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Generation of Temperature Sensitive Mutants of v-Ros, Oncogenic Insulin and Insulin-like Growth Factor I Receptors and Identification of the Insulin Receptor Interacting Proteins

by

Jing Chen

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degrees of Doctor of Philosophy, the City University of New York

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Date

Lu-Hai Wang, Ph.D.
Chair of Examining Committee

10/14/96

Date

Terry A. Krulwich, Ph.D.
Executive Officer

Irwin Gelman
Ron A. Kohanski
Lu-Hai Wang
Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

Generation of Temperature Sensitive Mutants of v-Ros, Oncogenic Insulin and Insulin-like Growth Factor I Receptors and Identification of the Insulin Receptor Interacting Proteins

by

Jing Chen

Advisor: Lu-Hai Wang, Ph.D., Professor

Receptor protein tyrosine kinases (RPTKs) are a family of transmembrane proteins with intrinsic tyrosine kinase activity. They play important roles in many cellular events including cell growth, metabolism and differentiation. My Ph.D. thesis work focuses on insulin receptor (IR), insulin-like growth factor I receptor (IGFR) and oncogene v-ros, all of which belong to a subfamily of RPTKs.

Oncogene v-ros, carried by avian sarcoma virus UR2, encodes a p68 gag-ros transforming protein with intrinsic tyrosine kinase activity. Temperature sensitive mutants of UR2 have been isolated in 1985. In this thesis, the molecular basis for ts251, a temperature sensitive (ts) mutant of UR2, has been identified. A point mutation in the catalytic loop of the kinase domain of UR2 is responsible for the ts phenotype and properties of ts251. The significance of the mutation is further demonstrated by introducing the same mutation into gag-IR and gag-IGFR receptor PTKs and obtaining a similar ts phenotype and properties of these receptor PTKs as well.

The second aspect of this thesis is the identification of Stat5, as a potential

physiological substrate of insulin receptor. Insulin receptor is very important in many biological functions including regulation of glucose homeostasis, stimulation of the uptake of amino acids, regulation of gene expression and promotion of cell proliferation and differentiation. Using yeast two hybrid system, C-terminal portion of human Stat5b (Stat5b-Ct) has been identified to interact with IR. Stat5b-Ct can be tyrosine phosphorylated in vitro and in vivo by IR. Furthermore, Stat5 is tyrosine phosphorylated and activated in insulin perfused livers and in fasted-and-refed mice. Taken together, Stat5 could be a potential physiological substrate of IR.

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This work is dedicated to my parents.

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1. Receptor PTKs and their signal transduction

Receptor protein tyrosine kinases (RPTKs) are a family of transmembrane proteins that possess intrinsic tyrosine kinase activity. These receptors are involved in the regulation of many cellular events including cell growth, differentiation, metabolism and cell death (Ullrich and Schlessenger, 1991; van der Geer and Hunter, 1994).

More than 50 RPTKs have been identified. Their structure can be generally divided into several domains: extracellular ligand binding domain; transmembrane domain; juxtamembrane domain, tyrosine kinase domain and C-terminal tail. According to the structural characteristics and sequence homology in their catalytic domains, the RPTKs are further divided into eleven subfamilies (van der Geer and Hunter, 1994). They are subfamilies of platelet derived growth factor receptor (PDGF), fibroblast growth factor receptor (FGFR), insulin receptor (IR), epidermal growth factor receptor (EGFR), nerve growth factor receptor (NGFR) and hepatocyte growth factor receptor (HGFR), of which ligands are already known and subfamilies of EPH, AXL, TIE, DDR and ROR, whose ligands remain unknown (van der Geer and Hunter, 1994).

Ligand binding to its cognate receptor induce conformational changes of the receptor and subsequent transduction of signal from surface to interior of cells. Several common

processes are summarized below: Step1. receptor dimerization after ligand binding; Step 2. activation of intrinsic tyrosine kinase activity by intermolecular phosphorylation; Step 3. recruitment and activation of downstream substrates important for signal transduction. Step1 and 2 are well established events and Step 3 represents the most studied area of signal transduction in recent years and much progress has been made in identification of the downstream molecules which interact with phosphorylated and activated receptors. The major downstream molecules are a family of proteins which contains SH2 (Src homology domain 2) and/or SH3 (Src homology domain 3) domains. SH2 domain (~100 amino acids), was first identified as a region of homology to Src and Fps that are not required for kinase activity (Sadowski et al., 1986; Pawson 1988). SH2 domain specifically interacts with phosphotyrosine containing proteins (Matsuda et al., 1991, Mayer et al.,1991); SH3 domain (~ 60 amino acids), was initially discovered as a region of homology between v-crk, PLCγ, and Src (Mayer et al., 1988; Stahl et al., 1988). They bind specifically to short proline-rich motifs. (Cicchetti et al., 1992).

The SH2 and SH3 domain-containing signaling molecules can be generally divided into two classes. Members of the first class possess enzymatic activities in their gene products. Non-receptor Src family kinases, phospholipase Cγ (PLCγ), Ras GTPase-activating protein (GAP), PI 3-kinase, Syp (tyrosine phosphatase) all belong to this class. In contrast, members of the second class do not possess enzymatic activities and only contain SH2 and SH3 domains. Therefore, they are also called adapter proteins. Members of this family include Grb2, Crk, IRS-1 (and 4PS), Shc and Nck.

Among members of the class I SH2/SH3-containing proteins, PLCγ, PI3 kinase and

Syp are among the best characterized. (1). PLCγ. Many RPTKs such as EGF, PDGF and FGF receptors can interact directly with PLCγ *in vivo* through SH2-phosphotyrosine interaction, followed by tyrosine phosphorylation of PLCγ, which is essential for its enzymatic activation (Schlessinger and Ullrich 1992). The function of PLCγ is to hydrolyze the phosphatidylinositol 4,5-diphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP3). DAG can activate protein kinase C whereas IP3 mobilizes Ca^{2+} release from intracellular stores and regulates Ca^{2+}- dependent kinase activity (Berridge and Irvine, 1989; Mignery and Sudhof, 1990). (2). PI 3-kinase. PI 3-kinase is a heterodimer composed of a 85 kDa regulatory subunit and a 110 kDa catalytic subunit (Carpenter et al., 1990). The p85 subunit contains both SH2 and SH3 domains. The p110 catalytic subunit catalyzes the phosphorylation of phosphotidylinositol (PI), phosphatidylinositol-4-phosphate (PI-4-P) and phosphatidylinositol-4,5-diphosphate (PI-4,5-P2) on the D3 position of the inositol ring to phosphatidylinositol 3-phosphate (PI-3-P). phosphatidylinositol 3,4-diphosphate (PI-3,4-P2) and phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P3) (Whitman et al., 1988; Escobedo et al., 1991; Skolnik et al., 1991; Otsu et al., 1991). Many receptor PTKs including PDGF, SCF, CSF, NGF, HGF, and insulin receptors have been shown to interact with PI3-kinase (van der Geer and Hunter, 1994). The mechanism of PI3-kinase activation in vivo is not clear. It has been shown that phosphotyrosine-containing peptides that bind to the SH2 domains of the regulatory subunit of PI 3-kinase can stimulate enzymatic activity (Backer et al., 1992; Carpenter et al., 1993). It is therefore thought that binding of the SH2 domain of p85 subunit of PI3-kinase to phosphotyrosine site of growth factor receptor (Escobeto et al., 1991, Otsu et al., 1991) will activate the p110 catalytic subunit of PI 3-kinase. In

addition, It has been proposed that tyrosine phosphorylation of p85 induced by growth factor may also increase the activity of PI3-kinase (Hayashi et al., 1992). (3). Syp Syp binds to PDGF, EGF, and SCF receptors *in vivo* (Feng et al., 1993; Lechleider et al., 1993). Activation of Syp could be through its association with activated receptors or through its tyrosine phosphorylation by activated receptors. Physiological downstream substrates of Syp remain unknown.

Members of the second class of SH2-domain containing proteins (adapter proteins) include Nck, Shc, Grb2 and IRS1. (1). Nck. Nck can bind to EGF and PDGF receptors via its SH2 domain in response to growth factor stimulation (Li et al., 1992). Overexpression of Nck in fibroblasts results in transformed phenotype and tumorigenicity, indicating that Nck might be involved in mitogenic signal transduction (Chou et al., 1992, Li et al., 1992). (2). Shc. Shc is another adaptor protein involved in mitogenesis. Overexpression of Shc induces transformation of NIH3T3 fibroblasts (Pelicci et al., 1992). Shc is tyrosine phosphorylated in response to cytokine and growth factor stimulation including EGF, PDGF, CSF-1, SCF, insulin, NGF, IL2 and IL3 (Burns et al., 1993; Cutler et al., 1993; Pelicci et al., 1992; Pronk et al., 1993; Ruff-Jamison et al., 1993a). Shc can associate with tyrosine phosphorylated EGF and erythropoietin receptors via its SH2 domain (Damen et al., 1993; Pelicci et al., 1992, Ruff-Jamison et al., 1993) and insulin and IGF1 receptors via its non-SH2 phosphotyrosine binding (PTB) domain (Craparo et al., 1995; Gustafson et al., 1995). (3). Grb2. Grb2 is a ~24 kDa protein composed of one SH2 domain flanked by two SH3 domains (Clark et al., 1992; Lowenstein et al., 1992; Matuoka et al., 1993). Grb2 can associate with tyrosine phosphorylated EGF, PDGF, and CSF-1 receptors in vivo via SH2-

phosphotyrosine interaction (Lowenstein et al., 1992; Matuoka et al., 1992; van der Geer and Hunter, 1993). This interaction will promote Sos (Son of Sevenless), which is pre-associated with Grb2, to translocate from the cytoplasm to plasma membrane where the Ras is located. Sos, a guanine nucleotide exchange factor, when located at the plasma membrane will promote the formation of GTP bound form of Ras and lead to activation of Ras\Raf\MAPKK\MAPK pathway (Nishida and Gotoh, 1993). Raf, a serine protein kinase, has been demonstrated to be the downstream target of Ras by *in vitro* and *in vivo* association and activation assays (Leevers and Marshall 1992; Wood et al., 1992; de Vries-Smits et al., 1992). Activated Raf then phosphorylates and activates mitogen-activated protein-kinase kinase (MAPKK, also known as MEK), a dual specificity protein kinase, which in turn phosphorylates and activates mitogen-activated protein kinase (MAPK) by threonine and tyrosine phosphorylation. MAPK then translocate into the nucleus, where it can phosphorylate and activate transcription factors such as Elk-1 and SRF (Blenis, 1993; Marshall, 1994; Treisman, 1994, Whitmarsh et al., 1995). Ras activation and MAP kinase pathway seems to be important in signal transduction for many RPTKs including PDGF, EGF, CSF-1 and insulin receptors (Burgering et al., 1989; Gibbs et al., 1990., Satoh et al., 1990). (3). IRS-1. IRS-1, the major substrate of IR which functions as a docking protein to recruit various SH2 domain-containing proteins including PI 3-kinase, Grb2, Syp and Nck and transmits the signals (White et al., 1994).

Recently, a new pathway, the Jak-Stat pathway was discovered. This pathway relays signal from the cell surface to nucleus and involves two essential players--Stats and Jaks.

Stat proteins are defined as a group of proteins by sequence similarities. All Stat

proteins have duel functions: signal transducer in cytoplasm and transcriptional activator in the nucleus. In general, in the absence of ligand stimulation, Stat proteins remain inactive. Upon ligands stimulation, these Stat proteins undergo tyrosine and serine phosphorylation and become activated. These activated Stat proteins, forming homo or heterodimers, translocate to nucleus and bind to specific DNA sequence and regulate gene transcription (Schindler and Darnell, 1995).

Stat proteins were first identified in interferon (IFN) induced signaling pathway. In response to IFNα, a protein complexes called interferon stimulated gene factor (ISGF3) (Levy et al., 1989) was formed, which is composed of Stat1 and Stat2 and a 48 kDa nuclear protein. ISGF3 binds to IFNα-stimulated response element (ISRE) (Reich et al., 1987; Levy et al., 1988; Kessler et al., 1988) and regulate IFNα specific gene transcription (Levy et al., 1989). IFNγ induces the formation of IFNγ activated factor (GAF) (Lew et al., 1991; Decker et al., 1991), a homodimer of Stat1. GAF binds to IFNγ activated site (GAS) sequence and regulates IFNγ specific gene transcription (Decker et al., 1989).

At present, six Stat proteins have been identified (Schindler and Darnell, 1995). These Stats share maximum sequence similarity in the SH2 domain. These SH2 domains function to bind tyrosine phosphorylated residues located in receptors or other Stat proteins. One of the phosphorylated tyrosine residue in response to ligand stimulation has been mapped to residue 701 in Stat1, 690 in Stat2 and 694 in Stat5. SH3 is less well conserved among Stat proteins. One of the contents of this thesis is the identification of Stat5, as a potential physiological substrate of insulin receptor. Stat5 was originally known as mammary gland factor (MGF), a transcription factor found in epithelial cells in the lactating

animals (Wakao et al., 1992, 1994). It is tyrosine phosphorylated and activated in different cell types in response to stimulation of cytokines such as IL-2, IL-4, IL-6, IL-9 and growth factors (Miyakawa et al., 1996; Nagata et al., 1995; Penta et al., 1995; Damen et al., 1995; Gilmour et al., 1995; Caldenhoven et al., 1995; Gouilleux et al., 1995; Johnston et al., 1995; Bacon et al., 1995; Mui et al., 1995; Pallard et al., 1995) such as EGF(Ruff-Jamison et al., 1995) and insulin (this study). The downstream target genes of Stat5 are unknown, however, Stat5 could be involved in many biological functions including cell growth, differentiation and metabolism.

Jak (Janus kinase) family is a family of soluble tyrosine kinases which is involved in signal transduction mediated by many members of cytokine family. There are four members of Jak family: Tyk2, Jak1, Jak2, Jak3. Their MW range is from 125 to135 kDa. They do not contain SH2 and SH3 domains and donot possess intrinsic tyrosine kinase activity. In the absence of ligand stimulation, they associate with cytoplasmic chain of receptor, however remain inactive. Ligand stimulation rapidly induce tyrosine phosphorylation within their kinase domains and lead to activation of their kinase activity. Genetic studies using various mutant cell lines lacking one of the Jak family members have indicated that Jak is required for Stat phosphorylation and activation induced by cytokine stimulation.

2. *V-ros*

V-Ros and c-Ros belong to the third subfamilies of RPTKs. V-*ros* oncogene is the counterpart of the proto-oncogene c-*ros* and is carried by avian sarcoma virus (ASV) UR2.

UR2, belonging to the group of acutely transforming RNA retroviruses, is a replication defective virus which was isolated together with its associated helper virus UR2AV from a spontaneous chicken tumor (Balduzzi et al., 1981). It induces sarcomas in chickens and efficiently transforms chicken embryo fibroblasts (CEF) to a distinct elongated morphology in culture.

The UR2 genome has been molecularly cloned from unintegrated circular viral DNAs derived from infected CEF (Neckameyer and Wang 1984). The genome length of UR2 is 3165 nucleotides with 1273 nucleotides of *v-ros* sequence inserted between the truncated *gag* gene coding for p19 and truncated *env* gene coding for gp37 of the UR2AV (Neckameyer and Wang, 1984).

The transforming protein of UR2 is *a gag-ros* fusion protein with apparent molecular weight of 68 kDa (P68). P68 contains 552 amino acids, of which 150 amino acids are encoded by gag and 402 amino acids are encoded by ros. This protein is associated with a PTK activity capable of autophosphorylation and phosphorylating other foreign substrates (Feldman et al 1982, Garber et al 1985). P68 is a transmembrane protein with the *gag* portion protruding extracellularly (Jong et al 1987).

In order to investigate the transforming function of P68, temperature sensitive (ts) mutants of UR2 were isolated several years ago (Garber et al., 1985). They were generated by treating the UR2 infected CEF with 5-azacytidine. Viruses obtained from the treated cultures were used for colony formation assay in soft agar. Three ts mutants ts251, ts252, ts253 were characterized. They are ts for cellular transformation by the criteria of morphological alteration and anchorage independent growth. They are ts for kinase

activity(Garber et al., 1985). These ts mutants provide genetic evidence that P68 kinase activity is crucial for cellular transformation by UR2.

I have identified a point mutation responsible for the ts phenotype of UR2. This C to G mutation resulting in alanine to glycine change in the catalytic loop of the kinase domain of v-Ros confers the ts phenotype of UR2. The important role of the catalytic loop for kinase activity is further demonstrated by introducing the same C to G point mutation to the oncogenic gag-IR and gag-IGFR receptor PTKs and obtaining the similar ts phenotype and property (see chapter 3).

3. IR and it signal transduction

Insulin elicits pleiotropic biological effects including uptake of glucose, ion and amino acids, modification of the enzyme activities involved in intermediary metabolism, regulation of cell growth and gene expression (Draznin et al., 1989). All these effects are mediated through insulin receptor (IR), a transmembrane glycoprotein with intrinsic protein tyrosine kinase activity.

The mature insulin receptor is a heterotetramer composed of two α-subunits and two β-subunits covalently linked by disulfide bonds. The α subunit has an MW ~135 kDa and located entirely extracellularly, whereas the β subunit is ~95 kDa and composed of a short extracellular domain, a 23 a.a. transmembrane domain (TM) domain and a cytoplasmic domain. The α subunit contains the ligand binding domain and the β subunit possesses the PTK domain.

Insulin binding to α subunit of IR will induce tyrosine autophosphorylation and

activation of the PTK activity in the β subunit of IR. Tyrosine kinase activity is required for insulin's metabolic and mitogenic functions (Chou et al., 1987; Ebina et al., 1987; McClain et al., 1987). At least six tyrosine residues in the cytoplasmic domain of the β-subunit have been shown to be tyrosine phosphorylated in response to insulin stimulation (White et al., 1988b; Goren et al., 1989). Tyr1158, Tyr1162 and Tyr1163, located in the PTK domain of IR, are the major sites of autophosphorylation and shown to regulate the kinase activity of IR (Goren et al., 1987; Ellis et al., 1986). Tyr1328 and Tyr1334, located in the C-terminal tail of IR β subunit, are minor phosphorylation sites (Tavare et al., 1988). Mutation of these sites does not impair kinase activity of IR (Tornqvist and Avruch, 1988). The biological role of these sites remain unclear. Tyr972 (in the context of NPEY), located in the juxtamembrane domain of IR, is responsible for interaction with IRS1 and Shc (White, 1988a; Craparo et al., 1995; Gustafson et al., 1995). The juxtamembrane region is also important for insulin stimulated receptor internalization (Backer et al., 1990). In addition to tyrosine phosphorylation, the IR β subunit is also phosphorylated in serine and threonine residues. Ser1305/1306 and Thr1348 are phosphorylated in response to insulin and phorbol esters (Lewis et al., 1990 a,b). However, the serine and threonine phosphorylation induced by phorbol ester is associated with a decreased IR PTK activity (Takayama et al., 1984; Haring et al., 1986). Therefore it has been proposed that serine, threonine phosphorylation negatively regulates signal transduction mediated by IR.

Similar to other RPTKs, the signal transduction of IR starts from insulin stimulation on the cell surface. Unlike other RPTKs such as EGF, PDGF and CSF-1 receptors, activated IR does not seem to have detectable association with various SH2-containing molecules such

as PI 3-kinase, Ras-GAP, PLCγ, Grb2, c-Fyn and c-Src. Instead IR phosphorylates the IRS-1 (White et al., 1985; Rothenberg et al., 1991; Sun et al., 1991), a major substrate of IR, which functions as a docking protein to recruit various SH2 domain-containing proteins and transmits the signals mediated by insulin.

IRS-1 is a ~185 kDa protein and the best characterized substrates of IR. The IRS1 does not contain SH2 and SH3 domains, but it contains a pleckstrin homology (PH) domain (Mayer et al., 1993) and a PTB domain at its N-terminal region (Voliovitch et al., 1995; Wolf et al., 1995). PH domain is an internal repeat in pleckstrin, which is the major substrate of protein kinase C in platelet. Function of PH domain is thought to mediate protein-protein interaction. IRS-1 contains 21 potential tyrosine phosphorylation sites and more than 30 potential Ser/Thr phosphorylation sites. Stimulation with insulin will cause substantial increase in both tyrosine and serine phosphorylation (Sun et al., 1992). The tyrosine phosphorylated sites are the potential binding sites for SH2 domain containing proteins. Several important SH2 domain containing proteins can associate with IRS-1: (1) the p85 subunit of PI 3-kinase. It has been shown that association of IRS-1 with PI3-kinase is a necessary step for activation of PI 3-kinase *in vitro* and *in vivo* (Backer et al., 1992; Kelly et al., 1993). The functions of PI3 kinase have been linked to many cellular events including cell growth (Kaplan et al., 1987; Valius, 1993), activation of p70 S6 kinase (Cheatham et al., 1994), prevention of apoptosis (Yao and Cooper, 1995), vesicle transporting and glucose transporters relocalization (Okada et al., 1994). (2) Grb2. Insulin stimulation will promote association of IRS-1 with Grb2 which lead to the activation of MAP kinase pathway. In addition to mitogenic response, the MAP kinase can phosphorylate and activate pp90 S6

kinase which in turn regulates the activity of PPG-1(glycogen associated protein phosphatase), a phosphatase involved in the regulation of glycogen metabolism (Dent et al., 1990; Crews and Erikson, 1993); (3) Syp. Syp is SH2-containing tyrosine phosphatase. It is a potential negative regulator of the insulin transmitted signals; (4). Nck is another SH2 and SH3 domains- containing adaptor protein which can bind to IRS-1 and may link IR to downstream pathways involved in cell growth.

Another less well characterized substrate of IR is IRS-2 (Sun et al., 1996). Transgenic mice lacking IRS-1 exhibit significant residual insulin action which corresponds to the presence of an alternative high MW substrate in liver and muscle (Araki et al., 1994). This high MW substrate called IRS-2, or 4PS, which has been cloned recently (Sun et al., 1996). IRS-1 and IRS-2 share extensive sequence homology, although immunologically distinct (Sun et al., 1996). Similar to IRS-1, activated IRS-2 has been shown to associate with p85 subunit of PI 3-kinase and Grb2 (Patti et al., 1995; Tobe et al., 1995).

The third substrate of IR is Shc. Shc can link IR to Ras/MAP kinase pathway by promoting formation of Shc/Grb2/Sos complex (Rozaki-Adcock et al., 1992; Skolnik et al., 1993).

Those different signaling molecules help to explain the pleiotropic effects of IR. However, other substrates of IR most likely exist.

The third and fourth parts of my thesis work have been devoted to identify the potential substrates of IR by using the yeast two hybrid system.

Chapter 2 Materials and Methods

Cells and viruses.

Chicken embryo fibroblasts (CEF) were maintained in F10 medium supplemented with 5% calf serum and 1% chicken serum. Virus stocks were obtained by collecting supernatant from the transformed cells. Avian sarcoma virus UR2 and its associated helper virus, UR2AV, temperature sensitive mutant of UR2-ts251 have been described elsewhere (Balduzzi et al., 1981; Wang et al., 1982; Neckameyer et al and Wang, 1984). UIR19t6 and NM1 are the retroviruses containing gag-IR and gag-IGFR oncogenic proteins respectively (Wang et al., 1987; Liu et al., 1992). COS1, COS7, HeLa and CHOIR (CHO cell overexpressing insulin receptors) were maintained in DMEM medium supplemented with 10% fetal calf serum.

Antibodies

Stat1 and Stat2 polyclonal antibodies were gifts from Dr Xinyuan Fu. Anti-Stat3, -Stat5, -JAK2, -JAK1 and -Tyk2 antibodies were purchased from Santa Cruz Biotech. Inc. Anti-phosphotyrosine (Ptyr) antibody RC20 was from Transduction Laboratories. Anti-phosphorylation antibody PY20 was from ICN. A monoclonal Ab 12CA5 against the influenza (Flu) hemagglutinin tag and the horse radish peroxidase (HRP)-coupled 12CA5 (12CA5-P) were purchased from Boehringer Mannheim.

RNA extraction and genomic DNA preparation

Approximately $4x10^6$ CEF (one 10cm dish) cells were infected with ts251 and helper UR2AV viruses. Infection was allowed to proceed overnight. Soft agar overlay was performed to amplify the transformed CEF. Two days later, the morphology of CEF

become fully transformed characterized by extremely elongated cell shape and higher refractivity compared with nontransformed CEF. The medium was then collected every 4 hours until a total volume of 400 ml was obtained. Cell debris was spun down at 900xg for 10 minutes. Equal volume of saturated ammonium sulfate (PH 7.0) was added to the supernatant containing the viruses and the mixtures were left on ice for 30 minutes to precipitate the viruses. The virus precipitates were centrifuged at 6500xg for 30 minutes, then resuspended in TEN buffer (10 mM Tris-HCl PH7.4, 1mM EDTA PH 8.0 and 100mM NaCl), followed by 20% to 60% sucrose density gradient separation at 39000 rpm for 1 hour at 4°C. The virus band was collected and the viruses were disrupted in TEN solution containing 1% SDS and 1% β-mercaptoethanol. The disrupted mixtures were extracted twice with phenol chloroform and once with chloroform to remove lipids and proteins. Viral RNAs were finally precipitated in 2.5 volume ethanol, pelleted and redissolved in TE buffer.

Genomic DNA preparation followed published protocol (Neckameyer and Wang 1984).

cDNA preparation and PCR amplification

The viral RNAs were used as the templates for cDNA synthesis. Two primers were used to generate cDNAs from viral RNAs: P2119 and P1395 (Figure3-1). P2119, 5'ttgcgcagctgtaccttga3', corresponds to nucleotide positions 2119 to 2100 of UR2 and contains a Mst I site (underlined). It is located at the junction site between *ros* and truncated gp37 *env* gene. P1395, 5'taagcagatctcctccttcc3', corresponds to nucleotide positions 1394 to 1375 and contains a Bgl II site (underlined). It is located in the kinase domain of the P68 gag-ros transforming protein. cDNAs were synthesized according to the following

conditions: 50 μl reaction mixtures containing 2 μg viral RNAs, 200 μM each dNTP, 10mM DTT, 40 units RNAsin, 20 units M-MLV reverse transcriptase and 400 ng primers (P1395 or P2119) were incubated at 37°C for 1 hour. The cDNAs synthesized with P2119 were for fragment III (742 bp) amplification using primers P2119 and P1378 and the cDNAs synthesized with P1395 were for fragment I (883 bp) and II (1134 bp) amplification using primers P512, P1395 and P261, P1395 respectively (Figure3-1). P512, 5'ttatattccccggggtcctg3' which corresponds to nucleotide positions 512 to 531 of UR2 and contains a Sma I site (underlined), is located in p19 *gag* region, 132 nucleotides downstream from the beginning of *gag* initiation site. P261, 5'ctactgcagggagccaacat3' which corresponds to nucleotides 261 to 280 and contains a Pst I site (underlined), is located in the 5' noncoding region, 100 nucleotides upstream from *gag* initiation codon. P1378, 5'aggaggagatctgctta3' which corresponds to nucleotides 1378 to 1395 and contains a Bgl II site (underlined), is located in the kinase domain of P68. The conditions for PCR amplification were as follows: 50 μl reaction mixtures containing 1 μl cDNA template, 300 ng each primer (two primers), 0.2 mM each of dNTP and 2.5 units Taq polymerase were set for 30 cycles of amplification (PERKIN ELEMER). Conditions for each cycle were: 94°C, 50 seconds, 55°C, 50 seconds and 72°C, 40 seconds (for fragment I and III) or 1 minute 10 seconds (for fragment II). For PCR amplification from genomic DNA template, the conditions were essentially the same with the following modification: 1 μg genomic DNA template was denatured at 95°C for 5 minutes before 30 cycles of PCR amplification.

PCR product cloning

Each PCR fragment was cloned into pBluescript (Stratagene) by two step blunt end

cloning method. The first step is to end-modify PCR products. To do this, 300 ng of PCR fragment was first incubated with T4 DNA polymerase (1.5 units) in the absence of dNTPs to remove the 3' protruding nucleotide at room temperature for 10 minutes. Then 0.2 mM each of dNTPs and 2.0 units T4 polymerase kinase were added to the mixtures and further incubated at 20°C for 40 minutes. The second step was the ligation reaction which was performed in 15 μl reaction mixtures containing 50 ng pBluescript vector (EcoR V precut and purified), 40 units T4 DNA ligase and the end-modified PCR fragment (300ng). Ligation was carried out at 20°C for 2 hours. The ligation mixtures were then used to transform XL1-Blue competent cells.

Sequencing

Sequencing was done using dideoxy chain termination method (Sanger et al 1977).

Construction of v-*ros* mutants

Sequencing results of pBluescript containing PCR fragment I/II and III derived from ts251 template indicated that two missense point mutations existed in ts251 (Figure3-2). One is located in PCR fragment I/II, which is a C to G mutation at nucleotide position 708 of UR2 resulting in substitution of arg for pro^{110} in the *gag* region. Another is located in PCR fragment III, which is also a C to G mutation resulting in substitution of gly for ala^{385} in the protein tyrosine kinase (PTK) domain. To construct v-*ros* mutants containing either single mutation (in the *gag* region or in the kinase domain) or double mutations (in both regions), the pBluescript vector containing PCR fragment III was cut with Bgl II and Apa I to release a 600 bp DNA fragment which contains the alanine to glycine mutation in the kinase domain and this fragment was cloned back to wt UR2 genome to obtain the mutants

containing the kinase domain mutation, or the pBluescript vector containing the PCR product I/II was cut with Sma I and Bgl II to release a 864 bp fragment which contains the proline to arginine mutation in the *gag* region and this fragment was cloned back into wt UR2 genome or mutant UR2 genome carrying mutation in the kinase domain to obtain the mutants containing *gag* region mutation or double mutations.

Transfection and selection of transformed cells and colony formation assay

The preparation of chicken embryo fibroblasts (CEF) and the colony formation assay have been described (Hanafusa 1969). 10 μg of mutant plasmid DNA or wild type UR2 DNA were transfected into CEF, together with 1 μg UR2AV helper DNA by polybrene method (Kawai and Nishizawa 1984). Transfected cells were cultured at 35°C and transformed CEF cells were selected by soft agar overlay for two to three cycles (Hanafusa 1969).

Extraction of cellular proteins

Cells were washed twice in Tris-Glu buffer and the cells were lysed on ice for 5 minutes in RIPA buffer containing 50mM Tris-HCl (PH 7.5), 150 mM NaCl, 5mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate, 1% Trasylol, 1mM phenylmethylsulfonyl fluoride (PMSF), 1 μM Leupeptin and 1 μM Antipain (0.5 ml RIPA buffer per 10cm dish). The lysed cells were scraped out by rubber policeman and centrifuged at 4°C, 12000 rpm for 10minutes. The supernatant of the cell extracts was ready for immunoprecipitation

Immunoprecipitation and *In vitro* kinase assay

Cell extracts prepared in 500 μl RIPA buffer were used for immunoprecipitation for 2 hours to overnight by adding 1μl polyclonal antibody. 20 μl protein-A sepharose beads

was then added to the mixtures for 1 hour at 4°C. For kinase assay, the immuno-precipitated pellet was washed twice in RIPA buffer, and twice in kinase reaction buffer containing 50 mM Tris-HCl (PH 8.0), 10 mM $MnCl_2$, 0.5 μM leupeptin and 0.5 μM antipain. After washing, the pellet was resuspended in 50 μl kinase reaction buffer. 1 μl (10 μCi) [γ-P^{32}] ATP was added to the mixtures to start the reaction. The reaction took 10 minutes at room temperature and was stopped by adding 0.4 ml RIPA buffer. The pellet was spun down and washed with RIPA buffer four times, followed by resuspension in 10μl (or 40μl depending on the gel loading capacity) 2x protein loading buffer and boiled for 5 minutes. The mixtures were then spun for 5 seconds at 10,000g and the supernatants were run on SDS-PAGE gel. The signals of phosphorylation was detected by autoradiography.

Western blotting for measuring intracellular autophosphorylation and PTK activity

The cells were treated with 200 μM sodium vanadate for 4 hours before extraction with RIPA buffer. Immunoprecipitation was then followed as described. The immune complexes were washed, and run on SDS-PAGE gel. The proteins were transferred to nitrocellulose membrane followed by Western blotting. Autoradiography or enzymatic color detection methods were used. For the first detection method, the filter was blocked for 1 hour at room temperature in the blocking solution containing 5% nonfat milk and 1XTTS (10 mM Tris-HCl PH 7.5, 0.1% Triton X-100, 0.9% NaCl, 0.02% sodium azide), followed by incubation with primary antiserum (polyclonal antiserum 1:1000 dilution or monoclonal anti-phosphotyrosine PY20 1:2000 dilution) at room temperature for 3 hours. The filter was then washed in 1XTTS buffer twice, five minutes each time. For monoclonal antibody PY20, a second rabbit anti-mouse serum (1:1000 dilution) was added and incubated at room

temperature for an additional 2 hours and washed again. After washing, 1 μl (1μCi) I^{125}-protein A was added and incubated at 37°C for 30 minutes. Finally, the filters were washed with 1XTTS at least twice, 15 minutes each time, and dried with an infrared light source and autoradiographed with an intensifying screen at -70°C. For the second detection method, the filter was blocked for 30 minutes in a blocking buffer containing 3% BSA, 1.5% tween 20, 50 mM Tris-HCl pH8.0. The primary antibody was added to a blocking buffer containing 0.1% BSA, 1.5% tween 20, 50 mM Tris-HCl and shaked for 1 hour at room temperature. If secondary Ab was needed, the filter was rinsed once in the blocking buffer before incubating with secondary Ab. Color detection was followed the conditions recommended by manufacturer (Transduction Laboratories)

For preparation of total cell lysates and Western blotting, the cells were treated with 200 μM sodium vanadate for 4 hours before extraction with Western extraction buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM Na_3VO_4, 100 μM Na_3Mo_4, 1% Trasylol,1% SDS and 1mM PMSF. Protein amounts were quantified using Bradford method. Cell lysates were analyzed by SDS-PAGE gel and Western blotting procedures were the same as described above.

Site directed mutagenesis of gag-IR and gag-IGFR fusion receptor PTKs.

For site directed mutagenesis of gag-IR fusion protein (Wang et al., 1987; Poon et al., 1991), four primers were used. The first pair of primers was: PIR1 5'ggtgtctcgagagaagatc3' corresponding to nucleotides 3189 to 3207 of insulin receptor (IR) (Ebina et al., 1985) and containing an Xho I site (underlined), and PIR2 5'agtttctg*ccggccaggtc3' corresponding to nucleotides 3631 to 3613 and containing a Nae

I site (underlined). The PCR product from this pair of primers was a 443 bp DNA fragment. Another pair of primers was: PIR3 5'gacctggccg*gcagaaactg3' corresponding to nucleotides 3613 to 3632 and containing a Nae I site (underlined) and PIR4 5'attagacaggccttggtaa3' corresponding to nucleotides 3864 to 3846 and containing a Stu I site (underlined). This pair of primers gave rise to a 251 bp DNA fragment. The nucleotide change leading to substitution of alanine by glycine is marked by an asterisks (*) at the beginning of the nucleotide. The two PCR fragments were cut with Nae I, purified and ligated together. 10 ng of the ligated DNA was used for PCR amplification again using primers PIR1 and PIR4 to obtain a 695 bp DNA product which was subcloned into the pBluescript vector and sequenced to confirm the mutation. Finally the 695 bp fragment was used to replace the corresponding DNA portion of the gag-IR containing vector pUIR19t6 (Poon et al., 1991).

For site directed mutagenesis of the gag-IGFR (Liu et al., 1992, 1993). The similar method was used. The four primers are the following: PIGFR1, 5'gcaagcatgcgtgagaggatt3', corresponds to nucleotides 3160 to 3180 of IGFR (Ullrich et al., 1985) and contains a Sph I site (underlined). PIGFR2, 5'aattccgg*ccggcaaggtc3', corresponds to nucleotides 3466 to 3448 and contains an Eag I site (underlined). PIGFR3, 5'cttgccg*gccggaattgc3', corresponds to nucleotides 3451 to 3468 and contains an Eag I site (underlined). PIGFR4, 5'gcggatcctacatgttctctggct3', corresponds to nucleotides 3945 to 3931. The 307 bp DNA fragment derived from PIGFR1 and PIGFR2 and 495 bp DNA fragment derived from PIGFR3 and PIGFR4 were cut with EagI, ligated, PCR amplified and used to replace the wild type sequence of gag-IGFR in NM1 (Liu et al., 1993).

Plasmids and yeast strain used in the two hybrid system

The yeast strains, plasmids and a HeLa cell-derived cDNA library were provided by Dr Roger Brent at Harvard Medical School. The yeast strain EGY48 [MATα, *trp1, ura3, His3, Leu2::pLexA-op6-LEU2*], containing an integrated copy of *leu2* gene with its upstream activating sequences (UAS) replaced by 6 copies of LexA binding sites, was used as a host for screening protein interaction. Three plasmids were used in this system (Figure4-2). First, the bait plasmid pEG202 which contains a *LexA* gene under the control of the constitutively activated promoter ADH1 followed by the multiple cloning sites (MCS), is used to make LexA-bait construct. Second, the reporter plasmid pSH18-34 which contains a *LacZ* gene under the control of Gal1 promoter in which its UAS has been replaced with 8 copies of LexA binding sites, is used to express β-galactosidase. Third, the library plasmid pJG4-5-cDNAs which contains a library of HeLa cell-derived cDNAs under the control of intact Gal1 promoter is used to express the potential interacting proteins.

Construction of bait plasmids for two hybrid screening

The pLexA-IR-S (Figure4-2) contains a 306-a.a. IR fragment cloned in frame to *LexA* in the bait plasmid pEG202. For pLexA-IR-L, two primers were used for generation of the fragment encoding IR-L (Figure 4-2). Primer 1: 5' CGGGATCCGTAGAAAGAGGCAGCCAG 3' and Primer 2: 5' CCACGCGTCGACTTAGGAAGGATTGGAC 3'. The 1.2 kb PCR fragment was cloned into pEG202 using BamHI and SalI sites.

Yeast cell transformation

The bait-IR fusion, reporter and library plasmids pLexA-IR(S/L), pSH18-34 and pJG4-5-cDNA were sequentially transformed into EGY48 (Gietz et al., 1985).

Extraction of DNA plasmids from yeast cells

2 ml yeast cells were pelleted at 12000 rpm for 10 minutes at 4°C. The cells were suspended in 0.5 ml sorbitol buffer containing 1M Sorbitol and 10 mM Tris-HCl (7.4). 50 µl zymolyase (0.5 mg/ml) and 50 µl β-mercaptoethanol (0.28M) were added. The tubes were incubated in a shaking incubator with 200 rpm for 1 hour at 37°C. The plasmid DNA was extracted with alkaline lysis method (see Maniatis et al-Molecular Cloning).

Extraction of proteins from yeast cells

Single colony or frozen cells was inoculated into 10 ml selection medium and grew overnight in a shaking incubator with 250 rpm at 30°C. The second day, the yeast cells were spun down at 4000 rpm for 10 minutes at 4°C. The yeast cells were broken up by the glass beads method (Cells were resuspend in 1 ml RIPA buffer per 20 ml of original yeast cell culture, 1g acid treated glass beads [0.45µm diameter] was added. The mixtures were vigorously vertaxed at 4°C, 1minute each time for 5 times). The cell debris were spun down. The supernatant can be used for immunoprecipitation followed by *in vitro* kinase assay or Western blotting.

Screening procedures for potential interacting proteins

The following is the details of screening steps using the kinase active LexA-IR fusion protein as an example. For kinase inactive LexA-IR fusion protein, screening processes are essentially the same. The flow diagram of screening is shown in Figure 4-5. Results of two hybrid screening for both constructs are in Table 4-1.

1. cDNA library plasmid transformation

Inoculated 100 µl EGY48 containing pLexA-IR-L and pSH18-34 into 10 ml selection

medium and let them grow overnight. The second day, inoculated all 10 ml medium to 100 ml selection medium, grew at 30°C.

	Starting OD595	Ending OD595
EGY48(pLexA-IR-L, pSH18-34) Growth Medium (H^-, U^-, T^-, Glu^+)	0.012	0.016
EGY48(pLexA-IR-L, pSH18-34) Growth Medium (H^-, U^-, Glu^+)	0.050	0.345

EGY48 containing pLexA-IR-L and pSH18-34 was transformed with 10μg library plasmid (pJG4-5-cDNA). The transformatants were plated on ten 15X150mm plate, plus two additional negative control. Three days later, checked the results of transformation.

plate order	1	2	3	4	5	6	7	8	9	10	11	12
selection medium	/---------- H^-, U^-, T^-, L^+, Glu^+----------/											
colony # per plate	/---------- TMTC----------/										5	4

plate #1 to #10: contain EGY48 (pLexA-IR-L, pSH18-34) transformed with 1 μg library plasmid.
plate#11: contains EGY48 (pLexA-IR-L, pSH18-34) transformed without library plasmid.
plate#12: contains EGY48 (pLexA-IR-L, pSH18-34) without doing transformation.
TMTC: too many to count.
H^-, U^-, T^-, L^+, Glu^+: selecting medium containing leucine, glucose, no histidine, no uracil and no tryptophan.

plate order	1	2	3	4
dilution ratio		1:1000		
colony # per plate	156	174	193	149

transformation efficiency= (156+174+193+149)/4X1000=$1.68X10^5$ colonies/μg

2. Storage of cDNA library transformants

Added 10 ml H_2O to each plate, shaked at plate shaker for 5 minutes or until all the colonies were loose. Combined yeast suspension, spun it at 2000 rpm for 5 minutes. The yeast cells were washed once with H_2O, once with H^-, U^-, T^-, L^-, Gal^+ medium and finally

resuspended in 10 ml freezing solution containing 65% glycerol, 0.1 M $MgSO_4$ and 25 mM Tris-HCl pH8.0 at -70°C. The final volume obtained was 12.5 ml. Aliquoted into 1 ml freezing tube and stored them at -70°C.

$1.68X10^5X10/12.5=1.3X10^5$colonies/ml freezing cells

3. Calculation of plating efficiency.

Took one aliquot from frozen cells, inoculated the freezing cells into H^-, U^-, T^-, L^-, Gal^+ selection medium, induce at 37°C for 4 hours and then plate them out on H^-, U^-, T^-, L^-, Glu^+ plate.

$$\text{Plating efficiency} = \frac{\#\text{ of colonies grown in } H^-, U^-, T^-, L^+, Glu^+}{\#\text{ of colonies calculated from transformation}} = \sim 800$$

Plating efficiency was a parameter used to calculate the volume of yeast cells to be plated out on one 15x150mm plate. For example, if $5X10^5$ colonies were plated on one 15x150 mm plate, the volume of yeast cells used is $V=(5X10^5)/(800X\ 1.3X10^5)=0.005$ ml

4.Selection of positive colonies

After induction in H^-, U^-, T^-, L^-, Gal^+ selection medium, the yeast cells were plated out in H^-, U^-, T^-, L^-, Gal^+ plate. $5X10^5$ colonies were plated per 15X150 mm plate. 20 plates were used.

# of colonies screened= $5X10^5X20=1.0X10^7$

Selection: 3 to 5 days after plating, the colonies grew up. Picked individual colonies (total # of colonies picked was ~600) , duplicated them in plate #1 containing H^-, U^-, T^-, L^-, Glu^+, PBS, X-gal and plate #2 containing H^-, U^-, T^-, L^-, Gal^+, PBS, X-gal. Let them grow at 30°C for 4 to 6 days (The blue color of the colonies would appear gradually). Pick those

colonies whose blue color was stringently dependent on the presence of galactose. 42 colonies were picked. These colonies were then grown in H^-, U^-, T^-, L^-, Gal^r selection medium for 2 days and the yeast plasmids were extracted using modified plasmid miniprep method. The purified plasmids were electroporated into DH5α competent cells. The library plasmid from each colony was then selected out against the bait and reporter plasmids by hybridization with ^{32}P labeled probe specific to a region of the library plasmid pJG4-5 (a 350 bp fragment cut from pJG4-5-cDNA with HindIII and EcoRI). The library plasmids obtained were to be used for sequencing or retransformation into yeast cells to confirm that they were true positives.

Construction of bacterial expression vectors of IR interacting proteins

pJG4-5-cDNAs encoding different interacting polypeptides of IR were cut with EcoRI and XhoI. These fragments thus obtained were ligated into pGST5x-1 (Pharmacia). The induction of GST-IRA3 (66kDa), GST7 (35 kDa) and GST12 (45 kDa) (Figure 4-12) and purification of these proteins followed the procedures recommended by the manufacturer (Pharmacia). For construction of His-tagged IRA3 expression vector, pGST-IRA3 was cut with BamHI and the 1.0 kb fragment released was ligated to the expression vector pQE32 (Qiagen) to obtain pQE32-IRA3 which was transformed into M15 (pREP4) cells for protein expression. His-IRA3 expression was induced by adding 1mM IPTG to bacterial cells in the log phase for 4 hours at 37°C and purified according to the denaturing conditions recommended by the manufacturer (Qiagen). The purified His-IRA3, GST-7 and GST-12 polypeptides were used for preparation of antisera.

Construction of mammalian expression vectors of IR interacting proteins

For construction of mammalian expression vectors of interacting proteins of IR, the cDNA fragments encoding the Flu HA tag and IR interacting polypeptides were amplified from the pJG4-5-cDNAs by PCR using library specific primers, primer1: 5' CCCAAGCTTACCATGGACTACCCTTATG 3' and primer2: 5' CCCAAGCTTAAGCTTCTCG 3'. These PCR fragments were cut with HindIII and then ligated to the expression vector pRcCMV (Invitrogen) to obtain the expression vector pRcCMV-IRA3, pRcCMV-7 and pRcCMV-12.

***In vitro* transcription and *in vitro* translation**

The linear fragments derived from pRcCMV-7, pRcCMV-22 and pRcCMV-IRA3 were purified by electroelution. Mixtures (20μl) containing 4 μl transcription buffer, 2 μl DTT(100 mM), 1μl RNasin(40 units), 1μl each of ATP,CTP,UTP and GTP (10 mM), 2μg purified linear template, 1μl T7 polymerase (50 units) and H_2O were subjected to the *in vitro* transcription reaction at 37°C for 1 hour. Mixtures (50 μl) containing 35 μl Rabbit Reticulocyte Lysate, 1μl amino acid mixtures, 4μl ^{35}S-Met, 1μl RNasin (40 units), 0.5 μl RNA template and H_2O were used in the *in vitro* translation reaction at 30°C for 2-3 hours.

***In vitro* phosphorylation of IRA3 by IR**

The cytoplasmic kinase domain (CKD) of IR (a.a.957-1355) was expressed in baculovirus and purified (kindly provided by Dr. R. A. Kohanski). Soluble CKD (0.3μg) and His-IRA3 proteins (0.75μg) were mixed and incubated in 50 μl kinase buffer containing 10 mM $MnCl_2$ and 50mM Tris-HCl pH 8.0. γ ^{32}P-ATP (10μCi) was added to the mixtures to start the reaction. The reaction time was 10 minutes at room temperature. 0.5 ml of RIPA buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100,

1% Sodium Deoxycholate, 1% Trasylol, 1 mM PMSF, 1 μM Leupeptin and 1 μM Antipain was added to the mixtures to stop the kinase reaction. Polyclonal anti-IR serum was used to immunoprecipitate the CKD protein. After pelleting the immunoprecipitate, the supernatant was collected and 20 μl 50% slurry of Ni-NTA-resin (Qiagen) was added and incubated at 4°C for several hours. The Ni-NTA-resin was washed 5 times with a buffer containing 6 M guanidine hydrochloride, 0.1 M NaH_2PO_4 and 0.01 M Tris-HCl (pH adjusted to 8.0) and twice with H_2O. The complexes were then boiled in 20 μl lammili buffer and analyzed by SDS-PAGE. The gel was transferred to nitrocellulose membrane and the signals were detected by autoradiography. The same filter was subsequently used for Western blotting with anti-IRA3 to detect the IRA3 protein.

Intracellular phosphorylation of IRA3 by IR

5 μg pRcCMV-IRA3 and 5 μg pECE-IR encoding the full length insulin receptor (Ebina et al, 1985a) were cotransfected into COS1 cells by lipofectamine transfection method (Gibco BRL). 48 hours later, COS 1 cells were starved in serum free DMEM medium for 2 hours. Insulin (50 nM) or IGF1 (20ng/ml) was added to cells for 15 minutes at 37°C. The cells were extracted in RIPA buffer and divided into two equal parts. The expression of HA-IRA3 was detected by anti-HA antibody conjugated with horseradish peroxidase (12CA5-P) antibody and tyrosine phosphorylated HA-IRA3 was detected by antiphosphotyrosine antibody.

Liver perfusion experiment

9 months old DBA2 mice, weighing 22-29 grams, were used in the perfusion study. Mice were anethesized by abdominal injection of appropriate amount of pentobarbital

according to the body weight. 10 ml Hanks solution containing 1mM sodium vanadate as a control or 1mM sodium vanadate plus 50 nM insulin was used as the perfusion solution. Perfusion was performed through the portal vein with a 31 G needle attached to a 10 ml syringe. Perfusion took ~ 2-3 minutes. After perfusion, the livers were removed and soaked in 5 ml perfusion solution for an additional 5 minutes at 37°C. The livers were then cut into pieces and dounce homogenized in RIPA buffer. The dounce homogenates were centrifuged at 12000 rpm for 10 minutes at 4°C. The proteins in the supernatant were quantified by Bradford method. 3 mg total proteins were used for immunoprecipitation with anti-Stat1, -3, -5, -IR or -IGFR antibody individually. Immunoblotting with various antibodies followed. The perfusion experiments were repeated three times with similar results.

Liver tissue fractionation

The liver tissue was dissociated using a polytron blender in isotonic buffer containing 20 mM Tris-HCl pH7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA, 1% trasylol and 1 mM PMSF. The dissociated liver cells were pelleted by centrifugation at 2500 rpm for 5 minutes. The pelleted liver cells were then swelled for 15 minutes on ice in a hypotonic buffer which was the same as isotonic buffer except without NaCl and then dounce homogenized (pestle B) to break up the cells. The nuclei were pelleted (1500 rpm,10 min) and nuclear proteins were extracted in a buffer similar to the isotonic buffer but containing 0.4 M NaCl for 30 minutes at 4°C.

Fasting and refeeding experiment

A pair of 9 weeks old mice was separated into different cages and starved for 2-3 days. Mouse food was given to one of the starved mice for 1 hour and 20 minutes and the

mice were then sacrificed. Various organs or tissues were immediately taken and frozen right away on dry ice. The tissues except muscle were first dissociated gently using a polytron homogenizer, followed by dounce homogenization. The muscle tissues were disrupted completely in a polytron homogenizer. The total proteins were assayed using Bradford method. The experiments were repeated several times with similar results.

Gel mobility shift assay

The DNA binding by gel mobility assay was done by Dr. H. Sadowski as described previously (Sadowski et al., 1993).

Chapter 3 Alanine to glycine mutation in the putative catalytic loop confers temperature sensitivity on ros, insulin receptor and insulinlike growth factor I receptor protein tyrosine kinases

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Abstract

The mutations of a temperature sensitive (ts) mutant of avian sarcoma virus UR2 coding a receptor protein tyrosine kinase (PTK) oncogene *ros* were identified. An ala^{385} to gly^{385} change mapping within the highly conserved RDLAARN motif in the *ros* kinase domain was shown to be responsible for the ts phenotype. Based on the sequence homology of all known protein kinases as well as the crystalline structure of the cAMP-dependent protein kinase (cAPK), this conserved region most likely represents the PTK catalytic loop. The same mutation when introduced into the human insulin and insulinlike growth factor I receptors made these PTKs ts in both biological function and kinase activity. Our results support the presumed catalytic role of this highly conserved sequence in PTKs. Due to its highly conserved nature, we predict that the same mutation would most likely confer temperature sensitivity on other PTKs as well.

Introduction

Protein phosphorylation mediated by tyrosine and serine/threonine kinases is an important step in many signal transduction pathways, including normal cell growth, embryonic development and morphogenesis, T cell, B cell and neutrophile activation, neurotransmitter signaling, metabolic processes and oncogenesis (Ullrich and Schlessenger, 1990; Hunter, 1991; Cantley et al., 1991; Pawson and Bernstein, 1990; Alexander and Cantrell, 1992; Veillette and Davidson, 1992). In spite of their enormous diversity of physiological functions, the over one hundred known protein kinases all share a conserved catalytic domain which is characterized by many highly conserved amino acid residues located in eleven major conserved subdomains (Hanks et al., 1988). Most of these residues directly take part in the phosphorylation processes including adenosine triphosphate (ATP) binding, substrate-peptide interaction and transfer of the phosphate group (Hanks et al., 1988; Knighton et al., 1991). Recently, analysis of the crystal structure of the catalytic subunit of the cAMP dependent protein kinase (cAPK) and insulin receptor (IR) (Hubbard et al., 1994) revealed two highly conserved loop structures (Knighton et al., 1991). The glycine loop (^{50}GXGXXG^{55}) located in the small lobe is responsible for anchoring the ATP moiety, whereas the catalytic loop (^{165}RDLKPEN^{171}) in the large lobe is essential for peptide binding and catalysis (Knighton et al., 1991). The corresponding sequences of the two loops in protein tyrosine kinases (PTKs) are GXGXXG and RDLAARN (for most PTKs) or RDLRAAN (for *src* family PTKs) respectively (Hanks et al., 1988; Knighton et al., 1991). The function of these conserved sequences can be studied by genetic approaches using engineered or spontaneous mutants such as temperature sensitive (ts) mutants defective in

kinase activity. Although it was shown that mutation of aspartic acid to asparagine in the putative catalytic loop of *c-kit and c-fms* resulted in severely diminished kinase activity (Tan et al., 1990; Reith et al., 1993), no mutations have been mapped to this region which resulted in ts phenotype. We report here that a conservative amino acid mutation in the putative catalytic loop of three receptor PTKs results in temperature dependent sensitivity in their enzymatic and biological activities. Our result strongly implies that the same mutation in other PTKs would have a similar effect as well.

Results and discussions

A ts mutant of *v-ros* of avian sarcoma virus UR2, ts251, was isolated and described previously (Garber et al., 1985). We have sequenced the entire open reading frame of the $P68^{gag\text{-}ros}$ transforming protein of ts251 using polymerase chain reaction (PCR) amplification method. The 1.7 Kb *gag-ros* coding sequence was divided into two 5' overlapping 883 bp(PCR fragment I) or 1134 bp fragment (PCR fragment II) and a 3' 742 bp fragment (PCR fragment III) for PCR amplification (Figure 3-1) from viral RNA or proviral DNA. Several independent clones from each PCR amplified fragment were chosen for subsequent sequencing. Comparison of the ts251 sequence with that of the wild type UR2 revealed two mutations (Figure 3-2), a C to G change at nucleotide position 708 resulting in amino acid substitution of proline by arginine in the *gag* region of $P68^{gag\text{-}ros}$ and a C to G change at nucleotide position 1533 resulting in amino acid substitution of alanine by glycine within the conserved kinase region of *ros* (Neckameyer and Wang, 1985). The latter mutation maps right in the presumed catalytic loop of the kinase domain (Figure 3-3). Both changes are from C to G transversion consistent with the use of 5-azacytidine for the UR2 mutants

isolation (Garber et al., 1985; Pathak and Temin, 1992). To discern which of the mutations is responsible for the ts lesion, either the individual or combined mutations were reconstructed into *gag-ros* sequence of UR2 DNA. These constructs were sequenced to confirm the changes. Transfection of the mutants into chicken embryo fibroblasts (CEF) demonstrated that the single mutation of ala^{385} to gly^{385} in the kinase domain is sufficient to confer ts properties on cell transformation and PTK activity to the $P68^{gag\text{-}ros}$ protein (Figure 3-4, 3-5). The transforming activity as reflected in morphological change and colony formation of infected cells correlated very well with *in vitro* and *in vivo* kinase activity of the mutant P68 at the permissive and nonpermissive temperatures. Aside from ts lesion, the alanine to glycine mutation also resulted in markedly reduced *in vitro* PTK activity even when the Ros proteins were isolated from the permissive temperature (Figure 3-5). This probably was due to increased lability in kinase activity of the mutant proteins during extraction and *in vitro* manipulation, which is the case with other PTKs ts mutants such as *v-src* (Maroney et al., 1992) and *v-abl* (Engelman and Rosenberg, 1990). There was little difference in intracellular kinase activity between the ts and wt Ros proteins as measured by autophosphorylation and phosphorylation of cellular substrates at the permissive temperature (Figure 3-5). The ts *ros* mutants have a very stringent temperature sensitivity: their transforming potential, *in vitro* and *in vivo* kinase functions are active at 35°C but not at 41°C (Figure 3-5). Mutation of amino acid 110 in *gag* from proline to arginine does not induce the ts phenotype by itself (data not shown). We conclude that ala^{385} to gly^{385} in *ros* is responsible for the ts lesion. The domain containing amino acids 381 to 387 corresponds to the catalytic loop amino acids 165 to 171 in cAPK and 1131 to 1137 in IR (Hubbard et

al., 1994) (Figure 3-3). The fact that a conserved single amino acid mutation within this region of Ros results in its ts lesion lends further support to the presumed catalytic role of this sequence in PTKs.

The PTK domain of Ros shares a close homology with those of human insulin and insulinlike growth factor I receptors (IR and IGFR) (Ebina et al., 1985a; Ullrich et al., 1985). By fusing the 3' portion of the IR and IGFR to the viral *gag*, we have previously generated transforming and tumorigenic gag-IR and gag-IGFR fusion receptors (Wang et al., 1987; Poon et al., 1991; Liu et al., 1992, 1993) (Figure 3-6). Aside from the 5' truncation, there are no mutations in the IR and IGFR fusion receptor PTK domains when compared with their native proteins. To determine whether the ts *ros* mutation would have a similar effect on those related PTKs, we engineered the alanine to glycine mutation in the gag-IR and gag-IGFR encoded by the viruses UIR19t6 and NM1 respectively and confirmed the change by sequencing (Figure 3-6). The mutant viruses, named tsIR and tsIGFR, displayed temperature sensitive cell transforming activity in regard to inducing morphological alteration and promoting colony formation of the transfected cells (Figure 3-7). The transforming potency of each mutant was indistinguishable from that of their parental viruses at the permissive temperature. Similarly, the *in vitro* and intracellular kinase activity of the mutant proteins were also temperature-sensitive. As with ts Ros, the *in vitro* kinase activity of the tsIR protein isolated from the permissive temperature was dramatically lower than that of the wild type protein. In contrast there was less difference between the activity of tsIGFR and its wt counterpart. Apparently, the mutation has subtle differential effect on the kinase stability of those two closely related PTKs (Figure 3-8, 3-9). In contrast to the *in vitro*

activity, the intracellular kinase activities of the mutant and the wild type IR and IGFR proteins were similar at the permissive temperature (Figures 3-8, 3-9).

Our observation demonstrates that removal of the methyl group from the alanine within the RDLAARN sequence renders the three related PTKs ts and further underscores the essential function of this presumed catalytic loop in their kinase activity. This residue maps right in the catalytic loop of cAMP and IR (Knighton et al., 1991; Hubbard et al., 1994). The catalytic loop of the cAPK and IR is thought to bind the substrate peptide and help orient the acceptor residues for the phosphate transfer. The glycine residue is known to interrupt α-helices and to increase the flexibility of the peptide backbone (Chou and Fasman, 1974). It is likely that substitution of alanine by glycine reduces the stability of the reaction complex and thus accounts for the temperature sensitivity. The mutation sites of other known ts mutants of PTK oncogenes vary broadly, mapping mostly in the large lobe (Welham and Wyke, 1988; Fincham and Wyke, 1986; Chen et al., 1986; Friel et al., 1987). Three *v-src* ts mutants, NY68, NY72-4 and PA104, each contains two mutations including a common val^{461} to met^{461} mutation (Mayer et al., 1986; Nishizawa et al., 1985) corresponding to the amino acid pro^{236} of cAPK which is involved in its high affinity substrate binding site interaction (Knighton et al., 1991). tsLA90 of *src* contains the glu^{280} to lys^{280} change (Maroney et al., 1992) mapping just after the GXGXXG glycine loop sequence. Physical analysis of the interaction between the ts mutant proteins described here and their respective substrate peptides at different temperatures would potentially be very useful for exploring the mechanism of the PTK catalytic function. With only a few exceptions, the sequences of the presumed catalytic loop of all PTKs can be grouped into 2

motifs, RDLRAAN for the *src* family and RDLAARN for the rest (which includes Ros, IR and IGFR). We predict that the same alanine to glycine mutation in the kinase domain of PTKs would have a similar effect and may even do so on the src family PTKs.

Figure 3-1. Schematic diagram of PCR amplification step.

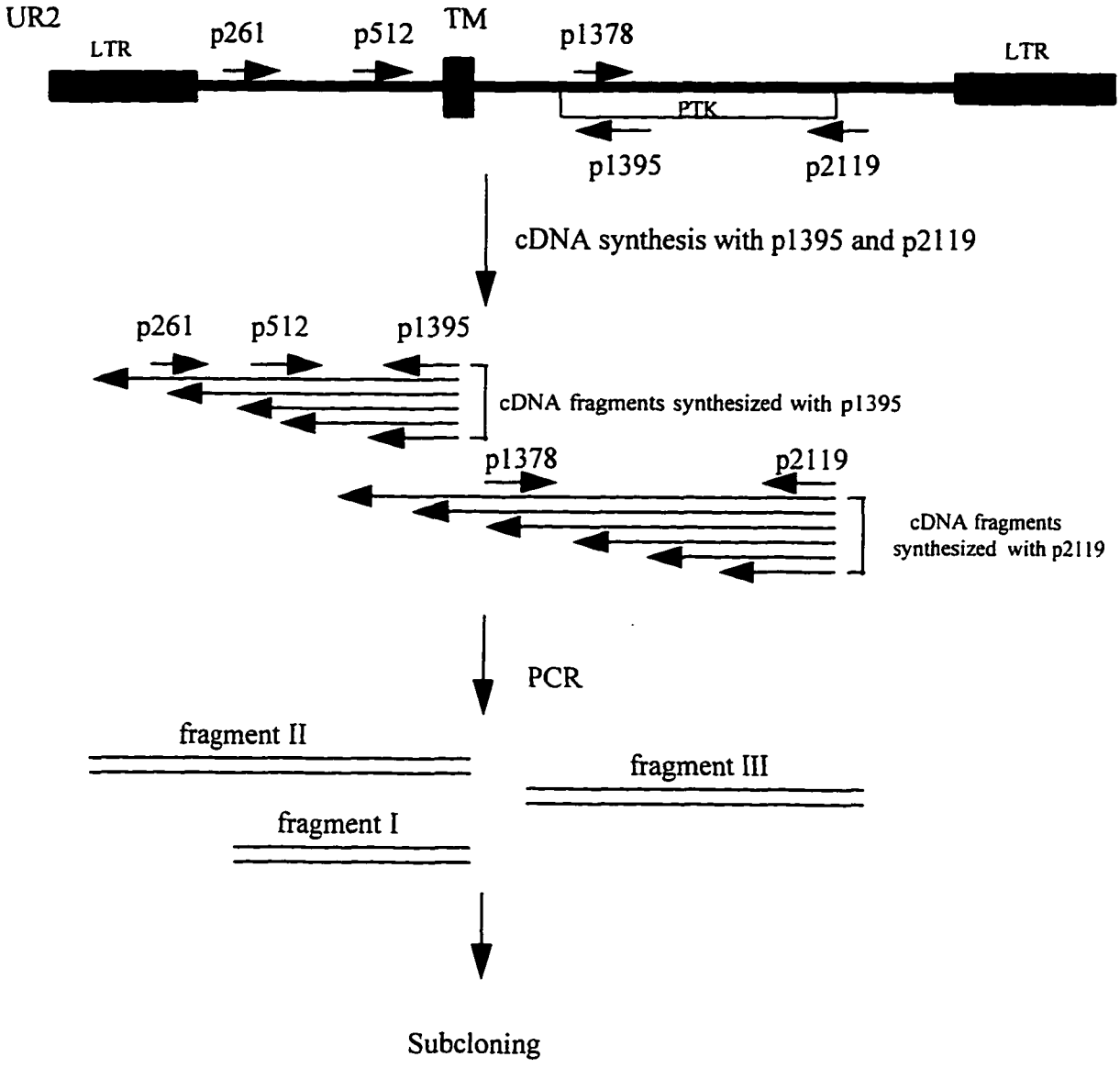


Figure 3-2. Summary of nucleotide changes found in ts251.

Nucleotide Position		Nucleotide 708	Nucleotide 1533
Nucleotide Change		C to G	C to G
Amino Acid Change		Pro to Arg	Ala to Gly
	6-513	+	NA
Independent	9-513	-	NA
clones	14-513	+	NA
	1st9	+	NA
WT clone	UR2	-	-
	3-2120	NA	-
	12-2120	NA	+
Independent	3'(#1)	NA	+
clones	3'(#2)	NA	+
	1(3')	NA	+
	9(3')	NA	+
WT clone	UR2	-	-

Pro:proline Arg:arginine Ala:alanine Gly:glycine NA: non applicable

6-513, 9-513 and14-513 are three independent pBluescript clones containing the PCR fragment I. 1st9 is the pBluescript clone containing the PCR fragment II. 3-2120, 12-2120, 3'(#1), 3'(#2), 1(3') and 9(3') are six independent pBluescript clones containing the PCR fragment III.

Figure 3-3. Amino acid sequences of the catalytic loops of cAPK, Ros, IR, IGFR and the ts mutants. The numbers are taken from published sequences of cAPK (Hanks et al., 1988), Ros (Neckameyer and Wang., 1985), IR (Ebina et al., 1985) and IGFR (Ullrich et al., 1985) respectively. The glycine mutation responsible for the ts phenotype is marked by an asterisk(*). The abbreviation for the amino acid residues are: A, alanine, D, aspartic acid, E, glutamic acid, G, glycine, K, lysine, L, leucine, N, asparagine, P, proline, R, arginine

cAPK	^{165}R D L K P E N^{171}
UR2ros	^{381}R D L A A R N^{387}
tsros	R D L A G* R N
IR	^{1131}R D L A A R N^{1137}
tsIR	R D L A G* R N
IGFR	^{1104}R D L A A R N^{1110}
tsIGFR	R D L A G* R N

Figure 3-4. Temperature dependent transforming activity of tsros mutants.

tsros101, 102 and 103 are three independent clones containing ala^{385} to gly^{385} mutation in the UR2 $P68^{gag\text{-}ros}$. Equal amount (10 μg) of plasmid DNA containing alanine to glycine mutation or wt UR2 genome was transfected into CEF, together with 1 μg UR2AV DNA, using polybrene method (Kawai and Nishizawa, 1984). Transfected cells were kept at 35°C and overlaid with soft agar medium two to three times upon each passage of the cells to achieve a fully transformed culture. At that point, cells were trypsinized and seeded either on regular dishes or in soft agar medium ($1x10^6$ cells/10 cm dish) to observe their monolayer morphology (A) and colony forming ability (B) at 35°C and 41°C. Pictures of the monolayer cultures were taken when cells reached confluence. Colonies shown were 17 days after plating.

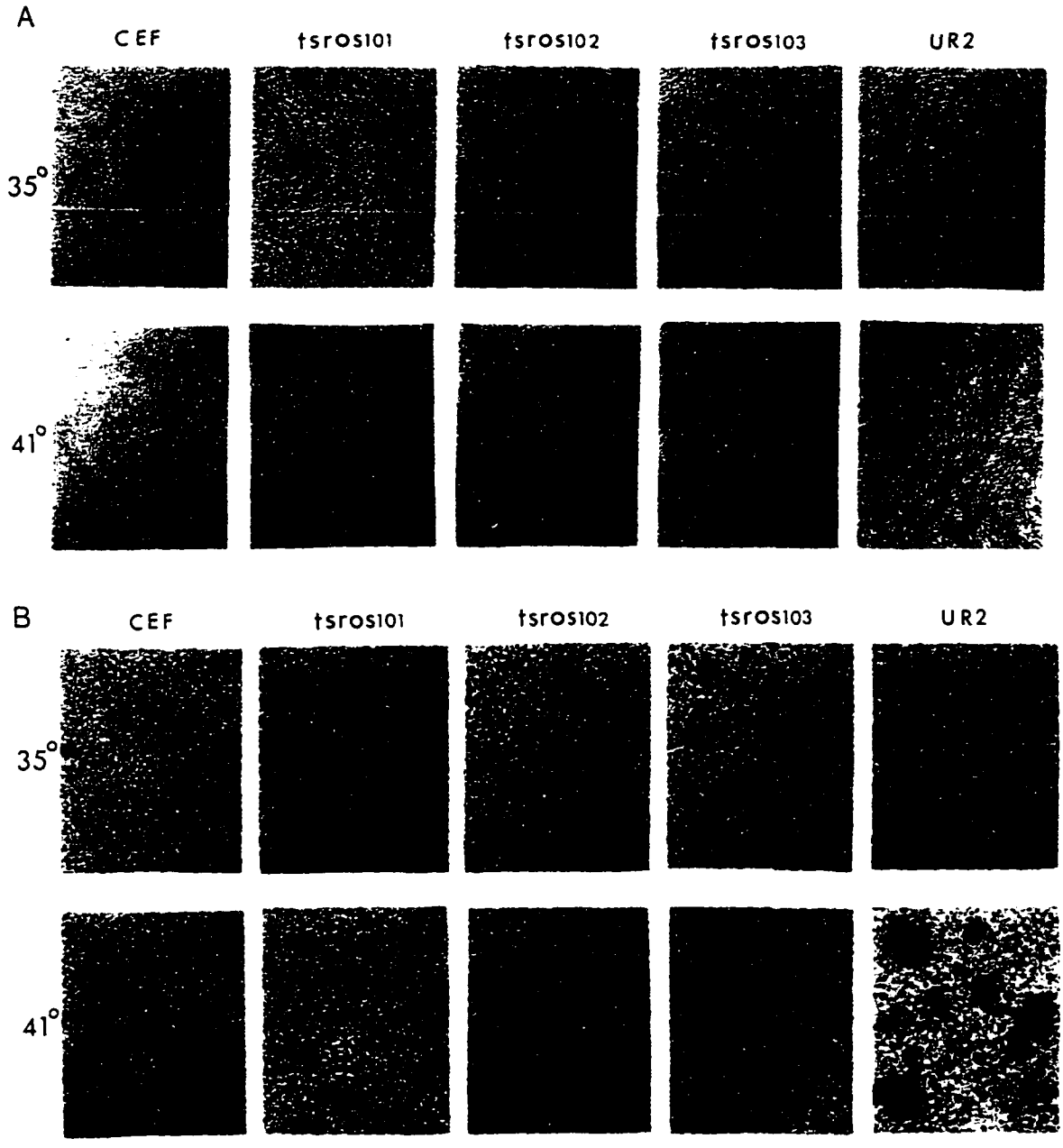


Figure 3-5. The *in vitro* and intracellular PTK activity of the ts Ros proteins.

tsros102 has been described in Figure3-4. tsros106 is UR2 mutant containing the combined mutations of pro^{110} to arg in the *gag* and ala^{385} to gly in the kinase domain. (A) *In vitro* autokinase assay of tsros102, 106 and wild type (wt) UR2 proteins. The upper two panels and lower two panels represent two independent experiments. In each experiment, the cell lysates from transfected cells were precipitated with an anti-ros serum. Precipitated immune complex was divided into two equal parts. One part was used for *in vitro* kinase assay (upper panel). Another was for Western blotting to monitor the amount of the $P68^{gag\text{-}ros}$ (lower panel). (B) Phosphotyrosyl protein patterns of tsros102, tsros106, ts251 infected cells at 35°C, 37°C and 41°C. Cultures were treated with 200 μM sodium vanadate for 4 hours before extraction. Cell lysates were analyzed by protein gel separation and immunoblotting with PY20. (C) Intracellular auto-phosphorylation of tsros102, tsros106, ts251 and wt UR2 proteins. Paralleled cultures kept at 35°C, 37°C or 41°C were treated with 200 μM sodium vanadate for 4 hours before extraction with RIPA buffer, followed by precipitation with anti-ros serum. The immune complex was divided into equal parts for gel separation and immunoblotting with either anti-phosphotyrosine antibody PY20 (ICN) (upper panel) or anti-ros serum (lower panel). The doublet of P68 in (**A**) through (**C**) reflected different degrees of phosphorylation.

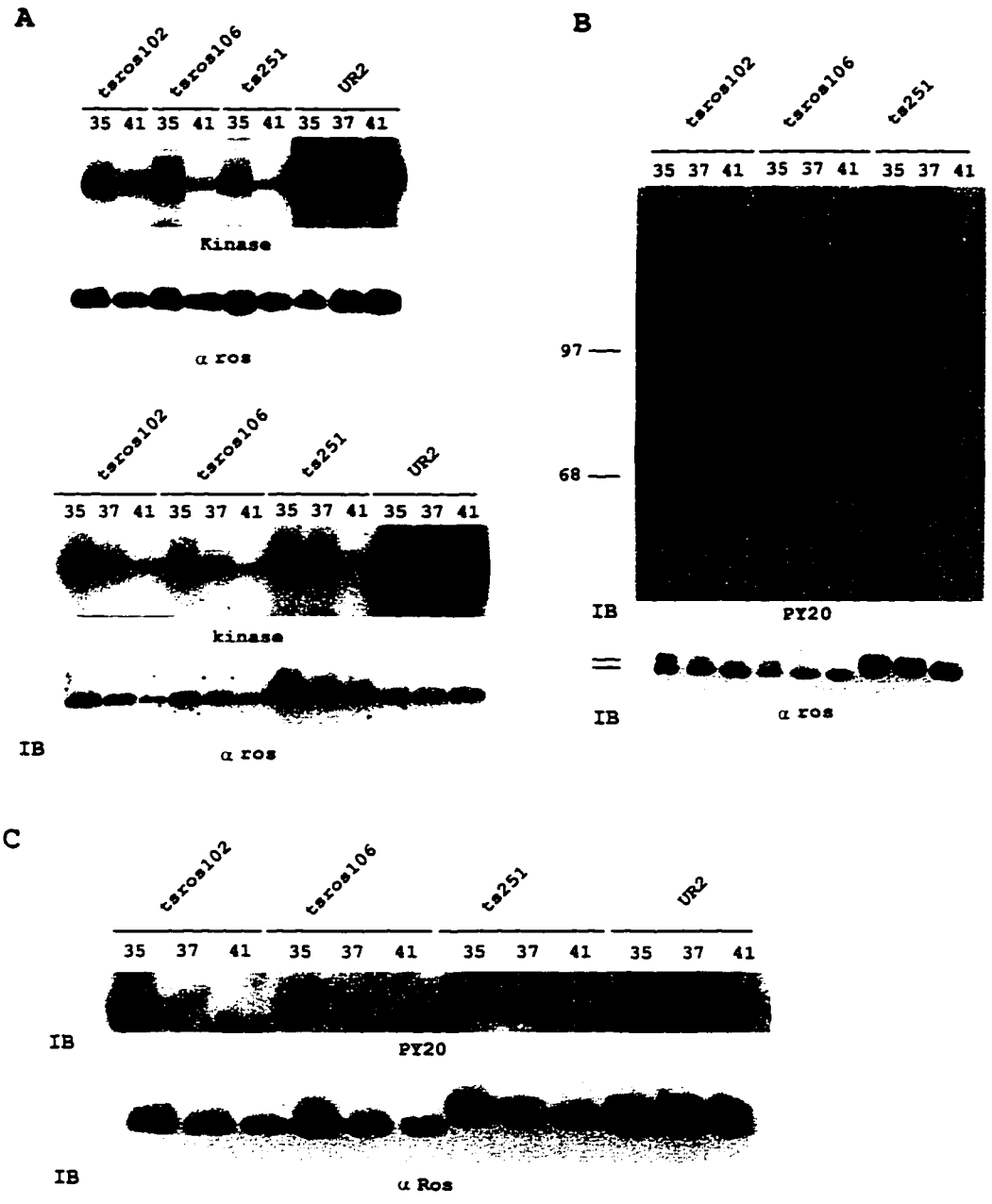


Figure 3-6. Schematic diagram of site directed mutagenesis by PCR.

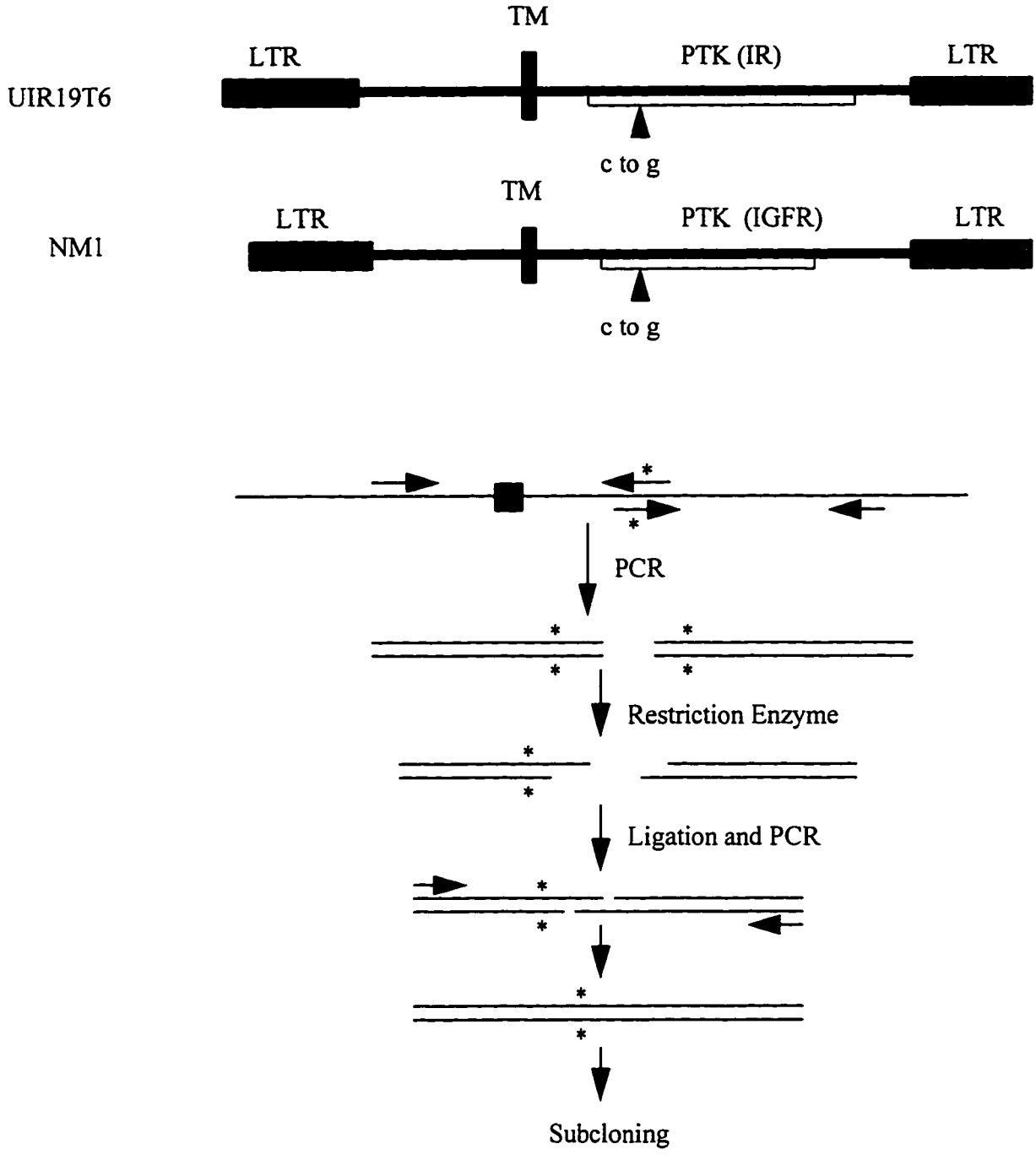


Figure 3-7. Temperature dependent transforming activity of tsIR and tsIGFR mutants. tsIR101,102 and tsIGFR101,102 are independent clones of gag-IR and gag-IGFR mutants engineered in this study. Each mutant contains an alanine to glycine mutation within the catalytic loop as shown in Figure3-6. Monolayer morphology of tsIR (A), tsIGFR (B), UIR19t (wt) (A, C) and colony forming ability of tsIR (C), tsIGFR (D), NM1 (wt) (B, D) at 35°C(upper panel) and 41°C (lower panel) are shown. Colony of tsIR and tsIGFR shown were 15 and 11 days after plating respectively.

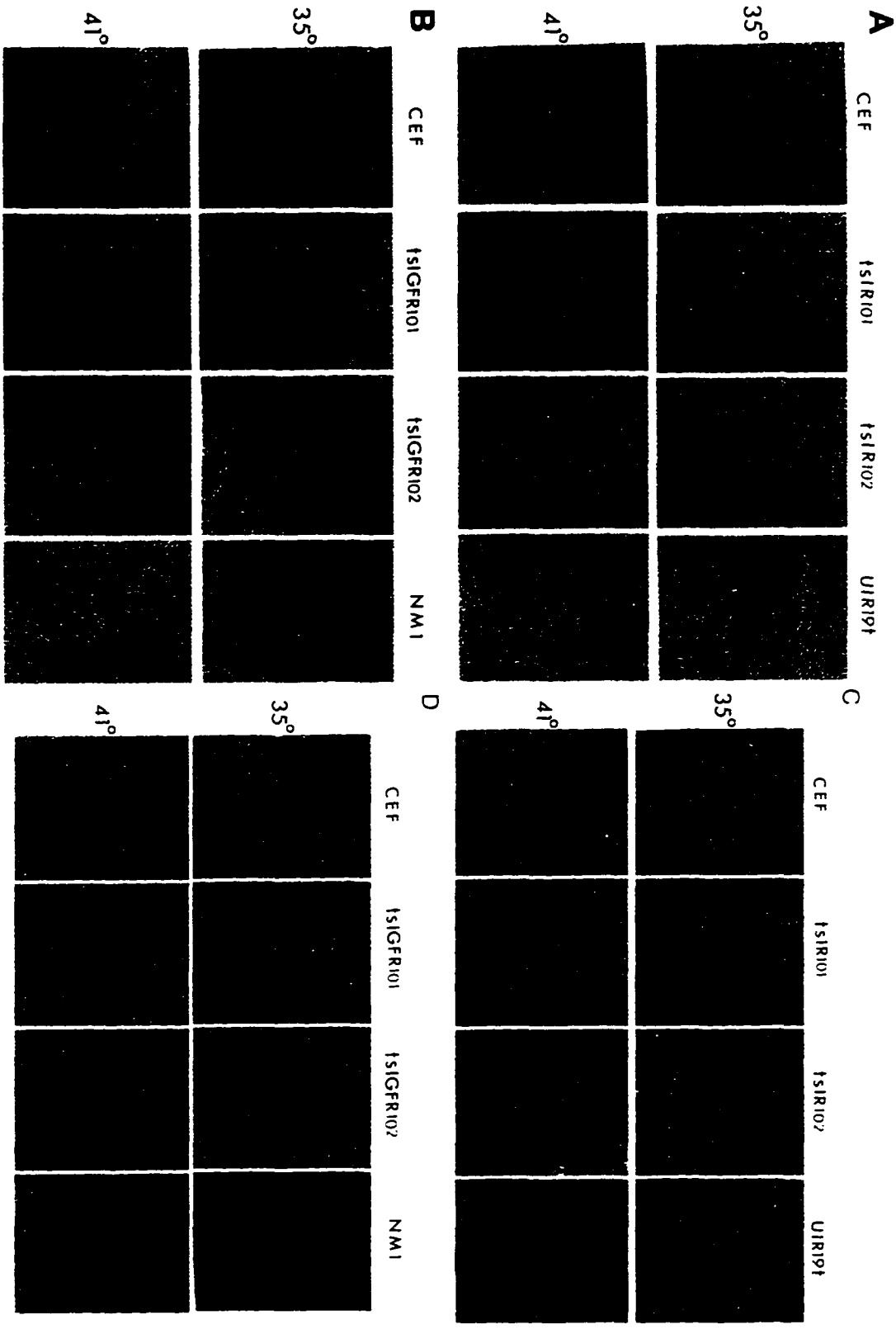


Figure 3-8. The *in vitro* and intracellular PTK activity of tsIR101, 102, 103 and UIR19t proteins. (A) *In vitro* kinase activity as assayed by autophosphorylation. Lower panel shows the IR protein amount by Western analysis. (B) Phosphotyrosyl protein patterns of tsIR101, 102, 103, 104 and UIR19t infected cells at 35°C and 41°C. Lower panel shows the amount of gag-IR proteins. tsIR101, 102 have been described in Figure3-7. tsIR103,104 are independent UIR19t6 mutants containing combined mutations of proline to arginine in the *gag* and alanine to glycine in the kinase domain. The doublet of P70 gag-IR reflected different degrees of phosphorylation.

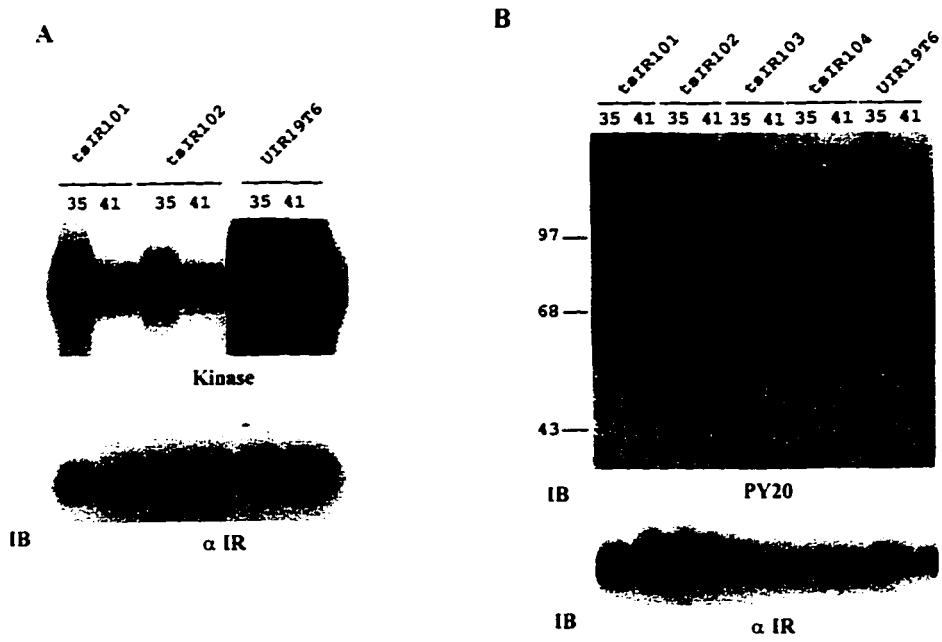
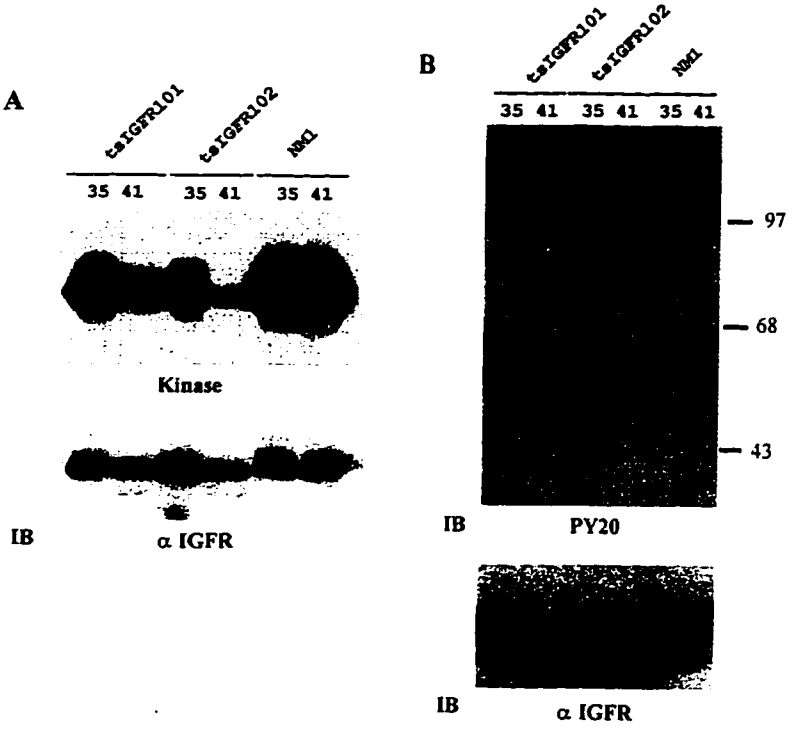


Figure 3-9. The *in vitro* and intracellular PTK activity of tsIGFR101, 102 and NM1 proteins.

(A) *In vitro* autophosphorylation activity (upper panel) and the corresponding gag-IGFR protein amount (lower panel). (B) Phosphotyrosyl protein patterns (upper panel) and corresponding gag-IGFR protein amount (lower panel). The multiple bands of P53 were due to different degrees of phosphorylation.



Chapter 4 Identification of interacting proteins of IR using yeast two hybrid system

Abstract

Using kinase inactive and kinase active IR as baits in the yeast two hybrid system, we have identified several potential interacting proteins of IR. Three proteins were shown to interact with kinase inactive IR in yeast cells. The first protein has no homology to any protein in the genebank. However, its 3' noncoding region shares high homology with glucose regulated protein-GRP78. The second protein is highly homologous to mitotic associated protein 2 (MAD2). The third protein also shares no homology with any protein known. Using kinase active IR as a bait, nine potential interacting proteins of IR have been identified. Four of them show no identity or homology to any protein in the gene bank. Among the remaining five, the first one encodes an unknown protein whose partial sequence has been deposited into the genebank; the second one encodes a transmembrane protein with unknown function; the third one is highly homologous to human enigma protein; the fourth encodes a protein highly homologous to mammary gland factor (MGF) (later called Stat5); the fifth encodes a protein which is highly homologous to cytochrome C oxidase subunit Vb. Attempts to demonstrate the stable interaction between IR (kinase positive or negative) and those proteins by other mthods failed, suggesting that interaction between these proteins and IR could be very transient. In this chapter, properties of some of these proteins will be discussed.

Introduction to the two hybrid system

The two hybrid system is a yeast based genetic system to detect protein-protein interaction *in vivo*. This system was first developed by Stanley Field's group at State University of New York (SUNY) and was later modified by Roger Brent's group at Harvard Medical School (Zervous et al., 1993).

The two hybrid system takes advantage of the modular nature of the yeast Gal4 transcriptional factor which can be divided into two separable functional domains: a specific DNA binding domain and a transcription activation domain. Functional transcriptional factor can be reconstituted when the DNA binding domain and activation domain are linked together by a pair of interacting proteins (Figure 4-1A) (Hope and Struhl, 1986; Ma and Ptashne, 1987).

Roger Brent's group modified this system as the following. 1). Instead of using Gal4 DNA binding domain, they use LexA protein as the DNA binding protein. LexA is a repressor of several chromosomal genes involved in the SOS response in E.coli (Ebina, et al., 1983). The specificity of LexA DNA binding is maintained by using LexA operator derived from colE1 gene in the promoter region of the reporter plasmid (Figure 4-1B); 2). The yeast strain EGY48 contain an integrated copy of *Leu2* gene, whose UAS in the promoter region has been replaced by LexA operator. Therefore, the expression of Leu2 is under the regulation of protein-protein interaction, which provides another criterion for selection of potential interaction proteins (growth selection) beside color selection (Figure 4-1B) (Zervous, et al., 1993); and 3). Stringent negative and positive controls to test properties of the bait fusion protein before performing library screening (Figure 4-2).

The two hybrid system has several advantages 1).It is highly sensitive allowing one to detect not only stable but also transient interaction. 2).The condition of testing protein interaction resembles the physiological environment within the eukaryotic cells. 3). It not only allows one to detect protein-protein interaction *in vivo*, but also to clone the genes of potential interacting proteins.

Results and discussions

In view of the diversified functions of insulin receptor, we suspect that it most likely involves signaling molecules in addition to a few that have been identified so far. Interaction between IR and its substrates studied so far appears to be very transient and difficult to detect by conventional coimmunoprecipitation. One way to circumvent this difficulty is to employ the yeast two hybrid system which has been successfully used to demonstrate interaction between IR and IRS-1 and between IR and Shc (Craparo et al., 1995; Gustafson et al., 1995). The bait plasmid pEG202, reporter plasmid pSH18-34 and library plasmid pJG4-5 as well as other plasmids used in the two hybrid system are shown in Figure 4-2. Plasmids pLexA-IR-S and pLexA-IR-L encoding IR bait proteins with different lengths (Figure 4-2) were used.

Before starting screening, several pretesting experiments were done. The results are shown in Figure 4-3. The purposes of the pretesting experiments are summarized below 1). Bait -fusion protein itself should not promote yeast cell growth in the absence of leucine and should not confer *LacZ* gene expression; 2). Bait protein can enter nucleus and bind LexA operator; and 3). Lex-IR fusion protein has the correct conformation inside the yeast cells. For the first part: pLexA-IR(S/L) together with pSH18-34 were transformed into EGY48

cells. The growth of EGY48 yeast cells containing pLexA-IR(S/L) and pSH18-34 in H^-,U^-, L^-,T^+, Glu^+ medium was compared with that in H^-, U^-, L^+, T^+, Glu^+ medium (Figure 4-3). This experiment was to confirm that LexA-IR do not support the growth of EGY48 in the absence of leucine. In addition, EGY48 containing plasmids pLexA-IR, pSH18-34 or pSH17-4 (a positive control plasmid containing both Gal4 DNA binding and activating domain) were plated onto X-Gal selection plate and assayed for the β-galactosidase activity. Negative result means that LexA-IR itself does not confer transcription activation of *LacZ* (Figure 4-3). pRFHM1 containing an inert protein of transcription activation was used as a negative control. For the second part, the nuclear localization, plasmids pLexA-IR and pJK101 were introduced into EGY48 cells and β-galactosidase activity was compared (Figure 4-3). pJK101 is a reporter plasmid whose *LacZ* gene product can be induced in the presence of galactose (Figure 4-2). However, when LexA-IR enters the nucleus and binds LexA operator in the promoter region of pJK101, the transcription activation will be repressed. This experiment was used to confirm that bait fusion protein can enter the nucleus. For the third part, the correct conformation of bait-IR fusion protein, there is no absolute criterion. Intracellular autophosphorylation and *in vitro* kinase assay were used as criteria to assess the conformation of the bait protein. LexA-IR-L has the autokinase activity and is tyrosine phosphorylated inside the yeast cells whereas the LexA-IR-S has neither activity (Figure 4-4 and data not shown). However, negative autokinase activity does not necessarily mean that the conformation of LexA-IR fusion protein is not correct. It is possibe that the conformation of LexA-IR-S is locked in the kinase inactive state. Therefore, both the kinase inactive and kinase active LexA-IR fusion proteins were used for the

screening.

The screening diagram is shown in Figure 4-5. The detailed steps in two hybrid screening are described in materials and methods. The summary of screening results for the kinase inactive and kinase active constructs is shown in Table 4-1. Using the kinase inactive IR as bait, three plasmids encoding potential interaction proteins of IR were identified. One of them (pJG4-5-7)appeared nine times out of 22 clones isolated and sequenced and the other two each appeared only once. They were named pJG4-5-7, pJG4-5-12, pJG4-5-17 containing approximately 700 bp, 1200 bp and 2000 bp cDNA inserts respectively. Nucleotide Sequence comparison of these cDNA inserts with gene bank (GCG program) revealed no identity to any gene known to date. However, the 700 bp cDNA insert derived from pJG4-5-7 has 97.2% identity between its 386 bp at 3' noncoding region and the nucleotide sequence of human 78 kelodalton glucose-regulated protein (GRP78) (Ting et al., 1988) (Figure 4-6). Nevertheless, the partial polypeptide (96 a.a.) predicted from the cDNA insert of pJG4-5-7 does not share homology with that of human GRP78. In addition, unlike human GRP78, this polypeptide (96 a.a.) does not have KDEL motif which is a marker for ER location and is present at the C-terminal region of human GRP78 protein (Figure 4-6). The cDNA inserts derived from pJG4-5-7, pJG4-5-12 were ligated into pGEX5x-1 to obtain GST-fusion plasmids which were then transformed into W3110 cells. These GST-fusion proteins were called GST7 and GST12 respectively. GST7 and GST12 expressed in W3110 cells were purified to homogeneity (Figure 4-7). Polyclonal antisera were made against bacterial purified GST7 and GST12 (Figure 4-8). Using antibody against GST7, we could detect a MW~68 kDa protein in various types of cells (Figure 4-9). This

68 kDa protein appeared not to be tyrosine phosphorylated and the protein levels remained constant in control CEF compared with those in cells transformed by T6 and NM1 encoding oncogenic gag-IR and gag-IGFR transforming proteins respectively (Figure 4-9).

The second one encodes a protein which is highly homologous to MAD2 (mitotic associated protein 2) (Figure 4-10), the third one encodes an unknown protein. Since there is not enough clues about the potential functions of these two proteins, we did not pursue further investigation of these two proteins.

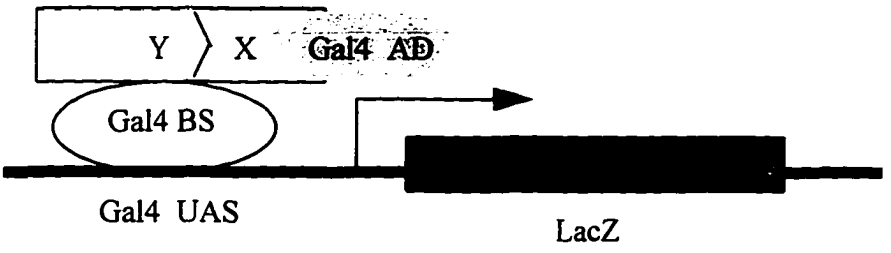
Using the kinase active IR as bait, nine strong positives were obtained (Table 4-1). However, most of them do not share homology to any protein in the genebank. Among the remaining clones, one of them has sequence homology to an unknown protein whose sequence has been deposited into the Gene Bank. The second one encodes a protein homologous to oxidase subunit Vb. The third encodes a protein whose sequence is homologous to enigma gene. The fourth encodes for an unknown transmembrane protein. The last one, which is also the focus in the next chapter, is the one whose encoded polypeptide is highly homologous to mammary gland factor (MGF) (later called Stat5) (Figure 4-11). We called this protein IRA3 for insulin receptor associated protein clone 3. GST-fusion protein and His-tagged fusion protein of IRA3 were made (Figure 4-7). The purified His-IRA3 was used for antiserum preparation (Figure 4-12).

The important question is to ask whether these potential IR interacting proteins can interact with IR outside the yeast system. Even though we repeatedly observed interaction between IR and these proteins in yeast cells, we could not demonstrate stable association by coimmunoprecipitation in animal cells. To increase the sensitivity of detection, *in vitro*

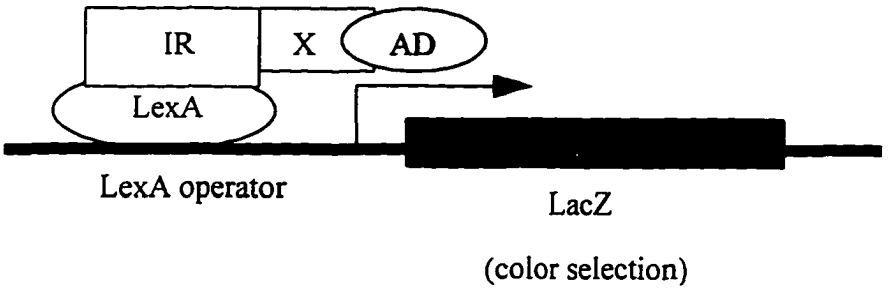
transcription and translation of these polypeptides were carried out (Figure4-13) and the products were incubated with cell lysates containing IR or partially purified IR (by immunoprecipitation), no stable association was detected. Failure to detect their association by traditional co-immunoprecipitation indicates that the interaction of IR with these proteins could be very transient; or the interaction is easily disrupted by the presence of detergent. The significance of these proteins in IR mediated functions remains open.

Figure 4-1. Principle of two hybrid system

A



B



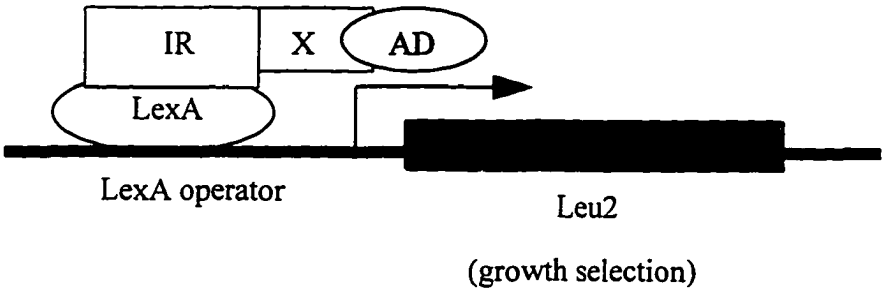
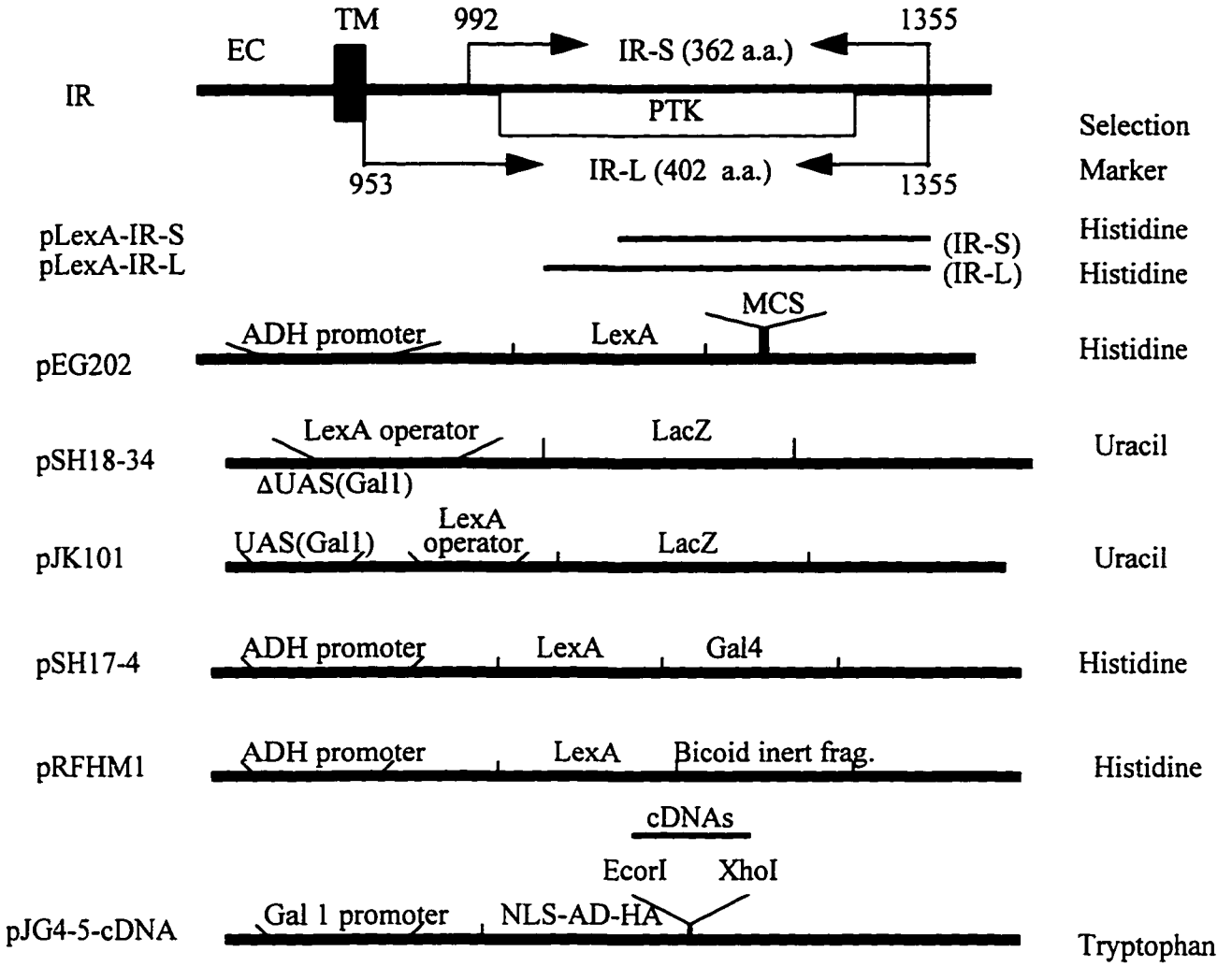


Figure 4-2. Schematic representation of plasmids used in the two hybrid system



Yeast Strain: EGY48 (MATα, *his3, trp1, ura3-52, Leu2::pLEU2-LexAop6*)

pLexA-IR-S and pLexA-IR-L: bait fusion plasmids containing the short or long fragment of IR β subunit respectively; pJG4-5-cDNAs: library plasmid; pSH18-34: reporter plasmid; Abbreviations: IR, insulin receptor; TM, transmembrane domain; PTK, protein tyrosine kinase; MCS, multiple cloning sites; NLS, nuclear localization signal; AD, activation domain; HA, hemagglutinin tag.

Figure 4-3. Results from pretesting experiments.

		Western Blotting		
Protein Expression	EGY48(pLexA-IR-S)	+		
	EGY48(pLexA-IR-L)	+		
		β-galactosidase activity	Growth in H-U-T+ L-, Glu	L+,Glu
Repression Assay	EGY48(pLexA-IR-S,pSH18-34)	-	-	+
	EGY48(pLexA-IR-L,pSH18-34)	-	-	+
	EGY48(pSH17-4,pSH18-34)	+	NA	NA
	EGY48(pRFHM1,pSH18-34)	-	NA	NA
		β-galactosidase activity		
Localization Assay	EGY48(pLexA-IR-S,pJK101)	+		
	EGY48(pLexA-IR-L,pJK101)	+		
	EGY48(pJK101)	+++		
	EGY48(pRFHM1,pJK101)	+		

Figure 4-4. Tyrosine kinase activity of LexA-IR-L bait protein in yeast EGY48 cells. 20 ml EGY48 containing pLexA-IR-L and pSH18-34 and control cells containing pSH18-34 only were grown in the glucose selection medium overnight. The yeast cells were broken up by glass beads method. The supernatants were immunoprecipitated with anti-IR antibody overnight, the immunoprecipitate was divided into duplicate aliquots. One part was for *in vitro* autokinase assay and the same filter was used for blotting with anti-IR antibody to detect the IR protein. Another part was for anti-Ptyr Western blotting to detect tyrosine phosphorylation. (A) *In vitro* kinase activity of LexA-IR-L protein expressed in EGY48 cells. Upper panel is the kinase activity, lower panel is its corresponding protein amounts. (B). Intracellular tyrosine phosphorylation of LexA-IR-L protein. C1, C2 are two independent control clones and LexA-IR-L1, Lex-IR-L2 are proteins expressed from the two independent positive clones. IP: immunoprecipitation; IB: immunoblotting.

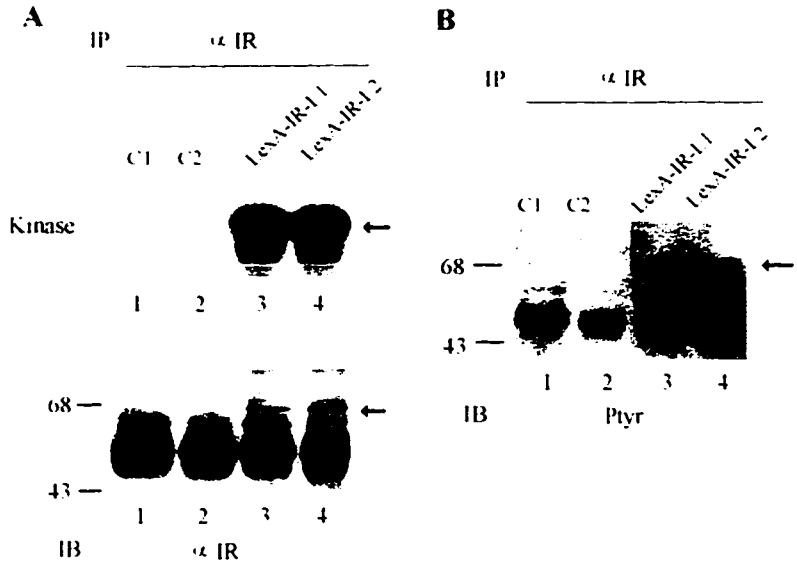


Figure 4-5. Flow diagram of screening process.

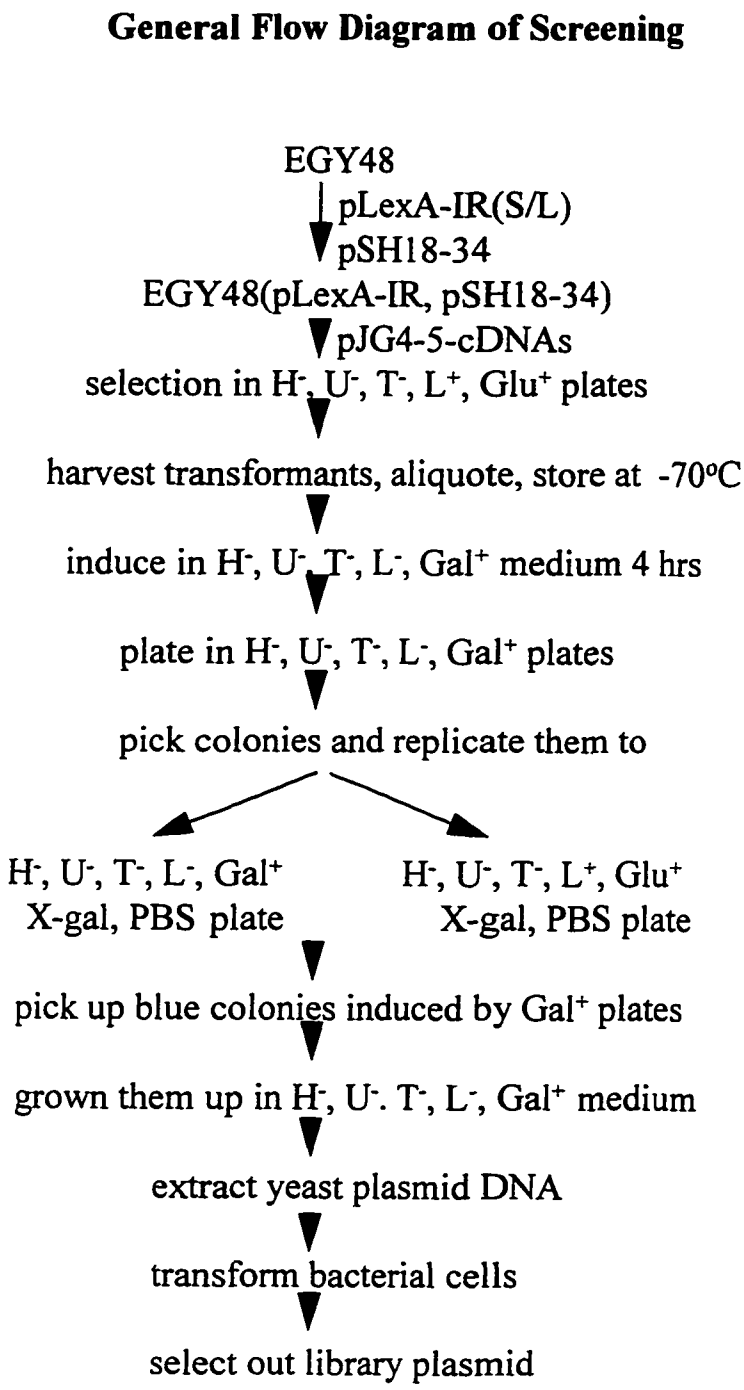


Figure 4-6. Nucleotide and amino acid sequences derived from cDNA insert of pJG4-5-7 and its comparison with *GRP78*.

```
HUMGRP78[Human 78 kdalton glucose-regulated protein (GRP78)] Length:5470 bp

ACCESSION   M19645  NID g183644
SCORES    97.2% identity in 386 bp overlap


pJG4-5-07 (240)TTAGTGAGGGTCAAGGAACAGCAGGGGAAGAAGAGGAGAAGAAAAAACAGAAGAAAAAGA
                                              ||  || |||||  | |||| |||||||||
Humgrp    (5030)CAAACTCTATGGAAGTGCAGGCCCTCCCCCAACTGGTGAAGAGGATACAGCAGAAAAAGA
pJG4-5-07       TGAGTTGTAGACACTGATCTGCTAGTGCTGTAATATTGTAAATACTGGACTCAGGAACTT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Humgrp          TGAGTTGTAGACACTGATCTGCTAGTGCTGTAATATTGTAAATACTGGACTCAGGAACTT
pJG4-5-07       TTGTTAGGAAAAAAATTGAAAGAACTTAAGTCTCGAATGTAATTGGAATCTTCACCTCAGA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Humgrp          TTGTTAGGAAAAAAATTGAAAGAACTTAAGTCTCGAATGTAATTGGAATCTTCACCTCAGA
pJG4-5-07       GTGGAGTTGAAACTGCTATAGCCTAAGCGGCTGTTTACTGCTTTTCATTAGCAGTTGCTC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Humgrp          GTGGAGTTGAAACTGCTATAGCCTAAGCGGCTGTTTACTGCTTTTCATTAGCAGTTGCTC
pJG4-5-07       ACATGTCTTTGGGTGGGGGGGGGAGAAGAAGAATTGGCCATCTTAAAAAGCGGGTAAAAA
                |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Humgrp          ACATGTCTTTGGGT--GGGGGGGAGAAGAAGAATTGGCCATCTTAAAAAGCGGGTAAAAA
pJG4-5-07       ACCTGGGTTAGGGTGTGTGTTCTCCTTCAAAATGTTCTATTTAACAACTGGGTCATGTGC
                |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Humgrp          ACCTGGGTTAGGGTGTGTGTTCACCTTCAAAATGTTCTATTTAACAACTGGGTCATGTGC
pJG4-5-07       ATCTGGTGTAGGAAGTTTTTTCTACCATAAGTGACACCAATAAATGTTTGTTATTT
                |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Humgrp          ATCTGGTGTAGGAGGTTTTTTCTACCATAAGTGACACCAATAAATGTTTGTTATTTACAC

Humgrp          TGGTCTAATGTTTGTGAGAAGCTT(5470)



Partial nucleotide and peptide sequence of pJG4-5-07;
Nucleotide length: 648  Polypeptide length:96

agcggggctgactgcaaaggagacccaaggaacagtgccaagttagatgccgattacccacttcgagtcctttat
S  G  A  D  C  K  G  D  P  R  N  S  A  K  L  D  A  D  Y  P  L  R  V  L  Y
tgtggagtctgttcattaccaacagagtactgtgaatatatgcctgatgttgctaaatgtagacaatggttagag
C  G  V  C  S  L  P  T  E  Y  C  E  Y  M  P  D  V  A  K  C  R  Q  W  L  E
aagaattttccaaatgaatttgcaaaacttactgtagaaaattcacccaaacaagaagctggaattagtgaggga
K  N  F  P  N  E  F  A  K  L  T  V  E  N  S  P  K  Q  E  A  G  I  S  E  G
caaggaacagcaggggaagaagaggagaagaaaaaacagaagaaaaagatgagttgtagacactgatctgctag
Q  G  T  A  G  E  E  E  E  K  K  K  Q  K  K  K  M  S  C  R  H  *
gctgtaatattgtaaatactggactcaggaacttttgttaggaaaaaattgaaagaacttaagtctcgaatgtaa

ttggaatcttcacctcagagtggagttgaaactgctatagcctaagcggctgtttactgcttttcattagcagtt

gctcacatgtctttgggtgggggggggagaagaagaattggccatcttaaaaagcgggtaaaaaacctgggttag

ggtgtgtgttctccttcaaaatgttctatttaacaactgggtcatgtgcatctggtgtaggaagttttttctacc

ataagtgacaccaataaatgtttgttattt
```

Figure 4-7. Purification of GST-fusion proteins and His-IRA3.

(A). Bacterial expression of GST-fusion proteins and His-tag fusion protein. 1mM IPTG was used for induction for 4 hours at 37°C. 5 μl of total cell lysates were loaded onto SDS-PAGE gel. Bacterial proteins were detected by Commassie blue staining. Arrows indicate the positions of induced bacterial fusion proteins. (B). Purification of GST and His-tag fusion proteins. 1 μg purified proteins were loaded onto gel and the proteins were detected by Commassie blue staining.

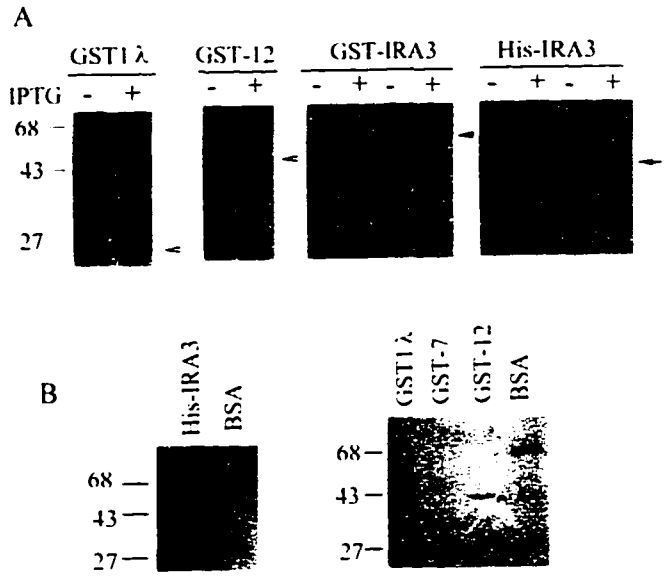


Figure 4-8. Anti-GST7 and anti-GST12 antibodies recognize their corresponding immunogens.

(A). Anti GST7 and anti-GST12 antisera recognize immunogens in the direct Western blotting. 1 μg, 0.1μg or 0.01μg of GST-fusion proteins were loaded onto SDS-PAGE gel. Western blotting was done with α7 (abbreviation for anti-GST7) or α12 (abbreviation for anti-GST12) Ab (1:500 dilution). (B). Anti-GST7 and anti-GST12 antisera immunoprecipitate their corresponding immunogens. 5 μg GST-fusion protein was used for immunoprecipitation, followed by Western blotting with anti-GST7 or GST12 antisera. Preα7 and preα12 are the preimmune sera of immunogen GST7 and GST12 respectively. α7 and α12 are the immune sera of GST7 and GST12 respectively. Arrows indicate the positions of the immunogens.

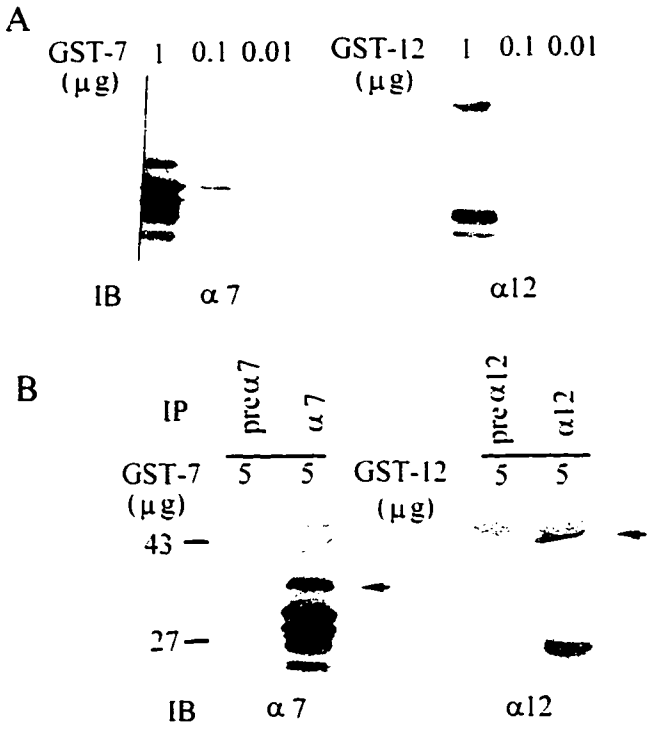


Figure 4-9. A 68 kDa endogenous protein is recognized by anti-GST7 antibody.

(A). A 68 kDa endogenous protein is immunoprecipitated by anti-GST7 antibody and the 68 kDa protein is not tyrosine phosphorylated in response to insulin stimulation. CHOIR cells were serum starved for 2 hours before insulin (50 nM) stimulation for 15 min. (B). The 68 kDa protein was expressed in human MCF7 cells. (C). Expression level of the 68 kDa protein remained constant at different temperatures in CEF cells transformed with ts mutant of Ros, T6 and NM1. (D). Expression level of the 68 kDa protein does not change in untransformed CEF cells compared to cells transformed with T6 and NM1. For all the experiments above, 0.5 mg proteins were used for each immunoprecipitation with different antibodies. NM1 and T6 are the retroviruses containing gag-IR and gag-IGFR transforming proteins respectively. tsRos, tsT6 and tsNM1 is the temperature sensitive mutants of wt UR2, T6 and NM1 respectively. The arrows indicate the position of the 68 kDa protein.

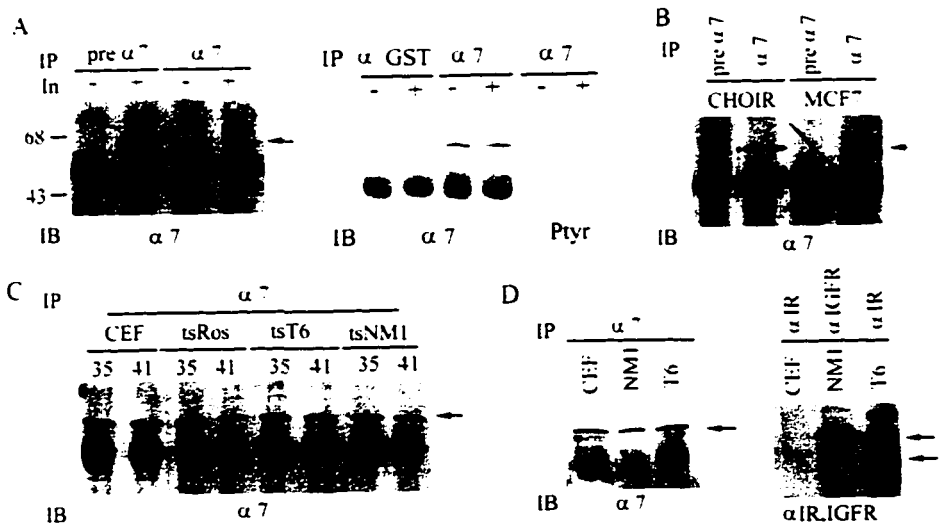


Figure 4-10. Nucleotide and amino acid sequences derived from cDNA insert of pJG4-5-12 and its comparison with MAD2.

```
Partial protein sequence of pJG4-5-12; Length:206
Partial nucleotide sequence of pJG4-5-12;  Length: 1315

gccatggcgctgcagctctcccgggagcagggaatcaccctgcgcgggagcgccgaaatcgtggccgagttc
A  M  A  L  Q  L  S  R  E  Q  G  I  T  L  R  G  S  A  E  I  V  A  E  F
ttctcattcggcatcaacagcattttatatcagcgtggcatatatccatctgaaacctttactcgagtgcag
F  S  F  G  I  N  S  I  L  Y  Q  R  G  I  Y  P  S  E  T  F  T  R  V  Q
aaatacggactcaccttgcttgtaactactgatcttgagctcataaaatacctaaataatgtggtggaacaa
K  Y  G  L  T  L  L  V  T  T  D  L  E  L  I  K  Y  L  N  N  V  V  E  Q
ctgaaagattggttattcaagtgttcagttcagaaactggttgtagttatctcaaatattgaaagtggtgag
L  K  D  W  L  F  K  C  S  V  Q  K  L  V  V  V  I  S  N  I  E  S  G  E
gtcctggaaagatggcagtttgatattgagtgtgacaagactgcaaaagatgacagtgcacccagagaaaag
V  L  E  R  W  Q  F  D  I  E  C  D  K  T  A  K  D  D  S  A  P  R  E  K
tctcagaaagctatccaggatgaaatccgttcagtgatcagacagatcacagctacggtgacatttctgcca
S  Q  K  A  I  Q  D  E  I  R  S  V  I  R  Q  I  T  A  T  V  T  F  L  P
ctgttggaagtttcttgttcatttgatctgctgatttatacagacaaagatttggttgtacctgaaaaatgg
L  L  E  V  S  C  S  F  D  L  L  I  Y  T  D  K  D  L  V  V  P  E  K  W
gaagagtcgggaccacagtttattaccaattctgaggaagtccgccttcgttcatttactactacaatccac
E  E  S  G  P  Q  F  I  T  N  S  E  E  V  R  L  R  S  F  T  T  T  I  H
aaagtaaatagcatggtggcctacaaaattcctgtcaatgactgaggatgacatgaggaaaataatgtaatt
K  V  N  S  M  V  A  Y  K  I  P  V  N  D  *
gtaattttgaaatgtggttttcctgaaatcaggtcatctatagttgatatgttttatttcattggttaattt

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ttgacttaccatggagttaacatcatgaatttattgcacattgttcaaaaggaaccaggaggttttttgtc

aacattgtgatgtatattccttgaagatagtaactgtagatggaaaaacttgtgctataaagctagatgctt

tcctaaatcagatgttttggtcaagtagtttgactcagtataggtaggagatatttaagtataaatacaaca

aaggaagtctaaatattcagaatctttgttaaggtcctgaaagtaactcataatctataaacaatgaaatat

tgcgtatagttcctttgaccttcatttcatgtatagttttccctattgaatcagtttccaattatttgact

ttaatttatgtaacttgaacctatgaagcaatgatatttgtactgtttaatgttctgtgatacagaactctt

aaaaatgtttttcatgtgttttataaaatcaagttttaagtgaaagtgaggaaataaagttaagtttgtttt
```

```
ID   MAD2_YEAST(MITOTIC MAD2 PROTEIN)   AC   P40958;
SCORES   42.8% identity in 194 aa overlap

pJG4-5-12        AMALQLSREQGITLRGSAEIVAEFFSFGINSILYQRGIYPSETFTRVQKYGLTL
                       |:|:|:||: :|:|||:::|||||||||:||:|:|: |:|||:|||
Mad2_Y                 MSQSISLKGSTRTVTEFFEYSINSILYQRGVYPAEDFVTVKKYDLTL
pJG4-5-12   LVTTDLELIKYLNNVVEQLKDWLFKCSVQKLVVVISNIESGEVLERWQFDIECDKTAKDD
            | | | || :|:::::  |:: ||:  : ::||: | : ::|||:||| |::: : :::::
Mad2_Y      LKTHDDELKDYIRKILLQVHRWLLGGKCNQLVLCIVDKDEGEVVERWSFNVQ-HISGNSN
pJG4-5-12   SAPREKSQKAIQDEIRSVIRQITATVTFLPLL--EVSCSFDLLIYTDKDLVVPEKWEESG
            ::    : :::|::||::||||::|||||  |  | :::|::| ||| |  || :|::|:
Mad2_Y      GQDDVVDLNTTQSQIRALIRQITSSVTFLPELTKEGGYTFTVLAYTDADAKVPLEWADSN
pJG4-5-12   PQFITNSEEVRLRSFTTTIHKVNSMVAYKIPVND
            :: |:::| |::::|:|: |||:: |:||
Mad2_Y      SKEIPDGEVVQFKTFSTNDHKVGAQVSYKY
```

Figure 4-11. Alignment of partial sequence of IRA3 with those of mouse Stat5a/5b and human Stat5a.

Amino acid differences among IRA3, mStat5a/5b and hStat5a are indicated by an asterisk (*) above each residue. The amino acid sequence position for each protein are indicated at the edge of each alignment. The highly conserved motif (GTFLLRFSD) and lysine residue in the SH2 domain, as well as the potential tyrosine phosphorylation site and its neighboring amino acids are marked by box A, italic letter and box B respectively.

```
IRA3       1                          DNNATATVLWDNAFAEPGRVPFAVPD  26
hStat5a  451  GSNELVFQVKTLSLPVVVIVHGSQDHNATATVLWDNAFAEPGRVPFAVPD 500
mStat5a  451  GSNELVFQVKTLSLPVVVIVHGSQDHNATATVLWDNAFAEPGRVPFAVPD 500
mStat5b  451  GGNELVFQVKTLSLPVVVIVHGSQDNNATATVLWDNAFAEPGRVPFAVPD 500

IRA3      27  KVLWPQLCEALNMKFKAEVQSNRGLTKENLVFLAQKLFNNSSSHLEDYSG  76
hStat5a  501  KVLWPQLCEALNMKFKAEVQSNRGLTKENLVFLAQKLFNNSSSHLEDYSG 550
mStat5a  501  KVLWPQLCEALNMKFKAEVQSNRGLTKENLVFLAQKLFNISSNHLEDYNS 550
mStat5b  501  KVLWPQLCEALNMKFKAEVQSNRGLTKENLVFLAQKLFNISSNHLEDYNS 550
                               |------------ SH3 -------------

IRA3      77  LSVSWSQFNRENLPGRNYTFWQWFDGVMEVLKKHLKPHWNDGAILGFVNK 126
hStat5a  551  LSVSWSQFNRENLPGWNYTFWQWFDGVMEVLKKHHKPHWNDGAILGFVNK 600
mStat5a  551  MSVSWSQFNRENLPGWNYTFWQWFDGVMEVLKKHHKPHWNDGAILGFVNK 600
mStat5b  551  MSVSWSQFNRENLPGRNYTFWQWFDGVMEVLKKHLKPHWNDGAILGFVNK 600
              ------------ SH3 ------------|          |--- SH2 ---

                            A
IRA3     127  QQAHDLLINKPDGTFLLRFSDSEIGGITIAWKFDSQERMFWNLMPFTTRD 176
hStat5a  601  QQAHDLLINKPDGTFLLRFSDSEIGGITIAWKFDSPERNLWNLKPFTTRD 650
mStat5a  601  QQAHDLLINKPDGTFLLRFSDSEIGGITIAWKFDSPDRNLWNLKPFTTRD 650
mStat5b  601  QQAHDLLINKPDGTFLLRFSDSEIGGITIAWKFDSQERMFWNLMPFTTRD 650
              ----------------------- SH2 -----------------------

                                                          B
IRA3     177  FSIRSLADRLGDLNYLIYVFPDRGKDEVYSKYYTPVPCESATAKAVDGYV 226
hStat5a  651  FSIRSLADRLGDLSYLIYVFPDRPKDEVFSKYYTPV.....LAKAVDGYV 695
mStat5a  651  FSIRSLADRLGDLNYLIYVFPDRPKDEVFAKYYTPV.....LAKAVDGYV 695
mStat5b  651  FSIRSLADRLGDLNYLIYVFPDRPKDEVYSKYYTPVPCEPATAKAADGYV 700
              -------- SH2 --------|

IRA3     227  KPQIKQVVPEFVNASADAGGGSATYMDQAPSPAVCPQAHYNMYPQNPDSV 271
hStat5a  696  KPQIKQVVPEFVNASADAGGSSATYMDQAPSPAVCPQAPYNMYPQNPDHV 745
mStat5a  696  KPQIKQVVPEFVNASTDAGAS.ATYMDQAPSPVVCPQPHYNMYPPNPDPV 744
mStat5b  701  KPQIKQVVPEFANASTDAGSG.ATYMDQAPSPVVCPQAHYNMYPPNPDSV 749

IRA3     272  LDTDGDFDLEDTMDVARRVEELLGRPMDS..QWIPHAQS            308
hStat5a  746  LDQDGEFDLDETMDVARHVEELLRRPMDSLDSRLSPPAGLFTSARGSLS  794
mStat5a  745  LDQDGEFDLDESMDVARHVEELLRRPMDSLDARLSPPAGLFTSARSSLS  793
mStat5b  750  LDTDGDFDLEDTMDVARRVEELLGRPMDS..QWIPHAQS            786
```

Figure 4-12. Anti-His-IRA3 polyclonal antibody recognizes its immunogen.

(A). Anti-His-IRA3 serum recognizes His-IRA3 in direct Western blotting. Different amounts of His-IRA3 protein were loaded onto SDS-PAGE gel. αIRA3 was used for direct Western blotting (1:500 dilution). (B). αHis-IRA3 immunoprecipitates its immunogen. 1 μg His-IRA3 was used for immunoprecipitation by different antibodies and immunoblotted with αIRA3 (left panel). 1 μg GST7 or GST12 was used for immunoprecipitation with different antibodies and immunoblotted with αGST (right panel).

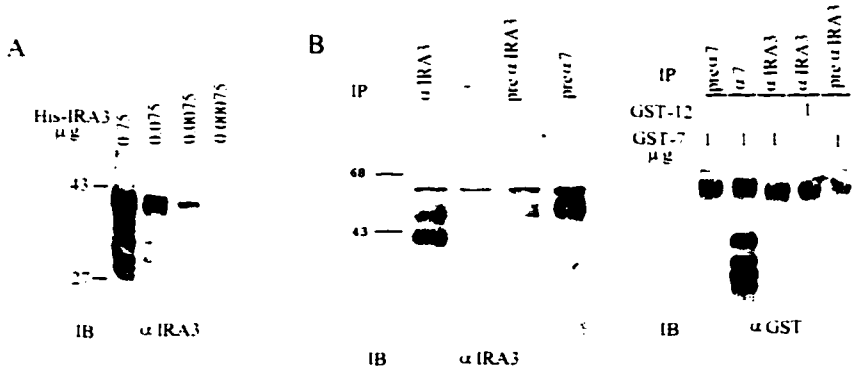


Figure 4-13. *In vitro* transcription and translation product of the potential interacting proteins.

The linear fragments derived from pRcCMV-7, pRcCMV-12, pRcCMV-IRA3 were purified and subjected to *in vitro* transcription and translation. 2 μl product was loaded onto SDS-PAGE gel. Signals were detected by autoradiography. C(-): *in vitro* translation without adding template; C(+): Luciferase RNA template control whose presumed *in vitro* translation product is 61 kDa. Arrows indicate the positions of *in vitro* translation products.

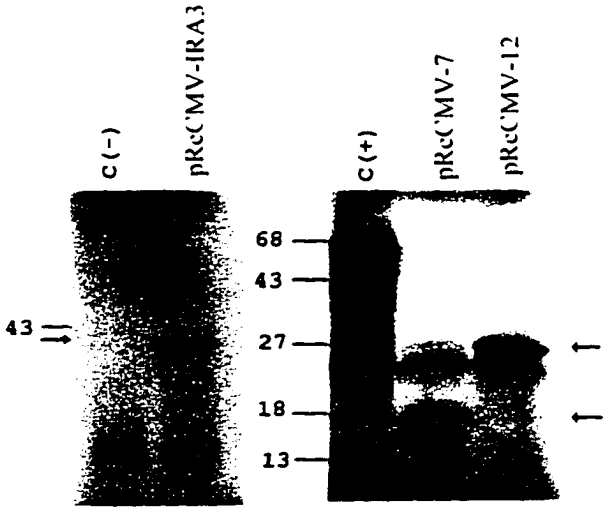


Table 4-1. Summary of screening results from kinase inactive and kinase active IR fusion proteins.

Bait protein	LexA-IR-S	LexA-IR-L
Transformation Efficiency (Colonies/μg)	$3.955X10^4$	$1.68X10^5$
Total pJG4-5-cDNAs Transformed (μg)	10	10
Total # of Colonies Screened	$9.0X10^5$	$1.5X10^7$
Total # of Colonies Selected in H-U-T-L-Gal+ plates	879	600
# of Positive Clones Sequenced	22	37
# of recomfirmed Strong Positives	3	9

Positives Screened from LexA-IR-S

Clone7: 97.7% identity in 384 bp overlap at 3' noncoding region to human 78 kDa GRP (glucose regulated protein).

Clone12: the partial cDNA sequence encodes 206 a.a. which is homologous to MAD2 protein (42.8% identity in 194 a.a. overlap).

Clone17: has ~ 2 kb cDNA insert, doesnot has any identity or homology to any sequence in the genebank.

Positives Screened from LexA-IR-L

Clone3: homologous to MGF(mammary gland factor)

Clone5: homologous to unknown homo sapiens transmembrane protein (92.1% identity in 127 bp overlap).

Clone6, 9, 11, 12: no identity or homology to any protein in the genebank.

Clone13: homologous to cytochrome C oxidase subunit Vb (CoxVb) (98% identity in 102 bp overlap).

Clone14: homologous to human enigma gene (89.3% identity in 75 bp overlap).

Clone20: homologous to homo sapiens partial cDNA sequence, clone c-Ooholl (95.7% identity in 139 bp overlap)

Chapter 5 Stat5 (MGF) is a potential physiological substrate of IR

Abstract

Using the cytoplasmic domain of insulin receptor(IR) as a bait in a yeast two hybrid system screening, we identified a cDNA clone encoding a polypeptide named IRA3 sharing over 95% homology with human and mouse Stat5 proteins. IRA3 is tyrosine phosphorylated *in vitro* by a purified soluble IR polypeptide with kinase activity, and is tyrosine phosphorylated intracellularly by cells overexpressing IR in response to insulin. Perfusion of mouse liver with insulin resulted in rapid and specific tyrosine phosphorylation as well as activation of DNA binding activity of both Stat5a and Stat5b but not of Stat1 or Stat3. Fasting and refeeding experiment demonstrated that Stat5 is tyrosine phosphorylated and activated in liver of refed mice. Taken together we have shown that IR is able to directly interact and phosphorylate Stat5 and that Stat5 could be a physiological downstream signaling molecule of insulin receptor.

Introduction

Insulin and insulin receptor (IR) are very important in regulation of cellular metabolism as well as cell growth and differentiation (Draznin et al., 1989). Their pleiotropic and complex biological functions have long been the focus of study in understanding the action of insulin.

Extensive progresses have been made in recent years in understanding the IR mediated signaling pathways. Binding of insulin to IR leads to crossphosphorylation of β subunits and activation of their protein tyrosine kinase (PTK) activity followed by phosphorylation of downstream signaling molecules (Cantley et al., 1991; Cheatham and Kahn., 1995). Unlike other PTKs, such as PDGF and EGF receptors, insulin receptor does not associate with multiple signaling components. Instead, the major substrate of IR called insulin receptor substrate 1 (IRS1) (Meyers et al., 1994; Sun et al., 1991; White., 1994) appears to serve as the main docking protein for recruiting other signaling proteins including PI 3-kinase (Backer et al 1992), Syp, a tyrosine phosphatase (Kuhue et al., 1993) and SH2/SH3-containing adaptor molecules Grb2 (Skolink et al., 1993) and Nck (Lee et al., 1993). In addition to IRS1, Shc, another SH2/SH3-containing adaptor, has also been shown to directly interact with IR (Craparo et al., 1995; Gustafson et al., 1995). Binding of IRS1 and Shc to Grb2 subsequently recruits effector molecule such as Sos, a Ras nucleotide exchange factor, to the plasma membrane, resulting in activation of Ras and the Ras/Raf/MAP kinase pathway (Nishida and Gotoh., 1993). The above studies have provided a significant insight for the mechanism of the action of insulin.

In the past few years, a distinct signaling pathway was discovered mainly from the

study of interferon and cytokine mediated signaling. This pathway distincts itself from the RPTK mediated cascade pathways in that it links receptor activation on the cell surface directly to gene transcription in the nucleus. The major players in this signaling process are a family of transcriptional factors called Stats (Signal transducer and activator of transcription) and a family of cytoplasmic protein tyrosine kinases known as Janus family kinases or JAKs (Schindler and Darnell, 1995). At present, there are at least 4 members of JAK kinases (JAK1. 2. 3 and Tyk2) and 6 members of Stats (Stat1, 2, 3, 4, 5 and 6) that have been identified (Schindler and Darnell, 1995). In addition to cytokines and their receptors, a number of receptor and non-receptor PTKs have been shown to cause phosphorylation and activation of various JAK and Stat proteins (Schindler and Darnell, 1995). Stat1 and Stat3 are tyrosine phosphorylated in response to EGF, PDGF and CSF-1 stimulation (Quelle et al., 1995; Ruff-Jamison et al., 1993b, 1994, 1995; Silvennoinen et al., 1993; Schindler et al., 1995). Stat3 is tyrosine phosphorylated and activated in v-Src transformed NIH3T3 cells (Cao et al., 1996; Yu et al., 199). JAK1, JAK3, Stat5 and Stat6 were shown to be activated in v-Abl transformed pre-B cells (Danial et al., 1995).

We have been interested in exploring the novel substrates of insulin receptor. Using the yeast two hybrid system, we have identified that Stat5 is capable of interacting with insulin receptor *in vitro* and intracellularly. Stat5 is tyrosine phosphorylated in response to insulin in cells overexpressing IR and is specifically activated in mouse liver perfused with insulin. Our result strongly suggest that Stat5 is a physiological substrate and downstream signaling molecule of insulin receptor.

Results and discussions

Using the kinase active LexA-IR-L as bait protein, we have identified a protein homologous to mouse Stat5a/5b and human Stat5a (Figure 4-8). We named this protein IRA3 for insulin receptor-associated protein clone 3. The β galactosidase activity induced by IRA3 is specific to the kinase active LexA-IR-L bait protein, since no blue colony was detected when LexA-IR-S (kinase inactive) was used as a bait protein (data not shown).

The cDNA insert from pJG4-5-IRA3 is 1.2 kb in length and encodes 308 amino acids. It represents the C-terminal 308 amino acids of recently cloned human Stat5b (Lin et al., 1996). Within this sequence, there is 95% identity between the IRA3 protein and mouse Stat5b. Motif sequence searching in the gene bank revealed that it has both SH2 and SH3 like domains (Figure 4-11). The SH2 motif GTFLLRFSXS and a highly conserved lysine residue, which is believed to be directly involved in the interaction with phosphotyrosine (Waksman et al., 1992, 1993), are found in the SH2 domain of IRA3. The putative tyrosine phosphorylation site, shown to be important for Stat protein dimerization (Shuai et al., 1994), and its neighboring amino acids in IRA3 are conserved among Stat proteins including Stat1 and Stat2 (Shuai et al., 1993).

In order to confirm the interaction between IR and IRA3 detected by the yeast two hybrid system, an *in vitro* assay of phosphorylation was set up. The purified His-IRA3 was subjected to *in vitro* phosphorylation by the catalytic kinase domain (CKD) derived from IR (CKD was purified from baculovirus and was a gift from Dr. R.A. Kohanski). As expected, CKD was able to autophosphorylate (Figure 5-1A) and it also phosphorylated His-IRA3 (Figure 5-1A, lane 7). Phosphorylation of IRA3 depended on the addition of both ATP and CKD. Since CKD and His-IRA3 migrated at similar positions in SDS gels, to rule out the

possibility that the IRA3 band seen in lane 7 of Figure 5-1A actually represented CKD brought down via association with IRA3 and Ni-NTA-resin, an additional experiment was performed, the result is shown in Figure 5-1B. In this experiment, IRA3 and Ni-NTA-resin were incubated with pre-autophosphorylated CKD. Figure 5-1B showed that no autophosphorylated CKD could be captured by Ni-NTA-resin and His-IRA3. We conclude that the band in lane7 Figure 5-1A in deed represents the IRA3 phosphorylated by CKD. Figure 5-2 showed that our polyclonal antibody αIRA3 recognized HA-IRA3 expressed intracellularly in COS cells. Figure 5-3 showed that oncogenic gag-IGFR proteins could also phosphorylate His-IRA3 *in vitro*.

Next the possible intracellular interaction between IR and IRA3 was tested. Expression plasmids pRcCMV-IRA3 and pECE-IR were co-transfected into COS1 cells. Both IR and HA-tagged IRA3 were expressed in COS cells (Figure 5-4). To demonstrate IRA3 phosphorylation in response to insulin is not through activated endogenous IGF1 receptor by insulin stimulation, the cells were stimulated with either insulin or IGF1 for 15 minutes. Figure 5-4 showed that IRA3 was tyrosine phosphorylated only in response to insulin but not IGF1. This co-transfection experiment, together with the data of *in vitro* phosphorylation and two hybrid interaction, indicate that insulin receptor can cause tyrosine phosphorylation of IRA3 through direct but transient interaction.

Sequence of IRA3 indicates that it represents the C-terminal 308 a.a. of human Stat5b (Lin et al., 1996; Silver et al., 1996). As expected, anti-Stat5 brought down a 92 kD protein recognized by anti-IRA3 in Western blotting (Figure5-5A). Conversely, anti-IRA3 brought down a 92 kD protein co-migrating with that recognized by the anti-Stat5 Ab. And the 92

kD protein could be competed away by exogenously added His-IRA3 (Figure5-5A). This provides evidence that IRA3 shares common epitope with human Stat5b.

So far the data strongly suggest that Stat5/IRA3 is a substrate of insulin receptor. To further test this possibility, tyrosine phosphorylation of the endogenous Stat5 in response to insulin stimulation in a CHO cell line overexpressing the native IR was examined. Figure 5-3 showed that insulin could stimulate tyrosine phosphorylation of Stat5 protein *in vivo* (Figure 5-5B). However, co-immunoprecipitation did not detect stable physical association between IR and Stat5 (data not shown). Besides Stat5, Stat3 was also tyrosine phosphorylated in response to insulin. There appeared to be a high basal level of Stat1 phosphorylation.

The physiological target tissues of insulin include liver, muscle and fat. Interestingly, repeated liver perfusion experiments with insulin revealed increased tyrosine phosphorylation of Stat5 in response to insulin (Figure 5-6). This response is specific to Stat5 and not to Stat1 or Stat3. The basel level of Stat5 tyrosine phosphorylation in control liver could be due to basel level of insulin or other stimulating factors existed in the bloodstream of experimental mice. This increase in Stat5 phosphorylation is accompanied by increased tyrosine phosphorylation of insulin receptor, but not IGF1 receptor (Figure 5-6). It has been shown that members of JAK family (JAK1, JAK2, JAK3 and TYK2) are responsible for phosphorylation of Stat proteins in response to cytokine stimulation. Therefore the activation of JAK kinases in perfused livers was tested. No increased tyrosine phosphorylation of the JAK kinases in insulin stimulated liver was detected (data not shown). Therefore, it is likely that insulin receptor is capable of phosphorylating Stat5

independent of activation JAK's. Cytosolic and nuclear extracts were prepared from the control and stimulated liver cells and tested for their DNA binding activity. Those phosphorylated Stat5 proteins can migrate to the nucleus and bind the Stat5 specific oligonucleotides shown in the gel shifting experiment (Figure 5-6). The DNA-Stat5 complexes could be supershifted with anti-Stat5 specific antibody, but not by anti-Stat3, anti-Stat1 antibody or normal rabbit serum. Moreover, supershift with antibodies specific to Stat5a or Stat5b demonstrated that both Stat5 proteins were present in the complexes. Control experiment shown in Figure 5-6B confirms that the specificity of the antibody supershift of Stat5a and Stat5b (The Gel shift experiment was done by Dr. H. Sadowski).

Finally, starvation-refeeding experiments were done. The result is shown in Figure5-7. Significant increase in the tyrosine phosphorylation of Stat5 in several tissues including muscle, fat and liver after refeeding could be detected. In contrast, no increase in Stat5 tyrosine phosphorylation was detected in brain and only a very slight increase of Stat5 tyrosine phosphorylation was detected in heart (faint signals in the original filter did not reproduce well). The low basel level of tyrosine phosphorylation of Stat5 could be due to low level of stimulating factors after starvation. Although the potential effects due to other hormones or factors that might be released after refeeding could not be ruled out, the simplest interpretation of these results is that secretion of insulin after refeeding is responsible for Stat5 activation in those target tissues. Gel-shift experiment was done to confirm that increased Stat5 DNA binding activity existed in refed mouse liver (Figure 5-7B). (The gel-shift experiment was done by Dr. H. Sadowski).

In summary, these results show that insulin receptor is able to interact directly with

Stat5 and induce its tyrosine phosphorylation and activation of DNA binding activity. Stat5 was initially known as mammary gland factor (MGF), a transcription factor originally found in mammary epithelial cells in the lactating animals (Wakao et al., 1992, 1994). Activated MGF recognizes a consensus sequence (TTCNNNGAA) in the promoter regions of milk protein genes, including β casein, WAP and lactoglobulin, and regulates their transcription during late pregnancy and lactation (Yoshimura et al., 1989). Similar sequence is also found in IFN, IL-6 and other cytokine responsive elements (Kotanides et al 1993; Pine et al., 1994). Stat5 is widely expressed in a variety of tissues and cell lines (Waoko et al., 1994; Liu et al., 1995 and data not shown) and can be activated by various cytokines (Miyakawa et al., 1996; Nagata et al., 1995; Penta et al., 1995; Damen et al., 1995; Gilmour et al., 1995; Caldenhoven et al., 1995; Gouilleux et al., 1995; Johnston et al., 1995; Bacon et al., 1995 Mui et al., 1995; Pallard et al., 1995; Wakao et al., 1995; Lin et al., 1995) and certain RPTKs such as EGF receptor (Ruff-Jamison et al., 1995) and insulin receptor shown in this study. The failure to detect tyrosine phosphorylation of JAKs is consistent with a recent report that none of the known members of JAKs was activated by insulin stimulation in lymphopoietic cells (Welham et al., 1995). Therefore, IR-mediated activation of Stat5 appears to be similar to the activation of Stat1 and Stat3 by PDGF and EGF receptors (Leaman et al., 1996; Vignais et al., 1996) and is distinct from interferon- and cytokine-mediated activation of JAK's and Stat's.

The known substrates of insulin receptor to date include IRS-1, IRS-2 (also called 4PS) and Shc (White et al., 1985; Pelicci et al., 1992; Sun et al., 1991, 1995). The finding of the interaction and activation of Stat5 adds to the list of IR substrates, in this case, a

nuclear effector. This may provide an additional pathway through which the IR signals to the nucleus. This new pathway may lead to differential activation of genes involved in metabolic activity and differentiation of cells.

Future Prospectives

So far Stat5 has been demonstrated to be the substrate of insulin receptor in vitro and in vivo and potentially could play some functional roles in insulin mediated metabolic activities. However, many questions remain unanswered. For examples, what is the downstream target genes of Stat5 in those insulin target tissues? How are those genes be regulated? What is the functional role of Stat5a and Stat5b? How is the activity of Stat5 regulated? Is there any modification other than tyrosine phosphorylation of Stat5 needed for its full activity? In addition, what are the interaction sites of IR and Stat5? Is NPEY motif of IR also involved in the interaction between IR and Stat5?

We will explore these questions using various techniques such as gene knockout mice, deficient cell lines lacking various components of signaling molecules and various constitutively activated (ca) mutants and dominant negative (dn) mutants.

Figure 5-1. *In Vitro* Phosphorylation of IRA3 by CKD.

(A). His-IRA3 can be *in vitro* phosphorylated by CKD. 0.3 μg CKD and 0.75 μg His-IRA3 proteins were incubated in 50 μl kinase buffer. [γ ^{32}P]-ATP (10 μCi) was added to start the reaction. At the end of reaction, 0.5 ml RIPA buffer was added and CKD protein was first immunoprecipitated by anti-IR (Upper panel), the same filter was used to immunoblot with anti-IR antibody to show the corresponding CKD immunoprecipitated by anti-IR (middle panel).. His-IRA3 protein was subsequently captured from the supernatant by Ni-NTA-resin and the phosphorylated His-IRA3 was detected by autoradiography (lower panel). (B). His-IRA3 does not coprecipitate with CKD *in vitro*. 0.75 μg His-IRA3 was incubated with pre-autophosphorylated CKD in RIPA buffer and precipitated with Ni-NTA-resin. The upper panel shows the signals brought down by His-IRA3 and/or Ni-NTA resin. The same filter was immunoblotted with αStat5b-Ct (lower panel) to show the corresponding His-IRA3 protein captured by Ni-NTA-resin. WB: Western blotting; His-Stat5b-Ct=His-IRA3; αStat5b-Ct=αIRA3.

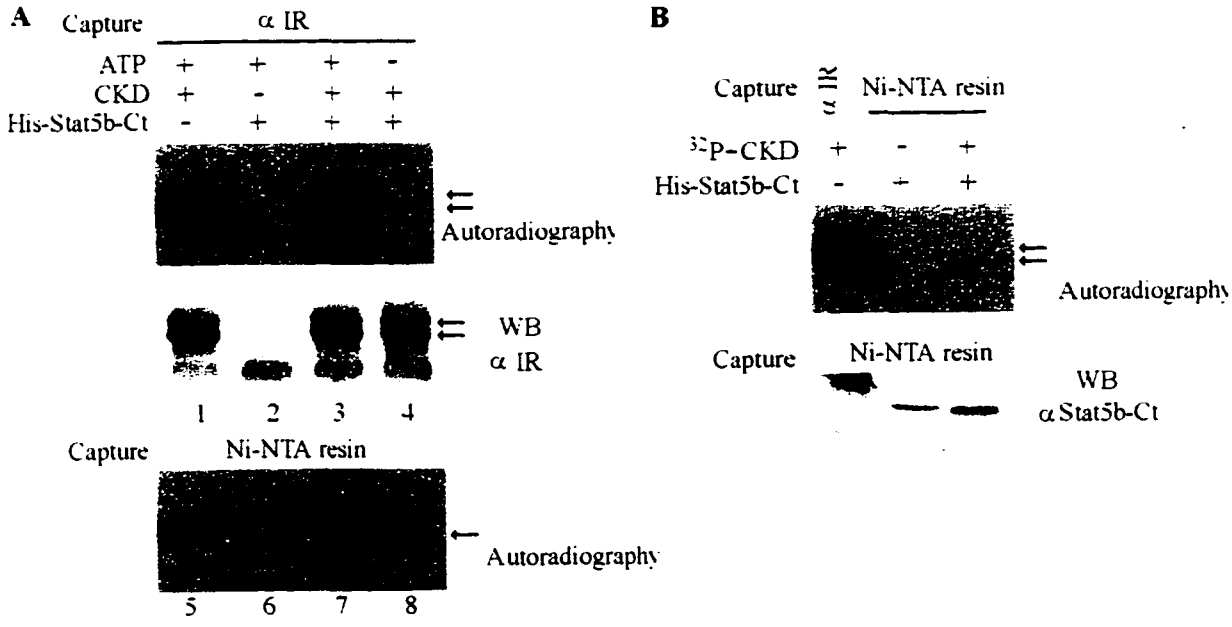


Figure 5-2. Polyclonal anti-IRA3 Ab recognizes IRA3 expressed intracellularly in COS cells.

(A). 12CA5 monoclonal antibody recognizes hemagglutinin (HA) epitope tagged IRA3 (HA-IRA3) expressed in COS cells *in vivo*. 5μg each of pRcCMV, pRcCMV-IRA3 were transfected into COS7 cells. 48 hours later, the COS7 cells were extracted with RIPA buffer and the HA-IRA3 was immunoprecipitated with 12CA5 monoclonal Ab. Western blotting was done with 12CA5-P (12CA5 conjugated with HRP) and the signal was detected with ECL. (B). αIRA3 recognizes HA-IRA3 expressed in COS7 cells. 5μg each of pRcCMV or pRcCMV-IRA3 were transfected into COS7 cells. 48 hours later, cells were extracted and the HA-IRA3 was immunoprecipitated with either 12CA5 or αIRA3 and immunoblotted with 12CA5-P or αIRA3. A monoclonal Ab 12CA5 against the influenza hemagglutinin tag and the house peroxidase (HRP)-coupled 12CA5 (12CA5-P) were purchased from Boehringer Mannheim.

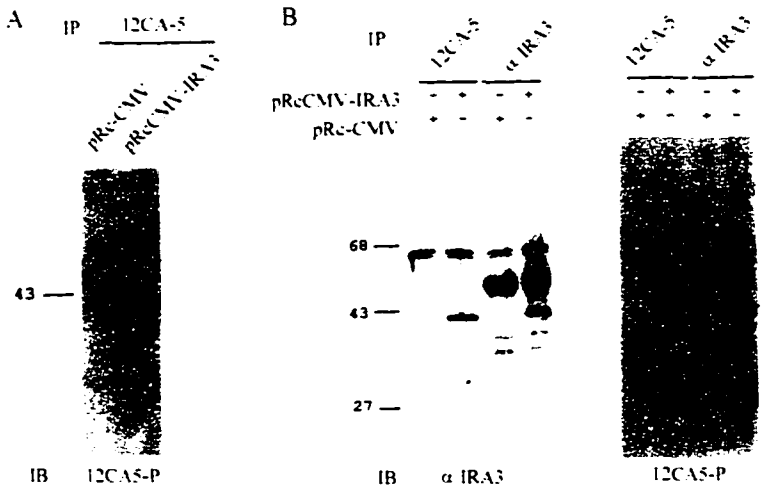


Figure 5-3. His-IRA3 can be tyrosine phosphorylated by gag-IGFR oncogenic protein. Total proteins were extracted from one 10cm dish CEF cells fully transformed with NM1 encoding oncogenic gag-IGFR transforming protein. The gag-IGFR protein was then immunoprecipitated with α IGFR. The immunocomplex was washed 3 times with RIPA buffer, 3 times with kinase buffer and triplicated equally. γ-^{32}PATP was added to the kinase reaction mixtures containing various combination of gag-IGFR and His-IRA3 proteins (Lane1,4: 1μg His-IRA3; Lane2,5: 0.1μg His-IRA3) to start the kinase reaction for 10 minutes at room temperature. After kinase reaction, the gag-IGFR was immunoprecipitated with αIGFR and the autophosphorylated signals were detected by autoradiography (Panel A). The nonabsorbed supernatant containing His-IRA3 was used to immunoprecipitate with αIRA3 and the phosphorylated His-IRA3 was detected by autoradiography (Panel B, upper panel). The same filter was then used to immunoblot with αIRA3 to detect corresponding His-IRA3 protein (Panel B, lower panel) Arrows indicate the positions of gag-IGFR and His-IRA3 proteins.

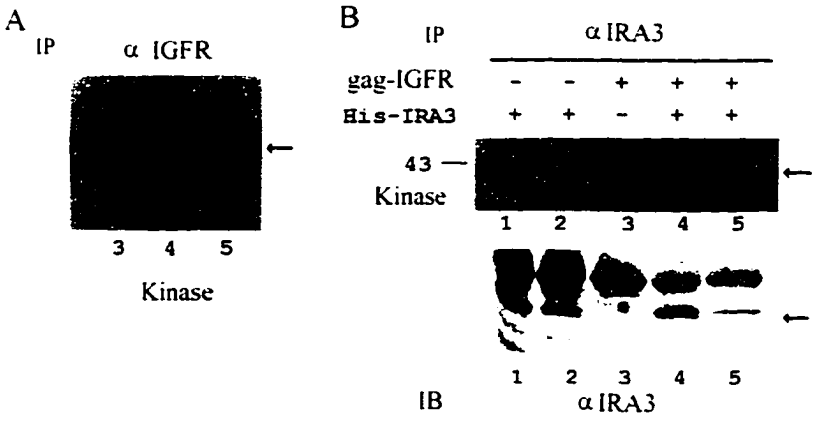


Figure 5-4. Intracellular phosphorylation of HA-IRA3 by the insulin receptor.

5 μg pRcCMV-IRA3 encoding HA-IRA3 and 5 μg pECE-IR encoding full length IR were co-transfected into COS1 cells. 48 hours later, cells were starved for 2 hours in serum free medium followed by stimulation with either insulin (50 nM) or IGF1 (20 ng/ml) for 15 minutes. Cells were extracted in RIPA buffer and the lysates were divided into duplicate aliquots. One aliquot was immunoprecipitated with anti-IR, followed by Western blotting with anti-IR (left panel). The other aliquot was immunoprecipitated with 12CA5 antibody and this immunoprecipitate was further divided into two aliquots for Western blotting with either anti-Ptyr (middle panel) or with HRP conjugated 12CA5 (right panel) respectively. Arrows indicate the positions of IR and HA-IRA3 expressed in COS cells.

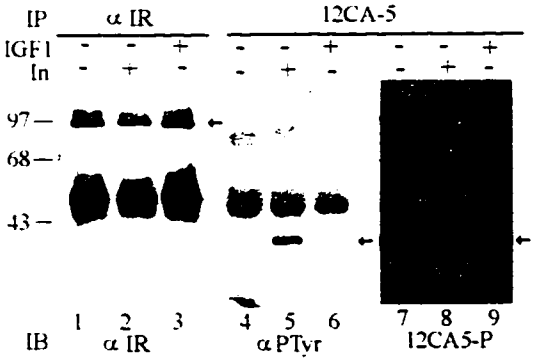


Figure 5-5. Endogenous Stat5 is phosphorylated in CHO-IR cells in response to insulin.

(A). Detection of a 92 kD protein in HeLa cells using anti-IRA3 antibody. HeLa cells were extracted with RIPA buffer and 1 mg protein of each cell lysate was immunoprecipitated with either anti-Stat5 or anti-IRA3 antibody, followed by Western blotting with anti-IRA3. HeLa cells used in lanes 1, 3, 5 and lanes 2, 4, 6 were from two independent sources. -, + indicate without or with addition of 1 μg His-IRA3 during immunoprecipitation. (B). Endogenous Stat5 and Stat3 were tyrosine phosphorylated in response to insulin. CHO cells overexpressing the native IR were starved in serum free medium for 4 hrs in the presence of 200 μM Na_3VO_4 and then stimulated with 50 nM insulin for 15 minutes. 1 mg protein of each cell lysate was immunoprecipitated with anti-Stat1, Stat3 or Stat5 antibody. Each immunoprecipitate was divided into two equal parts for detecting either Stat tyrosine phosphorylation (upper panel) or Stat proteins (lower panel).

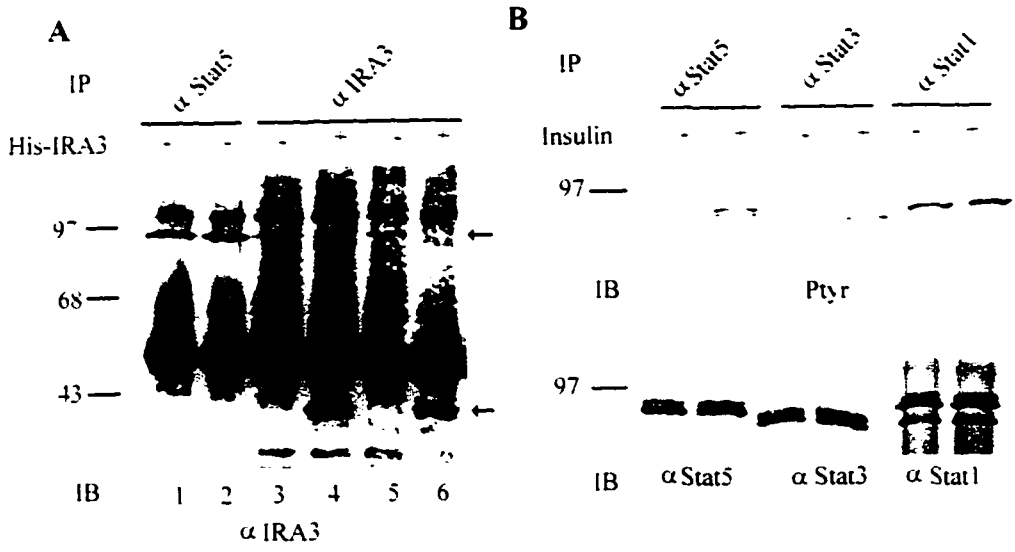


Figure 5-6. Endogenous Stat5 is specifically phosphorylated and activated in liver cells in response to insulin stimulation.

(A). Endogenous Stat5 is tyrosine phosphorylated in mouse liver perfused with insulin. 9 month old DBA2 mice were used for liver perfusion with 10 ml Hanks solution containing 1 mM Na_3VO_4 or 1mM Na_3VO_4 plus 50 nM insulin. 3 mg protein of each liver lysate was used in immunoprecipitation with anti-Stat1, -Stat3, -Stat5, -IR or -IGFR, and then divided into duplicate parts for Western blotting to detect tyrosine phosphorylation (upper lanes) or protein amount (lower lanes). The perfusion experiments were repeated three times with similar results. (B). Insulin stimulate Stat5 DNA binding activity. Nuclear proteins were extracted from the perfused mouse liver and subjected to gel shift assay using a ^{32}P-labeled double-stranded oligonucleotide containing a MGF/Stat5 binding site derived from β–casein promoter (Mui et al., 1995). Arrows indicate the specific insulin-inducible complexes and the asterisks indicate supershifted Stat5-containing complexes. Part B was done by Dr. H. Sadowski.

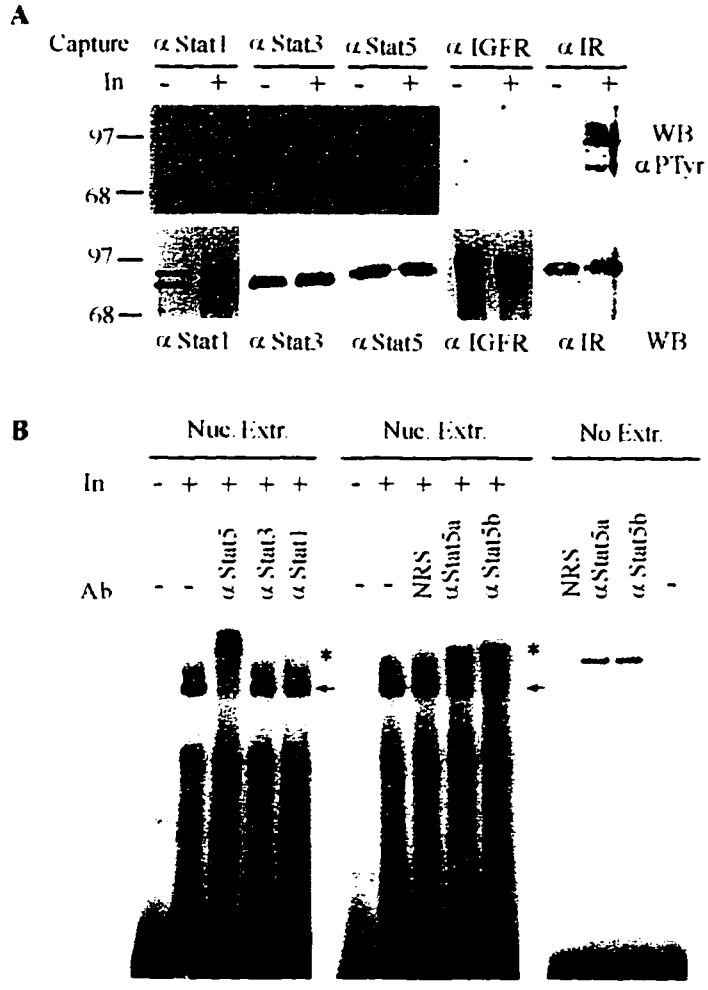
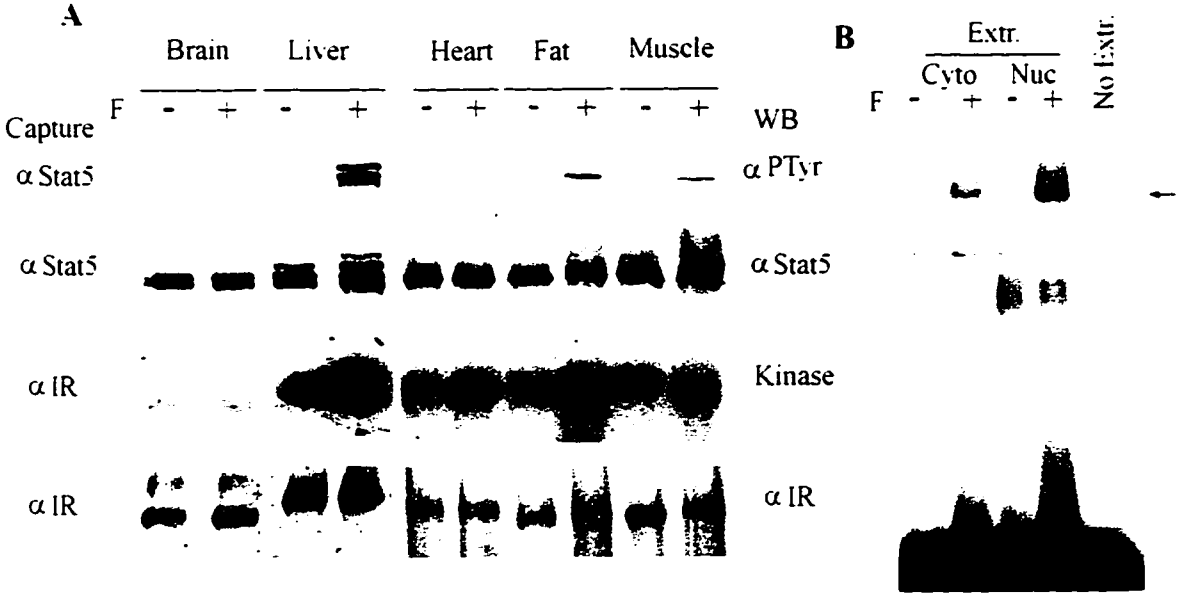


Figure 5-7. Stat5 is tyrosine phosphorylated and activated in insulin target tissues of refed mice. Mice were starved for 3 days, then one of them was allowed to feed for 80 minutes, and both were sacrificed. The whole lysates were prepared from the indicated tissues. 3 mg total proteins from different tissues were used for immunoprecipitation with anti-Stat5 first. A second immunoprecipitation from the nonabsorbed supernatant was done with anti-IR antibody. Immunoprecipitated complexes by anti-Stat5 were divided into two parts. One part was used for anti-Ptyr to detect tyrosine phosphorylation (Panel A, top panel), the other part was used for Stat5 protein detection with anti-Stat5 (Panel A, 2nd panel). The immunocomplexes brought down by anti-IR Ab was used for *in vitro* kinase assay (Panel A, 3rd panel). The same filter was then Western blotted with anti-IR antibody to detect amount of IR (Panel A, lower panel). Starvation and refeeding experiments were repeated several times with similar results. (B). Activation of Stat5 DNA binding activity in refed mouse. The arrow indicates the refeeding-induced complex. Part B was done by Dr. Sadowski.



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