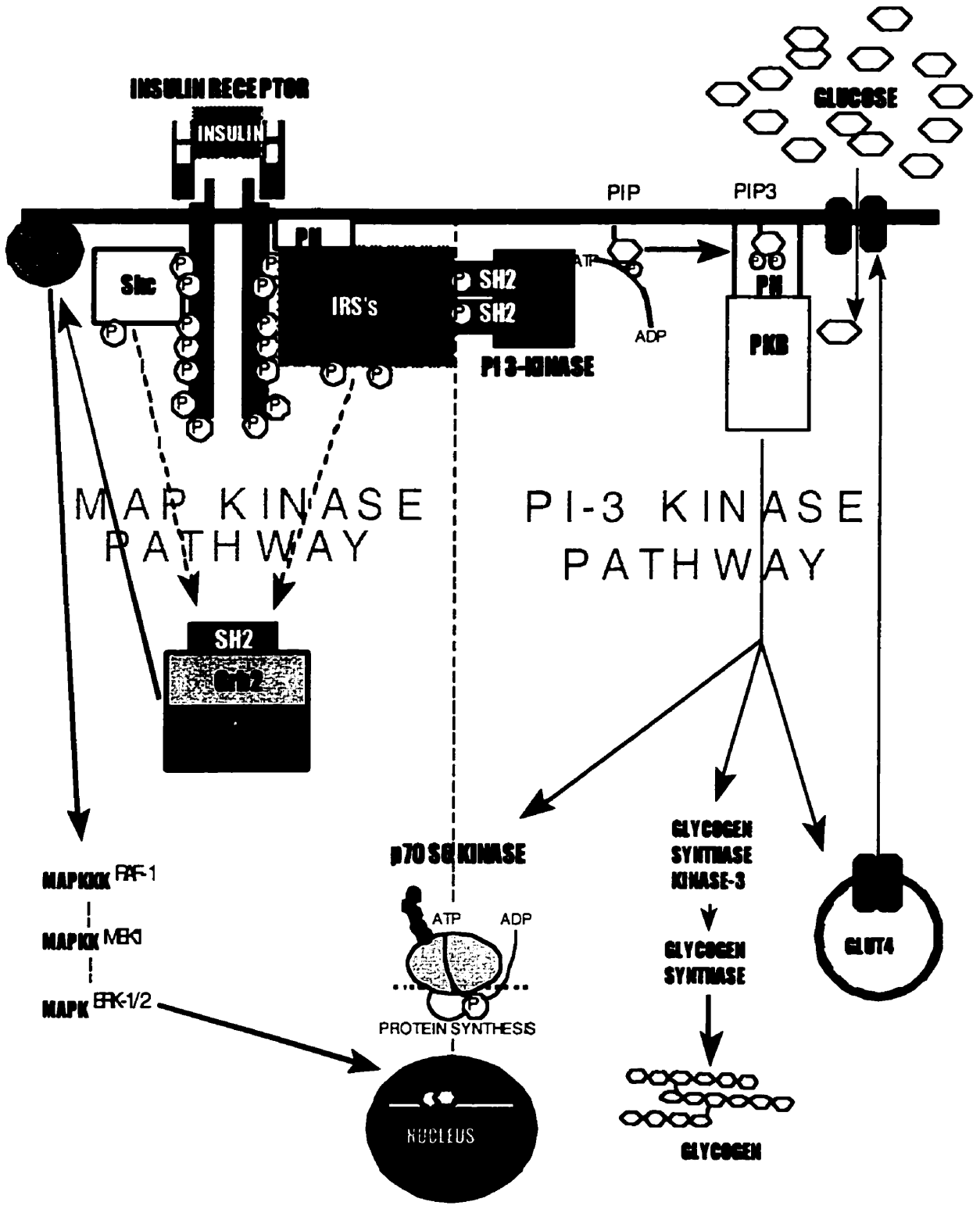
| Insulin induced Cellular Event | All Cells | Muscle | Adipose | Liver |
|---|---|---|---|---|
| Activation/inhibition of enzymatic activities | ⊗ | | | |
| Activation/inhibition of transcription | ⊗ | | | |
| Stimulation of Glucose uptake | | ⊗ | ⊗ | |
| Stimulation of Glucose incorporation to glycogen | | ⊗ | | ⊗ |
| Activation of lipogenesis | | | ⊗ | ⊗ |
| Activation of the Na+/K+ pump | | ⊗ | ⊗ | |
| Stimulation of amino acid uptake | ⊗ | | | |
| Stimulation of protein synthesis | ⊗ | | | |
| Induction into S-phase and cell growth | ⊗ | | | |
| Inhibition of apoptosis | ⊗ | | | |

### *1.4 The insulin signaling pathway*

Insulin alters cellular behavior in insulin sensitive tissues using a complex network of proteins (Figure 1). The extracellular signal is transmitted across the plasma membrane to a group of intracellular proteins that regulate both mitogenic and metabolic responses. The intracellular insulin signal begins after insulin binds to the extracellular domain of the insulin receptor (IR). This union activates the insulin receptor's intracellular tyrosine kinase resulting in the autophosphorylation of specific tyrosine residues found on the cytoplasmic segment of IR. The phosphorylated insulin receptor binds to, and phosphorylates, cytoplasmic proteins that activate, through the Ras protein, a signaling cascade called the MAP (mitogen activated protein) kinase pathway. Two proteins that have been shown to activate this pathway include Irs1 and Shc. Furthermore, Irs proteins can also initiate metabolic responses through the PI3–kinase (phosphatidylinositol 3-kinase) pathway. Lipid products produced by this pathway are essential second messengers for Protein Kinase B (Pkb/Akt), a protein that can target proteins involved in glucose-uptake, glycogen synthesis, and protein synthesis.

**Figure 1: Cellular events regulated by insulin**

Insulin binds to the extracellular domain of the IR activating its intracellular tyrosine kinase and initiating IR autophosphorylation. Tyrosine phosphorylated (P) insulin receptors recruit and phosphorylate Irs proteins and Shc, which act as docking sites for SH2 domain containing proteins including Grb2 and PI–3 kinase (PI-3K). The GTP exchanger Sos, which is constitutively bound to the Grb2 protein, associates with, and activates Ras, a potent activator of the MAP kinase pathway. Initiation of the MAP kinase pathway stimulates a sequential phosphorylation cascade that includes the proteins Raf1, Mek1, and Erk1/2. One endpoint for this pathway is the nucleus, where Erk1/2 activation has been shown to regulate gene expression. In response to IR phosphorylation, the PI-3 kinase pathway, stimulated by the binding of PI-3K to tyrosine phosphorylated Irs proteins, phosphorylate phosphatidylinositol phospholipids (PIP) at the 3' position of the inositol ring. PI-3K lipid products (PIP3) are secondary messengers essential for Pkb activation. The active Pkb has been shown to regulate several proteins including glycogen synthase kinase-3, Glut4, and $p70^{s6\ kinase}$, which are involved in glycogen synthesis, glucose-uptake, and protein synthesis respectively.


INSULIN RECEPTOR
INSULIN
GLUCOSE
PIP
PIP3
Shc
IRS's
SH2
SH2
PI 3-KINASE
ATP
ADP
PH
PKB
MAP KINASE PATHWAY
PI-3 KINASE PATHWAY
SH2
MAPKKK RAF-1
MAPKK MEK1
MAPK ERK-1/2
p70 S6 KINASE
ATP
ADP
P
PROTEIN SYNTHESIS
NUCLEUS
GLYCOGEN SYNTHASE KINASE-3
GLYCOGEN SYNTHASE
GLYCOGEN
GLUT4

### *1.4.1 The insulin receptor*

The insulin signal begins when insulin binds to the extracellular domain of the insulin receptor (IR). The insulin receptor, illustrated in Figure 2, is a hetero-tetrameric transmembrane glycoprotein protein that contains an intracellular tyrosine kinase. The IR heterotetramer ($\alpha_2ß_2$) is composed of two extracellular 135 kDa α-subunits (Herrera *et al.*, 1988; Shoelson *et al.*, 1988) that are linked by disulfide bonds to each other and to two 95 kDa ß-subunits (Roth and Cassell, 1983; Shia and Pilch, 1983; Petruzzelli *et al.*, 1984). The α-subunit, which binds to insulin, acts to inhibit the activity of the tyrosine kinase (Herrera *et al.*, 1988; Shoelson *et al.*, 1988). The binding of insulin to the α–subunits causes a conformational change in the IR, eliminating the inhibitory effects imposed by the α-subunits resulting in tyrosine kinase activity (Herrera *et al.*, 1988; Shoelson *et al.*, 1988).

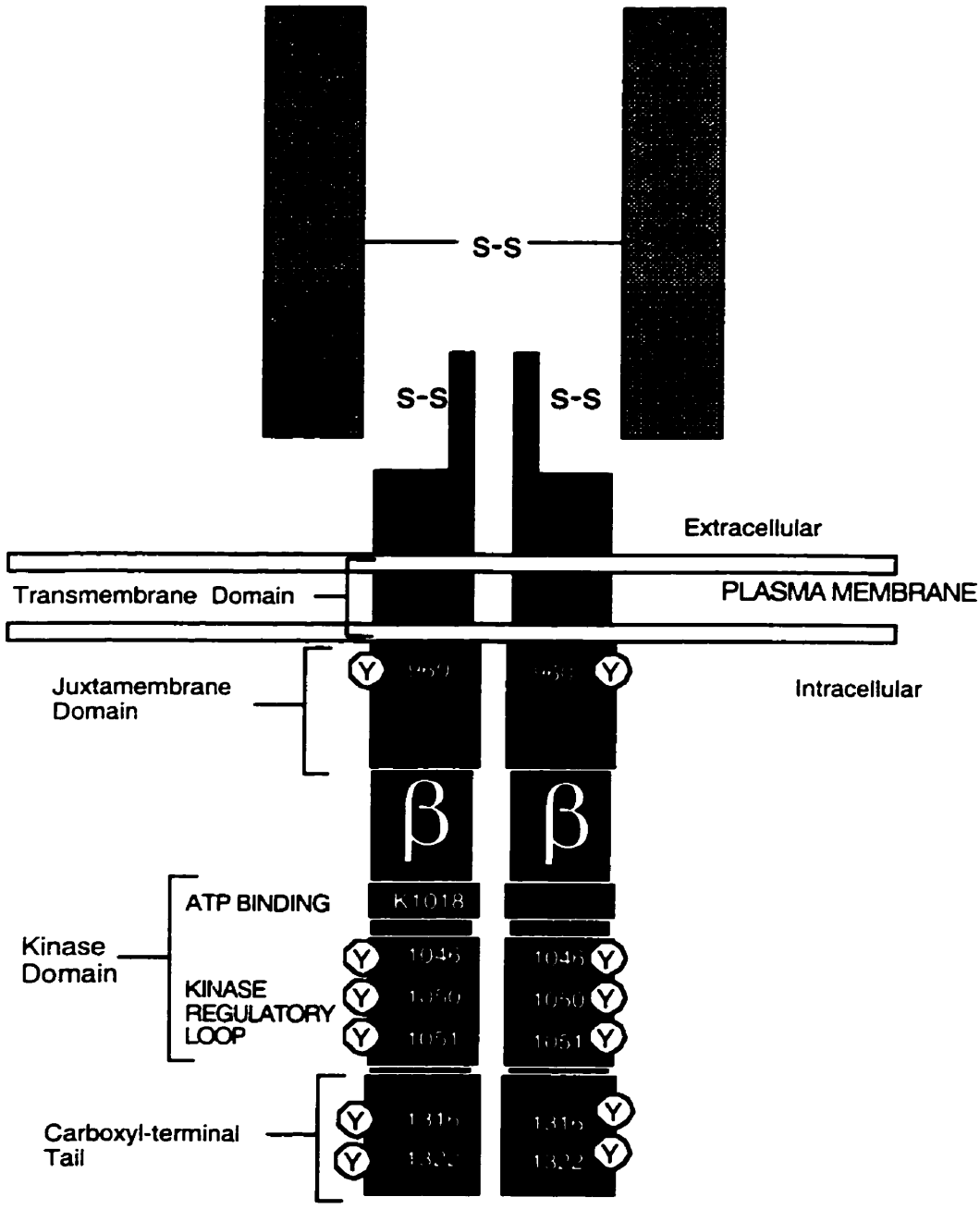
The insulin receptor's ß-subunit can be subdivided into: (i) the extracellular region, which links the ß-subunit to the α-subunit, (ii) the transmembrane domain, (iii) the juxtamembrane domain, which is involved signal transmission (White *et al.*, 1988; Kaburagi *et al.*, 1993), (iv) the tyrosine kinase domain, and (v) the carboxyl-terminal tail.

The activation of the intracellular tyrosine kinase is dependent on specific amino acid residues located within the kinase domain. These residues include the amino acid $K_{1018}$, which is essential for ATP binding, and $Y_{1046}$, $Y_{1050}$, and $Y_{1051}$ of the kinase regulatory loop, which augments kinase activity when phosphorylated (White *et al.*, 1988; Vogt *et al.*, 1991; Wilden *et al.*, 1993; Hubbard *et al.*, 1994).

The insulin receptor can modify its own tyrosine residues through a process called autophosphorylation. IR autophosphorylation occurs through a trans-mechanism whereby one of the ß-subunits from the heterotetramer phosphorylates the adjacent ß–subunit (Lee *et al.*, 1993). Autophosphorylation occurs on specific tyrosine residues that include: $Y_{960}$ of the juxtamembrane domain (Feener *et al.*, 1993), $Y_{1146}$, $Y_{1150}$, and $Y_{1151}$ of the kinase regulatory loop (Tavare *et al.*, 1988; White *et al.*, 1988; Wilden *et al.*, 1992), and $Y_{1316}$ and $Y_{1322}$ of the carboxy terminal tail (Herrera and Rosen, 1986; Tavare *et al.*, 1988). The phosphorylated insulin receptor binds to and phosphorylates cellular proteins that include Irs1 and Shc. When phosphorylated, both proteins act as docking sites for SH2 domain containing proteins that regulate signaling pathways essential for insulin action.

**Figure 2: The insulin receptor**

The insulin receptor is a heterotetrameric protein consisting of two α-subunits (grey) covalently linked by disulfide bonds (s-s) to each other and a ß-subunit (black). The extracellular α-subunits bind insulin. The ß-subunit consists of a short extracellular region, a transmembrane domain, and an intracellular domain that exhibits kinase activity. The intracellular domain can be subdivided into the juxtamembrane domain, the kinase domain, and the carboxyl-terminal tail. The amino acid residues K1018, Y1046, Y1050, and Y1051 are important for kinase activity. Several intracellular tyrosine residues (Y) undergo autophosphorylation. These include: Y960, Y1046, Y1050, Y1051, Y1316, and Y1322.


S-S
S-S
S-S
Extracellular
Transmembrane Domain
PLASMA MEMBRANE
Juxtamembrane Domain
Intracellular
β
β
ATP BINDING
K1018
Kinase Domain
KINASE REGULATORY LOOP
1046
1050
1051
Carboxyl-terminal Tail
1316
1322

### *1.4.2 Insulin receptor substrates (Irs)*

The most prominent group of IR binding proteins are the insulin receptor substrates. Irs1, a member of this group, was the first substrate identified for the insulin receptor (Sun *et al.*, 1991). Shortly after the cloning of Irs1, several Irs1–like proteins were identified and appropriately named Irs2 (Sun *et al.*, 1995), Irs3 (Sciacchitano *et al.* 1997; Lavan *et al.*, 1997a), and Irs4 (Lavan *et al.*, 1997b; Fantin *et al.*, 1999).

Irs proteins contain several domains and motifs involved in mediating biological interactions (Figure 3). Two domains found in all known Irs proteins include the phosphotyrosine binding domain (PTB) and a pleckstrin homology (PH) domain (Wolf *et al.*, 1995; Sun *et al.*, 1995; Sun *et al.*, 1995; Sciacchitano *et al.* 1997; Lavan *et al.*, 1997a; Lavan *et al.*, 1997b; Fantin *et al.*, 1999). These domains are believed to mediate protein:protein or protein:lipid interactions respectively (Myers *et al.*, 1995; Voliovitch *et al.*, 1995; Lemmon *et al.*, 1996). A third domain, found in Irs2, called the KRLB (kinase regulatory loop binding) domain, binds to the phosphorylated IR kinase regulatory loop (He *et al.*, 1996). Irs proteins also carry several carboxyl-terminal tyrosines that are substrates for receptor tyrosine kinases (Yenush and White, 1997).

Tyrosine phosphorylated Irs proteins act as docking sites for proteins containing phosphotyrosine binding domains called Src Homology-2 (SH2) domains. These interactions couple IR activation with signaling proteins that include Grb2, PI 3-Kinase, and Shp2 (Backer *et al.*, 1992; Myers *et al.*, 1994; Kuhne *et al.*, 1993) which are involved in regulating cellular responses. Two pathways downstream of Irs proteins, are the Map Kinase pathway and the phosphatidylinositol-3 kinase (PI-3 kinase) pathway. The MAP kinase pathway is also activated by another substrate of the insulin receptor called Shc.

**Figure 3: Structural characteristics of Irs1 and Irs2**

Two domains common to Irs proteins, shown here in Irs1 and Irs2, include an amino terminal Pleckstrin homology (PH) domain and a Phosphotyrosine binding domain (PTB). A third domain found in Irs2 is the kinase regulatory loop binding (KRLB) domain. Vertical lines indicate tyrosine residues that can be phosphorylated by receptor tyrosine kinases, including the IR.

IRS1

IRS2

### *1.4.3 The Shc protein binds to the insulin receptor*

In addition to Irs proteins, the Shc (Src homologous and collagen) protein has also been shown to be tyrosine phosphorylated by the IR in response to insulin (Pronk *et al.*, 1992; Kovacina and Roth, 1993). Two isoforms of Shc, with predicted molecular masses of 46.8 kDa and 51.7 kDa, have been identified (Pelicci *et al.*, 1992). Shc consists of an amino-terminal PTB domain, a carboxyl-terminal SH2 domain, a proline rich motif and several tyrosine phosphorylation sites (Pelicci *et al.*, 1992; Gustafson *et al.*, 1995). Shc, like Irs1, is a docking site for the Grb2 protein (Ravichandran *et al.*, 1995; Páez-Espinosa *et al.*, 1998), and has been proposed to be the major activator of the Map kinase pathway (White and Yenush, 1998).

### *1.4.4 The MAP kinase pathway is activated by insulin*

Insulin activates a three-component signaling cascade called the mitogen–activated protein (MAP) kinase pathway. The activation of the MAP kinase pathway, illustrated in Figure 1, occurs when Grb2 binds to tyrosine phosphorylated Irs1 or Shc (Skolnik *et al.*, 1992a; Skolnik *et al.*, 1992b; Egan *et al.*, 1993). This association has been shown to activate the GTP-exchanger Sos, which subsequently stimulates the GTPase Ras (Rozakis-Adcock *et al.*, 1992), a potent activator of the Map Kinase pathway (Marais *et al.*, 1995).

MAP kinases belong to a family of serine/threonine kinases that are primarily involved in regulating cellular events through gene expression (Haruta *et al.*, 1995; Azpiazu *et al.*, 1996; Widman *et al.*, 1999). The Map kinase pathway involves the sequential activation of three kinases generically named: (i) Map kinase kinase kinase (Mapkkk), (ii) Map kinase kinase (Mapkk), and (iii) Map kinase (Mapk). The first

protein in this cascade, Mapkkk, is stimulated by Ras activation (Widman *et al.*, 1999). Several Mapkkk have been described including: Raf1 (Bonner *et al.*, 1986), Mekk2/3 (Blank *et al.*, 1996) and Mst (Dorow *et al.*, 1995). One of the most responsive Mapkkk in the insulin signaling pathway is the Raf1 protein (Lee *et al.*, 1991; Koide *et al.*, 1993; Schaap *et al.*, 1993). Raf1 has been shown to phosphorylate Mek1 (Crews *et al.*, 1992). The activation of Mek1 is followed by phosphorylation of Erk1 or Erk2 (Gonzalez *et al.*, 1992; Charest *et al.*, 1993), two Mapk proteins that phosphorylate cytoplasmic and nuclear proteins (Widmann *et al.*, 1999).

### *1.4.5 PI3-kinase in insulin signaling*

The insulin signal is also transduced into the phosphatidylinositol-3 kinase (PI-3 kinase) pathway through Irs proteins. PI-3 kinase is a cytoplasmic heterodimer consisting of a p85 adapter subunit and a p110 kinase subunit (Escobedo *et al.*, 1991; Otsu *et al.*, 1991; Skolnik *et al.*, 1991; Hiles *et al.*, 1992). Activation of the p110 kinase occurs when both SH2 domains of the p85 subunit bind to tyrosine phosphorylated Irs1 or Irs2 (Backer *et al.*, 1992; Yonezawa *et al.*, 1992; Yamada *et al.*, 1997). The active p110 kinase phosphorylates the hydroxyl group at position 3 on the inositol ring of phosphatidylinositol phospholipids (Carpenter and Cantley, 1990; Fry and Waterfield, 1993). The lipid products produced by PI 3-kinase are known to stimulate the Pkb (Protein Kinase B; Akt; Rac-pk) protein (Downward, 1998). When activated, Pkb has been shown to regulate various cellular responses including glycogen synthesis (Cross *et al.*, 1995; Cohen *et al.*, 1997), Glut4 mediated glucose-uptake (Kanai *et al.*, 1993) and p70 $^{S6\ kinase}$ regulated protein synthesis (Somwar *et al.*, 1998).

### *1.5 Alternative insulin receptor binding partners*

In addition to Irs proteins and Shc, several other proteins have been shown to bind to the activated IR. These proteins include Gab1 (Holgado-Madruga *et al.*, 1996), Dos (Raabe *et al.*, 1996), Enigma (Wu *et al.*, 1996) Dok (Yamanashi and Baltimore, 1997), PSM (Riedel *et al.*, 1997), SH2B-α (Kotani *et al*, 1998), and APS (Moodie *et al.*, 1999). Interestingly, two adaptor proteins structurally similar to PSM, SH2B-α and APS, called Grb10 and Grb14, have also been shown to interact with the activated IR (Lui and Roth, 1995; Kasus-Jacobi *et al.*, 1998). To date, the role of Grb10 and Grb14 in insulin signaling is unclear. Clarifying the role of these proteins in insulin signaling will help researchers understand insulin's effect on the cell.

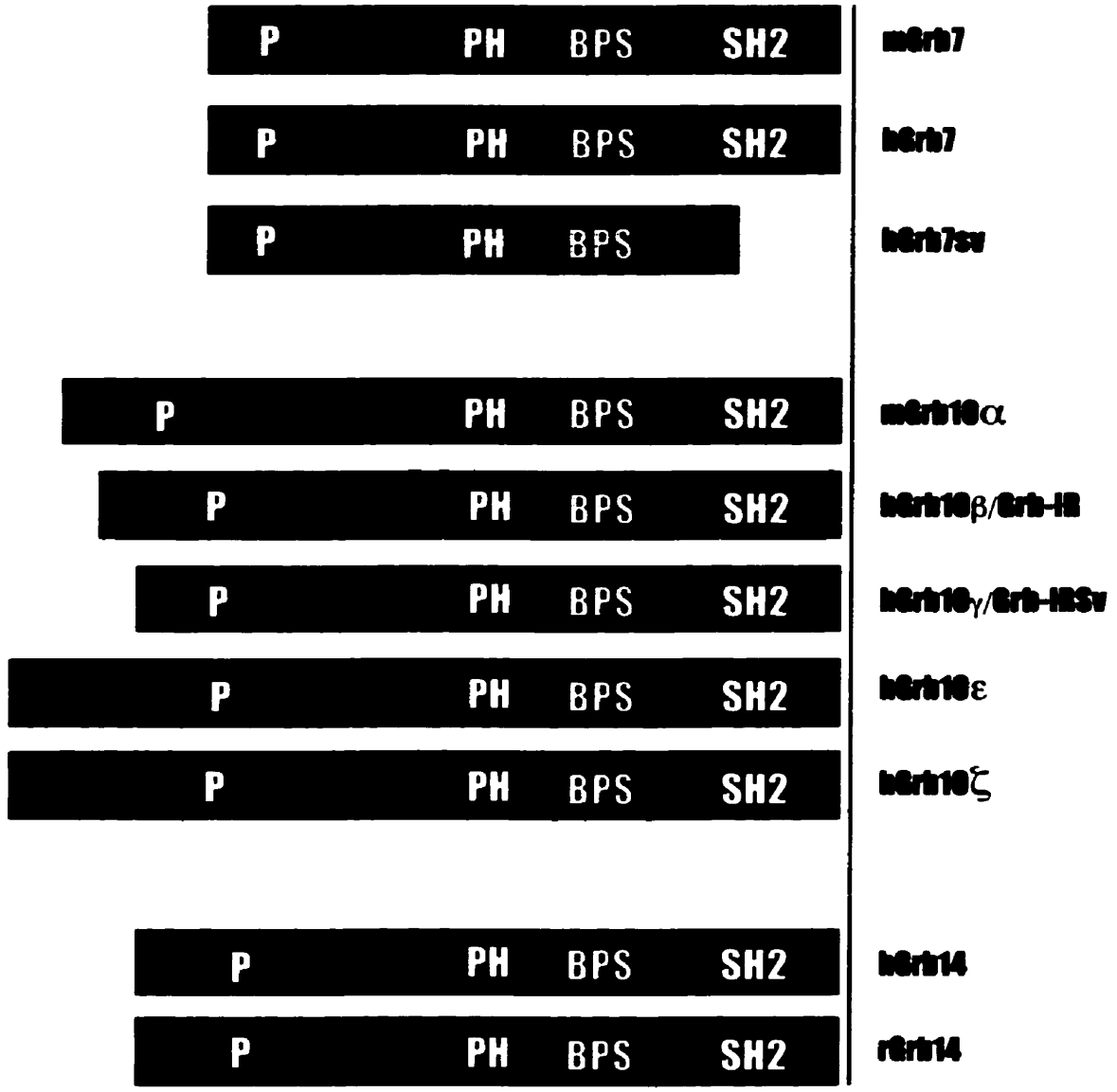
### *1.6 The Grb7 family of adapter proteins*

Recently, a new family of growth factor receptor binding proteins called the Grb7 (growth factor receptor bound-7) protein family has emerged. Currently, there are 3 members in this protein family: Grb7, Grb10, and Grb14. The Grb7 family members do not appear to exhibit enzymatic activity and are thus believed to act as adaptor proteins (Daly, 1998). All members in this family contain protein modules known to mediate protein:protein or protein:lipid interactions. These modules include an amino-terminal proline rich motif (PS/AIPNPFPEL), an internal PH (pleckstrin homology) domain, a BPS domain (Between Pleckstrin and Src Homology), and a carboxyl-terminal SH2 (Src Homology 2) domain (see Figure 4). To date, only Grb10 and Grb14 have been shown to interact with the activated IR (Lui and Roth, 1995; Kasus-Jacobi *et al.*, 1998) suggesting that both proteins participate in insulin signaling.

**Figure 4: The Grb7 protein family**

The structure of several Grb7 family members is illustrated. The members of this protein family carry several conserved sequences that encode an amino-terminal proline-rich motif (P), an internal domain PH (pleckstrin homology), a Between PH and SH2 (BPS) domain, and a SH2 (Src homology-2) domain. Several variants resulting from alternative splicing are also shown.

m=*Mus musculus*; r=*Rattus norvegicus;* h=*Homo sapien*


P PH BPS SH2
P PH BPS SH2
P PH BPS
P PH BPS SH2
P PH BPS SH2
P PH BPS SH2
P PH BPS SH2
P PH BPS SH2
P PH BPS SH2

### *1.6.1 Growth factor receptor bound-10 (Grb10)*

The Grb10 protein was first identified as a binding partner for the epidermal growth factor receptor (EGFR) using the CORT (cloning of receptor targets) methodology (Ooi *et al.*, 1995). The human homologue for Grb10, mapped to 7p11.2-7p12 (Jerome *et al.*, 1997), consists of 15 exons (Angrist *et al.*, 1998) that are alternatively spliced (Liu *et al.*, 1995; Ooi *et al.*, 1995; O'Neill *et al.*, 1996; Dong *et al.*, 1997; Frantz *et al.*, 1997; Laviola *et al.*, 1997).

Grb10 has been shown to associate with both cytoplasmic and membrane bound proteins. The membrane bound proteins are primarily receptors which include: EGFR (Ooi *et al.*, 1995), IR (Lui and Roth, 1995), ret receptor (Pandey *et al.*, 1995), insulin-like growth factor receptor-1 (Morrione *et al.*, 1996), Elk receptor (Stein *et al.*, 1996), and growth hormone receptor (Moutoussamy *et al.*, 1998). Grb10 can also bind to cytoplasmic proteins including Janus kinase (Moutoussamy *et al.*, 1998), Bcr-Abl (Bai *et al.*, 1998), and the Map kinase kinase kinases Raf1 and Mekk1 (Nantel *et al.*, 1998). In addition, recent data suggests that Grb10 may self-associate to form a tetrameric complex (Dong *et al.*, 1998).

The cellular role of Grb10 remains controversial. A report indicating that Grb10 overexpression has a positive stimulatory role in insulin signaling has been reported (Wang *et al.* 1999). However, several lines of evidence, determined in cells

overexpressing Grb10, also suggest that Grb10 may be involved in down-regulating the insulin signal.

Specific effects of hGrb10 α/Grb-IR overexpression include reduced tyrosine phosphorylation of Irs1 and $pp60^{GAP}$, reduced PI–3 kinase activity (Lui and Roth, 1995), limited c-fos phosphorylation by the Tec kinase (Mano *et al.*, 1998), and decreased enzyme activity from proteins encoded by genes regulated by the c-fos or the GH response element-2 (Moutoussamy *et al.*, 1998). Similarly, when the Grb10 BPS domain is overexpressed, it inhibits insulin stimulated mitogenesis (He *et al.*, 1998). Interestingly, other evidence suggests that Grb10 may also be involved in apoptosis. Nantel *et al.* (1998) has shown that the injection of a hGRB10ς mutant protein into HTC–IR and COS-7 cells induced apoptosis in these cells.

### *1.6.2 Growth factor receptor bound-14 (Grb14)*

The Grb14 protein is another member of the Grb7 protein family. It was initially identified by screening a human epithelial breast cDNA library with the carboxyl–terminus of the EGF receptor using CORT methodology (Daly *et al.*, 1996). The *GRB14* gene maps to 2q22-24 (Baker *et al.*, 1996) and encodes a 540 amino acid protein with a predicted molecular weight of 60.9 kDa. Phosphoamino acid analysis of human Grb14 (hGrb14) indicates that it is weakly phosphorylated on serine residues in the absence EGF stimulation (Daly *et al.*, 1996). Furthermore, Hemming *et al.*

(unpublished data) have shown that hGrb14 interacts directly with the activated insulin receptor in the two-hybrid system and in CHO-IR cells overexpressing hGrb14 tagged with the FLAG epitope. This result was verified with the rat homologue of Grb14 (rGrb14) which was also shown to bind the IR *in vivo* (Kasus-Jacobi, 1998). In the latter study, rGrb14 overexpression decreased both thymidine incorporation during DNA synthesis and glycogen production (Kasus-Jacobi, 1998). To date, the cellular function of hGrb14 in insulin signaling is unclear. Identifying binding partners for the hGrb14 protein with the aid of the two-hybrid system (Figure 5) may aid in defining its role in this signaling pathway.

### *1.7 Thesis objectives*

The objective of this project was to utilize the two-hybrid system (Fields and Song, 1989), co-expressing a constitutively active ß-IR subunit in the cellular background, to identify human Grb14 interacting proteins (GIP). This thesis describes the characterization of one such protein, Tax1bp1, and its interaction with hGrb14 in the yeast two-hybrid system and a mammalian cell system.

**Figure 5: The two-hybrid and modified two-hybrid systems**

(A) *The yeast two-hybrid system.* The yeast two-hybrid system is a cellular assay used to study protein:protein interactions. This system is based on two hybrid proteins: one consisting of a DNA binding domain (BD) fused to a protein of interest (X), and the other consisting of a transcriptional activation domain (AD) fused to a second protein of interest (Y). The resulting fusion proteins are BD:X and AD:Y. The BD binds to promoter sequences. The AD can activate transcription when it is localized to a promoter sequence. When protein X and Y do not interact, transcription of the reporter gene is not detected because the AD does not localize to the promoter. However, when X and Y interact, the localization of the transcriptional activating domain (AD) to the promoter stimulates the transcription of a reporter gene (indicated by the arrow) that can be detected phenotypically. The two reporter genes used in this system are the *lacZ* and the *HIS3* reporter genes. The activation of the reporter genes are detected chromogenically using the compound X-Gal (5-bromo-4-chloro-3-indoyl-ß-D-galactoside) or by growth on a medium lacking histidine respectively.

(B) *A modified two-hybrid screen.* The modified two-hybrid system, designed to detect phosphotyrosine dependent interactions, expresses the intracellular portion of the IR (ß–IR), fused to the $lexA_{(aa1-202)}$ protein. To screen for possible interacting proteins, a library of *AD:FUSION* genes is first constructed by fusing a library of cDNA sequences (here numbered 1-6) to the AD. The resulting hybrid proteins are tested against the BD:X hybrid protein (bait protein) for reporter gene activation. Any AD:hybrid clones activating the reporter gene(s) are potential interacting partners. A $Gal4_{BD}$:$hGrb14_{(aa1-540)}$ hybrid protein was utilized to screen an adult human liver cDNA library with the lexA:ßIR $_{(aa941-1343)}$ hybrid protein expressed in the background.

**A) Two-Hybrid System**


**B) Modified Two-Hybrid system**

# 2. MATERIALS AND METHODS

## *2.1 Plasmids*

Plasmids not created in this project are listed in Table 2.

**Table 2: Plasmids**

| Plasmid Name | Source | Reference |
|---|---|---|
| pBTM116A | R.D. Gietz | Bartel *et al.*, 1993 |
| pGBT9B | R.D. Gietz | Bartel *et al.*, 1993 |
| PACTIIC | R.D. Gietz | Durfee *et al.*, 1993 |
| pDB169 | R.D. Gietz /B.Triggs-Raine | Hemming *et al.*, unpublished results |
| pDB202 | R.D. Gietz /B.Triggs-Raine | Hemming *et al.*, unpublished results |
| pcDNA3 | B.Triggs-Raine | Invitrogen |
| pBSK-TAX1BP1 | K.T. Jeang | |
| pBTR406 | R.D. Gietz /B.Triggs-Raine | Hemming *et al.*, unpublished results |

[A,B,C] are illustrated in Figure 6.

## *2.2 Bacterial strains*

The bacterial strains utilized for this project are listed in Table 3.

**Table 3: *E.coli* Strains**

| Designation | Genotype |
|---|---|
| DH5α | F-, *recA*1, *endA*1,*gyrA*96,*thi*, *hsdR*17, *supE*44, *relA*1, Δ(*arg lacZYA*) U169 (ϕ80d*lacZ*ΔM15)λ- |
| GM2163 | F- *dam*-13::*Tn9 dcm*-6 *hsd*R2 *leu*B6 *his*-4 *thi*-1 *ara*-14 *lacY*1 *galK2 galT*22 *xyl*-5 *mtl*-1 *rps*L136 *ton*A31 *tsx*-78 *supE*44 McrA- McrB- |
| KC8 | *hsd*R, *leu*B600, *trp*C9830, *pyrF*::Tn5, *his*B463, *lacD*X74, *strA*, *gal*U,K |

### *2.3 Two-hybrid yeast strains*

The two-hybrid strains utilized for this project are listed in Table 4.

**Table 4: *S. cerevisiae* Strains**

| Strain | Genotype | Source |
|---|---|---|
| KGY37 | MATa ade2 gal4 gal80 his3-Δ200 trp1-Δ901, leu2::pUC18 ura3::GAL1-lacZ, lys2::UASG 17 MERS(x3) GAL1-HIS3 | K.C. Graham, 1996 |
| KGY94 | MATa, ade2, trp1-Δ901,leu2-3.112, his3-Δ200,gal80,gal4 ura3-52, leu2::pUC18 URA3::GAL1-lacZ lys2::lexAop(x3) GAL1-HIS3 | K.C. Graham, 1996 |

### *2.4 Genetically modified Chinese Hamster Ovary (CHO) Cell lines*

Cell lines utilized in this study are listed in Table 5.

**Table 5: Cell lines**

| Designation | Tissue of Origin | Overexpressed Proteins | Source |
|---|---|---|---|
| CHO-IR | Chinese Hamster Ovary | Human IR | R. Roth |
| CHO26 | Chinese Hamster Ovary | Human IR /Human Grb14:FLAG | B.Triggs-Raine |

### *2.5 Antibodies*

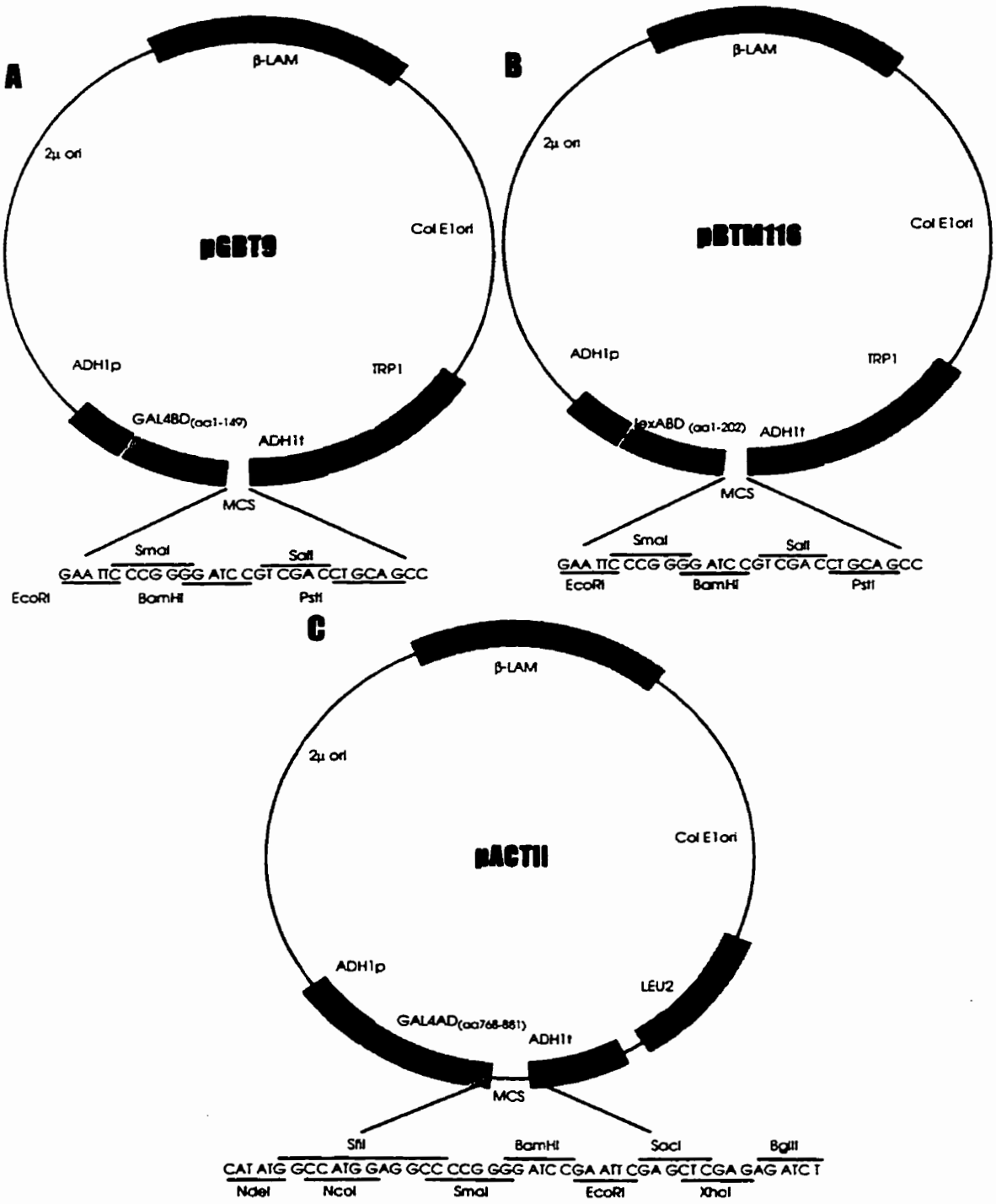
Antibodies utilized in this study are listed in Table 6.

**Table 6: Antibodies**

| Name | Type | Recognition sequence | Source |
|---|---|---|---|
| Anti-Tax1bp1 | polyclonal | DYDQQVFERHVQTHFDQNVLNFD | K.T Jeang |
| M2 Anti-FLAG | monoclonal | DYKDDDDK | Kodak |

**Figure 6: Two-hybrid plasmids**

The plasmids: (A) pGBT9, (B) pBTM116 (C) pACT II are illustrated. Each shuttle vector carries a bacterial origin of replication (Col E1 ORI), the ampicillin resistance gene ß-lactamase (ß-LAM), the yeast alcohol dehydrogenase promoter (ADH1p) and terminator (ADH1t), and a 2μ-circle origin of replication. The plasmids pGBT9 and pBTM116 both carry the yeast *TRP1* gene, which is used to complement yeast strains carrying a *trp1* mutation. The vectors pGTB9 and pBTM116 also carry the $Gal4_{(aa1-149)}$ or the $lexA_{(aa1-202)}$ DNA binding domains (BD), respectively. Both BD are flanked at the 3' end with a multiple cloning site (MCS). The vector pACTII utilizes the yeast *LEU2* gene for selection, and encodes the $Gal4_{(aa768-881)}$ transcriptional activation domain (AD). The restriction sites for each MCS are indicated below each vector. The nucleotide triplets found in the MCS correspond to the reading frame at the fusion junction in each vector. To construct fusion genes in these vectors, the reading frame of the gene of interest must coincide with the reading frame found in the MCS from each vector.


A
β-LAM
2μ ori
pGBT9
Col E1ori
ADH1p
TRP1
GAL4BD(aa1-147)
ADH1t
MCS
SmaI
SalI
GAA TTC CCG GGG ATC CGT CGA CCT GCA GCC
EcoRI
BamHI
PstI
B
β-LAM
2μ ori
pBTM116
Col E1ori
ADH1p
TRP1
LexABD (aa1-202)
ADH1t
MCS
SmaI
SalI
GAA TTC CCG GGG ATC CGT CGA CCT GCA GCC
EcoRI
BamHI
PstI
C
β-LAM
2μ ori
pACTII
Col E1ori
ADH1p
LEU2
GAL4AD(aa768-881)
ADH1t
MCS
SfiI
BamHI
SacI
BglII
CAT ATG GCC ATG GAG GCC CCG GGG ATC CGA ATT CGA GCT CGA GAG ATC T
NdeI
NcoI
SmaI
EcoRI
XhoI

### *2.6 Transformation of Saccharomyces cerevisiae*

Plasmid introduction into *S. cerevisiae* was performed using the method of Gietz *et al.* (1995). A 5ml volume of YPAD or 10 ml volume of synthetic complete omission medium (SC-) was inoculated with a single yeast colony and incubated overnight at 30 °C on a shaker cycling at 200 RPM. The following day, a 50 ml volume of fresh liquid YPAD was inoculated, using a sample of the overnight culture, to a final concentration of $5x10^6$ cells. The inoculum was then grown for approximately 3 hr to a final cell concentration of $2 \times 10^7$ cells/ml. Cells were then collected by centrifugation at 3000 x*g* for 5 min at room temperature. Cell pellets were resuspended in 25 mls of sterile Nanopure™ water, and harvested by centrifugation as described above. Following the removal of the water, cell pellets were pre-treated with 1 ml of 100 mM LiAc, collected by centrifugation at 13 000 x*g* for 15 s in a Biofuge A microcentrifuge, and resuspended to a final concentration of $2.0x10^9$ cells/ml with 100 mM LiAc. For each transformation, $1.0 \times 10^8$ cells was collected, from a 50 µl sample of the previous solution, by centrifugation. The cells were incubated in a solution containing 34% $PEG_{(Mr\ 3350)}$ (w/v), 0.1 M LiAc, 50 µg of denatured salmon sperm DNA in a final volume of 351 µl for 30 min at 30 °C and heat shocked for 20 min at 42°C. Cells treated with the transformation mixture were collected by centrifugation at 8000 RPM for 15 s using a Biofuge A microcentrifuge and resuspended in 1 ml of sterile Nanopure™ water. Samples of various sizes were plated onto the required SC- media and incubated at 30 °C for 2 to 4 days.

### *2.7 Library screen transformations*

The transformation protocol utilized for library screening was performed essentially as described in section 2.6. After determining the quantity of DNA providing the optimal transformation efficiency, a 120 x scaled-up high-efficiency transformation was performed. The cells were then plated onto SC- omission media lacking the amino acids histidine (H), leucine (L), tryptophan (T), and uracil (U) and containing 5 mM 3-amino-1,2,4-triazole or 3-AT (Sigma) and incubated for up to 7 days at 30°C. The 2 to 7 day old $His^+$ yeast colonies were then selected and streaked onto SC-H-L-T-U containing 5 mM 3-AT to reverify its $His^+$ phenotype. To estimate the total number of transformants screened samples of 1µl and 10µl were plated onto SC–T–L-U media.

### *2.8 Plasmid recovery from yeast*

Plasmid recovery from yeast cells was performed using the method of Hoffman and Winston (1987). A yeast colony harboring the desired plasmid was inoculated into 5 ml of SC–T-L-H media and grown for 16 to 18 hr at 30 °C with shaking at 200 RPM. The cells from the overnight culture were collected by centrifugation, and resuspended with 50 µl of yeast cracking buffer [2% (v/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 20 mM Tris-Cl (pH 8.0), 10 mM EDTA]. Cells were then mixed with an equal volume of 425 to 600 µm acid washed glass beads (Sigma) and 1 volume of phenol:chloroform. This solution was then mixed with a vortex for 30 s three times. The cell lysates were clarified by centrifugation at 4 °C for 2 min and ethanol precipitated

(section 2.12). The DNA solutions were used to transform *E.coli* for plasmid amplification.

## *2.9 Transformation E. coli*

### *2.9.1 Preparation of electrocompetent cells*

The preparation of electrocompetent *E. coli* was performed as described by Dower *et al.* (1988). Cells were prepared by growing a 1L culture of LB medium (see Appendix) inoculated with the strain of interest to an $OD_{600}$ between 0.5 and 1.0. The culture was equilibrated in an ice-water bath and harvested by centrifugation at 4000 x*g* for 15 min at 4°C. Cell pellets were resuspended in 1L of sterile ice-cold Nanopure™ water, collected as above, resuspended in 500 mls of sterile ice-cold Nanopure™ water and harvested as above. The cells were then resuspended in 20 ml ice-cold sterile 10% glycerol (v/v), again collected by centrifugation, and finally resuspended with 2 mls of 10% glycerol (v/v). The cells were aliquoted as 25 µl samples, frozen with liquid nitrogen, and stored at –80°C.

### *2.9.2 Electroporation*

Introduction of plasmid DNA into *E. coli* was performed by electroporation (Dower *et al.*, 1988). A frozen 25µl aliquot of electrocompetent cells was thawed on ice and mixed with 2 µl (up to 1 µg) of DNA. The DNA/cell mixture was then inserted into an ice-cold electroporation cuvette (BIO-RAD) with a 0.1 cm gap and pulsed using a

BIORAD Gene Pulser set at 1.25 kV, 25 μF, with a 400 Ω resistor in parallel to the sample. The pulse duration ranged from 7-9 ms. The cells were immediately resuspended in 1 ml of room temperature SOC medium (see Appendix) and incubated in a 37 °C waterbath for 30 min. Samples of 20 μl and 200 μl were spread onto LB plates (see Appendix) containing carbenicillin (20 μg/ml) and incubated at 37 °C for 12 to 16 hr.

### *2.9.3 Identification of KC8 E.coli colonies containing library plasmid DNA*

The library plasmids responsible for reporter gene activation were isolated from yeast and amplified in bacteria to determine the identity of the inserted cDNA. Plasmid DNA was isolated from the yeast cells (section 2.8), transformed into the *E. coli* strain KC8, which carries a mutated LeuB gene, by electroporation (section 2.9.2). The transformants were plated onto LB medium containing 20 μg/ml of carbenicillin and incubated at 37 °C for 15 to 18 hr. Carbenicillin resistant colonies were replica plated onto M9 medium containing all the essential amino acids except leucine, and grown at 37 °C for 15-18 hr to select for bacteria containing the pACTII derived library plasmid. Cells carrying the pACTII derived library plasmid can grow on this medium because these plasmids carry the *LEU2* gene, which can complement the *leuB* mutation present in the KC8 *E. coli* strain. The $Leu^+$ KC8 colonies were inoculated into 2 mls of LB containing 50 μg/ml of ampicillin and grown overnight for plasmid DNA extraction.

### *2.10 Isolation of plasmid DNA from E.coli*

Purification of plasmid DNA was accomplished using a modification of the method of Birnboim & Doly (1979). Two millilitres of LB medium containing ampicillin (50 μg/ml) was inoculated with an *E.coli* colony harboring the plasmid to be purified. The cells were incubated, with shaking, for 8 to 18 hr at 37 °C and collected by centrifugation at top speed for 1 min. The medium was then removed by aspiration. The cell pellet was then resuspended in 100 μl of TGE [10 mM Tris-Cl (pH 8.0), 20%(w/v) glucose, 1 mM EDTA]. Cells were lysed by mixing 200 μl of lysis buffer [1% (w/v) SDS, 0.2 N NaOH] with the previous solution and incubating the mixture for 5 min on ice. The cell lysate was neutralized by the addition of 150μl of 3M potassium acetate (pH 4.8) followed by an incubation on ice for 2.5 min. The tubes were centrifugated at 13 0000 x*g* for 5 min at 4 °C and the supernatant transferred to a fresh tube.

### *2.11 Phenol:chloroform extraction of DNA*

DNA preparations were purified by phenol:chloroform extraction. An equal volume of Tris-buffered phenol chloroform (1:1) was added to the DNA sample and mixed using a vortex for 30 seconds. The aqueous and organic phases were separated by centrifugation at 13 000 x*g* for 1 minute with a Biofuge A microcentrifuge. The aqueous phase was then transferred to a fresh tube. The DNA was precipitated from this solution using ethanol.

### *2.12 Ethanol precipitation*

DNA samples were concentrated by ethanol precipitation. The nucleic acids were precipitated by adding a 1/10 volume of 3.0 M sodium acetate (pH 6.0) and 2.5 volumes of absolute ethanol to the nucleic acid solution. The samples were mixed and incubated at –20 °C for at least 1 hr. The nucleic acid precipitate was collected by centrifugation at 13 000 x*g* for 5 minutes at 4°C. The pellet was rinsed with 150 µl of 70% ethanol, dried for 15 min at room temperature, and resuspended in an appropriate volume of TE [10 mM Tris-Cl (pH 8.0), 1 mM EDTA].

### *2.13 DNA precipitation with Polyethylene Glycol (PEG)*

RNA oligonucleotides were removed from DNA solutions with polyethylene glycol essentially as described in Sambrook *et al.* (1989). A 50µl sample was treated with 33 µl of $PEG_{Mr\ 8000}$/NaCl solution [20 % (w/v) polyethylene glycol (Mr 8000), 5 M NaCl] and incubated on ice-water for 1 hr. The precipitate was collected by centrifugation at 13 000 x*g* at 4 °C for 5 min. The pellet was rinsed with 100 µl of room temperature 70% ethanol, dried for 30 min at room temperature, and resuspended in 75 µl of TE. The DNA sample was then purified by phenol extraction (section 2.11) and concentrated by ethanol precipitation (section 2.12).

### *2.14 DNA sequencing*

DNA sequencing was performed with the T7 Sequenase version 2.0 sequencing kit (Amersham), which uses a modification of method of Sanger *et al.* (1977). Fifty microlitres (5-10 μg) of plasmid DNA was treated with 3 μl of Ribonuclease A (1mg/ml) (Sigma) for 30 min at 37 °C. The sample was then treated with PEG as described in section 2.13. The DNA was collected by centrifugation at 13 000 x*g* for 5 minutes at 4 °C, washed with 70% ethanol, dried for 30 min at room temperature, and resuspended in 20μl of TE [10 mM Tris-Cl (pH 8.0), 1mM EDTA].

Purified plasmid DNA was denatured by the addition of 18 μl of DNA to 2 μl of 2 N NaOH and incubating the solution for 5 min at room temperature. The sample was neutralized with the addition of 8 μl of 5 M ammonium acetate and then precipitated with the addition of 100 μl of absolute ethanol. The sample was incubated in ethanol for at least 1 hr at –20 °C and collected by centrifugation at 13 000 x*g* for 5 min at 4 °C. The pellet was washed with 150 μl of 70% ethanol, air dried for 30 minutes, and dissolved in 7 μl of sterile Nanopure™ water. Six nanograms of sequencing primer and 2 μl of 5x Sequenase reaction buffer [200mM Tris-Cl (pH 7.5), 100 mM $MgCl_2$, 250 mM NaCl] were added to the denatured template. The primer was annealed to the template at 65 °C for 15 min and slowly cooled to room temperature.

Primer extension was initiated by adding 1 μl of 1.0 M DTT, 2 μl of 5x dGTP labeling mix [7.5 μM dCTP, 7.5 μM dGTP, 7.5 μM dTTP], 0.5 μl of [$\alpha$ $S^{35}$] dATP (1000-1500 Ci/mmol), and 2.0 μl (26 U) of T7 polymerase (Sequenase 2.0, Amersham).

The reaction was allowed to proceed at room temperature for 2.5 min. Dideoxynucleotide mediated termination was initiated by mixing 3.5 µl of the above reaction mixture with 2.5 µl of a solution containing one of the four termination nucleotides (ddATP, ddCTP, ddGTP, ddTTP), previously aliquoted in separate tubes. Each reaction was incubated for 5 min at 37 °C and terminated with 4 µl of Stop solution (95% formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF). The samples were either stored at -20°C or prepared for electrophoresis.

DNA sequencing products were separated in denaturing acrylamide gels (6% (19:1) acrylamide, 7 M urea, 0.089 M Tris-borate, 0.001 M EDTA) prepared in a Sequigen Sequencing Cell (BIO-RAD). Prior to loading, samples were incubated in a boiling water bath for 3 min and then placed on ice. A 3.0 µl sample of each sequencing reaction was loaded onto gels pre-warmed to 55 °C. The DNA products were separated at 60 W for 2 to 12 hr. After electrophoresis, the gels were transferred to 3MM Whatmann paper and dried at 80 °C for 2 to 4 hr using a Savant gel dryer.

### *2.15 Autoradiography*

Autoradiography was utilized to visualize radioactive molecules. Biomax MR or X-Omat XB-1 film (Kodak) was placed directly onto the dried gel and exposed for 0.4 to 14 days at –80 °C. The exposed film was then processed manually by placing the film in GBX developer (Kodak) for 1 to 5 min, 1.5% (v/v) acetic acid for 1 min, and GBX fixer

(Kodak) for 5 min. The film was washed with water for 5 min and allowed to dry at room temperature.

### *2.16 PCR (Polymerase chain reaction)*

The Polymerase chain reaction (PCR) was performed with the Expand High Fidelity PCR amplification kit (Boehringher-Mannheim). Approximately 100 ng of template DNA was mixed with a solution containing 200 μM dNTPs, 300 nM of each primer, 1.5 mM $MgCl_2$, 1X Expand Buffer, and 2.6 units of the Pwo:Taq enzyme mixture prepared to a final volume of 100 μl in a 0.25 ml microcentrifuge tube (BIO-RAD). Reactions were overlaid with mineral oil. Thermal cycling was performed using a Perkin-Elmer Cetus thermocycler as follows: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 40 s, elongation at 74 °C for 2 min; and a final extension at 74 °C for 4 min. The amplified fragments were phenol extracted (section 2.11) and concentrated by ethanol precipitation (section 2.12).

### *2.17 Preparation of plasmid DNA for cloning*

#### *2.17.1 Restriction endonuclease digestion*

Restriction endonucleases, obtained from New England Biolabs, GIBCO–BRL, Pharmacia Biotech, Boehringer-Mannheim, and MBI-Fermentas were utilized according to the manufacturer's recommendations. Nucleic acids were digested in a 500 μl solution

containing 1X reaction buffer, 6 ng/µl Ribonuclease A (Sigma), 9 to 60 units of each restriction enzyme, and 2 to 10 µg of plasmid DNA. Reactions were incubated at the enzymes optimal temperature for 1 to 6 hours. When necessary, BSA (New England Biolabs) was added to the reaction mixture to a final concentration of 0.1 µg/µl. Following digestion, DNA fragments were concentrated by ethanol precipitation (section 2.12) and resuspended in 50 µl of TE [10 mM Tris-Cl (pH 8.0), 1 mM EDTA].

### *2.17.2 Purification of DNA from agarose gels*

Purification of DNA fragments was performed using a modification of the method of Girvitz *et al.* (1980). DNA fragments were first separated by agarose gel electrophoresis. A vertical incision was made with a scalpel into the gel between the DNA fragment to be purified and the anode. A piece of 3MM Whatmann paper, backed with dialysis membrane (Gibco-BRL) was inserted into the incision, orienting the Whatmann paper between the desired DNA fragment and the dialysis membrane. Electrophoresis was continued at 200 V for 10 min to allow the fragment to migrate into the paper/dialysis membrane dam. The dam was then removed from the gel, and transferred to a decapitated 0.5 ml microfuge tube that was previously punctured in the bottom with an 18 gauge needle and placed inside a decapitated 1.5 ml microfuge tube. The DNA dam was then soaked with 100 µl of Band Elution buffer [50 mM Tris-Cl (pH 7.6), 200 mM NaCl, 1 mM EDTA, and 0.1% (w/v) SDS] and incubated for 2 min. The elution buffer was collected by centrifugation at 2000 RPM in a Biofuge A microfuge for 10 s, and transferred to another tube. This process was repeated 3 times. The remaining

elution buffer was collected by centrifugation at top speed for 30 s. The DNA was then purified by phenol extraction (section 2.11), and precipitated with ethanol (section 2.12).

### *2.17.3 Phosphatase treatment of DNA fragments*

Calf–intestinal alkaline phosphatase or CIAP (Boehringer-Mannheim), an enzyme which removes 5' terminal phosphates from DNA, was utilized to prevent intramolecular and intermolecular ligation of vector DNA. Forty-three microlitres of linearized plasmid DNA, previously treated with PEG (section 2.13), was mixed with 5 µl of React III buffer (Boehringer-Mannheim) and 2 µl of CIAP (1 U/µl) to initiate the reaction. The reaction was incubated at 37 °C for 1 hr, terminated by phenol extraction, (section 2.11), and ethanol precipitated (section 2.12).

## *2.18 DNA ligations*

### *2.18.1 Cohesive-end ligations*

DNA fragments were ligated to linearized plasmids that contained compatible-ends essentially as described in Maniatis *et al.* (1982). Linearized plasmids were first prepared as described in sections 2.13 and 2.17.3. Approximately 200 ng of linearized plasmid DNA was used for each reaction. The DNA fragment was added to the plasmid DNA to maintain a 3:1 molar ratio, respectively. Two microlitres of 10X ligation buffer [6.6 mM Tris-Cl (pH 7.5), 50 mM $MgCl_2$, 50 mM dithiothreitol, 10 mM ATP] and 1 Wiess unit of T4 DNA ligase (Boehringer-Mannheim) was added to the previous solution

and brought to a final volume of 20 µl. Each reaction was incubated for 1 to 3 hours at room temperature.

### *2.18.2 Blunt-end ligations*

DNA fragments with incompatible-ends were treated with the *E. coli* Klenow polymerase to create blunt-ends for ligation. Approximately 200 ng of linearized plasmid DNA was used for each reaction. The DNA fragment was added to the plasmid DNA to maintain a 3:1 molar ratio respectively. This was then added to a solution containing 50 mM Tris-Cl (pH 7.2), 10 mM $MgCl_2$, 100 µM dATP, 100 µM dCTP, 100 µM dGTP, 100 µM dTTP. The ends were treated with 5 units of *E. coli* Klenow fragment (New England Biolabs) for 20 min at room temperature. The DNA molecules were then ligated by adding 0.5 µl of 50 mM ATP, 0.5 µl of 250 mM DTT, and 5 Weiss units of T4 DNA ligase to the solution and incubating the final solution at 12 °C for 16 to 18 hr in a ligation cooler (Boekel).

## *2.19 ß-galactosidase assays*

### *2.19.1 The Chloroform filter assay*

Qualitative measurements of ß-galactosidase activity were performed using a modification of the method of Breeden and Nasmyth (1985). Transformants were patched onto the appropriate SC- omission media and grown for 2 to 4 days at 30 °C. The cells were transferred to sterile 70 mm Whatmann filter disks (Whatmann #2) by

carefully laying the filter paper onto the colonies and quickly peeling the filter disks from the plates. The filters were submersed in chloroform for 3 min and dried inside a fumehood at room temperature for 5 min. The dried filters were overlaid onto a second filter, previously saturated in 1.25 mls of a Z-buffer/X-Gal solution [100 mM $Na_2HPO_4$, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 38 mM ß-mercaptoethanol, 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-ß-galactoside)] pre-warmed to 37 °C. The filters were incubated for 0.5 to 2 hrs and then dried in fumehood at room temperature.

### *2.19.2 Liquid ß-galactosidase assays*

Quantitative measurements of ß-galactosidase activity were performed using a modification of the method of Miller (1972). Transformants were inoculated into 2 mls of the appropriate SC omission media and grown for 18 to 20 hr at 30 °C on a shaker cycling at 200 RPM. When the culture was ready to be assayed, 5 µl of the culture was diluted in 995 µl of Nanopure™ water to determine its $OD_{600}$. One-hundred microlitres of the culture was mixed with 700 µl of Z–buffer [100 mM $Na_2HPO_4$ (pH 7.0), 10 mM KCl, 1 mM $MgSO_4$, 38 mM ß–mercaptoethanol] pre-warmed to 37 °C. The yeast cells were lysed by adding 50 µl of chloroform and 50 µl of 0.1% (w/v) SDS to the previous solution and mixing the final solution with a vortex for 30 s. The reaction was initiated by the addition of 100 µl of 4 mg/ml ortho-nitro-pyranogalactoside (ONPG). This solution was incubated at 37°C until a yellow color developed. Reactions were terminated by the addition of 400 µl of 1 M $Na_2CO_3$. The cellular debris was separated by centrifugation at room temperature in a Biofuge A microfuge at 13 000 x*g*. Each

clarified solution was then transferred to a fresh 1.5 ml microfuge tube, and the $A_{420}$ measured for each solution.

The ß-galactosidase activity, measured in Miller Units, was determined using the following equation:

$$\text{Miller Unit} = \frac{(A_{420})(1000)}{(Vol)(t)(OD_{600})}$$

where $A_{420}$ is the absorbance at 420 nm, $t$ is the incubation time in minutes, $OD_{600}$ is the optical density of the cell culture, and *Vol* is the volume of culture utilized in the assay.

### *2.20 Liposome mediated transfection*

The DOSPER liposome delivery system (Boehringer-Mannheim) was utilized to introduce DNA into Chinese hamster ovary (CHO) cells. CHO-IR or CHO26 cell lines were prepared for transfection by growing them to 80 to 90% confluence in HAM's F12 medium (Gibco-BRL) containing 10% fetal calf serum (FCS) (Gibco-BRL), 0.1 µg/ml streptomycin (Gibco-BRL), and 0.1 U/ml penicillin (Gibco-BRL) at 37 °C in a humidified incubator containing a $CO_2$ tension of 5%. Approximately two to three hours prior to transfection, the growth medium was replaced with 2.5 mls of OPTI-MEM Reduced Serum Medium (Gibco-BRL). The DNA:micelle complexes were prepared by combining 80 µl of a 0.05 µg/µl DNA solution containing 10 mM HEPES Buffered Saline (pH 7.0) with 80 µl of 0.2 µg/µl DOSPER solution containing 10 mM HEPES Buffered Saline (pH 7.0). This mixture was incubated at room temperature for 20 min.

Cells were transfected by dispersing the transfection reagent as drops throughout the dish and incubating the cells for 6 hr at 37 °C in a humidified incubator containing a 5% $CO_2$ tension. Cells were then supplemented with 2.5 mls of HAM's F12 medium containing 10% FCS (Gibco-BRL) and incubated for 24 hr.

### *2.21 Insulin stimulation*

Cell lines were grown in a medium lacking serum and then stimulated with insulin. Transfected and untransfected cells were serum starved by growing them in HAM's F12 medium lacking serum for 24 hr. The cells were then stimulated with 2.5 mls HAM's F12 medium containing 100 nM bovine or porcine insulin (Eli-lily) for 7 to 10 min. The cells were then lysed to analyze cellular proteins.

### *2.22 Cell lysis*

Cells were lysed with the aid of detergents to extract intracellular components. Prior to lysis, cells were washed three times with ice-cold PBS [80 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$ (pH 7.5), 100 mM NaCl] to remove any contaminating extracellular material. Cells were lysed by flooding the plate with 700 µl of ice-cold lysis buffer containing various protease inhibitors [50 mM Tris (pH 7.6), 150 nM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100/NP-50, 1 mM $NaVO_4$, 10 mM NaF, 10 mM pyrophosphate, 1.0 mM EDTA, 1 mM $MgCl_2$, 1mM PMSF, 0.5 µM aprotinin, 0.5 µM leupeptin, 0.5 µM E64] for 10 min on ice. Cellular debris was dislodged from the dish using a cell scraper (Corning). The cell lysate was then transferred into a fresh 1.5 ml

microfuge tube, incubated for 10 min on ice-water, and clarified by centrifugation at 13 000 x*g* for 10 min at 4 °C. The clarified lysates were finally transferred to a fresh 1.5 ml microfuge tube and stored at –80 °C.

### *2.23 Bradford protein assay*

Protein concentrations were determined using a modification of the method of Bradford (1976). Cell lysates were prepared by mixing 2.5 µl of cell lysate with 797.5 µl of sterile Nanopure™ water and 200 µl of Bradford reagent. The absorbance at 595 nm was measured for each sample and the protein concentrations extrapolated from a standard curve.

### *2.24 Co-immunoprecipitation*

Co-immunoprecipitation of specific proteins was performed to investigate protein interactions in mammalian cells. Cell lysates were pre-adsorbed with 50 µl of a 20% (w/v) slurry of protein-A or protein-G sepharose beads in PBS [80 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$, 100 mM NaCl, (pH 7.5)] for 30 min at 4 °C to remove proteins that bind to the sepharose beads. The sepharose beads were collected by centrifugation at 13 000 x*g* for 10 s and the supernatant transferred to a fresh 1.5 ml microfuge tube. The pre-adsorption was then repeated. The cell lysates were mixed with 1 µg of antibody and incubated at 4 °C for 4 hr. Immune-complexes were harvested by mixing the samples with 50µl of a

20% (w/v) slurry of protein-A or protein-G sepharose beads in PBS azide [80 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$ (pH 7.5), 100 mM NaCl, 0.02% sodium azide] for 1 hr at 4 °C. The sepharose beads were collected by centrifugation at 13 000 x*g* for 10 s and washed three times with 450 µl of cold lysis buffer [50 mM Tris-Cl (pH 7.6), 150 nM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA] and once with 450 µl of cold PBS [80 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$, 100 mM NaCl, (pH 7.5)] to remove unbound contaminants. After removing the final wash solution, the beads were mixed with 30 µl of 2X sample loading buffer [50 mM Tris-Cl (pH 6.8), 100 mM DTT, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol] and incubated in a boiling water bath for 5 minutes. The boiled solution was then separated from the beads and transferred to a fresh 1.5 ml microfuge tube for storage at –80 °C.

### *2.25 SDS-PAGE electrophoresis*

Separation of protein samples was performed using SDS-PAGE (Laemmli, 1970). A discontinuous acrylamide gel system, consisting of an upper stacking gel [4.5% acrylamide (29:1); 127 mM Tris-Cl (pH 6.8), 0.1% (w/v) SDS, 1.3 mM APS, and 9.1 mM TEMED] and a lower resolving gel [7.5% acrylamide (29:1), 375 mM Tris-Cl pH 8.8, 0.1% (w/v) SDS, 2.2 mM APS, 3.9 mM TEMED] was prepared in a Miniprotean

electrophoresis system (BIO-RAD). Proteins samples were prepared by adding an equal volume of 2X sample loading buffer [50 mM Tris-Cl (pH 6.8), 100 mM DTT, 2% (w/v) SDS, 0.1% bromophenol blue, 10% (v/v) glycerol] and incubating this mixture in a boiling waterbath for 5 min. Samples were cooled at room temperature for 5 min and centrifugated at 13 000 x*g* for 10 s. Samples of approximately equivalent protein mass were loaded into the wells of the stacking gel and separated by electrophoresis at 100 to 150 volts for 1 to 4 hr.

## *2.26 Western blotting*

### *2.26.1 Protein transfer to nitrocellulose*

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes using a modification of the method of Towbin (1979). Protein transfer was prepared in a Mini-transblot transfer cell (BIO-RAD) as outlined by the manufacturer's instructions. The components and the orientation of the components in the gel holder cassette are illustrated in Figure 7. The gel cassette, carrying the acrylamide gel and nitrocellulose, was immersed in a tank containing transfer buffer [25 mM Tris-Cl, 192 mM glycine, 20%

(v/v) methanol] and transferred for 1 hr at 100 volts. The membrane was then recovered, dried for 10 min at room temperature, and prepared for immunoblotting.

### *2.26.2 Antibody mediated protein detection*

Detection of membrane bound proteins was accomplished using an antigen specific antibody (primary Ab). The dried membrane, carrying the protein(s) of interest, was soaked in water for 5 min and then equilibrated in TBS-T [20 mM Tris-Cl (pH 7.6), 150 mM NaCl, 0.1% (v/v) Tween-20] for 5 min. Each membrane was incubated in 15 mls of 5% (w/v) Skim Milk (Carnation) dissolved in TBS-T for 1 hr at room temperature to block non-specific antibody adsorption sites. This was replaced by 15 mls of the same solution containing the primary Ab and incubated at room temperature for 1 to 2 hours. The membrane was rinsed on a shaker with copious amounts of TBS-T for 10 minutes three times to remove unbound contaminants.

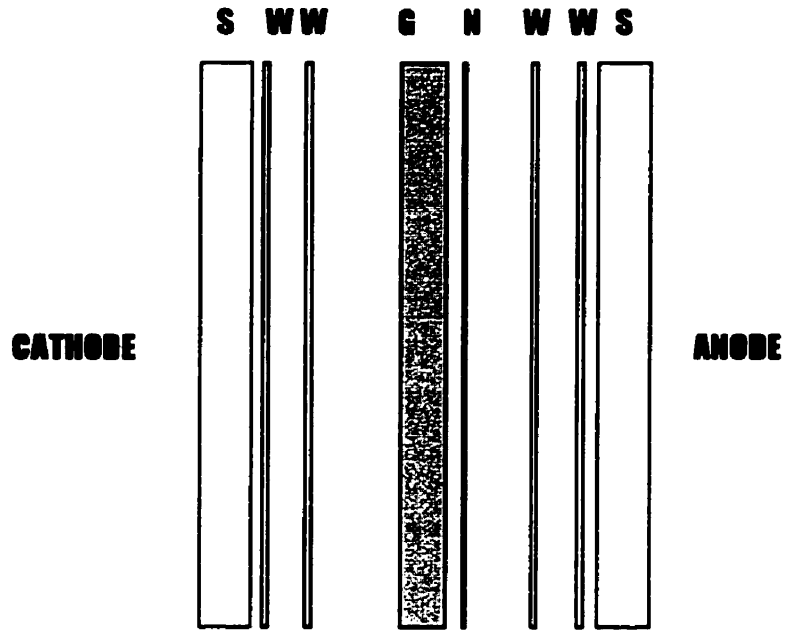
To assist in immune-complex detection, a second, species specific anti-IgG antibody, conjugated with horseradish peroxidase (Jackson Laboratories) was used. The membrane was incubated in 15 mls of 5% (w/v) skim milk dissolved in TBS-T, containing 1.5 µl of the secondary Ab, for 60 minutes with shaking. The membrane was finally washed on a shaker using copious amounts of TBS-T three times for 10 minutes.

### *2.27 Enhanced chemiluminescence*

The ECL (Enhanced Chemiluminescence) kit (Amersham) was used to detect horseradish peroxidase conjugated antibodies. Briefly, the antibody treated blots were flooded with a 1:1 mixture of ECL reagents 1 and 2 and incubated for 1 minute at room temperature. The excess ECL reagent was removed from the membrane with a paper towel. The membrane was then wrapped in plastic wrap (AEP-Borden) and exposed to Biomax X-Omat film (Kodak).

**Figure 7: Transblot apparatus**

The transfer of proteins from an acrylamide gel to nitrocellulose was accomplished using the transblot apparatus. The components and arrangement of the gel holder cassette from the Transblot apparatus is illustrated. S=Scotchbrite pad, W=Whatmann Paper, G=Acrylamide gel, N=Nitrocellulose.


S W W G N W W S
CATHODE
ANODE

# 3. RESULTS AND DISCUSSION

## *3.1 Modified Two-hybrid Screening*

Two-hybrid system screens (Chien *et al.*, 1993) have been used by many researchers to identify biologically important protein:protein interactions (Harper *et al.*, 1993; Freed *et al.*, 1994; Chinnaiyan *et al.*, 1995; Umar *et al.*, 1996; Tsang *et al.*, 1997; Takekawa *et al.*, 1998; Zhu *et al.*, 1999). However, modifications to this system allowing the detection of protein:protein interactions that are dependent on phosphorylation by the IR have not been developed. A modified two-hybrid system, allowing the detection of these types of interactions, was designed to characterize the phosphotyrosine dependent interaction between Irs1 and Shp2 (Sun *et al.*, 1993; Knells, 1996; Gietz, unpublished results). In this system, the expression of a constitutively active lexA:ßIR$_{(aa\ 941\text{-}1343)}$ fusion protein allowed IR mediated tyrosine phosphorylation of the Gal4$_{AD}$:Irs1 fusion protein in yeast. When the Gal4$_{BD}$:Shp-2 fusion protein was co-expressed in the same yeast cell containing both the Gal4$_{AD}$:Irs1 and the lexA:ßIR hybrid constructs, activation of the reporter gene could be detected (Knells, 1996; Gietz, unpublished results). No reporter gene activity was observed in the absence of the lexA:ßIR$_{(aa\ 941-1343)}$ hybrid

protein suggesting that an IR dependent interaction between $Gal4_{AD}$:Irs1 (pY) and the $Gal4_{BD}$:Shp2 hybrid proteins was occurring in this system. This system was utilized in this project to identify novel proteins that may require tyrosine phosphorylation by the IR to interact with hGrb14.

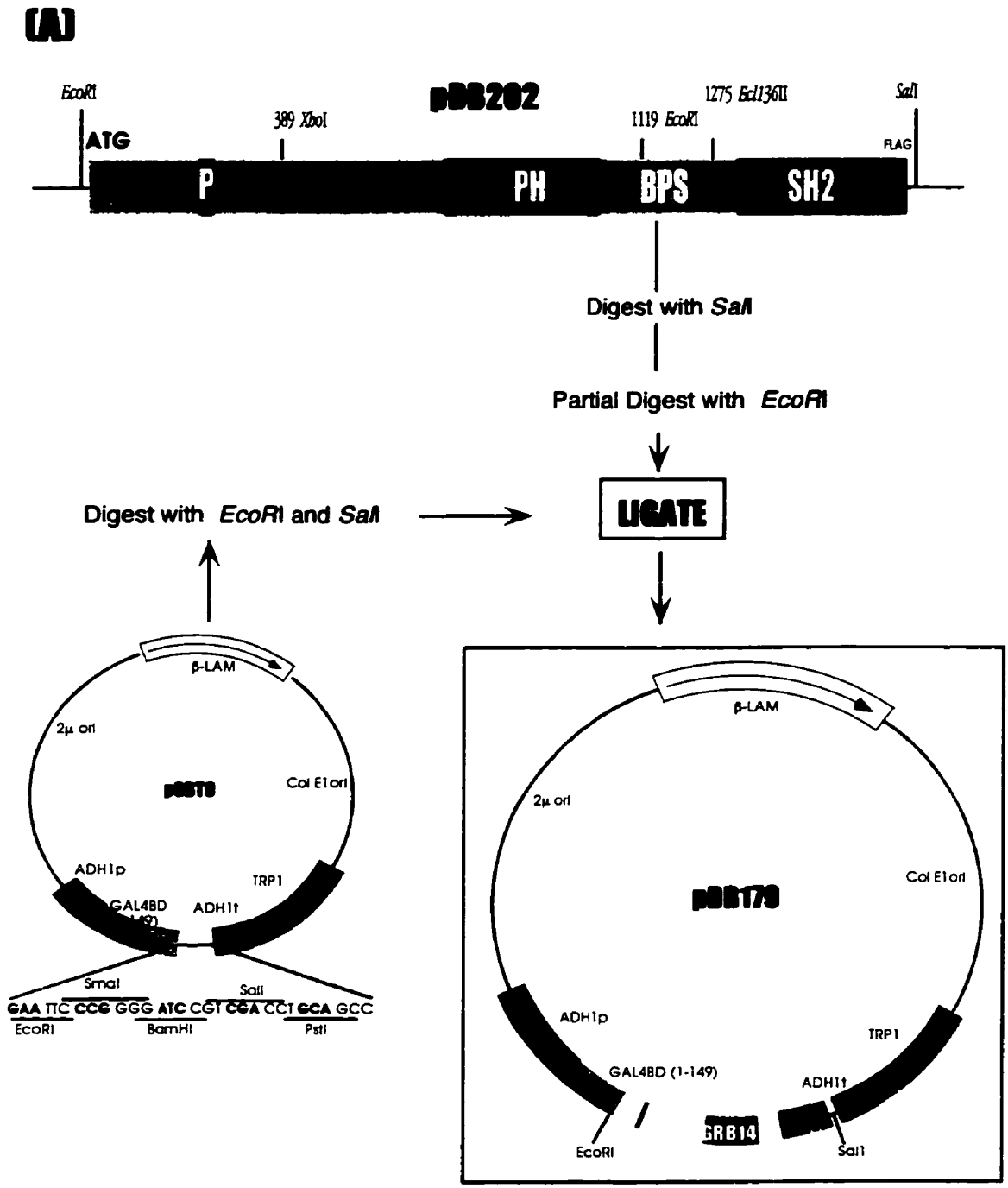
### *3.1.2 Construction of pDB179[$Gal4_{BD}$:$hGrb14_{(aa1-540)}$]*

A bait plasmid encoding a *$GAL4_{BD}$:hGRB14* fusion gene was constructed (Figure 8) to screen for hGrb14 interacting partners in the two-hybrid system. The vector pDB202 was first digested with *Sal*I. The linearized plasmid was then partially digested with *Eco*RI liberating a 1.6 kb *Eco*RI-*Sal*I fragment containing the entire 1620 bp *hGBR14* ORF carrying the FLAG epitope at the 3' end of the gene (Daly *et al.*, 1996). This DNA fragment was isolated (section 2.17.2) and ligated (section 2.18.1) into the *Eco*RI-*Sal*I digested vector pGBT9[$Gal4_{BD(aa1-149)}$] creating pDB179[$Gal4_{BD}$:$hGrb14_{(aa1-540)}$].

**Figure 8: Construction of pDB179 [*GAL4*$_{BD}$:h*GRB14* ]**

(A) A 1.6 kb *EcoR*I-*Sal*I Grb14 fragment was isolated from pDB202[Gal4$_{AD}$:hGrb14$_{(aa1-540)}$FLAG] and ligated with *EcoR*I-*Sal*I digested pGBT9[Gal4$_{BD(aa1-149)}$], creating pDB179[Gal4$_{BD}$:hGrb14$_{(aa1-540)}$FLAG].

(B) The nucleotide sequence at the *GAL4*$_{BD}$:*hGRB14* fusion junction and the carboxyl–terminus of the fusion gene is shown. The reading frames of the GAL4$_{BD}$ and the *GRB14* ORF are represented as nucleotide triplets. The DNA sequence of a portion of the *GAL4*$_{BD}$ and the pGBT9 MCS is italicized. The nucleotide sequence for *hGRB14* is in boldface. In addition, the nucleotide sequence of the FLAG epitope and the in-frame stop codon are listed. The *EcoR*I recognition sequence in pDB179 is underlined.


(A)
EcoRI
pBB202
1275 Ecl136II
SalI
389 XbaI
1119 EcoRI
ATG
FLAG
P
PH
BPS
SH2
Digest with SalI
Partial Digest with EcoRI
Digest with EcoRI and SalI
LIGATE
β-LAM
2μ ori
pBBT8
Col E1 ori
ADH1p
TRP1
GAL4BD
ADH1t
SmaI
SalI
GAA TTC CCG GGG ATC CGT CGA CCT GCA GCC
EcoRI
BamHI
PstI
β-LAM
2μ ori
pBB179
Col E1 ori
ADH1p
TRP1
GAL4BD (1-149)
ADH1t
GRB14
EcoRI
SalI
(B)
GAA TTC ccg gag ctt aca ATG...GRB14...GCA GCA gac tac aag gac gat gac gac aag TGA
D Y K D D D D K
FLAG

### *3.1.3 Library screen*

Since the liver is an important target of insulin action, an adult human liver matchmaker cDNA library (Cat#HL4024AH, lot#52057, Clontech) was chosen for the two-hybrid screen. The cDNA library, constructed in the vector pACTII (Figure 6), had a complexity of 3 $x10^6$ independent clones with an average insert size of 1.3 kb and an estimated insert frequency of 90%. The yeast strain (DBY275) utilized for this modified two-hybrid screen was prepared by co-transforming the bait plasmid pDB179 [$Gal4_{BD}$:hGrb14$_{(aa1-540)}$] and the IR plasmid pBTR406 [lexA:ßIR$_{(aa941-1343)}$] into the yeast strain KGY37 (Graham, 1996). The strain DBY275 was grown onto either SC–T-L or SC-T-L-H+5 mM 3-AT and tested for reporter gene autoactivation using the filter assay or by assaying for growth on a medium lacking histidine. DBY275 did not activate either reporter gene under these conditions, and was deemed suitable for a library screen (data not shown). Approximately 10 µg of the library plasmid DNA was transformed into DBY275 [KGY37+[$Gal4_{BD}$:hGrb14$_{(aa1-540)}$]+[lexA:ßIR$_{(aa941-1343)}$]). An estimated 1.1 x $10^7$ transformants were plated onto SC-T-L-U-H media containing 5 mM 3-AT and incubated at 30 °C for 2 to 10 days.

Sixteen yeast colonies capable of activating the *HIS3* and *lacZ* reporter genes were identified. Plasmid DNA from each yeast colony was recovered (section 2.8) and transformed into the *E.coli* strain KC8 (section 2.9.2). Four bacterial colonies were selected from each transformation (section 2.9.3) because yeast colonies isolated from the library screen often carry multiple library plasmids (Gietz, personal communication).

The plasmid DNA was recovered (section 2.10) from each clone and transformed into DBY275(KGY37+pDB179[$Gal4_{BD}$:$hGrb14_{aa1-540}$]+pBTR406[lexA:ßIR$_{aa941-1343}$]). Several yeast colonies from each yeast transformation were then tested for reporter gene activity to identify the library plasmid responsible for reporter gene activation. The cDNA insert from the library plasmid capable of activating both reporter genes was sequenced using a $GAL4_{AD}$ primer 5' GAAGATACCCCACCAAAC 3'. The DNA sequence of each clone was then screened against the Genbank database to see if identical or homologous sequences existed.

### *3.14 hGrb14 interacting proteins*

The DNA sequence of each cDNA clone was used to search the EST (expressed sequence tag) and non-redundant databases of Genbank (http://www.ncbi.nlm.nih.gov/blast/). This search disclosed three distinct open reading frames from the sixteen clones identified (Table 7). Two of the three open reading frames matched existing Genbank entries.

**Table 7: Modified Two-hybrid Screen Results**

| Clone Name | Genbank Accession Number | # of Times Identified |
|---|---|---|
| GIP1 (GIPM4) | N/A | 1 |
| KIAA0093 (Nedd4 homologue) | D42055 | 2 |
| *TAX1BP1* | NM006024 | 13 |

*3.1.4.1 GIP1 (GRB14 INTERACTING PROTEIN-1)*

One cDNA sequence was named *GIP1* for Grb14 Interacting protein-1. This sequence (see appendix 5.5) shared no significant similarity with any known nucleic acid or protein sequences in the non-redundant and/or EST databases. This clone was not pursued.

*3.1.4.2 KIAA0093*

Two non-identical cDNA clones contained sequence information identical to the *KIAA0093* ORF (accession # D42055). This sequence was similar to the ubiquitin-ligase Nedd4. Both clones showed an autoactivation phenotype when incubated overnight with an empty $Gal4_{BD}$ vector suggesting that these clones are false positives. However, in the presence of the $GAL4_{BD}$:*hGRB14* plasmid, both clones were able to activate the *lacZ* reporter gene in 30 min. The rapid response observed in the presence of the $GAL4_{BD}$:*hGRB14* plasmid suggests that this may be a genuine interaction, however, because of its autoactivation phenotype this clone was not pursued.

Interestingly, a report describing a Grb10:Nedd4 interaction was recently published (Morrione *et al.*1999) supporting the observations found here. Grb10 and Grb14 are likely functionally related, as both proteins have similar primary sequences and bind to the activated IR.

Nedd4 is a ubiquitin-ligase which can add ubiquitin moieties to proteins targeted for 26s proteasome mediated proteolytic degradation (Hershko, *et al.*, 1992). The study presented by Morrione *et al.* (1999) indicates that the BPS and SH2 domains of mGrb10

are involved in mediating mGrb10's interaction with Nedd4. Since no evidence for *in vivo* ubiquitination of mGrb10 was observed (Morrione *et al.*, 1999), the authors have hypothesized that this interaction may be a mechanism to target Grb10 binding partners for proteolytic degradation. Further investigation into the hGrb14:KIAA0093 interaction may help define the role this protein has in signal transduction.

*3.1.4.3 TAX1BP1 (TAX1 BINDING PROTEIN-1)*

Thirteen identical sequences corresponding to the 3' end (nucleotides 709 to 2241) of the *TAX1BP1/TXBP151* ORF (accession number NM006024) were also identified. Tax1bp1 was first identified in a two-hybrid screen as a binding partner for the HTLV- 1 (human T-cell leukemia virus-type 1) Tax1 protein (Jin *et al.*, 1997).

Tax1 is a protein product of the HTLV-1 virus, which is believed to be a causative agent in Adult T-cell Leukemia (ATL) and HTLV-1 associated myelopathy (HAM)/Tropical spastic paraparesis (Poiesz *et al.*, 1980; Yamamoto *et al.*, 1982; Gessain *et al.*, 1985; Osame *et al.*, 1986). Tax1's role in cellular immortalization is evident as it has been shown to transform cells in culture (Grassmann *et al.*, 1989; Tanaka *et al.*, 1990). Tax1 has also been shown to target the mitotic checkpoint protein hsMAD1 (also Txbp181), and appears to abrogate mitogenesis by this process (Jin *et al.*, 1998). Similarly, it has also been shown to bind Mekk1, a protein involved in the Map kinase cascade (Yin *et al.*, 1998). Tax1's role in targeting signaling proteins implies that Tax1bp1 may also be involved in cell signaling. From the three genes identified in this two-hybrid screen, Tax1bp1 was selected for further analysis.

### *3.2 The IR is not required for hGrb14:Tax1bp1 association*

The hGrb14:Tax1bp1 interaction was tested in the two-hybrid system to determine if this interaction was dependent on the presence of the activated insulin receptor. This was achieved by evicting the plasmid pBTR406[lexA:ßIR$_{(aa941-1343)}$], a plasmid carrying the *URA3* gene in place of the *TRP1* gene, from the yeast strain DBY275 containing pRA38[Gal4$_{AD}$:Tax1bp1$_{(aa236-747)}$]. pBTR406 was evicted from this strain utilizing 5-floro-orotic acid (5-FOA) an analogue of orotic acid, which is toxic to cells expressing the *URA3* gene product (Boeke *et al.*, 1984). Cells were grown onto SC–T-L FOA medium to select for the clones that have evicted the *URA3* plasmid pBTR406. The FOA$^R$ yeast strain containing only pRA38[Gal4$_{AD}$:Tax1bp1$_{(aa236-747)}$] and pDB179 [Gal4$_{BD}$:hGrb14$_{(aa1-540)}$] was used to test for reporter gene activation. In this strain, reporter gene activity was not observed (Figure 9).

This strain was also transformed with the vector pDB169 [lexA:ßIR$_{MUT}$], which encodes the lexA protein fused to the ß-subunit of the IR carrying three mutations: Y1146F, Y1150F, and Y1151F. These mutations, all found in the tyrosine kinase regulatory loop, hamper the activation of the kinase (White *et al.*, 1988; Vogt *et al.*, 1991; Wilden *et al.*, 1993; Hubbard *et al.*, 1994). The yeast strain carrying the vector pDB169 [lexA:ßIR$_{MUT}$] was also tested for reporter gene activation. Reporter gene activity was not observed in the strain carrying the mutant receptor. The presence of activity in both strains suggest that the hGrb14:Tax1bp1 interaction is not dependent on the presence of the activated ßIR in yeast .

**Figure 9. hGrb14 does not require the activated IR for Tax1bp1 binding**

The yeast strain KGY37, carrying pDB179 [$Gal4_{BD}$:$hGrb14_{(aa1-540)}$FLAG] or pGBT9 and pRA38 [$Gal4_{AD}$:$Tax1bp1_{(aa236-747)}$], was tested for ß-galactosidase activity using the filter assay in the presence (+IR) and the absence (-IR) of the activated insulin receptor (pDB406). Cells expressing the *lacZ* gene turn blue when the *lacZ* gene product ß-galactosidase is incubated in the presence of X-gal.

| | | | |
|---|---|---|---|
| pGBT9 | | | + |
| pGAL4 | + | + | |
| pSV40 | + | + | + |
| pVA3-1 (H) | - | + | |

### *3.3 Analysis of the Tax1bp1 primary sequence*

The primary amino acid sequence of Tax1bp1 was analyzed to determine if any known structural elements found in other proteins were present in Taxbp1. The entire *TAX1BP1* cDNA, obtained from Dr K.T. Jeung (National Institutes of Health, Bethesda, MD), contains an open reading frame comprised of 2241 nucleotides. The *TAX1BP1* ORF encodes a 747 amino acid protein (Figure 10) with a predicted molecular weight of 86.2 kDa. Examination of the full-length amino acid sequence using the BLAST 2.0 algorithm (Altschul *et al.*, 1997) to screen the Genbank non-redundant database revealed similarities with several proteins containing coiled-coil domains (section 5.4). The Ndp52 protein, which also carries a carboxyl-terminal LIM domain and leucine zipper (Koriath *et al.*, 1995) showed the highest similarity sharing 29% identity and 44% similarity with the Tax1bp1 primary sequence.

The functional role of Ndp52 is currently unknown. Ndp52 shows both cytoplasmic and nuclear distribution, and was believed to localize to a poorly understood multi-protein nuclear domain called the Nuclear dot-10 (ND10) domain (Koriath *et al.*, 1995). Whether NDP52 localizes to the ND10 domain remains controversial since Sternsdorf *et al.* (1997) did not observe similar localization patterns. The high similarity between the primary sequences of both proteins suggests that Tax1bp1 may also contain similar localization sequences and may thus share similar localization patterns.

### *3.3.1 Tax1bp1 contains a coiled-coil domain*

The primary sequence of Tax1bp1 was analyzed using a coiled-coil prediction algorithm (Lupas *et al.*, 1991). The output from this algorithm indicates that amino acids 140 to 592 have a high probability of forming coiled-coils (Figure 11a), suggesting that Tax1bp1 possess this domain.

Coiled-coils consist of two intertwining alpha-helical structures carrying heptad repeats (Branden and Tooze, 1991). These domains usually mediate protein multimerization with proteins carrying similar domains. The presence of this domain in Tax1bp1 suggests that Tax1bp1 may self-associate. This hypothesis is probable, since Ndp52 was also found to self-associate in the two-hybrid system (Sternsdorf *et al.*, 1997).

Coiled-coil domains are involved in a variety of biological processes. Proteins containing these structures are involved in cellular ultrastructure (Steinert *et al.*, 1988), muscular contraction (Gulick and Rayment, 1997), vesicular fusion (Skehel and Wiley, 1998) and mitotic regulation (Jin *et al.*, 1998). Furthermore, several proteins carrying coiled-coil domains have also been shown to be involved in signal transduction. These proteins include the: TRAF (Tumor necrosis factor receptor-associated factor) proteins (Rothe *et al.*, 1994; Rothe *et al.*, 1995; Sato *et al.*, 1995; Ishida *et al.*, 1996), the RBCK1 (RBCC-finger protein interacting with Pkc1) protein (Tokunaga *et al.*, 1998), the STAT (signal transduction and activator of transcription) proteins (Zhu *et al.*, 1999), and the Fer tyrosine kinase (Craig *et al.*, 1999).

### *3.3.2 Tax1bp1 contains a LIM domain*

Several carboxyl-terminal amino acid residues (aa 648-747) of Tax1bp1 share similarity with the LIM domain of Ndp52. To verify the presence of this domain in Tax1bp1, the Tax1bp1 primary sequence was compared to the LIM consensus sequence (Sanchez-Garcia and Rabbits, 1994). Figure 11b shows that a good alignment was observed with both sequences indicating that the amino acid residues 648-747 of Tax1bp1 likely form a LIM domain structure.

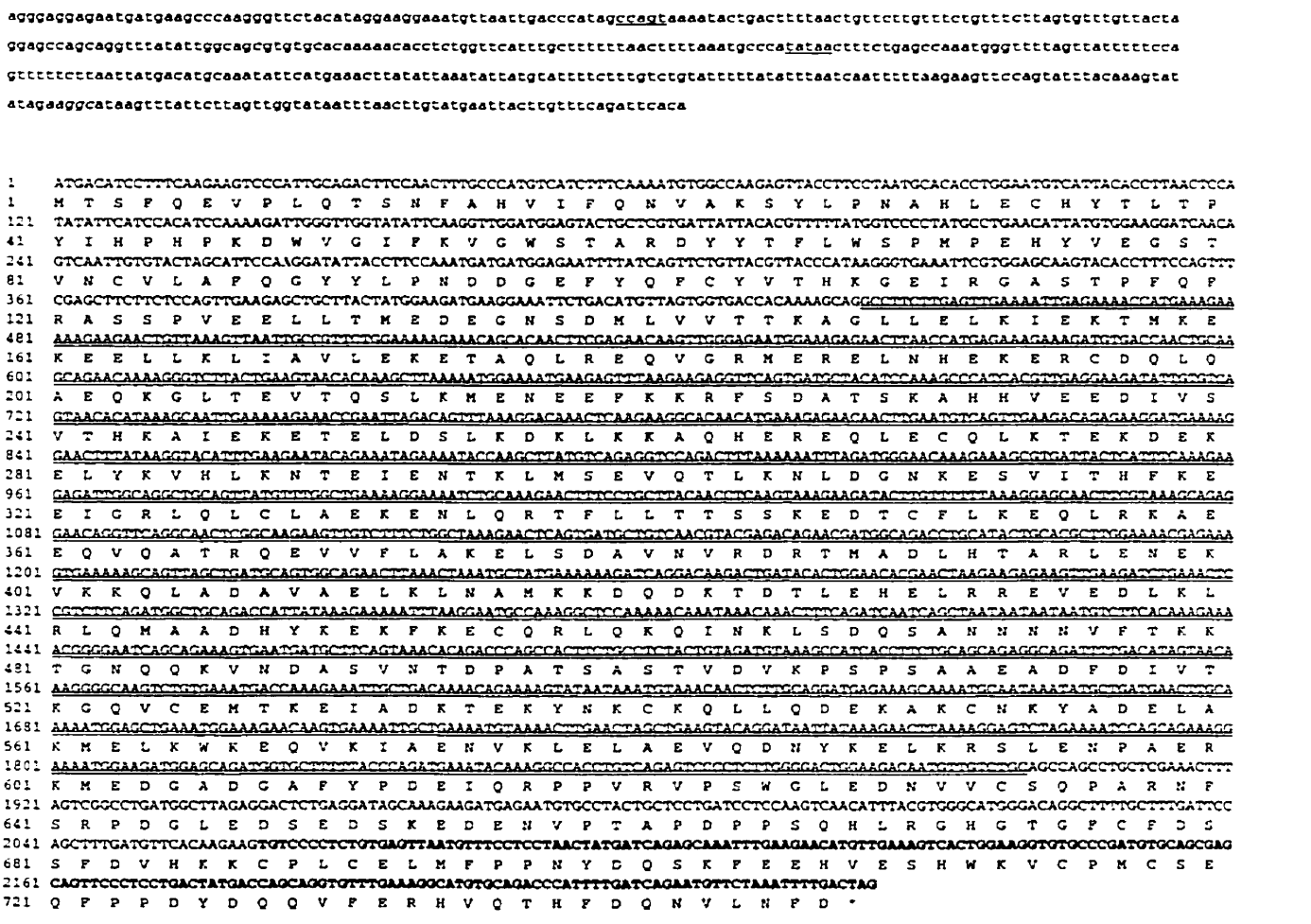
LIM domains are versatile protein modules that direct several cellular responses through protein:protein or protein:DNA interactions. LIM is an acronym for the three transcription factors first described to contain this domain: Lin-1 (Freyd *et al.*,1990), Isl–1(Karlsson *et al.*, 1990), Mec3 (Way *et al.*, 1988). Proteins containing LIM domains also have several functions that include: transcriptional regulation (Freyd *et al.*, 1990; Jurata *et al.*, 1999), cell-cell adhesion (Brown *et al.*, 1998), developmental regulation (Curtiss and Heilig, 1998), and Erk1 and Erk2 activation (Goyal *et al.*, 1999). Furthermore, two LIM domain containing proteins: (i) the Isl-1transcription factor and (ii) the Enigma protein have been shown to participate in insulin signaling. The Isl-1 protein binds to the enhancer region of the insulin gene regulating its expression (Riggs *et al.*, 1995), while Enigma has been found to directly interact with the ß-subunit of the IR (Wu *et al.*, 1996).

### *3.3.3 Tax1bp1 and Ndp52 share a novel domain*

During the BLAST analysis of Tax1bp1, the amino-terminal region of Tax1bp1 showed high similarity with the same region of the Ndp52 protein (Figure 11c). This sequence of amino acids was unique to Tax1bp1 and Ndp52 as no other proteins showed significant similarity with subsequent BLAST 2.0 analysis. This novel sequence may define a previously uncharacterized domain, so far unique to these proteins.

**Figure 10: Tax1bp1 nucleotide and protein sequence**

The human *TAX1BP1* coding sequence contains 2241 bp that encodes a 747 amino acid protein. Uppercase characters correspond to the *TAX1BP1* ORF. Lowercase characters correspond to 5' and 3' genomic sequences. The predicted amino acid sequence is found directly below the nucleotide sequence. Underlined =coiled-coil domain. Boldface characters =LIM-like domain.



agggaggagaatgatgaagcccaagggttctacatagggaaggaaatgttaattgacccatagccagtaaaatactgacttttaactgttcttgtttctgtttcttagtgtttgttacta

ggagccagcaggtttatatttggcagcgtgtgcacaaaaacacctctggttcatttgctttttaacttttaaatgcccatataactttctgagccaaatgggttttagttatttttcca

gtttttcttaattatgacatgcaaatattcatgaaacttatattaaatattatgtcattttctttgtctgtatttttatatttaatcaatttttaagaagttccagtatttacaaagtat

atagaaggcataagtttattcttagttggtataatttaacttgtatgaattacttgtttcagattcaca

1 ATGACATCCTTTCAAGAAGTCCCATTGCAGACTTCCAACTTTGCCCATGTCATCTTTCAAAATGTGGCCAAGAGTTACCTTCCTAATGCACACCTGGAATGTCATTACACCTTAACTCCA
1 M T S F Q E V P L Q T S N F A H V I F Q N V A K S Y L P N A H L E C H Y T L T P
121 TATATTCATCCACATCCAAAAGATTGGGTTGGTATATTCAAGGTTGGATGGAGTACTGCTCGTGATTATTACACGTTTTTATGGTCCCCTATGCCTGAACATTATGTGGAAGGATCAACA
41 Y I H P H P K D W V G I F K V G W S T A R D Y Y T F L W S P M P E H Y V E G S T
241 GTCAATTGTGTACTAGCATTCCAAGGATATTACCTTCCAAATGATGATGGAGAATTTTATCAGTTCTGTTACGTTACCCATAAGGGTGAAATTCGTGGAGCAAGTACACCTTTCCAGTTT
81 V N C V L A F Q G Y Y L P N D D G E F Y Q F C Y V T H K G E I R G A S T P F Q P
361 CGAGCTTCTTCTCCAGTTGAAGAGCTGCTTACTATGGAAGATGAAGGAAATTCTGACATGTTAGTGGTGACCACAAAAGCAGGCCTTCTTGAGTTGAAAATTGAGAAAACCATGAAAGAA
121 R A S S P V E E L L T M E D E G N S D M L V V T T K A G L L E L K I E K T M K E
481 AAAGAAGAACTGTTAAAGTTAATTGCCGTTCTTGGAAAAAGAAACAGCACAACTTCGAGAACAAGTTGGGAGAATGGAAAGAGAACTTAACCATGAGAAAGAAAGATGTGACCAACTGCAA
161 K E E L L K L I A V L E K E T A Q L R E Q V G R M E R E L N H E K E R C D Q L Q
601 GCAGAACAAAAGGGTCTTACTGAAGTAACACAAAGCTTAAAAATGGAAAATGAAGAGTTTAAGAAGAGGTTCAGTGATGCTACATCCAAAGCCCATCACGTTGAGGAAGATATTGTCCA
201 A E Q K G L T E V T Q S L K M E N E E F K K R F S D A T S K A H H V E E D I V S
721 GTAACACATAAAGCAATTGAAAAAGAAACCGAATTAGACAGTTTAAAGGACAAACTCAAGAAGGCACAACATGAAAGAGAACAACTTGAATGTCAGTTGAAGACAGAGAAGGATGAAAAG
241 V T H K A I E K E T E L D S L K D K L K K A Q H E R E Q L E C Q L K T E K D E K
841 GAACTTTATAAGGTACATTTGAAGAATACAGAAATAGAAAATACCAAGCTTATGTCAGAGGTCCAGACTTTAAAAAATTTAGATGGGAACAAAGAAAGCGTGATTACTCATTTCAAAGAA
281 E L Y K V H L K N T E I E N T K L M S E V Q T L K N L D G N K E S V I T H F K E
961 GAGATTGGCAGGCTGCAGTTATGTTTGGCTGAAAAGGAAAATCTGCAAAGAACTTTCCTGCTTACAACCTCAAGTAAAGAAGATACTTGTTTTTTTAAAGGAGCAACTTCGTAAAGCAGAG
321 E I G R L Q L C L A E K E N L Q R T F L L T T S S K E D T C F L K E Q L R K A E
1081 GAACAGGTTCAGGCAACTCGGCAAGAAGTTGTCTTTCTGGCTAAAGAACTCAGTGATGCTGTCAACGTACGAGACAGAACGATGGCAGACCTGCATACTGCACGCTTGGAAAACGAGAAA
361 E Q V Q A T R Q E V V F L A K E L S D A V N V R D R T M A D L H T A R L E N E K
1201 GTGAAAAAGCAGTTAGCTGATGCAGTGGCAGAACTTAAACTAAATGCTATGAAAAAAGATCAGGACAAGACTGATACACTGGAACACGAACTAAGAAGAGAAGTTGAAGATCTGAAACTC
401 V K K Q L A D A V A E L K L N A M K K D Q D K T D T L E H E L R R E V E D L K L
1321 CGTCTTCAGATGGCTGCAGACCATTATAAAGAAAAATTTAAGGAATGCCAAAGGCTCCAAAAACAAATAAACAAACTTTCAGATCAATCAGCTAATAATAATAATGTCTTCACAAAGAAA
441 R L Q M A A D H Y K E K F K E C Q R L Q K Q I N K L S D Q S A N N N N V F T K K
1441 ACGGGGAATCAGCAGAAAGTGAATGATGCTTCAGTAAACACAGACCCAGCCACTTCTGCCTCTACTGTAGATGTAAAGCCATCACCTTCTGCAGCAGAGGCAGATTTTGACATAGTAACA
481 T G N Q Q K V N D A S V N T D P A T S A S T V D V K P S P S A A E A D F D I V T
1561 AAGGGGCAAGTCTGTGAAATGACCAAAGAAATTGCTGACAAAACAGAAAAGTATAATAAATGTAAACAACTCTTGCAGGATGAGAAAGCAAAATGCAATAAATATGCTGATGAACTTGCA
521 K G Q V C E M T K E I A D K T E K Y N K C K Q L L Q D E K A K C N K Y A D E L A
1681 AAAATGGAGCTGAAATGGAAAGAACAAGTGAAAATTGCTGAAAATGTAAAACTTGAACTAGCTGAAGTACAGGATAATTATAAAGAACTTAAAAGGAGTCTAGAAAATCCAGCAGAAAGG
561 K M E L K W K E Q V K I A E N V K L E L A E V Q D N Y K E L K R S L E N P A E R
1801 AAAATGGAAGATGGAGCAGATGGTGCTTTTTACCCAGATGAAATACAAAGGCCACCTGTCCAGAGTCCCCCTCTTGGGGACTGGAAGACAATGTTGTCCTGCAGCCAGCCTGCTCGAAACTTT
601 K M E D G A D G A F Y P D E I Q R P P V R V P S W G L E D N V V C S Q P A R N F
1921 AGTCGGCCTGATGGCTTAGAGGACTCTGAGGATAGCAAAGAAGATGAGAATGTGCCTACTGCTCCTGATCCTCCAAGTCAACATTTACGTGGGCATGGGACAGGCTTTTGCTTTGATTCC
641 S R P D G L E D S E D S K E D E N V P T A P D P P S Q H L R G H G T G F C F D S
2041 AGCTTTGATGTTCACAAGAAGTGTCCCCTCTGTGAGTTAATGTTTCCTCCTAACTATGATCAGAGCAAATTTGAAGAACATGTTGAAAGTCACTGGAAGGTGTGCCCGATGTGCAGCGAG
681 S F D V H K K C P L C E L M F P P N Y D Q S K F E E H V E S H W K V C P M C S E
2161 CAGTTCCCTCCTGACTATGACCAGCAGGTGTTTGAAAGGCATGTGCAGACCCATTTTGATCAGAATGTTCTAAATTTTGACTAG
721 Q F P P D Y D Q Q V F E R H V Q T H F D Q N V L N F D *

ttacttttattatgagttaatatagtttagcagtaaaaaaaaaaaaaaaaaaccacacctaaaatagaccactgaggagaccatagagcggatgctttcatgcacccctttactg
cactttctgaccaggagctactttgagtttggtgttactaggatcagggtcagtctttggcttatcaataaatttaatctctgttaatcttacctgctttaaaaaaaagttct
tgtgtgttcgtatctttatttattccctagtttgcagaactgtctgaataaaggatacaaggattatttcaatgttactgcactgaaaaacgtgtatgtattagtgtgctagat
tatttagcagaatattcacaagtttctgttgaccttgttgattgagcatgactactaaatattatgtaataaaaagcatttgtcataacagtcttatgaagtagttcttcgaat
atagaaagttctataatttagcccatgaaatgataggtttttaatttcagaaatggagctgcatgtagaatgagatcacatgcttttatatgtgaaatattggttttagcaat
taacagaaggcatactttgctaatttttatggcaaaatttagaataacctgaatgattatttttaaactatcttgaagttgtatgtatatatctaatgggaaaatggaacaaga
gatgtcagtataattgttttcctattaaatgatctcataacaggggtgtttgtggcataa

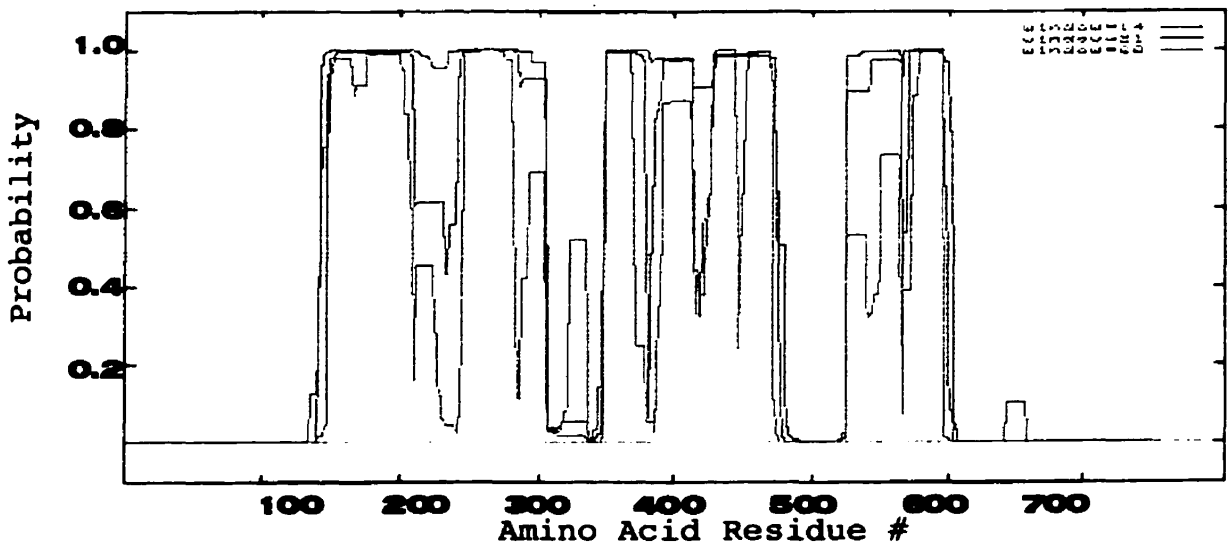
**Figure 11: Structural analysis of the Tax1bp1 protein**

(A) Probability output from the coiled-coiled prediction algorithm (Lupas *et al.*, 1991)

(B) The putative LIM sequence of Tax1bp1 aligned with a LIM domain consensus sequence (Sanchez-Garcia and Rabbits, 1994).

(C) Tax1bp1/NDP52 sequence alignment of their amino-terminal regions. The human (*Homo sapien)* and bovine *(Bos taurus)* sequences are shown.

a)




b)

```
Tax1bp1          CPLCELMFPPNYDQSKFEEHVESHWKVESHWKVCPMCSEQFPPDYDQQVFERHVQTH
                 1----------------16---20-----26---30----------------51---54
LIM Consensus    CXXCXXXXX16-23XXXXXHXX-CXX---CXX-CXXXXXXXX16-21XXXC/CXX-H/D
```

c)

```
Tax1bp1(Homo sapien)  VPLQTSNFAHVIFQNVAKSYLPNAHLECHYTLTPYIHPHPK
Ndp52  (Homo sapien)  VLLDHCHFSQVIFNSVEKFYIPGGDVTCHYTFTQHFIPRRK
Ndp52  (Bos taurus)   VLLDHCHFSQVIFNSVEKFYIPGGDITCYYTLTQHFIPRRK
```

### *3.4 Analysis of the Tax1bp1:Grb14 interaction in the two-hybrid system*

To determine if interaction studies in mammalian cells should be pursued, the entire Tax1bp1 sequence was fused to the $Gal4_{AD}$ and tested for interaction with hGrb14 in the two-hybrid system. In addition, various Tax1bp1 deletion mutants were constructed and analyzed in the two-hybrid system to characterize the portions of the Tax1bp1 protein involved in hGrb14 binding.

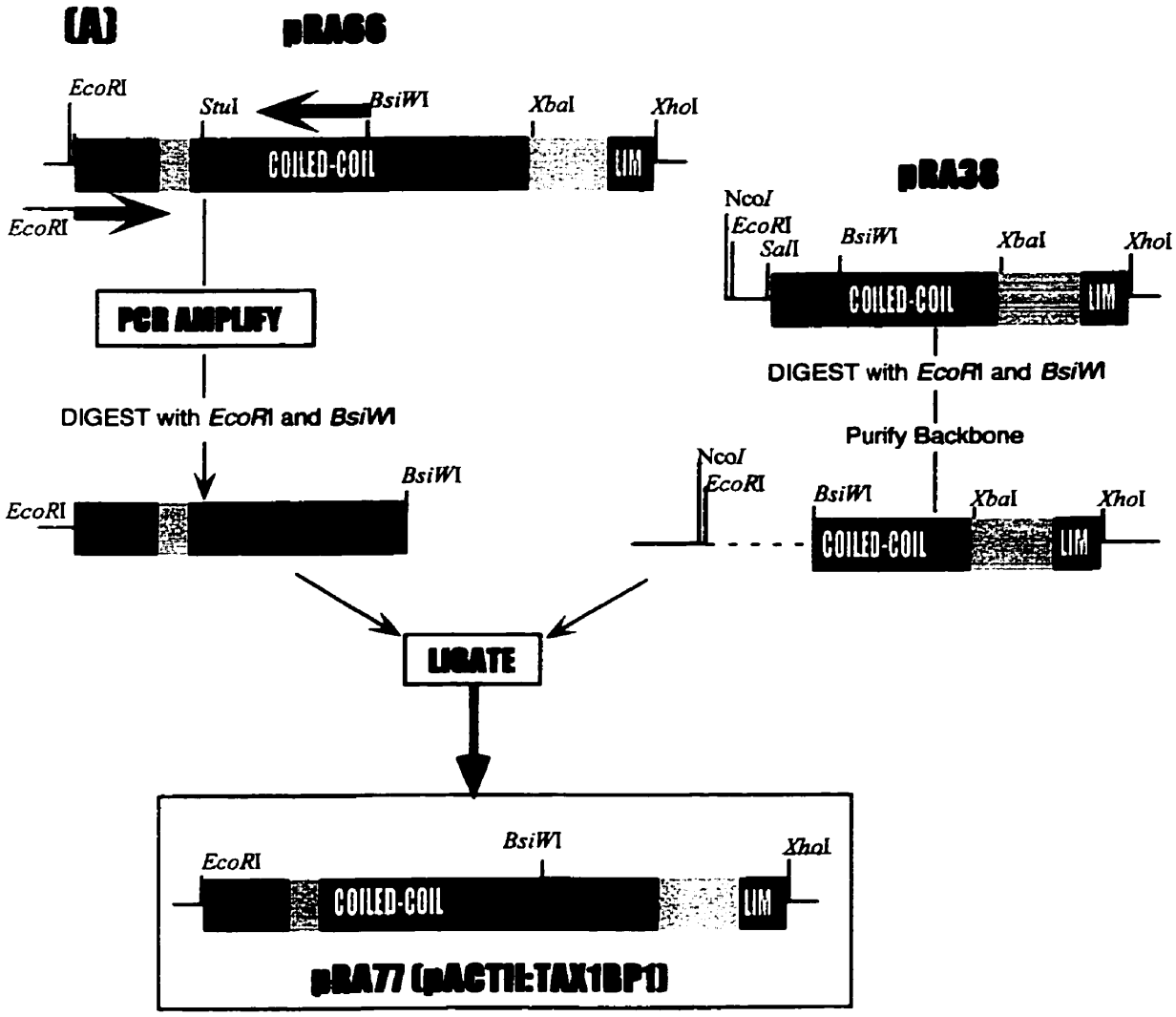
#### *3.4.1 Construction of pRA77 [$Gal4_{AD}$:Tax1bp1$_{(aa1-747)}$]*

To construct the *GAL4*$_{AD}$*:TAX1BP1* fusion gene carrying the entire *TAX1BP1* ORF, a 1.1 kb *TAX1BP1* fragment (aa 1-382), containing the missing 706 nucleotides (aa1-235), was PCR amplified from the plasmid pRA66 which carries the entire *TAX1BP1* cDNA. The amplification was performed as described in section 2.16 with the primers RAO1-5' C CGA ATT CAA ATG ACA TCC TTT CAA GAA GTC 3', and RAO2-5' GCG CGT ACG TTG ACA GCA TCA CTG 3'. The amplified fragments were digested with the enzymes *EcoR*I and *BsiW*I, purified (section 2.17.2), and ligated (section 2.18.1) with pRA38 [$Gal4_{AD}$:Tax1bp1$_{(aa236-747)}$], one of the Tax1bp1 clones identified in screen, which had been previously digested with *EcoR*I and *BsiW*I (Figure 12A). This produced pRA77 [$Gal4_{AD}$:Tax1bp1$_{(aa1-747)}$], a plasmid containing the *GAL4*$_{AD}$*:TAX1BP1* fusion gene carrying the entire *TAX1BP1* ORF. To verify that the *TAX1BP1* reading frame was maintained, the fusion junction was sequenced using a $GAL4_{AD}$ primer 5' GAAGATACCCCACCAAAC 3' (Figure 12B).

**Figure 12: Construction of pRA77 [*GAL4$_{AD}$:TAX1BP1*]**

(A) A 1146 bp *TAX1BP1* fragment, containing the missing nucleotides 1 to 706, was PCR amplified using the vector pRA66, containing the full-length *TAX1BP1* cDNA, as a template. The amplified fragment was digested with *EcoR*I and *BsiW*I, purified, and ligated with *EcoR*I-*BsiW*I digested pRA38 [Gal4$_{AD}$:Tax1bp1$_{(aa236-747)}$]. The resulting construct, containing the *GAL4$_{AD}$* fused in-frame with the entire *TAX1BP1* ORF, was named pRA77.

(B) The *GAL4$_{AD}$:TAX1BP1* fusion junction was determined by DNA sequencing. The reading frames of the *GAL4$_{AD}$* and the *TAX1BP1* are represented as nucleotide triplets. Italicized characters represent the *GAL4$_{AD}$* and the pACTII MCS. Boldface characters represent the *TAX1BP1* ORF. The *EcoR*I recognition site from the primer RAO1 is indicated in red.




**(B)**

*GGT CAT ATG GCC ATG GAG GCC CCG GGG ATC CGA ATT CAA* **ATG ACA TCC TTT...**

### *3.4.2 Construction of pRA20 [lexA$_{BD}$:Grb14FLAG]*

The *hGRB14* ORF was fused to the *lexA* ORF to analyze the Tax1bp1:hGrb14 interaction in the two-hybrid yeast strain KGY94. KGY94 utilizes the lexA operator, which is recognized by the lexA protein encoded within pBTM116 (Figure 6), to regulate *lacZ* gene expression. The vector pDB202 was first digested with *Sal*I. The linearized plasmid was then partially digested with *Eco*RI liberating a 1.6 kb *Eco*RI-*Sal*I fragment containing the entire 1620 bp *hGBR14* ORF carrying the FLAG epitope at the 3' end of the gene (Daly *et al.*, 1996). This DNA fragment was isolated (section 2.17.2) and ligated (section 2.18.1) into the *Eco*RI-*Sal*I digested vector pBTM116[lexA$_{(aa1-149)}$] creating pRA20[lexA:hGrb14 $_{(aa1-540)}$] (Figure 13).

### *3.4.3 Tax1bp1$_{(aa1-747)}$ interacts with hGrb14 in the two-hybrid system*

The full-length Tax1bp1 protein was tested against hGrb14 in the two-hybrid system to determine if studies in mammalian cells should be pursued. The vectors pRA77 [Gal4$_{AD}$:Tax1bp1$_{(aa1-747)}$] or pRA38 [Gal4$_{AD}$:Tax1bp11$_{(aa236-747)}$] were co-transformed (section 2.6) into the yeast strain KGY37 with either pDB179 [Gal4$_{BD}$:hGrb14$_{(aa1-540)}$FLAG] or pGBT9 [Gal4$_{BD(aa1-149)}$] and grown for 3-5 days on SC–T–L. Individual transformants were subsequently patched onto SC-T-L medium and assayed for activation of the *lacZ* gene product (ß-galactosidase) using the filter assay

(section 2.19.1). Transformants were also patched onto SC-T-L-H+5 mM 3-AT medium to assay for growth stimulated by the expression of the *HIS3* gene product (imidazole-glycerol-P-dehydratase).

An increase in ß-galactosidase activity and growth on medium SC medium lacking histidine (SC-T-L-H+5 mM 3-AT) was observed when either pRA77[$Gal4_{AD}$:$Tax1bp1_{(aa1-747)}$] or the original library clone pRA38[$Gal4_{AD}$:$Tax1bp1_{(aa236-747)}$] was co-transformed into KGY37 with pDB179 [$Gal4_{BD}$:$hGrb14_{(aa1-540)}$]. Furthermore, no increase in ß-galactosidase activity or growth on SC medium lacking histidine (SC-T-L-H+5 mM 3-AT) was observed when pRA38[$Gal4_{AD}$:$Tax1bp1_{(aa236-747)}$] or pRA77[$Gal4_{AD}$:$Tax1bp1_{(aa1-747)}$] were co–transformed with pGBT9, indicating that the increased reporter gene activity was due to the presence of the $Gal4_{BD}$:Grb14 hybrid protein and not the $Gal4_{BD}$. (Figure 14).

Type II two-hybrid system false positives are $Gal4_{AD}$ hybrid proteins which activate reporter genes in the presence of any binding domain hybrid protein (Bartel *et al.*, 1993; Gietz *et al.*, 1997). The Tax1bp1 plasmids pRA38 ($Gal4_{AD}$:$Tax1bp1_{[aa563-747]}$) and pRA77 ($Gal4_{AD}$:$Tax1bp1_{[aa1-747]}$) were tested with the unrelated plasmid pRA4 ($Gal4_{BD}$:Rad18) to determine if they were type II false positives. In each case, the $Gal4_{AD}$:Tax1bp1 fusion proteins did not activate either reporter gene, showing that the interaction between Tax1bp1 and hGrb14 is specific in the two hybrid system. This encouraged me to pursue this interaction.

**Figure 13: Construction of pRA20 *[lexA:hGRB14]***

(A) A 1.6 kb *EcoR*I-*Sal*I Grb14 fragment was isolated from pDB202[Gal4$_{AD}$:hGrb14$_{(aa1-540)}$FLAG] and ligated with *EcoR*I-*Sal*I digested pBTM116[lexA$_{(aa1-149)}$], creating pRA20[lexA:hGrb14$_{(aa1-540)}$FLAG].

(B) The nucleotide sequence at the *lexA:hGRB14* fusion junction and the carboxyl-terminus of the fusion gene is shown. The reading frames of the *lexA* and the h*GRB14* ORF are represented as nucleotide triplets. The DNA sequence of a portion of the *lexA* and the pBTM116 MCS is italicized. The nucleotide sequence for *hGRB14* is in boldface. In addition, the nucleotide sequence of the FLAG epitope and the in-frame stop codon are listed. The *EcoR*I recognition sequence in pRA20 is underlined.

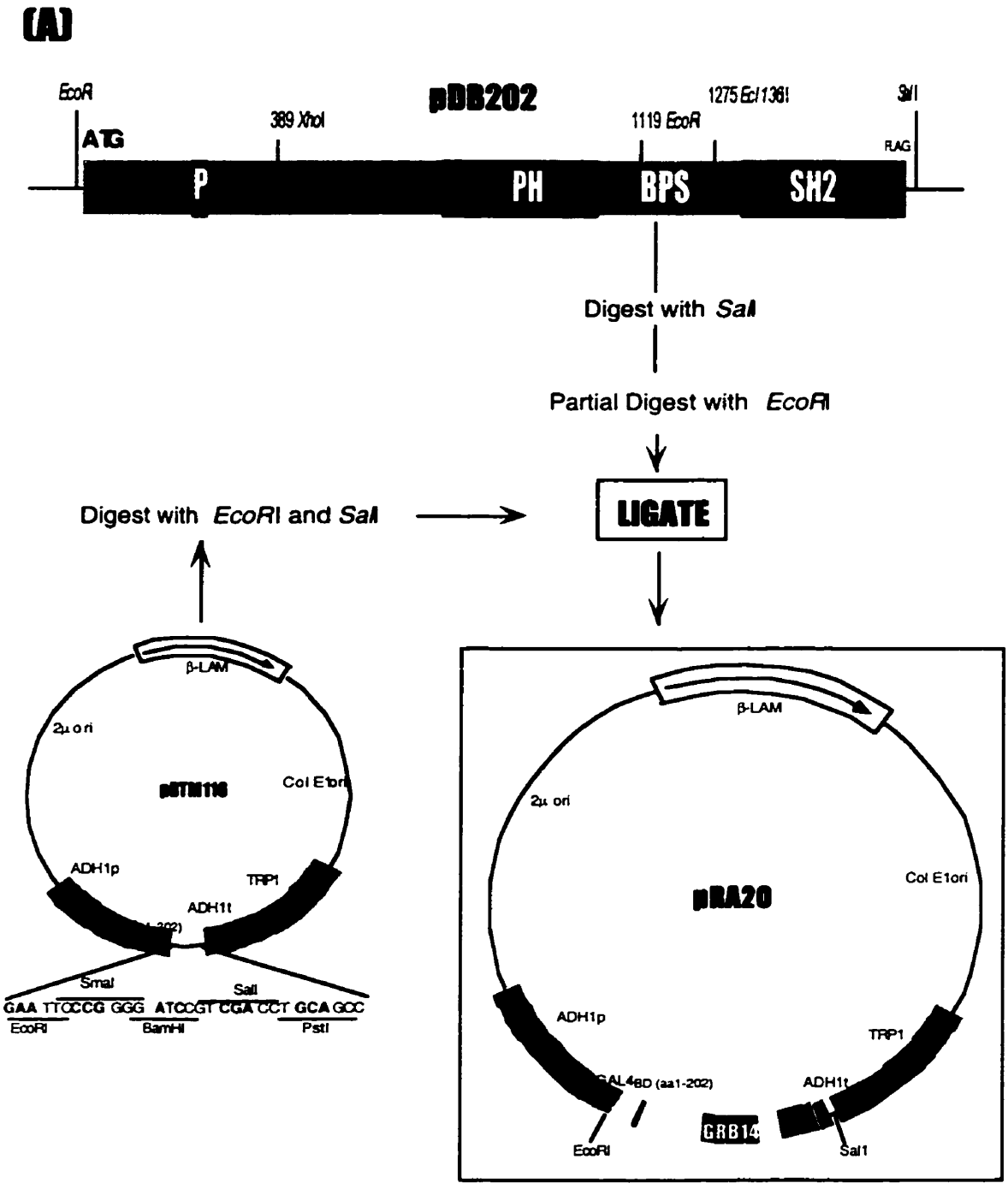
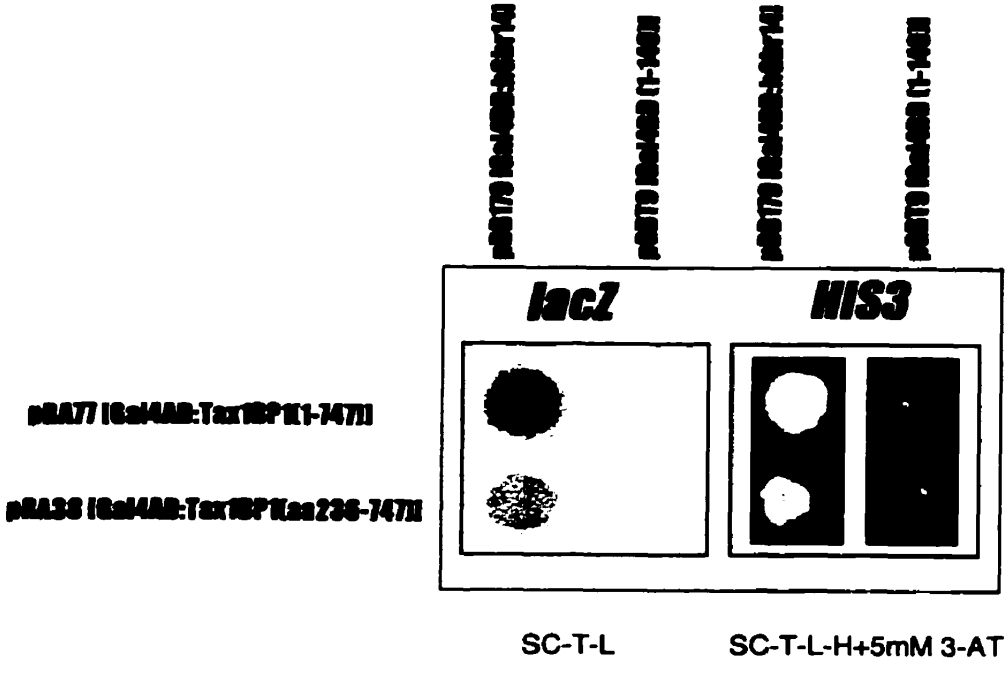

(A)
EcoR
ATG
pDB202
389 XhoI
1119 EcoR
1275 Ecl1361
SalI
FLAG
P
PH
BPS
SH2
Digest with Sal
Partial Digest with EcoRI
LIGATE
Digest with EcoRI and Sal
β-LAM
2μ ori
Col E1ori
ADH1p
TRP1
ADH1t
SmaI
SalI
GAA TTCCCG GGG ATCCGT CGA CCT GCA GCC
EcoRI
BamHI
PstI
pRA20
GAL4BD (aa1-202)
GRB14
EcoRI
SalI
(B)
GAA TTC ccg gag ctt aca ATG...GRB14...CGA GCA gac tac aag gac gat gac gac aag TGA
D Y K D D D D K
FLAG

**Figure 14: Tax1bp1 interacts with hGrb14 in the Two-Hybrid system**

The vectors pRA38 [$Gal4_{AD}$:$Tax1bp1_{(aa236-747)}$] or pRA77 [$Gal4_{AD}$:$Tax1bp1_{(aa1-747)}$] were co-transformed into KGY37 with either pDB179 [$GAL4_{BD}$:$hGrb14_{(aa1-540)}$FLAG] or pGBT9[$Gal4_{BD(aa1-149)}$] and grown onto SC-T-L media for 3 to 5 days. Transformants were patched onto SC-T-L and SC-T-L-H + 5 mM 3-AT and tested for *lacZ* or *HIS3* reporter gene activity using a filter assay or by growth SC medium lacking histidine, respectively. The results from the lacZ filter assay and the HIS3 reporter assays are presented in the respective panels. The $GAL4_{BD}$ plasmid present in each yeast colony is indicated on the top of each panel. The $GAL4_{AD}$:*TAX1BP1* plasmid present in each yeast colony is indicated on the left side.


lacZ
HIS3
SC-T-L
SC-T-L-H+5mM 3-AT

### *3.4.4 Tax1bp1 deletion analysis in the two-hybrid system*

Deletion mutants were constructed from the vector pRA38 (Figure 15B) to identify the domains of Tax1bp1 involved in hGrb14 binding.

#### *3.4.4.1 Construction of pRA83[Gal4$_{AD}$:Tax1bp1$_{(aa236-593)}$]*

The plasmid carrying a portion of the Tax1bp1 coiled-coil domain (Figure 15C) was constructed to determine if this domain was important for hGrb14 binding. This construct was produced by digesting the vector pRA38 with *Xho*I, followed with a partial digestion using the enzyme *Xba*I. The 9201 bp fragment lacking the Tax1bp1 LIM domain, was purified (section 2.17.2) and blunt-end ligated (section 2.18.2) onto itself to form vector pRA83 [Gal4$_{AD}$:Tax1bp1 $_{(aa\ 236\text{-}593)}$].

#### *3.4.4.2 Construction of pRA81 [Gal4$_{AD}$:Tax1bp1$_{(aa593-747)}$]*

The plasmid carrying a DNA fragment containing the LIM domain (Figure 15D) was also constructed to determine if this domain was important for hGrb14 association. This construct was produced by first digesting the plasmid pRA38 with *Nco*I; this was followed with a partial digestion with *Xba*I. The 8598 bp DNA fragment, containing the sequence encoding amino acids 593 to 747 of Tax1bp1, was isolated (section 2.17.2) and blunt-end ligated (section 2.18.2) on itself to form pRA81 [Gal4$_{AD}$:Tax1bp1$_{(aa593-747)}$]. To verify that the *TAX1BP1* reading frame was maintained with the *GAL4$_{AD}$*, the fusion junction was sequenced using a GAL4$_{AD}$ primer 5' GAAGATACCCCACCAAAC 3'.

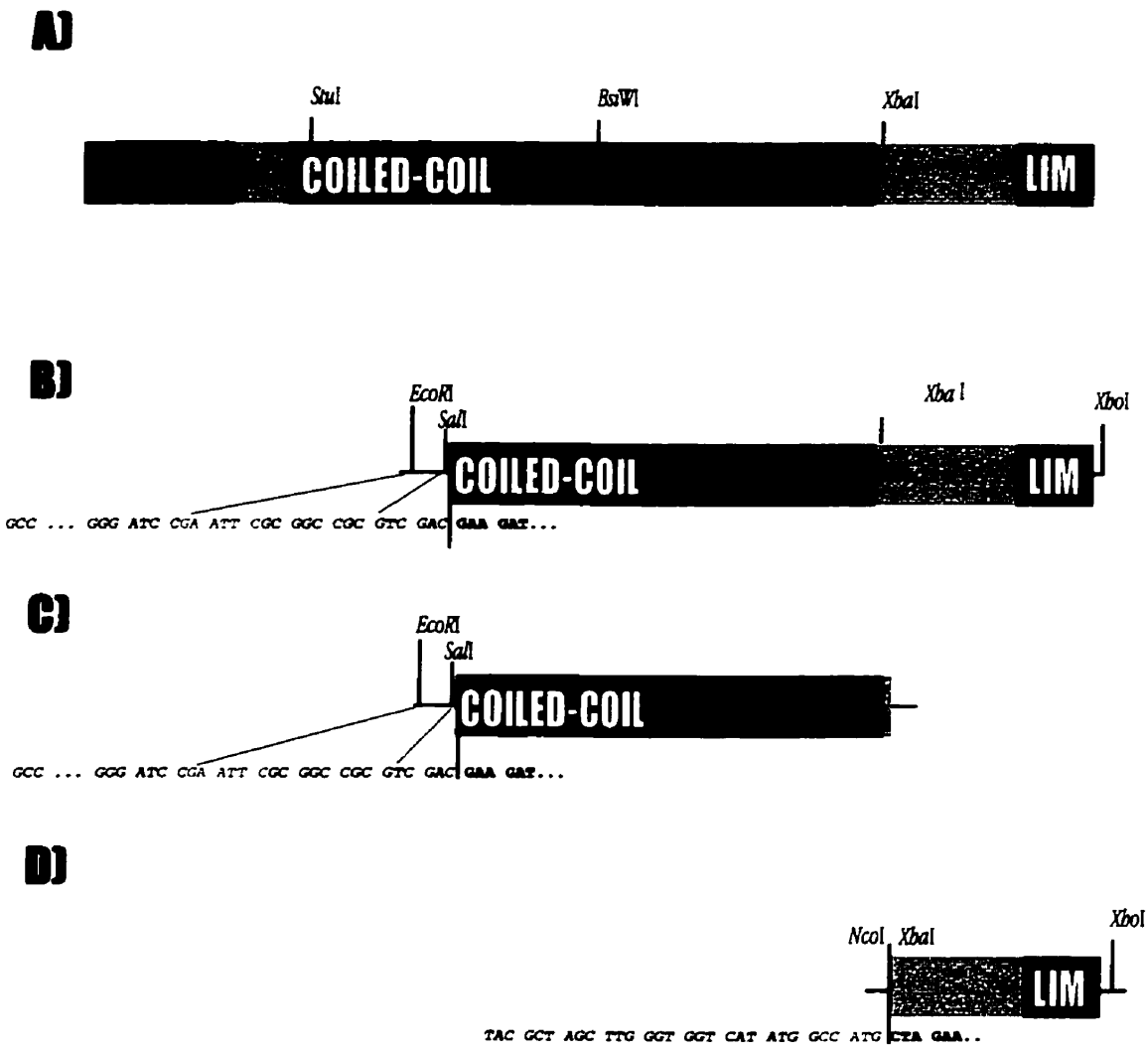
**Figure 15: Gal4$_{AD}$:Tax1bp1 deletion mutants**

(A) The positions of the putative coiled-coil domain, the LIM domain, and selected restriction sites, on the entire *TAX1BP1* ORF is illustrated.

(B) A representation of the Tax1bp1 fragment in the library vector pRA38 [Gal4$_{AD}$:Tax1bp1$_{(aa236-747)}$] is illustrated. The nucleotide sequence presented is found at the *GAL4$_{AD}$:TAX1BP1* fusion junction. Nucleotide triplets represent the *GAL4$_{AD}$* and *TAX1BP1* reading frames. The *GAL4$_{AD}$* and pACTII MCS nucleotide sequence is italicized. The *TAX1BP1* nucleotide sequence is in boldface.

(C) A representation of the Tax1bp1 fragment in the vector pRA83 [Gal4$_{AD}$:Tax1bp1$_{(aa236-593)}$] is illustrated. The nucleotide sequence presented is found at the *GAL4$_{AD}$:TAX1BP1* fusion junction. Nucleotide triplets represent the *GAL4$_{AD}$* and *TAX1BP1* reading frames. The *GAL4$_{AD}$* and pACTII MCS nucleotide sequence is italicized. The *TAX1BP1* nucleotide sequence is in boldface.

(D) A representation of the Tax1bp1 fragment in the vector pRA81 [Gal4$_{AD}$:Tax1bp1$_{(593-747)}$] is illustrated. The nucleotide sequence presented is found at the *GAL4$_{AD}$:TAX1BP1* fusion junction. Nucleotide triplets represent the *GAL4$_{AD}$* and *TAX1BP1* reading frames. The *GAL4$_{AD}$* and pACTII MCS nucleotide sequence is italicized. The *TAX1BP1* nucleotide sequence is in boldface.


A)
StuI
BsiWI
XbaI
COILED-COIL
LIM
B)
EcoRI
SalI
XbaI
XhoI
COILED-COIL
LIM
GCC ... GGG ATC CGA ATT CGC GGC CGC GTC GAC GAA GAT...
C)
EcoRI
SalI
COILED-COIL
GCC ... GGG ATC CGA ATT CGC GGC CGC GTC GAC GAA GAT...
D)
NcoI XbaI
XhoI
LIM
TAC GCT AGC TTG GGT GGT CAT ATG GCC ATG CTA GAA..

*3.4.4.3 The putative coiled-coil domain of Taxbp1 binds to hGrb14*

To define the region(s) of Tax1bp1 involved in hGrb14 binding, each Tax1bp1 deletion mutant was tested with the $Gal4_{BD}$:hGrb14 hybrid protein for reporter gene activation in the two-hybrid system. The vectors pRA81 [$Gal4_{AD}$:Tax1bp1$_{(aa593-747)}$], and pRA83 [$Gal4_{AD}$:Tax1bp1$_{(aa236-593)}$] were co-transformed into the yeast strain KGY37 with either pDB179 [$GAL4_{BD}$:hGrb14$_{(aa1-540)}$ FLAG] or pGBT9 [$Gal4_{BD(aa\ 1-149)}$] and grown onto SC-T-L media at 30 °C for 3 to 5 days. Individual transformants were subsequently patched onto SC-T-L or SC-T-L-H+5 mM 3-AT, grown for 1-2 days at 30°C, and assayed for ß-galactosidase activity (*lacZ* gene product) using the filter assay (section 2.19.1) or for growth stimulated by the activation of the *HIS3* gene product respectively. The results are presented in Figure 16A. Reporter gene activity was observed in the yeast strains containing the Tax1bp1 clones carrying the coiled-coil domain and hGrb14. No increase in reporter gene activity was observed in yeast strains harboring the plasmids encoding the Tax1bp1 LIM domain and hGrb14FLAG. The minimal Tax1bp1 fragment capable of interacting with hGrb14 is encoded by the amino acids 236 to 593 of the predicted coiled-coil domain. No increase in reporter gene activity was observed when each clone was tested against the empty vector pGBT9.

A measurement of ß-galactosidase activity was performed to quantitatively compare the levels of reporter gene activation induced by each Tax1bp1 hybrid protein (Figure 16B). The plasmids, pRA77, pRA38, pRA81, and pRA83 were co-transformed into KGY94 (Graham, 1996) with either pRA20 [lexA:hGrb14$_{(aa1-540)}$FLAG] or pBTM116 [lexA $_{(aa1-202)}$]. This yeast strain was used because it exhibited much higher

levels of ß-galactosidase activity, in response to protein interactions using the liquid ß-galactosidase assay than did KGY37. Furthermore, this strain exhibited low levels of background activity providing the best signal to noise ratio for this assay. Unlike KGY37, this strain utilizes the bacterial lexA operator to regulate the *lacZ* reporter gene. The lexA operator is recognized by the lexA (aa1-202) protein, which is encoded in the two-hybrid system plasmid pBTM116 (Figure 6).

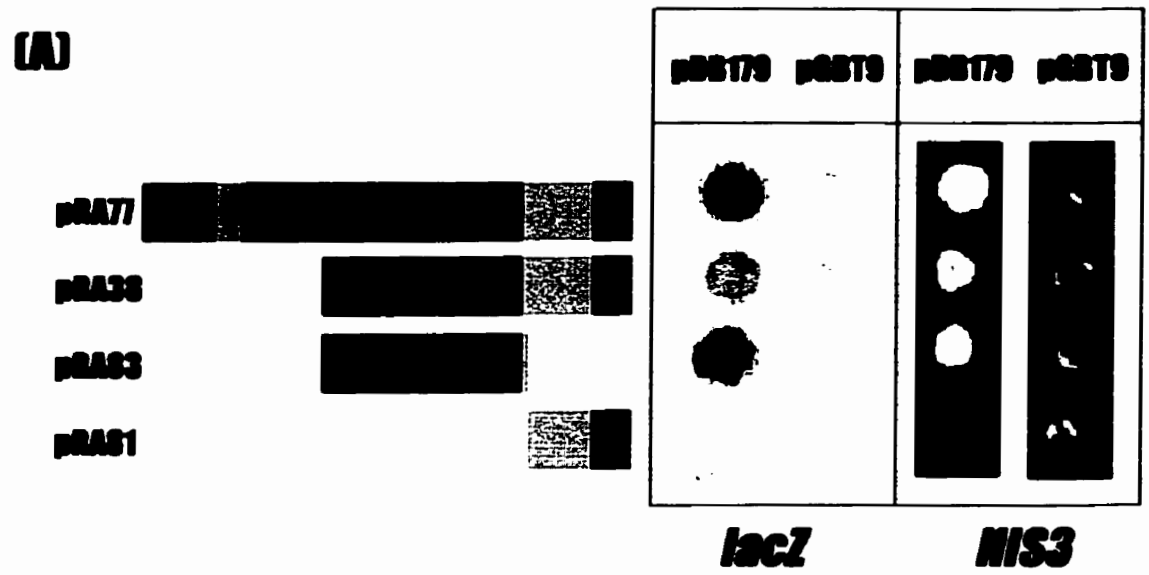
The ß-galactosidase activity was determined for each yeast strain. The highest levels of ß-galactosidase activity were found in yeast colonies containing the vectors pRA77 (18.89 Miller units), pRA38 (24.11 Miller units), and pRA81 (160.94 Miller units) which all contained at least a portion of the predicted coiled-coil domain. Interestingly, a lower level of ß–galactosidase activity (6.45 Miller units) was also observed with the fragment carrying the LIM domain, suggesting that this region may play a minor role in hGbr14 binding. Surprisingly, these results also suggest that the fragment containing the LIM domain may sterically hinder hGrb14 binding, since the removal of this fragment resulted in a 6.8 fold increase in ß–galactosidase activity, as compared to its parental derivative-pRA38. Whether or not protein folding or protein expression levels significantly contribute to these results are unknown.

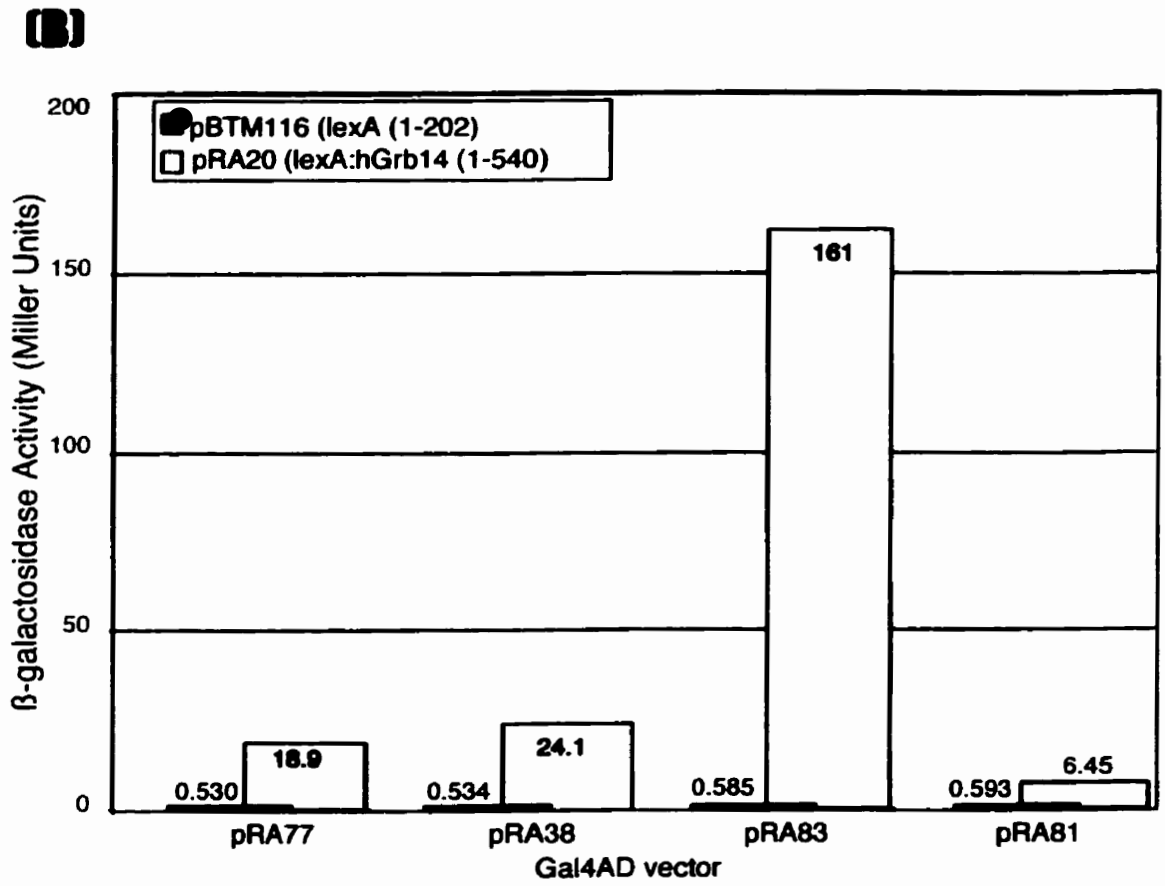
In short, these results indicate that the amino acids 236 to 593 of the predicted coiled-coil domain is important for hGrb14 binding, while amino acids 593 to 747 play a minor role in this interaction. Further deletion analysis in the two-hybrid system will be needed to refine the boundaries involved in hGrb14 binding.

**Figure 16: The predicted coiled-coil domain binds to hGrb14.**

(A) The ß-galactosidase activity was determined, using the filter assay, for the each *GAL4$_{AD}$:TAX1BP1* plasmid in the strain KGY37 harboring pDB179[Gal4$_{BD}$:hGrb14]. The vectors pRA77, pRA38, pRA83, and pRA81 were co-transformed into the strain KGY37, with either pDB179 or pGBT9 and grown onto SC-T-L media for 3 to 5 days. The results from the lacZ filter assay and the HIS3 reporter assays are found in their respective panels. Representations of the Tax1bp1 fragment encoded by the indicated vectors are located to the left of the panels. Each colony represents KGY37 harboring the indicated plasmids. In the lacZ panel, the filter assay was continued for 60 min. Cells expressing the ß-galactosidase protein convert X-Gal into a blue compound. The yeast cells tested for HIS3 reporter activity were grown for 8 days at 30°C on SC-T-L-H + 5mM 3-AT medium. The small amount of cells seen with each strain carrying pGBT9 represents the initial inoculum and not growth. **pDB179** [Gal4$_{BD}$:hGrb14 $_{(aa1-540)}$FLAG]; **pGBT9**(Gal4$_{BD(aa1-149)}$); **pRA77**[Gal4$_{AD}$:Tax1bp1$_{(aa1-747)}$]; **pRA38**[Gal4$_{AD}$:Tax1bp1$_{(aa236-747)}$]; **pRA83**[Gal4$_{AD}$:Tax1bp1$_{(aa236-593)}$]; **pRA81**[Gal4$_{AD}$:Tax1bp1$_{(aa593-747)}$].

(B) Quantitative analysis of ß-galactosidase activity was performed using the liquid ß–galactosidase assay. The vectors pRA77, pRA38, pRA83, and pRA81 were co-transformed into the yeast strain KGY94, with the vectors pRA20 or pBTM116 and grown onto SC-T-L media for 3 to 5 days. The ß-galactosidase assays were repeated four times using four separate transformants. The value listed is an average of the four assays. The calculation of ß-galactosidase activity, measured in Miller Units, was performed as previously described. **pBTM116** [lexA$_{(aa1-202)}$]; **pRA20** [lexA:hGrb14$_{(aa1-540)}$FLAG]; **pRA77**[Gal4$_{AD}$:Tax1bp1$_{(aa1-747)}$]; **pRA38**[Gal4$_{AD}$:Tax1bp1$_{(aa236-747)}$]; **pRA83**[Gal4$_{AD}$:Tax1bp1$_{(aa236-593)}$]; **pRA81**[Gal4$_{AD}$:Tax1bp1$_{(aa593-747)}$].


(A)
pRA77
pRA38
pRA83
pRA81
lacZ
HIS3


(B)
pBTM116 (lexA (1-202)
pRA20 (lexA:hGrb14 (1-540)
ß-galactosidase Activity (Miller Units)
200
150
100
50
0
0.530
18.9
0.534
24.1
0.585
161
0.593
6.45
pRA77
pRA38
pRA83
pRA81
Gal4AD vector

### *3.5 Tax1bp1 self-associates in the two-hybrid system*

Tax1bp1 self–association was investigated since coiled-coil domains have been shown to mediate protein multimerization (Jin *et al.*, 1998; Málnási-Csizmadia *et al.*, 1998; Sternsdorf *et al.*, 1997).

#### *3.5.1 Construction of pRA88 [lexA:Tax1bp1$_{(aa1-747)}$]*

A *lexA:TAX1BP1* fusion gene was constructed to investigate Tax1bp1 self–association. The *TAX1BP1* ORF was PCR amplified (section 2.16) with the primers: RAO8 - 5' CC GAA TTC ATG ACA TCC TTT CAA GAA 3' and RAO5 - 5' GCG GGA TCC CTA GTC AAA ATT TAG AAC ATT CTG 3' using pRA66 as a template. The amplified fragment was digested with *EcoR*I and *BamH*I, and ligated (section 2.18.1) to *EcoR*I–*BamH*I digested pBTM116 creating the vector pRA88 (Figure 17).

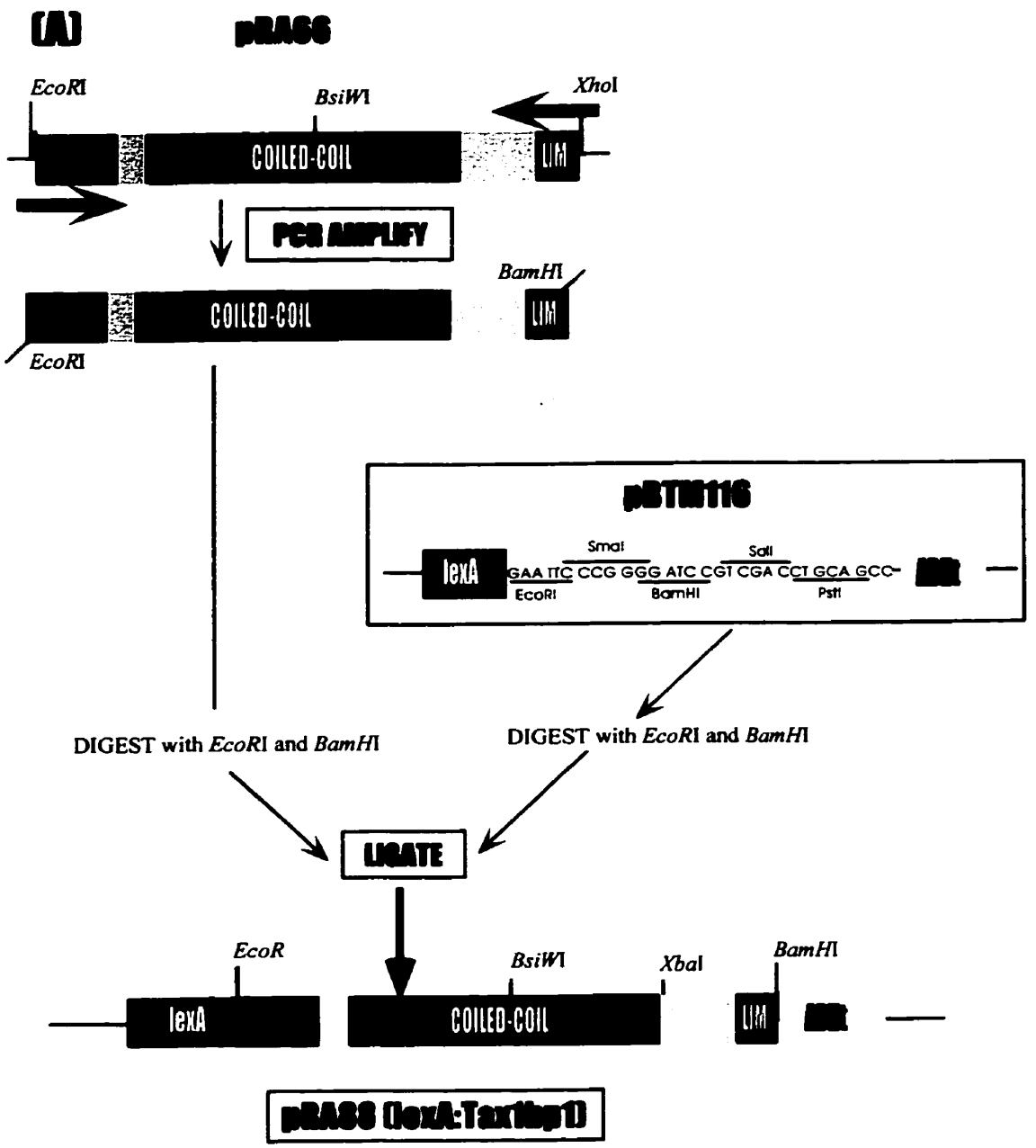
#### *3.5.2 Tax1bp1 self-association is mediated by its coiled-coil domain*

To test for Tax1bp1 self-association, the vectors pRA77[Gal4AD:Tax1bp1$_{(aa1-747)}$], pRA83[Gal4AD:Tax1bp1$_{(aa236-593)}$], and pRA81[Gal4AD:Tax1bp1$_{(aa593-747)}$] were co-transformed (section 2.6) into the KGY94 with either pRA88[lexA:Tax1bp1$_{(aa1-747)}$] or pBTM116[lexA$_{(aa1-202)}$], and grown onto SC–T-L media for 3 to 4 days. Transformants were then patched onto the same medium, grown for 1 to 2 days at 30 °C, and assayed for ß–galactosidase activity using a filter assay (section 2.19.1). Figure 18 shows that ß-galactosidase activity was present only in the strains carrying the GAL4$_{AD}$ vectors encoding the coiled-coil domain and pRA88[lexA:Tax1bp1$_{(aa1-747)}$]. These results show that Tax1bp1 can self-associate in the two-hybrid system, and that a carboxyl-terminal portion of its putative coiled-coil domain (amino acids 236-593) can mediate this interaction.

**Figure 17: Construction of pRA88 [*lexA:TAX1BP1*]**

A. The *TAX1BP1* ORF was PCR amplified, with the primers RAO8 (1) and RAO5 (2) using pRA66 as a template. The PCR products were purified, digested with *EcoR*I and *BamH*I, and ligated with *EcoR*I-*BamH*I digested pBTM116. This gave rise to pRA88, which encodes the lexA:Tax1bp1$_{(aa1-747)}$ hybrid protein.

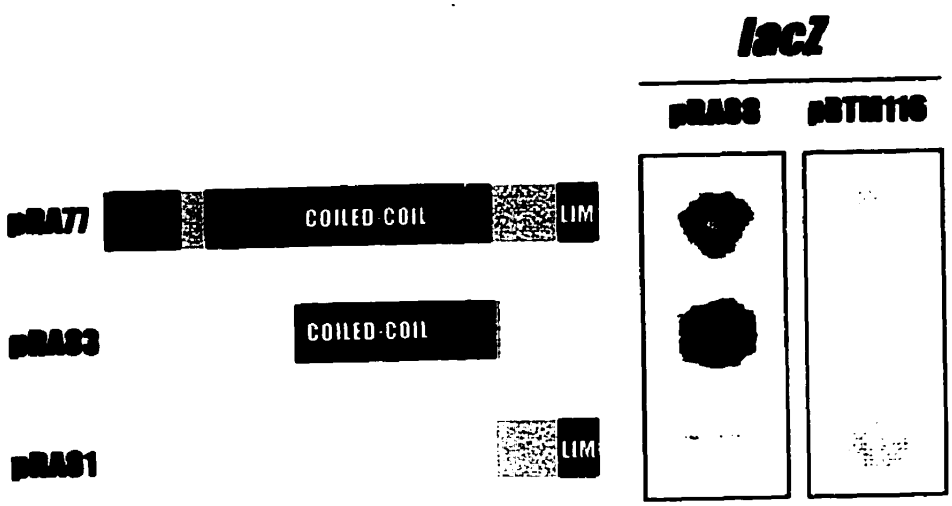
B. The nucleotide sequence from *lexA:TAX1BP1* fusion junction in the plasmid pRA88 is shown. Nucleotide triplets represent the *lexA* and *TAX1BP1* reading frames. The nucleotide sequence of the *lexA* gene and pBTM116 MCS are italicized. The *TAX1BP1* nucleotide sequence is in boldface.




(B) *GTT ATT CGC AAC GGC GAC TGG CTG GAA TTC* ATG ACA TCC

**Figure 18: The putative coiled-coil domain mediates Tax1bp1 dimerization.**

The ß-galactosidase activity was determined for the each *GAL4$_{AD}$:TAX1BP1* plasmid using the filter assay. The results from the lacZ filter assays are shown in the panels presented. A representation of the Tax1bp1 fragment encoded by their respective vector is found to the left of the panel. The *lexA* plasmids found in each yeast strain are indicated on the top of each panel. The *GAL4$_{AD}$:TAX1BP1* plasmid present in each yeast colony is indicated on the left of the panels. Each colony represents the yeast strain KGY94 co-transformed with the indicated plasmids. For each panel, the lacZ filter assay was continued for 60 min. Cells activating the *lacZ* reporter gene can convert X-Gal into a blue compound. **pRA88** [lexA:Tax1bp1$_{(aa1-747)}$]; **pBTM116** [lexA$_{(aa1-202)}$]; **pRA77** [Gal4$_{AD}$:Tax1bp1$_{(aa1-747)}$]; **pRA83** [Gal4$_{AD}$:Tax1bp1$_{(aa236-593)}$]; **pRA81** [Gal4$_{AD}$:Tax1bp1$_{(aa593-747)}$].


lacZ
COILED-COIL
LIM
COILED-COIL
LIM

### *3.6 The hGrb14:Tax1bp1 interaction in mammalian cells*

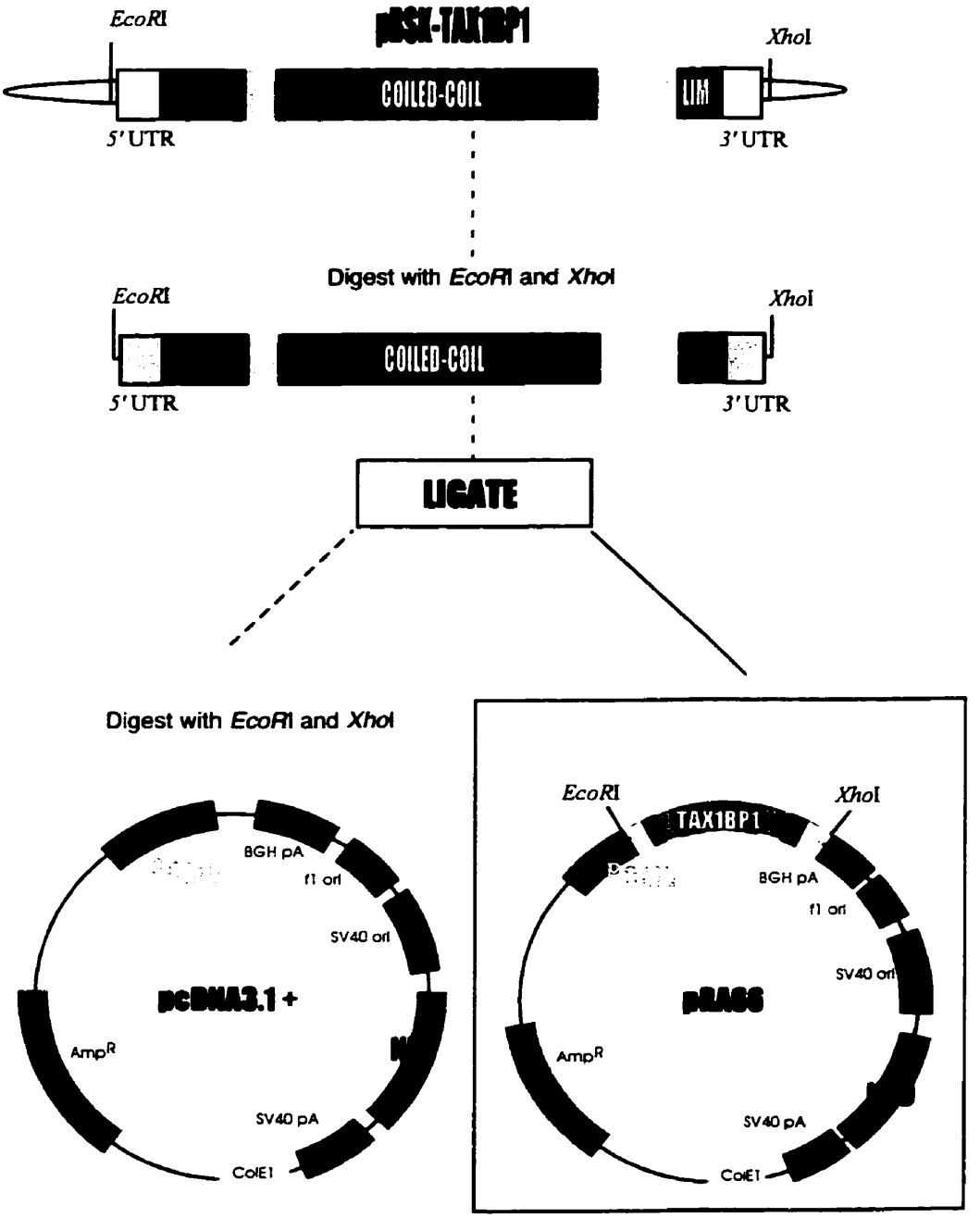
To substantiate the Tax1bp1:hGrb14 interaction observed in the yeast two-hybrid system, an alternative approach using cultured mammalian cells was taken. The CHO26 cell line (Hemming *et al.*, unpublished results) which overexpresses both the human IR and human Grb14FLAG was the cell line chosen to reconfirm the interaction. The CHO26 cell line was transfected with a plasmid allowing constitutive Tax1bp1 protein expression. Co–immunoprecipitation was then used to confirm the hGrb14:Tax1bp1 interaction in these cells.

#### *3.6.1 Construction of pRA66 [CMVp:Tax1bp1$_{(aa1-747)}$]*

The *TAX1BP1* cDNA was cloned into the plasmid pcDNA3.1 (Figure19), which uses the constitutively active CMV (cytomegalovirus) promoter, to obtain detectable levels of Tax1bp1 protein in the cultured cells. The vector pBluescript SK(-)*TAX1BP1* (A gift from Dr. K.T. Jeung, NIH, Bethesda, M.D) was digested with *EcoR*I and *Xho*I, releasing a 2.4 kb fragment carrying entire *TAX1BP1* cDNA. The fragment was then purified (section 2.17.2) and ligated (section 2.18.1) into the *EcoR*I and *Xho*I sites of pcDNA3.1 giving rise to pRA66.

**Figure 19: Construction of pRA66 [*CMVp:TAX1BP1*]**

The construction of the plasmid pRA66 is illustrated. The vector pBluescript SK(–)*TAX1BP1*, containing the entire *TAX1BP1* cDNA, was digested with the restriction enzymes *EcoR*I and *Xho*I, liberating a 2.4 kb fragment containing 5' and 3' UTR and the *TAX1BP1* ORF. This 2.4 kb fragment was purified and ligated with *EcoR*I-*Xho*I digested pcDNA3.1 creating the vector pRA66.


EcoRI
pBSK-TAX1BP1
XhoI
COILED-COIL
LIM
5'UTR
3'UTR
Digest with EcoRI and XhoI
EcoRI
XhoI
COILED-COIL
5'UTR
3'UTR
LIGATE
Digest with EcoRI and XhoI
BGH pA
f1 ori
SV40 ori
pcDNA3.1+
AmpR
SV40 pA
ColE1
EcoRI
XhoI
TAX1BP1
BGH pA
f1 ori
SV40 ori
pRAGS
AmpR
SV40 pA
ColE1

### *3.6.2 hGrb14 co-immunoprecipitates with Taxlbp1*

Co-immunoprecipitation assays were performed using cell lysates from CHO26 cells transiently expressing Taxlbp1. The CHO26 cell line was selected since no other cell lines expressing detectable levels of Grb14 were available. The CHO26 cell line, overexpressing the IR and hGrb14FLAG, was transfected (section 2.20) with pRA66. The cells were serum starved in HAM's F12 medium for 24 hrs, stimulated with 100 nM insulin for 7 min, and lysed (section 2.22). Taxlbp1 was immunoprecipitated from the cell lysates (section 2.24) with 1 μg of polyclonal anti–Taxlbp1 antibody (A gift from Dr. K.T. Jeung, NIH, Bethesda, MD). The immunoprecipitates were separated using SDS-PAGE (section 2.25), transferred to nitrocellulose (section 2.26.1), and immunoblotted with the M2 anti-FLAG monoclonal antibody (Kodak, USA) to detect the FLAG tagged hGrb14 protein.

Figure 20 shows that hGrb14 co-immunoprecipitates with Taxlbp1 in CHO26 cells transiently expressing Taxlbp1. This is evident because the hGrb14FLAG protein (predicted Mr=62.2 kDa), detected in the control lysate (Lane A), co–migrates with a protein beleieved to be Grb14FLAG immunoprecipitated by the Taxlbp1 antibody from cell lysates overexpressing both hGrb14FLAG and Taxbp1 (Lane D). This band is not

detected in the immunoprecipitates obtained from the cell lines not expressing hGrb14:FLAG indicating that hGrb14 co-immunoprecipitates with the Tax1bp1 protein. The large smear in lanes B through D, found below the hGrb14 band, represents the detection of the anti-Tax1bp1 heavy chain. Together, the hGrb14:Tax1bp1 interaction observed in the two-hybrid system, and the preliminary results observed in this assay, support the hypothesis that hGrb14 and Tax1bp1 interact in a cellular system. Whether this interaction can be observed at physiological levels in insulin responsive tissues is currently unknown.

Immunoprecipitation of Grb14:FLAG using the M2 antibody was also performed to determine if Tax1bp1 could co-immunoprecipitate with Grb14:FLAG (Figure 21). Conclusions to from this data were difficult to interpret as the 86 kDa band was found in both the negative control and the experimental lanes. Whether this band represents Tax1bp1 is currently unknown. Further analysis with another antibody specific for Tax1bp1 is necessary to verify the Tax1bp1:hGrb14 interaction.

The effect of insulin on the hGrb14:Tax1bp1 interaction in mammalian cells is currently unknown. The results presented in Figure 20 lane D suggest that insulin may be necessary for this interaction. However, this experiment is lacks immunoprecipitates

from unstimulated CHO26 cell expressing Tax1bp1. Unfortunately, an experiment characterizing this interaction in the absence and presence of insulin stimulation was not performed due to time constraints. Similarly, experiments to determine if non-specific binding of hGrb14FLAG to the Tax1bp1 antibody or the sepharose beads needs to be determined.

The results observed in the two-hybrid system indicate that this interaction is not dependent on the presence of the activated IR in yeast. The effect of IR activation on the hGrb14:Tax1bp1 interaction in mammalian systems may not be consistent with the results observed in the two-hybrid system since IR activation may indirectly regulate this interaction through cellular proteins not expressed in the yeast system. Experiments determining if insulin stimulation facilitates, abrogates, or has no role in the Tax1bp1:hGrb14 interaction are necessary to answer this question.

**Figure 20: hGrb14 co-immunoprecipitates with Tax1bp1.**

Cell lysates from CHO-IR cells, CHO-IR cells transfected with pRA66 (CMVp–*TAX1BP1*), and CHO26 cells transfected with pRA66 (CMVp-*TAX1BP1*) were immunoprecipitated (IP) with 1 μg of polyclonal anti-Tax1bp1 antibody. The immunoprecipitates were captured with protein-G-Sepharose beads, separated by SDS–PAGE, and transferred to nitrocellulose. The membrane was treated with a 1:3000 dilution of the M2 monoclonal anti-FLAG antibody. The FLAG antibody was detected by ECL using a goat anti-mouse HRP conjugated antibody.

**Lane A**: Cell lysate from CHO26 cells expressing Tax1bp1.

**Lane B**: Anti-Tax1bp1 immunoprecipitates from CHO-IR cell lysate.

**Lane C**: Anti-Tax1bp1 immunoprecipitates from serum starved (-) CHO-IR cells expressing Tax1bp1.

**Lane D**: Anti-Tax1bp1 immunoprecipitates from insulin-treated (+) CHO26 (CHO-IR + hGrb14FLAG) expressing Tax1bp1.

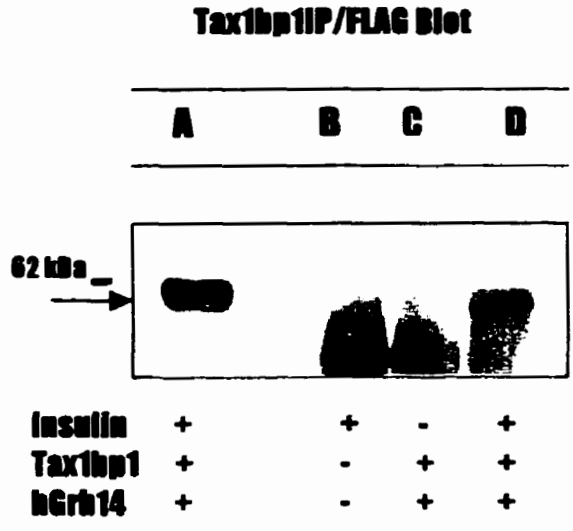

Tax1bp1IP/FLAG Blot
A
B
C
D
62 kDa
Insulin + + - +
Tax1bp1 + - + +
hGrb14 + - + +

**Figure 21: Evidence of Tax1bp1 from hGrb14FLAG immunoprecipitates.**

Cell lysates from CHO-IR cells, CHO-IR cells transfected with pRA66 (CMVp–*TAX1BP1*), and CHO26 cells transfected with pRA66 (CMVp-*TAX1BP1*) were immunoprecipitated (IP) with 1 μg of monoclonal M2 anti-FLAG antibody. The immunoprecipitates were captured with protein-G-Sepharose beads, separated by SDS–PAGE, and transferred to nitrocellulose. The membrane was treated with a 1:1000 dilution of the polyclonal anti-tax1bp1 antibody. The secondary polyclonal anti-tax1bp1 antibody was detected by ECL using a goat anti-mouse HRP conjugated antibody.

**Lane A**: Cell lysate from CHO26 cells expressing Tax1bp1.

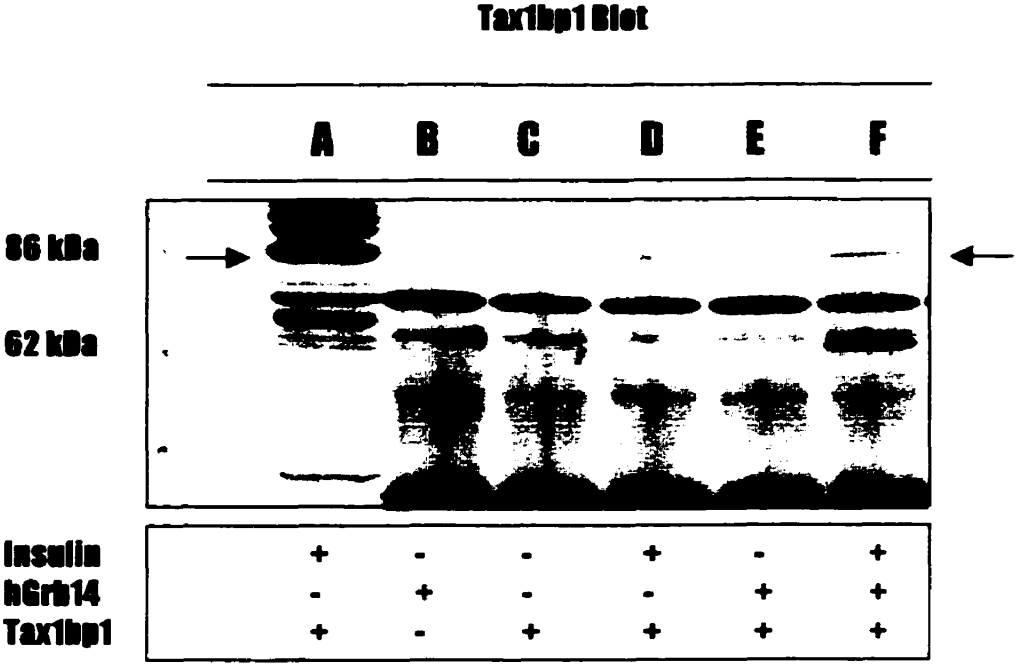
**Lane B**: M2 anti-FLAG immunoprecipitates from untransfected CHO-IR cell lysates.

**Lane C**: M2 anti-FLAG immunoprecipitates from serum starved (-) CHO-IR cells expressing Tax1bp1.

**Lane D**: M2 anti-FLAG immunoprecipitates from insulin stimulated (+) CHO-IR cells expressing Tax1bp1

**Lane E** M2 anti-FLAG immunoprecipitates from serum starved (-) CHO26 (CHO-IR + hGrb14FLAG) expressing Tax1bp1

**Lane F** M2 anti-FLAG immunoprecipitates from insulin stimulated (+) CHO26 (CHO-IR + hGrb14FLAG) expressing Tax1bp1.


Tax1bp1 Blot
A
B
C
D
E
F
86 kDa
62 kDa
Insulin
hGrb14
Tax1bp1
+ - - + - +
- + - - + +
+ - + + + +

### *3.7 Tax1bp1 as a signaling molecule*

The interaction observed with hGrb14, a protein involved in the insulin signal, and Tax1bp1, in both the yeast two-hybrid and mammalian systems suggests that Tax1bp1 may partake in the regulation of the intracellular insulin signal through its interaction with hGrb14. Grb14, which has been shown to directly interact with the IR (Kasus-Jacobi *et al.*, 1998, Hemming *et al.*, unpublished results), can down-regulate glycogen synthesis and decrease thymidine incorporation into DNA when it is overexpressed in CHO-IR cells (Kasus-Jacobi *et al.*, 1998). This suggests that Grb14 may be involved in down-regulating the insulin signaling pathway. Tax1bp1's interaction with hGrb14 may implicate it, through association, in the regulation of glycogen synthesis or mitogenesis. Whether Tax1bp1 participates in these or any cellular events regulated by the insulin signal is currently unknown.

Tax1bp1 has also been shown to interact with A20, an anti-apoptotic protein implicated in TNF (tumor necrosis factor) signaling (Opipari *et al.*, 1992; Van Antwerp *et al.*, 1996; Beg and Baltimore, 1996; Natoli *et al.*, 1998; De Valck *et al.*, 1999). The A20 gene encodes a 790 amino acid protein containing seven carboxyl-terminal zinc-finger motifs (Opipari *et al.*, 1990). The A20 protein is found downstream of Traf1 (Song *et al.*, 1996) and Traf2 (Heyninck *et al.*, 1999) which belong to the Traf protein family. Coincidentally, Traf proteins also carry an internal coiled-coil domain, flanked by two globular domains; one of which, like the Tax1bp1 LIM domain, bind zinc ions.

The Taxbp1 protein was found to interact with the amino acids 373 to 790 of A20, the region containing the zinc-finger heptad (De Valck *et al.*, 1999). Like A20, Tax1bp1 appears to be a barrier for apoptosis in specific cell lines. When overexpressed, Tax1bp1 was shown to inhibit TNF and Fas induced apoptosis in NIH3T3 cells (De Valck *et al.*, 1999). In addition, Tax1bp1 is degraded *in vivo* during apoptosis. This may be mediated by an apoptotic cysteine protease, as three of these proteases, caspase-3, caspase-6, and caspase-7 were capable of degrading Tax1bp1 *in vitro* (De Valck *et al.*, 1999). The mechanism which Tax1bp1 inhibits apoptosis is currently unknown.

Tax1bp1's association with hGrb14, an insulin signaling molecule, and A20, a TNF signaling molecule suggests that cross-talk between both signaling pathways may be occurring. The anti-apoptotic properties of Tax1bp1 may be relevant to the insulin signaling pathway since insulin has also been shown to inhibit apoptosis (Rodriguez-Tarduchy *et al.*, 1992; Rampalli and Zellenka, 1995;Tanaka *et al.*, 1995). Tax1bp1 degradation through the insulin signaling pathway may be mediated by caspase-3 since insulin stimulated caspase-3 activation can occur through the PI-3 kinase pathway (Godbout *et al.*, 1999). Furthermore, the association of Tax1bp1 with hGrb14 may also implicate hGrb14 in programmed cell death. This possibility is supported by evidence presented by Nantel *et al.* (1998) who have shown that Grb10, a Grb14 homologue and an IR binding partner, also participates in apoptosis. Studies characterizing the role of Tax1bp1 in cell signaling may aid in defining Grb14's role in the insulin signaling pathway.

### *3.8 Gene Structure of TAX1BP1*

The *TAX1BP1* gene structure was produced *in silico* as the sequence of the *TAX1BP1* cDNA and the genomic regions encoding the *TAX1BP1* cDNA was known. The Genbank database was screened with the *TAX1BP1* cDNA identifying two bacterial artificial chromosomes (BAC) clones (Accession number: RG318C11 & RG459N13) that mapped to chromosomal region 7p14-7p15. The cDNA sequence was compared to the DNA sequences of both BAC clones using the BLAST 2.0 algorithm (Altschul *et al.*, 1997) to identify the identical regions. The intron-exon boundaries were identified by manual inspection using the consensus splice donor and acceptor sites (Table 8) giving rise to the *TAX1BP1* gene structure. The *TAXBP1* gene contains 16 exons ranging from 46 to 225 nucleotides in length that span a 70 kb region (Figure 22).

The OMIM (Online-Mendelian Inheritance of Man) database (http://www3.ncbi.nlm.nih.gov/Omim/) was used to identify genetic diseases related to diabetes that mapped to the chromosomal location encoding the *TAX1BP1* gene (7p14–7p15). One form of type II diabetes mapped to the chromosomal region 7p13-7p15 (Elbein *et al.*, 1993). The susceptibility gene, mapped to this region called *MODY1* (Maturity Onset Diabetes of the Young), is associated with early-onset type II diabetes.

The defective gene is believed to be Glucokinase (Stoffel *et al.*, 1992; Froguel *et al.*, 1993; Sun *et al.*, 1993), a protein involved in glucose-6-phosphate synthesis. This carbohydrate precursor is necessary for glycogen synthesis, and is also an important molecule regulating pancreatic insulin secretion (Gidh-Jain *et al.*, 1993; German *et al.*, 1993; Sun *et al.*, 1993). Whether Tax1bp1 is involved in the onset of NIDDM is currently unknown.

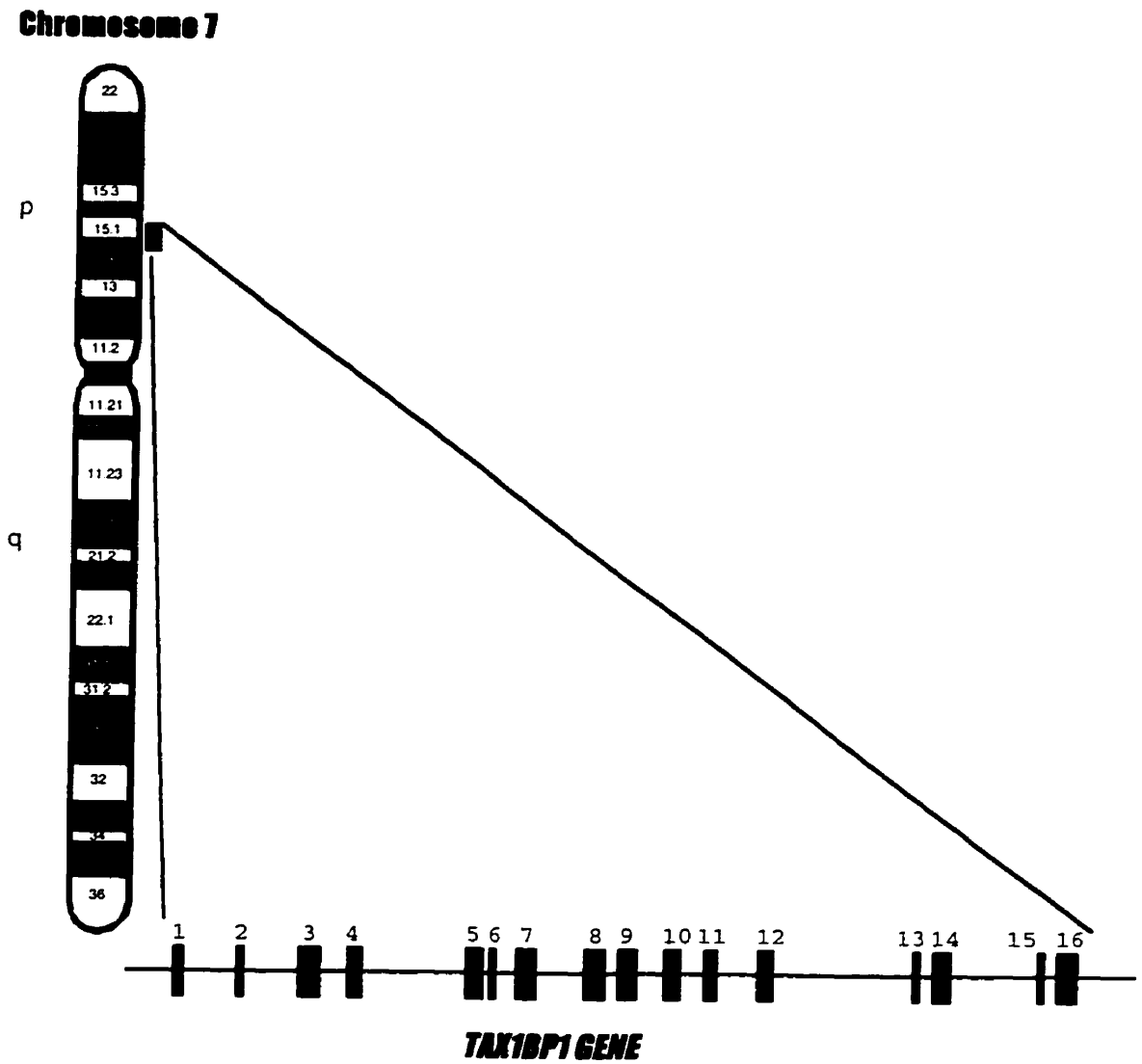
**Table 8: TAX1BP1 intron-exon boundaries**

| Exon # | Exon Size(bp) | Splice Acceptor | Splice Donor |
|---|---|---|---|
| **1** | 162 | 5′ UTR | TATTCAAG**gt**aagaaa |
| **2** | 103 | ccctttt**ag**GTTGGATG | ATTCCAAG**gt**aaggac |
| **3** | 188 | cctctt**ag**GATATTAC | TTCTTGAG**gt**tggtgt |
| **4** | 159 | ctttat**ag**TTGAAAAT | AACAAAAG**gt**tagttg |
| **5** | 149 | cttctc**ag**GGTCTTAC | TTAGACAG**gt**atttct |
| **6** | 91 | tatctt**ag**TTTAAAGG | TTTATAAG**gt**aattta |
| **7** | 186 | cattac**ag**GTACATTT | CAAGTAAA**gt**aagtac |
| **8** | 225 | ttcttc**ag**GAAGATAC | AAGATCAG**gt**aaaaca |
| **9** | 147 | ctttct**ag**GACAAGAC | ATCAATCA**gt**aagtat |
| **10** | 124 | ttgaat**ag**GCTAATAA | TTCTGCAG**gt**aaaaat |
| **11** | 104 | ttctac**ag**CAGAGGCA | TCTTGCAG**gt**aagtta |
| **12** | 126 | tcatttt**ag**GATGAGAA | ATTATAAA**gt**aagttt |
| **13** | 46 | aattttt**ag**GAACTTAA | AATGGAAG**gt**cagaat |
| **14** | 149 | tattac**ag**ATGGAGCA | TAGCAAA**gt**aaattg |
| **15** | 83 | tcccct**ag**GAAGATGA | GATTCCAG**gt**agtttt |
| **16** | 199 | tctttaa**g**CTTTGATG | 3′ UTR |

Intron-Exon Boundaries of Tax1bp1. Intron-Exon boundaries were determined by comparing the cDNA and a genomic sequence of *TAX1BP1*. Uppercase characters indicate exon DNA sequence. Lower case characters represent intron DNA sequences. Boldface characters represent the donor-acceptor splice dinucleotides. The *TAX1BP1* gene contains 16 exons, which range in size from 46-225 bp that span a region of approximately 70 kb.

**Figure 22: The gene structure of *TAX1BP1*.**

An ideogram of chromosome 7 is illustrated. The genetic position of the *TAX1BP1* gene (7p14-15) is indicated in by the vertical bar The predicted gene structure of *TAX1BP1* is also shown. The exons of the *TAX1BP1* gene are indicated (▮).


Chromosome 7
p
q
22
15.3
15.1
13
11.2
11.21
11.23
21.2
22.1
31.2
32
36
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
TAX1BP1 GENE

# 4. CONCLUSION

To help define hGrb14's cellular function, a modified two-hybrid screen was utilized to identify hGrb14 binding partners. A novel interaction involving hGrb14 and Tax1bp1 was identified and characterized. Tax1bp1 is a 747 amino acid protein encoded by a gene mapped to 7p14-7p15. The Tax1bp1 protein appears to belong to a novel protein family that includes Ndp52. Tax1bp1 and Ndp52 share several domains including a novel amino-terminal domain, an internal coiled-coil domain, and a carboxyl–terminal LIM domain. Furthermore, Tax1bp1's association with hGrb14, a protein implicated in insulin signaling, and with A20, a protein implicated in TNF signaling, suggest that Tax1bp1 is a signaling molecule. Tax1bp1 has been shown to exhibit anti-apoptotic properties, a trait which may be of relevance in the insulin signaling pathway, as insulin has also been shown to inhibit apoptosis (Rampalli and Zellenka, 1995;Tanaka *et al.*, 1995). Tax1bp1's possible role in apoptosis may also implicate hGrb14 in programmed cell death; a likely possibility, since Grb10 was also found to participate in apoptosis (Nantel *et al.*, 1998). New studies will be required to determine if hGrb14 has an effect on cellular survival.

The biological relevance of the Tax1bp1:hGrb14 interaction to the cell, and more importantly to the multicellular organism is unclear. This interaction may belong to a previously uncharacterized insulin signaling transduction pathway involved in cellular survival. Understanding hGrb14 and Tax1bp1 should provide new insights into the cellular mechanisms regulated by insulin, which will hopefully lead to the identification of the cellular agents involved NIDDM.

# 5. APPENDIX

## *5.1 Bacterial Media*

The bacterial medium was prepared as described by Sambrook *et al.* (1991) in a 600 ml volume carrying the appropriate components. The medium was autoclaved for 20 min at 121°C and 15 pounds per square inch.

### *5.1.1 Luria Bertani (LB)*

| Components | Mass (g) per 600 ml | Final Concentration g/L |
|---|---|---|
| Difco Bacto-yeast extract | 3 | 5 g/L |
| Difco Bacto-tryptone | 6 | 10 g/L |
| NaCl | 6 | 10 g/L |
| *Difco Bacto-agar | 9 | 16.67 |

Luria Bertani medium was used to grow *E.coli*. The above ingredients were added to distilled water to a final volume of 600 mls. The pH was titrated to 7.0. The antibiotics were added after sterilization. Ampicillin was added to a final concentration of 50μg/ml. The antibiotic carbenicillin was added to a final concentration of 20 μg/ml.

* Bacto-agar was added to the solution when solid plates were required.

### *5.1.2 SOC*

| Components | Mass (g)/ 600 ml | Final Concentration g/L |
|---|---|---|
| Difco Bacto-yeast extract | 12 | 20 g/L |
| Difco Bacto-tryptone | 3 | 5 g/L |
| NaCl | 0.36 | 0.6 g/L |
| KCl | 0.108 | 0.18 g/L |
| Dextrose | 1.2 | 2 g/L |

SOC medium was used to supply nutrients to electrocompetent cells that were transformed by electroporation. The above ingredients were added to distilled water and brought to a final volume of 600 mls. The SOC media was sterilized using a filters containing 0.22 μm pores (Nalgene). Sterilized solutions were supplemented with 1 M $MgCl_2$ and 1M $MgSO_4$ to a final concentration of 0.5 M $MgCl_2$: $MgSO_4$. Filter sterile glucose was added to a final concentration of 0.002% (w/v).

### *5.1.3 M9 minimal medium:*

| Components | Mass / 600 ml | Final Concentration g/L |
|---|---|---|
| $Na_2HPO_4$ | 3.6 g | 6 g/L |
| $KH_2PO_4$ | 1.8 g | 3 g/L |
| NaCl | 0.3 g | 0.5 g/L |
| $NH_4Cl$ | 0.6 g | 1 g/L |
| Dextrose | 1.2 g | 2 g/L |
| $MgSO_4$ | 0.6 g | 1 g/L |
| $CaCl_2$-$2H_2O$ | 3.3 mg | 5.5 mg/L |
| Thiamine | 1.2 mg | 2.0 mg/L |
| $FeCl_3$ | 0.32 mg | 0.533 g/L |
| *Each Supplement | 12 mg | 20 mg/L |

To produce the media required for plasmid selection, the appropriate amino acid synthesized by the plasmid was ommited. The medium was prepared in a 600 ml volume and autoclaved for 20 min at 121°C and 15 pounds per square inch.

*The supplements methionine, arginine, histidine, leucine, proline, threonine, tryptophan, and uracil were added from a 2 mg/ml solution carrying the respective component.

### *5.2 Yeast Media*

Yeast media was prepared as described by Sherman *et al.* (1991). The media was prepared in 600 ml volumes. The addition of 16.67 g/L of Bacto-agar was added as a solidification agent. All media was sterilized in an autoclave for 20 min at 121°C and 15 pounds per square inch.

#### *5.2.1 YPAD (Yeast, Peptone, Adenine, Dextrose)*

| Components | Mass/600 ml | Final Concentration g/L |
|---|---|---|
| Difco Bacto-yeast extract | 6 g | 6.67 g/L |
| Difco Bacto-tryptone | 12 g | 20g/L |
| Dextrose | 12 g | 20g/L |
| Adenine hemisulphate | 60 mg | 100 mg/L |
| * Difco Bacto-Agar | 10 g | 16.67 g/L |

YPAD media was prepared in 600 ml volumes containing the above components, brought to a pH of 6.0 and sterilized by autoclavation.

* Difco Bacto-Agar was added if growth on a solid matrix was required.

### *5.2.2 Synthetic Complete Omission Media*

| Components | Mass/600 ml | [Final] g/L |
|---|---|---|
| Difco Bacto-yeast nitrogen Base (without amino acids or $(NH_4)_2SO_4$) | 1 g | 6.67 g/L |
| Ammonium Sulfate | 3 g | 5 g/L |
| Dextrose | 12 | 20g/L |
| Amino acid drop-out mix | 350 mg | 583 mg/L |
| * Difco Bacto-Agar | 10 | 16.67 g/L |

***Amino acid drop-out mixture composition:***

4 g Adenine sulfate(46 mg/L), 2 g Arginine HCl (23 mg/L), 2 g Aspartic acid (23 mg/L), 2 g Glutamic Acid (23 mg/L), 2 g Histidine HCL 23 mg/L, 2.0 myo-Inositol (23 mg/L), 2 g Isoleucine (23 mg/L), 2.0 g Leucine (23 mg/L), 2 g Lysine HCl(23 mg/L), 2 g methionine (23 mg/L), 0.2 g p-aminobenzoic acid (PABA) (2 mg/L), 3.0 g Phenylalanine (35 mg/L), 2 g serine (23 mg/L), 6 g homoserine (70 mg/L), 2 g Tyrosine (23 mg/L), Tyrosine 2 g (23 mg/L), 2 g Uracil (23 mg/L), 9.0 g Valine (105 mg/L).

To prepare synthetic complete omission media, the above components were added minus the amino acid synthesized by the selected vector.

The above components were added to distilled water. The final volume of the solution was 600 ml. The pH was titrated with 10 N NaOH to a final pH of 5.6. The media was sterilized in an autoclave as described above.

* Difco Bacto-Agar was added if growth on a solid matrix was required.

*5.2.2.1 3-aminotriazole (3-AT)*

The insecticide 3-aminotrizole or 3-AT (Sigma) was added to the media to inhibit the activity of background imidazole-glycerol-P-dehydratase (*HIS3* gene product). 3-AT is a competitive inhibitor of the imidazole-glycerol-P-dehydratase enzyme (Kishore and Shah, 1988). 3-AT was added at various concentrations.

*5.2.2.2 5-flouroorotic acid (FOA)*

5-flouroorotic acid (FOA) was used to select for cells lacking URA3 gene expression. 600 mg of FOA was added to 300 mls of Nanopure™ water. This solution was filter sterilized and added to a 300 ml volume of SC- medium previously autoclaved.

## *5.3 Mammalian Cell Culture Media*

Ham's F12 medium was used for the propagation of CHO-IR and CHO26 cell lines. An prepared mixture was purchased for Gibco-BRL. Various antibiotics were used for selection. Fetal calf-serum was added to a final concentration of 5-10% (v/v).

### *5.3.1 HAM's F12*

8.9 mg/ml L-Alanine, 211 mg/ml L-arginine-HCl, 15.0 mg/ml L-asparagine-$H_2O$,13.3 mg/ml L-aspartic acid, 35.1 mg/mlL-cysteine HCl-$H_2O$,14.7 L-glutamic acid, 146 mg/ml L-glutamine,7.5 mg/l glycine,21 mg/l L-histidine HCl-$H_2O$, 3.94 mg/l L-isoleucine, 13.1 L-leucine, 36.5 mg/l L-lysine HCl,4.48 mg/l L-methionine, 4.96 mg/l L-phenyalanine, 34.5 mg/l L-proline, 10.5 mg/l L-serine, 11.9 mg/l L-threonine, 2.4 mg/l L-tryptophan, 5.40 mg/l L-tyrosine, 11.7 mg/l L-valine. 0.0073 mg/l biotin, 0.480 mg/l D-CA pantothenate, 14.0 mg/l cholinechloride, 1.30 mg/l folic acid, 17.0 mg/l I-inositol, 0.04 mg/l nnicotinamide, 0.062 mg/l pyridoxal HCl, 0.038 mg/l riboflavin, 0.34 mg/l thiamin HCl, 1.36 mg/l Vitamin $B_{12}$, 0.062 mg/l pyridoxine HCl, 44.0 mg/l $CaCl_2$-$2H_2O$, 224 mg/l KCl, 122 mg/l $MgCl_2$-6 $H_2O$, 7599 mg/l NaCl, 1,176 mg/l $NaHCO_3$, 268 mg/l $Na_2HPO_4$-2 $H_2O$, 0.00249 mg/l$CuSO_4$.5 $H_2O$, 0.834 mg/l$FeSO_4$-7 $H_2O$, 0.863 mg/l $ZnSO_4$-7 $H_2O$, 1,802 mg/l D-glucose, 0.21 mg/l lipoic acid, 12 mg/l phenol red, 110 mg/l sodium pyruvate, 4.10 mg/l hyoxanthine, 0.161 mg/l putrescine 2 HCl, 0.73 mg/l thymidine, 5% $CO_2$, pH 7.0.

Media was prepared as described by manufacturers instructions. The medium was filter sterilized with filters containing 0.22μm pores. Selective antibiotics G418 (Geneticin) was added to a final active concentration of 200 μg/L and Puromycin to a final concentration of 50 μg/L, by adding to media prior to filter sterilization. Other antibiotics including penicillin and streptomycin was added from a 100x stock preparation (GIBCO-BRL) to a final concentration of 0.1 U/L and o.1 μg/L. FCS was supplemented at a final concentration of 5-10%v/v.

## *5.4 Tax1bp1 BLAST 2.0 Query Results*

Sequences producing significant alignments:

| | Score | E-Value |
|---|---|---|
| (Query Date September 17, 1999) | | |
| ref\|NP_006015.2\|PTAX1BP1\| Tax1 (human T-cell leukemia virus... | 1477 | 0.0 |
| gi\|3046307 (AC004549) TXBP151 [Homo sapiens] | 1144 | 0.0 |
| gb\|AAD15412\| (AC005091) similar to nuclear domain 10 protei... | 323 | 4e-87 |
| dbj\|BAA23587\| (AB008852) NDP52 [Bos taurus] | 158 | 1e-37 |
| ref\|NP_005822.1\|PNDP52\| nuclear domain 10 protein >gi\|10826... | 150 | 3e-35 |
| gi\|1353761 (U43192) myosin II heavy chain [Naegleria fowleri] | 90 | 7e-17 |
| gi\|1850913 (L03534) myosin heavy chain [Entamoeba histolytica] | 89 | 1e-16 |
| sp\|P08799\|MYS2_DICDI MYOSIN II HEAVY CHAIN, NON MUSCLE >gi\|... | 85 | 2e-15 |
| ref\|NP_005187.1\|PCENPF\| centromere protein F (400kD) >gi\|13... | 83 | 9e-15 |
| gi\|1000094 (U30872) mitosin [Homo sapiens] | 83 | 9e-15 |
| ref\|NP_001804.1\|PCENPE\| centromere protein E >gi\|399227\|sp\|... | 83 | 9e-15 |
| emb\|CAA19588.1\| (AL023860) putative nuclear pore complex-as... | 82 | 2e-14 |
| emb\|CAB40350.1\| (AJ131892) Hyperion protein, 419 kD isoform... | 82 | 2e-14 |
| prf\|\|1819485A CENP-E protein [Homo sapiens] | 81 | 3e-14 |
| gi\|735904 (L41069) testicular protein [Mus musculus] | 81 | 3e-14 |
| sp\|Q62209\|SCP1_MOUSE SYNAPTONEMAL COMPLEX PROTEIN 1 (SCP-1 ... | 80 | 4e-14 |
| sp\|Q15431\|SCP1_HUMAN SYNAPTONEMAL COMPLEX PROTEIN 1 (SCP-1 ... | 80 | 8e-14 |
| dbj\|BAA78718.1\| (AB019691) Centrosome- and Golgi-localized ... | 79 | 1e-13 |
| dbj\|BAA13639\| (D88539) synaptonemal complex protein 1 [Mus ... | 79 | 1e-13 |
| gi\|2773363 (AF041382) microtubule binding protein D-CLIP-19... | 78 | 2e-13 |
| gi\|2781381 (AC004013) Similar to rabbit A-kinase-anchoring ... | 78 | 2e-13 |
| pir\|\|S03166 myosin heavy chain, gizzard smooth muscle - chi... | 78 | 2e-13 |
| dbj\|BAA34523.1\| (AB018346) KIAA0803 protein [Homo sapiens] | 78 | 2e-13 |
| sp\|P10587\|MYSG_CHICK MYOSIN HEAVY CHAIN, GIZZARD SMOOTH MUSCLE | 78 | 2e-13 |
| sp\|P39922\|MYS3_HYDAT MYOSIN HEAVY CHAIN, CLONE 203 >gi\|5312... | 78 | 2e-13 |
| gb\|AAD22767.1\|AF083037_1 (AF083037) A-kinase anchoring prot... | 78 | 2e-13 |
| emb\|CAB40713.1\| (AJ131693) AKAP450 protein [Homo sapiens] | 78 | 2e-13 |
| emb\|CAA09361\| (AJ010770) Hyperion protein [Homo sapiens] | 78 | 2e-13 |
| gb\|AAD39719.1\|AF091711_1 (AF091711) splice variant AKAP350 ... | 78 | 2e-13 |
| gi\|3660672 (AF055895) nonmuscle myosin II heavy chain A [Xe... | 78 | 3e-13 |
| emb\|CAA99841\| (Z75538) similar to myosin heavy chain; cDNA ... | 77 | 4e-13 |
| sp\|Q05000\|MYS_PODCA MYOSIN HEAVY CHAIN >gi\|9808\|emb\|CAA4879... | 77 | 4e-13 |
| sp\|P02566\|MYSB_CAEEL MYOSIN HEAVY CHAIN B (MHC B) >gi\|71606... | 77 | 5e-13 |
| emb\|CAB04089.1\| (Z81499) similar to myosin heavy chain; cDN... | 77 | 5e-13 |
| pir\|\|S44243 endosomal protein - human >gi\|475934\|emb\|CAA556... | 77 | 5e-13 |
| emb\|CAA49154\| (X69292) smooth muscle mysosin heavy chain [... | 77 | 5e-13 |
| pir\|\|S21801 myosin heavy chain, neuronal - rat | 76 | 7e-13 |
| gi\|553596 (M81105) cellular myosin heavy chain [Homo sapiens] | 76 | 7e-13 |
| sp\|P35579\|MYSN_HUMAN MYOSIN HEAVY CHAIN, NONMUSCLE TYPE A (... | 76 | 7e-13 |
| pir\|\|S60943 hypothetical protein YOR216c - yeast (Saccharom... | 76 | 7e-13 |
| gi\|2104553 (AF001548) Myosin heavy chain (MHY11) (5'partial... | 76 | 7e-13 |
| emb\|CAB05105\| (Z82215) dJ6802.2 [Homo sapiens] | 76 | 7e-13 |
| gi\|189036 (M31013) nonmuscle myosin heavy chain (NMHC) [Hom... | 76 | 1e-12 |

### *5.5 GIP1 (Grb14 Interacting Protein-1 coding sequence) -Clone M4*

```
GGT GCA GAC CCA ACA CTG CTC AAT TGT CAC AAT AAA AGT GCT ATA GAC TTT GGC
G   A   D   P   T   L   L   N   C   H   N   K   S   A   I   D   F   G

TCC ACA CAA CAG TTA AAA GAA AGA TTA GCA TAT GAT TTA AA
S   T   Q   Q   L   K   E   R   L   A   Y   D   L
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