1-1-2012

Activation Of Insulin Receptor Signaling Pathway Using Ligand Microbeads In Mammalian Cells And Isolation Of Receptor-Bead Complex

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ACTIVATION OF INSULIN RECEPTOR SIGNALING PATHWAY USING LIGAND MICROBEADS IN MAMMALIAN CELLS AND ISOLATION OF RECEPTOR-BEAD COMPLEX

by

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B. Tech, Heritage Institute of Technology, India, 2006

A thesis

presented to Ryerson University

in partial fulfillment of the

requirements for the degree of

Master of Science

in the Program of

Molecular Science

Toronto, Ontario, Canada, 2012

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Activation of Insulin Receptor Signaling Pathway using Ligand Microbeads in Mammalian Cells and Isolation of Receptor-Bead complex

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ABSTRACT

The study of cell surface receptors and their associated signaling pathways on the plasma membrane are vital in understanding cellular responses. The insulin receptor is a tyrosine kinase that is activated in response to insulin microbeads presented to three mammalian cell lines: CHO, NIH-3T3 and COS-7. Phosphatidylinositol 3-kinase (PI3K) signaling, a major insulin receptor signaling pathway, phosphorylates the 3-position hydroxyl group of the inositol ring of phosphatidylinositol-4,5-biphosphate, resulting in phosphatidylinositol-3,4,5-triphosphate (PIP3) that acts by recruiting specific pleckstrin homology (PH) domain containing proteins to cell membranes**.** Akt (protein kinase B) containing PH domain gets activated, binds to PIP3 and localizes to the site of receptor activation. The PH domain of Akt was fused to green fluorescent protein (GFP) to create a biosensor for phosphatidylinositol. Confocal microscopy confirmed Akt-PH recruitment to the cell membrane. Immunofluorescence staining (IF) and western blots confirmed insulin receptor and phosphotyrosine activity in cells. The activated insulin receptor complex was captured and isolated by insulin coated microbeads on the surface of cells.

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor, Dr. John Marshall, for his guidance, support and motivation all throughout my research period. I have learnt a lot from him. He has a lot of amazing experience to share and new concepts to talk about. I was fortunate enough to take every opportunity to learn from him.

I would also like to thank my co-workers, fellow graduate students and Marshall lab members, especially, Angelique Florentinus, Monika Tucholska and Jeff Howard. Thank you for all your help and assistance in the lab. All the time we spent together will be forever cherished.

I would sincerely like to thank the members of my Supervisory committee, Dr. Roberto Botelho and Dr. Jeffrey Fillingham for their helpful insights, comments and guidance.

Last but not least, I would like to thank my loving husband Mezbauddin Mahtab, my parents and family for their constant support, prayers and encouragement especially during times when I needed them the most. They have played a huge role in helping me obtain my achievements.

Once again, thank you all.

TABLE OF CONTENTS

INDEX OF FIGURES

INDEX OF APPENDICES

LIST OF SELECTED ABBREVIATIONS

AGC	cAMP-dependent, cGMP-dependent and protein kinase C
CR	Cysteine-rich domain
CT	Carboxy-terminal tail
DFP	Diisopropylfluorophosphate
DTT	Dithiothreitol
EGFP	Enhanced Green Fluorescent Protein
ECL	Enhanced chemiluminescence
Fn0, Fn1, Fn2	Fibronectin type III domains
GFP	Green Fluorescent Protein
GLUT4	Glucose transporter 4
IDDM	Insulin Dependent Diabetes Mellitus
IF	Immunofluorescence
Ins	Insert in Fn1 domain
IR	Insulin receptor
IRS-1	Insulin Receptor Substrate-1
JM	Juxtamembrane domain
L1	large domain 1 (leucine-rich repeats)
L2	large domain 2 (leucine-rich repeats)
LAC	Ligand Affinity Chromatography
LB	Luria Bertani
NIDDM	Non-Insulin Dependent Diabetes Mellitus
PBS	Phosphate Buffered saline

PBST	Phosphate Buffered saline Triton/Tween
PDK1	3-Phosphoinositide dependent Kinase 1
PH	Pleckstrin Homology
PI3K	Phosphatidylinositol 3-Kinase
PIP2	Phosphatidylinositol-4,5-biphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKB	Protein Kinase B
PtdIns	Phosphatidylinositol
PVDF	Polyvinylidene difluoride
RTK	Receptor Tyrosine Kinase
SDS-PAGE	Sodium Dodecyl Sulphate- Poly Acrylamide Gel Electrophoresis
SH2	Src Homology Domain 2
SH3	Src Homology Domain 3
TCA	Trichloroacetic acid
TK	Tyrosine-kinase domain
TM	Transmembrane domain
TE	Tris-EDTA

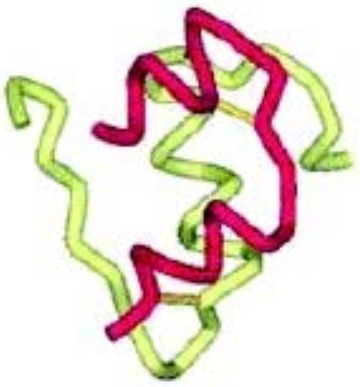
INTRODUCTION

Insulin

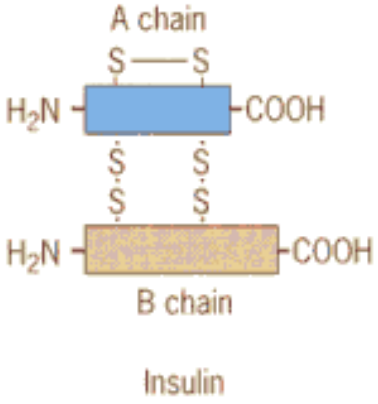
Insulin is an important hormone that is anabolic in nature and has extensive effects on growth and metabolism. It is vital for the proper development of tissues including growth and maintenance of glucose homeostasis in the body (Virkamäki et al., 1999). Insulin is secreted by the β cells of the pancreatic islets of Langerhans in response to increased levels of nutrients, such as glucose, in the blood (Pessin & Saltiel, 2000). It has the ability to stimulate glucose uptake in the liver, muscle, and fat tissue cells, which is then stored as glycogen and thereby used as their source of energy (Sheikh-Ali & Chehade, 2009; Cushman & Wardzala, 1980; Suzuki & Kono, 1980). Insulin also plays an important role in lipid metabolism as it increases lipid synthesis in liver and fat cells and attenuates the release of fatty acids from triglycerides in fat and muscle (Pessin & Saltiel, 2000).

Structure

Insulin (Figure 1) is a 5.8 kDa molecule that consists of two short peptide chains linked by two disulfide bridges (Sanger, 1959). The A chain is 21 amino acids in length that contains an internal disulfide bridge and the B chain is 30 residues long (Humbel et al., 1972; De Meyts, 2004).



a)



b)

Figure 1: Insulin structure. a) Structure obtained by X-ray crystallography and NMR spectroscopy (Baker, 1988). Red: A chain, Green: B-chain, b) Chemical model of Insulin (Snustad et al., 1997).

History

Insulin was first discovered by Banting, F. G. and Best, C. H. in 1922 (Litwack, 2009; Steiner et al., 1985) and one of the first proteins to be crystallized in pure form in 1926 (Murnaghan, 1967). The crystalline form allowed researchers to study its structure with a technique known as X-ray crystallography that helped in approximating its three-dimensional shape (Smith et al., 1984; Baker et al., 1988; Smith & Dodson, 1992; Smith & Ciszak, 1994; Whittingham et al., 1995). Insulin was the first protein fully sequenced using Edman sequencing (Sanger, 1959). It has become a significant model protein for structural studies mainly because of its small size, ease of availability and wide medical importance (Steiner et al., 1985).

Diseases

Insulin is used medically to treat some forms of diabetes mellitus (Galloway & Bressler, 1978). Type 1-diabetes, also known as insulin dependent diabetes mellitus (IDDM), occurs due to autoimmune destruction of the pancreatic beta cells causing the loss of insulin production. Type-1 diabetes is usually diagnosed in childhood or adolescence and requires lifelong insulin treatment (Torpy & Golub, 2011). Type-2 diabetes, also known as non-insulin dependent diabetes mellitus (NIDDM), is a common type of diabetes (Mbanya & Ramiaya, 2006) that occurs due to insulin resistance, abnormal insulin secretion and defect in signal transduction (Pessin & Saltiel, 2000). Patients with Type-2 diabetes mellitus are insulin resistant and suffer from insulin deficiency. Insulin may be required by Type-2 diabetic patients in order to control and improve blood glucose levels in the body (Torpy & Golub, 2011).

Insulin Receptor

The insulin receptor belongs to the family of receptor tyrosine kinases (Ullrich et al., 1985; Yarden & Ullrich, 1988). Studies have shown that the receptor tyrosine kinase plays a critical role in transmission of insulin signal (Chou et al., 1987; Ebina et al., 1987). Receptor tyrosine kinases (RTKs) are also major targets for anticancer therapy (De Meyts et al., 2007). Insulin receptor is thus a transmembrane protein with tyrosine kinase activity. Tyrosine kinase phosphorylates a tyrosine residue in a protein. The insulin receptor is found to be present in almost all vertebrate tissues. There may be as few as 40 receptors on circulating erythrocytes to more than 200,000 receptors on adipocytes and hepatocytes (White & Kahn, 1994). Insulin

receptors are extremely important and fundamental players in the regulation of cell differentiation, growth, and metabolism. They have a number of distinctive biochemical and physiological properties that differentiates them from other members of this large well-studied receptor family. The major physiological role of the receptor is the regulation of metabolism. On the other hand, all other receptor tyrosine kinases are mostly engaged in regulating cell growth and differentiation (Lee & Pilch, 1994). Insulin receptor has two basic functions. It recognizes the hormone among the various substances present in the blood and binds to it with high affinity and a high degree of specificity. Secondly, it creates a signal that is sent across the membrane which effects intracellular metabolism and mediates the action of the hormone (Kahn & White, 1988).

Structure

Insulin receptor (Figure 2) is a hetero- tetramer of two α and two β subunits (Pessin & Saltiel, 2000). The α subunit is extracellular and contains the major insulin binding region of the receptor (Ullrich et al., 1985; Ebina et al., 1985). The β subunit is a transmembrane protein that contains a tyrosine specific protein kinase (Kasuga et al., 1982) and is autophosphorylated (Ullrich et al., 1985; Ebina et al., 1985; Hedo & Simpson, 1984).

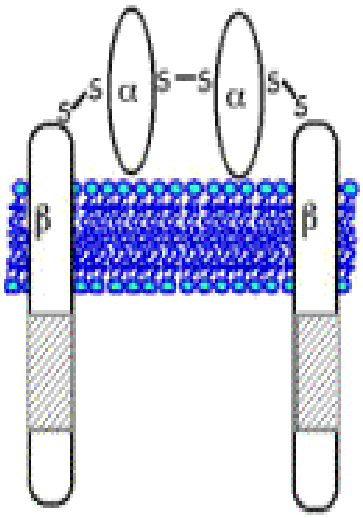


Figure 2: Structure of Insulin receptor (Hancock, 2005).

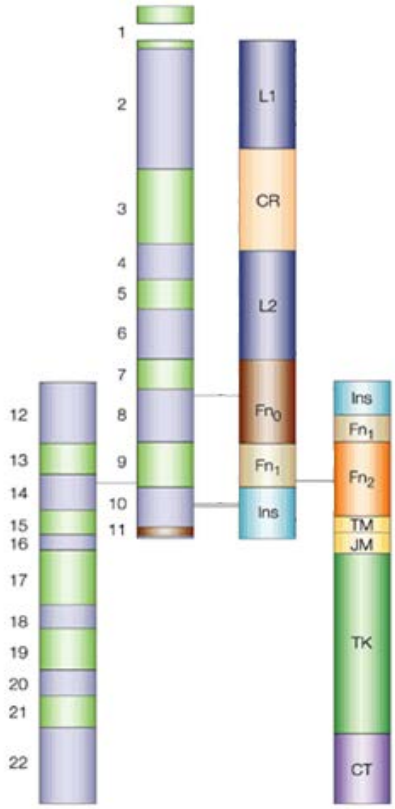


Figure 3: Modular structure of the insulin receptor (De Meyts & Whittaker, 2002).

The modular structure of the insulin receptor is encoded by a gene containing 22 exons and 21 introns (Seino et al., 1990). The exon 11 is alternatively spliced, resulting in two isoforms (A and B) (De Meyts et al., 1994). Both isoforms have slightly different binding affinities toward insulin (McClain, 1991; Yamaguchi et al., 1991; Gu & Meyts, 1991). The synthesis of these receptors begins as preproreceptors that have a single chain which is later processed, glycosylated, folded and dimerised to produce the mature $\alpha_2\beta_2$ receptor (De Meyts, 2004; De Meyts & Whittaker, 2002).

As shown in Figure 3, the N-terminal half of the α subunit comprises of two homologous large globular domains (L1 and L2), that include a cysteine-rich (CR) region in between (Bajaj et al., 1987). Studies about the crystal structure of this domain helped immensely in confirming the predicted boundaries of the protein modules (De Meyts et al., 2007). The C-terminal half of the receptors consists of three fibronectin type III (FnIII) domains (Fn_1, Fn_2 and Fn_0) (Marino-Buslje et al., 1998; Mulhern et al., 1998; Ward, 1999). The second FnIII domain forms the C-terminal part of the α subunit and the N-terminal part of the β subunit. It contains a large insert domain (Ins) of about 120-130 residues containing the site of cleavage between α and β subunits. The intracellular portion of the β subunit contains a transmembrane domain (TM) and a tyrosine kinase catalytic domain (TK) flanked by two regulatory regions, a juxtamembrane (JM) region involved in docking insulin receptor substrates (IRS) and a C-terminal tail (CT) containing two phosphotyrosine binding sites (De Meyts & Whittaker, 2002; De Meyts et al., 2007).

Insulin Receptor Signaling Events and Phosphotyrosine Activity

Receptor activation upon insulin binding gives rise to multiple signaling cascades to elicit its metabolic actions (Thong et al., 2007). Binding of the insulin ligand to the extracellular α subunit brings about a conformational change that promotes autophosphorylation, and activates the intrinsic tyrosine kinase domain of the β subunit (Kasuga et al., 1982; Van Obberghen & Kowalski, 1982; Lee et al., 1993). The activated receptor recruits and regulates target proteins, thereby transmitting the signal downstream (White & Kahn, 1994). Phosphorylation of proteins is one of the most common mechanisms for regulating protein function (Roach, 1990) that is known to play a significant role in controlling a variety of biological functions in many organisms (Hubbard et al., 1993; Manning et al., 2002; Koch et al., 1991). Phosphorylation is simply the addition of a phosphate group to a protein. Within a protein, phosphorylation can occur on several amino acids. Phosphorylation on serine is the most common (Whiteside and Israel, 1997), followed by threonine (Moeslein et al., 1999). Phosphoserine, phosphothreonine and phosphotyrosine residues play critical roles in the regulation of many cellular processes (Diella et al., 2004).

Tyrosine phosphorylation forms a major part of signal transduction and enzymatic activity regulation (Moeslein et al., 1999). Tyrosine phosphorylation of proteins occurs after the activation of the insulin receptor tyrosine kinase as it unleashes the intracellular transmission of insulin signals (Thirone et al., 2006; Ushiro & Cohen, 1980). The receptor is subjected to a series of intramolecular trans-phosphorylation mechanisms (Pessin & Saltiel, 2000). It undergoes autophosphorylation on multiple tyrosine residues (Virkamäki et al., 1999). Different tyrosine residues perform distinctive functions. Phosphorylation of C-terminal tyrosines directs the

actions of insulin hormone. The phosphorylated tyrosine residues present in the juxtamembrane domain help in insulin receptor substrate binding. Phosphorylation of tyrosine residues of the kinase catalytic domain stimulates the catalytic activity of the β subunit of the receptor (Pessin & Saltiel, 2000). Phosphorylation activity and the resultant protein–protein or protein-lipid interactions act as indispensable tools to transmit and compartmentalize the insulin signal from the surface receptor to the final cellular effect (Virkamäki et al., 1999), such as stimulation of glucose transport, protein and glycogen synthesis, inhibition of lipolysis, regulation of gene transcription and translation, and cell growth and proliferation (Brazil et al., 2002; Cantley 2002; Myers et al., 1994; Vivanco and Sawyers, 2002).

Phosphoinositide 3-Kinase (PI3K)

Phosphoinositide 3-kinases, also called phosphatidylinositol 3-kinases (or PI3Ks) are a family of lipid kinases that act as signal transducer enzymes and play a crucial role in the regulation of many cellular processes (Stein, 2001). They are activated by insulin and are capable of phosphorylating the 3-position hydroxyl group of the inositol ring (Figure 4) of phosphatidylinositols (PtdIns) (Hirsch et al., 2009).

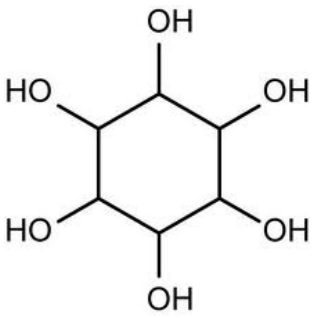


Figure 4: Inositol ring (Cyclohexane-1,2,3,4,5,6-hexol).

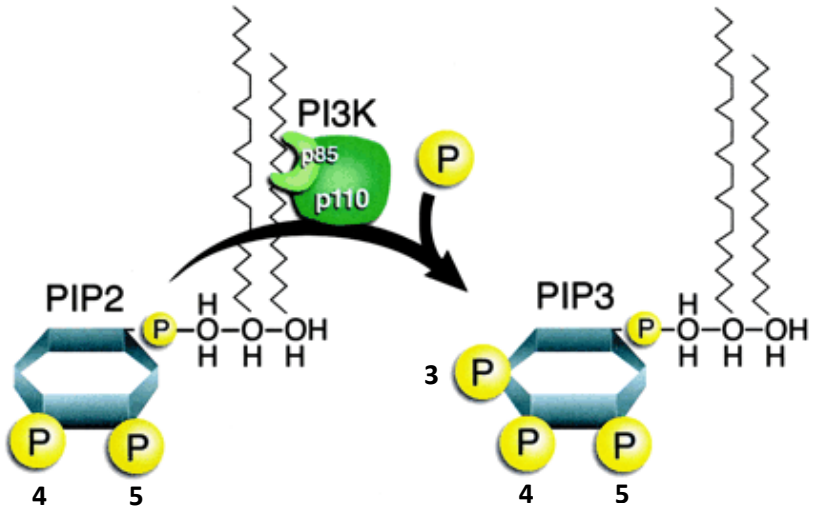


Figure 5: PI3K activity: PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate or PIP2 to phosphatidylinositol-3,4,5-trisphosphate or PIP3 (Isabelle et al., 2004).

PI 3-kinase is a heterodimeric enzyme comprising of a regulatory subunit (p85) responsible for binding to insulin receptor substrates and a catalytic subunit (p110) that phosphorylates the phosphatidylinositols present in cell membranes (Virkamäki et al., 1999). The p85 regulatory subunit stimulates the intrinsic PI 3-kinase activity of the enzyme (Backer et al., 1992, Carpenter et al., 1993; Baltensperger et al., 1994). It contains two Src homology 2 (SH2) domains and one

Src homology 3 (SH3) domain (Escobedo et al., 1991; Otsu et al., 1991; Skolnik et al., 1991). The SH2 domains are required for the association of the kinase with tyrosine phosphorylated proteins, such as the insulin receptor substrate IRS-1 (Sun et al., 1991), whereas, SH3 mediates interactions with proline-rich regions (Anderson et al., 1990; Moran et al., 1990; Ren et al., 1993). PI3K was the first SH2 protein found to associate with IRS-1. Both SH2 domains associate specifically with phosphorylated IRS-1 (White & Kahn, 1994). The insulin receptor substrate 1 is phosphorylated in response to insulin on several tyrosine residues that serve as binding sites for proteins containing the SH2 domain (Myers et al., 1994). As shown in Figure 5, PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate or PtdIns(4, 5)P_2 or PIP2 to form the lipid second messenger, phosphatidylinositol-3,4,5-trisphosphate or PtdIns(3, 4, 5)P_3 or PIP3 (Yuan & Cantley, 2008). PtdIns(3, 4, 5)P_3 acts as a second messenger in the regulation of cellular processes such as metabolism, proliferation, and survival through interactions with proteins that contain Pleckstrin homology (PH) domains (Cantrell, 2001). PH domains consist of well-characterized modules of approximately 120 amino acids and have a high affinity binding with phosphoinositides that are phosphorylated forms of phosphatidylinositol (Lemmon, 2007). They are found in various signaling proteins that play an important role in cytoskeletal remodeling, cellular signaling and intracellular trafficking (Gibson et al., 1994) by mediating protein-lipid (Frech et al., 1997) and protein-protein interactions (Datta et al., 1995).

Akt (PKB)

Some of the best studied targets for PIP3 belong to the AGC (cAMP-dependent, cGMP-dependent and protein kinase C) family of protein kinases (Alessi & Downes, 1998; Williams et

al., 2000). These include PDK1 (3-phosphoinositide-dependent kinase 1) and PKB (protein kinase B) or Akt that contain a PH domain. Akt is the cellular homologue of the viral oncoprotein v-Akt which is a 60 kDa serine/threonine kinase (Figure 6) (Bellacosa et al., 1991; Coffer & Woodgett, 1991; Jones et al., 1991; Staal et al., 1977). It is widely expressed in tissues, including the liver (Chan et al., 1999; Song et al., 2005) and important in cellular signaling.

Figure 6: Akt domain structure.

PI3K/Akt Signaling Pathway

Upon insulin stimulation at the cell surface, PIP3 binds to the PH domain of PDK-1 and Akt, promoting their translocation to the membrane (McManus et al., 2004; Alessi et al., 1997; Brazil et al., 2002; Cantley, 2002; Stokoe et al., 1997; Vivanco and Sawyers, 2002). Thus, association with PIP3 at the membrane recruits and localizes these proteins (Akt and PDK1) to the cell surface and facilitates phosphorylation and activation of Akt by PDK1 (Lawlor & Alessi, 2001; Cohen et al., 1997; Coffer & Woodgett, 1998; Mora et al., 2004; Engelman et al., 2006). PDK1 acts as a master kinase which is crucial for the activation of Akt/PKB (Mora et al., 2004; Storz & Toker, 2002; Vanhaesebroeck & Alessi, 2000; Wick & Liu, 2001). The PI3K/Akt upstream signaling (Figure 7) is one of the major insulin signaling cascades. PI3K/Akt downstream

signaling is the activation of Akt leading to translocation of GLUT4 molecule, activation of glycogen synthase and other proteins required for metabolic effects of insulin (Jensen & De Meyts, 2009). The PI3K/Akt signaling pathway has been known to perform wide range of cellular activities such as cellular proliferation and survival (Rong et al., 2001).

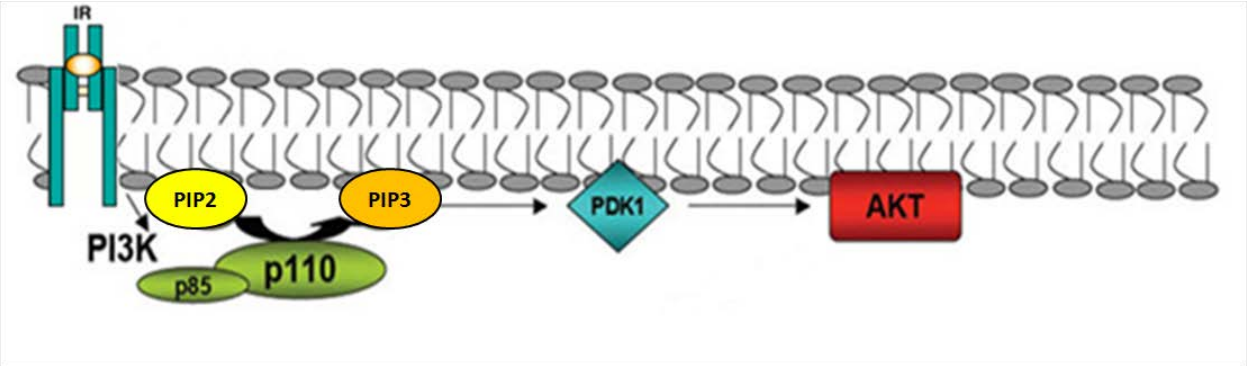


Figure 7: Upstream PI3K/Akt signaling pathway as adapted from Viglietto et al., 2011.

PI 3-kinase activity can be inhibited by the drugs wortmannin and LY294002 (Stein, 2001; O'Gorman et al., 2000; Kim & Denkers, 2006; Vanhaesebroeck et al., 1997). PI 3-kinase suppression affects various cellular events of insulin such as insulin-stimulated glucose uptake, translocation of GLUT4 vesicle to the plasma membrane, activation of fatty acid synthesis, glycogen synthase, Akt phosphorylation and stimulation of protein and DNA synthesis (Alessi & Downes, 1998).

Orthovanadate

Orthovanadate is a known insulinomimetic agent, which acts on glucose metabolism (Dubyak & Kleinzeller, 1980; Shechter & Karlish, 1980), glycogen synthase activity (Tamura et al., 1984) and on cell growth and transformation (Smith, 1983; Wice et al., 1987; Klarlund, 1985). Orthovanadate is an inhibitor of tyrosine phosphatase activities (Heffetz et al., 1990) and therefore, acts as a valuable tool in the investigation of the role of phosphorylation in kinase activities (Scimeca et al., 1992). Studies have shown that vanadate and hydrogen peroxide are well known insulin-mimicking agents (Czech et al., 1974; Dubyak & Kleinzeller, 1980; May & de Haen, 1979; Schechter & Karlish, 1980). Vanadate has also been shown to trigger the insulin receptor tyrosine kinase activity (Tamura et al., 1984). Pervanadate is a peroxidized form of vanadate which is formed by mixing vanadate with hydrogen peroxide and catalase (Kadota et al., 1987). Pervanadate mimics insulin biological effects (Fantus et al., 1989).

In View of Modular Domains

Many cellular proteins are composed of domains that have modular structures which helps them to retain their structure and biochemical properties so that they can function and exist on their own. These domains possess catalytic functions, such as protein kinase activity or lipid kinase activity, or can help in molecular interactions with other proteins (Pawson, 1995). The N- and C-termini of these interaction domains are closely spaced which allows them to be integrated into a host protein while preserving their ligand binding properties (Pawson et al., 2002). Known as the ‘Plug and Play’, this feature helps these proteins to be incorporated into pre-existing proteins and acquire new biological functions (Pawson et al., 2002). They play a

critical role in signal transduction in the activation of signaling pathways, recruitment of target proteins to activate receptors and formation of signaling complexes (Pawson & Scott, 1997; Kuriyan & Cowburn, 1997). Protein interaction domains can determine the kinetics and dynamic state of the cell, and mediate signaling pathways by controlling cellular activities like gene expression, protein synthesis, metabolism and cytoskeletal architecture (Pawson, 2003; Pawson & Nash, 2003).

In the study of the insulin receptor pathway, the role of interaction domains is a very important one. Interaction domains can regulate the localization of signaling proteins with the help of modules such as PH domains to bind phosphoinositides such as PIP3. The activation of PI 3-kinase increases the concentration of PIP3 in the plasma membrane, which consequently recruits the PKB/Akt, and its activating kinase PDK1, both of which have PH domains that selectively bind PIP3. Once activated at the membrane, PKB is a key regulator of cell growth, survival and metabolism. Interaction domains also regulate the effects of post-translational modifications such as phosphorylation (Pawson, 2003).

Isolation of the Receptor Complex on Ligand Coated Beads

Insulin receptor complex is activated and captured by insulin coated microbeads on surface of live cells. After the cells are disrupted, the receptor complexes are isolated by washing and sucrose gradient ultracentrifugation. Proteins associated with the receptor complex can be subjected to liquid chromatography and mass spectrometry for identification. This novel procedure called Ligand Affinity Chromatography (LAC) confirms recruitment and monitors receptor function. Thus, the recruitment of protein subunits and the penetration and efficacy of

drugs against receptor pathways may be quantified in live cells. LAC can help to detect receptor isoforms and proteins close to specific receptors that are important targets for the treatment of diseases. Because the ligand of the insulin receptor (insulin) is a treatment for some diseases like diabetes, the use of insulin-coated microbeads to capture a receptor complex further conceptually establishes that an antibody, protein ligand, or therapeutic molecule may be used to capture a receptor complex from the cell surface (Jankowski et al., 2008).

RATIONALE

Uniform polystyrene microspheres have been used recently in biological applications like drug delivery, bioimaging and biosensing (Kiani et al., 2002; Lu et al., 2008; Elsom et al., 2008). In this research study, the use of microbeads was helpful to display insulin to three different mammalian cells. Upon ligand-receptor binding, intracellular protein–protein interactions transmitted the signal from the activated receptor to the intracellular effects. Members of the receptor complex recruit upon ligand binding. Studies were concentrated on proteins that localized or translocate to the plasma membrane of the cell in response to insulin. It was easy to visualize the effects using the confocal microscope to target the beads on the cells and confirm recruitment of Akt protein to the activated receptor complex on the cell membrane. The main objective of this study was to isolate the insulin receptor complex from live cells by coating microbeads with insulin, binding to the cells, lysing and extracting the resultant protein bound microbeads. The isolated receptor-bead complex can be further introduced to mass spectrometry analysis in order to identify novel proteins in the insulin receptor signaling pathway that could help in the investigation of diseases.

MATERIALS AND METHODS

Cell lines

CHO cells (Chinese hamster ovary) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). COS-7 cells (Monkey kidney fibroblast) were a gift from Dr K. Siminovitch (Mount Sinai Hospital, Toronto, ON, Canada) and NIH-3T3 cells (Mouse embryo fibroblast) were a gift from R. Collins (Cell Biology, Hospital for Sick Children, Toronto, ON, Canada).

Antibodies

Anti-phosphotyrosine, clone 4G10 antibody (05-321X) was obtained from Millipore (Billerica, MA, USA). Insulin antibody (NBP1-45662) was obtained from Novus Biologicals Canada (Oakville, ON, Canada). Anti-insulin Rβ (C-19) (sc-711) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Cy3 and HRP conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA).

Protease inhibitors

Diisopropylfluorophosphate or DFP (protease inhibitor) and eukaryotic protease inhibitor cocktail was obtained from Sigma–Aldrich (St. Louis, MO, USA).

Nucleic acids

Akt-PH-GFP was constructed using pEGFP-C1 vector (Haugh et al., 2000). pEGFP was obtained from Clontech Laboratories, Inc. (California).

Experimental Procedures

Preparation of competent E. coli (DH5α) cells

E. coli DH5α stock was inoculated in LB broth and incubated at 37°C overnight with shaking at 200 rpm. A streak plate was made from the overnight culture onto LB media plate and incubated overnight. Fresh colony was picked and inoculated in 5 ml LB media and grown for 2 hours at 37°C with shaking at 200 rpm. Again 1 ml of culture was inoculated in 100 ml pre-warmed media and grown at 37°C for 2-3 hours until an optical density of 0.45 was reached at 600 nm. The culture was chilled on ice, centrifuged at 3500 rpm at 4°C for 5 minutes. The supernatant was discarded and pellet was re-suspended in 2/5th original volume of Tfb 1 buffer (30 mM potassium acetate, 100 mM RbCl2, 10 mM CaCl2.2H20, 50 mM MnCl2.4H20, 15% glycerol, pH 5.8) and chilled on ice for 5 minutes followed by centrifugation at 3500 rpm at 4°C for 5 minutes. The pellet was again re-suspended in 1/25th original volume of Tfb 2 buffer (10 mM MOPS, 75 mM CaCl2.2H20, 10 mM RbCl2, 15% glycerol, pH 6.5) and chilled on ice for 15 minutes. Competent cells were stored at -80°C in 200 µl aliquots.

Bacterial transformation with Akt-PH-GFP plasmid

Competent DH5α cells were transformed with Akt-PH-GFP. 100 µl of competent DH5α cells were thawed and incubated with 1 mg/ml DNA on ice for 30 minutes. The cells were heat shocked at 42°C for 90 seconds and chilled for 1-2 minutes on ice. To the cell suspension, 1 ml of LB was added and cells were incubated at 37°C for 1 hour with shaking at 200 rpm. The cell

suspension was plated on LB agar plate alone, LB containing 50 µg/ml kanamycin and LB containing 100 µg/ml ampicillin and left overnight to grow at 37° C. Colonies grown on plates showing kanamycin resistance (LB containing kanamycin) were looped off and inoculated in 2.5 ml LB containing kanamycin (50 µg/ml) and incubated at 37° C while shaking at 200 rpm for 3 hours. 1 ml of the culture was grown in 100 ml LB with same antibiotic conditions overnight. The DNA plasmid was then extracted and purified using the QIAfilter Plasmid Maxi Kit (QIAGEN, Valencia, California). The DNA concentration in TE buffer was determined at a wavelength of 260 nm. The DNA sample was run on an agarose gel (1%) alongside GeneRuler 1 kb Plus DNA Ladder (75-20,000 bp) to analyze DNA yield and quality.

Preparation of insulin coated beads: Microbead adsorption

10 mg of insulin (SAFC Biosciences, Cat. # 91077C) were dissolved in 50 ml of water, with pH adjusted to 2-3 using 0.1 N HCl (according to insulin manufacturer's instructions). The solution was subjected to centrifugal force, vacuum and heat to help speed evaporation using a centrivap. Purified insulin (100 µg) was dissolved in 100 µl of 1XPBS. 2 µm polystyrene beads from a 10% (v/v) suspension stock (Bang's Laboratories, Fishers, IN, USA) were diluted to 1% solids with 1XPBS. 10 µl of the microbead suspension was added to the dissolved insulin and incubated for 30 minutes at room temperature with constant rotation (Figure 8). The beads were washed 3 times each with 100 µl 1XPBS to remove any unbound insulin by pelleting beads in a microcentrifuge at 8000 rpm for 2 minutes. The washed and coated beads were re-suspended in 100 µl of 1XPBS.

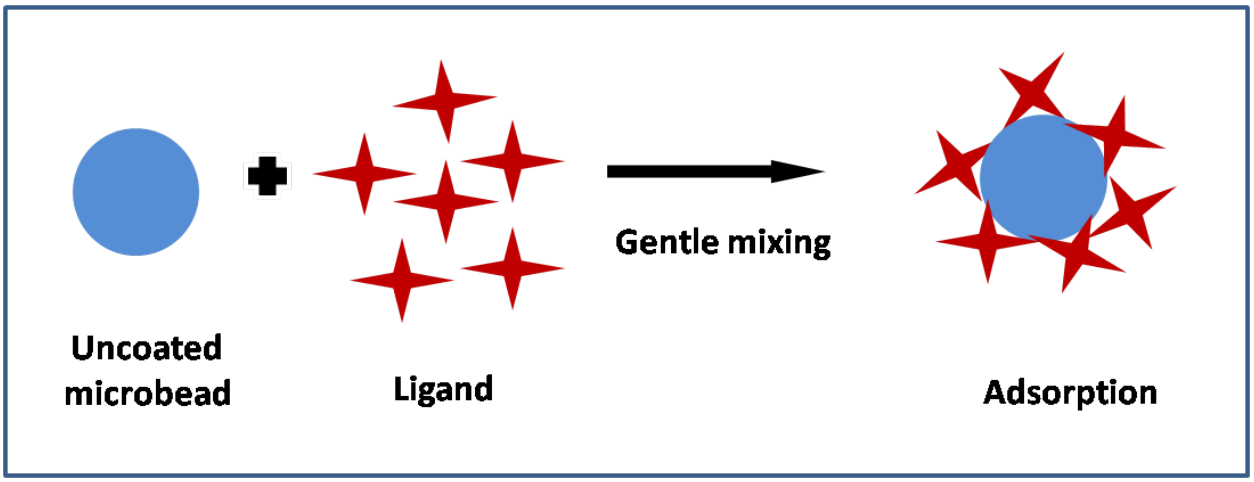


Figure 8: Adsorption of ligand onto microbead.

SDS PAGE analysis of insulin coated beads

Insulin coated beads were prepared as mentioned above. Samples were loaded on a tris-tricine based 15% separating gel, 4% stacking gel and run for 30 minutes at 30 V and at 125 V for 60 minutes or until the tracking dye has just run off. Marker used was an ultra-low range molecular weight marker (M. W. 1,060-26,600) from Sigma Aldrich (Cat. # M3546). The gel was silver stained.

Cell culture

CHO, COS-7 and NIH-3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS) in 75-cm^2 flasks to confluency.

Cell lysate preparation and Western blot for detection of insulin receptor (IR)

Cells were grown to confluency, washed 3 times with PBS and treated with DFP on ice. They were lysed in boiling 2XSDS sample buffer and scraped. SDS PAGE analysis was done with 10% separating gel and 4% stacking gel alongside a molecular weight marker. Proteins from the gel were transferred to Immobilon-P Transfer (PVDF) membrane (Millipore, Bedford, MA) which was first soaked in 100% methanol for activation. The membrane was stained with Coomassie stain without acetic acid, destained with 50% methanol, dried and marked with a pencil to indicate the location of the marker. The membrane was then soaked in 100% methanol and blocked with 5% skim milk in PBST (PBS+ 0.1% Tween) for 1 hour. The membrane was washed 1 time with PBST and incubated with insulin receptor beta (C-19) primary antibody at 1:1000 dilution for 1 hour and again washed 3 times with PBST. It was then incubated with goat anti-rabbit IgG HRP secondary antibody at 1:10000 dilution for 45 minutes. The membrane was washed with PBST and developed using ECL (enhanced chemiluminescence) developing solution (Haan & Behrmann, 2007).

Preparation of cell lysates and Western blot confirmation of phosphotyrosine (YP) activity

Cells were grown to confluency, serum starved overnight and incubated with insulin coated beads for 5, 10 and 15 minutes. They were washed 3 times with PBS and fixed with 10% TCA on ice for 15 minutes. Cells were lysed using lysis buffer (50 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1 mM PMSF; 1 mM Na3VO4; 1 mM NaF) and scraped. Cell lysates were mixed with 2XSDS sample buffer and boiled prior to performing SDS PAGE on a 9% resolving, 4% stacking gel, followed by

western blot, as described before, using 5% BSA in PBST (PBS+ 0.1% Tween) as blocking solution, anti-phosphotyrosine primary antibody (4G10) at 1:1000 and goat anti-mouse IgG HRP secondary antibody at 1:10000 dilution.

Cell transfection and confocal microscopy

CHO, COS-7 and NIH-3T3 cells were plated on glass cover slips to approximately 20% confluence and left to grow for 48 hours before removing all of the old medium and replacing it with fresh 10% FBS in DMEM. Plasmid encoding Akt-PH-GFP was transfected using Fugene 6 (Roche Diagnostics, USA). After 24–48 hours, cells were incubated with insulin coated beads for 15 minutes and then used to image the recruitment of Akt-PH-GFP at the site of receptor activation by the ligand-coated microbeads at the plasma membrane on the cell surface. The cells were then imaged at 37°C in DMEM buffered with 140 mM NaCl, 10 mM sucrose and 1 mM each of CaCl2 and MgCl2 buffered to pH 7.4 with 20 mM Hepes. Images of cells binding ligand-coated beads were recorded on a 510 meta laser confocal microscope with Argon 488-nm lasers (Carl Zeiss Microscopes, Jenna, Germany). Magnification: 100x oil immersion objective.

Immunofluorescent staining for Phosphotyrosine activity, Insulin Receptor and Insulin

CHO, COS-7 and NIH-3T3 cells were scraped and grown on 2.5 cm glass cover slips for 24-48 hours until 50-60% confluency. The cells were serum starved (overnight) and incubated with insulin coated beads for 15 minutes. Then they were fixed with 4% paraformaldehyde for 30

minutes, washed, and quenched with 5% glycine in PBS before blocking with 3% BSA and 0.1% Triton X-100 in PBS for 1 hour. Cells were incubated with anti-phosphotyrosine primary antibody (4G10) used at 1:25 dilution for 1 hour, then washed in PBS. Cells were incubated with Cy3 conjugated donkey anti-mouse IgG secondary antibody used at 1:10,000 dilution for 30 minutes in dark. Experiment was repeated for anti-insulin receptor (1:100) and anti-insulin antibody (1:100) with Cy3 conjugated donkey anti-rabbit IgG (1:10000) as the secondary antibody. Blocking solution used was 3% milk in PBST (PBS+ 0.1% Triton). The cells were washed three times with PBS in dark and mounted with a Dako fluorescent mounting medium (Dako, Denmark). Images of cells binding insulin-coated beads were recorded on a 510 meta laser confocal microscope with Ar 488-nm and He/Ne 543-nm lasers (Carl Zeiss Microscopes, Jenna, Germany).

Isolation of Insulin receptor complex on the beads

Cells were grown to 60-75% confluency in 75 cm^2 flasks and gently washed 3 times with ice cold 1XPBS. 200 µl of insulin coated beads was added to each flask in experimental medium (10 mM HEPES, 135 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM sucrose, pH 7.4) on ice for 15 minutes before washing away the free beads with ice cold PBS. Cells were warmed at 37°C for 15 minutes and the experiment was quenched with ice-cold PBS. Cells were treated with DFP, fixed with 10% TCA for 15 minutes on ice and washed 3 times with PBS. Homogenization buffer (20 mM Tris-HCl pH 7.4, protease inhibitor cocktail, 5 mM pervanadate, AEBSF, PMSF, DNAse and RNAse) was added and cells were scraped. Cells were disrupted in a pre-cooled French

press (FA-078A, Thermo Electron, Waltham MA, USA) at a pressure of 400 psi, allowing cell suspension to equilibrate for 10 seconds. Collected sample was placed on ice and efficiency of disruption was confirmed using light microscope. The beads with attached receptor complex were isolated through a sucrose gradient in a 1 ml sample volume using a high speed ultra centrifuge (Beckman, USA). The final pellet containing the receptor bound to the bead was dissolved in 2XSDS sample buffer and boiled prior to performing the SDS-PAGE and western blot for insulin receptor and phosphotyrosine activity detection.

RESULTS

Cell transformation

Competent E. coli (DH5α) cells were prepared and transformed with Akt-PH-GFP. Bacterial colonies that grew in LB plates containing kanamycin were resistant to the antibiotic since these cells had incorporated the Akt-PH-GFP (Figure 9). The plasmid for Akt-PH-GFP construct (pEGFP-C1) confers kanamycin resistance to the bacterial cells.

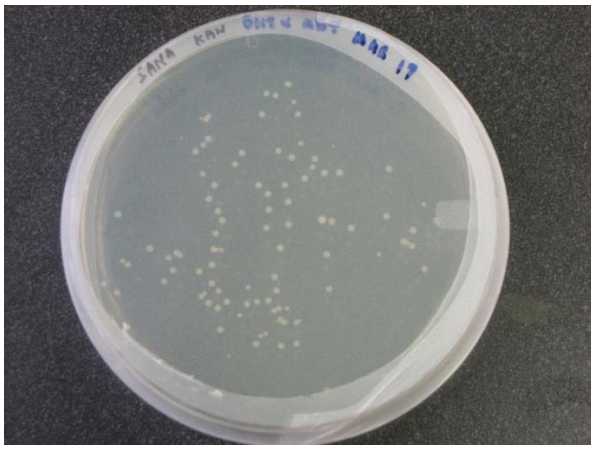


Figure 9: Competent DH5α cells that were transformed with Akt-PH-GFP grow on LB agar plate containing kanamycin.

DNA agarose gel analysis

The plasmid DNA was extracted and purified using maxi prep followed by agarose gel analysis on a 1% gel. As shown in Figure 10, two bands were obtained in lanes 1 (cleared lysate containing supercoiled, open circular plasmid DNA and degraded RNA) and 4 (pure plasmid

DNA). The bands were observed at about 5000 bp. The pEGFP-C1 plasmid size was 4700 bp and insert size of Akt-PH was 457 bp. See Appendix A and B.

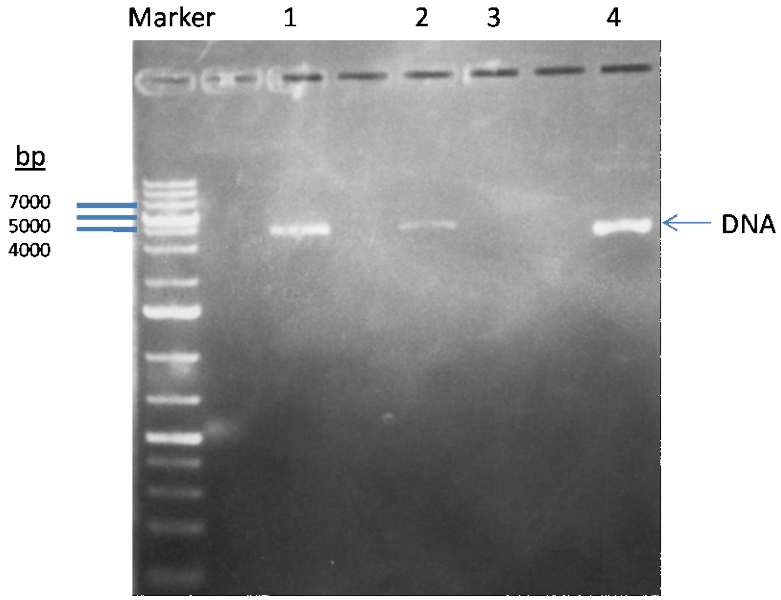


Figure 10: Agarose gel analysis of DNA on 1% gel. Pure DNA band observed at 5000 bp. Lane 1: cleared lysate containing supercoiled, open circular plasmid DNA and degraded RNA; lane 2: Flow through obtained after cleared lysate passes through resin and degraded RNA is depleted; lane 3: Combined wash fractions that contain no DNA; lane 4: Pure plasmid DNA. Marker: GeneRuler 1 kb Plus DNA ladder.

SDS-PAGE analysis of insulin coated beads

SDS-PAGE analysis was done to confirm the binding of insulin on the beads. High percentage (15%) Tris-tricine gel was used for detection of insulin which is a low molecular weight protein (5.8 kDa). Coomassie staining of the gel was done but there were no visible bands. Silver

staining produced the expected bands, thereby confirming binding of insulin on beads (Figure 11). Lane 1 is the control lane containing the adsorption buffer PBS. No band was formed. Lane 2 is insulin solution in PBS and lane 3 is the supernatant obtained after incubation of beads with insulin solution and centrifugation which contained unbound insulin. Lanes 2 and 3 showed bands for insulin. Lanes 4, 5 and 6 are the washes of beads with PBS for removal of any unbound insulin. These lanes did not show any band confirming there is less than microgram amounts of insulin in the wash fractions. Lane 7 is the final sample obtained after the three washes with PBS that showed a band for insulin confirming its presence and binding onto the beads.

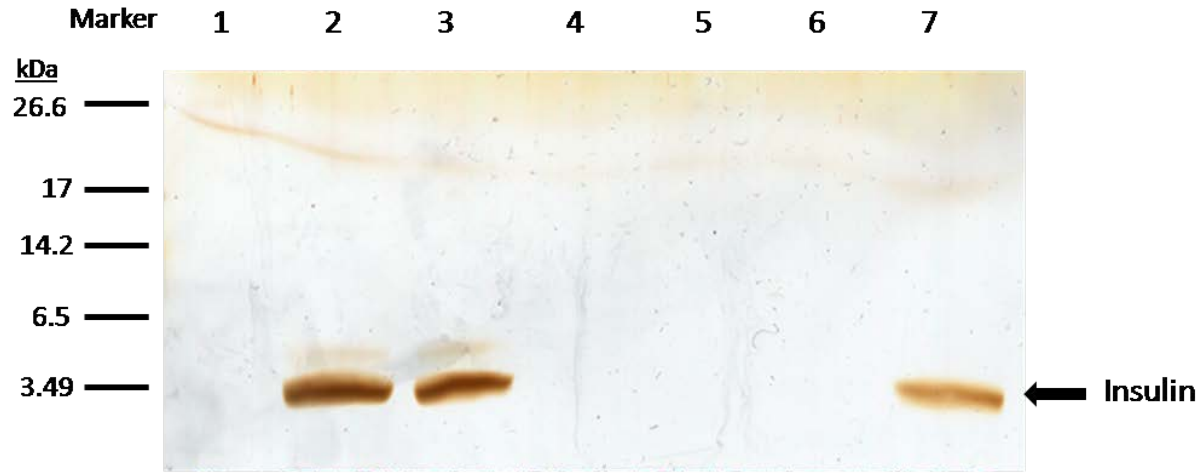


Figure 11: SDS-PAGE analysis confirming binding of insulin on beads. 20 µl of each sample was loaded on a 15% Tris-tricine gel and the gel was silver stained. Lane 1: (control) PBS; lane 2: Insulin solution (1 µg/µl x 20 µl = 20 µg) in PBS; lane 3: Supernatant obtained after incubation of beads with insulin solution and centrifugation; lane 4, 5, 6: Washes with PBS; lane 7: Sample of beads coated with insulin. Marker: Ultra-low range molecular weight marker. Black arrow: Band obtained for insulin at 3.49 kDa.

Western blot for detection of insulin receptor (IR) in cell lysates

The presence of insulin receptors in NIH-3T3, COS-7 and CHO cell lysates was confirmed using western blot. Cells, when treated with DFP (Figure 12, a), showed a band at 95 kDa (shown by a black arrow) upon incubation with insulin receptor beta (C-19) primary antibody against the receptor indicating the presence of the receptor in the cell lysates. Experiment was also repeated without DFP treatment (Figure 12, b), where the CHO and COS-7 cells did not show strong bands for insulin receptor, whereas the NIH-3T3 cells showed a band.

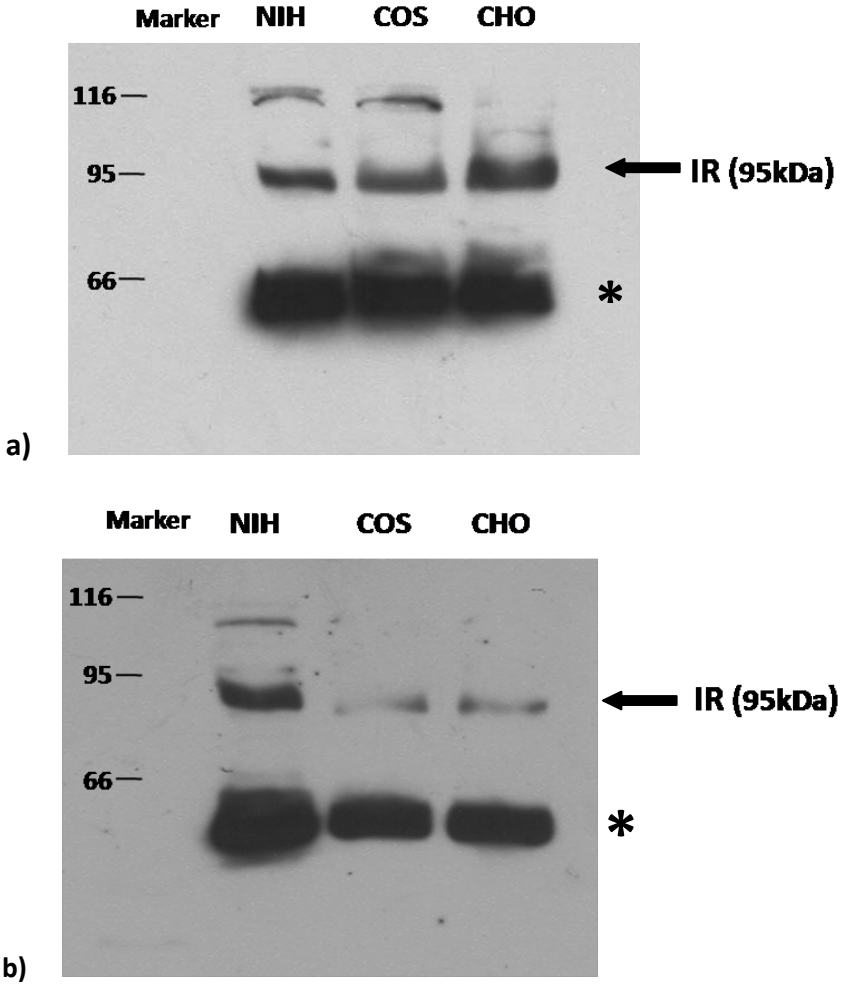


Figure 12: Western blot detection of insulin receptor in NIH-3T3, COS-7 and CHO cell lysates. Cells were treated a) with DFP, b) without DFP, and lysed in boiling 2XSDS sample buffer.

Samples were run on a 10% SDS PAGE gel followed by western blot transfer, blocking of transfer membrane with 5% skim milk in PBST and incubation with insulin receptor beta (C-19) primary antibody and goat anti-rabbit IgG HRP secondary antibody. The blot was developed using ECL solution. Black arrow: Bands obtained for insulin receptor at 95 kDa. *****: A splice variant.

Western blot confirmation of phosphotyrosine (YP) activity in cell lysates

Western blot was performed on CHO (Figure 13, a), COS-7 (Figure 13, b) and NIH-3T3 (Figure 13, c) cell lysates using the anti-phosphotyrosine (4G10) antibody in order to examine the tyrosine phosphorylation activity in response to insulin since it is an important step in insulin signal transduction. Three time points were performed where cells were incubated with insulin coated beads for 5, 10 and 15 minutes alongside two controls, C1 and C2. Control C1 cells were not treated with insulin or beads (0 minute). Control C2 cells were incubated with only beads for 15 minutes. Proteins that were phosphorylated were detected by the antibody and appeared on the blot. Compared to the control lanes, the 10 and 15 minutes incubation lanes produced more bands, indicating that more proteins were phosphorylated in the process of insulin signaling for these times.

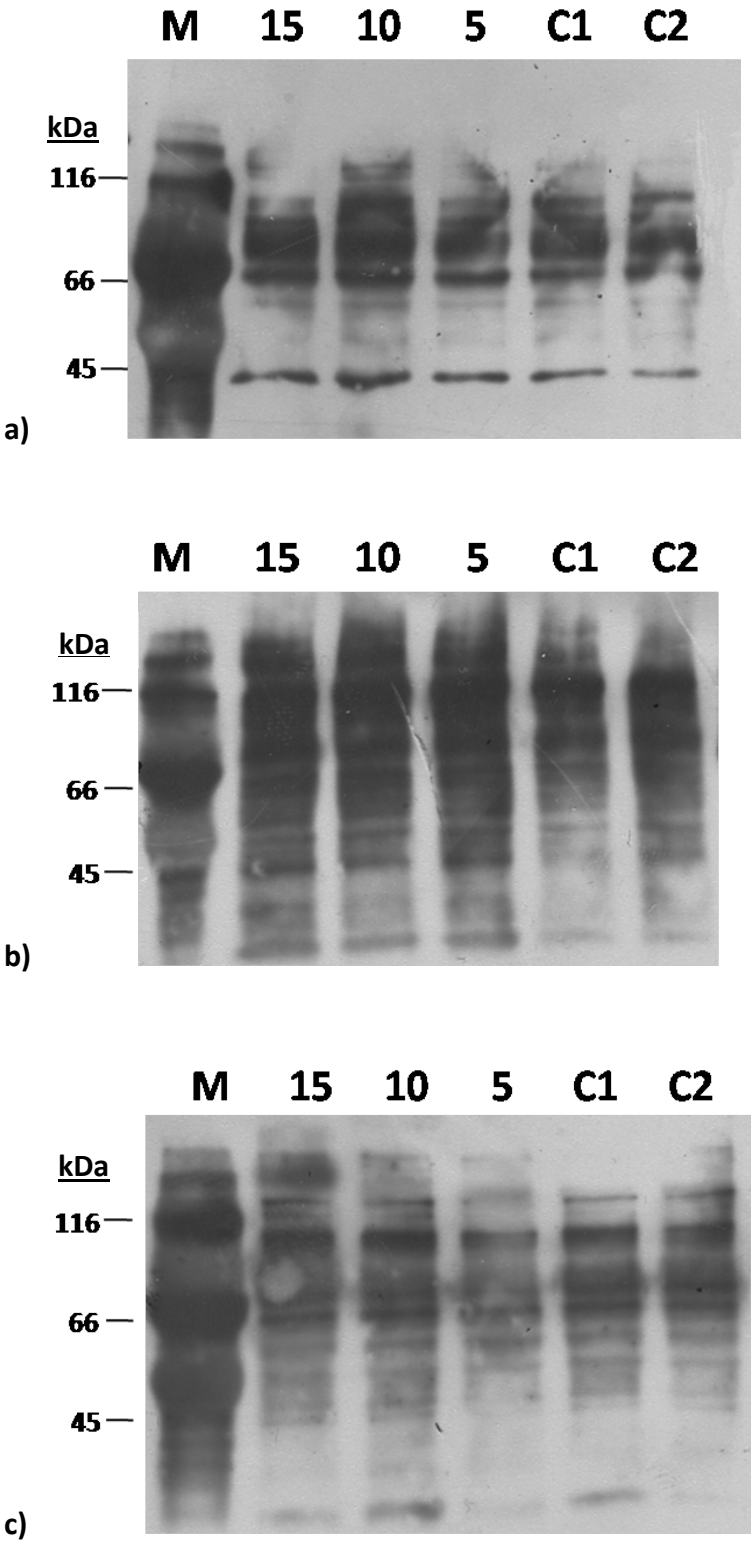


Figure 13: Western blot confirmation of phosphotyrosine activity in a) CHO, b) COS-7 and c) NIH-3T3 cell lysates. Cells were serum starved overnight and incubated with insulin coated

beads for 15, 10 and 5 minutes, fixed with TCA, lysed using lysis buffer containing vanadate and protease inhibitors. Samples were boiled in 2XSDS sample buffer and run on a 9% gel, followed by western blot transfer, blocking of transfer membrane with 5% BSA in PBST and incubation with anti-phosphotyrosine (4G10) antibody and goat anti-mouse IgG HRP secondary antibody. The blot was developed using ECL solution. M: Marker; 15, 10, 5: Cells incubated with insulin coated beads for 15, 10 and 5 minutes; C1: Untreated cells (0 minute); C2: Cells treated with only beads for 15 minutes.

Confocal microscopy of Akt-PH-GFP

Cells were transfected with Akt-PH-GFP to confirm its localization to the site of receptor activation by insulin coated microbeads and to observe its distribution with the help of confocal microscopy. GFP or green fluorescent protein helped to visualize and monitor the distribution. CHO cells that were not transfected with Akt-PH-GFP acted as the control cells (Figure 14, panel A) and showed no signal showing that the cells had no detectable fluorescence at these settings. Transfected CHO cells (Figure 14, panel B) showed cytosolic signal throughout the cell with slight increase in intensity around the nucleus. Transfected cells treated with insulin coated beads for 15 minutes (Figure 14, panel C) showed bright fluorescence at the cell membrane confirming that insulin coated beads induced insulin receptor signaling resulting in the localization of the Akt-PH-GFP to the cell membrane. See Appendix F for images of COS-7 and Appendix G for NIH-3T3 cells that showed similar results as CHO cells for each of the above stated conditions.

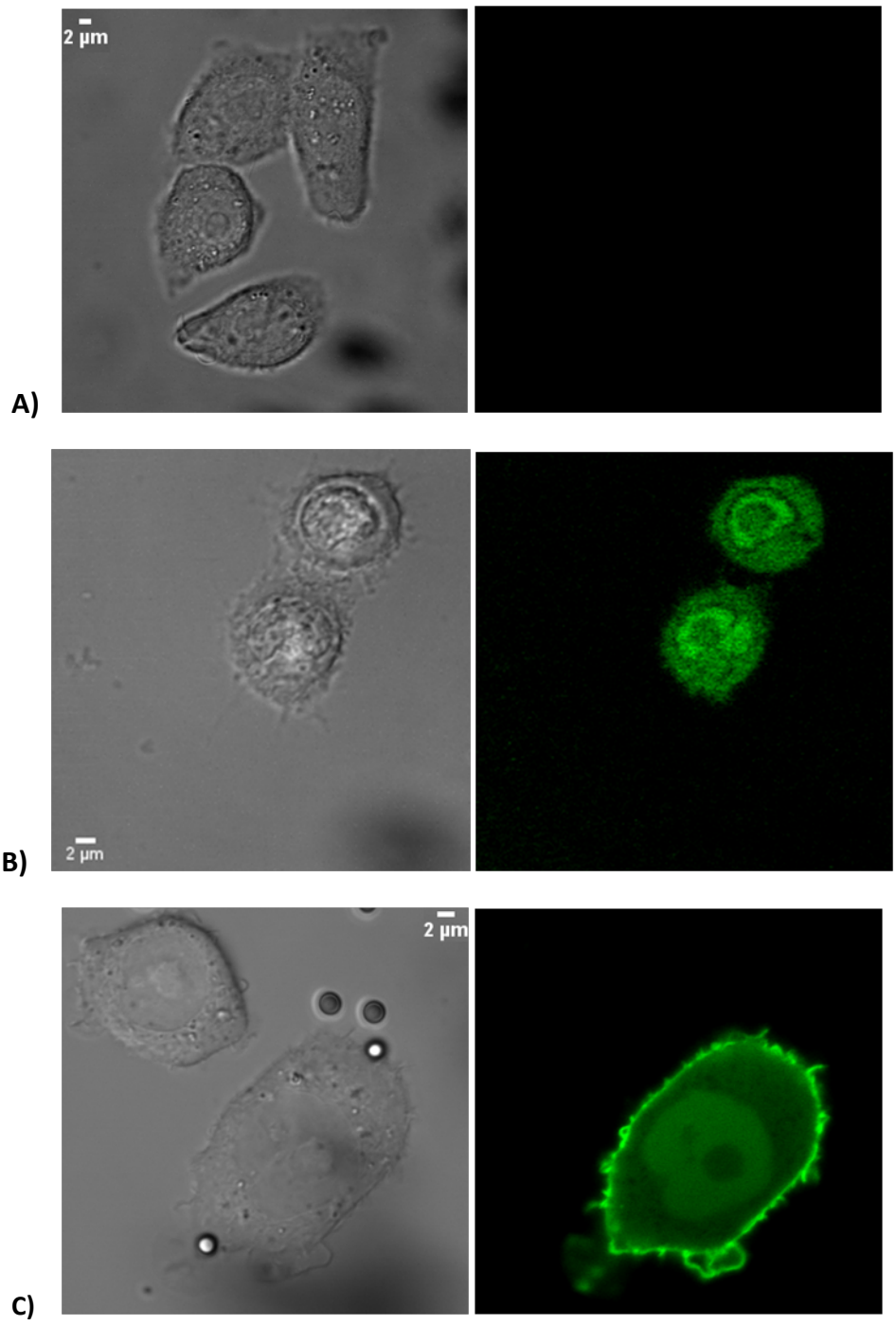


Figure 14: Transfection of CHO cells with Akt-PH-GFP to confirm its recruitment to the cell membrane in response to receptor activation by insulin coated microbeads. Cells were transfected with Akt-PH-GFP using Fugene 6 transfection reagent, incubated with insulin coated beads for 15 minutes and imaged at 37° C in DMEM buffered with 20 mM Hepes containing sucrose. Images were recorded on a 510 meta laser confocal microscope with Ar 488-nm lasers.

Magnification: 100x oil immersion objective. A) Non-transfected cells (control); B) Transfected cells (expression of Akt-PH-GFP); C) Localization of Akt-PH-GFP after incubation of transfected cells with insulin coated beads for 15 minutes.

Immunofluorescence staining for Phosphotyrosine, Insulin Receptor and Insulin

Immunofluorescence studies helped to detect the phosphotyrosine activity which is an important step in insulin signaling and the presence of insulin receptors and insulin distribution in cells through staining of formaldehyde fixed cells with appropriate primary antibodies and their respective Cy-3 conjugated secondary antibodies. Incubation of CHO cells with anti-phosphotyrosine 4G10 antibody (Figure 15, panel A) showed fluorescence indicating phosphorylation of proteins at tyrosine residues. Incubation of CHO cells with insulin receptor beta (C-19) antibody (Figure 16, panel A) showed bright fluorescence around the cell membrane confirming the presence of insulin receptors at the surface. Incubation of CHO cells with insulin antibody (Figure 17, panel A) produced fluorescence over the entire cell body showing the presence of insulin. There were three types of control sets in the experiment that showed very minimal fluorescence. Images of control experiments were taken with the same setting as those when the primary antibodies were used. Control 1 (C1) cells (panel B in Figures 15, 16 and 17) were not treated with insulin or beads (0 minute). Control 2 (C2) cells (panel C in Figures 15, 16 and 17) were treated with insulin coated beads for 15 minutes and incubated with Cy-3 conjugated secondary antibody alone in order to account for any non specific binding and to confirm that cell staining and fluorescence was produced due to the specific binding of the antibody to its target protein. Control 3 (C3) cells (panel D in Figures 15, 16 and 17) were treated only with beads, without any insulin to confirm that the presence of insulin ligand is a significant factor in the production and transmission of signal to the cell. See Appendix J for images of COS-7 and Appendix K for NIH-3T3 cells that showed similar results as CHO cells.

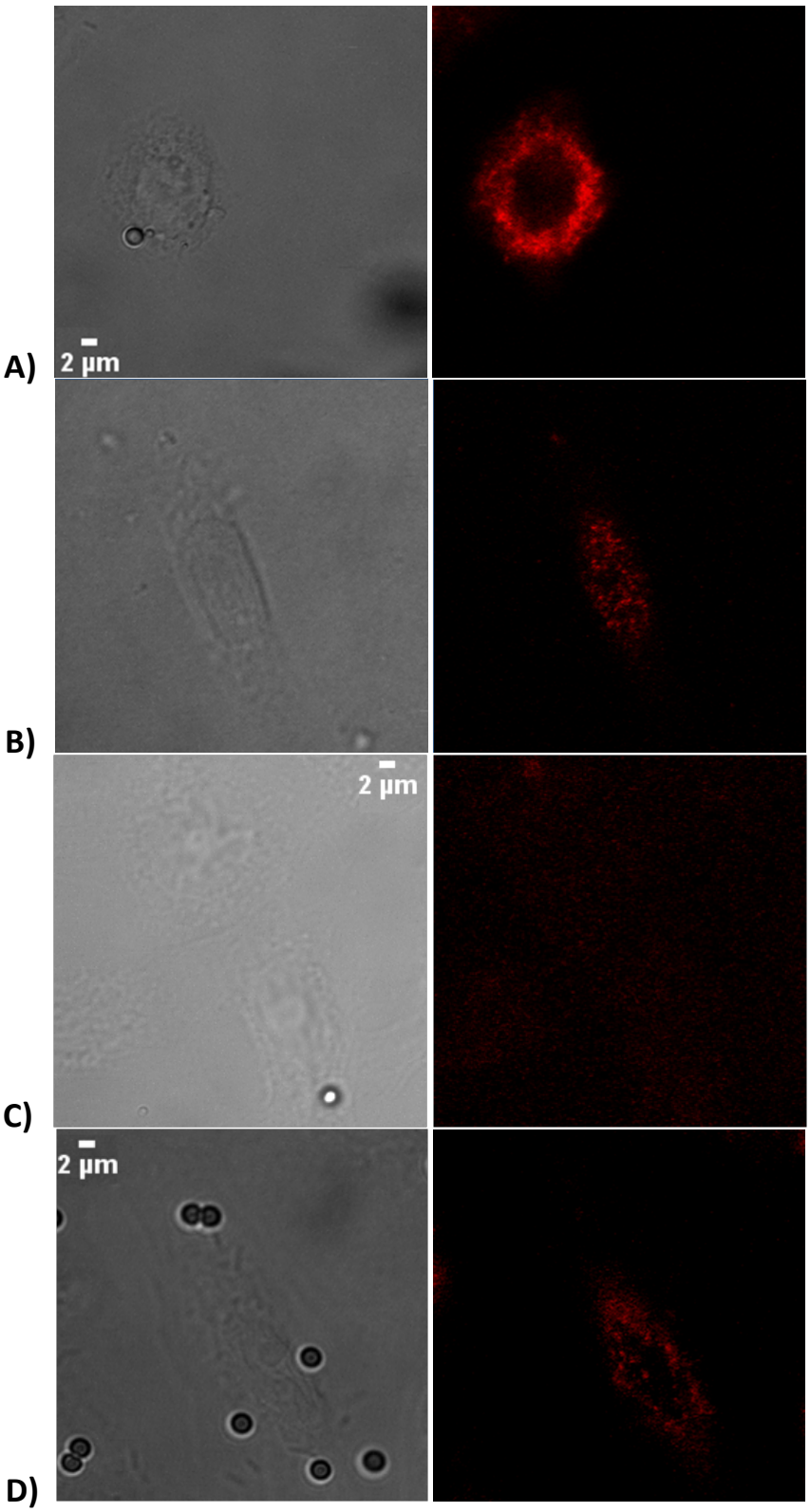


Figure 15: Immunostaining images of phosphotyrosine activity in CHO cells. Cells were serum starved overnight, incubated with insulin coated beads for 15 minutes, fixed with 4% PFA for 30

minutes, washed, quenched with 5% glycine and blocked with 3% BSA in PBST for 1 hour. Cells were incubated with anti-phosphotyrosine primary antibody (4G10) for 1 hour, washed and then incubated with Cy3 conjugated donkey anti-mouse IgG secondary antibody for 30 minutes in dark before mounting with Dako fluorescent mounting medium. Images were recorded on a 510 meta laser confocal microscope with Ar 488-nm and He/Ne 543-nm lasers. A) 15 minutes incubation with insulin coated beads; B) No incubation with beads or insulin, 0 minute (C1); C) 15 minutes incubation with insulin coated beads, stained with only secondary antibody, no primary antibody (C2); D) 15 minutes incubation with only beads, without insulin (C3).

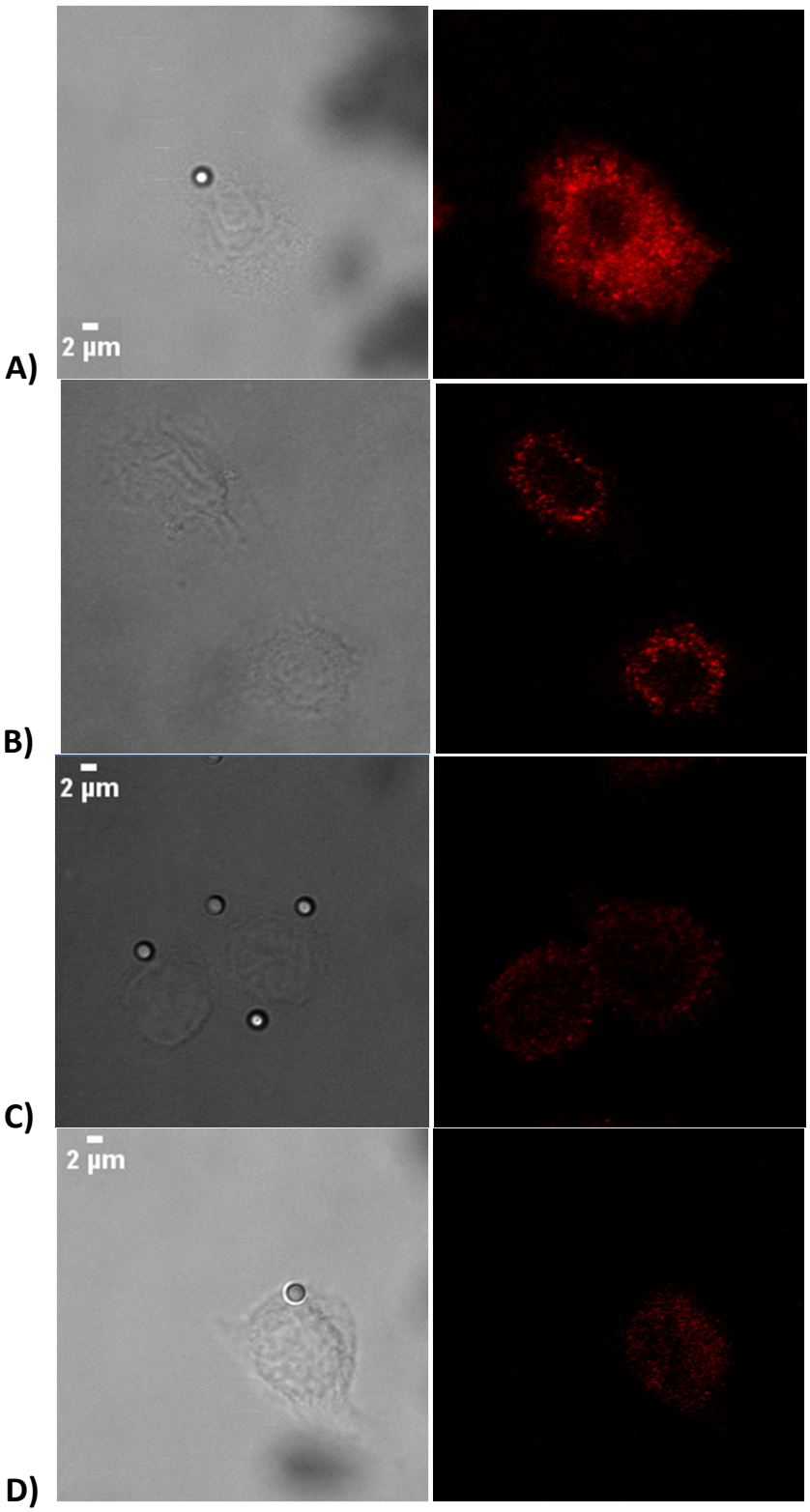


Figure 16: Immunostaining images of insulin receptor in CHO cells. Cells were serum starved overnight, incubated with insulin coated beads for 15 minutes, fixed with 4% PFA for 30

minutes, washed, quenched with 5% glycine and blocked with 3% milk in PBST for 1 hour. Cells were incubated with insulin receptor beta (C-19) primary antibody for 1 hour, washed and then incubated with Cy3 conjugated donkey anti-rabbit IgG secondary antibody for 30 minutes in dark before mounting with Dako fluorescent mounting medium. Images were recorded on a 510 meta laser confocal microscope with Ar 488-nm and He/Ne 543-nm lasers. A) 15 minutes incubation with insulin coated beads; B) No incubation with beads or insulin, 0 minute (C1); C) 15 minutes incubation with insulin coated beads, stained with only secondary antibody, no primary antibody (C2); D) 15 minutes incubation with only beads, without insulin (C3).

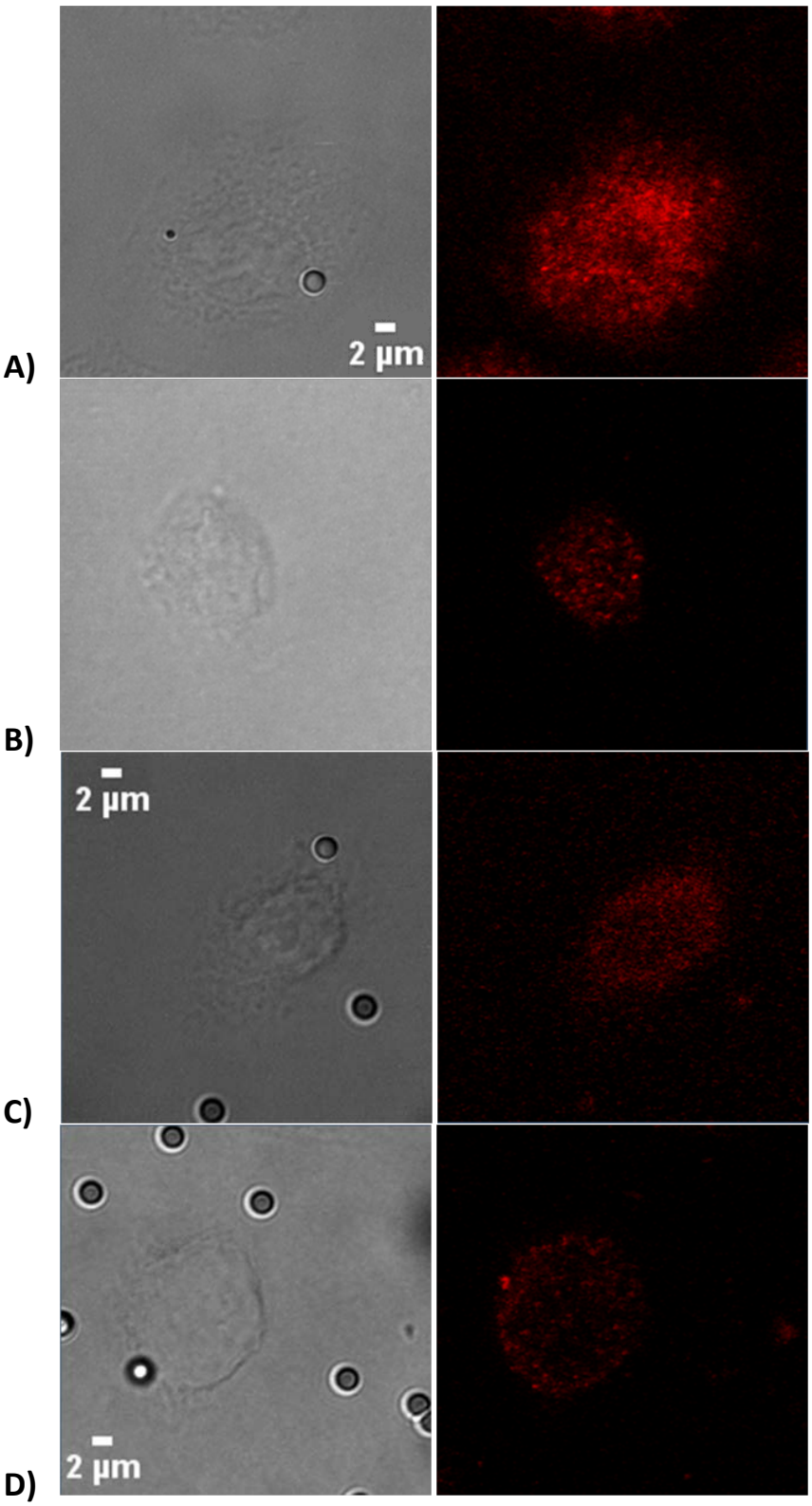


Figure 17: Immunostaining images of insulin in CHO cells. Cells were serum starved overnight, incubated with insulin coated beads for 15 minutes, fixed with 4% PFA for 30 minutes, washed,

quenched with 5% glycine and blocked with 3% milk in PBST for 1 hour. Cells were incubated with anti-insulin primary antibody for 1 hour, washed and then incubated with Cy3 conjugated donkey anti-rabbit IgG secondary antibody for 30 minutes in dark before mounting with Dako fluorescent mounting medium. Images were recorded on a 510 meta laser confocal microscope with Ar 488-nm and He/Ne 543-nm lasers. A) 15 minutes incubation with insulin coated beads; B) No incubation with beads or insulin, 0 minute (C1); C) 15 minutes incubation with insulin coated beads, stained with only secondary antibody, no primary antibody (C2); D) 15 minutes incubation with only beads, without insulin (C3).

Isolation of Insulin receptor complex on the beads: LAC (Ligand Affinity Chromatography)

Insulin receptor complex was activated and captured by insulin coated microbeads on the surface of CHO, COS-7 and NIH-3T3 cells. Confirmation of the presence of insulin receptor (IR) and phosphotyrosine activity of proteins that may be present in the complex was done using western blot for all three cell lines. Two sets of experiments were done, one that included DFP treatment of cells and the other did not include treatment with DFP. In both cases, blots for samples incubated with insulin receptor antibody (Figure 18) showed expected bands (marked by a black arrow) for the receptor at 95 kDa (the weight of the receptor) for cells incubated with insulin coated beads (for 15 minutes), confirming that the isolated complex contained the receptor protein. There were two controls, C1 and C2. C1 were cells that were not treated with insulin or beads (0 minute). C2 cells were incubated with only beads for 15 minutes. The control cells that were treated with DFP (Figure 18, A, C, E) produced bands for the receptor and those that were not treated with DFP (Figure 18, B, D, F) did not produce any band.

The blots for samples incubated with the 4G10 antibody for phosphotyrosine (Figure 19) showed bands for phosphorylated proteins that were present in the receptor-bead complex. Cells that were incubated with insulin coated beads for 15 minutes and treated with DFP produced more bands as compared to those that were not treated with DFP. The two control lanes (C1 and C2) in both cases showed fewer bands than the lane containing cells that were incubated with insulin coated beads for 15 minutes.

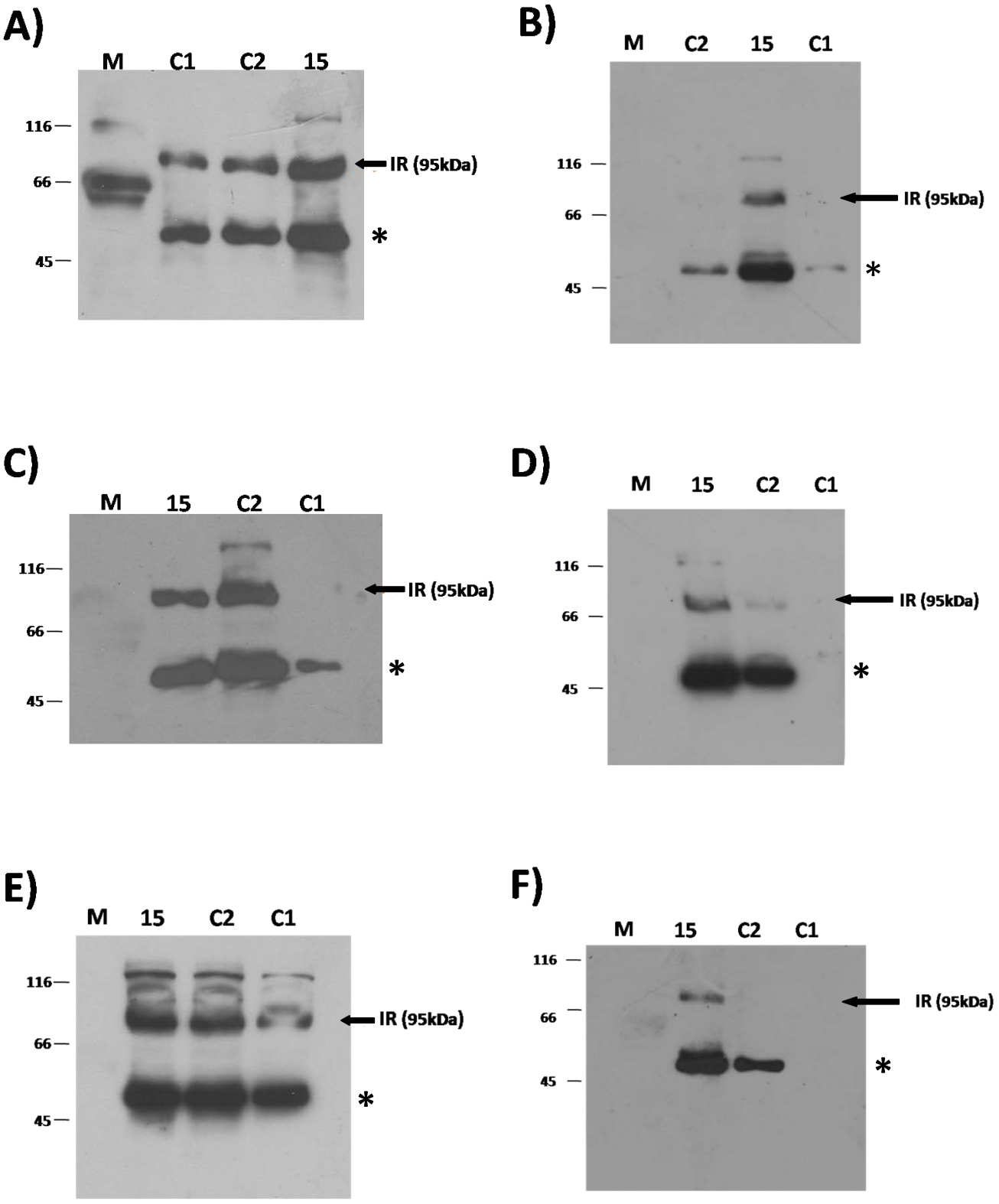


Figure 18: Western blot of LAC samples of CHO (A, B), COS-7 (C, D) and NIH-3T3 (E, F) cells for insulin receptor detection. Cells that were treated with DFP: A, C, E and cells that were not

treated with DFP: B, D, F. Cells were fixed with 10% TCA for 15 minutes, washed, homogenized using buffer containing protease inhibitors and vandate and disrupted in a French press. Receptor-bead complex was isolated by washing and sucrose gradient ultracentrifugation. The pellet was dissolved in 2XSDS sample buffer and boiled prior to performing SDS-PAGE (9% gel) and western blot using insulin receptor beta (C-19) primary antibody and goat anti-rabbit IgG HRP secondary antibody. C1: Cells not treated with insulin or beads (0 minute); C2: Cells treated with only beads for 15 minutes; 15: Cells incubated with insulin coated beads for 15 minutes. Black arrow: Bands obtained for insulin receptor at 95 kDa. *****: A splice variant.

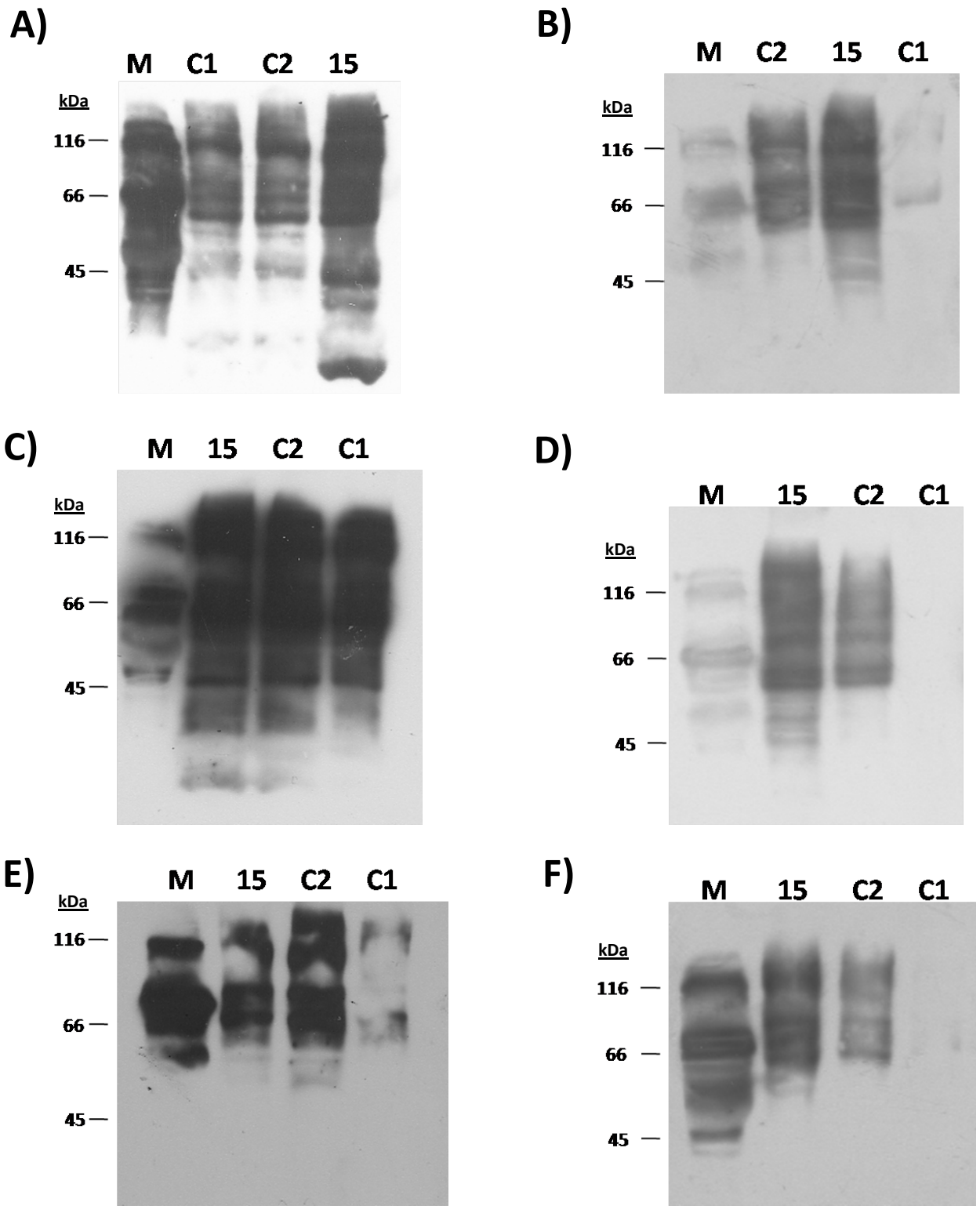


Figure 19: Western blot of LAC samples of CHO (A, B), COS-7 (C, D) and NIH-3T3 (E, F) cells for phosphotyrosine activity. Cells that were treated with DFP: A, C, E and cells that were not treated with DFP: B, D, F. Cells were fixed with 10% TCA for 15 minutes, washed, homogenized

using buffer containing protease inhibitors and vandate and disrupted in a French press. Receptor-bead complex was isolated by washing and sucrose gradient ultracentrifugation. The pellet was dissolved in 2XSDS sample buffer and boiled prior to performing SDS-PAGE (9% gel) and western blot for detection of tyrosine phosphorylated proteins using anti-phosphotyrosine primary antibody (4G10) and goat anti-mouse IgG HRP secondary antibody. C1: Cells not treated with insulin or beads (0 minute); C2: Cells treated with only beads for 15 minutes; 15: Cells incubated with insulin coated beads for 15 minutes.

DISCUSSIONS

SDS-PAGE analysis of insulin coated beads and silver staining

The SDS-PAGE analysis for confirming insulin binding on beads was performed under reducing and denaturing conditions in the presence of dithiothreitol (DTT). DTT resulted in the disruption of the disulfide bonds of insulin molecule (5.8 kDa), breaking it into its chains. The band obtained on the gel was at 3.49 kDa that corresponds to the B chain of the insulin molecule.

Insulin cannot be visualized by Coomassie even at high concentrations (Fujita et al., 1984). Diamine silver staining is reported to be sensitive to about 50 ng or less depending on the protein (Dionne et al., 1994; Chevallet et al., 2006). Sensitivity depends on the nature of the protein and insulin shows relatively poor sensitivity (Morrissey, 1981). Insulin was applied to the beads in a solution of 1 µg/µl and it was apparent that microgram amounts of insulin remained on the beads after washing. In previous studies, insulin receptor activation has been reported to occur at levels ranging from 0.1 to 500 ng/ml (Klein et al., 1986), which is likely well below the amount of insulin that might be detected in 20 µl of the washing buffer. Therefore, we cannot rule out that sufficient insulin to activate the insulin receptor was released from the beads during the experiment and this interpretation is consistent with images showing the translocation of Akt to the membrane even where no insulin coated bead is in contact with the cell.

Western blot for detection of insulin receptor (IR) in cell lysates

Western blot was performed to confirm the presence of insulin receptors in the cell lysates. Cells treated with DFP (a potent irreversible inhibitor of serine proteases that prevents degradation of proteins), produced clear bands at the 95kDa position (molecular weight of the receptor). The band for CHO cells appeared to be thicker than the other two cell lines. Cells that were not treated with DFP showed very light bands in the case of CHO and COS-7. However, NIH-3T3 did show a strong band. DFP treated cells showed better results compared to those that were not. The comparison of results with and without DFP seem to indicate that IR is rapidly degraded in crude cell extracts. In both cases, a second thick band was observed below 66 kDa which corresponds to a protein of unknown identity (O'Neill et al., 1996) that could be a splice variant also. The reason for the extra small band at the top (~116 kDa) could be attributed to the secondary antibody. See Appendix D for loading controls.

Western blot confirmation of phosphotyrosine activity in cell lysates

Western blot was performed to observe phosphorylated proteins in the cell lysates in response to insulin. Cells incubated with insulin coated beads for 10 and 15 minutes, showed more bands as compared to the controls, C1 and C2, thereby detecting more proteins that were phosphorylated due to presence of insulin.

TCA (trichloroacetic acid) fixes proteins on cells and helps preserve cell structure. It inactivates tyrosine phosphatases (Hayashi et al., 1999). Vanadate is also an inhibitor of tyrosine

phosphatase activities which acts to preserve the phosphorylation of proteins of interest (Heffetz et al., 1990). It mimics insulin activity (Czech et al., 1974; Dubyak & Kleinzeller, 1980; May & de Haen, 1979; Schechter & Karlish, 1980). The control cells, C1 and C2 showed bands due to the presence of vanadate which may have prevented dephosphorylation in the cells. Another reason for C2 showing bands is that the beads that were present are hydrophobic in nature and so they attached to other proteins. The use of hydrophobic beads as a non-specific binding reference might provide a more meaningful control and this can be achieved by pre-blocking the beads with an inert control protein with physical properties similar to insulin. See Appendix E for quantification of bands on western blots using densitometric plots.

Cell transfection and confirmation of Akt-PH-GFP recruitment to cell membrane by confocal microscopy

Previous studies have been done showing the recruitment of Akt-PH-GFP to quantify the production of phosphorylated inositol (PIP3) at the activated receptor complex using IgG-coated beads in Fc Receptors (Jankowski et al., 2008). It was observed that the expression of the Akt-PH-GFP yielded intense fluorescent signals at the ligand microbeads and indicated an accumulation of PIP3 at the site of the activated receptor where ligand coated microbeads bound to the cell.

In the case of insulin receptors, the observation that Akt translocated to the plasma membrane may be explained by insulin dissolving from the beads and activating the IR over the cell surface. Loss of insulin into the medium might be prevented by treating the coated beads with

a cross-linking agent. This possibility might be confirmed by incubating beads in experimental medium, removing the beads by centrifugations and testing to see if the supernatant will activate the cells. Alternatively, the PIP3 produced at the site of IR activation rapidly diffuses around the cell membrane. These two possibilities are not mutually exclusive. There were a few instances where the insulin receptor appeared to collect around insulin coated beads and this is consistent with the nature of the IR that is known to be freely mobile (Lange, 2002). However as noted above, if the insulin receptors had already bound the activating ligand from the pool dissolved in the medium, then there would be no reason to collect around the ligand beads as observed.

A control experiment was done for a GFP-only plasmid in CHO cells (Appendix H). Fluorescence of high intensity was observed evenly throughout the cell, as compared to Akt-PH-GFP transfected cells where the fluorescence signal was slightly more around the nucleus, showing the location of Akt. The option of having another set of control cells that are transfected and treated with only beads can be proposed for future experiments which could provide information on how the beads alone act in the environment of cells transfected with Akt-PH-GFP. See Appendix I for more images of transfected CHO cells where fluorescence is intense at the site of beads.

Immunofluorescence staining for Phosphotyrosine, Insulin Receptor and Insulin

The failure to detect the insulin in the beads by immunostaining, as compared to Western blot, seems to indicate that the insulin antibody was not effective to bind insulin in the native state.

Although it remains possible that some insulin washed away from the beads, the pool of insulin that must have remained bound to the beads showed no reactivity at all with the antibody. Alternatively, it might be that the anti-insulin antibody was sterically hindered in its binding to insulin adsorbed to the beads. The failure of the insulin coated beads to act as a positive control make the insulin staining observed through the cell difficult to interpret. It may be possible that the insulin observed throughout the cell resulted from its binding to the receptor followed by internalization (Di Guglielmo et al., 1998).

The observation of a bright phosphotyrosine signal in the cells treated with insulin coated beads seems directly interpretable as the result of the well known chain of IR receptor phosphorylation followed by the phosphorylation of its cellular targets (Di Guglielmo et al., 1998).

The observation that the IR was detected with a strong signal in the positive cells but the signal was markedly reduced in the control cells may indicate that the antibody binds with a great affinity to the receptor after it has changed its conformation upon binding its ligand or perhaps after phosphorylation. However, taken together the observations that insulin in the cell but not the beads, the anti-phosphotyrosine and anti-IR antibodies, all showed stronger signalling in the positive experiments but not in the control, may indicate the presence of some sort of systemic bias in the experiments that remains to be defined. See Appendix L for quantification of fluorescence signal intensity for immunostain images of CHO cells.

Isolation of Insulin receptor complex on the beads

The apparent successful capture of the insulin receptor in cells without treatment by DFP seems on the surface to be a validation of the approach of ligand affinity capture of the IR complex. However the observations may also be interpreted to indicate that IR is rapidly degraded in crude cell extracts and does not survive long enough to non-specifically adsorb to the polystyrene beads. Alternatively, the result may indicate that the insulin receptor may be more resistant to degradation when bound to its ligand on microbeads. Taken together, these results indicate that it may be necessary to block the insulin coated beads with another inert protein to prevent non-specific background binding in the controls beads.

A second thick band was observed below 66 kDa for all the cell lines in both DFP treated and untreated conditions, which is a protein of unknown identity (O'Neill et al., 1996) that could be a splice variant also. See Appendix O for densitometric plots.

CONCLUSION

For the purpose of this study, three mammalian cell lines were used, each belonging to a different organ, species and having different morphologies. These cell lines were not over-expressed or mutated with insulin receptors and were also easy to transfect. All the cell lines produced close to similar results, however, the CHO cells were easier to grow and work with. Compared to COS-7 and NIH-3T3, the CHO cells also produced a thicker band for insulin receptor in the western blot experiment for detection of the receptor. Western blots helped to detect presence of insulin receptor in the cells in the presence of DFP and helped to confirm that tyrosine phosphorylation occurs in response to insulin which is a crucial step in insulin signaling process. The best and maximum phosphorylation occurred at 15 minutes incubation of cells with insulin coated beads as shown by phosphotyrosine blots and immunostain images. The PH domain of Akt was fused to green fluorescent protein (GFP) to monitor the distribution of phosphoinositide (PIP3) in live cells acting as a biosensor for phosphatidylinositol. Confocal microscopy helped to analyze the translocation of the protein at the site of insulin receptor activation. Microbeads coated with insulin ligand proved helpful in regulating protein-phospholipid interaction in mammalian cells. The main objective of this study was to use the above mentioned experimental tools in order to develop a model to isolate activated insulin receptor and identify novel proteins to investigate mechanism of diseases like diabetes. Activated receptor complex from the surface of live cells was successfully captured and isolated using ligand-coated microbeads (LAC) which could further lead to identification of members of the receptor complex or pathway by liquid-chromatography and mass spectrometry. LAC could provide useful information on the isoforms of the protein families involved in insulin receptor

function. Similar model of study could be applicable to a variety of other cell surface receptors for understanding their ligand-receptor complexes in cells.

FUTURE OBJECTIVES

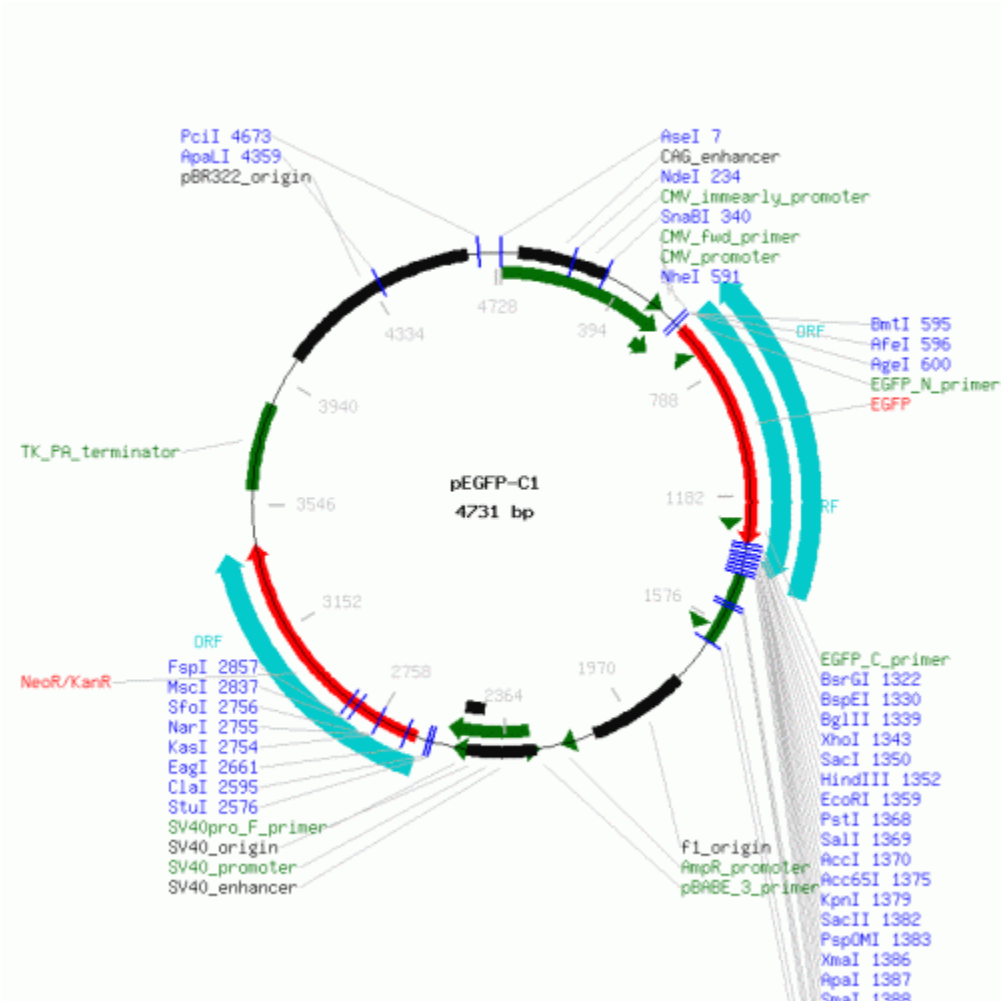
An issue of concern that arose from the results of immunostaining and cell transfection experiments was the leaking out or washing away of the insulin ligand from the beads. An alternative approach to overcome this problem in future experiments could be the use of chemical agents for cross-linking the insulin ligand to the beads instead of merely incubating the beads with insulin which was done in this study.

The isolated and captured insulin receptor complex can be subjected to mass spectrometry for further analysis. Mass spectrometry is a well-known method used for the identification and analysis of proteins in cell lysates. It is a fast, powerful, selective, sensitive and an ideal tool for the analysis of post-translational modifications (McLachlin & Chait, 2001; Annan et al., 2001; Mann et al., 2002), since that cannot be determined from DNA sequence alone. Phosphorylation on serine, threonine or tyrosine residues is an important and very common post-translational modification of proteins (Benjamin et al., 2005). There are several proteins (members of the receptor family) attached to the receptor complex that could be identified using the mass spectrometer.

APPENDICES

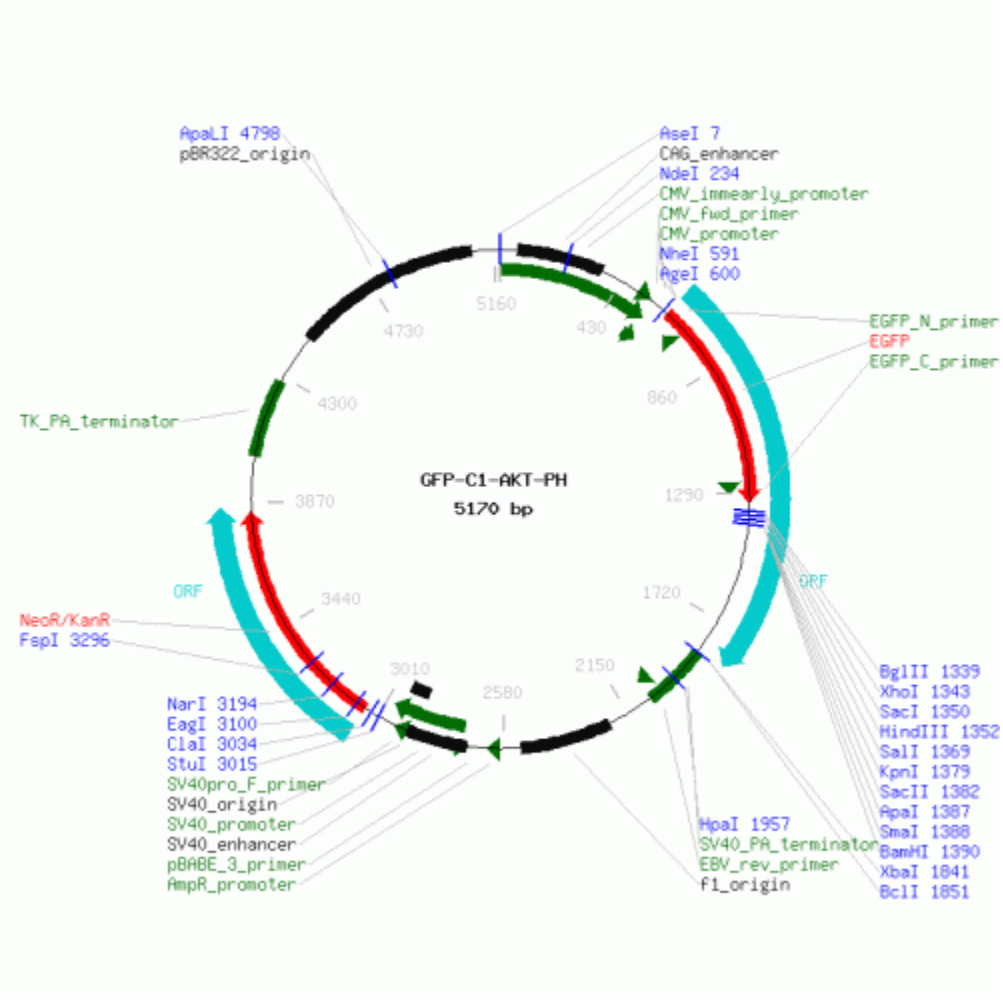
APPENDIX A- Plasmid information: pEGFP-C1

Plasmid name	pEGFP-C1
Source/Vendor	Clontech
Plasmid type	Mammalian
Plasmid size	4700
Bacterial Resistance	Kanamycin



APPENDIX B- Akt insert information

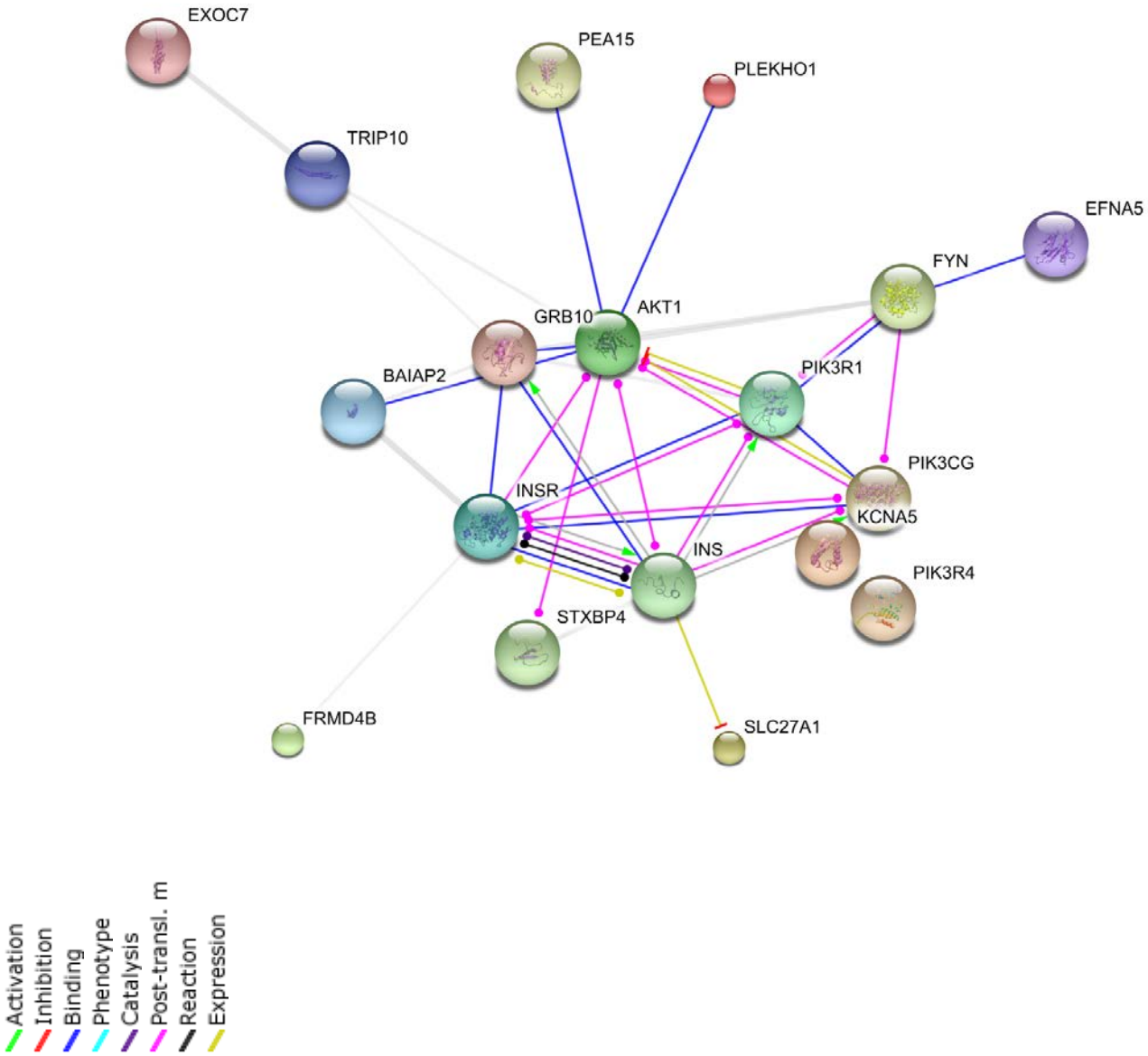
Gene/insert name:	AKT-PH
Insert size (bp):	457
Species of gene(s):	M. musculus (mouse)
Fusion proteins or tags:	GFP
Vector backbone:	C1
Backbone manufacturer:	Clontech
Type of vector:	Mammalian expression
Backbone size (bp):	4700
Bacteria resistance:	Kanamycin



Taken from Lablife Addgene Vector database.

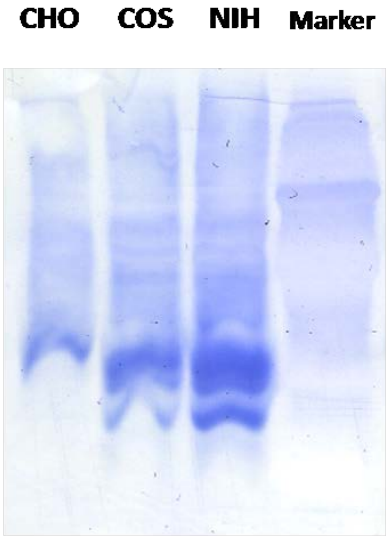
APPENDIX C- STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) diagram

A database of known and predicted protein interaction that displays proteins that interact with the receptor in many ways by binding, linking, activating, catalyzing, inhibiting etc. to give an outlook of what kind of interacting proteins may be available in the insulin receptor complex.

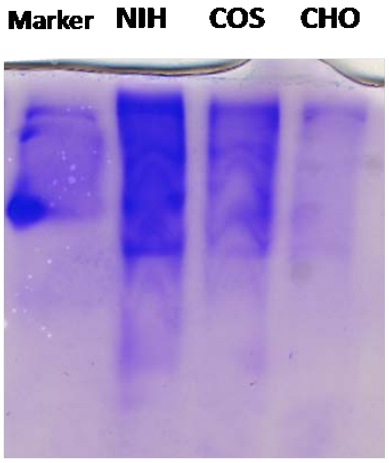


APPENDIX D- Loading controls for western blot detection of insulin receptor in cell lysates. Coomassie stained a) PVDF transfer membrane and b) gel.

a)

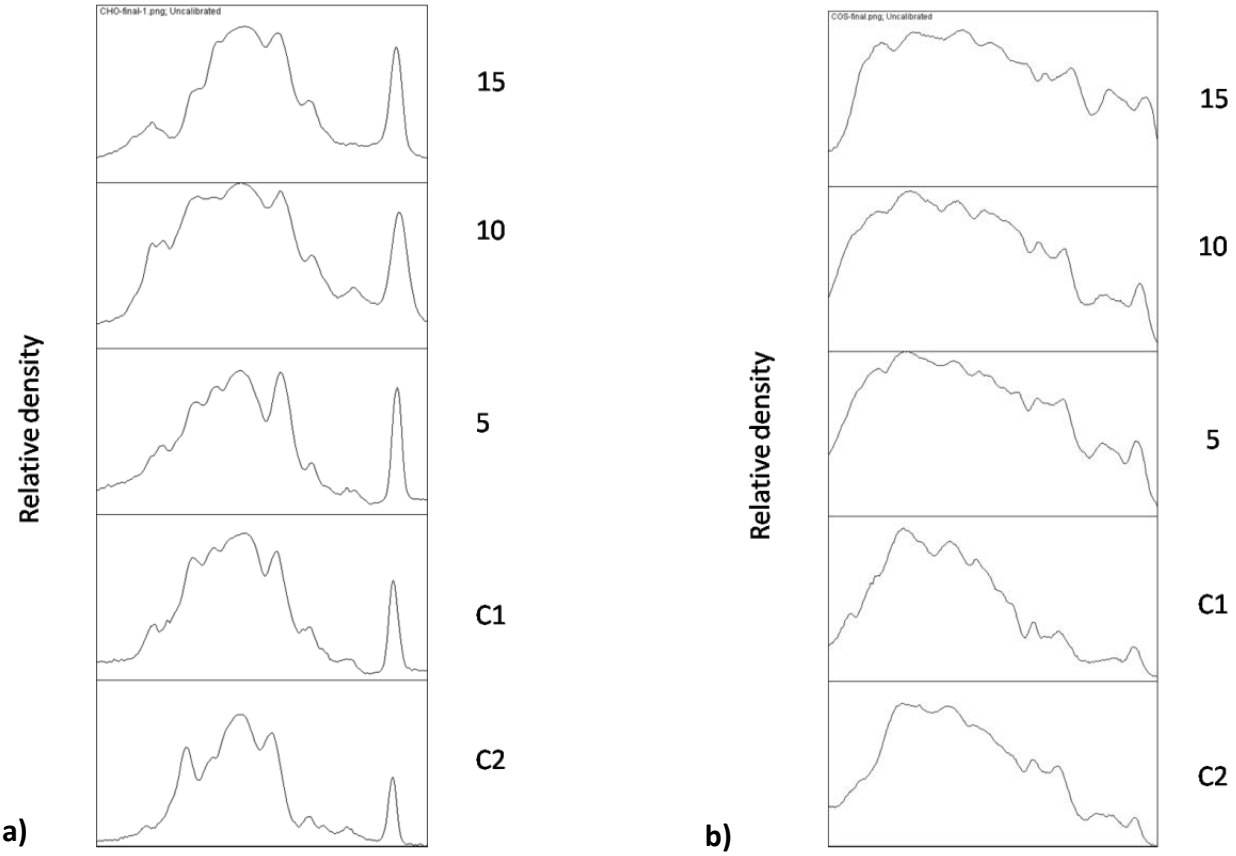


b)

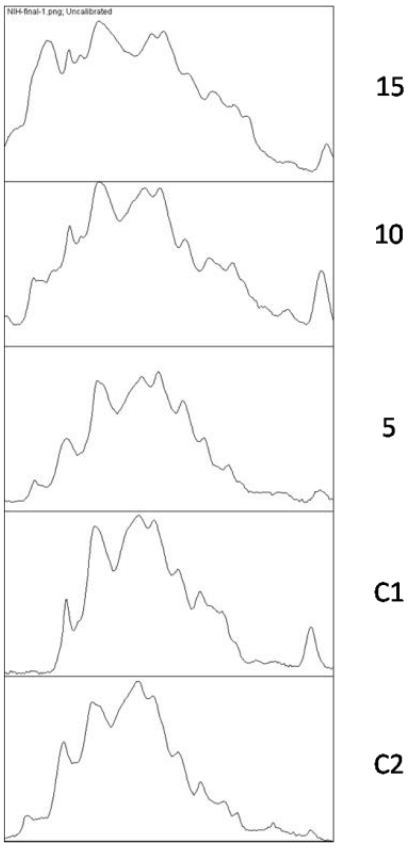


APPENDIX E- Densitometric plots showing quantification of western blots of phosphotyrosine activity in cell lysates: a) CHO, b) COS-7 and c) NIH-3T3

Densitometry was performed using Image J analysis software (NIH). The profile plot represents the relative density of the contents of the rectangle over each lane. Higher peaks represent darker bands. Wider peaks represent bands that cover a wider size range on the original gel.

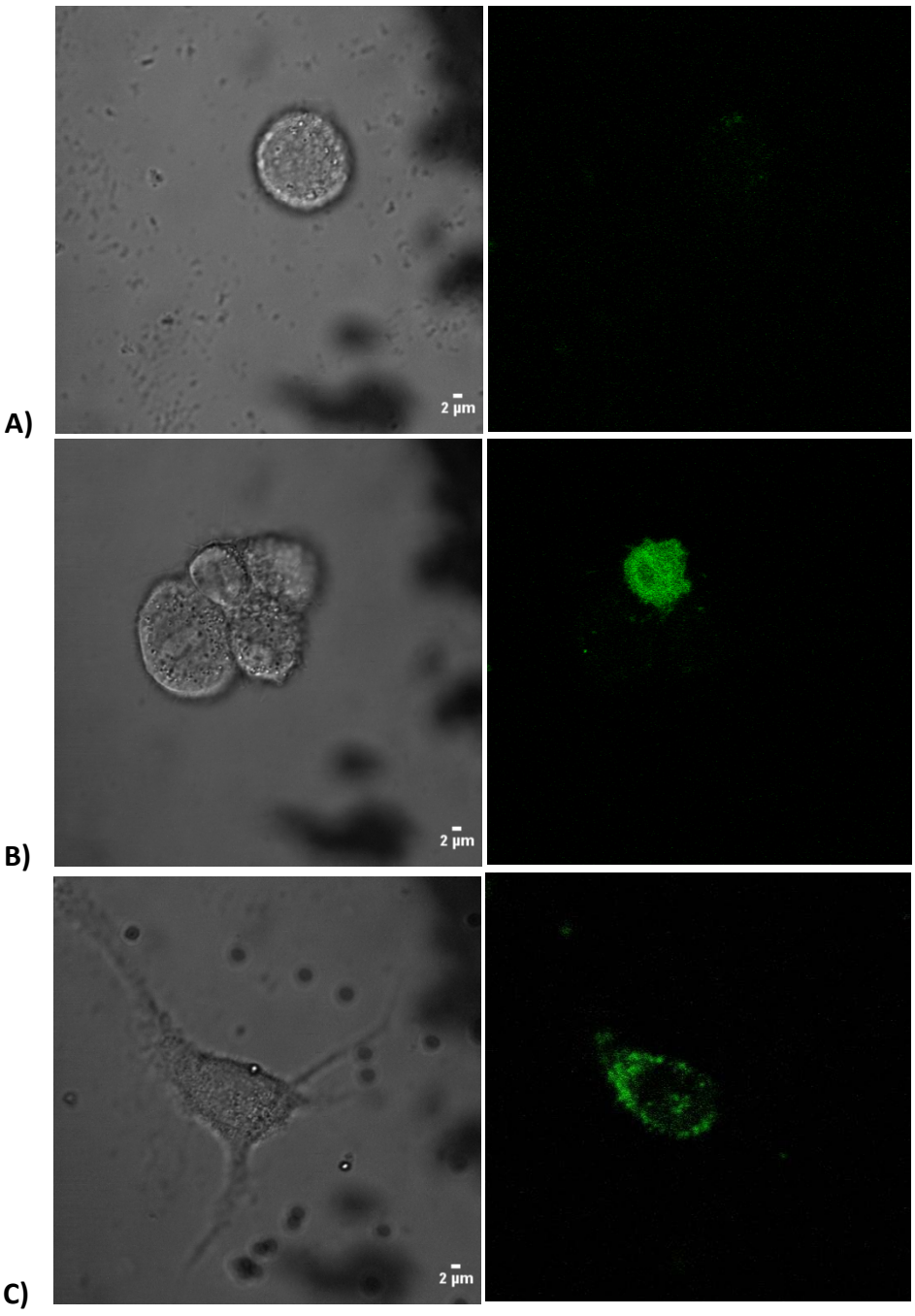


c)



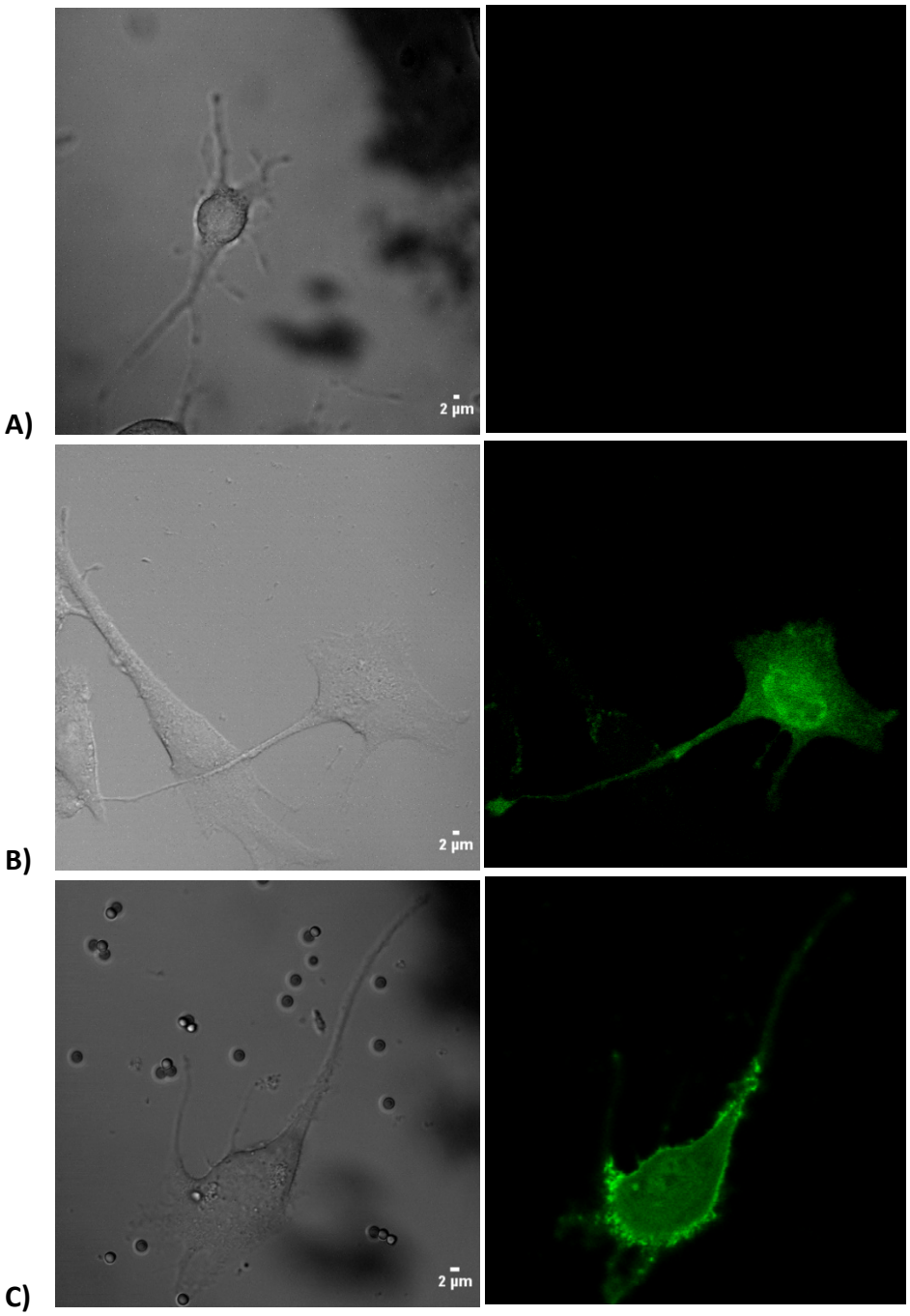
APPENDIX F- Transfection of COS-7 cells with Akt-PH-GFP to confirm its recruitment to the cell membrane in response to receptor activation by insulin coated microbeads.

A) Non-transfected cells (control); B) Transfected cells (expression of Akt-PH-GFP); C) Localization of Akt-PH-GFP after incubation of transfected cells with insulin coated beads for 15 minutes.

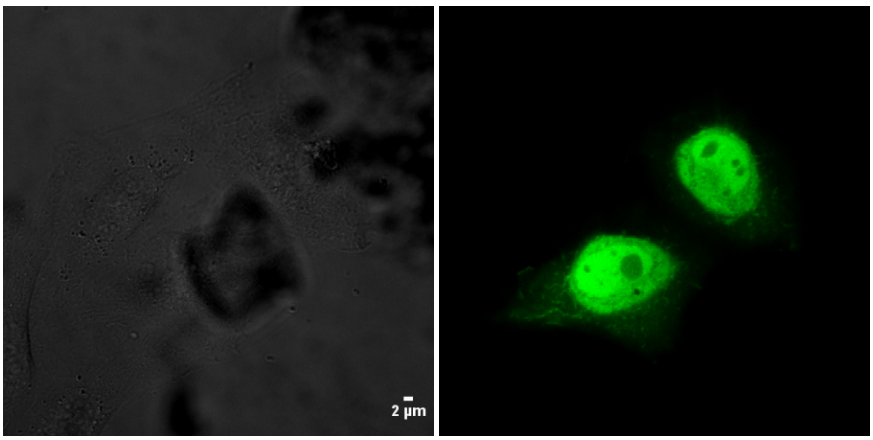


APPENDIX G- Transfection of NIH-3T3 cells with Akt-PH-GFP to confirm its recruitment to the cell membrane in response to receptor activation by insulin coated microbeads.

A) Non-transfected cells (control); B) Transfected cells (expression of Akt-PH-GFP); C) Localization of Akt-PH-GFP after incubation of transfected cells with insulin coated beads for 15 minutes.

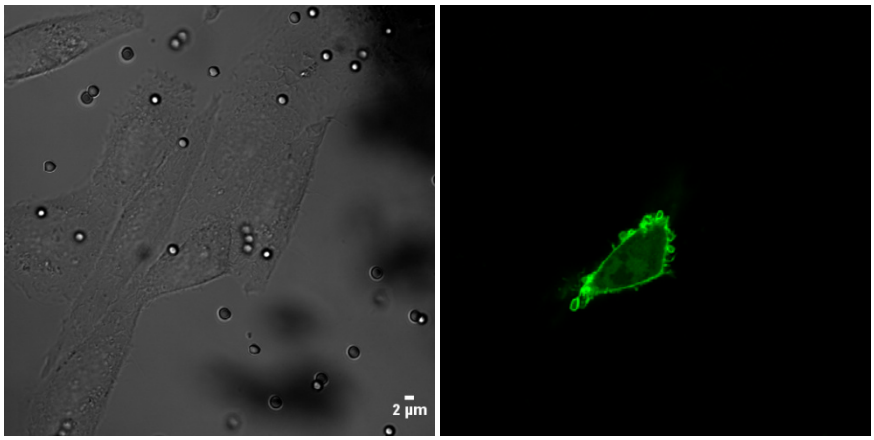


APPENDIX H- GFP only test

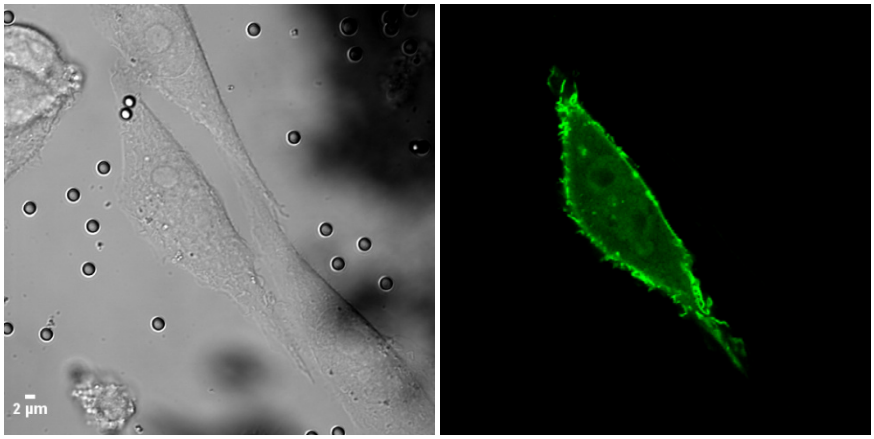


APPENDIX I- CHO cell transfections with insulin coated beads showing intense fluorescence at the beads.

A)



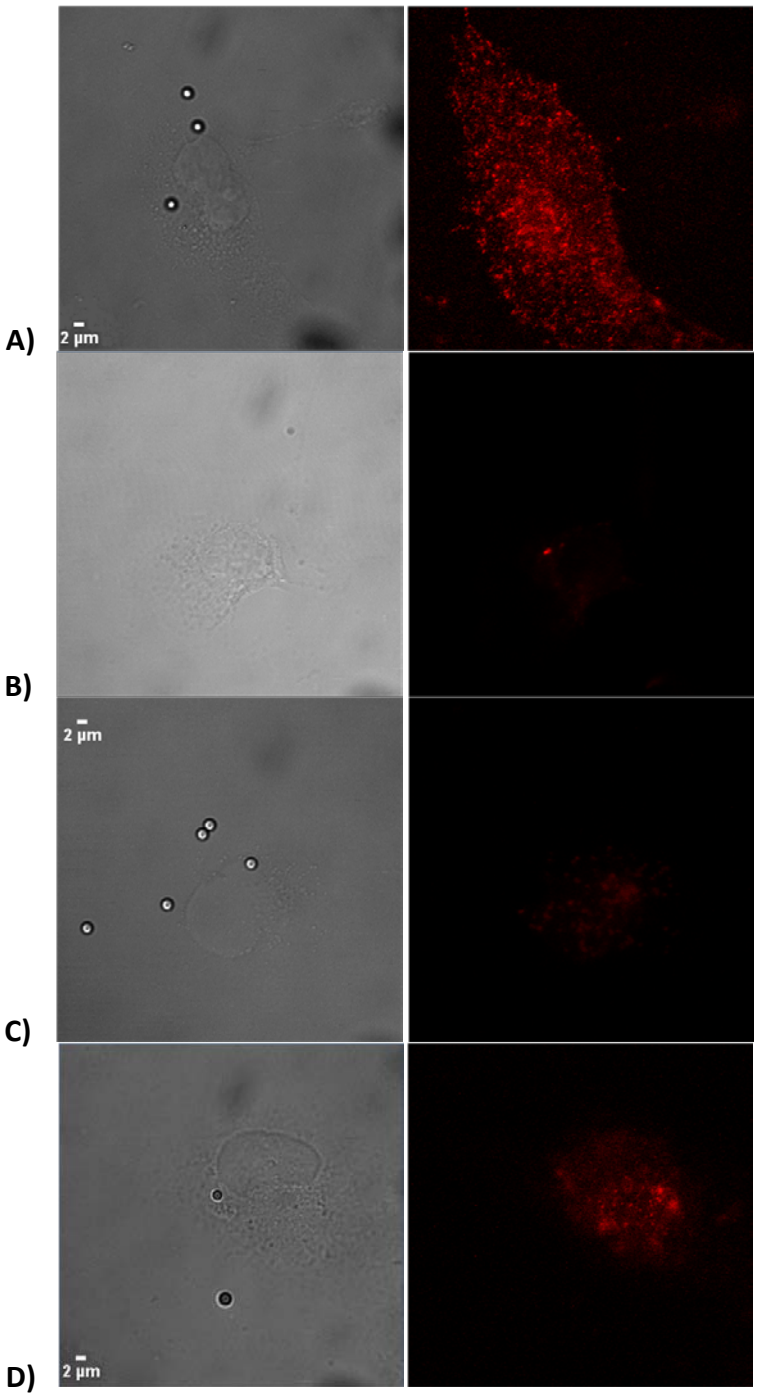
B)



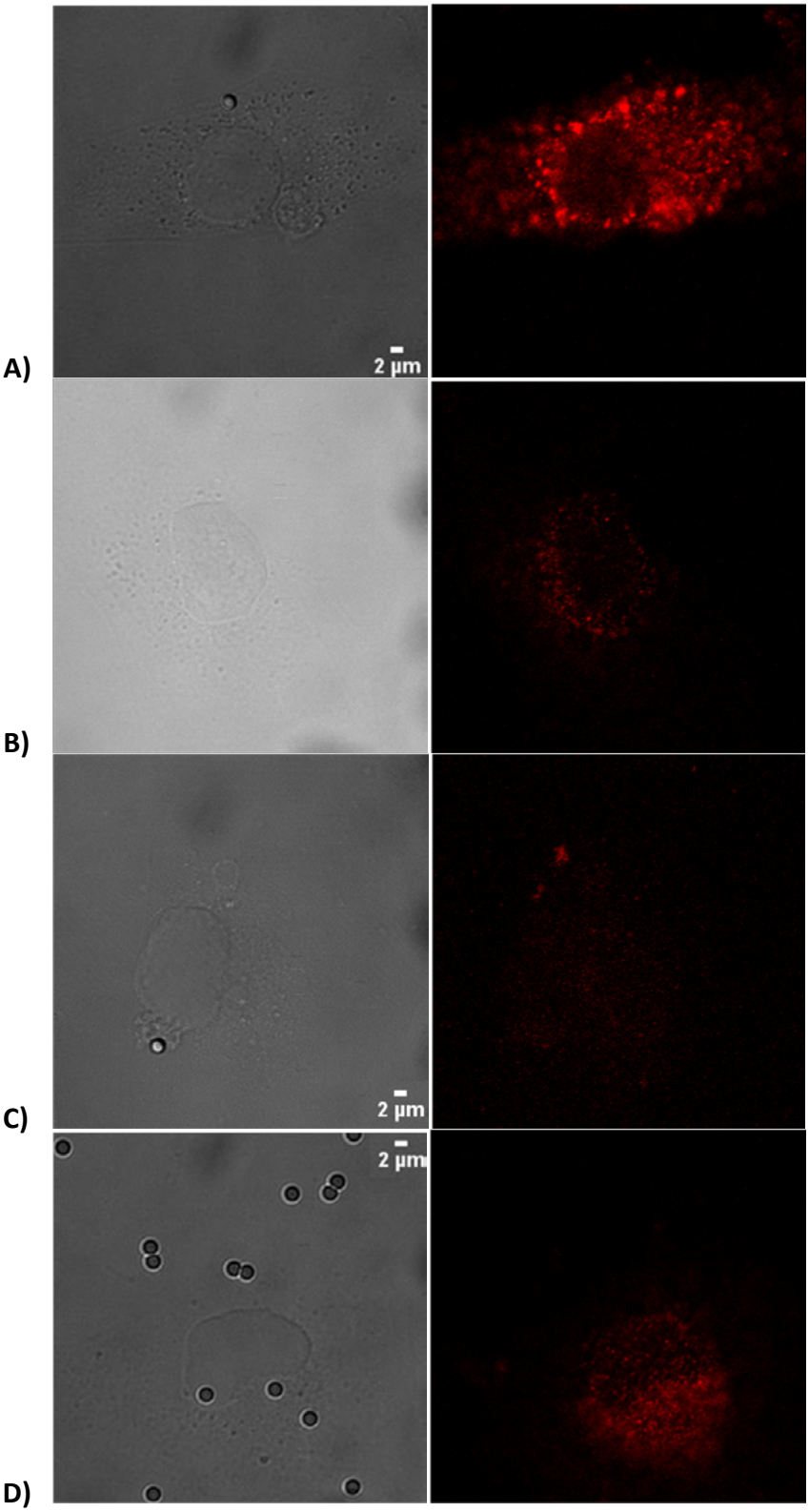
APPENDIX J- Immunostaining images of COS-7 cells.

A) 15 minutes incubation with insulin coated beads; B) No incubation with beads or insulin, 0 minute (C1); C) 15 minutes incubation with insulin coated beads, stained with only secondary antibody, no primary antibody (C2); D) 15 minutes incubation with only beads (C3).

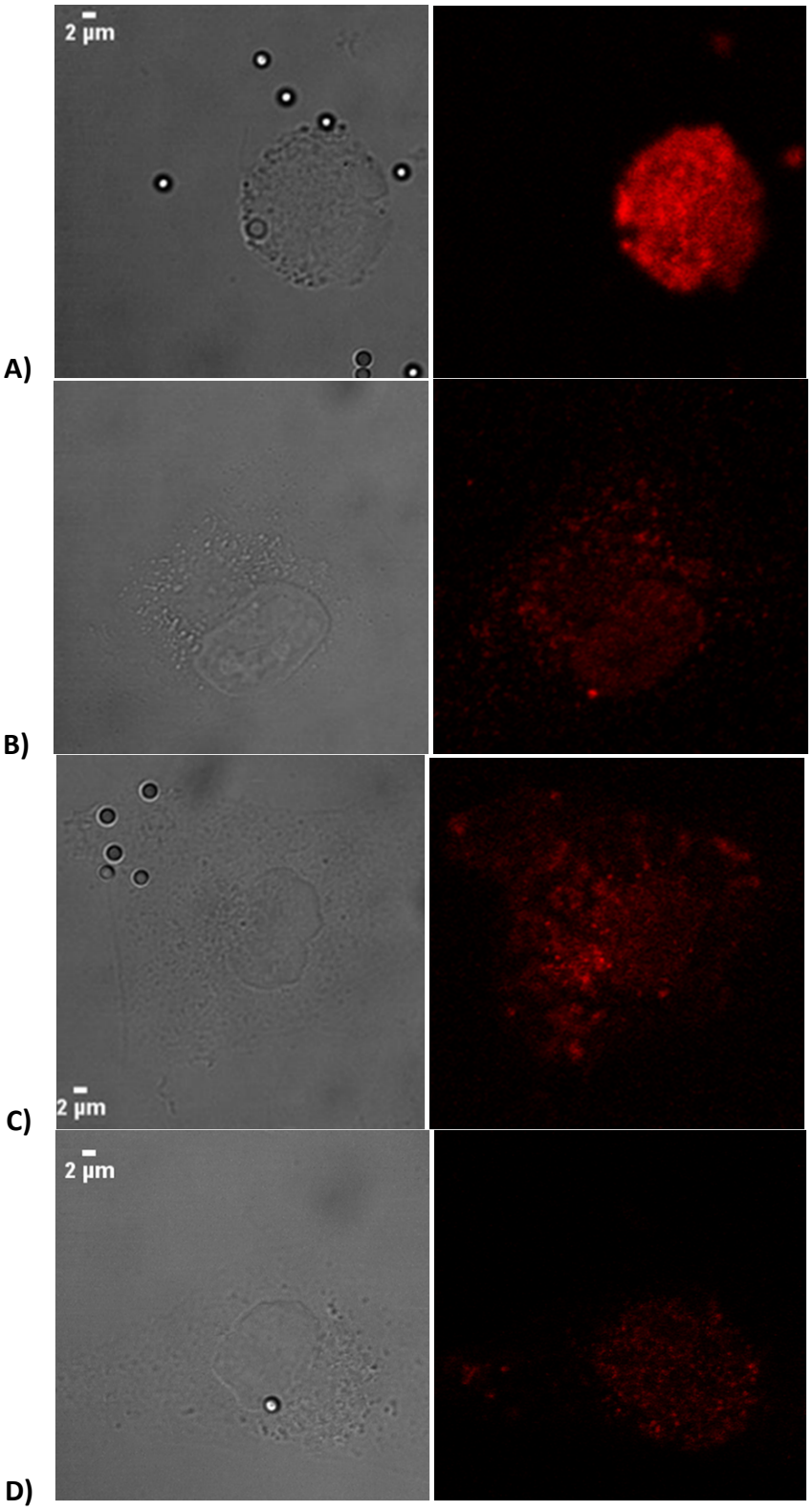
1. **Phosphotyrosine activity:**



2. Insulin receptor:



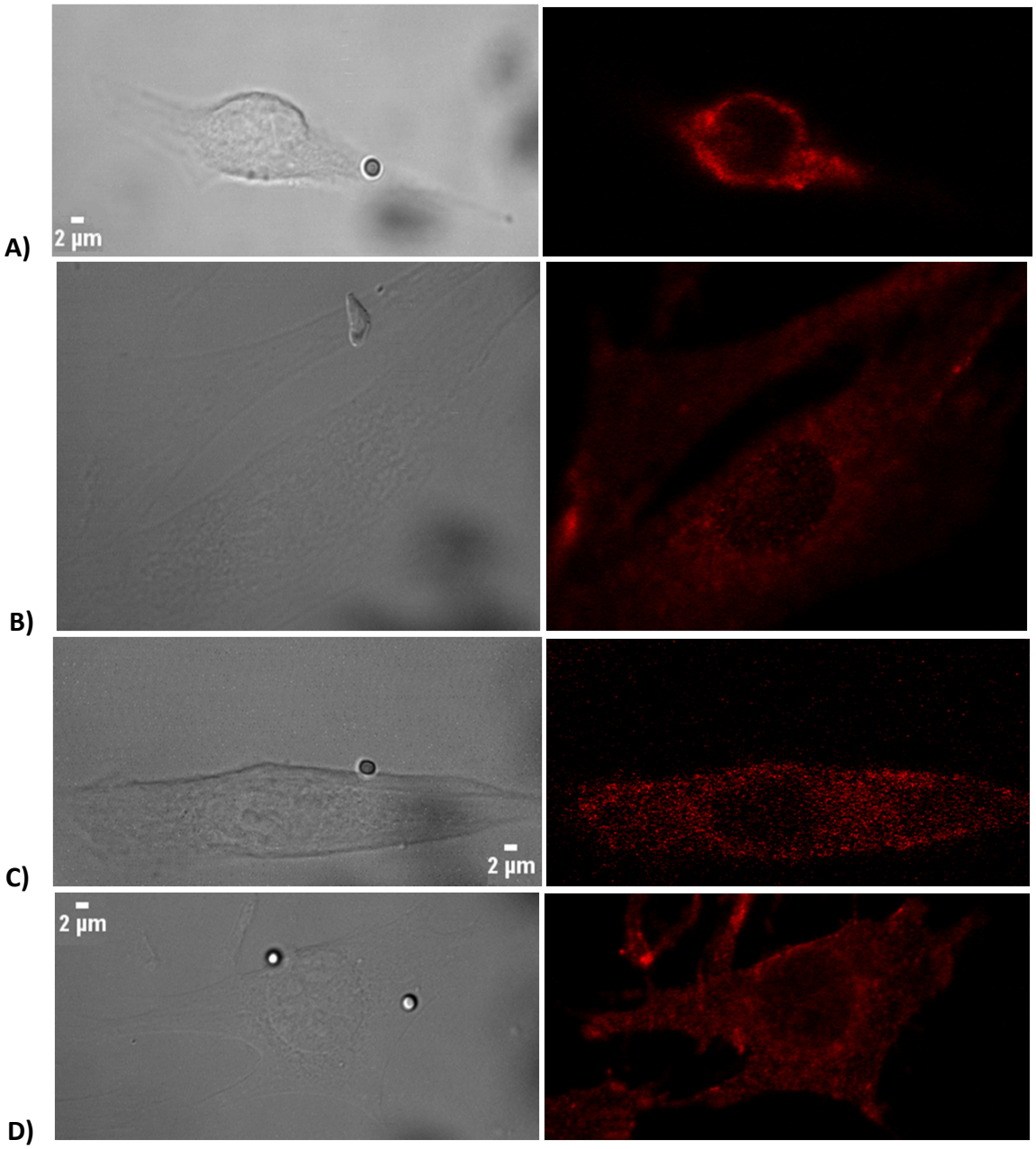
3. Insulin:



APPENDIX K- Immunostaining images of NIH-3T3 cells.

A) 15 minutes incubation with insulin coated beads; B) No incubation with beads or insulin, 0 minute (C1); C) 15 minutes incubation with insulin coated beads, stained with only secondary antibody, no primary antibody (C2); D) 15 minutes incubation with only beads (C3).

1. **Phosphotyrosine activity:**



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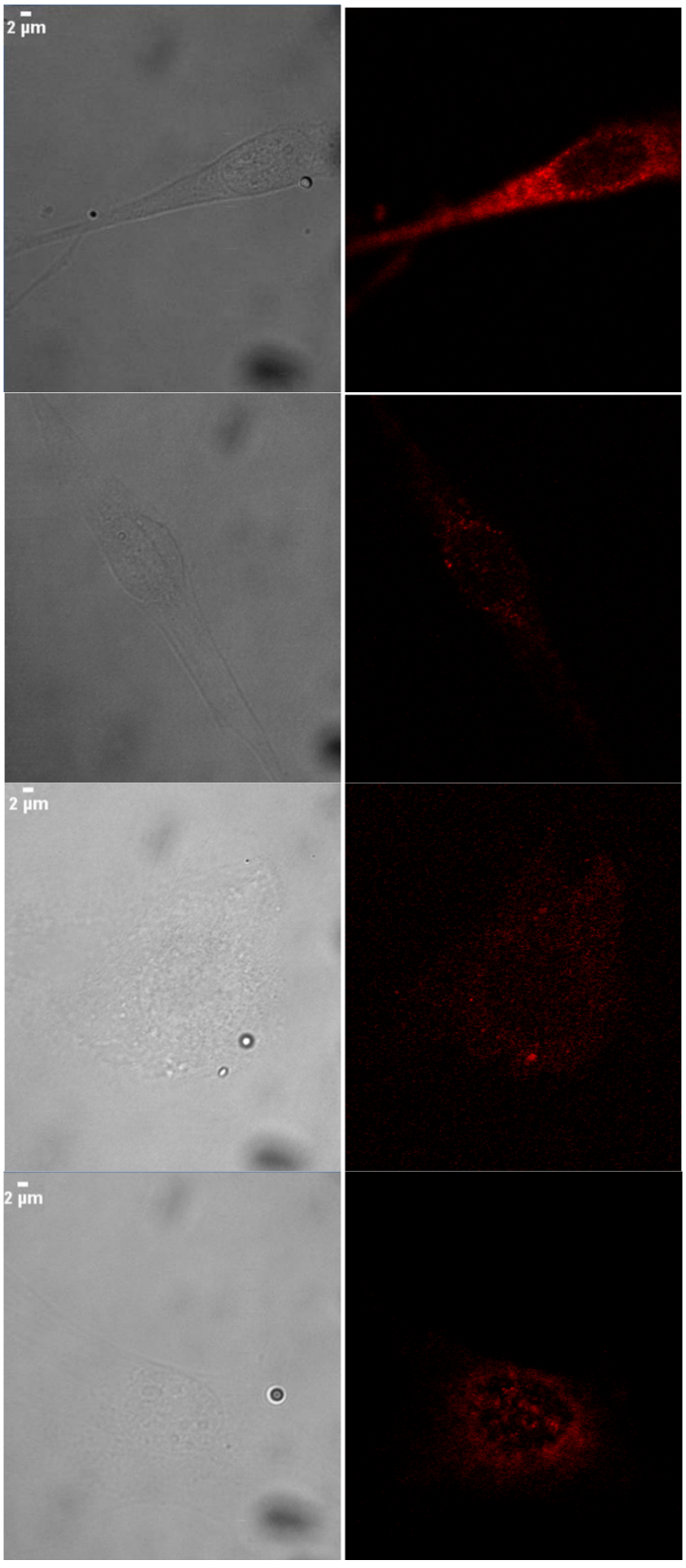

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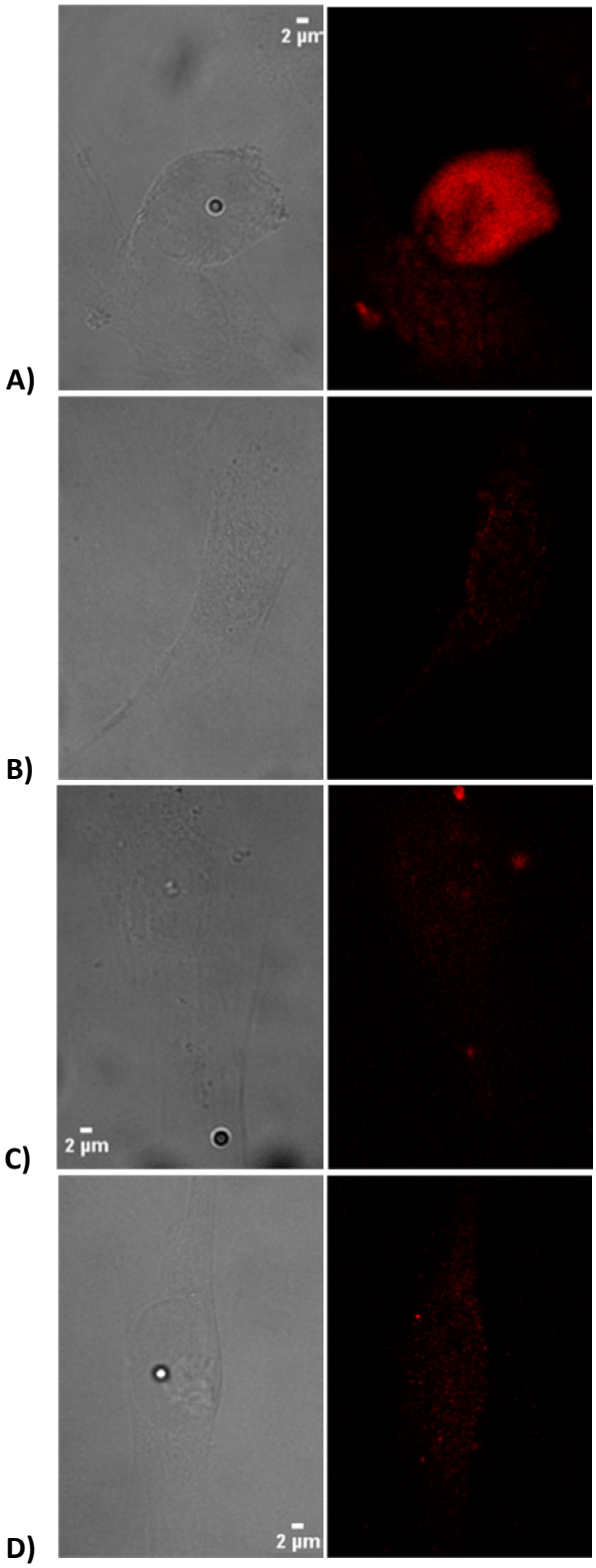
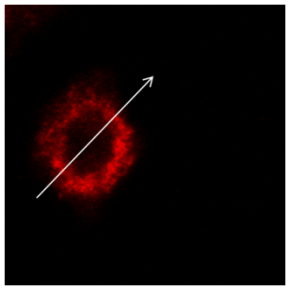


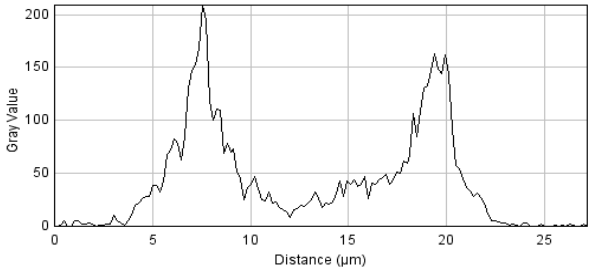
APPENDIX L- Quantification of fluorescence signal intensity for immunostain images of CHO cells.

A) 15 minutes incubation with insulin coated beads; B) No incubation with beads or insulin, 0 minute (C1); C) 15 minutes incubation with insulin coated beads, stained with only secondary antibody, no primary antibody (C2); D) 15 minutes incubation with only beads, without insulin (C3). Performed using Image J analysis software (NIH). The signal intensity graph measures intensity of fluorescence along the length of the white arrow on the microscope image.

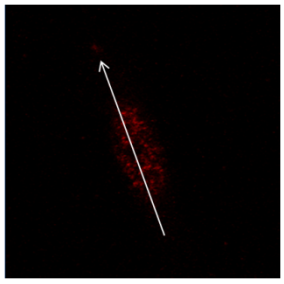
1. **Phosphotyrosine activity:**

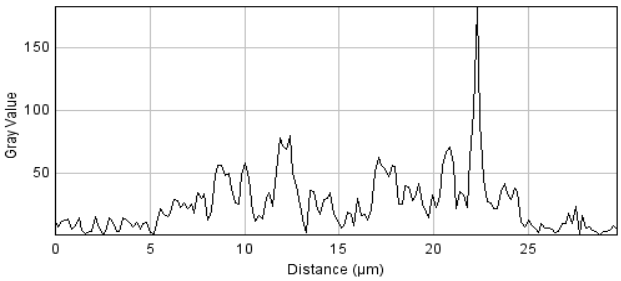
A)





B)





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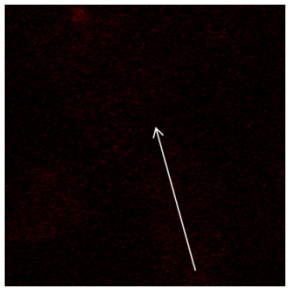
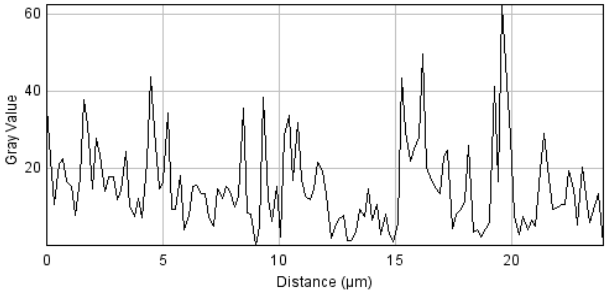
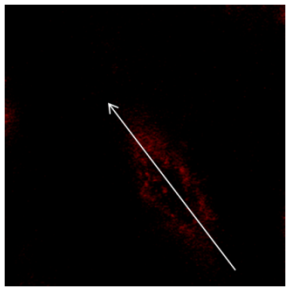
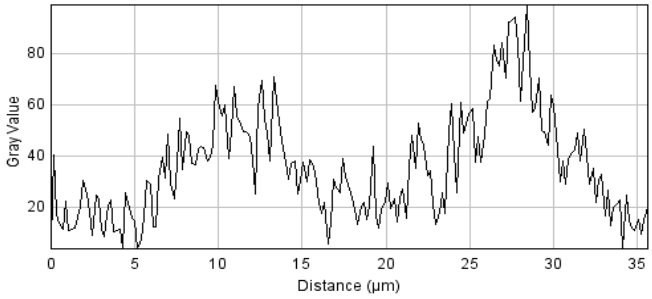




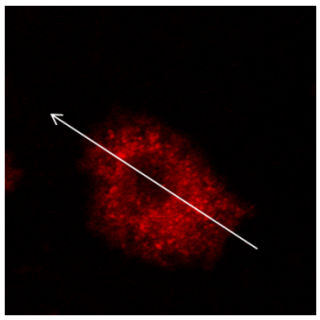
D)

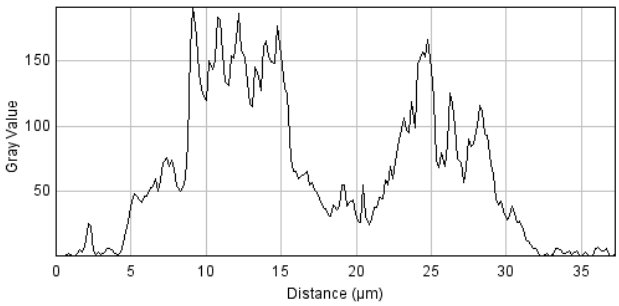




2. Insulin receptor:

A)





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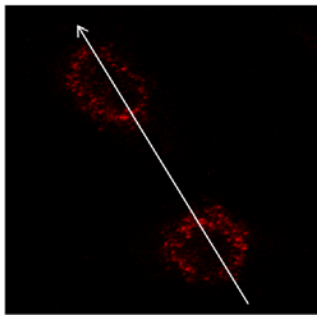
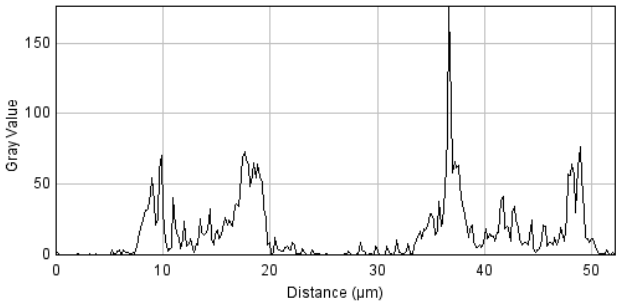
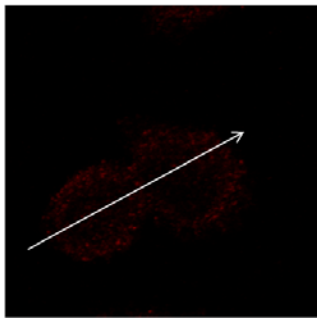
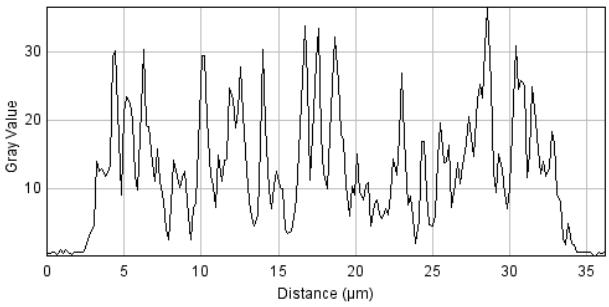


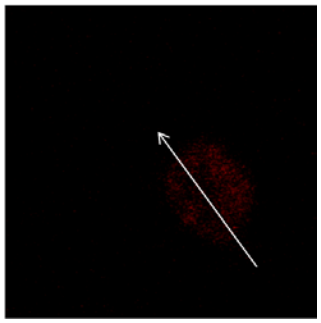


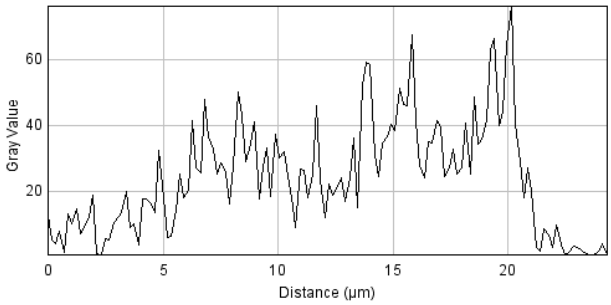
C)





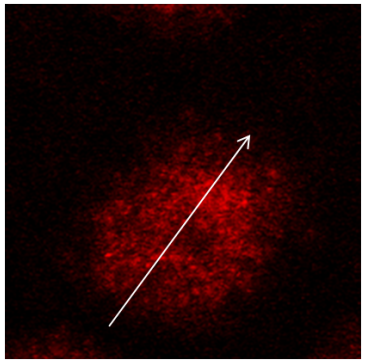
D)

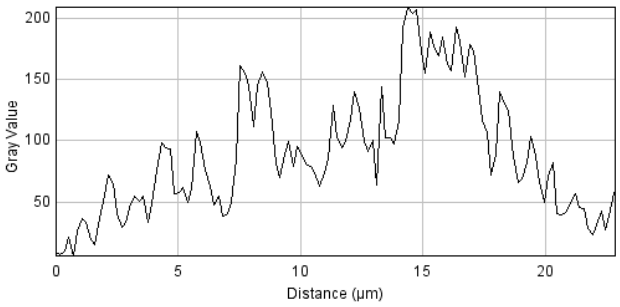




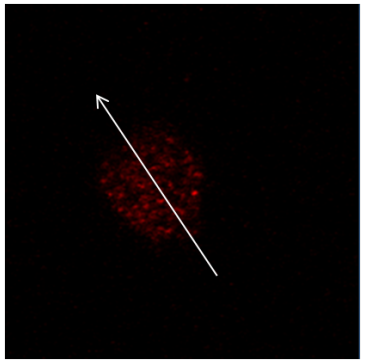
3. Insulin:

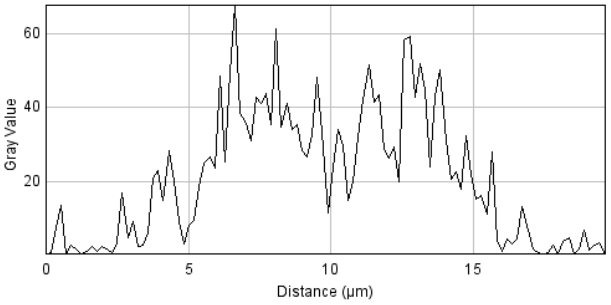
A)



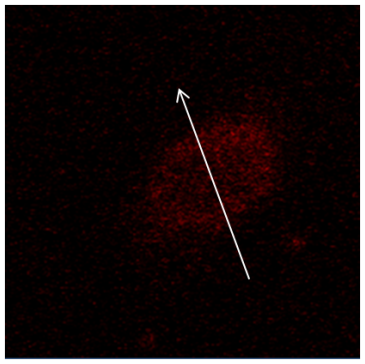


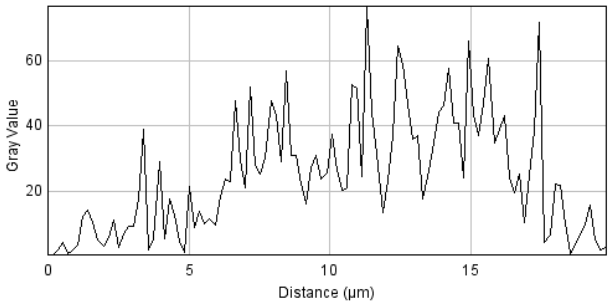
B)



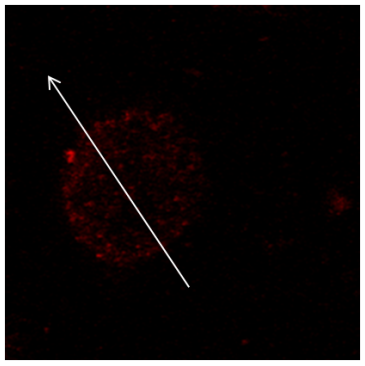


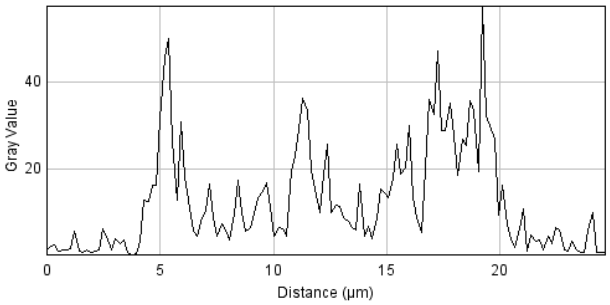
C)





D)

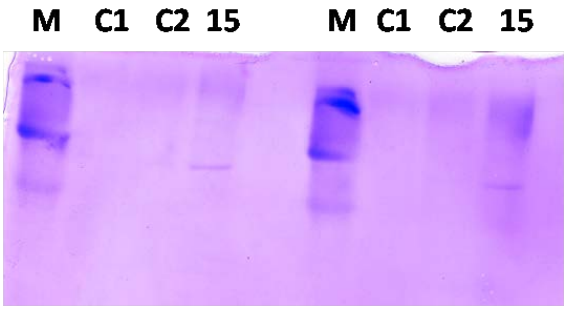


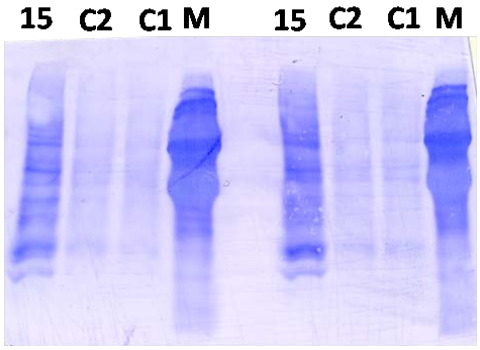


APPENDIX M- Loading controls for western blots of LAC samples for a) CHO and b) NIH-3T3 cell lysates treated with DFP.

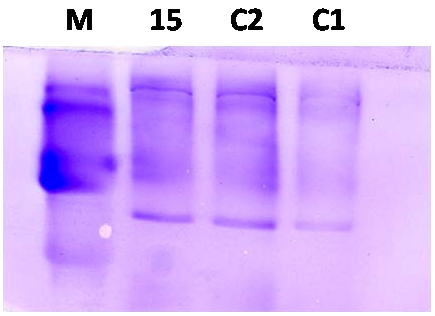
Coomassie stained 1) gel and 2) PVDF transfer membrane.

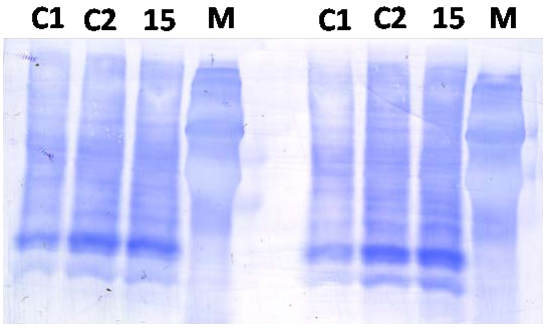
a)

1. 

2. 

b)

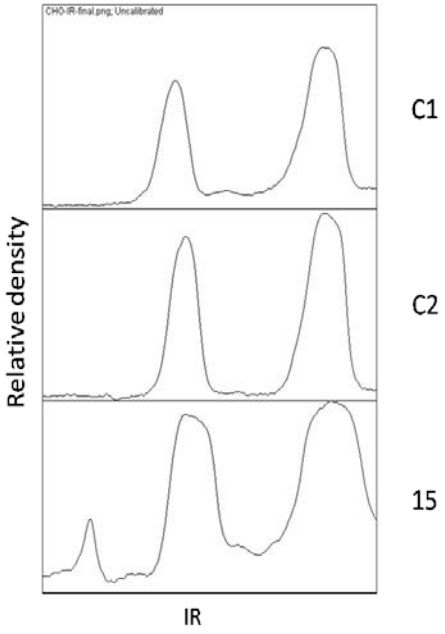
1. 

2. 

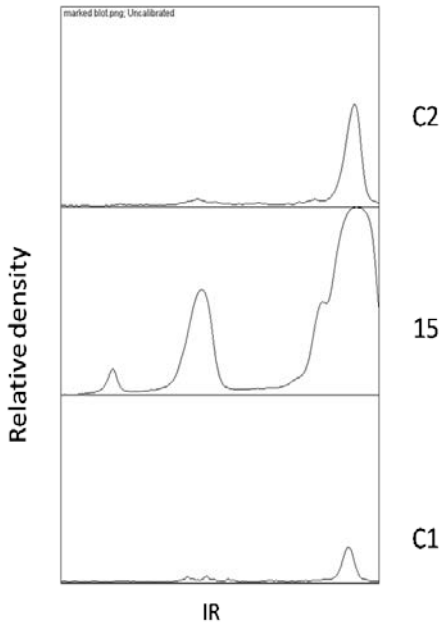
APPENDIX N- Densitometric plots for western blots of LAC samples for detection of insulin receptor in A) CHO, B) COS-7 and C) NIH-3T3 cell lysates treated with 1) DFP and 2) without DFP.

Densitometry was performed using Image J analysis software (NIH). The profile plot represents the relative density of the contents of the rectangle over each lane. Higher peaks represent darker bands. Wider peaks represent bands that cover a wider size range on the original gel.

A) CHO

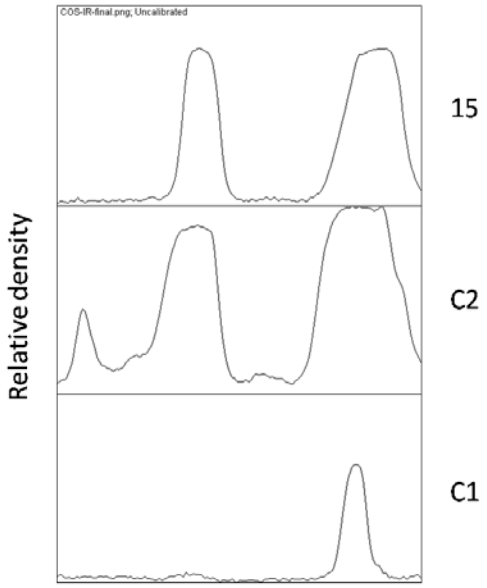


1.

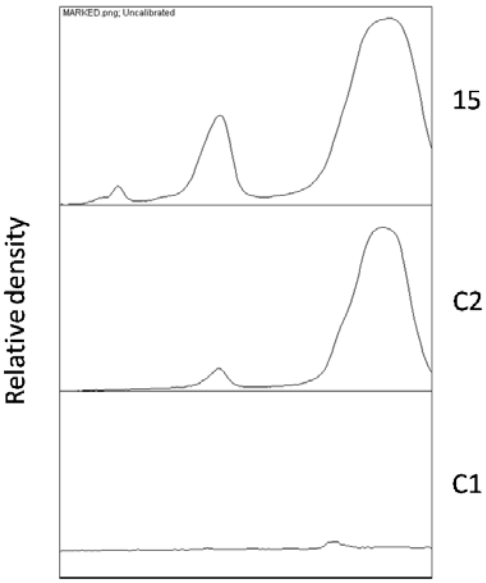


2.

B) COS-7

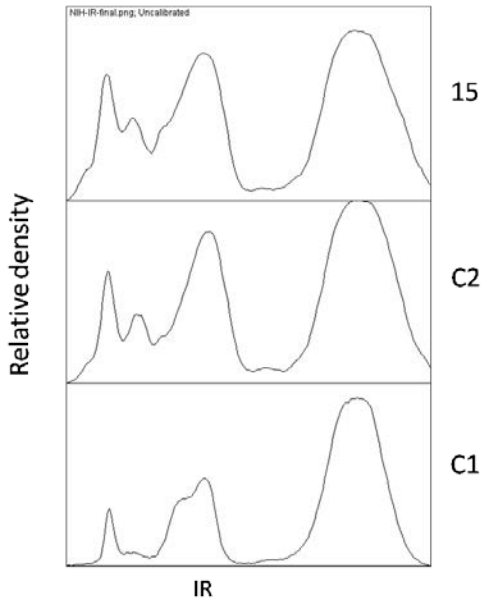


1.

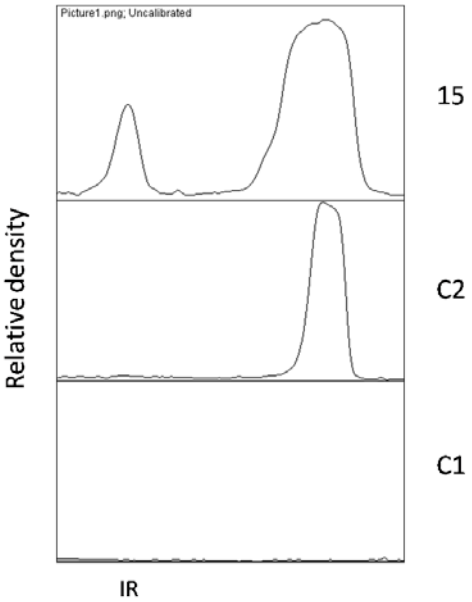


2.

C) NIH-3T3



1.

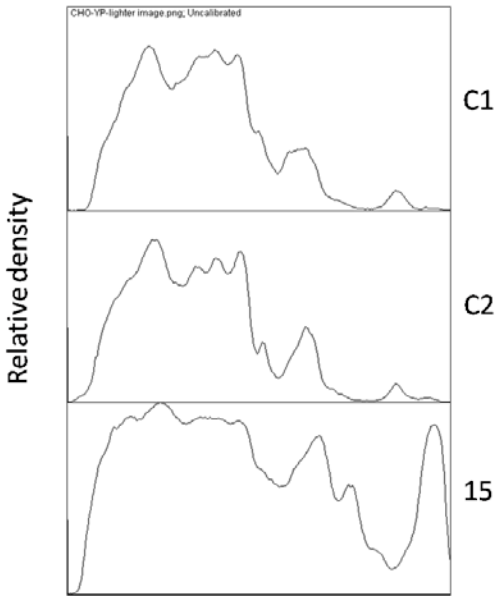


2.

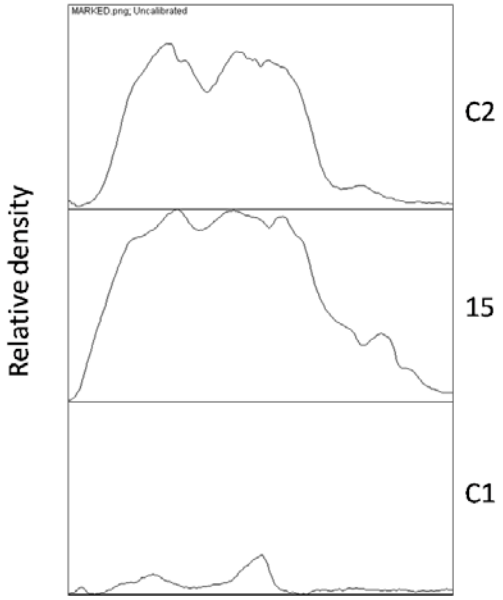
APPENDIX O- Densitometric plots for western blots of LAC samples for detection of phosphotyrosine activity in A) CHO, B) COS-7 and C) NIH-3T3 cell lysates treated with 1) DFP and 2) without DFP.

A) CHO

1.

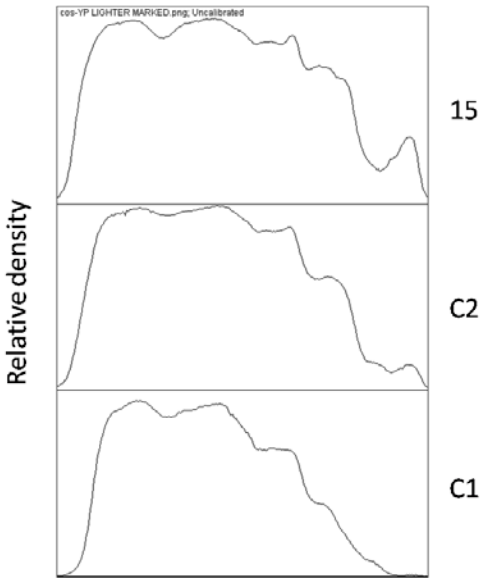


2.

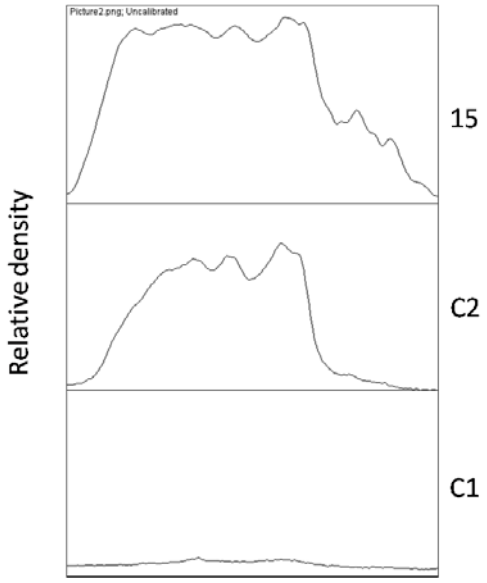


B) COS-7

1.

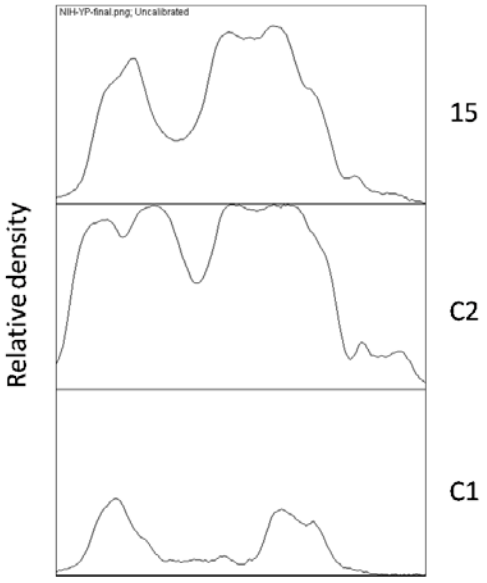


2.

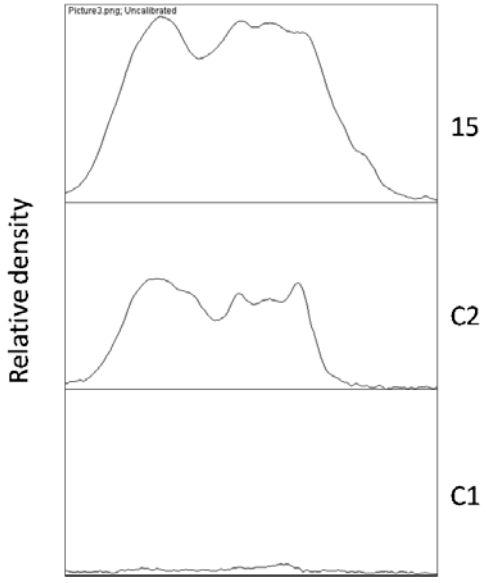


C) NIH-3T3

1.



2.



REFERENCES

1. Accili, D., Frapier, C., Mosthaf, L., McKeon, C., Elbein, S. C., Permutt, M. A., Ramos, E., Lander, E., Ullrich, A., Taylor, S. I. (1989). A mutation in the insulin receptor gene that impairs transport of the receptor to the plasma membrane and causes insulin-resistant diabetes. *EMBO J.,* 8(9), 2509-17.
2. Alessi, D. R. and Downes, C. P. (1998). The role of PI 3-kinase in insulin action. *Biochim Biophys Acta.*, 1436, 151-64.
3. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol.*, 7, 261-269.
4. Anderson, D., Koch, C. A., Grey, L., Ellis, C., Moran, M. F. and Pawson, T. (1990). Binding of SH2 domains of phospholipase C-yl, GAP, and Src to activated growth factor receptors*. Science,* 250, 979-982.
5. Annan, R. S., Huddleston, M. J., Verma, R., Deshaies, R. and Carr, S. A. (2001). A multidimensional electrospray MS-based approach to phosphopeptide mapping. *Anal. Chem.* 73, 393-404.
6. Backer, J. M., Myers, M. G., Jr., Shoelson, S. E., Chin, D. J., Sun, X. J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J. and White, M. F. (1992). Phosphatidylinositol 3-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J.,* 11, 3469-3479.

7. Bajaj, M., Waterfield, M. D., Schlessinger, J., Taylor, W. R. and Blundell, T. (1987). On the tertiary structure of the extracellular domains of the epidermal growth factor and insulin receptors. *Biochim Biophys Acta*, 916, 220-226.
8. Baker, E. N., Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. C., Hubbard, R. E., Isaacs, N. W., Reynolds, C. D., Sakabe, K., Sakabe, N. and Vijayan, N. M. (1988). The structure of 2Zn pig insulin crystals at 1.5 A resolution. *Phil Trans R Soc Lond B*, 319: 369-456.
9. Baltensperger, K., Kozma, L. M., Jaspers, S. R. and Czech, M. P. (1994). Regulation by insulin of phosphatidylinositol 3-kinase bound to *α*- and β-isoforms of p85 regulatory subunit. *The Journal of Biological Chemistry*, 269, 46, 28937-28946.
10. Bellacosa, A., Testa, J. R., Staal, S. P. and Tsichlis, P. N. (1991). A retroviral oncogene, Akt, encoding a serine-threonine kinase containing an SH2-like region. *Science*, 254, 274-277.
11. Bernier, M., Liotta, A. S., Kole, H. K., Shock, D. D. and Roth, J. (1994). Dynamic regulation of intact and C-terminal truncated insulin receptor phosphorylation in permeabilized cells. *Biochemistry,* 33, 4343-4351.
12. Brazil, D.P., Park, J. and Hemmings, B.A. (2002). PKB binding proteins. Getting in on the Akt. *Cell*, 111, 293-303.
13. Cantley, L.C. (2002). The phosphoinositide 3-kinase pathway. *Science*, 296, 1655-1657.
14. Cantrell, D. A. (2001). Phosphoinositide 3-kinase signaling pathways. *J Cell Sci.*, 114 (8), 1439-45.

15. Carpenter, C. L., Auger, K. R., Chanudhuri, M., Yoakim, M., Schaffhausen, B., Shoelson, S. and Cantley, L. C. (1993). Phosphoinositide 3-kinase is activated by phosphopeptides that bind to the SH2 domains of the 85 kD subunit. *J. Biol. Chem.,* 268, 9478-9483.

16. Chevallet, M., Luche, S. and Rabilloud, T. (2006). Silver staining of proteins in polyacrylamide gels. *Nat Protoc*., 1(4): 1852–1858.

17. Chou, C. K., Dull, T. J., Russell, D. S., Gherzi, R., Lebwohl, D., Ullrich, A. and Rosen, O. M. (1987). Human insulin receptors mutated at the ATP-binding site lack protein tyrosine kinase activity and fail to mediate postreceptor effects of insulin. *J Biol Chem*, 262, 1842-1847.

18. Coffer, P. J. and Woodgett, J. R. (1991). Molecular cloning and characterization of a novel protein-serine-threonine kinase related to the cAMP-dependent and protein kinase C families. *Eur J Biochem.,* 201, 475-481.

19. Coffer, P. J., Jin, J. and Woodgett, J. R. (1998). Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem J*, 335, 1-13.

20. Cohen, P., Alessi, D. R. and Cross, D. A. (1997). PDK1, one of the missing links in insulin signal transduction? *Growth Regul*., 410, 3-10.

21. Cushman, S. and Wardzala, L. (1980). Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell. Apparent translocation of intracellular transport systems to the plasma membrane. *J. Biol. Chem.,* 255, 4758-4762.

22. Czech, M. P., Lawrence, J. C., Jr., and Lynn, W. E. (1974). Hexose transport in isolated brown fat cells. A model system for investigating insulin action on membrane transport. *J. Biol. Chem*. 249, 5421-5427.

23. Datta, K., Franke, T. F., Chan, T. O., Makris, A., Yang, S. I., Kaplan, D. R., Morrison, D. K., Golemis, E. A. and Tsichlis, P. N. (1995). AH/PH domain-mediated interaction between Akt molecules and its potential role in Akt regulation. *Mol Cell Biol.*, 15, 2304-2310.

24. Dawson, K., and Aviles-Hernandez, A. (2001). Insulin-regulated trafficking of dual-labeled glucose. *Biochemical and Biophysical Research Communications,* 287, 445-454.

25. De Meyts, P. (2004). Insulin and its receptor: structure, function and evolution. *BioEssays*, 26, 1351-1362.

26. De Meyts, P. and Whittaker, J. (2002). Structural biology of insulin and IGF1 receptors: Implications for drug design. *Nat Rev Drug Disc,* 1, 769-783.

27. De Meyts, P., Sajid, W., Palsgaard, J., Theede, A. M., Gauguin, L., Aladdin, H. and Whittaker, J. (2007). Insulin and IGF-I receptor structure and binding mechanism. *Mechanisms of Insulin Action,* 1, 1-32.

28. De Meyts, P., Wallach, B., Christoffersen, C. T., Ursø, B., Grønskov, K., Latus, L. J., Yakushiji, F., Ilondo, M. M. and Shymko, R. M. (1994). The insulin-like growth factor-I receptor. Structure, ligand binding mechanism and signal transduction. *Hormone Research*, 42, 152-169.

29. Di Guglielmo, G. M., Baass, P. C., Authier, F., Posner, B. I., Bergeron, J. J. M. (1998). Insulin receptor internalization and signalling. *Molecular and Cellular Biochemistry,* 182 (1-2), 59-63.

30. Diella, F., Cameron, S., Gemünd, C., Linding, R., Via, A., Kuster, B., Sicheritz-Pontén, T., Blom, N. and Gibson, T. J. (2004). Phospho.ELM: A database of experimentally verified phosphorylation sites in eukaryotic proteins. *BMC Bioinformatics*, 5(79), 1471-2105.

31. Dionne, R., Forest, J. C., Moutquin, J. M., De Grandpré, P., Massé, J. (1994). Electrophoretic method for separating small peptides in serum without extraction of macromolecules: application to the detection of preeclampsia. *Clin Biochem.,* 27(2), 99-103.

32. Dubyak, G. R. and Kleinzeller, A. (1980).The insulin-mimetic effects of vanadate in isolated rat adipocytes. *J Biol Chem*, 255, 5306-5312.

33. Ebina, Y., Araki, E., Taira, M., Shimada, F., Mori, M., Craik, C. S., Siddle, K., Pierce, S. B., Roth, R. A. and Rutter, W. J. (1987). Replacement of lysine residue 1030 in the putative ATP-binding region of the insulin receptor abolishes insulin- and antibody-stimulated glucose uptake and receptor kinase activity. *Proc Natl Acad Sci, USA,* 84, 704-708.

34. Ebina, Y., Ellis, L., Jarnaghin, K., Edery, M., Graf, L., Clauser, E., Ou, J., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A. and Rutter, W. J. (1985). The human insulin receptor cDNA: the structural basis for hormone activated transmembrane signaling. *Cell,* 40, 747-758.

35. Elsom, J., Lethem, M. I., Rees, G. D. and Hunter, A. C. (2008). Novel quartz crystal microbalance based biosensor for detection of oral epithelial cell–microparticle interaction in real-time. *Biosensors and Bioelectronics,* 23, 1259–1265.

36. Engelman, J. A., Luo, J. and Cantley, L. C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet*, 7, 606-619.

37. Escobedo, J. A., Navankasattusas, S., Kavanaugh, W. M., Milfay, D., Fried, V. A. and Williams, L. T. (1991). cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF β-receptor. *Cell*, 65, 75-82.

38. Fantus, I. G., Kadota, S., Deragon, G., Foster, B. and Posner, B. I. (1989). Pervanadate [peroxide(s) of vanadate] mimics insulin action in rat adipocytes via activation of the insulin receptor tyrosine kinase. *Biochemistry*, 28, 8864-8871.

39. Frech, M., Andjelkovic, M., Ingley, E., Reddy, K. K., Falck, J. R. and Hemmings, B. A. (1997). High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of RAC/protein kinase B and their influence on kinase activity*. J Biol Chem.,* 272, 8474-8481.

40. Fujita, T., Toda, T. and Ohashi, M. (1984). Silver stain for proteins on a cellulose acetate membrane. *Analytical Biochemistry*, 139, 463-467.

41. Galloway, J. A., and Bressler, R. (1978). Insulin treatment in diabetes. *The Medical Clinics of North America*, 663-680.

42. Garcia, B. A., Shabanowitz, J, Hunt, D. F. (2005). Analysis of protein phosphorylation by mass spectrometry. *Methods,* 35, 256-264.

43. Gibson, T. J., Hyvonen, M., Musacchio, A., Saraste, M. and Birney, E. (1994). PH domain: the first anniversary. *Trends Biochem. Sci.*, 79, 349-353.

44. Gu, J. L., and De Meyts, P. (1991). The structure and function of the insulin receptor. In: Zai-Ping, L., Zi-Xian, L., You-Shang-Z, eds. Retrospect and Prospect of Protein Research. *Singapore: World Scientific Publishing Co*, 120-125.

45. Haan, C. and Behrmann, I. (2007). A cost effective non-commercial ECL-solution for Western blot detections yielding strong signals and low background. *J Immunol Methods*, 318 (1-2), 11-9.

46. Hancock, J. T. (2005) *Cell Signaling,* 2nd edition. Oxford University Press, USA, 9.

47. Haring, H., Kasuga, M., White, M. F., Crettaz, M. and Kahn, C. R. (1984). Phosphorylation and dephosphorylation of the insulin receptor: evidence against an intrinsic phosphatase activity. *Biochemistry, 23,* 3298-3306.

48. Haugh, J.M., Codazzi, F., Teruel, M. and Meyer, T. (2000). Spatial sensing in fibroblasts mediated by 3 phosphoinositides. *J. Cell Biol.* 151, 1269-1279.

49. Hayashi, K., Yonemura, S., Matsui, T., Tsukita, S. and Tsukita, S. (1999). Immunofluorescence detection of ezrin/radixin/moesin (ERM) proteins with their carboxyl-terminal threonine phosphorylated in cultured cells and tissues. *Journal of Cell Science*, 112, 1149-1158.

50. Hedo, J. A., and Simpson, I. A. (1984). Internalization of insulin receptors in the isolated rat adipose cell. Demonstration of the vectorial disposition of receptor subunits. *J. Bio. Chem.*, 259, 11083-11089.

51. Heffetz, D., Bushkin, I., Dror, R. and Zick, Y. (1990). The insulinomimetic agents H_2O_2 and vanadate stimulate protein tyrosine phosphorylation in intact cells. *J Biol Chem*, 265, 2892-2902.

52. Hirsch, E., Braccini, L., Ciraolo, E., Morello, F. and Perino, A. (2009). Twice upon a time: PI3K's secret double life exposed. *Trends Biochem Sci,* 34, 244-248.

53. Hubbard, M. J. and Cohen, P. (1993). On target with a new mechanism for the regulation of protein phosphorylation. *Trends Biochem. Sci.* 18, 172-177.

54. Humbel, R. E., Bosshard, H. R. and Zahn, H. (1972). Chemistry of insulin. In *Handbook of Physiology: Endocrinology,* ed. Steiner, D. F., Freinkel, N., 1, 111-32. Baltimore, Md: Williams & Wilkins.

55. Jacobs, A. R., LeRoith, D. and Taylor, S. I. (2001). Insulin receptor substrate-1 pleckstrin homology and phosphotyrosine-binding domains are both involved in plasma membrane targeting. *Journal of Biological Chemistry*, 276 (44), 40795-40802.

56. Jankowski, A., Zhu, P., Marshall, J. G. (2008). Capture of an activated receptor complex from the surface of live cells by affinity receptor chromatography. *Analytical Biochemistry* 380, 235–248.

57. Jensen, M. and De Meyts, P. (2009). Molecular mechanisms of differential intracellular signaling from the insulin receptor. *Vitam Horm.,* 80, 51-75.

58. Jones, P. F., Jakubowicz, T., Pitossi, F. J., Maurer, F. and Hemmings, B. A. (1991). Molecular cloning and identification of a serine/threonine protein kinase of the second messenger family. *Proc Natl Acad Sci USA,* 88, 4171-4175.

59. Kadota, S., I., Fantus, I. G., Deragon, G., Guyda, H. J. and Posner, B. I. (1987). Stimulation of insulin-like growth factor II receptor binding and insulin receptor kinase activity in rat adipocytes. *J. Biol. Chem*, 262, 17, 8252-8256.

60. Kahn, C. R., and White, M. F. (1988). The insulin receptor and the molecular mechanism of insulin action*. J. Clin. Invest.,* 82, 1151-1156.

61. Kasuga, M., Karlsson, F. A. and Kahn, C. R. (1982). Insulin stimulates the phosphorylation of the 95,000-Dalton subunit of its own receptor. *Science*, 215, 185-188.

62. Kiani, M. F., Yuan, H., Chen, X., Smith, L., Gaber, M. W. and Goetz, D. J. (2002). Targeting microparticles to select tissue via radiation-induced upregulation of endothelial cell adhesion molecules. *Pharmaceutical Research,* 19, 9.

63. Kim, L. and Denkers, E. Y. (2006). Toxoplasma gondii triggers Gi-dependent PI 3-kinase signaling required for inhibition of host cell apoptosis. *Journal of Cell Science,* 119, 2119-2126.

64. Klarlund, J.K. (1985). Transformation of cells by an inhibitor of phosphatises acting on phosphotyrosine in proteins. *Cell*, 41, 707-717.

65. Klein, H. H., Freidenberg, G. R., Kladde, M. and Olefsky, J. M. (1986). Insulin activation of insulin receptor tyrosine kinase in intact rat adipocytes. *The Journal of Biological Chemistry*, 261, 10, 4691-4697.

66. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C. and Pawson, T. (1991). SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science*, 252, 668-674.

67. Kuriyan, J. and Cowburn, D. (1997). Modular peptide recognition domains in eukaryotic signaling. *Annu. Rev. Biophys. Biomol. Struct.,* 26, 259-288.

68. Lange, K. (2002). Role of microvillar cell surfaces in the regulation of glucose uptake and organization of energy metabolism. *Am J Physiol Cell Physiol,* 282, C1-C26.

69. Lawlor, M. A. and Alessi, D. R. (2001). PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J. Cell Sci.,* 114, 2903.

70. Lee, J. and Pilch, P. F. (1994). The insulin receptor: structure, function, and signaling. *American Journal of Physiology - Cell Physiology*, 266 (2 35-2), C319-C334.

71. Lee, J., O'Hare, T., Pilch, P. F. and Shoelson, S. E. (1993). Insulin receptor autophosphorylation occurs asymmetrically. *J. Biol. Chem.*, 268 (6), 4092-4098.

72. Lemmon, M. A. (2007). Pleckstrin homology (PH) domains and phosphoinositides. *Biochem Soc Symp.*, 74, 81-93.

73. Litwack, G. (2009). Insulin and IGFs*. Vitamins & Hormones*, 80, 21-22.

74. Lu, F., Zheng, W. and Huang, Z. (2008). High contrast bioimaging using interferometric polarization coherent anti-Stokes Raman scattering microscopy, *Proc. SPIE* 6860, 68600Z.

75. Mann, M., Ong, S. E., Gronborg, M., Steen, H., Jensen, O. N. and Pandey, A. (2002). Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. *Trends Biotechnol*, 20, 261–268.

76. Manning, G., Whyte, D. B., Martinez, R., Hunter, T. and Sudarsanam, S. (2002). The protein kinase complement of the human genome. *Science*, 298, 1912-1934.

77. Marino-Buslje, C., Mizuguchi, K., Siddle, K. and Blundell, T. L. (1998). A third fibronectin type III domain in the extracellular region of the insulin receptor family. *FEBS Lett*, 441, 331-336.

78. Marshall, J. G., Booth, J. W., Stambolic, V., Mak, T., Balla, T., Schreiber, A. D., Meyer, T. and Grinstein, S. (2001). Restricted accumulation of phosphatidylinositol-3-kinase

products in a plasmalemmal subdomain during Fc gamma receptor mediated phagocytosis. *J. Cell Biol.*, 153, 1369-1380.

79. May, J. M., and de Haen, C. (1979). The insulin-like effect of hydrogen peroxide on pathways of lipid synthesis in rat adipocytes. *J. Biol. Chem*. 254, 9017-9021.

80. Mbanya, J. C. and Ramiaya, K. (2006). Diabetes Mellitus. *Disease and mortality in Sub-Saharan Africa;* Jamison, T., 2nd edition, 267-287.

81. McClain, D. A. (1991). Different ligand affinities of the two human insulin receptor splice variants are reflected in parallel changes in sensitivity for insulin action. *Mol Endo,* 5, 734-739.

82. McLachlin, D. T. and Chait, B. T. (2001). Analysis of phosphorylated proteins and peptides by mass spectrometry. *Curr. Opin. Chem. Biol*. 5, 591–602.

83. McManus, E. J., Collins. B. J., Ashby, P. R., Prescott, A. R., Murray-Tait, V., Armit, L. J., Arthur, J. S. and Alessi, D. R. (2004). The in vivo role of PtdIns(3,4,5)P3 binding to PDK1 PH domain defined by knockin mutation. *EMBO J.*, 23(10), 2071-82.

84. Moeslein, F. M., Myers, M. P. and Landreth, G. E. (1999). The CLK family kinases, CLK1 and CLK2, phosphorylate and activate the Tyrosine Phosphatase, PTP-1B. *The Journal of Biological Chemistry,* 274, 26697-26704.

85. Mooney, R. A. and Anderson, D. L. (1989). Phosphorylation of the insulin receptor in permeabilized adipocytes is coupled to a rapid dephosphorylation reaction. *The Journal of Biological Chemistry,* 264, 12, 6850-6857.

86. Mora, A., Komander, D., van Aalten, D. M. and Alessi, D. R. (2004) PDK1, the master regulator of AGC kinase signal transduction. *Semin Cell Dev Biol,* 15, 161-170.

87. Moran, M. F., Koch, C. A., Anderson, D., Ellis, C., England, L., Martin, G. S. and Pawson, T. (1990). Src homology region 2 domains direct protein-protein interactions in signal transduction. *Proc. Natl. Acad. Sci. USA,* 87, 8622-8626.

88. Morrissey, J. H. (1981). Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Analytical Biochemistry*, 117, 307-310.

89. Mulhern, T. D., Booker, G.W. and Cosgrove, L. A. (1998). Third fibronectin-type-III domain in the insulin-family receptors. *TIBS*, 23, 465-466.

90. Murnaghan, J. H. and Talalay, P. (1967) John Jacob Abel and the crystallization of insulin. *Perspect Biol Med,* 10, 334.

91. Myers, M. G., Jr., Sun, X. J., and White, M. F. (1994). The IRS-1 signaling system. *Trends Biochem. Sci.,* 19, 289-293.

92. O'Gorman, D. M., McKenna, S. L., McGahon, A. J., Knox, K. A. and Cotter, T. G. (2000). Sensitisation of HL60 human leukaemic cells to cytotoxic drug-induced apoptosis by inhibition of PI3-kinase survival signals. *Leukemia*, 14, 602-611.

93. O'Neill, T. J., Rose, D. W., Pillay, T. S., Hotta, K., Olefsky, J. M. and Gustafson, T. A. (1996). Interaction of a GRB-IR Splice Variant (a Human GRB10 Homolog) with the Insulin and Insulin-like Growth Factor I Receptors. *The Journal of Biological Chemistry,* 271, 37, 22506-22513.

94. Otsu, M., Hiles, I., Gout, I., Fry, M. J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A. D., Morgan, S. J., Courtneidge, S. A., Parker, P. J.

and Waterfield, M. D. (1991). Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60$^{c\text{-}src}$" complexes,and PI3-kinase. *Cell,* 65, 91-104.

95. Pawson, T. (1995). Protein modules and signalling networks. *Nature*, 373, 573-580.
96. Pawson, T. (2003). Organization of cell-regulatory systems through modular protein interaction domains. *Phil. Trans. R. Soc. London. A,* 361, 1251-1262.
97. Pawson, T. and Nash, P. (2003). Assembly of cell regulatory systems through protein interaction domains. S*cience,* 300, 445-452.
98. Pawson, T. and Scott, J.D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. *Science,* 278, 2075-2080.
99. Pawson, T., Raina, M., Nash, P. (2002). Interaction domains: from simple binding events to complex cellular behavior, *FEBS Letters*, 513, 1, 2-10.
100. Pessin, J. E. and Saltiel, A. R. (2000). Signaling pathways in insulin action: molecular targets of insulin resistance. *Journal of Clinical Investigation*, 106 (2), 165-169.
101. Ren, R., Mayer, B. J., Cicchetti, P. and Baltimore, D. (1993). Identification of a ten-amino acid proline-rich SH3 binding site. *Science,* 259, 1157-1161.
102. Roach, P. J. (1990). Control of glycogen synthase by hierarchal protein phosphorylation. *The FASEB Journal*, 4, 2961-2968.
103. Rong, S. B., Hu, Y., Enyedy, I., Powis, G., Meuillet, E. J., Wu, X., Wang, R., Wang, S. and Kozikowski, A. P. (2001). Molecular modeling studies of the Akt PH domain and its interaction with phosphoinositides. *J Med. Chem.*, 44(6), 898-908.
104. Sanger, F. (1959). Chemistry of insulin. *Science*, 129: 1340-44.

105. Sansal, I. and Sellers, W. R. (2004). The biology and clinical relevance of the PTEN tumor suppressor pathway. *Journal of Clinical Oncology*, 22, 14, 2954-2963.

106. Scimeca, J. C., Ballotti, R., Filloux, C. and Obberghen, E. V. (1992). Insulin and orthovanadate stimulate multiple phosphotyrosine-containing serine kinases. *Molecular and Cellular Biochemistry,* 109, 139-147.

107. Seino, S., Seino, M. and Bell, G. I. (1990). Human insulin receptor gene. *Diabetes*, 39, 129-133.

108. Shechter, Y. and Karlish, S. J. D. (1980). Insulin-like stimulation of glucose oxidation in rat adipocytes by vanadyl (IV) ions. *Nature,* 284, 556-558.

109. Sheikh-Ali, M. and Chehade, J. M. (2009). A Rational approach to the initiation of insulin therapy in older adults. *Geriatrics and Aging*, 12 (3), 135-140.

110. Skolnik, E. Y., Margolis, B., Mohammadi, M., Lowenstein, E., Fischer, R., Drepps, A., Ullrich, A. and Schlessinger, J. (1991). Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/ cloning of target proteins for receptor tyrosine kinases. *Cell*, 65, 83-90.

111. Smith, G. D., Swenson, D. C., Dodson, E.J., Dodson, G. G. and Reynolds, C. D. (1984). Structural stability in the 4-zinc human insulin hexamer. *Proc Natl Acad Sci USA,* 81, 7093-7097.

112. Smith, G.D. and Ciszak, E. (1994). The structure of a complex of hexameric insulin and 4'-hydroxyacetanilide. *Proc Natl Acad Sci USA,* 91, 8851-8855.

113. Smith, G.D. and Dodson, G. G. (1992). The structure of a rhombohedral R6 insulin/phenol complex. *Proteins Struct Funct Genet,* 14, 401-408.

114. Smith, J. B. (1983). Vanadium ions stimulate DNA synthesis in Swiss mouse 3T3 and 3T6 cells. *Proc Natl Acad Sci USA,* 80, 6162-6166.

115. Snustad, D. P., Simmons, Mf. J. and Jenkins, J. (1997). *Principles of Genetics.* John Wiley and Sons Inc., New York, NY.

116. Song, G., Ouyang, G. and Bao, S. (2005). The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med,* 9(1), 59-71.

117. Staal, S., Hartley, J. and Rowe, W. (1977). AKT-8, an acute transforming retrovirus isolated from a rodent T-cell lymphoma. *Proc Natl Acad Sci USA*, 74, 3065-3070.

118. Stein, R. C. (2001). Prospects for phosphoinositide 3-kinase inhibition as a cancer treatment. *Endocrine-Related Cancer,* 8, 237-248.

119. Steiner, D. F., Chan, S. J., Welsh, J. M. and Kwok, S. C. M. (1985). Structure and evolution of the insulin gene. *Ann. Rev. Genet.*, 19, 463-84.

120. Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F. and Hawkins, P. T. (1997). Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science,* 277, 567–570.

121. Storz, P. and A. Toker. (2002). 3′-phosphoinositide-dependent kinase-1 (PDK-1) in PI 3-kinase signaling. *Front Biosci,* 7, 886-902.

122. Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J., and White, M. F. (1991). Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature*, 362, 73-77.

123. Suzuki, K., and Kono, T. (1980). Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. *Proc. Natl. Acad. Sci. U. S. A.,* 77, 2542-2545.

124. Tamura, S., Brown, T. A., Whipple, J. H., Fujita-Yamaguchi, Y., Dubler, R. E., Cheng, K. and Lamer, J. (1984). A novel mechanism for the insulin-like effect of vanadate on glycogen synthase in rat adipocytes. *J Biol Chem,* 259, 6650-6656.

125. Thirone, A. C. P., Huang, C. and Klip, A. (2006). Tissue-specific roles of IRS proteins in insulin signaling and glucose transport. *TRENDS in Endocrinology and Metabolism*, 17, 2, 72-78.

126. Thong, F. S. L., Bilan, P. J. and Klip, A. (2007). The Rab GTPase-activating protein AS160 integrates Akt, Protein Kinase C, and AMP-Activated Protein Kinase signals regulating GLUT4 traffic. *Diabetes*, 56, 2, 414-423.

127. Torpy, J. M. and Golub, R. M. (2011). JAMA patient page. Diabetes. *JAMA,* 305(24), 2592.

128. Tung, O. C., Susan, E. R. and Philip, N. T. (1999). Akt/PKB and other D3 phosphoinositide-regulated kinases: Kinase activation by phosphoinositide-dependent phosphorylation. *Annual Review of Biochemistry*, 68, 965-1014.

129. Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. D., Gray, A., Coussens, L., Liao, Y. C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M. and Ramachandran, J. (1985). Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature*, 313, 756-761.

130. Ushiro, H. and Cohen, S. (1980). Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes *J. Biol. Chem.*, 255, 8363-8365.

131. Van Obberghen, E. and Kowalskl, A. (1982). Phosphorylation of the hepatic insulin receptor. *FEBS Lett*, 143, 179-182.

132. Vanhaesebroeck, B. and D. R. Alessi. (2000). The PI3K-PDK1 connection: more than just a road to PKB. *Biochem. J.,* 346, 561-576.

133. Vanhaesebroeck, B., Welham, M. J., Kotani, K., Stein, R., Warne, P. H., Zvelebil, M. J., Higashi, K., Volinia, S., Downward, J. and Waterfield, M. D. (1997). p110δ, a novel phosphoinositide 3-kinase in leukocytes. *Proc. Natl. Acad. Sci. USA*, 94, 4330-4335.

134. Viglietto, G., Amodio, N., Malanga, D., Scrima, M. and De Marco, C. (2011). Contribution of PKB/AKT signaling to thyroid cancer. *Front Biosci*., 16, 1461-87.

135. Virkamäki, A., Ueki, K. and Kahn, C. R. (1999). Protein–protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. *J. Clin. Invest.,* 103, 931-943.

136. Vivanco, I. and Sawyers, C. L. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer*, 2, 489-501.

137. Ward, C. W. (1999). Members of the insulin receptor family contain three fibronectin type III domains. *Growth factors*, 16, 315-322.

138. White, M. F. and Kahn, C. R. (1994). The insulin signaling system*. Journal of Biological Chemistry*, 269(1), 1-4.

139. Whiteside, S. T. and Israel, A. (1997). IκB proteins: structure, function and regulation. *Seminars in* CANCER BIOLOGY, 8, 75–82.

140. Whittingham, J. L., Chaudhurri, S., Dodson, E. J., Moody, P. C. E. and Dodson, G. G. (1995). X-ray crystallographic studies on hexameric insulins in the presence of helix-stabilizing agents, thiocyanate, methylparaben, and phenol. *Biochemistry,* 34, 15553-15563.

141. Wice, B., Milbrandt, J. and Glasser, L. (1987). Control of muscle differentiation in BC3H1 cells by fibroblast growth factor and vanadate. *J Biol Chem,* 262, 1810-1817.

142. Wick, K. L., and Liu, F. (2001). A new molecular target of insulin action: regulating the pivotal PDK1. *Curr. Drug Targets Immune Endocr. Metabol. Disord*., 1, 209-221.

143. Williams, M. R., Arthur, J. S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P. and Alessi, D. R. (2000). The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Curr Biol.,* 10(8), 439-48.

144. Yamaguchi, Y., Flier, J. S., Yokota, A., Benecke, H., Backer, J. M. and Moller, D. E. (1991). Functional properties of two naturally occuring isoforms of the human insulin receptor in chinese hamster ovary cells. *Endocrinology,* 129, 2058-2066.

145. Yarden, Y. and Ullrich, A. (1988). Growth factor receptor tyrosine kinases. *Ann Rev Biochem,* 57, 443-478.

146. Yuan, T. L. and Cantley, L. C. (2008). PI3K pathway alterations in cancer: variations on a theme. *Oncogene,* 27, 5497-5510.