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IDENTIFICATION OF RHO-ASSOCIATED PROTEIN KINASEα AS AN INSULIN RECEPTOR SUBSTRATE-1 BINDING PROTEIN

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Thesis submitted to the Department of Biochemistry in partial fulfillment of the requirements for the degree of Master of Science

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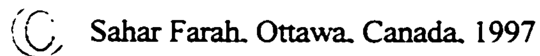
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To my husband and son
who have given me their love and support

Abstract

Insulin receptor substrate-1 (IRS-1) is phosphorylated on multiple tyrosine residues by ligand-activated insulin receptor. These tyrosine phosphorylation sites serve to dock several SH2-containing signaling proteins. In addition, IRS-1 also contains several protein modules that have been implicated in protein-protein or protein-lipid interactions. In an attempt to identify novel proteins that may interact with these IRS-1 protein modules, yeast two-hybrid screening was employed. The bait, corresponding to the N-terminal 500 amino acids of the *Xenopus* IRS-1 (XIRS-1), was comprised of a pleckstrin homology (PH) domain, a phosphotyrosine binding (PTB) domain and a SAIN (Shc and IRS-1 NPXY binding) domain. Screening of a *Xenopus* oocyte cDNA library with the bait resulted in the isolation of a partial *Xenopus* cDNA, XROKα. The cloned cDNA contains an open reading frame of about 500 amino acids (in frame with the N-terminal GAL4 activating domain) which are 90% identical to the C-terminus of the recently identified RhoA-associated protein kinase α (ROKα). The partial XROKα cDNA contains the putative Rho binding domain as well as the C-terminal pleckstrin homology/cysteine rich domain (PH/CRD) but lacks the N-terminal serine/threonine kinase domain. Using the yeast two-hybrid system, we showed that XROKα interacts strongly with a constitutively active form of RhoA (V14-RhoA). Further analysis indicated that the XIRS-1 PTB domain is specifically involved in binding to XROKα. To further characterize the potential role of XROKα in insulin signalling, I have cloned the entire coding sequence of XROKα by a combination of hybridyzation screening and PCR amplification. I have also generated XROKα specific polyclonal antibodies. Using these antibodies I have detected endogenous XROK α protein by both Western blotting and

by immune kinase assay in vitro. Taken together. these studies have identified an IRS-1 binding serine/threonine kinase which may play an important role in modulating insulin signalling.

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List of Abbreviation

AD	activating domain
BD	binding domain
cAMP	cyclic AMP
DMK	myotonic dystrophy kinase
GAP	GTP ase-activating protein
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
GVBD	germinal vesicle breakdown
HA	hemagglutinin
IGF-1	insulin like growth factor-1
IR	insulin receptor
IRS-1	Insulin receptor substrate-1
MAP	mitogen-activated protein
MLC	myosin light chain
MPF	maturation-promoting factor
PH/CRD	pleckstrin homology / cysteine rich domain
PH	pleckstrin homology
PI 3-kinase	phosphatidylinositol 3-OH kinase

PKA	protein kinase A
PTB	phosphotyrosine binding
ROKα	RhoA-associated protein kinase α
SAIN	Shc and IRS-1 NPXY binding
SH2	Src homology 2
Sos	son-of-sevenless
TNFα	tumor necrosis factor α
UTR	untranslated region
XIRS-1	*Xenopus* IRS-1

Chapter One

INTRODUCTION

My thesis work was directed towards identification of novel proteins that may interact with the N-terminal portion of *Xenopus* IRS-1 (XIRS-1), which contains several identifiable protein modules. In particular, I will describe identification of *Xenopus* homolog of a Rho-associated kinase (XROKα) as an IRS-1 binding protein using the yeast two-hybrid screening. Work described in this thesis, together with related work carried out by others in our laboratory, strongly suggests that ROKα, which is known as an effector of Rho GTPases in modulating actin cytoskeleton, has a novel function in regulating insulin signalling through its direct interaction with IRS-1. Therefore, I will review literature in insulin signalling in some details but also will briefly review literature on Rho GTPases and Rho-associated protein kinases and their roles in regulating actin cytoskeleton.

1.1 Insulin Signalling

1.1.1 Insulin and IGF-1

Insulin and insulin like growth factor (IGF-1) are closely related polypeptide hormones capable of inducing pleiotropic responses in many cell types, including activation of metabolic processes (glucose and amino acid uptake, glycogen synthesis etc) and stimulation of cell growth and differentiation (Rechler and Nissley, 1985). It is believed that *in vivo*, insulin mediates mostly metabolic effects, whereas IGF-1 acts as a potent growth and

differentiation factor. *In vitro* however the two often have related and overlapping cellular effect which is at least in part due to the fact that insulin is capable of binding and activating IGF-1 receptor at higher (non-physiological) concentrations, and vise versa.

1.1.2 Insulin receptor and IGF-1 receptor

Insulin and IGF-1 initiate cellular functions by activating their homologous receptors containing protein-tyrosine kinase activity (Kasuga, *et al.*, 1982, White & Kahn, 1986, Rosen, 1987, Czech *et al.*, 1989, Pawson, 1992, White & Kahn, 1994, Lienhard, 1994). These receptors are disulphide-linked heterotetramers containing two identical α subunits and two β-subunits (Hedo *et al.*, 1981). The α and β subunits of the respective receptor are encoded by the same gene and made as a precursor protein containing both subunits in tendon. Post translational proteolytic cleavage of the receptor precursor resulted in the α and β subunits which are then assembled at the cell surface (Rechler and Nissley, 1990). The α-subunits are located entirely outside of the cell and contain the insulin binding site(s), whereas the intracellular portion of the β-subunit contains the insulin-regulated tyrosine protein kinase. Insulin-induced tyrosine autophosphorylation of the receptors within the kinase domains stimulates kinase activity towards exogenous substrates (White *et al.*, 1988a). Cross-reactivity of insulin and IGF-1 with the two related receptor is perhaps best illustrated in the example of *Xenopus* oocyte maturation which is the model system used in our laboratory. *Xenopus laevis* oocyte maturation can be induced *in vitro* by insulin or IGF-1. The relatively low potency of insulin (EC_{50}=12nM) compared to the much higher potency of IGF-1 (EC_{50}=0.4nM) suggests that insulin/IGF-1-induced oocyte maturation is mediated

by endogenous IGF-1 receptor (Maller and Koontz, 1981). Work in our laboratory (Cummings *et al.*, 1996; Zhu, *et al.*, Endocrinology in press) have demonstrated that indeed *Xenopus* IGF-1 receptor is responsible for insulin/IGF-1-induced oocyte maturation.

1.1.3 Insulin receptor substrate-1 as an SH2 docking protein

Insulin receptor substrate-1 (IRS-1) was first identified as a 180 kDa phosphotyrosine-containing protein in insulin-stimulated cells (White, 1985). Protein purification and molecular cloning studies have revealed that IRS-1 contains multiple potential tyrosine phosphorylation sites but lacks any identifiable enzymatic domains (Sun *et al.*, 1991). Indeed, following insulin stimulation, IRS-1 is rapidly phosphorylated on multiple tyrosine residues (Sun *et al.*, 1993). Tyrosine phosphorylated IRS-1 binds several proteins containing Src homology 2 (SH2) domains (Anderson *et al.*, 1990). These include the lipid kinase phosphatidylinositol-3-OH kinase (PI 3-kinase) (Backer et al.,1992), a protein phosphotyrosine phosphatase SHP2 (Plutzky *et al.*, 1992 and Kuhné *et al.*, 1993), a complex that catalyses the release of GDP from Ras (Grb2-Sos) (Baltensperger *et al.*, 1993), and a protein with yet unknown function (Nck) (Lee *et al.*, 1993). Binding to each of these SH2 containing proteins to IRS-1 is directed through a specific tyrosine phosphorylation site.

The PI 3-kinase was the first SH2 containing protein found to bind IRS-1. This enzyme consist of two subunits, p85 regulatory subunit and p110 catalytic subunit. It phosphorylates the D3 position of phosphotidylinositol (PI), PI 4 monophosphate or PI (4,5) bisphosphate to produce PI 3 P, PI(3,4)P_2 or PI(3,4,5)P_3 repectively (Carpenter *et al.*, 1990). These 3-phosphoinositides are thought to act as some form of second messengers capable

of inducing multiple downstream responses. The best known of these is perhaps Akt or PKB (Burgering and Coffer. 1995). PI 3-kinase is activated when phosphorylated IRS-1 binds to the SH2 domains in its p85 regulatory subunit (Backer *et al.*. 1992). The IRS-1 tyrosine phosphorylated Tyr^{608} and Tyr^{628} in pYMXM motif are double binding sites for the two SH2 domains of p85 (Sun *et al.*, 1993).

SHP2. a protein tyrosine phosphatase that contains two SH2 domains. preferentially binds to Tyr^{1172} in IRS-1. The association of SHP2 with IRS-1 results in the activation of the phosphatase (Lechleider *et al.*. 1993). although the in vivo significance of SHP2 in insulin signalling remains unknown.

Grb2 is a small cytoplasmic protein that contains one SH2 domain and two Src homology 3 (SH3) domains (Mayer *et al.*. 1988). The Grb2 SH3 domains was found to bind a gaunine nucleotide exchange factor for $p21^{ras}$ termed mSOS [homologous to the Drosophila protein. son-of-sevenless(Sos)]. Since phosphorylated Tyr^{895} IRS-1 binds to SH2 domain of Grb2. Grb2-Sos complex protein serves as an adaptor molecule linking phosphorylated IRS-1 to Ras pathway (Baltensperger *et al.*,1993). Microinjection of Grb2 or $p21^{ras}$ alone into fibroblasts has no effect on DNA synthesis. whereas co-injection of Grb2 and $p21^{ras}$ stimulates cell proliferation (Lowenstein *et al.*. 1992). In addition to IRS-1. another insulin receptor substrate. Shc. may also play a role in linking IR to Ras activation. Shc is phosphorylated by IR and tyrosine-phosphorylated Shc binds Grb2/mSos and hence provides the coupling (Sasaoka *et al.*. 1994). To complicate thing even more. a recent study using yeast two-hybrid system and in vitro interaction assay demonstrates a direct interaction between Shc and IRS-1 (Kasus-Jacobi *et al.*. 1997).

1.1.4 Other IRS-1 protein modules (PH, PTB,and SAIN domains).

In addition to the multiple tyrosine phosphorylation sites which serve to dock SH2 domain containing proteins. IRS-1 contains other potential protein binding domains including pleckstrin homology (PH) domain. a phosphotyrosine binding (PTB) domain and Shc and IRS-1 NPEY binding (SAIN) domain (Pawson. 1995) (Fig.1).

The PH domain was first described in 1993 (Haslam *et al.*. 1993 and Mayer *et al.*. 1993) and was so called because it was originally detected as an internal repeat in pleckstrin. a 47 kDa protein that is the major substrate of protein kinase C in platelets. PH domains are comprised of about 100 amino acids which later were detected in a number of proteins with diverse cellular functions. The PH domain seems to bind phosphoinositides. particularly $PI(3.4.5)P_3$ (Lemmon *et al.*. 1996) and therefore may play an important role in propagating signals downstream of PI 3'-kinase. Deletion of the IRS-1 PH domain results in a decrease of its phosphorylation by the insulin receptor which suggests that IRS-1 PH domain may play an important role in mediating IRS-1 interaction with the the receptor or with membrane (Myers *et al.*. 1995).

The PTB domain was first reported in Shc protein as a phosphotyrosine binding domain that are structurally distinct from SH2 domains which also bind phosphotyrosines (Kavanaugh and Williams. 1994. Bork and Margolis. 1995). While SH2 domain binding is determined by amino acids consensus sequence C-terminal to phosphotyrosine (Songyang *et al.*. 1993). PTB domain specifically recognize NPXpY motif . The presence of a PTB domain in IRS-1 explains the earlier observation that mutation of NPEY-950 (or Y-960 of

human IGF-1 receptor) autophophorylation site diminished IRS-1 phosphorylation (White *et al.*, 1988b). Mutation studies suggest that amino acids 162-265 of rat IRS-1 are the required component of the PTB domain that binds the NPEpY motif of the insulin receptor (Wolf *et al.*, 1995). This was confirmed by studying the crystal structures of the IRS-1 PTB domain, alone or in complex with the juxtamembrane region of the insulin receptor containing the NPXpY motif (Eck *et al.*, 1996). However, using the yeast two-hybrid assay, Gustafson's group have identified additional amino acid sequence carboxy-terminal to the PTB domain in IRS-1 to be involved in binding to insulin receptor NPEpY autophosphorylated site. This motif was called SAIN domain and is located within amino acids 313-462 on IRS-1 (O'Neill *et al.*, 1994, Gustafson *et al.*, 1995, He *et al.*, 1995, He *et al.*, 1996).

1.1.5 IRS-1 serine threonine phosphorylation.

IRS-1 is also prominently phosphorylated on serine/threonine residues, both at basal level as well as following insulin stimulation (Sun *et al.*,1991, Myers *et al.*,1996). Many kinases, including casein kinase II (Tanasijevic *et al.*, 1993), PI 3-kinase (PI 3-kinase also has intrinsic serine kinase activity) (Tanti *et al.*, 1994b), and MAP kinase (Mothe and Van obberghen, 1996) are capable of phosphorylating IRS-1, although the physiological significance of these modifications are unknown.

An insulin-resistance state (a smaller than normal response to given amount of insulin) is reached after treating cells with either okadaic acid (Tanti *et al.*,1994a) or tumor necrosis factor TNFα (Hotamisligil *et al.*,1996). In okadaic acid treated 3T3-L1 adipocytes

a decrease in IRS-1 tyrosine phosphorylation was linked to increased phosphorylation on serine/threonine residues. (Tanti *et al.*,1994a). Treatment of cultured murine adipocytes with TNFα was shown to induce serine phosphorylation of IRS-1 and convert IRS-1 into an inhibitor of the IR tyrosine kinase activity in vitro (Hotamisligil *et al.*, 1996). In both cases serine/threonine phosphorylation of IRS-1 appears to inhibit its tyrosine phosphorylation by IR, thus providing a possible negative regulation mechanism for insulin action.

1.1.6 Other IRS proteins

Two groups have recently generated mice homozygous for the *IRS-1* gene (Tamemoto *et al.*,1994, Araki *et al.*,1994). Two important conclusions can be drawn from theses studies. The first is the existence of other IRS-1-like genes in mice. Unlike mice homozygous for IGF-1 receptor which are severely retarded during gestation and died shortly after birth (Liu *et al.*, 1993), the IRS-1 knockout mice suffer relatively mild retardation during gestation and live to adulthood. In addition, while a lack of insulin or its receptor causes diabetes, mice lacking IRS-1 did not develop any form of diabetes. These strongly suggest that other IRS-1-like gene(s) may compensate for the loss of IRS-1 function in mediating insulin/IGF-1 signalling in the knockout mice. The second conclusion is that IRS-1 is important in insulin signalling, since the knockout mice developed significant insulin resistance.

Indeed, several other IRS proteins have since been discovered. IRS-2, which is slightly larger than IRS-1, was found to be a protein having a role in both insulin signalling and in cytokine signalling (IL-4) (Sun *et al.*,1995, He *et al.*,1996). Function of the two

newest members, a 60kD IRS-3 (Lavan *et al.*,1997a) and 160 kDa IRS-4 (Lavan *et al.*,1997b), are not known. All three new IRS proteins, like IRS-1, possesss highly conserved PH and PTB domains at the N terminus of the proteins and various tyrosine phosphorylation sites spread over the C-terminal portion of the proteins (Fig.1).

1.2 *Xenopus* oocytes as a model to study insulin signalling

Fully grown oocytes of *Xenopus laevis* are physiologically arrested at the G_2/M transition of the first meiotic prophase. Oocyte maturation is the release of G2/M arrest causing oocytes to progress through meiosis until the second meiotic metaphase where oocytes await fertilization. This process occurs just prior to ovulation and is believed to be triggered by progesterone, which is produced by the surrounding follicle cells in response to a pre-ovulatory gonadotropin surge. In addition to progesterone, a variety of substances can induce oocyte maturation *in vitro*. Of particular interest is the observation that insulin and IGF-1 are potent inducer of *Xenopus* oocyte maturation (El-Etr *et al.*,1979, Maller and Koontz *et al.*,1981).

The relatively low potency of insulin (EC_{50}=12nM) compared to the much higher potency of IGF-1 (EC_{50}=0.4nM) in meiosis induction suggests that insulin/IGF-1-induced oocyte maturation is mediated by endogenous IGF-1 receptor (Maller and Koontz, 1981). Our laboratory has recently cloned the full-length cDNA encoding *Xenopus* IGF-1 receptor (Zhu, *et al.* Endocrinology in press). Sequence analysis reveals high percentage identity (90%) between the human (Ulrich *et al.*, 1986) and *Xenopus* IGF-1 receptors with all important elements conserved. These include the three catalytically important

autophosphorylation sites within the kinase domain and the NPEY motif (another autophosphorylation site) which is apparently important for insulin-induced phosphorylation of IRS-1 (White *et al.*, 1988b and Yamasaki *et al.*, 1992).

Earlier studies by Chuang *et al.* demonstrated that recombinant IRS-1 potentiates insulin signalling in *Xenopus* oocytes (Chaung *et al.*, 1993), suggesting the presence of an endogenous IRS-1 protein. Multiple anti-IRS-1 antibodies or antibodies against phosphotyrosine, however, failed to detect any such proteins. To address this issue, our laboratory set out to clone cDNA for *Xenopus* IRS-1-like proteins. A candidate XIRS-1 was cloned and it encodes a protein similar in size and predicted amino acid sequence to mammalian IRS-1. XIRS-1 expressed in a baculovirus expression system was readily phosphorylated by recombinant insulin receptor and the phosphorylated XIRS-1 bound PI 3-kinase (Liu, *et al.*, 1995).

1.3 Rho family GTP-binding proteins

The Rho family small GTPases (Rho, Rac, and Cdc 42) are known to function in reorganization of the actin cytoskeleton. Like other small GTPases in the Ras superfamily, these GTPases also function as molecular switches having two nucleotide-bound states. At basal state, Rho exists in the biologically inactive GDP-bound state. In response to an up-stream signal (growth factors, lysophosphatidic acid etc), Rho undergoes GDP release and followed by binding to GTP, a process catalysed by guanine nucleotide releasing proteins (Ridley and Hall, 1992). The GTP-bound Rho then triggers downstream signals by binding and activating effector molecules. Hydrolysis of the bound GTP by its intrinsic GTPase

activity which is enhanced in vivo by GTPase activating proteins (GAP) returns Rho protein back to the GDP-bound state. Such a G protein cycle is often disrupted by mutation in the GTPases. The most significant mutation, replacing Gly-14 with valine (a mutation equivalent to that found in viral Ras protein), abolishes the intrinsic GTPase activity of Rho thereby resulting in a constitutively active Rho protein. On the other hand, Rho proteins can be specifically inhibited by a bacterial toxin, *clostridium botulinum* C3 transferase, which ADP ribosylates Asn41 in the effector region of RhoA. Different members of the Rho subfamily GTPases regulate distinct cellular processes involving actin polymerization. For example, Rac proteins are involved in growth factor induced membrane ruffling (Ridley *et al.*,1992), whereas Rho is required for the formation of stress fibres and focal adhesion (Ridley and hall,1992). Cdc 42 activation leads to the formation of filopodial protrusions at cell periphery (Nobes and Hall, 1995).

1.4 ROK proteins

RhoA-binding kinase (ROK) is the prototype of a family of newly identified serine/threonine kinases that bind to, and are activated by, GTP-bound Rho. ROKα was first cloned by screening an expression library using GTP-RhoA as a probe (Leung *et al.*, 1995). The isolated rat ROKα cDNA encodes a 160 kDa protein with an N-terminal serine/threonine kinase domain. This protein also contains a coiled-coil domain, RhoA binding domain, and a C-terminal PH domain that is split by cysteine rich sequence (PH/CRD). Several other proteins with very similar sequence and binding properties have been identified (Mukai and Ono,1994, Mukai *et al.*,1995, Palmer *et al.*,1995, Watanabe *et*

al., 1996). As expected, ROK proteins promoted formation of stress fibers and focal adhesion (Leung *et al.*, 1996 and Amano *et al.*, 1997, Ishizaki *et al.*, 1996). In these studies, overexpression of the catalytic kinase domain of ROK proteins induced the formation of stress fibers and focal adhesion. In contrast, dominant negative mutants of ROKα blocked stress fiber formation induced by either the activated Rho or by external stimuli (growth factors and lysophosphatidic acid).

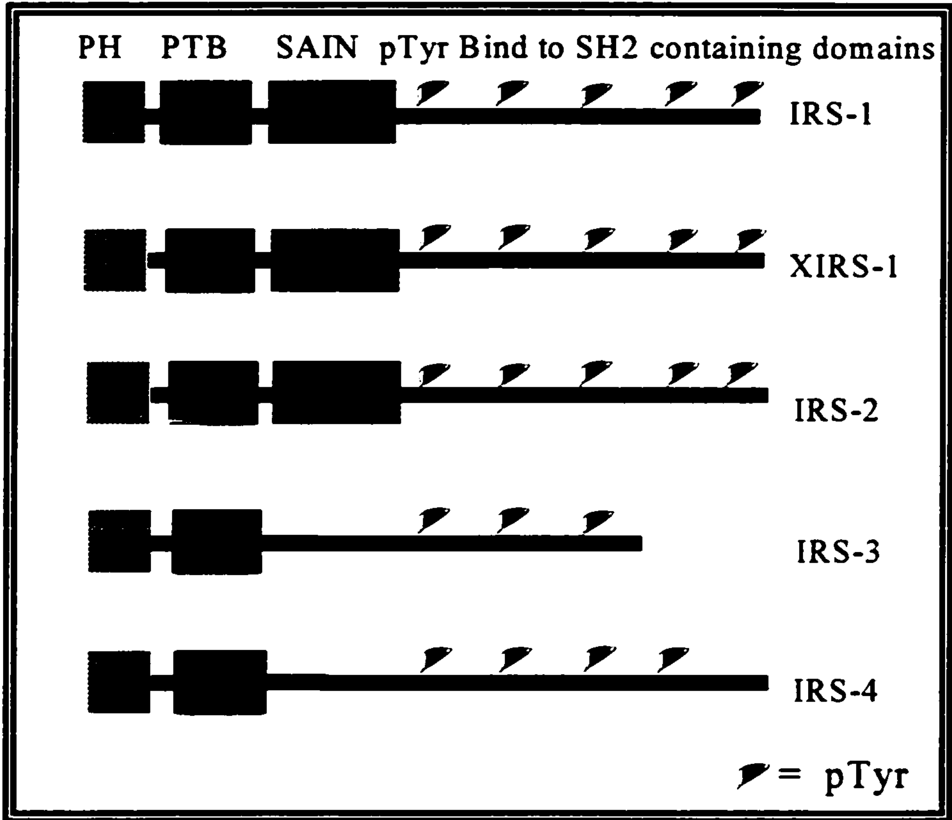

PH
PTB
SAIN
pTyr Bind to SH2 containing domains
IRS-1
XIRS-1
IRS-2
IRS-3
IRS-4
= pTyr

Figure 1:

Structure of IRS proteins

Schematic representation of the various IRS proteins. The N-terminus contains the PH. PTB. and SAIN domain (only in IRS-1. XIRS-1 and IRS-2). whereas the C-terminus contains the phosphotyrosine reisdues implicated in binding SH2 domains in signalling proteins.

Chapter Two

ROKα is an IRS-1 binding protein

Insulin and IGF-I initiate cellular functions by activating their homologous tyrosine kinase receptors. In most mammalian cell types, this results in rapid tyrosine phosphorylation of a high-molecular-weight substrate termed IRS-1. In addition, IRS-1 contains several protein modules implicated in protein-protein or protein-lipid interaction.

In an attempt to detect proteins interacting with these protein modules the yeast two-hybrid screening strategy was employed. In this chapter I described the identification of Xenopus ROKα as an IRS-1 binding protein. Further analysis showed that IRS-1 PTB domain is mainly responsible for the interaction with XROKα.

Overview of the Yeast two-hybrid system

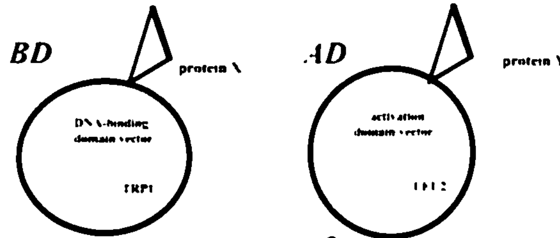
The two-hybrid system is based on the two-hybrid assay developed by Fields and co-workers (Bartel *et al.*, 1993, Chien *et al.*, 1991, Fields and Song, 1989). The two-hybrid assay is a sensitive *in vivo* method for identifying genes encoding proteins that interact with a protein of interest and is well-suited for detecting weak or transient interactions (Guarente, 1993). Moreover, since the assay is performed in living cells, the proteins in question are more likely to be in their native conformation.

The yeast two-hybrid method is built on the restoration of transcriptional activation to indicate interaction between two known proteins. The yeast GAL4 transcription activator consist of two physically discrete domains, with one acting as the DNA binding domain and the other as the activator domain. The yeast two-hybrid system take advantage of the concept that the two GAL4 sub-domains need to be fused for normal activation. The DNA-binding domain vector is used to generate a fusion of the GAL4 DNA-binding domain and a target protein X, while the activation domain is used to generate a fusion of the GAL4 activation domain and a target protein Y. The two hybrids are cotransformed into a yeast host strain containing reporter genes (*LacZ* and *HIS3*) with upstream GAL4 binding sites. In these yeast strains the endogenous gal4, gal80 (inhibitor of GAL4) and *his* genes are mutated or deleted. When an interaction occurs between the target protein X and the candidate protein Y, the two GAL4 domains are fused together resulting in the restoration of transcriptional activation. Thus expression of the reporter genes indicates an interaction of proteins X and Y.

The yeast two-hybrid system is also used for screening GAL4 activation domain libraries to identify genes encoding proteins that interact with a target protein (Fig.2). The cDNA of the protein of interest is used to make a fusion protein with a GAL4 DNA binding domain in a cloning vector containing the GAL4 DNA binding domain. The DNA binding hybrid protein cannot activate transcription since it does not contain a transcriptional activation domain. The GAL4 activation domain/cDNA fusion library, can activate transcription but cannot itself interact with DNA. The two types of hybrid are then cotransformed into the yeast host strain, which has the two reporter genes (*HIS3* and *LacZ*).

Clone gene for target protein (X) into DNA-binding domain vector

Clone random collection of cDNAs (Y) into the activation domain vector



Expression of both hybrid proteins in the same cell

DNA-binding domain/Protein X hybrid protein

\+

Activation domain/Protein Y hybrid protein

Assay for B-galactosidase activity to confirm interacting proteins

<u>Figure 2:</u>
Screening GAL4 AD fusion libraries for proteins that interact with a target protein
Adapted from the MATCHMAKER Library Protocol (see text for detail).

Thus, if the target protein interacts with a library encoded protein, then a functional GAL4 activator is reconstituted, and the expression of the *HIS3* reporter gene is activated. Primary His+ transformants are tested for expression of the second reporter gene, *LacZ* to reduce the background of false positive arising in the His selection.

2.1 Material and Methods

2.1.1 Yeast Strains and plasmids

Sarccharomyces cervisiae yeast strains HF7c (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS2::GAL1-HIS3, URA3::(GAL4 17-mer)*$_3$ *-CYC1-lacZ*) and Y190 strain (*MATa, leu2-3, 112, ura3-52, trp1-901, his3-200, ade2-101, gal4D, gal80D, URA3::GAL1-lacZ, LYS2::GAL1-HIS3, cyh*^{r}) were used. Yeast expression plasmids (Clontech) pAS1-CYH2 (pAS2) and pGAD10 were used with Y190 and HF7c yeast strains containing a *LacZ* gene downstream of the Gal4 binding sequence. pAS2 contains Gal4 DNA binding domain (Gal4 BD), tryptophan as a marker gene, and a hemagglutinin (HA) tag (Fig.3). pGAD10 contains the Gal4 activation domain (Gal4 AD) and leucine marker gene (Fig.4).

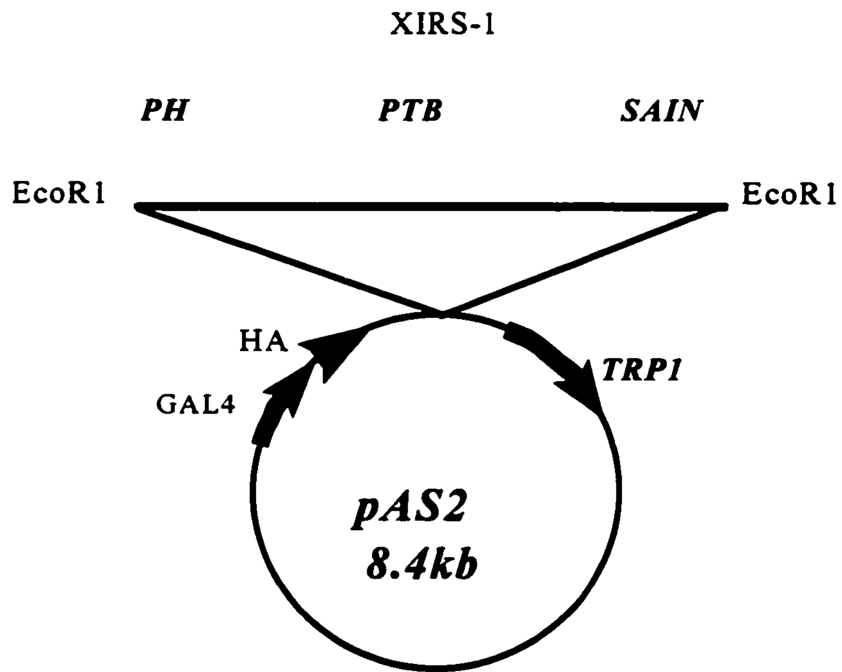

XIRS-1
PH
PTB
SAIN
EcoR1
EcoR1
HA
GAL4
TRP1
pAS2
8.4kb

Figure 3:
The N-terminal XIRS-1 in pAS2 construct
The N-terminus of XIRS-1 containing the PH, PTB, and SAIN domains were subcloned into the pAS2 vector. pAS2 is a yeast GAL4 DNA-binding domain cloning vector designed for use in the yeast two hybrid system. pAS2 contains the *Trp1* gene (for selection in yeast strains deficient in tryptophan) and the hemagglutinin (HA) epitope tag.

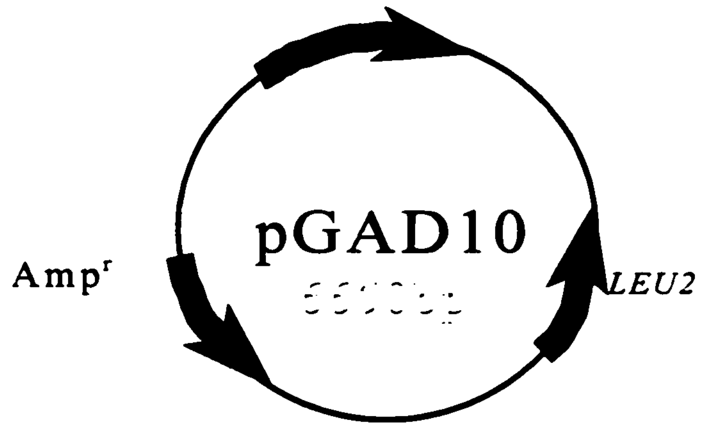

GAL4 AD
pGAD10
Amp^{r}
LEU2

Figure 4:
pGAD 10 yeast activating domain cloning vector.
pGAD10 is a yeast GAL4 activation domain designed for use with GAL4-based MATCHMAKER two hybrid system. This vector is used to generate fusions of a protein with the GAL4 activation domain. The *LEU2* gene allows selection in yeast host strains.

2.1.2 Bait Construction

The N-terminal XIRS-1 (amino acids 3-500) was used as bait to screen an oocyte cDNA library. 1500 nucleotides from the 5' end of XIRS-1 cDNA were amplified by the polymerase chain reaction (PCR) with primers Y2HN and Y2HC as 5' and 3', containing BamH1 and Sal1 sites, respectively (Table1). The PCR fragment was agarose gel purified using the geneclean kit (Bio 101) and digested by BamH1 and Sal1 restriction enzymes using appropriate buffer (Gibco). This digested PCR product was then subcloned into a pAS2 vector, in-frame with the GAL4 DNA binding domain cDNA (Fig.3). HF7c competent yeast cells were transformed with the resultant BD/XIRS-$1_{3\text{-}500}$ plasmid.

2.1.3 Library preparation

The MATCHMAKER *Xenopus laevis* oocyte cDNA library was constructed in the pGAD10 vector (AD/library; Clontech). The library was titered and amplified to obtain sufficient plasmid for yeast transformation according to the manufacturer's protocol (Clontech). Library derived plasmids were isolated from *E. coli* using the cesium chloride gradient purification method (Maniatis,1989). Briefly, the *E. coli* culture cells were collected by centrifugation and resuspended in sucrose-TE (0.44M sucrose, 50mM Tris-HCl pH 8.5 and 50mM EDTA). The cells were lysed with lysozyme and lysis solution (0.1% Triton X-100, 50mM Tris-HCl pH 8.5 and 50mM EDTA) and cell debris was cleared by centrifugation at 15,000 g for 45 min at 4°C. Cesium chloride and ethidium bromide were added to the supernatant to a final concentration of 0.95g/ml and 0.12mg/ml, respectively. The mixture

was then centrifuged at 24.000 g for 20h at 20°C. Two DNA bands were visible under UV illumination. the above one represent nicked or linear DNA. whereas the lower band is the supercoiled DNA. The lower band was drawn out using a 20g needle and the DNA was extracted by adding cesium chloride and water saturated iso-butanol. The library DNA was then precipitated with two volumes of 95% ethanol and the pellet was washed with 70% ethanol and resuspended in Tris-EDTA (10mM Tris-HCl and 1mM EDTA).

2.1.4 Yeast transformation

2.1.4.1 Library transformation

To screen the *Xenopus* oocyte cDNA library using the yeast two-hybrid system. competent yeast cells were first transformed with the bait. (see section 2.1.2) and then with the cDNA library (see section 2.1.3) using the lithium acetate method developed by Ito *et al.*. (1983) and modified by Gietz *et al.*. (1992). The HF7c yeast strain containing the BD/XIRS-1_{3-500} was inoculated into synthetic dropout media lacking tryptophan (SD/-Trp) and incubated overnight at 30°C to select for the presence of pAS2. Cells were harvested by centrifugation. and washed by resuspending them in 0.5% volumes of distilled deionized water followed by centrifugation. The purified *Xenopus* cDNA library DNA was added to the prepared competent cells to a final concentration of 0.17μg/μl along with DNA-carrier mix (salmon sperm DNA .0.022mg/ml . 34% PEG. and 0.1M lithium acetate). Cells were heat-shocked at 42°C for 30 min. and collected by centrifugation. Transformants were spread onto plates containing SD medium lacking histidine (-His). leucine (-Leu). and tryptophan (-Trp). supplemented with 3-aminotriazole (3AT). The plates were incubated at 30°C for 10

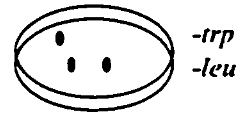
days to select for colonies expressing interacting hybrid proteins until His+ colonies were approximately 1mm in diameter.

2.1.4.2 Elimination of False Positives

Several different AD/library plasmids may have been present in each β-galactosidase positive colony. Restreaking each of the positive colonies on SD/-Trp/-Leu/-His selection plates will, in many cases, allow multiple AD/library plasmids to segregate. To verify the β-galactosidase-positive phenotype, completely isolated colonies were reassayed by colony lift assay.

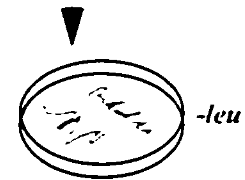
To eliminate the DNA-BD/target plasmid, plasmids in yeast were segregated by removing the Trp selection. Positive colonies were streaked on Leu- plates. Under these conditions, the AD/library plasmid carrying the *LEU2* gene was maintained but the DNA-BD/target plasmid carrying TRP1 was randomly lost from some transformants. The same step was repeated several times until a colony lost β-galactosidase activity. Colonies were plated on two SD plates, one -Leu/-Trp and the other -leu only. Trp auxotrophs which were able to grow on +trp but not on -trp medium had lost the DNA-BD/target plasmid with TRP1 but retained the AD/library plasmid with *LEU2*. The colonies were further assayed for lacZ function by β-galactosidase activity. Negative colonies were isolated and these AD/library plasmids were considered candidates for encoding a genuine interacting protein (Fig.5). Trp auxotrophs that still retained β-galactosidase activity most likely contained an AD/library plasmid encoding a transcriptional activator that recognized the GAL1 promoter and were discarded. Library-derived plasmids were rescued directly from Trp auxotrophs negative for β-galactosidase.

1. Segregate multiple AD/Library plasmids within a single colony

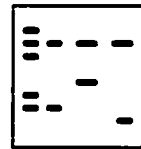


2. Sort persistent positive clones into groups.

3. generating clones containing only the AD plasmids



4. Transform those plasmids in E. coli.



5. Recotransform posive clones with the bait

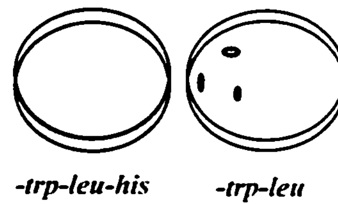


Figure 5:
Procedure for eliminating false positives.
Adapted from the MATCHMAKER Library Protocol
(see text for explanation)

To verify positive results, AD/library plasmids extracted from all positive colonies were co-transformed with the XIRS-1 bait in Y190 yeast cells using purified plasmids. The ability of transformants to grow on SD/-Trp/-Leu/-His and to express β-galactosidase were confirmed (see below).

2.1.4.3 Cotransformation method

To confirm interaction between the positive clones and the bait, the isolated library plasmid and BD/XIRS-1 $_{3\text{-}500}$ plasmid were cotransformed in Y190 competent cells. Y190 competent cells were prepared by growing a single colony of Y190 yeast cells overnight at 37°C in YEPD media (peptone, 20g/L, yeast extract, 10g/L, and 2% dextrose; Difco) YEPD media was innoculated with the overnight culture and grown to the mid-log phase (OD_{600} 1.0). Cells were harvested, washed with LiAcTE (100mM lithium acetate, 10mM Tris-HCl pH 8, 1mM EDTA), and resuspended in LiSORB (LiAcTE + 1M sorbitol). Both BD/XIRS-$1_{3\text{-}500}$ DNA of and its interacting plasmid (isolated from the library) were added to the yeast cell suspension along with salmon sperm DNA (0.022 mg/ml) followed by the addition of 40% PEG-3350 in LiAcTE. Cells were heat-shocked at 42°C for 10 min and transferred to selective media (SD/-Trp/-Leu/-His). The culture was incubated at 30°C for 3-4 h, collected by centrifugation, and spread onto plates containing selective media (SD/-Trp/-Leu/-His) and 2% agar.

2.1.5 Yeast β-Galactosidase Assay (lift assay)

To assay for β-galactosidase activity by blue/white screening, the colony lift β-galactosidase assay was used. Dry Whatman Grade 413 filter papers were layed onto yeast plates. The filters were peeled off, with yeast colonies sticking to them, and were immersed into a pool of liquid nitrogen for 30 s to permeabilize the cells. Then the filters were thawed and laid onto whatman 3MM paper saturated with X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 0.1% in N,N-dimethylformamide (DMF)] in Z buffer solution (16.1g/L Na_2HPO_4 $7H_2O$, 5.5g/L NaH_2PO_4 H_2O, 0.75g/L KCl, and 0.246g/L $MgSO_4$ $7H_2O$) with 0.027ml β-mercaptoethanol. This was incubated overnight at 30°C for blue color development. Colony color assays were performed on at least five separate colonies from each transformation to reduce variability.

2.1.6 Yeast β-Galactosidase Assay (liquid assay)

For quantitative analysis, a liquid culture β-galactosidase assay with 0-nitrophenyl β-D galactopyranoside (ONPG) as substrate was used. A saturated yeast culture was diluted in selective media (SD/-Trp/-Leu /-His) and grown at 30°C to the mid-log phase (A_{600} = 0.5 to 0.8). Cells were harvested, resuspended in pre-mix buffer [0.1 M NaPi pH 7.5, 100XMg (100mM $MgCl_2$, 4.5M β-mercaptoethanol), and β-galactosidase substrate 0-nitrophenyl β-D galactopyranoside (133mM ONPG in DMF)]. The mixture was incubated at 37°C for 30 min. or until a yellow colour appeared. The reaction was stopped by adding Na_2CO_3 to 6.25mM and β-galactosidase activity was measured spectrophotometrically by reading the absorbance at 420nm. One unit of β-galactosidase was defined as the amount of enzyme which

hydrolyzes 1 μmole of ONPG to o-nitrophenol and D-galactose per minute at 37°C (Miller. 1972) and β-galactosidase activity was calculated as follows:

β-galactosidase activity = $1000 \times OD_{420}/(t \times V \times OD_{600})$

where: t = elapsed time (in min) of incubation

V= 0.1ml X concentration factor

$OD_{600} = A_{600}$ of 1 ml of culture

2.1.7 Fast Yeast Plasmid Rescue

Yeast colonies of 3-5 mm were picked from recently streaked plates into rescue buffer (100mM NaCl. 10 mM Tris pH 8.0. 1mM EDTA. 0.1% SDS). Glass beads (0.45mm) were added and the mixture was vortexed. The plasmids derived from yeast cells were extracted with phenol. followed by extraction with chloroform/isoamyl alcohol (24:1). DNA was precipitated with 95% ethanol and the pellet was washed with 70% ethanol and resuspended in Tris-EDTA.

2.1.8 Sequence analysis.

DNA sequence analysis was carried out using the Licor automated sequencer. Briefly. Infrared fluorescent dye IRD41 Labelled primers were employed in sequithermal cycle sequencing (Licor). The resultant labelled PCR products were resolved on a Long Ranger 4% polyacrylamide gel and sequenced using the Licor automated sequencer. In some cases manual sequencing was also carried out following the protocol provided in the USB sequencing kit.

2.1.9 N-terminal XIRS-1 subclones

To delineate the domains of XIRS-1 responsible for interacting with clones isolated from the library, several subclones of the N-terminus of XIRS-1 were constructed in the pAS2 plasmid. The various regions of XIRS-1 (Fig. 11) were amplified by PCR using specific primers (Table 1), which introduced a BamH1 and Sal1 sites at the ends of the cDNA fragments to allow an in frame insertion into pAS2. The PCR fragments were gel-purified and digested with BamH1 and SalI and subcloned into a pAS2 vector previously digested with BamH1 and Sal1. These constructs were cotransformed with the previously isolated AD/library plasmid in Y190 yeast cells to run the two hybrid assay (described in section 2.1.4.3).

2.1.10 Western Blot Analysis of Hybrid Protein Expression

Yeast cells transformed with subdomains of the N-terminus XIRS-1 in pAS2 were grown in selctive media (SD/-Trp /-Leu) to the mid-log phase. Cells were lysed in sample buffer (20% glycerol, 4% SDS, 0.005% Bromophenol blue, 125mM Tris, and 10% beta-mercaptoethanol) with protease inhibitors (1mM PMSF, 10μg/L leupeptin) and glass beads. Proteins were denatured by boiling at 95°C for 5-10 min. and resolved on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). Gels were electroblotted to nitrocellulose membranes in transfer buffer (48mM Tris, 39mM glycine, 0.0375% SDS and 20% methanol) using a Biorad semi-dry transfer apparatus. Membranes were blocked for at least 1h in TBST (50mM Tris-HCl pH 7.5,150mM NaCl, 0.5% Tween 20) plus 5% skim milk powder and

immunoblotted with anti-hemagglutinin (HA) monoclonal antibody (in 5% skim milk) for 2h. The blots was then washed with several rinses of TBST and probed with horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody. The blot was washed again with TBST and developed by enhanced chemiluminescence (ECL) (Amersham) reaction. Membranes were exposed to XAR-5 Kodak film to visualize immunoreactive bands.

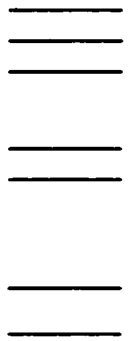
2.2 Results

2.2.1 Identification of XROKα as an IRS-1 binding protein

Amino acids 3 to 500 of XIRS-1 containing the PH. PTB and SAIN domains. were PCR amplified and subcloned into the pAS2 vector. The resulting plasmid encoded a fusion protein containing a hemagglutinin (HA) epitope tag. and the Gal4 DNA-binding domain. followed by the XIRS-1 sequence. This plasmid. BD/XIRS-1 $_{3\text{-}500}$. was introduced into the HF7c yeast strain.

To determine if the BD/XIRS-1 $_{3\text{-}500}$ fusion protein was being expressed in the transformed HF7c cells. Western blot analysis using an anti-HA antibody was employed. As a positive control. SF9 insect expressed HA tagged XIRS-1 protein lacking the PH domain (Liu *et al.*, 1995) was also blotted (Fig.6). As can be seen in Figure 6. a 100 kDa protein was detected in the HF7c yeast cells transformed with the BD/XIRS-1$_{3\text{-}500}$ plasmid whereas this protein was not detected when SF7 was cotransformed only with the BD vector. In the positive control lane. the HA antibody detected the expected 120 kDa HA/XIRS-1 protein. as well as two bands which probably represented degradation products.

To demonstrate that the bait was not able to serve as a transcription factor. which would produce false positives (i.e. β-galactosidase activity). transformants expressing BD/XIRS-1 $_{3\text{-}500}$ hybrid protein were analysed by the colony lift assay for β-galactosidase activity (see section 2.1.5). No β-galactosidase activity was observed (data not shown).


180
116
84
58
48
36
26

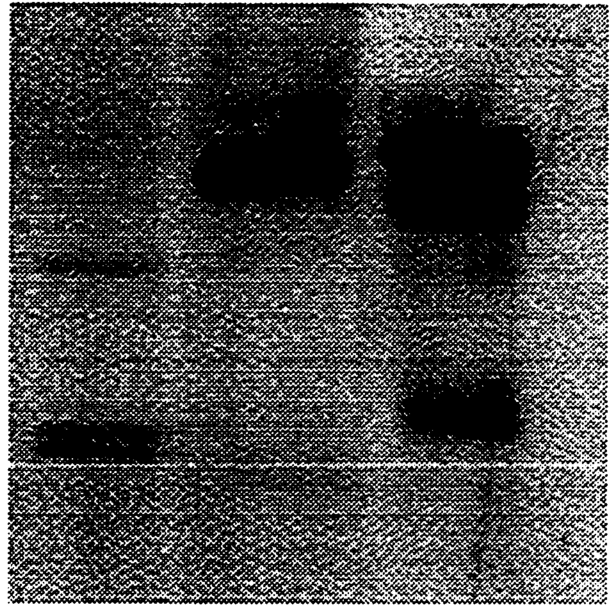

1
2
3

Figure 6:

Immunoblot analysis of expression of the XIRS-1 hybrid protein

Expression of the XIRS-1 hybrid was analysed by immunoblotting with the hemagglutinin epitope tag antibody as described in Material and Methods.Lane 1 contains only the pGAD10 vector. whereas Lanes 2 is the bait. XIRS-1 N-terminal sequence 3-500 amino acids. lane 3 is HA/XIRS-1 lacking the PH domain expressed in SF9 cells. used as positive control. The extracts used for immunoblotting were derived from the same yeast cultures that were used in the solution β-galactosidase assays. Sizes are indicated in kilodaltons according to prestained protein markers.

The *Xenopus Laevis* MATCHMAKER AD/library were amplified to obtain sufficient plasmid for yeast transformations. The purified library DNA was introduced into the HF7c yeast strain previously transformed with the bait plasmid. A total of 7.5×10^5 transformants were plated and screened on appropriate media (SD/-Trp/-Leu/-His) to select for colonies expressing interacting hybrid proteins. Following 10 days, histidine prototrophs were screened by a colony lift assay. Four positive clones (SF7, SF77, SF107A, and SF107B) were identified..

Since more than one AD/library plasmids may be present in each β-galactosidase positive colony, re-streaking of each of the positive colonies on SD/-Trp/-Leu/-His selection plates was used to segregate multiple AD/library plasmids (Fig.5). As well, to eliminate the bait plasmid BD/XIRS-$1_{3\text{-}500}$ the positive colonies were re-streaked several times on SD/-Leu media, removing the Trp selection, to select for Trp auxotrophs. These colonies would have then presumably lost the bait plasmid (*TRP1)* yet still have had the AD/library plasmid with *(LEU2)*.

2.2.2 The two hybrid assay demonstrates the specific interaction of XIRS-1 with XROKα

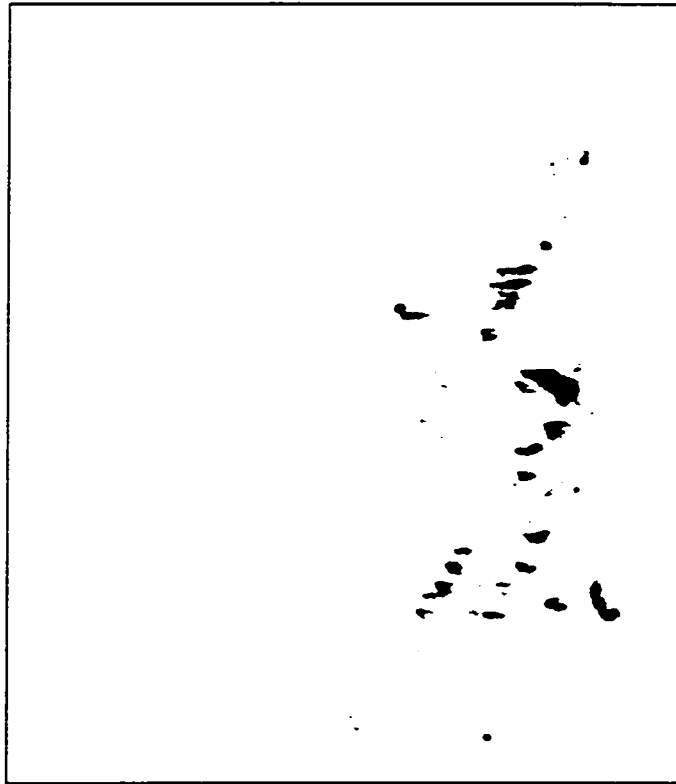
To confirm the positive results obtained, AD/library plasmid DNA extracted from all positive colonies were co-transformed with the BD/XIRS-1 $_{3\text{-}500}$ in an other yeast strain, Y190. Y190 transformants containing BD/XIRS-$1_{3\text{-}500}$ together with each of the four clones

(SF7, SF77, SF107A, and SF107B) were able to produce blue colour by the colony lift assay indicating β-galactosidase activity, confirming the results obtained in HF7c cells.

An EcoR1 digest of the positive clones revealed that two positive clones (SF7 and SF107A) contained similar 3.5 kb inserts, whereas the remaining two clones contained 0.7 kb inserts. Sequence analyses of both plasmids containing the insert SF7 and SF107A showed that they were identical. The SF7/ SF107 clones encodes an open reading frame of 500 amino acids fused in frame with the GAL4 activating domain.

To ensure that the SF7 was not interacting with the pAS2 portion of the bait, it was co-transformed with a non-specific bait VAMP1 constructed in the same vector (Calakos and Scheller, 1994; provided by Dr. J. Ngsee). As can be seen in Figure 7, colony lift assays for β-galactosidase activity revealed no activity with this combination. While SF7 with the original bait BD/XIRS-1_{3-500} showed β-galactosidase activity, demonstrating that SF7 interacted specifically with the XIRS-1 portion and not the pAS2 portion of the bait.

The DNA sequence of the SF7 clone was compared to reported sequences in the databases. This analysis revealed that the 5' end of the cDNA encodes an open reading frame (ORF) of 500 amino acid which was highly homologous to the C-terminus of rat ROKα with 80% and 90% identity in nucleotide and amino acid sequences, respectively (Fig.8). Compared to rat ROKα, the N-terminus of SF7 encodes the putative RhoA binding domain and the PH/CR domains. The rest of the insert presumably was derived from the 3' non-translated region of the mRNA (Fig.9).



VAMP1 *XIRS-L*

Figure 7:

SF7 interacts specifically with N-terminal XIRS-1 in the two hybrid assay.

SF7 induced β-galactosidase activity when cotransformed with the GAL4/XIRS-1 fused plasmid, but not when cotransformed with a vector containing an unrelated insert (VAMP1), confirming that the β-galactosidase activity was dependent on the bait. β-galactosidase activity, reflecting an interaction between hybrid proteins, was analysed by colony Lift assay using X-Gal as substrate.

The yeast strainY190, transformed with SF7 plasmid was cotransformed with VAMP1, in pAS2, plasmid in lane 1 and with XIRS-1 in, pAS2, in lane 2, both transformants were streaked on SD /-Trp /-Leu media

```
SF7     1 ............................GCGGCTCAGCTGGAGATCACA 21
ROKα 2751 AGGATTTACAGGATGAAAGAGACTCCTTGGCTGCCCAGCTGGAGATCACC 2800

       22 TTGACCAAGGCCGACTCCGAGCAGCTGGCACGTTCCATCGCTGAGGAACA 71
     2801 CTCACCAAAGCAGACTCTGAGCAGCTGGCTCGCTCCATTGCTGAGGAGCA 2850

       72 GTACTCCGACCTGGAGAAAGAGAAGATCATGAAGGAACTAGAGATAAAGG 121
     2851 ATACTCTGACTTGGAAAAAGAGAAGATCATGAAAGAGCTGGAAATCAAAG 2900

      122 AGATGATGGCGCGGCACAAGCAGGAACTGGCTGAAAAATACGCTACAATT 171
     2901 AGATGATGGCTAGACACAAACAAGAGCTTACTGAAAAGGACGCTACAATT 2950

      172 ACTTCTTTGGAGGAAACTAACAAAACACTGACTATCGATGTGGGTAATTT 221
     2951 GCATCCCTTGAAGAAACAAATAGAACACTAACTAGTGATGTTGCAAATCT 3000

      222 GGCTAATGAGAAGGAGGACTTAAATAACAGGCTGAAAGAAGCCCATGAAC 271
     3001 TGCAAACGAGAAAGAAGAACTAAACAACAAGTTGAAAGATACCCAAGAAC 3050

      272 AAATTCAGAGGCTGAAAGAGGAAGAAAACAGCGTTGTTACCATCAAGACA 321
     3051 AACTGTCAAAGTTGAAAGATGAAGAGATCAGTGCAGCGGCTATTAAAGCA 3100

      322 CAGTTTGAGAAGCAGCTGTTGACTGAGAGGACTCTGAAGACACAGGCTGT 371
     3101 CAGTTTGAGAAGCAGCTGCTGACTGAGCGAACACTCAAGACTCAAGCTGT 3150

      372 GAACAAGCTAGCGGAGATCATGAACCGCAAGTTACCCACCAAGAGAGGGC 421
     3151 GAATAAGTTGGCAGAGATCATGAATCGAAAAGAACCTGTCAAGCGTGGTA 3200

      422 CGGACACAGATGTACGAAGGAAGGAAAAGGAGAACCGCAAACTGCAGCTT 471
     3201 GTGACACAGATGTGCGAAGAAAAGAAAAGGAAAACAGAAAATTACATATG 3250

      472 GACTTGAAATCGGAGCGTGAGAAATTCACCCAGCTTGTCATCAAGTACCA 521
     3251 GAGCTTAAGTCTGAACGTGAAAAATTGACACAACAGATGATCAAATATCA 3300

      522 GAGGGAGATGAATGATATGCAGGCGCAAATAGCAGATGAGAACCAAGTCC 571
     3301 GAAAGAACTGAATGAAATGCAGGCTCAAATAGCTGAAGAGAGCCAAATTC 3350

      572 GAATCGAACTGCAGATGGCGCTAGATAGCAAAGACAGTGACATCGAGCAG 621
     3351 GAATAGAACTGCAGATGACCCTGGACAGTAAAGACAGTGACATTGAACAG 3400

      622 CTCCGGTCGCAAATGC............TCGGCCTCGACAGCACGAGTAT 659
     3401 CTTCGGTCGCAGCTCCAGGCCTTGCATATTGGTATGGACAGTTCCAGTAT 3450

      660 CGGCAGTGGACACGGGGACACAGATGCTGAAGATGGCTTTCCAGAATCCA 709
     3451 AGGCAGTGGACCAGGGGATGCTGAGCCTGATGACGGATTTCCAGAATCAC 3500

      710 GGTTGGAAGGATGGTTGTCACTTCCTTTGCG...CAACGCCAAAAAGTTT 756
     3501 GATTAGAAGGATGGCTGTCGTTGCCTGTGCGGAACAACACTAAAAAGTTT 3550
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 757 GGTTGGAATAAGAAGTATGTAGTGGTAAGCAGCAGGAAGaTCCTGTTTTA 806

3551 GGATGGGTTAAGAAGTATGTGATTGTAAGCAGCAAGAAGATTCTTTTCTA 3600

 807 TGACAGTGAGCAGGATAAGGAGCTGTCAAACCCATCCATGGTGCTGGACA 856

3601 TGATAGTGAACAAGATAAAGAGCAGTCTAACCCTTACATGGTGTTAGACA 3650

 857 TTGATAAATTGTTCCATGTGCGACCAGTTACTCAGACGGATGTATATCGA 906

3651 TAGACAAGCTATTTCATGTCCGACCTGTTACTCAGACAGATGTATACAGA 3700

 907 GCCGATGCCAAAGAGATTCCACGGATATTCCAGATTCTATACGCCAATGA 956

3701 GCAGATGCTAAAGAAATTCCAAGGATATTTCAGATCCTGTATGCCAATGA 3750

 957 AGNAGAAAGCAAGaAAGAACAAGAGTTCCAAGTGGATCCCCT...AGAGA 1003

3751 AGGAGAAAGTAAAAAGGAACCAGAATTTCCAGTGGAACCAGTGGGAGAAA 3800

1004 AGTCCAATTACATATGTCACAAAGGACACGAGTTCATCCCCACACTCTAC 1053

3801 AATCTAATTATATTTGCCACAAAGGACATGAGTTTATTCCTACTCTTTAT 3850

1054 CATTTCCCCACTAGCTGTGACGCTTGTATGAAGCCCCTGTGGCACATGTT 1103

3851 CACTTCCCAACCAACTGTGAGGCCTGTATGAAGCCACTGTGGCACATGTT 3900

1104 TAAACCCCCCGCTGCCCTGGAGTGTCGGCGCTGCCATATCAAGTGCCACA 1153

3901 TAAACCTCCTCCTGCTCTAGAGTGCAGTAGATGCCACATTAAATGTCATA 3950

1154 AGGATCACATGGACAAGAAAGAAGAGATAATAGCACCTTGCAAAGTGAAC 1203

3951 AAGATCACATGGACAAAAAGGAAGAGATCATAGCCCCCTGCAAAGTTTAT 4000

1204 TATGATATTTCCACCGCAAAGAATCTACTATTACTAGCCAACTCCACAGA 1253

4001 TATGATATATCATCGGCAAAGAATCTGTTGTTATTAGCAAATTCAACGGA 4050

1254 AGAACAGCAGAAGTGGGTGAGTCGACTGGTGAAAAAGATCCCCAAGAAGC 1303

4051 AGAACAGCAGAAATGGGTTAGTCGGTTGGTGAAAAAGATACCTAAAAAGC 4100

1304 CCCCAGCCTCTGAGCATCAAGCTCGGTCCTCTCCACGGCCTCCTGCTAAG 1353

4101 CTCCAGCTCCAGACCCTTTTGCCCGATCATCCCCTAGAACCTCAATGAAA 4150

1354 GCTTCGCTAAACCAGTCCATGAGGCGGCCAAGCCGACAGCTTCCTCCAAA 1403

4151 ATACAACAAAACCAGTCCATTCGGCGGCCCAGTCGACAGCTTGCTCCAAA 4200

1404 TAAACCCAGCTAAC.TGCCTTCCGTGAATGCAGTCCCCATTTCAAGACGT 1452

4201 CAAACCAAGCTAACTTGCCTTCTATGAAAGCAGTTATTGAAGGTGATCAT 4250

1453 TCGTTT............................................ 1458

4251 ATTCTTCCATCCACAAGACTGAAATATGGTTTCTCAAAACTTAACTAAAA 4300
```

Figure 8A: .

Nucleotide sequence comparison between XROKα and rat ROKα clones.

Nucleotide sequence of SF7 was compared to that of ROKα using Bestfit program (Genetic Computer Group, Madison, Wisconsin)

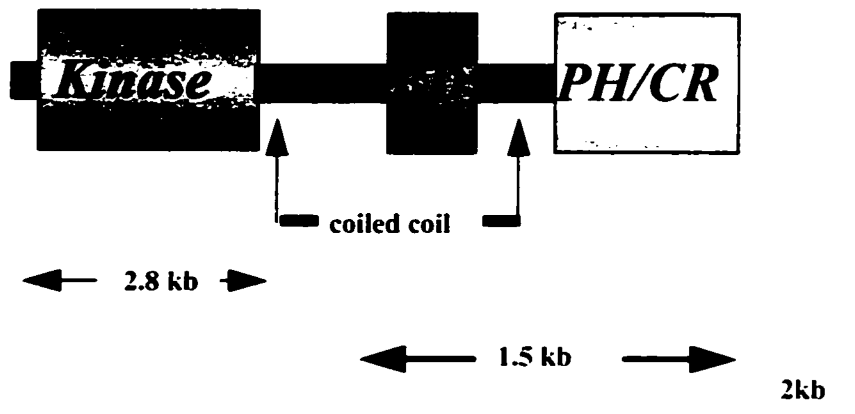
```
SF7     1 ................EFAAASTLKEECEVKGKMYKEVQQKVQELQDERD 34

ROKα  851 KELQDQLEAEQYFSTLYKTQVRELKEENEEKTKLCKELQQKKQDLQDERD 900

       35 SLAAQLEITLTKADSEQLARSIAEEQYSDLEKEKIMKELEIKEMMARHKQ 84

      901 SLAAQLEITLTKADSEQLARSIAEEQYSDLEKEKIMKELEIKEMMARHKQ 950

       85 ELAEKYATITSLEETNKTLTIDVGNLANEKEDLNNRLKEAHEQIQRLKEE 134

      951 ELTEKDATIASLEETNRTLTSDVANLANEKEELNNKLKDTQEQLSKLKDE 1000

      135 ENSVVTIKTQFEKQLLTERTLKTQAVNKLAEIMNRKLPTKRGPDTDVRRK 184

     1001 EISAAAIKAQFEKQLLTERTLKTQAVNKLAEIMNRKEPVKRGSDTDVRRK 1050

      185 EKENRKLQLDLKSEREKFTQLVIKYQREMNDMQAQIADENQVRIELQMAL 234

     1051 EKENRKLHMELKSEREKLTQQMIKYQKELNEMQAQIAEESQIRIELQMTL 1100

      235 DSKDSDIEQLRSQM....LGLDSTSIGSGHGDTDAEDGFPESRLEGWLSL 280

     1101 DSKDSDIEQLRSQLQALHIGMDSSSIGSGPGDAEPDDGFPESRLEGWLSL 1150

      281 PLR.NAKKFGWNKKYVVVSSRKILFYDSEQDKELSNPSMVLDIDKLFHVR 329

     1151 PVRNNTKKFGWVKKYVIVSSKKILFYDSEQDKEQSNPYMVLDIDKLFHVR 1200

      330 PVTQTDVYRADAKEIPRIFQILYANEXESKKEQEFQVDPL.EKSNYICHK 378

     1201 PVTQTDVYRADAKEIPRIFQILYANEGESKKEPEFPVEPVGEKSNYICHK 1250

      379 GHEFIPTLYHFPTSCDACMKPLWHMFKPPAALECRRCHIKCHKDHMDKKE 428

     1251 GHEFIPTLYHFPTNCEACMKPLWHMFKPPPALECSRCHIKCHKDHMDKKE 1300

      429 EIIAPCKVNYDISTAKNLLLLANSTEEQQKWVSRLVKKIPKKPPASEHQA 478

     1301 EIIAPCKVYYDISSAKNLLLLANSTEEQQKWVSRLVKKIPKKPPAPDPFA 1350

      479 RSSPRPPAKASLNQSMRRPSRQLPPNKPS*LPSVNAVPISRRSF...... 522

     1351 RSSPRTSMKIQQNQSIRRPSRQLAPNKPS*LAFYES.SY*R*SYSSIHKT 1399
```

Figure 8B:

Predicted Amino acids sequence of SF7

Comparison of the deduced amino acid sequence from SF7 to that of XROKα (Bestfit).

rat ROKa



SF7

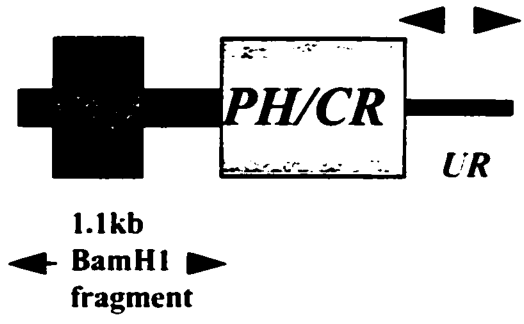


Figure 9:
Schematic comparison between SF7 and rat ROKα
Representation of the linear peptide sequence of rat ROKα and the sequence encoded by the partial cDNA sequence of XROKα, SF7, obtained by yeast two-hybrid screening.
BD = binding domain, PH/CR = pleckstrin homology and cysteine rich domain.

In order to determine whether SF7 interacted with activated RhoA. SF7 was co-transformed with pAS2/V14-RhoA. (provided by Dr. J. Ngsee). Both filter lift assay and liquid assay for β-galactosidase demonstrated that SF7 interacted strongly with V14-RhoA (Fig.10)

2.2.3 Identification of specific XIRS-1 domains interacting with XROKα.

Having demonstrated interaction between XIRS-1 and SF7 in the two-hybrid system (see section 2.2.2). I went on to delineate the domain of XIRS-1 $_{3-500}$ that was responsible for interacting with SF7. A series of deletion mutants were generated. These Included PH (2-140). PH/PTB (2-324). PTB (136-324). PTB/SAIN (136-500). and SAIN (307-500) domains inserted into the pAS2 vector. These constructs were cotransformed with SF7 (Fig. 11).

There was no interaction between the PH domain and SF7 despite high-levels of protein expression (see below: Fig.12). The highest binding to SF7 was found to occur with the XIRS-1$_{3-500}$, PTB or PTB/SAIN domains. while the SAIN domain displayed less binding to the SF7. This suggested that the major XIRS-1 domain interacting with SF7 was primarily located within the PTB domain. RhoA was used as positive control and the pAS2 as a negative control (Fig.11)

BD hybrid	X	AD hybrid	Colony lift assay	β-galactosidase activity (units)
BD/XIRS-1 $_{(3\text{-}500)}$	X	—	—	0.15
BD/XIRS-1 $_{(3\text{-}500)}$	X	AD/SF7	++	8.4
BD/RhoA	X	AD/SF7	+++	45.2

Figure 10:
SF7 interacts with RhoA
Interactions of indicated constructs were determined by both filter assay and liquid assay for β-galactosidase activity (see Material and Methods). The colony colour assay showed either white (-), blue (++), or dark blue (+++) colonies. β-galactosidase (β-GAL) activity is reported by Miller units as mean (Miller, 1972).

XIRS-L subclones in pAS1
and
XROKa in pGAD10

pGAD10 vector

PH

PH PTB

PH

RhoA

Colony colour intensity

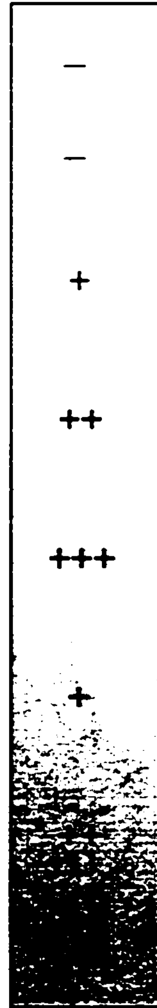
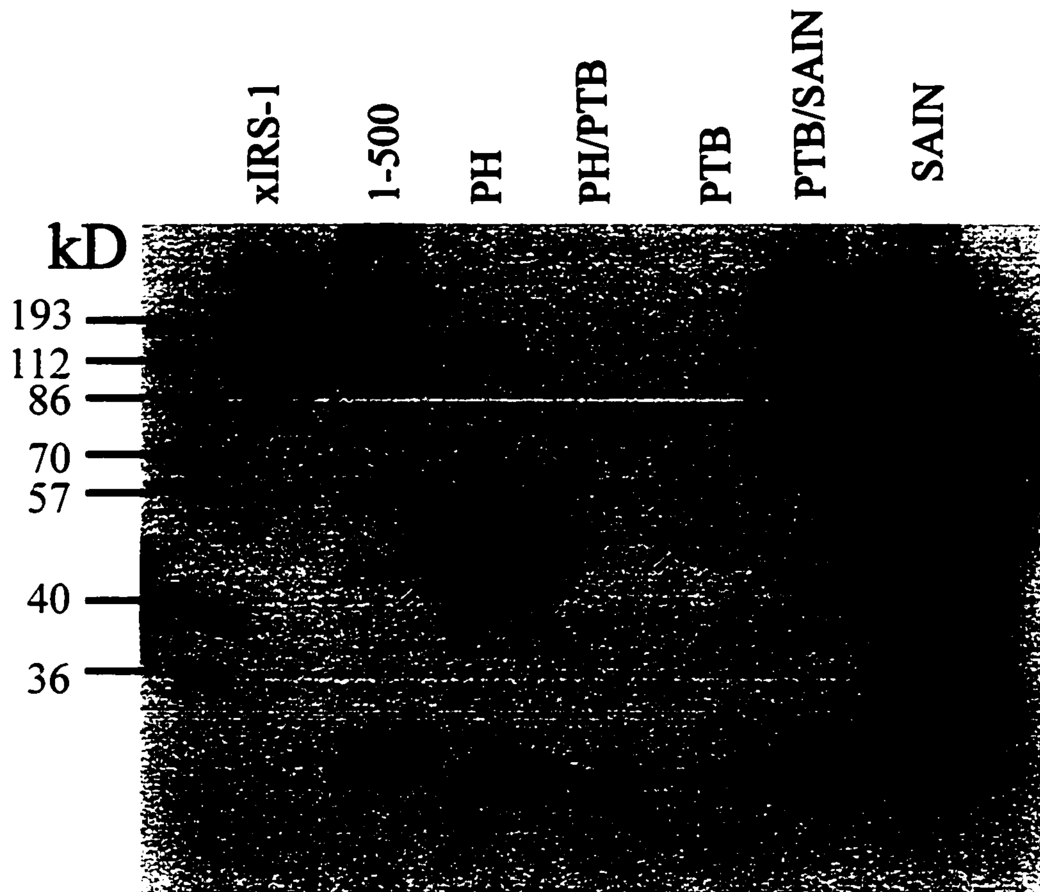


Figure 11:

The PTB/SAIN of XIRS-1 interacts with the SF7

Schematic representation of five XIRS-1 activation domain hybrids used in the two hybrid assay. Following cotransformation of plasmids encoding for these hybrid proteins the colony colour assay showed either white (-), very light blue (+), blue (++), or dark blue (+++). (5 independent colony were used for each separate subclone)



xIRS-1
1-500
PH
PH/PTB
PTB
PTB/SAIN
SAIN
kD
193
112
86
70
57
40
36

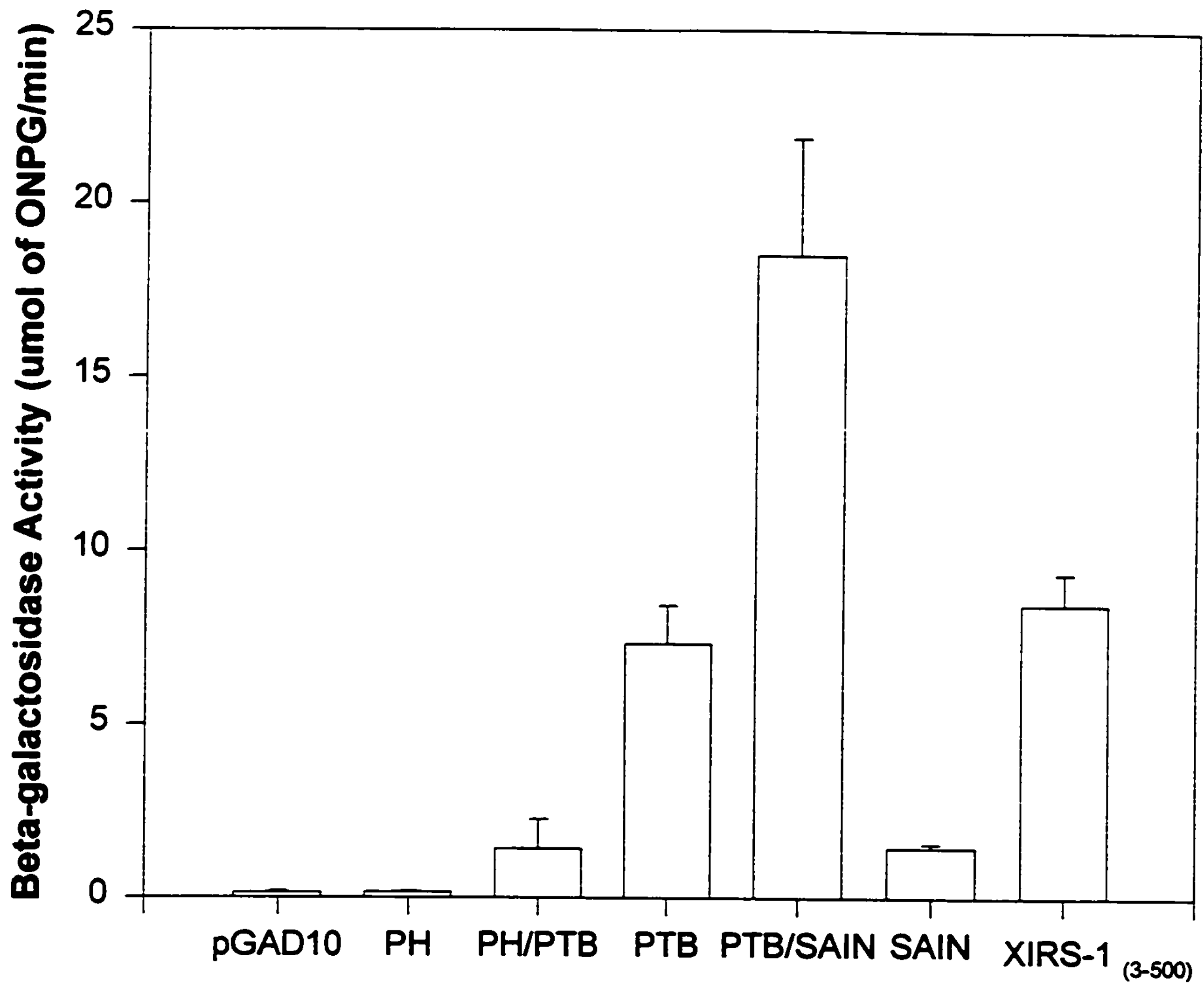
Figure 12:

Immunoblot analysis of expression of the XIRS-1 fusion protein

Immunoblot analysis of expression of the XIRS-1 domain hybrid proteins using HA antibody. Equal amount of cell lysate were analysed for all samples as described in Material and Methods. Lane 1 HA tagged XIRS-1 expressed in Sf9 cells as a control (Liu *et al.*, 1995); lane 2 N-terminal XIRS-1 hybrid 3-500; lane 3, PH domain hybrid; lane 4, PH/PTB hybrid; lane 5, PTB hybrid; lane 6, PTB/SAIN hybrid; and lane 7 SAIN hybrid. The extracts used for immunoblotting were derived from the same yeast cultures that were used in the solution β-galactosidase assays (Fig. 13). Sizes are indicated in kilodaltons.

To confirm the expression of the deletion mutants in the Y190 yeast cells. yeast lysates were resolved on an SDS-PAGE gel and then Western blotted with HA antibody. While all constructs expressed proteins of anticipated molecular weight (Fig. 12). the levels varied considerably. The PTB domain. although expressed at a relatively low level. activated β-galactosidase to a similar level as the original bait (XIRS-1_{3-500}) (Fig. 12). The PH domain. in contrast. showed no β-galactosidase activity despite a considerably higher level of expression. The SAIN domain was expressed to an extremely high level. making it difficult to interpret the relatively weak binding observed (Fig.11).

As a more quantitative method to determine the degrees of binding with SF7. six representative colonies from each deletion mutants co-transformed with SF7 were assayed for β-galactosidase activity. using a liquid assay. When β-galactosidase activity obtained from these transformants was analysed (Fig.13). the PTB/SAIN domain demonstrated about twice as much as XIRS-1_{3-500}. However. the PH domain did not show any interaction with XROKα. The PTB domain interacted with XROKα almost at the same level as the XIRS-1_{3-500} (Fig.13).



Beta-galactosidase Activity (umol of ONPG/min)
25
20
15
10
5
0
pGAD10
PH
PH/PTB
PTB
PTB/SAIN
SAIN
XIRS-1 (3-500)

Figure 13:

Binding of the various N-terminal XIRS-1 domains to SF7 was assessed by liquid β-gal assay

Shown are average of 6 separate transformants with standard errors.

Chapter Three

Cloning and characterization full length XROKα

In the preceding chapter. I described the identification of XROKα as an IRS-1 binding protein. In order to carry out functional studies of XROKα in insulin signalling. I decided to clone the full length cDNA of XROKα and to generate XROKα specific antibodies.

3.1 Materials and Methods

3.1.1 Library Screening

A λgt10 *Xenopus laevis* oocyte cDNA library was provided by D.A. Melton (Dumont. 1971). Recombinant λgt10 phage were allowed to adsorb to an overnight culture *E. coli* C600 bacteria for 10 min at 37°C. plated (20,000 pfu/15cm plate). and incubated for 8-12 hr at 37°C. Nitrocellulose filters were overlayed on the plates for 1 min. and then soaked in denaturing solution (1.5M NaCl. 0.5M NaOH) for 5min. They were then neutralized in 1.5 M NaCl and 0.5 M Tris-HCl pH 8. for 5 min. and finally rinsed in 0.2 M Tris-HCl pH 7.5 and 2X SSC (1X SSC is 0.15M NaCl and 0.01M soduim citrate) for 30 sec. The DNA was fixed to the filters by baking them at 80°C for 2 hr. The filters were then probed with specific probes derived from XROKα cDNA. These probes were labelled with α^{32}P dCTP using a random primed extension labelling kit (Boehringer). Prehybridization was carried out

in 50% formamide. 5X SSPE (1X SSPE is 0.18 M NaCl. 10mM NaH_2PO_4. and 1mM EDTA pH 8). 5X Denhardt's solution (50X Dnehardt's is 1% ficoll. 1% polyvinyl pyrrolidone. 1% bovine serum albumin) and 0.1% SDS. The hybridization buffer consisted of 50% formamide. 5X SSPE. 1X Denhardt's solution. 90 mg of dextran sulfate per ml. and 0.1% SDS. Hybridization was carried out at 42°C for overnight. The filters were washed several times with large volumes of increasingly dilute SSC containing 0.1% SDS at increasing temperatures. then autoradiographed by exposure to Kodak XAR-5 X-ray film. Positive clones were confirmed and purified by secondary and tertiary screening using the same XROKα probe used for the primary screening.

3.1.2 Isolation of bacteriophage DNA

To verify the positive clones obtained from screening. bacteriophage DNA was isolated using Dario Grossberger's method (1987). An isolated λgt10 phage plaque was picked from the plates using the small end of a pasture pipette and resuspended in SM buffer (0.09M NaCl. 8mM $MgSO_4 \cdot 7H_2O$. 50mM Tris-HCl (pH 7.5). and 0.01% gelatin solution; Maniatis. 1989). The phage were allowed to adsorb to an overnight culture of *E. coli* (C600) liquid culture for 10 min at 37°C. Ten milliliters of LB broth containing 10 mM $MgCl_2$ and 0.1% glucose was added to the phage/bacterium mixture and the tubes were shaken overnight in a 37°C during which cell lysis occur. The cell debris were pelleted by two low speed centrifugation. The phage particles were then pelleted by high speed centrifugation (50.000g for 1h) and resuspended in SM buffer. Proteinase K was added to a final concentration of 50μg/ml and the samples were incubated at 37°C for 2 h. Following this incubation. the

samples were extracted once with phenol and once with chloroform. The DNA was precipitated with 0.3M ammonium acetate and 100% ethanol. The precipitated DNA was pelleted by centrifugation, washed with 70% ethanol, dried, and dissolved in TE (10mM Tris-HCl pH 7.4 and 1mM EDTA pH 8.0)

3.1.3 Southern blot analysis

Purified bacteriophage DNA was digested with EcoR1 restriction enzymes to excise the insert. The electrophoretically separated DNA fragments were transferred by capillary blotting for 16 h to Hybond-N membranes (Amersham) following standard procedure (Maniatis, 1989). The DNA was cross-linked to the membranes by exposing them to 120 mJ ultraviolet light using a GS Gene linker UV chamber (Biorad). Prehybridization of the membrane was carried out in 50% formamide, 5X Denhardt's solution, 5X SSPE, 0.1% SDS and 10μg/ml heat-denatured salmon sperm DNA for 4 to 16 h at 42°C. Hybridization was carried out in the above mentioned prehybridization buffer with the addition of 1X Denhardt's solution and 90 mg/ml of dextran sulfate. A random-primed α^{32}P-labelled DNA probe (using Boehringer kit) with a specific activity between 3-20 X 10^6 cpm/ml was added to the hybrid mix and then incubated at 42°C for 8 to 12 h. Filters were washed in 3 times using of 2X SSC and 0.1% SDS at room temperature for 2 h, followed by several washes in 0.2% SSC and 0.1% SDS at 42°C for 2h to remove non-specifically bound probe. The filters were then exposed to Kodak (XAR-5) films.

3.1.4 Oocyte Protein Isolation

Stage VI *Xenopus* oocyte (Dumont, 1971) were isolated from pregnant mare serum gonadotropin (PMSG)-primed female. Oocytes were lysed by forcing them through pipette tips in PBS lysis buffer (10 mM sodium phosphate, pH 7.5, 150mM NaCl, 1% Triton X-100, 10μg/ml each of leupeptin and aprotinin, 1mM phenylmethylsulfonate and 1mM sodium orthovanadate; 10μl/oocyte). The homogenate was centrifuged at 15000 rpm for 15 min at 4°C. Under these conditions, the yolk protein (vitellogenin) was not solubilized and was discarded as a pellet. The cell lysates, which were usually clouded due to the presence of lipids, were suitable for immunoprecipitation and western blotting.

3.1.5 Generation of an XROKα antibody

A 1100 bp fragment of SF7 was isolated by digestion with BamH1 restriction enzyme (Fig.9). The gel-purified fragment was subcloned into a BamH1 digested pGEX vector. The pGEX vector allowed the fusion of a gene of interest to the C-terminus of glutathione S-transferase (GST). The GST fusion proteins were produced by isopropyl thio-β-D-galactoside (IPTG) induction and the fusion protein was isolated by affinity purification according to Frangioni and Neel (1993) method.

The purified GST fusion proteins were used to immunize rabbits. For the first injection each rabbit received 200μg of purified protein mixed with complete Freund's adjuvant. The rabbit was injected 4 weeks later using half as much antigen mixed with

incomplete Freund's adjuvant. 10-14 days later the rabbit was bled. The blood was incubated at 37°C for 1h to allow clogs to develop. After a low speed centrifugation the serum was recovered and used directly without further processing.

3.1.6 XROKα Immunoprecipitation and kinase assay

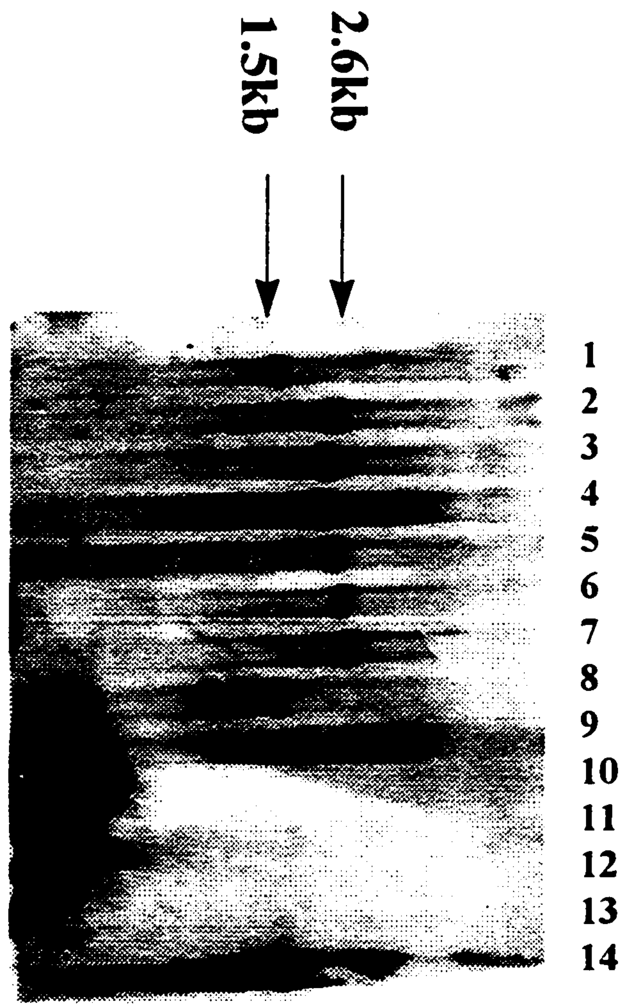
Oocytes were lysed with lysis buffer [25mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES: pH 7.3), 0.3M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 20mM sodium β-glycerophosphate, 1 mM Sodium vanadate, 0.5% Triton X-100, and 5% glycerol]. For immunoprecipitation, extracts containing 2mg proteins were incubated with 5 μl of preimmune-serum and immune-serum generated against GST-XROKα fusion protein, described above, for 2 h before collection on protein A sepharose beads. After several washes with lysis buffer a kinase reaction was carried out in buffer (50 mM HEPES (pH 7.3), 10mM $MgCl_2$, 2mM $MnCl_2$, 1mM dithiothreitol, and 0.05% Triton X- 100 with 10μM [γ-^{32}P] ATP and 5μg of myelin basic protein (MBP). After 30 min, the reaction was stopped by sample buffer (20% glycerol, 4% SDS, 0.005 %Bromophenol blue, 125mM Tris (pH=6.8), and 10% beta-mercaptoethanol) . Samples were denatured by boiling and resolved by SDS-PAGE on 7.5% and 15% polyacrylamide gels to reveal autophosphorylation and phosphorylation of MBP respectively.

3.2 Results

3.2.1 Cloning full length XROKα.

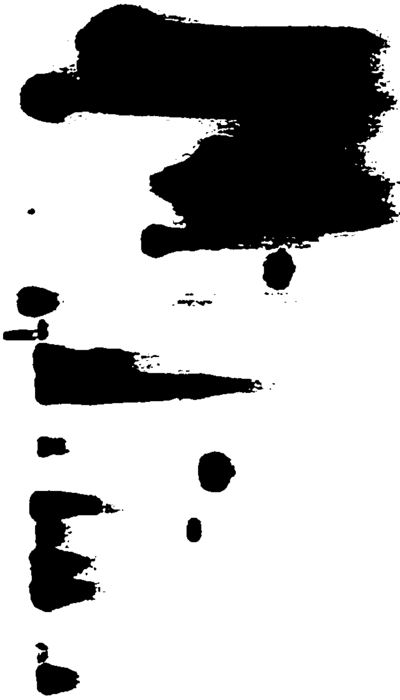
In order to clone the remaining coding region of XROKα, the 1100 base pair (b.p) BamH1 fragment (Fig. 4), of (SF7) was used to screen a λgt10 *Xenopus* ovary cDNA library. Through the screening of 0.6 x 10^6 recombinant phage plaques, 22 clones that hybridized with the probe were isolated. To confirm that these were true positive clones, inserts were excised by EcoR1 digest, Southern blotted with the same probe (Fig.14A). Almost all of the 22 clones hybridized with the partial sequence of XROKα. The longest clone of 3.5 kb (clone 45) was sequenced and found to contain an additional 1 kb sequence 5' to SF7 (Fig.16).

A subsequent screening of the same λgt10 *Xenopus* ovary cDNA library, using a 1.5 kb DNA fragment derived from the 5' end of clone 45 (Fig. 16), identified 14 positive clones. Restriction digests revealed that the longest clone, clone 63, contained a 2.6 kb insert. Further confirmation was obtained from a southern blot and probed with a radioactive labelled 1.5 kb 5' sequence of clone 45 (Fig.14B). Sequence comparison of clone 63 to rat ROKα, showed that clone 63 contained the entire kinase domain of XROKα; however, it was still missing 40 or 50 amino acids by comparison to ROKα.



B

3.5kb



A

Figure 14:

Southern Blot analysis

A) Positive clones from first round hybridization screening. the clone in lane 2 was subcloned and sequenced (clone 45).

B) Positive clones from the second round of hybridization screening, the clone in lane 3 was subcloned and sequenced (clone 63)

3.2.2 PCR amplification of the 5' end of XROKα

The remaining DNA sequence of the 5' coding region of XROKα was obtained using polymerase chain reaction (PCR) on the original Clontech's pGAD10 *Xenopus laevis* oocyte library. To do this, two nested primers (xrok63-2 and xrok63-4) antisense to the 5' end of clone 63 were designed (table1). These two primers were used in combination with pGAD10 forward or reverse primers in PCR reaction. A fragment of 450 bases was amplified by xrok63-4 and the pGAD10 reverse primer (Fig.15). The specificity of this amplification was confirmed by re-amplification using xrok63-2, which is antisense to a region of XROKα 100 b.p. 3' to xrok63-4. This resulted in the amplification of an expected 350 b.p. fragment. The 450 bp PCR product was cloned and sequenced. This clone, PCR L1, contains the missing N-terminus of XROKα.

3.2.3 Characterization of XROKα in *Xenopus* oocytes

To characterize endogenous XROKα, I produced polyclonal antisera in rabbits. Amino acids (994-1364) of XROKα (Fig.17) was subcloned in pGEX kt vector (Guan, 1991). Following induction with IPTG in transformed bacteria, a GST fusion protein containing the XROKα sequence (~80 kDa) was detected by both coomasie blue staining and by Western blotting using anti-GST antibody. This protein was subsequently purified by binding to glutathione beads. The purified protein was used to immunize two rabbits. The antisera obtained from the immunized rabbits were used out for further processing.

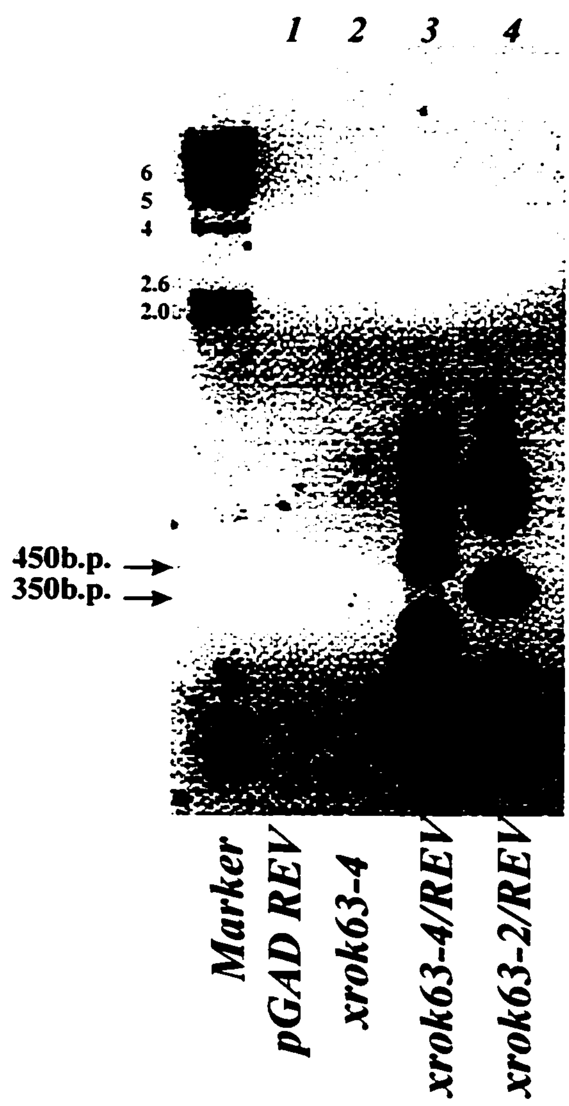
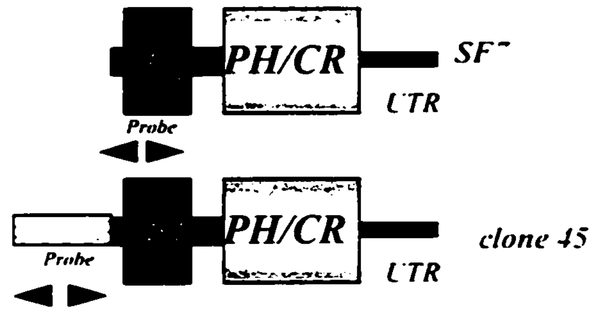

1
2
3
4
6
5
4
2.6
2.0
450b.p.
350b.p.
Marker
pGAD REV
xrok63-4
xrok63-4/REV
xrok63-2/REV

Figure 15:

PCR Amplification of the extreme 5' end of the XROKα from *a Xenopus* library

Using xrok63-4 and pGAD10 reverse primers, 450 b.p. were amplified from the pGAD10 *Xenopus* cDNA library (lane 3). Re-amplifying this band by xrok63-2 and pGAD10 reverse resulted in 350 b.p (lane 4). Lane 1 and 2 were two negative control for the PCR using single primers.

XROKa



PCR L1

Figure 16:
Diagramatic representation of the various XROKα subclones.
(See text for details)

Since ROKα was reported earlier (Leung *et al.*.1995. Ishizaki *et al.*. 1996. Amano *et al.*. 1996) to be capable of autophosphorylation and phosphorylation of MBP. we were interested in detecting if XROKα exhibits a kinase activity. This possibility was examined by immunoprecipitating endogenous XROKα with anti-XROKα antiserum. using preimmune serum as a negative control. from oocyte lysates. The kinase assay was carried out using these immunoprecipitates in the presence of [γ-^{32}p]ATP and myelin basic protein (MBP). As shown in Figure 19 immunoprecipitated XROKα was able to phosphorylate MBP. however this phosphorylation was not observed when pre-immune serum was used. Moreover the autophosphorylation of a 200 kDa protein was also detected (Fig. 18). Western blotting with the XROKα antibody revealed that the p200 was XROKα (Fig.18). This result suggested that like ROKα (Leung.T.1996). XROKα is also a protein kinase.

Primer	Sequence	specification
xrok63-2	5'-CA GGG AAA TCA AGG TCC AG-3'	PCR primer 100 bp apart from the 5' end of clone 63
xrok63-4	5'-CTT CGG CTT TCA TTT GAA GCT TGC GG-3'	PCR primer at the 5' end of clone63
T3	5'-ATTAACCCTCACTAAAG-3'	sequencing primer for pbluescript
pGADfor	5'CTATTCGATGAAGATACCCCACCAAAC-3'	PCR and seq.
pGADrev	5'GTGAACTTGCGGGGTTTTTCAGTATCTACG-3'	PCR and seq.
Primer	Sequence	specification
Y2HN	5'TATGGATCCCTAGCCCCACAGATC-3'	PCR for XIRS-1
Y2HC	5'TATGTCGACTGAGGAGTGAGTCC 3'	PCR for XIRS-1
Y2HC1	5'TATGTCGACATCTCCATTTAAGC	PCR for XIRS-1
Y2HN1	5' TATGGATCCATGATGGCTTAAATGG 3'	PCR for XIRS-1
Y2HC2	5' TATGTCGACACTAGATGCCCGG 3'	PCR for XIRS-1
Y2HN2	5' TATGGATCCCACCTGCTGGGGGTG 3'	PCR for XIRS-1
pGADfor	5'CTATTCGATGAAGATACCCCACCAAAC-3'	sequencing primer
pGADrev	5'GTGAACTTGCGGGGTTTTTCAGTATCTACG-3'	sequencing primer

Table 1:
Primers used for both sequencing and PCR purposes through out the thesis

```
GAAAAGAGCAATATGGAAATAGATATGaCCTACAAACTGAAAGCACTACAGCAAAGCGTT
E  K  S  N  M  E  I  D  M  T  Y  K  L  K  A  L  Q  Q  S  V    689
GAGAAAGAGGAATCTGAACATAAAGCCACAAAGGCTCGGCTTGCTGACAAGAACAAAATC
E  K  E  E  S  E  H  K  A  T  K  A  R  L  A  D  K  N  K  I    709
TATCAGTCCATCGAGGAGACCAAGTCAGAAGCAATGAAAGACATGGAGAAGAAGCTTCAG
Y  Q  S  I  E  E  T  K  S  E  A  M  K  D  M  E  K  K  L  Q    729
GAGGAACGCGTGGCCAAGCAGAGGCTGGAGAATAATTTGCTGGAGACAGAGAAACAATAC
E  E  R  V  A  K  Q  R  L  E  N  N  L  L  E  T  E  K  Q  Y    749
TCCATGTTGGACTGTGACCTCAAACAAGCAAAGCAAAAGATCAATGAGTTGGAAGCACTC
S  M  L  D  C  D  L  K  Q  A  K  Q  K  I  N  E  L  E  A  L    769
AAAGACAAACTCAGTGAGGATATTAAAAACTTAACGCTAAAAGCAGAACAAGAGACGCAA
K  D  K  L  S  E  D  I  K  N  L  T  L  K  A  E  Q  E  T  Q    789
AAGCGCAGCCTGTCGCAGAATGATCTGAAGATGCAGCTGCAGCAAGTGAATTGCCTGAAG
K  R  S  L  S  Q  N  D  L  K  M  Q  L  Q  Q  V  N  C  L  K    809
ATGTCTGAGAAGCAGCTGAAGCAAGAGGTCAACCATCTGACCGAGATCAAACTGAACCTG
M  S  E  K  Q  L  K  Q  E  V  N  H  L  T  E  I  K  L  N  L    829
GAAAAGCAGAACAACGAGTTGCGCAAAGAAAGAGTAGATGCGGATGGACAGATGAAGGAG
                                   (xROK-K)λ63-
E  K  Q  N  N  E  L  R  K  E  R  V  D  A  D  G  Q  M  K  E    849
CTTCAAGACCAGTTGGAGGCTGAGCAGTATTTCTCAACACTGTATAAGACCCAGGTACGG
L  Q  D  Q  L  E  A  E  Q  Y  F  S  T  L  Y  K  T  Q  V  R    869
GAGTTGAAAGAGGAGTGTGAGGtCAAGGGCAAGATGTACAAAGAAGTGCAGCAGAAGGTC
                             -SF7(xROK-C)
E  L  K  E  E  C  E  V  K  G  K  M  Y  K  E  V  Q  Q  K  V    889
CAAGAACTCCAGGATGAGAGAGACTCACTGGCGGCTCAGCTGGAGATCACATTGACCAAG
Q  E  L  Q  D  E  R  D  S  L  A  A  Q  L  E  I  T  L  T  K    909
GCCGACTCCGAGCAGCTGGCACGTTCCATCGCTGAGGAACAGTACTCCGACCTGGAGAAA
A  D  S  E  Q  L  A  R  S  I  A  E  E  Q  Y  S  D  L  E  K    929
GAGAAGATCATGAAGGAACTAGAGATAAAGGAGATGATGGCGCGGCACAAGCAGGAACTG
E  K  I  M  K  E  L  E  I  K  E  M  M  A  R  H  K  Q  E  L    949
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A  E  K  Y  A  T  I  T  S  L  E  E  T  N  K  T  L  T  I  D    969
GTGGGTAATTTGGCTAATGAGAAGGAGGACTTAAATAACAGGCTGAAAGAAGCCCATGAA
V  G  N  L  A  N  E  K  E  D  L  N  N  R  L  K  E  A  H  E    989
CAAATTCAGAGGCTGAAAGAGGAAGAAAACAGCGTTGTTACCATCAAGACACAGTTTGAG
Q  I  Q  R  L  K  E  E  E  N  S  V  V  T  I  K  T  Q  F  E    1009
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K  Q  L  L  T  E  R  T  L  K  T  Q  A  V  N  K  L  A  E  I    1029
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M  N  R  K  L  P  T  K  R  G  P  D  T  D  V  R  R  K  E  K    1049
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E  N  R  K  L  Q  L  D  L  K  S  E  R  E  K  F  T  Q  L  V    1069
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I  K  Y  Q  R  E  M  N  D  M  Q  A  Q  I  A  D  E  N  Q  V    1089
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R  I  E  L  Q  M  A  L  D  S  K  D  S  D  I  E  Q  L  R  S    1109
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Q  M  L  G  L  D  S  T  S  I  G  S  G  H  G  D  T  D  A  E    1129
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D  G  F  P  E  S  R  L  E  G  W  L  S  L  P  L  R  N  A  K    1149
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S  E  Q  D  K  E  L  S  N  P  S  M  V  L  D  I  D  K  L  F    1189
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H  V  R  P  V  T  Q  T  D  V  Y  R  A  D  A  K  E  I  P  R    1209
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D  P  L  E  K  S  N  Y  I  C  H  K  G  H  E  F  I  P  T  L    1249
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S  *
```

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M S P R Q D E Y M 9
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-63(xROK-K)
E G L L D G L N S I V L D L D F P A L R 49
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Y D V P E K W A K F Y T A E V V L A L N 189
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K Y G H L K L A D F G T C M K M D Q T G 229
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H A K N L I C A F L T D R E V R L G R N 329
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I R E T V A P V V P E L A S D I D T S N 369
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N L Q S A L E S E R R D R T Q G S E V I 629
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S D L Q G R I S V L E E D L K K G K E L 649
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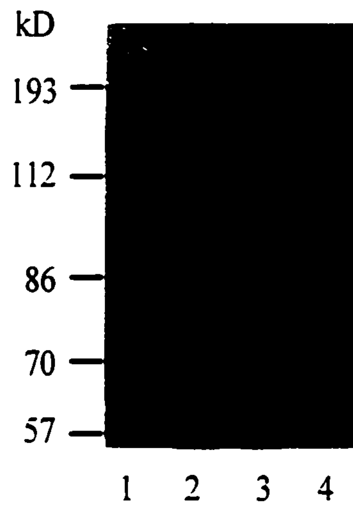
L A R A D A E K Q Q L H E R L A I L E K 669

Figure 17:

The XROKα full length nucleotides and amino acid sequence

The coding sequence of XROKα with the corresponding amino acid sequence (in one letter code). The bold-face regions (from amino-terminus to carboxy-terminus) correspond to the kinase domain. Rho-binding domain and PH/CDR respectively. The boundaries of clones λ63 and SF7 are indicated.

A. in vitro kinase



B. anti-xROKα blot

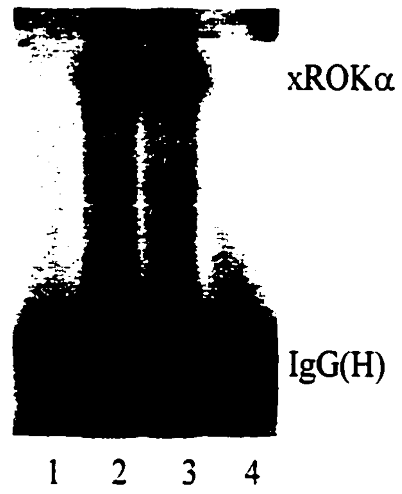


Figure 18:

Characterization of endogenous XROKα in Xenopus oocytes

Immunoprecipitation was carried out using either anti-XROKα, lane 2 and 3, or their respective pre-immunesera, lane 1 and 4.(A) In vitro kinase assay showed autophosphorylation of 200kDa band. (B) western blot analysis using anti XROKα, arrows are pointing to endogenous XROKα

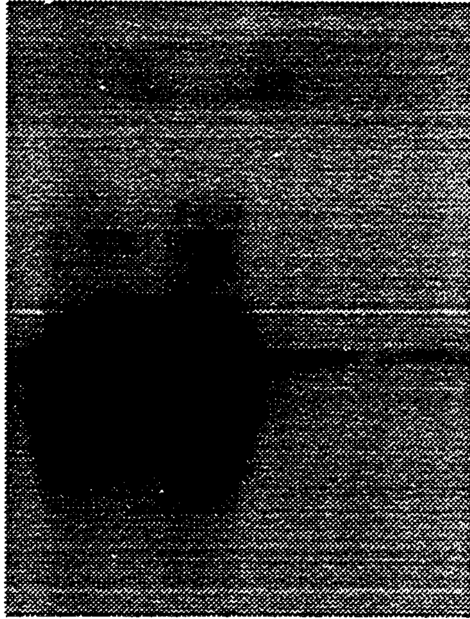

1
2
3
4
MBP

Figure 19:

Kinase assays with myelin basic protein (MBP) as an exogenous substrate.
Endogenous XROKα protein was immunoprecipitated from oocyte lysate. Kinase activity was performed using MBP as a substrate and [γ^{32}P] ATP. In lanes 1 and 2 anti-XROKα antibody was used. whereas preimmune serum was used in both lanes 3 and 4.

Chapter Four

DISCUSSION

4.1 XROKα as an IRS-1 binding protein

In addition to the multiple tyrosine phosphorylation sites, IRS-1 contains several identifiable protein modules (PH, PTB and SAIN) implicated in protein-protein and protein-lipid interaction. Furthermore, these domains are the most highly conserved regions among different members of the IRS family (Lavan *et al.*, 1997). For example, while the overall homology between XIRS-1 and rat IRS-1 is about 65% identical in amino acid sequence, the PH and PTB domain of XIRS-1 are more than 90% identical (Liu *et al.*, 1995). The highly conserved nature of these protein modules prompted us to search for novel proteins interacting with these domains. In this study, I describe the identification of Xenopus ROKα as the first serine/threonine kinase that physically interacts with XIRS-1 PTB domain.

Many control experiments were carried out in this study to ensure that the interaction between XIRS-1 and XROKα is specific. Before I started using the bait construct, two experiments were carried out to ensure its suitability for the screening. The first was to determine that XIRS-$1_{3\text{-}500}$, when transformed into yeast alone, did not activate β-galactosidase. Second, Western blot analysis was carried out to determine that a fusion protein [HA-tagged GAL4-DB/XIRS-$1_{3\text{-}500}$] of expected size was indeed expressed in the transformed yeast. The purpose of the first control was to ensure that XIRS-$1_{3\text{-}500}$ alone did

not produce background false positive; that of the second control was to make sure that I was indeed searching for proteins interacting with XIRS-1(3-500) and not with a truncated or mutated protein, due to errors either in PCR amplification of XIRS-1 or during subcloning.

Following isolation of the partial XROKα (SF7) as an IRS-1 binding proteins, several more control experiments were carried out to verify the specificity of this interaction. First, co-transformation was carried out using purified plasmids in two different yeast strains (HF7c and Y190) having slightly different genetic make-up. The co-transformation experiments demonstrated that SF7 alone did not activate β-galactosidase expression. Rather, its ability to activate β-galactosidase expression was dependent on the presence of bait construct in the same cells. In other word, it was the interaction of XIRS-1(3-500) and SF7 that was responsible for the activation. Second, I carried out co-transformation using either the vector (pAS2) or pAS2/VAMP1 in combination with pGAD10/SF7 which was negative inβ-galactosidase induction. These experiments suggest that SF7 did not interact non-specifically with either the HA tag or the GAL4 DNA binding domain, but rather it interacts with the XIRS-1 sequence.

In addition, *in vivo* confirmation of this interaction was done later in our lab by injecting oocytes with Myc-tagged SF7 mRNA followed by immunoprecipitation using anti-XIRS-1, but not a control antibody (anti-GST) resulted in co-immunoprecipitation of Myc-SF7 detected by anti Myc antibody (Farah *et al.*., in press).

These data suggest a direct binding between XIRS-1 and XROKα, although we cannot exclude the possibility of accessory proteins that may mediate this binding. However the presence of these accessory proteins in yeast seems unlikely, since yeasts have not been

found to have signalling systems such as the insulin receptor and IRS-1. Furthermore, if these signalling accessory proteins were to exist in *S. cerevisiae*, it would be unlikely that they localized in the nucleus where binding of the two hybrids occurs.

4.2 Cloning and characterization of full-length XROKα

The best evidence that support the notion that we have cloned Xenopus ROKα is perhaps the fact the cloned sequence interacts strongly with an active form of RhoA (V14-RhoA) (Fig. 10). The full-length *Xenopus* ROKα predicts a protein of 1370 amino acids with calculated molecular weight of 159034 Daltons. This prediction is based on an initiation codon (ATG) which is downstream from several in frame termination codons and immediately preceded by a good Kozak consensus sequence (GAA) (see Fig 17) (Kozak, 1987). The termination codon following amino acid 1370 appears correct as well since multiple termination codons are present in all three reading frames immediately after that (not shown). Although the original SF7 clone contains at least 1.3 kb sequence 3' to the termination codon, I did not complete sequence analysis of the non-coding region. Therefore it remains unknown at this point whether this region contains a polyadenylation signal (AATAAA) or an actual polyA sequence typical of eukaryotic mRNA.

The predicted XROKα contains 1370 amino acids, a number almost matches that of rat ROKα (1379; leungh *et al.*, 1995). However, unlike rat ROKα, which was reported to be a 160 kDa protein on SDS-PAGE, XROKα detected in Xenopus oocytes are over 200 kDa by Western blotting (Fig.18). The reason for this discrepancy is not clear. We believe that the >200 KD protein detected in Xenopus oocytes indeed represents XROKα. First, while

this protein was recognized by immune sera from two different rabbits. neither pre-immune sera from the same two rabbits detected it (Fig. 18). Second. the >200 KD protein. like rat ROKα. known to be serine threonine kinase. was capable of autophosphorylation and phosphorylation of MBP (Fig. 19).

4.3 XIRS-1 PTB domain is responsible for binding XROKα

The yeast two-hybrid test indicates that the minimum region of XIRS-1 capable of efficient binding to XROKα reside at amino acids 136-324. roughly corresponding to the PTB domain. However. we do not yet know whether it is a functional PTB domain or a smaller motif within it. is the binding domain. The distinction of these two possibilities must await further investigation. For example. critical residues in the IRS-1 PTB (Eck et al. 1996) domain could be mutated to determine whether a functional PTB is indeed involved in binding. Alternatively. phosphotyrosine peptides corresponding to the NPEpY autophosphorylation site of insulin receptor can be used in competition experiments once an in vitro binding assay is established. Nonetheless. it is interesting to note that the construct containing both PH and PTB was much less potent than PTB alone in binding to SF7 XROKα. This may suggest that PH domain negatively regulates binding of the PTB to XROKα.

PTB domain was originally identified as a protein module that recognizes phosphotyrosines within the amino acid sequence NPXpY (Bork and Margolis. 1995. Kavanaugh and Williams.1994). Recently. it has been reported that some PTB domains bind YENPTY. regardless if the protein contains phosphotyrosine (Borg *et al.*.1996). Neither

NPXY nor YENPTY were present in the XROKα (Fig. 17). This suggests that XIRS-1 PTB domain may interact with a yet unidentified motif in XROKα. Supporting this, several recent studies have reported the involvement of PTB domains in a non-phosphotyrosine interactions (Charest *et al.*, 1996, Borg, J.-P., 1996, Kasus-Jacobi, 1997). One of these studies described a phosphotyrosine-dependent interaction between IRS-1 and Shc. In addition, deletion analysis of the proteins to map the domains implicated in this interaction shows that the PTB of Shc binds to the region of IRS-1 comprising amino acids 583-661 (Kasus-Jacobi, A. 1997).

In an attempt to identify region within SF7 that is responsible for XIRS-1 binding, I generated several pGAD10 fusion containing various sub-regions within SF7. Co-transformation of each of these constructs with BD/XIRS-$1_{3\text{-}500}$ (the "bait") was carried out. Unfortunately, none of these constructs was able to induceβ-galactosidase in yeast when co-transformed with the bait, whereas co-transformation of the bait with pGAD10-SF7 induced β-galactosidase in the same experiments (not shown). Similar attempt was made to determine whether any of these constructs express the anticipated GAL4-AD fusion was also unsuccessful due to the poor quality of the commercial anti-GAL4-AD antibodies (Santa Cruz). Further studies will therefore be needed to identify the motif in SF7 responsible for binding XIRS-1.

In a related study carried out by Dr. Yehenew Agazie (Farah, *et al.*, in press), injection of cRNA corresponding to SF7 (the original "prey" isolated from yeast) has produced a protein that bound endogenous XIRS-1 and inhibited insulin-induced MAP kinase activation with a concomitant inhibition of oocyte maturation. In contrast, a construct

containing the entire kinase domain (clone 63, Fig. 16) enhanced insulin signalling in Xenopus oocytes. These data seem to suggest that full-length XROKα may participate positively in insulin signalling leading the activation of MAP kinase (Farah *et al.* in press). How do we explain these findings, particularly the role of XIRS-1 PTB domain in protein-protein interaction ? Although it is possible that XROKα binding to XIRS-1 may interfere with the presumed interaction of XIRS-1 with XIGF-1 receptor (a PTB-NPEpY interaction discussed in Chapter 1) we consider the following more likely. We suggest that the PTB domain may serve a dual, but sequential, function in linking both XIGF-1 receptor and XROKα. For example, the PTB domain may mediate a transient interaction between XIRS-1 and the receptor (NPEpY autophosphorylation site) which results in tyrosine phosphorylation of XIRS-1. Tyrosine phosphorylated XIRS-1 may in turn bind XROKα via another PTB-mediated interaction resulting in modulation of XROKα kinase activity and/or subcellular localization. Alternatively, XIRS-1 may have to be phosphorylated by XIGF-1 receptor and, subsequently, by XROKα and both phosphorylation events are mediated by XIRS-1 PTB domain (in sequence).

In summary, we have identified the first serine/threonine kinase (apart from PI 3-kinase which also possesses protein kinase activity and which binds IRS-1 through its non-catalytic p85 subunit) capable of interacting with IRS-1 both physically and functionally. Further studies using the reagents generated through these studies will undoubtedly reveal novel and significant information on insulin signalling.

REFERENCES

Amano. M.. Ito. M.. Kimura. K.. Fukata. Y.. Chihara. K.. Nakano. T.. Matsuura. Y. And Kaibuchi. K. (1996) Phosphorylation and Activation of Myosin by Rho-associated Kinase (Rho-kinase). J. Biol. Chem. 271. 34: 20246-20249.

Amano. M.. Chihara. K. . Kimura. K.. Fukata. Y. . Nakamura. N. . Matsuura. Y. and Kaibuchi K (1997) Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase Science. 275. 1308-11

Anderson. D.. Koch. C.A.. Grey. L.. Ellis. C.. Moran. M. F. and Pawson. T. (1990). Binding of SH2 domains of phospholipase C gamma 1. GAP. and Src to activated growth factor receptors Science. 250. 979-982.

Araki. E.. Lipes. M.. Mary-Elizabeth. P.. Bruning. J.. Haag III. B.. Johnson. R. and Kahn. C. (1994) Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. Nature 372.186-190.

Backer. J. M.. Myers. M. G.. Jr.. Shoelson. S. E.. Chin. D. J.. Sun. X.. Miralpeix. M.. *et al.*. (1992). Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. EMBO J. 11. 3469-3479.

Baltensperger. K.. Kozma. L. M.. Cherniack. A. D.. Klarlund. J. K.. Chawla. A.. Banerjee. U. and Czech. M. P. (1993). Binding of the Ras activator son of sevenless to insulin receptor substrate-1 signaling complexes. Science 260. 1950-1952.

Bartel. P. L.. Chien. C.--T.. Stemglanz. R. and Fields. S. (1993) using the two-hybrid system to detect protein-protein interactions. In Cellular interactions in development: A practical approach.. D.A. Hartley. ed. (Oxford University Press. Oxford). 153-179.

Borg. J.-P.. Ooi. J.. Levy. E., and Margolis. B. (1996) The phosphotyrosine interaction domains of X11 and FE65 bind to distinct sites on the YENPTY motif of amyloid precursor protein. Mol. Cell. Biol. 16. 6229-6241.

Bork. P. and Margolis. B. (1995) A Phosphotyrosine Interaction Domain. Cell 80. 693-694.
Burgering. B.. and Coffer. P. (1995) Protein kinase B (c-AKT) in phosphatidylinositol-3-OH kinase signal transduction Nature 376. 599-602

Calakos, N. And Scheller, R. H. (1994) Vesicle-associated membrane protein and synaptophysin are associated on the synaptic vesicle. J. Biol. Chem. 269, 24534-24537.

Carpenter, L. C., Duckworth, B. C., Auger, K. R., Cohen, B., Schaffhausen, B. S. and Cantley, L. C. (1990) Purification and characterization of phosphoinositide 3-kinase from rat liver. J. Biol. Chem. 265, 19704-19711.

Charest, A., Wagner, J., Jacob, S., Mcglade, C. J., and Tremblay, M.L. (1996) Phosphotyrosine-independent binding of SHC to the NPLH sequence of murine protein-tyrosine phosphatase-PEST. Evidence for extended phosphotyrosine binding/phosphotyrosine interaction domain recognition specificity. J. Biol. Chem. 271, 8424-8429.

Chaung, L. -M., Myers, Jr. M. G., Seidner, G. A., Birnbaum, M. J., White, M. F. and Kahn, C. R. (1993) Insulin receptor substrate 1 mediates insulin and insulin-like growth factor I-stimulated maturation of *Xenopus* oocytes. Proc. Natl. Acad. Sci. USA 90, 5172-5175.

Chien, C. T., Bartel, P. L., Sternglanz, R. and Fields, S. (1991) The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. Proc. Nat. Sci. USA 88, 9578-9582.

Cummings, C., Zhu, L., Sorisky, A., and Liu, X. J. (1996) A peroxovanadium compound induces *Xenopus* oocyte maturation: Inhibition by a neutralizing anti-insulin receptor antibody. Developmental Biology 175, 338-346.

Czech, m. P. (1989) Signal transmission by the insulin-like growth factors (Review) Cell 59:235-238.

Dario Grossberger (1987). Minipreps of DNA from bacteriophage lambda. Nucleic Acids Research, 15.

Dumont, J. N. (1971) Oogenesis in *Xenopus laevis* (Daudin). I.Stages of oocyte development in laboratory maintained animals. Morph. 136, 153-180

Eck, M., Dhe-Paganon, S., Trüb, T., Nolte, R. T. and Shoelson, S. E. (1996) Structure of the IRS-1 PTB domain bound to the juxtamembrane region of the insulin receptor. Cell. 85, 695-705.

El-Etr, M., Schorderet-Slatkine, S., and Baulieu, E. E. (1979) Meiotic maturation in *Xenopus laevis* oocytes initiated by insulin Science 205, 1397-1399

Farah, S., Agazie, Y., Ohan, N., Ngsee, J. K. and Liu, X.J. A Rho-associated protein kinase, ROKα, binds insulin receptor substrate-1and modulates insulin signalling. (Submitted).

Fields, S. and Song, O. (1989) A novel genetic system to detect protein-protein interactions. Nature. 340, 245-247.

Frangioni, J. V. and Neel, B. G. (1993) Solubilization and purification of enzymatically active Glutathione S-transferase (pGEX) fusion proteins Analytical Biochemistry 210, 179-187

Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Improved method for high efficiency transformation of intact yeast cells. Nuc Acids Res. 20,1425-1425.

Guan, K. and J.E. Dixon (1991) Eukaryotic proteins expressed in Escherichia coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal. Biochem 192, 262-267

Guarente, L. (1993) Strategies for the identification of interacting proteins. Proc. Natl. Acad. Sci. USA 90, 1639-1641.

Gustafson, T. A., He, W., Craparo, A., Schaub, C. D. and O'Neill, T. J. (1995) Phosphotyrosine-dependent interaction of SHC and insulin receptor substrate 1 with the NPEY motif of the insulin receptor via a novel non-SH2 domain. Mol. Cell. Biol. 15, 2500-2508.

Haslam, R. J., Kolde, H.B. and Hemmings, B. A. (1993) Pleckstrin domain homology. Nature 363, 309-310

He, W., Craparo, A., Zhu, Y., O'Neill, T., Wang, L-M., Pierce, J. and Gustafson, T. (1996) Interaction of insulin receptor substrate-2 (IRS-2) with the insulin and insulin-like growth factor I receptors. J. Biol. Chem. 271, 11641-11645.

He, W., O'Neill, T. J. and Gustafson, T. A. (1995) Distinct modes of interaction of SHC and insulin receptor substrate-1 with the insulin receptor NPEY region via non-SH2 domains. J. Biol. Chem. 270, 23258-23262.

Hedo, J., Kasuga, M., Van obberghen, E., Roth, J. and Kahn, C. R. (1981) Direct demonstration of glycosylation of insulin receptor subunits by biosynthetic and external labeling: evidence for heterogeneity. Proc. Natl. Acad. Sci. USA 78, 4792-4795

Hotamisligil, G. S., Peraldi, P., Budavari, A., Ellis, R., White, M.F. and Spiegelman, B. M. (1996). IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-α- and obecity-induced insulin resistance. Science 271, 665-668.

Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N. and Narumiya, S. (1996) The Small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase EMBO J. 15, 1885-1893.

Ito, H., Fukada, Y., Murata, K. and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153, 163-168.

Kasuga, M., Karlsson, F. and Kahn, C. R. (1982) Insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. Science. 215, 185-189.

Kasus-Jacobi, A., Perdereau, D., Tartare-Deckert, S., Van Obberghen, E., Girard, J., and Burnol, A-F. (1997) Evidence for direct interaction between insulin receptor substrate-1 and Shc. J. Biol. Chem. 272, 17166-17170

Kavanaugh, W. M. and Williams, L. T. (1994) An alternative to SH2 Domains for binding tyrosine-phosphorylated proteins. Science 266, 1862-1865.

Kozak,M (1987) An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. 15, 8125-8132

Kuhné, M. R., Pawson, T., Lienhard, G. E. and Feng, G.-S. (1993) The insulin receptor substrate 1 associates with the SH2-containing phosphotyrosine phosphatase Syp. J. Biol. Chem. 268, 11479-11481.

Lavan, B., Lane, W. and Lienhard, G. (1997a) The 60-kDa phosphotyrosine protein in insulin-treated adipocytes is a new member of the insulin substrate family. J. Biol. Chem. 272, 11439-11443

Lavan, B., Fantin, V., Chang, E., Lane, W., Keller, S. and Lienhard, G. (1997b) A novel 160-kDa phosphotyrosine protein in insulin-treated embryonic kidney cells is a new member of the insulin receptor substrate family. J. Biol. Chem. 272, 21403-21407.

Lechleider, R. J., Sugimoto, S., Bennett, A. M., Kashishian, A. S., Cooper, J. A., Shoelson, S. E., Walsh, C. T. and Neel, B. G. (1993) Activation of the SH2-containing phosphotyrosine phosphatase SH-PTP2 by its binding site, phosphotyrosine 1009, on the human platelet-derived growth factor receptor. J. Biol. Chem. 268, 21478-21481.

Lee, C.-H., Li, W., Nishimura, R., Zhou, M., Batzer, A. G., Myers, Jr. M.G., White, M. F., Schlenssinger, J. and Skolnik, E. Y. 1993. Nck associates with the SH2 domain-docking protein IRS-1 in insulin stimulated cells. Proc. Natl. Acad. Sci. USA 90, 11713-11717.

Lemmon, M. A., Ferguson, K. M. and Schlessinger, J. (1996) PH Domains: Diverse Sequences with a common fold recruit signaling molecules to the cell surface. Cell. 85, 621-624.

Leung, T., Chen, X., Manser, E. and Lim, L. (1996) The p160 RhoA-binding kinase ROKα is a member of a kinase family and is involved in the reorganization of the cytoskeleton. Mol. Cell. Biol. 16, 5313-5327.

Leung, T., Manser, E., Tan, L. and Lim, L. (1995) A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. J. Biol. Chem. 270, 29051-29054.

Lienhard, G. (1994) Insulin signalling and the stimulation of glucose transport. Biochem. Soc. trans. 22, 676-680.

Liu, J. X., Sorisky, A., Li, Z. and Pawson, T. (1995) Molecular cloning of an amphibian insulin receptor substrate 1-like cDNA and involvement of phosphotidylinositol 3-kinase in insulin-induced *Xenopus* oocyte maturation. Mol. Cell. Biol. 15, 3563-3570.

Liu, J-P., Baker, J., Perkins, AS., Robertson, EJ., and Efstratiadis, A. (1993) Mice carrying null mutations of the genes encoding insulin-like growth factor I (*igf1* and type 1 IGF receptor (*igf-1r*). Cell 75, 59-72

Lowenstein, E. J., Daly, R. J., Batzer, A. G., Li, W., Margolis, B., Lammers, R., Ulrich, A., Skolnik, E. Y., Bar-Sagi, D. and Schlessinger, J. (1992) The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell. 70, 431-442.

Maller, J. L. and Koontz, J. W. (1981). A study of induction of cell division in amphibian oocytes by insulin. Dev. Biol. 85, 309-316.

Maniatis, T. *Et al.*. (1989) molecular cloning. A laboratory Manual. Cold Sring Harbor Laboratory, Cold Spring Harbor, NY.

Mayer, B. J., Hamaguchi, M. & Hanafusa, H.(1988) Nature 332, 272-275

Mayer, B. J., Ren, R., Clark, K. L. and Baltimore, D. (1993) A putative modular domain present in diverse signaling proteins [letter]. Cell. 73, 629-630.

Miller, J. H. (1972) Experiments in molecular genetics. Cold Spring Harbor laboratory, Cold Spring Harbor, NY.

Mothe, I. and E. Van Obberghen. (1996) Phosphorylation of insulin receptor substrate-1 on multiple serine/threonine residues, 612, 632, 662, and 731, modulates insulin action. J. Biol. Chem. 271, 11222-11227.

Mukai, H. and Ono, Y. (1994) A novel protein kinase with leucine zipper-like sequences : its catalytic domain is highly homologous to that of protein kinase C Biochem. Biophy. Res. Com 199, 897-904.

Mukai, H., Mori, K., Takanaga, H., Kitagawa, M., Shibata, H., Shibata, H., Shimakawa, M., Miyahara, M. and Ono, Y. (1995) *Xenopus* PKN: cloning and sequencing of the cDNA and identification of conserved domains. Biochimica. Biophysica. Acta 1261, 296-300.

Myers, M. G. Jr., Grammar, T. C., Brooks, J., Glasheen, E. M., Wang, L. M., Sun, X. J., Blenis, J., Pierce, J. H. and White, M. F. (1995). The pleckstrin homology domain in IRS-1 sensitizes insulin signaling. J. Biol. Chem. 270, 11715-11718.

Myers, Jr. M. G., Zhang,Y., Aldaz, G. A. L., Grammer, T., Glasheen, E. M., Yenush, L., Wang, L. W., Sun, X. J., Blenis, J., Pierce, J. H. and M. F. White. (1996)YMXM motifs and signaling by an insulin receptor substrate 1 molecule without tyrosine phosphorylation sites. Mol. Cell. Biol. 16, 4147-4155.

Nobes, C. D. and Hall, A. (1995) Rho, Rac and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia and filopodia. Cell. 81, 53-62.

O'Neill, T. J., Craparo, A. and Gustafson, T. A. (1994). Characterization of an interaction between insulin receptor substrate 1 and the insulin receptor by using the two hybrid system. Mol. Cell. Biol. 14, 6433-6442.

Palmer, R., Ridden, J. and Parker, P. (1995) Cloning and expression patterns of two members of a novel protein-kinase-C-related kinase family. Eur. J. Biochem. 227, 344-351.

Pawson T. (1992)Cell signalling. Conviction by genetics [news; comment]. Nature 356, 285-286.

Pawson, T. (1995) Protein modules and signalling networks. Nature 373, 573-579.

Plutzky, J., Neel, B.G. and Rosenberg, R. D. (1992). Isolation of Src homology 2-containing tyrosine phosphatase. Proc. Natl. Acad. Sci. USA 89, 1123-1127.

Rechler, M. M., and S. P. Nissley. (1990) Insulin-like growth factors, p. 263. In M. B. Sporn and A. B. Roberts (ed), Handbook of experimental pharmacology, vol.95/I, peptide growth factors and their receptors I. Springer-Verlag, Berlin.

Rechler. M. M. and Nissley. S. P. (1985)The nature and regulation of the receptors for insulin-like growth factors. [Review] Annu. Rev. Physiol. 47. 425-467.

Ridley. A. J. and Hall. A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell. 70. 389-399.

Ridley. A. J.. Paterson. H. F.. Johnston. C. L.. Diekmann. D. and Hall. A. (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell. 70. 401-410.

Rosen. O. M. (1987)After insulin binds. [Review] Science 237. 1452-1458.

Sanger. F.. Nicklen. S. and coulson. A.. (1977)DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci.USA 74. 5463-5467.

Sasaoka. T.. Draznin. B.. Leitner. J. W.. Langlois. W. J. and Olefsky. J. M. (1994) Shc is the predominant signaling molecular coupling insulin receptors to activation of guanine nucleotide releasing factor and p21 ras-GTP formation J. Biol. Chem. 269. 10734-10738.

Songyang. Z.. Shoelson. S. E.. Chaudhuri. M.. Gish. G.. Pawson. T.. King. F.. Roberts. T.. Ratnofsky. S.. Schaffhausen. B. and Cantley. L. C. (1993) SH2 domains recognize specific phosphopeptide sequences. Cell. 72. 767-778.

Sun. X. J.. P. Rothenberg. C. R. Kahn. J. M. Backer. E. Araki. P. A. Wilden. D. A. Cahill. B. J. Goldstein and White. M. F. (1991). Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature. 352. 73-77.

Sun. X. J.. Crimmins. D. L.. Myers. Jr.. Glasheen. E. M.. Miralpeix. M. and White. M.F. (1993) Pleiotropic insulin signals are engaged by multisite phosphorylation of IRS-1. Mol. Cell. Biol. 13. 7418-7428.

Sun. X.. Wang. L-M.. Zhang. Y.. Yenush. L.. Myers. M. Jr. Glasheen. E.. Lane. W.. Pierce. J. and White. M. (1995) Role of IRS-2 in insulin and cytokine signalling Nature. 377. 173-177.

Tamemto. H.. Kadowaki. T.. Tobe. K.. Yagi. T.. Sakura. H.. Hayakawa. T.. Terauchi. Y.. Ueki. K.. Kaburagi. Y.. Satoh. S.. Sekihara. H.. Yoshioka. S.. Horikoshi. H.. Furuta. Y.. Ikawa. Y.. Kasuga. M.. Yazaki. Y. and Aizawa. S. (1994) Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature. 372. 182-186.

Tanasijevic. M. J.. Myers. M. G. Jr.. Thoma. R. S.. Crimmins. D. L.. White. M. F. and Sacks. D. B. (1993). Phosphorylation of the insulin receptor substrate IRS-1 by casein kinase II. J. Biol. Chem. 268. 18157-18166.

Tanti, J. F., Gremeaux, T., Van Obberghen, E. and Le Marchand-Brustel, Y. (1994a). Serine/threonine phosphorylation of insulin receptor substrate 1 modulates insulin receptor signaling. J. Biol. Chem. 269, 6051-6057.

Tanti, J. F., Gremeaux, T., Van Obberghen, E. and Le Marchand-Brustel, Y. (1994b). Insulin receptor substrate 1 is phosphorylated by the serine kinase activity of phosphatidylinositol 3-kinase. Biochem. J. 304, 17-21.

Ulrich, A., GRAY, A., Tam, A. W., Yng-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., LeBon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J., and Fugita-Yamaguchi, Y. (1986) Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. EMBO J. 5, 2503-2512.

Watanabe, G., Saito, Y., Madaule, P., Toshimasa, I., Fujisawa, K., Mrii, N., Mukai, H., Ono, Y., Kakizuka, A. and Narumiya, S. (1996) Protein kinase N (PKN) and PKN-related protein Rhophilin as targets of small GTPase Rho. Science, 271, 645-647.

White M. F. Maron R. Kahn CR. (1985) Insulin rapidly stimulates tyrosine phosphorylation of a Mr-185,000 protein in intact cells Nature. 318, 183-186.

White, M. F. and Kahn, C. R. (1986) in The enzymes, eds. Boyer, P.D. & Krebs, E. G. Academic, Orlando, FL 247-302.

White, M. F., Shoelson, S. E., Keutmann, H. and Kahn, C. R. (1988a)A cascade of tyrosine autophosphorylation in the beta-subunit activates the phosphotransferase of the insulin receptor. J. Biol. Chem. 263, 2969-2980.

White, M., Livingston, J., Backer, J. M., Lauris, V., Dull, T.J., Ullrich, A. and Kahn, C. R. (1988b) Mutation of the insulin receptor at tyrosine 960 inhibits signal transmission but does not affect its tyrosine kinase activity. Cell, 54, 641-649.

White, M. F. and Kahn, C. (1994) The insulin signaling system J. Biol. Chem. 269, 1-4.

Wolf, G., Trüb, T., Ottinger, E., Groninga, L., Lynch, A., White, M. F., Miyazaki, M., Lee, J. and Shoelson, E. (1995). PTB domains of IRS-1 and Shc have distinct but overlapping binding specificities. J. Biol. Chem. 270, 27407-27410.

Yamasaki, H., Prager, D., Gebremedhin, S. and Melmed, S. (1992) Human insulin-like growth factor I receptor 950 tyrosine is required for somatotroph growth factor signal transduction. J. Biol. Chem. 267, 20953-20958

Zhu, L., Ohan, N., Agazie, Y., Cummings, C., Farah, S. and Liu, X.J. Molecular cloning and characterization of *Xenopus* IGF-1 receptor: role in mediating insulin-induced *Xenopus* oocyte maturation and expression during embryogenesis. (In press).

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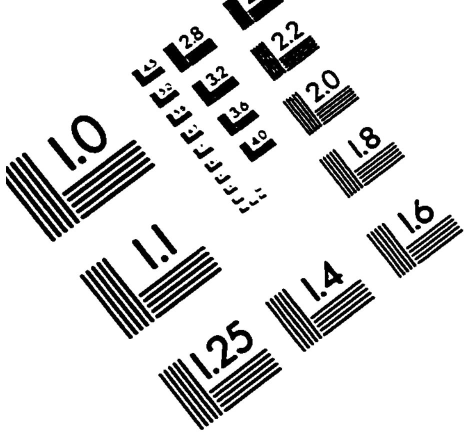

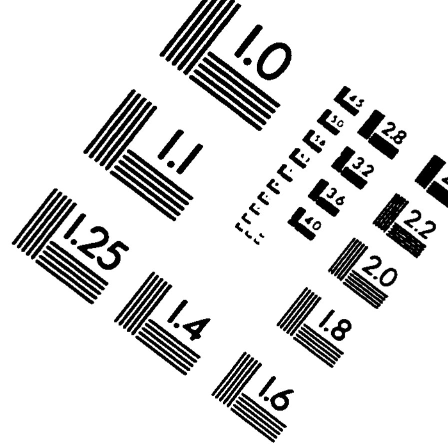

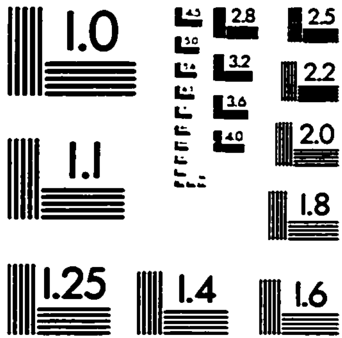

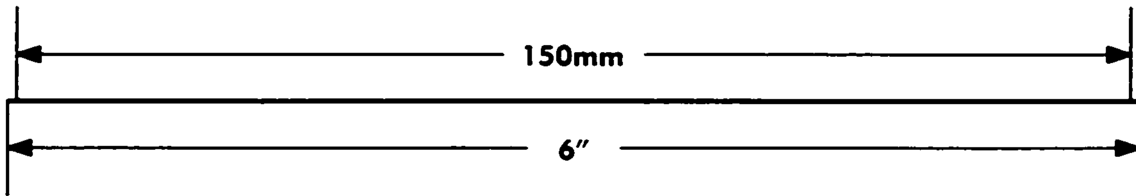

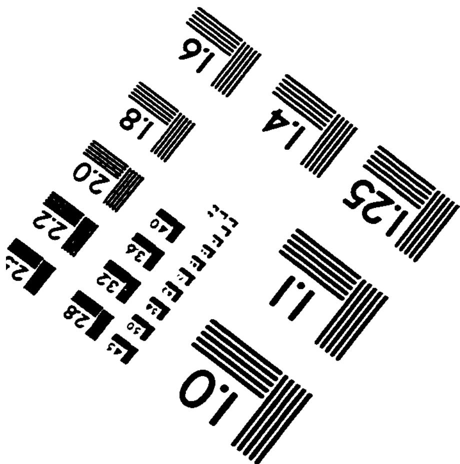

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