University of Alberta

Platelet-derived growth factor receptor-β internalization is modulated by intrinsic kinase activity, receptor dimerization and internalization codes

by

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Dedication

I dedicate this work to those who have been directly and indirectly affected by receptor tyrosine kinase associated pathogenesis.

Preface

Organisms, whether unicellular or multi-cellular, have acquired at one point or another, the advantage of communicating with the extracellular environment. Prokaryotes can sense light or chemical cues enabling migration toward microbial utopias with the promise of survival. Eukaryotic cells have developed tools to interact with neighbouring cells or organisms to ensure aberrant growth is avoided, to become active, or to trigger cell-mediated cell death cascades. All of these coordinating events occur due to the intricate choreography of molecular events where proteins are, to list a few processes, created and degraded, myristoylated, phosphorylated, and translocated. Surface receptors are important mediators of cell events and are present in all eukarya. This thesis focuses on the plasma membrane of cells, where surface receptors called receptor tyrosine kinases (RTKs) directly interact with their environment using their extracellular domain. These interactions elicit changes in the intracellular portion of the RTK which can result in dramatic cell changes such as those listed above. Environmental cues can also initiate a process where cells take the RTK in from the cell surface into intracellular compartments, an event coined receptor mediated endocytosis. This process is very important for controlling cellular responses and in the event where atypical RTK activity is sustained, diseases such as cancer or fibrosis become established.

Abstract

Surface receptor internalization is an essential event in cells that controls many cellular processes. This thesis explores some of the mechanisms involved in platelet-derived growth factor receptor (PDGFR) endocytosis. Analysis of the involvement of kinase activity in PDGFR internalization suggests that kinase activity participates in the endocytosis process, but is not necessary. Receptor dimerization is shown to be directly involved in PDGFR's internalization, and data obtained during this study further supports the hypothesis that receptor dimerization is the basis for RTK internalization. Lastly, this thesis defines putative internalization motifs in the carboxyl-terminus of PDGFR. Amino acids 952-965 make up a hydrophobic region that when removed by truncation or deletion, results in reduced PDGFR internalization. When brought together, these data provide evidence that PDGFR employs intrinsic kinase activity as an non-essential modulator of internalization, and that endocytic codes and receptor dimerization are compulsory for PDGFR internalization.

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List of Symbols, Abbreviations and Nomenclature

(#)'.....(duration) minutes
α.....anti-/alpha
AA (#).....AP20187+AG1296 (duration) treated cells
AG1296.....PDGFR kinase inhibitor
AP (#).....AG1296+PDGF (duration) treated cells
AP20187.....FKBP cross-linking reagent
ATP.....adenosine triphosphate
AXL.....AXL receptor tyrosine kinase
bp.....base pair
BV.....baculovirus
°C.....degrees Celsius
CCV. .....clathrin-coated vesicle
CDR.....circular dorsal ruffles
CT141. .....141 amino acid PDGFR truncation mutant
CT155. .....155 amino acid PDGFR truncation mutant
Da.....Dalton
DMSO. .....dimethyl sulfoxide
DNA.....deoxyribonucleic acid
DDR. .....discoidin domain receptor
DSS.....di-succinimidyl suberate
EEA-1. .....early endosomal autoantigen 1
EGF.....epidermal growth factor
EGFR.....epidermal growth factor receptor
EM.....electron microscopy
Ep. .....EGFR with PDGFR hydrophobic swap
EPH. ..... discovered in **e**rythropoietin-**p**roducing human **h**epato-carcinoma cell line
ER.....endoplasmic reticulum
ERK. .....extracellular signal-regulated kinase
ES.....endosome
F442. .....NIH-3T3-F442 murine cell line

FBS.. .....fetal bovine serum
FC.....flow cytometry
FGFR. .....fibroblast growth factor receptor
FITC.....fluorescein isothiocyanate
FKBP. ..... FK506 binding protein
FRET. .....fluorescence resonance energy transfer
GAP.....GTPase activating protein
GDP.....guanosine diphosphate
GEF.....guanosyl exchange factor
GFP.....green fluorescent protein
eGFP.....enhanced green fluorescent protein
Glc.....glucose
GM1.....monosialotetrahexosylganglioside
GPCR. .....G-protein coupled receptor
Grb2. .....growth factor receptor bound protein 2
GST.....glutathione-S-transferase
GTP.....guanosine triphosphate
GTPase.....guanosine triphosphatase
HepGP. .....HepG2 cell stably transfected with wt-PDGFR
HepGR.....HepG2 cell stably transfected with kinase dead-PDGFR
HepGW.....wild type HepG2 cells
HGFR. .....hepatocyte growth factor receptor
HRP.....horseradish peroxidise
IB. .....immuno blot
IFM.....immunofluorescence microscopy
Ig.....immunoglobulin
IR. .....insulin receptor
IPTG.....isopropyl-γ-D-thiogalactoside
KD.....kinase dead
KLG.....KLG receptor tyrosine kinase
LR.....leucine-rich
LTK.....leukocyte tyrosine kinase

MAPK.....mitogen-activated protein kinase
MEK.....MAPK kinase
mRNA.....messenger RNA
MuSK.....muscle skeletal receptor tyrosine kinase
OD.....optical density
p.....phospho
P(#).....PDGF (duration) treatment
PAGE.....polyacrylamide gel electrophoresis
PBS. .....phosphate buffered saline
PCR.....polymerase chain reaction
PDGF.....platelet-derived growth factor
PDGFR.....platelet-derived growth factor receptor
Pe.....PDGFR w/ EGFR hydrophobic swap
PH.....pleckstrin homology
PI3K. .....phosphatidyl inositol 3-kinase
PIAP.....primary internalization associated proteins
PM.....plasma membrane
PM:ES.....plasma membrane to endosome ratio
PTB.....phosphotyrosine binding
QDM.....quantitative deconvolution microscopy
R.....arginine
RET.....rearranged during transformation receptor tyrosine kinase
RFP.....red fluorescent protein
RNA.....ribonucleic acid
ROR.....Ror receptor tyrosine kinase
RTK.....receptor tyrosine kinase
RYK.....RYK receptor-like tyrosine kinase
SF..... .....serum free
SDM.....site directed mutagenesis
SDS.....sodium dodecyl sulphate
SH2.....Src homology 2
SH3.....Src homology 3

SOS.....son-of-sevenless

TIE.....tyrosine kinase with Ig and EGF homology domains 1

TLR.....toll-like receptor

TR-EGF.....Texas red-conjugated EGF

TRK.....tropomyosin-related kinase

V.....volt

VEGFR.....vascular endothelial growth factor receptor

WCL.....whole cell lysate

WHMIS.....workplace hazardous material information system

wt.....wild type

eYFP.....enhanced yellow fluorescent protein

Y.....tyrosine

Chapter One:

Introduction to Receptor Tyrosine Kinases

1.1 Receptor tyrosine kinase structure and activation

1.1.1 Perspective

Every human being is composed of trillions of cells. Our bodies are an intricate network of cells that rigorously coordinate their own growth, movement, differentiation, and death. In order for multi-cellular organisms to equilibrate into homeostasis, react sensibly to their environment, or protect themselves from pathogens, cells utilize integral membrane proteins called surface receptors (Figure 1). Seven major classes of surface receptors exist: G protein-coupled receptors (GPCR), cytokine receptors, receptor tyrosine kinases (RTKs), tumor growth factor-β receptors, hedgehog receptors, wnt receptors, and notch receptors (Lodish *et al.*, 2004). Surface receptors transcend both the interior and exterior of the cell cortex. The structure of the extracellular portion of the protein can be modulated by the presence or absence of a particular physical cue in the environment. Of particular importance to this thesis, extracellular proteins called ligands directly bind to RTKs which result in cellular changes (Heldin, 1995). The intracellular segment of most surface receptors is capable of directly interacting with intracellular proteins. These interactions result in the initiation of signalling cascades that dictate cellular responses (Figure 1, 2b). It is therefore important that surface receptors function properly in order to maintain cell control as well as elicit suitable responses when interacting with the surrounding environment.

The inability of cells to properly communicate with each other or the environment results in aberrant cellular activity and frequently leads to establishment of diseases.

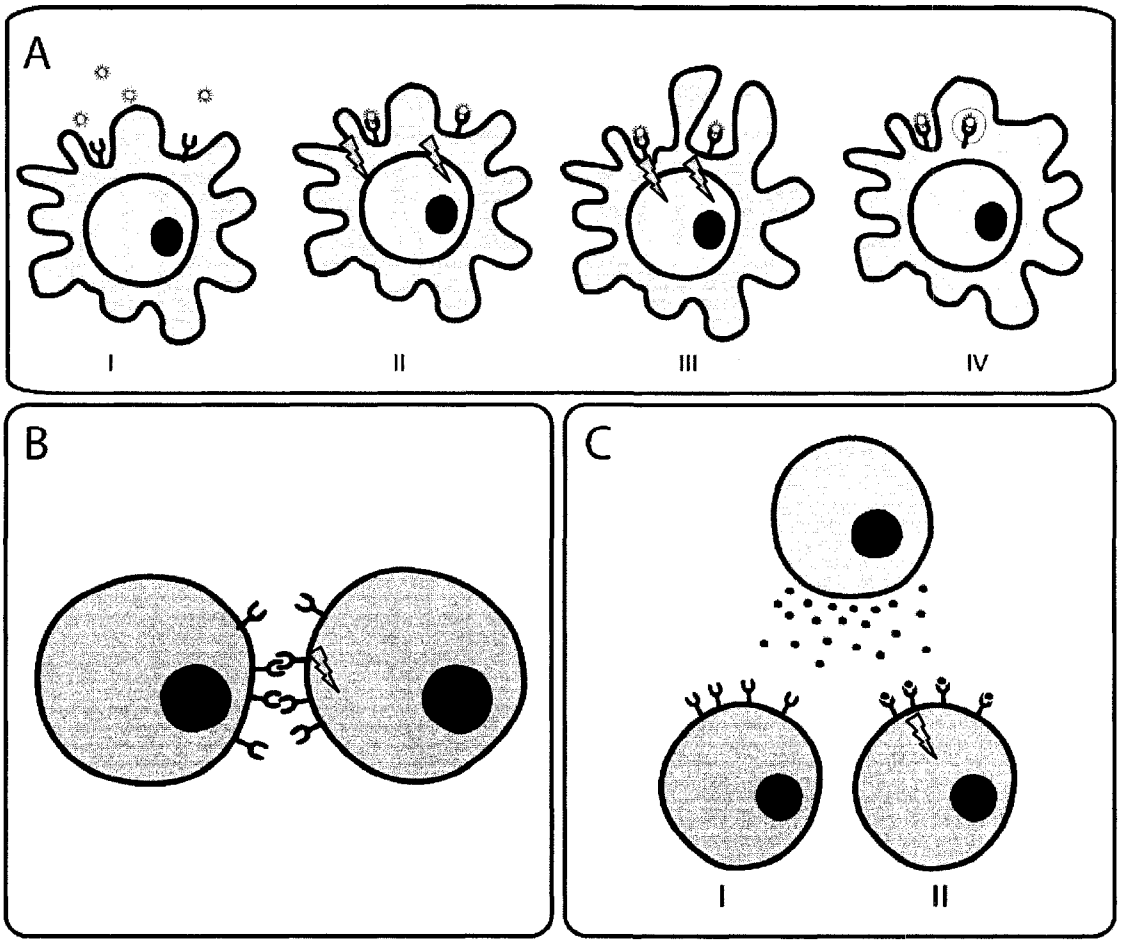


Figure 1 – Surface receptors enable cells to interact with each other and the surrounding environment. A) Leukocytes use surface receptors to detect pathogens (orange) in the environment (I). Once the cell detects a pathogen via direct surface receptor binding (II), cell structure rearrangements occur, enabling phagocytosis (III). The pathogen is eventually completely endocytosed and degraded (IV). B) Cells rely on cell-cell interactions via surface receptors to control cell growth and movement. C) Environmental cues, such as proteins or substrates secreted from near or distant cells, can change the status of cells from inactive (I) to active (II) or *vice versa*. Lightning bolts indicate transduction of intracellular signals.

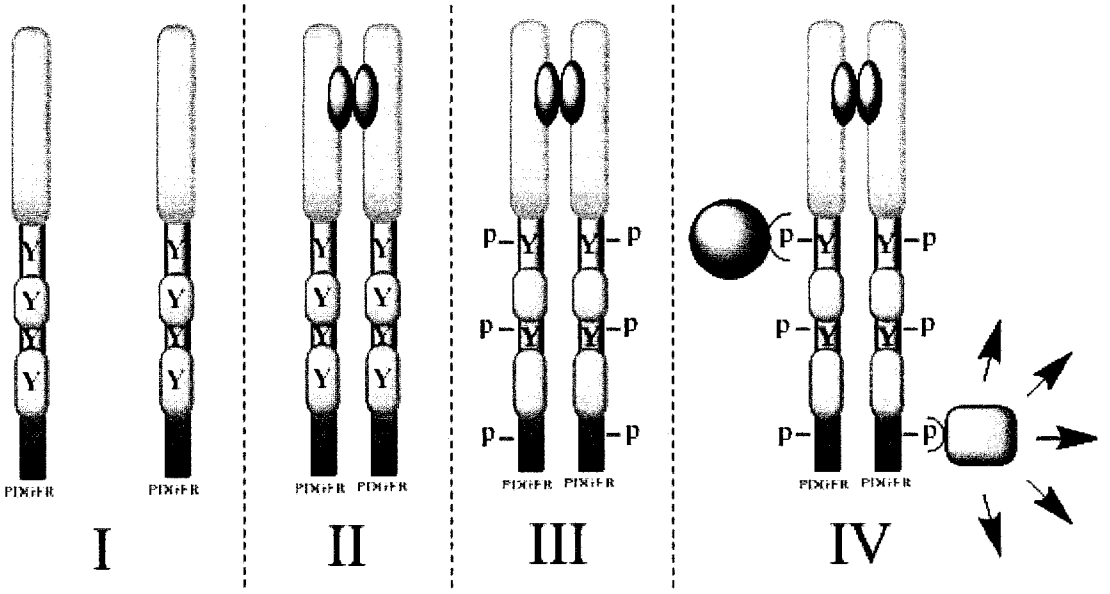


Figure 2 – Receptor tyrosine kinase (RTK) dimerization and activation. Ligand-induced PDGFR activation. After proper protein folding and transport to the plasma membrane, most RTKs, are in non-activated monomeric states (I). Binding of the ligand (blue) causes two receptors to come into close proximity, a process called dimerization (II). The act of dimerization elicits conformational changes in the kinase domains of the RTK causing the phosphorylation in trans of select tyrosine residues on the cytosolic portion of the RTK (III). The presence of phosphorylated tyrosine residues increases the binding affinity of other cytosolic proteins (grey and red) to the RTK causing accumulation of factors at the plasma membrane (IV). This accumulation of proteins at the plasma membrane initiates signalling cascades that propagate throughout the cell resulting in unique cell responses (IV).

Ailments such as insulin resistance, papillary renal-cell carcinomas, breast cancer, venous malformation, gastrointestinal stromal tumors, mastocytosis, and lymphedema are only a fraction of the human diseases linked to uncharacteristic surface receptor activity (Robertson *et al.*, 2000; Apperley *et al.*, 2002; Cools *et al.*, 2003; Tan, 2006; Amit *et al.,* 2007; Stumvoll *et al.*, 2007). While we have sufficient information to diagnose many human diseases, scientists require a lot more data before we can treat the disorders. In order to prevent and treat disease, scientists must deduce disease-associated cellular events and the mechanisms that govern them. The resultant knowledge can then be applied to develop new technologies and theories that in turn converge on patient care. In short, we need to fully understand how cellular events occur in order to safely and confidently develop and administer treatments.

This thesis focuses on elucidating the principles that govern how surface receptors translocate from the cell surface into the cell interior. The process is called receptor-mediated endocytosis and it must occur in order to maintain cell stability. Cells use this process to modulate the number of receptors on the cell surface, the total number of receptors in the cell, and the intensity and duration of surface receptor signals that are propagated throughout the cells. When the process of receptor-mediated endocytosis is interrupted, cells experience aberrant growth and migration which leads to the establishment of disease (Grandal *et al.*, 2007). The material presented in this thesis further refines our understanding of the endocytic process.

1.1.2 Receptor tyrosine kinase structure and function

Receptor tyrosine kinases (RTKs) are a genetically diverse subgroup of cell surface receptors that exist only within the metazoan clade (Robinson *et al.*, 2000). The described functions of these glycoproteins include, but are not limited to: cell-cell signalling, mitogenesis, protein expression modulation, cell differentiation, whole organism development, cell motility, apoptosis, and cell adhesion (McKay and Morrison, 2007; Robinson *et al.*, 2000; Heldin and Westermark, 1999). Twenty are included in the RTK protein subgroup, characterized by similarities in protein structure, most of which are depicted in Figure 3 (Robertson *et al.*, 2000; Pawson, 2002). These similarities include the presence of extracellular domains which are composed of immunoglobulin-like (Ig-like) domains, fibronectin repeats, or cysteine rich regions (Figure 3). These structures are involved in the process of initiating receptor dimerization (Figure 2; Robertson *et al.*, 2000; Heldin, 1995). Each RTK also has an alpha-helix transmembrane domain and a conserved cytosol kinase domain (Robertson *et al.*, 2000) (Figure 3). The structural similarity that ties RTK families into the RTK protein subgroup alludes to the possibility that family members function similarly. In a broad sense, this is correct. RTKs are initiators of signalling cascades whereby receptor activation results in cellular changes. While the details of the elicited cellular changes are unique to each receptor, which may be attributed to the fact that RTKs are generally expressed in a cell type-dependent manner, there are striking similarities in the downstream substrates that are affected by RTK activation.

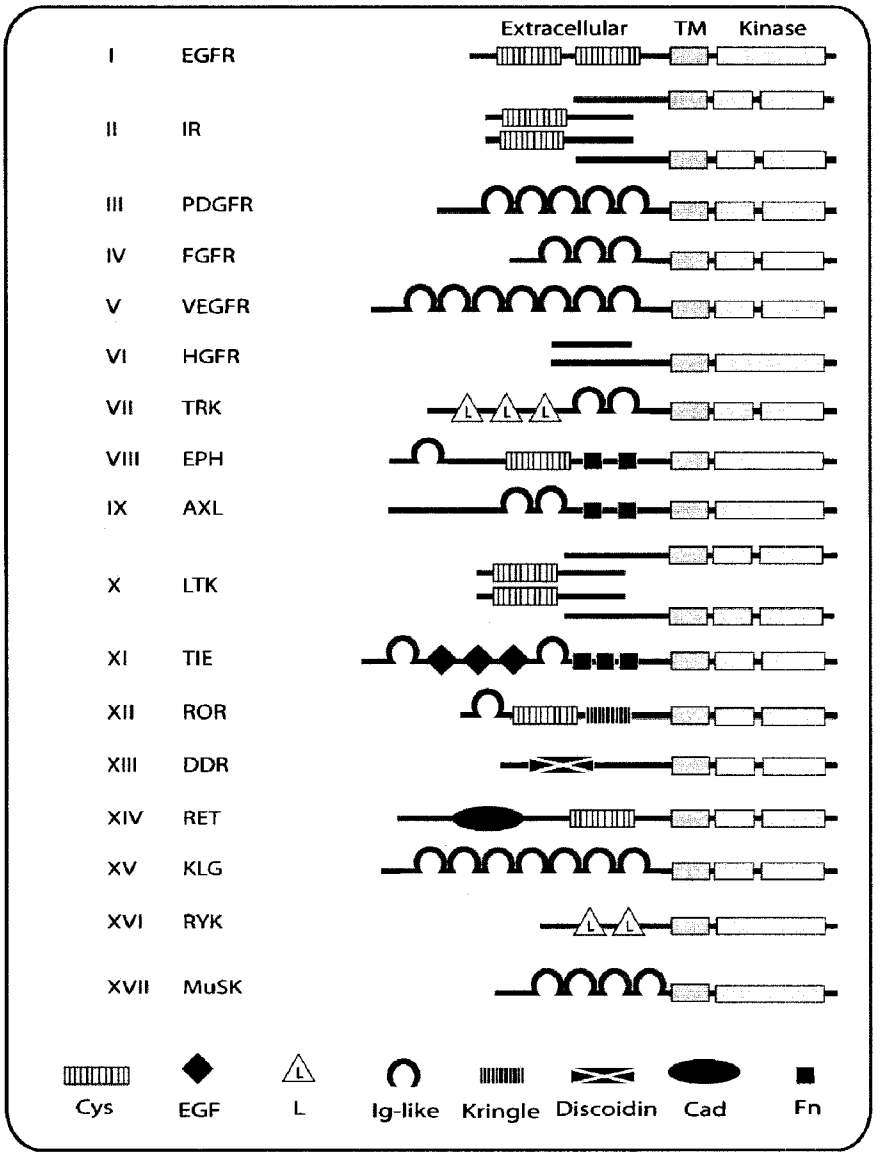


Figure 3 - Receptor tyrosine kinase family members are comprised of unique and conserved structural components. RTKs are composed of an extracellular domain, single transmembrane domain (TM), juxtamembrane domain, kinase domain, and carboxyl terminal domain. Note that the RTK intracellular portion (kinase domain) is highly conserved ,while the extracellular structural properties vary greatly. Adapted from Robertson *et al.*, 2000.

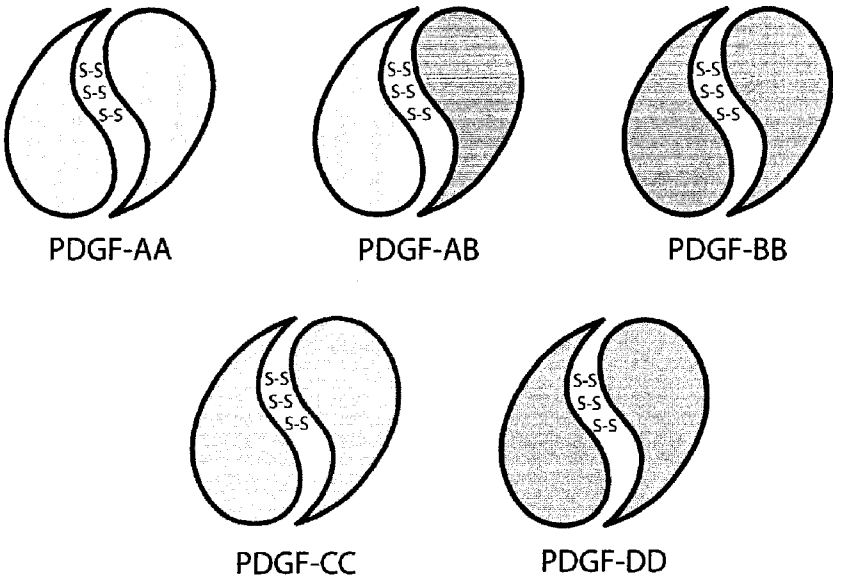


Figure 4– Platelet-derived growth factor structure and multiformity. Each PDGF dimer is composed of two anti-parallel PDGF monomers covalently bound together by three disulfide bridges (-S-S-). Five distinct PDGF ligands exist and each one binds with unique affinity to PDGFR-α and PDGFR-β. Differences in PDGFR: PDGF association results in varied cellular responses.

1.1.3 Receptor tyrosine kinase activation

Initiators of RTK activation are protein ligands. RTK ligands are sometimes monomeric, homodimeric, or heterodimeric. For example, platelet-derived growth factors (PDGFs) exist as both homo- and hetero-dimeric proteins. There are five different PDGFs: PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC, and PDGF-DD (Figure 4). PDGF's are part of a ligand subgroup called the cysteine knot family of proteins that include many different RTK ligands such as nerve growth factor, transforming growth factor-β and human chorionic gonadotropin (McDonald and Hendrickson, 1993; Sun and Davies, 1995). Each ligand dimer is composed of two monomer subunits that are bound together in an anti-parallel fashion by three disulfide bridges (Sun and Davies, 1995; Reigstad *et al.*, 2005) (Figure 4). Binding of PDGFs to platelet-derived growth factor receptors (PDGFRs) induces PDGFR dimerization and kinase activation, which is broadly referred to as ligand-induced RTK activation (Figure 2).

Although the precise mechanism of RTK activation varies between each receptor, each RTK, with the exceptions of insulin receptor (IR) and leukocyte tyrosine kinase (LTK), exists as a monomer that resides in the plasma membrane of cells (Schlessinger, 2000) (Figure 2, I). A RTK's respective ligand binds directly to the RTK extracellular domain. Even though ligand structures vary, the resulting interaction of the ligand with its respective RTK, drives dimerization of a receptor pair (Schechter *et al.*, 1979; Reviewed in Heldin, 1995; Schlessinger, 2000) (Figure 2, II). Dimerization drives a process called trans-autophosphorylation where each receptor phosphorylates tyrosine amino acid residues on its partner (Ullrich and Schlessenger, 1990). This action results in

enhanced kinase activity and further propels trans-autophosphorylation of the receptor pair (Heldin, 1995) (Figure 2, III). Phosphorylation of tyrosine residues is particularly important for binding of cytosolic proteins to activated receptors, but evidence suggests that serine/threonine residues also play a role in subsequent signal transduction (Pawson, 2002) (Figure 2, IV).

1.1.4 Receptor tyrosine kinase activated signalling and cellular outcomes

Although the precise mechanism of subsequent events differs from receptor to receptor, once the kinase domain of the RTK becomes activated, tyrosine residues throughout the cytoplasmic domain become phosphorylated which allows it to interact with cytosolic signalling proteins (Robertson *et al.*, 2002). These interactions occur through Src-homology 2 (SH2) domains or phospho-tyrosine binding (PTB) domains present on the cytosolic proteins (McKay and Morrison, Robertson *et al.*, 2002; Pawson, 2002; Schlessinger, 2000). These interactions are essential for proper signal transduction in cells.

Conformational changes in the cytosolic RTK structure subsequent to receptor activation may also allow for interaction with other protein domains in cytosolic proteins such as Src-homology 3 (SH3) domains, proline rich domains, hydrophobic domains or PDZ domains. These structural changes may also allow for non-phosphorylation based post-translational modification of the RTK (such as palmitoylation). Unfortunately, complete crystal structures of active and inactive RTKs have not been obtained, so the role of conformational changes in RTKs is unclear.

The cellular outcome of each RTK activation scheme is complex. Activation of different RTKs in the same cell type can elicit different, and in some cases, contrasting cellular responses. For example, activation of PDGFR with subsequent activation of IR in the same cell results in reduced mitogenesis compared to cellular mitogenesis detected when only PDGFR is activated (Cirri *et al.*, 2005). This interaction between PDGFR and IR signalling is perplexing considering that both pathways, when exclusively activated, induce cell mitogenesis (Cirri *et al.*, 2005). Furthermore, it was recently discovered that in order to stimulate human smooth muscle cell proliferation via PDGFR activation, fibroblast growth factor receptor-1 must be trans-activated by PDGFR (Millette *et al.*, 2005). Another example involves hetero- and homo-dimeric PDGFR-α/β dimers. Data suggest that RTK family members can differentially activate signalling cascades depending on which receptors dimerize. For example, PDGFR-ββ homo-dimers do not elicit as strong of a mitogenic response as PDGFR-α/β hetero-dimers (Pawson, 2002; Heldin and Westermark, 1999).

Cellular proteins seemingly outside of the RTK circle of influence have also been implicated in the activation of RTKs. Epidermal growth factor receptors (EGFRs) are now known to be activated during innate immune response (Koff *et al.*, 2008). This activation is not simply due to standard EGFR activation, but is induced by toll-like receptor activity (Koff *et al.*, 2008).

1.1.5 Platelet-derived growth factor receptor (PDGFR)

Originally identified due to the isolation of PDGF, PDGFR is now a classic

member of the RTK family (Ross *et al.*, 1974; Heldin *et al.*, 1981). It consists of an extracellular domain with five Ig-like domains, a single trans-membrane domain, and a cytosolic domain housing a split kinase domain (Figure 3, III). PDGFR converts extracellular stimuli in the form of PDGF binding to transducible cytosolic signals (Lokker *et al.*, 1997; Heldin and Westermark, 1999) (Figure 2). PDGF receptor is able to affect many cellular characteristics such as cell chemotaxis, cytoskeletal manipulation, differentiation, division, mitogenesis, and motility (Heldin *et al.*, 1998; Alvarez *et al.*, 2006).

The binding of the PDGF ligand to the extracellular domain of PDGFR induces dimerization and subsequent autophosphorylation in trans (Kelly *et al.*, 1991). This autophosphorylation of many characterized tyrosine sites on PDGFR increases interactions between PDGFR and numerous cytosolic proteins at specific docking sites (Heldin *et al.*, 1998) (Figure 2). Resultant interactions include (but are not limited to) the following proteins: phophatidylinositol 3' kinase (PI3K), phospholipase C-γ (PLC-γ), Src kinases, SHP-2, Ras-GAP, Grb2, Shc, Nck, Grb7, and Crk (Heldin *et al.*, 1998). Aberrant activation or expression of PDGFR or these cytosolic proteins has been shown to be associated with many different cancers and pathological fibrotic diseases (Apperley *et al.*, 2002; Cools *et al.*, 2003; Heldin *et al.*, 1998; Rocconi *et al.*, 2008, Fleming *et al.*, 1992). Because of this, deciphering the mechanism of action of PDGFR is necessary to treat PDGFR-associated pathogenesis.

1.2 Mechanism involved in RTK endocytosis – rationale and hypotheses

1.2.1 Introduction

Internalization of cell-surface receptor proteins from the plasma membrane of cells into cytoplasmic compartments is a characteristic of many receptors. The internalization process may be a means for down-regulating surface receptors for cellular desensitization or receptor degradation (Samanta *et al.* 1990; Ercolani *et al.,* 1985; Yarden 2001; Grandal *et al.*, 2007). Recent evidence has reinforced the notion that the initiation and transduction of receptor signals does not occur only at the plasma membrane, but also occurs in endosomes; a phenomenon referred to as the 'signalling endosome hypothesis' (Wang *et al.,* 2004; Pennock and Wang, 2003; Holegoua *et al.*, 1991, Shao *et al.,* 2002). This has led to the hypothesis that the transition from surface to endosomal signal transduction is a means for modulating the intensity or directionality of cellular outcomes (Burke *et al.,* 2001). Moreover, mutant receptors strongly associated with cancer and fibrotic diseases have been shown to remain catalytically active on the plasma membrane rather than internalizing upon ligand binding (Grandal *et al.*, 2007). In support of this hypothesis, several viral proteins have been shown to stabilize signalling receptors on the plasma membrane, thereby increasing the host cell's mitogenic potential and ultimately leading to aberrant cell growth and metastasis (Yarden, 2001). Being able to understand and resolve these pathogenic mechanisms demands that we obtain a higher resolution of events that occur during receptor-mediated endocytosis.

In order to fully understand and delineate the process of PDGFR internalization, it is necessary to define the beginning and the end of the endocytic process. With respect to the material presented in this thesis, we characterize PDGFR endocytosis as follows: the physical location of PDGFR endocytic initiation is the plasma membrane of the cell where the receptors exist as monomers (Figure 5, I). PDGF binding results in the dimerization of the receptors (Figure 5, II). Receptor dimerization is followed by the collection of PDGF receptors into either caveolae, where signalling cascades are thought to be initiated, or clathrin-coated pits (Liu *et al.*, 1996; Liu *et al.*, 2000; Sorkin *et al.*, 1991; Kapeller *et al.*, 1993; Joly *et al.*, 1994) (Figure 5, III). The molecular events that initiate PDGFR internalization (the nucleation of clathrin-coat assembly, for example) are unknown. Although the activity of caveolae-associated uptake is not well defined, it has been established conclusively that clathrin-coated pits pinch off from the plasma membrane into clathrin-coated vesicles due to dynamin fission activity (Rappoport, 2008) (Figure 5, IV). Dissolution of the coated vesicle's coats occurs and the result of the endocytic process is the accumulation of PDGFR in punctate cytosolic bodies called endosomes (Figure 5, V). Although many different species of endosomes exist, we will define the completion of PDGFR internalization as the ingression into early endosomal structures. Succeeding endocytosis is the dissociation of the PDGFR dimer complex, which either recycles back to the plasma membrane, or is degraded in lysosomes or via cytoplasmic degradation by proteosomes after ubiquitination (Mori *et al.*, 1992; Mori *et al.*, 1995; Sorkin *et al.*, 1991; Heldin and Westermark, 1999) (Figure 5, VI).

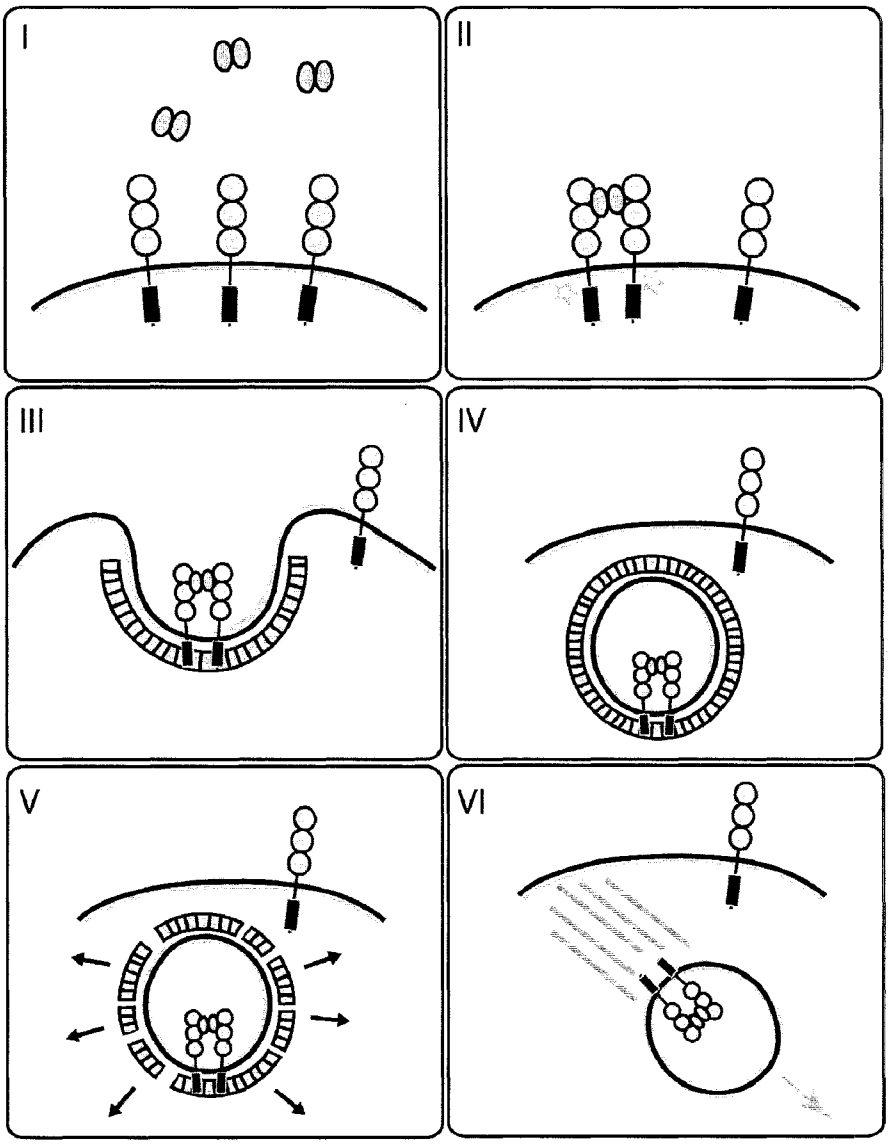


Figure 5 – Receptor-mediated endocytosis. RTKs exist as monomers on the plasma membrane of cells (I). Interaction between an extracellular ligand(s) (purple) and the RTK results in dimerization and subsequent kinase activation (stars) of the RTK (II). Factors and events that are not yet fully understood result in the recruitment of internalization machinery. These factors drive membrane curvature, creating a coated pit (III). The coated pit pinches off from the plasma membrane and is now a coated vesicle (IV). The coating disbands (V), leaving a vesicle called an endosome, which is then transported to various areas of the cell (VI).

Now that the limits of PDGFR internalization have been bounded, the principal hypotheses that are tested in this thesis are discussed in the next three sections. These hypotheses focus on three general RTK categories that are all related – namely, kinase activity, receptor dimerization, carboxyl terminal internalization motifs, and their involvement in receptor-mediated endocytosis of PDGF receptors.

1.2.2 Intrinsic kinase activity and PDGFR endocytosis

Work from the 1980s and 1990s suggested that RTK endocytosis is largely dependent on intrinsic kinase activity (Sorkin *et al.*, 1991; Mori *et al.*, 1994; Dougher and Terman, 1999; Glenney *et al.*, 1988; Welsh *et al.*, 1994). For example, studies implicated a requirement for a juxtamembrane tyrosine residue, which when mutated, decreased the efficiency of PDGFR internalization (Mori *et al.,* 1993). This residue is phosphorylated upon kinase activation and thus, kinase activity is suggested to be required in PDGFR internalization. PI3K has also been shown to have an effect on PDGFR internalization. This was suggested when decreased PDGFR internalization was detected when PDGFR amino acids residues important for PI3K:PDGFR interaction, were mutated (Joly *et al.,* 1994). These residues are also phosphorylated upon kinase activation, indirectly suggesting the need for PDGF receptor kinase activity during PDGFR internalization.

There is also direct evidence suggesting that kinase activity is involved in the process of PDGF receptor internalization. In porcine aortic endothelial cells not expressing endogenous PDGFR, the relative rate of receptor internalization in kinase-dead (K634A) PDGFR receptor expressing cells was slower relative to wild-type kinase-

active receptor expressing cells (Sorkin *et al.*, 1991). With the subsequent development of more sensitive technology, the involvement of PDGFR kinase activity in PDGFR internalization can be further investigated at higher resolution.

More recent evidence suggests that kinase-independent RTK internalization also exists (Wang *et al.*, 2005). While earlier studies suggested that internalization of EGFR could occur in the absence of kinase activity, receptor sorting rather than receptor internalization was the central focus of analysis (Felder *et al.*, 1990). Wang *et al.* (2005) utilized biochemical and visual methods to characterize the internalization pattern of EGFR, and it was determined that kinase activity is not involved in the internalization of EGFR (Wang, *et al.*, 2005). It was not clear whether this finding could be generalized to all RTKs, so PDGFR is used in this study as a second model system.

These data led to the first hypothesis I set out to test as part of my thesis: **Similar to what was seen with EGF receptor, PDGF-mediated PDGFR internalization will occur independently of PDGFR intrinsic kinase activity.**

1.2.3 Involvement of receptor dimerization in PDGFR internalization

PDGF receptor oligomerization was found to occur after ligand binding (Heldin *et al.*, 1989; Ullrich, and Schlessinger, 1990; Claesson-Welsh, 1994). The downstream effects of receptor dimerization are not well defined. Arguably, the single most cited and described repercussion of PDGFR dimerization is the activation of intrinsic kinase activity; yet very few publications point out other roles for receptor dimerization. As

mentioned in the previous section, Wang *et al,* (2005) provide a strong argument that in addition to activating RTK kinase activity, receptor dimerization is also a central factor in directly driving receptor internalization. In these experiments, inhibiting receptor dimerization virtually eliminated the internalization of EGF receptor (Wang *et al.*, 2005). To investigate whether dimerization is a driving force for RTK internalization, the PDGFR is utilized as a second model system to confirm or reject the universality of this hypothesis.

These data led to the second hypothesis I set out to test as part of my thesis: **Similar to what was seen with EGFR internalization, PDGF-mediated PDGFR internalization is driven primarily by PDGFR dimerization.**

1.2.4 Involvement of internalization codes in PDGFR internalization

Endocytic RTK cytosolic amino acid "codes" have been shown to mediate the process of RTK internalization (Backer *et al.,* 1990; Chang *et al.*, 1993; Welsh *et al.*, 1994). This is not surprising as many other protein trafficking processes also make use of conserved motifs. Well known trafficking codes include KDEL and KKXX sequences that if present in a particular region of a protein result in the protein being retained in the endoplasmic reticulum (ER) and/or recycled back to the ER from the Golgi in mammalian cells (Murshid and Presley, 2004). Additional examples include conserved nuclear localization signals (NLS) or peroxisomal localization signal (PLS) (Lange, *et al.*, 2007; Olivier and Krisans, 2000). It is likely that other protein trafficking codes remain undefined and RTK endocytic codes are not an exception.

Previous work has characterized at least three separate endocytic codes in both EGFRs and IRs. Consisting of less than a dozen amino acids in size (NPXT in IR, NPXY in low density lipoprotein receptor, YTRF in transferrin receptor, and QQGFF, FYRAL or LL in EGFR), each endocytic code elicits different strengths of internalization for different receptors (Bansal and Gierasch, 1991; Rajagopalan *et al.*, 1991; Backer *et al.*, 1992; Backer *et al.*, 1990; Wang *et al.*, 2005; Chang *et al.*, 1993). On the other hand, internalization code(s) within PDGFRs have not been well characterized. Early work by Mori *et al.* (1991) raised the possibility that a short 14 amino acid region C-terminal of the kinase domain is involved in PDGFR internalization. Analysis of truncation mutants with the 14 amino sequence removed suggested that the sequence is required for PDGFR internalization (Mori *et al.*, 1991). With this in mind, the third part of this project aimed to confirm these data, as well as to provide direct evidence with regards to whether or not the intervening 14 amino acid sequence is involved in PDGFR endocytosis.

These data led to the third hypothesis I set out to test as part of my thesis: **Similar to what was seen with EGFR internalization, PDGF-mediated PDGFR internalization is dependent on the presence of intrinsic endocytic codes in the carboxyl terminus of PDGFR.**

Chapter Two:

Materials and Methods

2.1 Methods

2.1.1 Cell Lines

The cell lines that were used for data collection in this thesis are the following: Mouse NIH-3T3-F442 (F442) fibroblast cell line expressing wild type endogenous PDGFR-β (expressing 50,000-100,000 receptors/cell), human HepG2 cell lines stably transfected with wild type PDGFR-β (HepGP) or kinase dead K634R PDGFR-β (HepGR) (expressing ~500,000 receptors/cell) (Vaillancourt *et al.*, 1996; Baxter *et al.*, 1998; Montmayeur *et al.*, 1997; Klinghoffer *et al.*, 1996)), wild type HepG2 (HepGW) cell lines with no detectable endogenous PDGFR-β expression (Richard Lehner, University of Alberta), and a Human Embryonic Kidney cell line containing simian virus 40 T-antigen (293T). One clone of each of the stably transfected kinase-active and kinase-dead PDGFR HepG2 cell lines was obtained from Dr. Andrius Kazlauskas (Harvard University).

Cells expressing kinase-active PDGFR (HepGP and F442 cells in the absence of kinase inhibitor AG1296) will be referred to in this thesis as kinase-active cells, whereas cells expressing kinase-inactive PDGFR (HepGR as well as HepGP and F442 treated with kinase inhibitor AG1296) are called kinase-inactive or kinase-dead cells.

2.1.2 Preparation of cell culture

Prior to each experiment, F442 cells were grown to 40-60% confluency in 10% fetal bovine serum (FBS) (Sigma) containing Dulbecco's modified eagle medium

(DMEM) (Gibco) and subsequently serum starved for 12 hours in 2% FBS-containing medium followed by complete starvation in DMEM (0% FBS) for 4 hours. Stably transfected HepG2 cells were grown to 40% confluency in 5% FBS-containing DMEM, serum starved for 24 hours in 1% FBS-containing medium follow by complete serum starvation in DMEM for a further 12 hours, rendering a final confluency of 60-80%. HepGW cells were grown to 60-80% confluency, transiently transfected in serum-free medium using Lipofectin (Invitrogen), and subsequently serum starved for a further 24 hours before analysis. 293T cells were grown to 50% confluency, transiently transfected in serum-free medium using $CaCl_2$ and BES (Table 2.2.8), followed by further growth in 5% FBS-containing DMEM and subsequent serum starvation for 12-18 hours before analysis.

2.1.3 PDGFR immunofluorescence and/or Western Blot assay

Cells were grown as described in Section 2.1.2 and treated with 10 ng/ml PDGF-BB (Biosource) to induce PDGFR-β internalization for the allotted time period. PDGF (-), also called serum-free (SF) samples, were not treated with ligand. For PDGFR kinase inhibitor experiments, cells were pre-incubated with 50 μM AG1296 (Calbiochem) at 37° C for 45 minutes in DMEM, the medium aspirated, and pre-warmed DMEM containing 10 ng/ml PDGF-BB and 50 μM AG1296 was added for the indicated time period. Following treatment, cells were lysed using Mammalian Protein Extraction Reagent (M-PER) (Pierce) and Nonidet P40 (NP-40) (BDH) with protease inhibitor cocktail (Table 2.2.8). Lysates were centrifuged at 4° C for 30 minutes at 17, 000 x *g*.

Protein concentration from the supernatant was quantified using the Bradford protein dye assay (Bradford, 1976). Protein concentration was calculated using the 595 nm absorbance value as measured by a Beckman DU 640 spectrophotometer (Beckman Instruments). The lysate was boiled for 5 minutes in loading buffer and analyzed with PAGE and Western blot using anti-PDGFR (sc-339) (Santa Cruz), anti-phospho-PDGFR (sc-12907) (Santa Cruz) or anti-GFP (Luc Berthiaume, University of Alberta).

Immunofluorescence was completed in order to visualize PDGFR within cells. Cells were grown (Section 2.1.2) and treated (Section 2.1.3) on cover slips and then fixed in 4% paraformaldehyde or -20° C methanol for 10 or 4 minutes at room temperature, respectively. Cells were then permeabilized with 0.4% Triton-X for 5 minutes and incubated with PDGFR specific primary antibodies (sc-339, sc-6252, sc-19995, sc-6414 or sc-12907) for 1 hour at room temperature or overnight at 4° C. The cover slips were briefly washed with PBS (Table 2.2.8) and secondary antibodies FITC-α-rabbit or rhodamine-α-mouse applied for 1 hour at room temperature and mounted on slides. Transfections were carried out by using transient calcium phosphate transfection in 293T cells or Lipofectin in HepG2 wild type cells. The cells were prepared and treated as mentioned above and fixed with -20° C methanol for 4 minutes and mounted. The cells were viewed using an Olympus IX71 microscope with a CoolSnap HQ2 camera (Applied Precision) and the data were analyzed using Delta Vision softWoRx software.

2.1.4 Cross-linking assays

In order to study the dimerization of PDGFRs, samples were prepared as described in Section 2.1.2 and treated as listed in Section 2.1.3. After treatment, intact cells were collected and treated with 0.01-1 mM di-succinimidyl suberate (DSS) (Calbiochem) at 4° C. After one hour, 1M Tris-HCl, pH 7.5, was added to the cells to a final concentration of 20 mM in order to quench the cross-linking reaction. This was followed by addition of Triton X-100 (BDH) and NP-40 to a concentration of 1% each and extensive vortexing. The lysate was analyzed via PAGE and Western blot.

2.1.5 Ligand independent dimerization assay

In order to artificially dimerize PDGFRs, a chimeric protein consisting of PDGFR-β with FKBP at the carboxy terminus was generated using the ARGENT^{TM} Regulated Homodimerization Kit 2.0 plasmid pC_4-F_v1E vector (ARIAD). The vector was transiently transfected into 293T or HepGW as previously mentioned. The cells were treated with PDGF-BB (15 ng/mL) or the FKBP cross-linker AP20187 (50 nM, ARIAD) for 30 minutes followed by fixation using -20° C methanol for 4 minutes and further prepared for PDGFR immunofluorescence visualization as previously described (sc-339 antibody). Images were obtained as previously described.

2.1.6 Flow cytometry

PDGFR and EGFR internalization was assessed using flow cytometry. Cells were prepared as mentioned above. For treatment with kinase inhibitor, cells were incubated at 4° C for 10 minutes followed by incubation with Alexa 647-PDGF-BB and AG1296 (50 μM) at 4° C for 45 minutes to allow for binding of the ligands without induction of internalization. Alexa 647-EGF (Molecular Probes) was used as the ligand for EGFR internalization studies. The cells were washed once with cold PBS and once with room temperature PBS, and 37° C DMEM (+/- AG1296) was added to the culture. The cells were incubated at 37° C for the indicated times. Residual membrane-bound ligand was stripped from the sample using acid stripping buffer (Table 2.2.8) for 3 minutes at 4° C and washed with 4° C PBS. The total fluorescence sample was not acid stripped and only washed after pre-incubation, while the background fluorescence negative control sample was washed and acid stripped in a similar manner to the experimental samples. The cells were suspended in FACS buffer (Table 2.2.8) and FACS fixation buffer (Table 2.2.8) and 10,000 cells were analyzed using a BD Bioscience FACScanto. Mean and standard deviation were calculated from three independent experiments. Receptor internalization was computed as

$$\% \text{ internalized} = \frac{(\text{sample fluorescence} - \text{background fluorescence})}{(\text{total fluorescence} - \text{background fluorescence})} \times 100.$$

2.1.7 Quantitative deconvolution microscopy (QDM)

HepGP and HepGR cells were infected with Rab5a-GFP Organelle lights (Invitrogen), incubated for 16 hours in 5% FBS-containing medium, and subsequently serum starved and treated with ligand as mentioned in previous sections. Immediately after ligand treatment, cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells were permeabilized using 0.2% Triton X-100 in PBS, and PDGF receptors were labelled with α-PDGFR primary antibody (sc-339) overnight at 4° C followed by secondary staining with rhodamine anti-rabbit antibodies for 1 hour at room temperature. The cells were then mounted and viewed using bright field microscopy. The contrast of fluorescent images was enhanced to assist in defining the location of plasma membrane, while Rab5a-GFP was used as a marker for endosomes. Plasma membrane and endosomal locations in each *z*-stack were separated. These separated regions are called masks. The masks were then applied to PDGFR fluorescence in the cell, creating two discernable PDGFR populations. A ratio of total receptor fluorescence within the confines of the plasma membrane mask (receptors on the plasma membrane), and the receptor fluorescence in the Rab5a mask (receptors in the endosomes) was generated. The data was represented as a ratio of plasma membrane receptors to endosome localized receptors. See Box 1 and Figures 6 and 7 for a step-by-step description of data capture.

Six cells from each sample cover slip were analyzed. These 6 cells originated from at least 3 different images taken from different areas of the cover slip. This entire experiment was completed twice (two cover slips) for each sample and the mean ratios considered independent values. Selected cells were chosen primarily due to clarity and

Box 2.1 – A Step-by-step protocol that describes how data were obtained using quantitative deconvolution microscopy. Box 1 should be used in conjunction with Figure 6 and Figure 7.

1) Take the picture and deconvolve it (encompasses the entire depth of the cell)
2) Choose and crop a single cell
3) Enhance the contrast of fluorescence (only alters display parameters)
4) Manually define the region of the PM using endosomes as an additional guide
5) Separate the PM mask from the original image (only λ = rhodamine) (image a)
6) Separate everything outside the mask from the original image (λ = rhodamine and eGFP) (image b)
7) Manually define the cytoplasm of the cell from image b by encapsulating everything within the PM mask cut-out area → (image c)
8) Use auto polygon finder (Softworx plug in) to define the area of the eGFP endosomes and cut mask of only the λ = rhodamine spectrum (image d)
9) Use auto polygon finder to define the PM (image a) →(image e) and apply the same threshold to image (d) → (image f)
10) Change the header of image e to one that differs from λ = rhodamine, (618 nm for example) → (image g)
11) Use image fusion to compile images g and e together →image h
12) Project the fused *z*-stack image h → (image i) to sum all fluorescent intensity into a single plane
13) Record the total intensity values for each wavelength
14) Compute a ratio of the two values

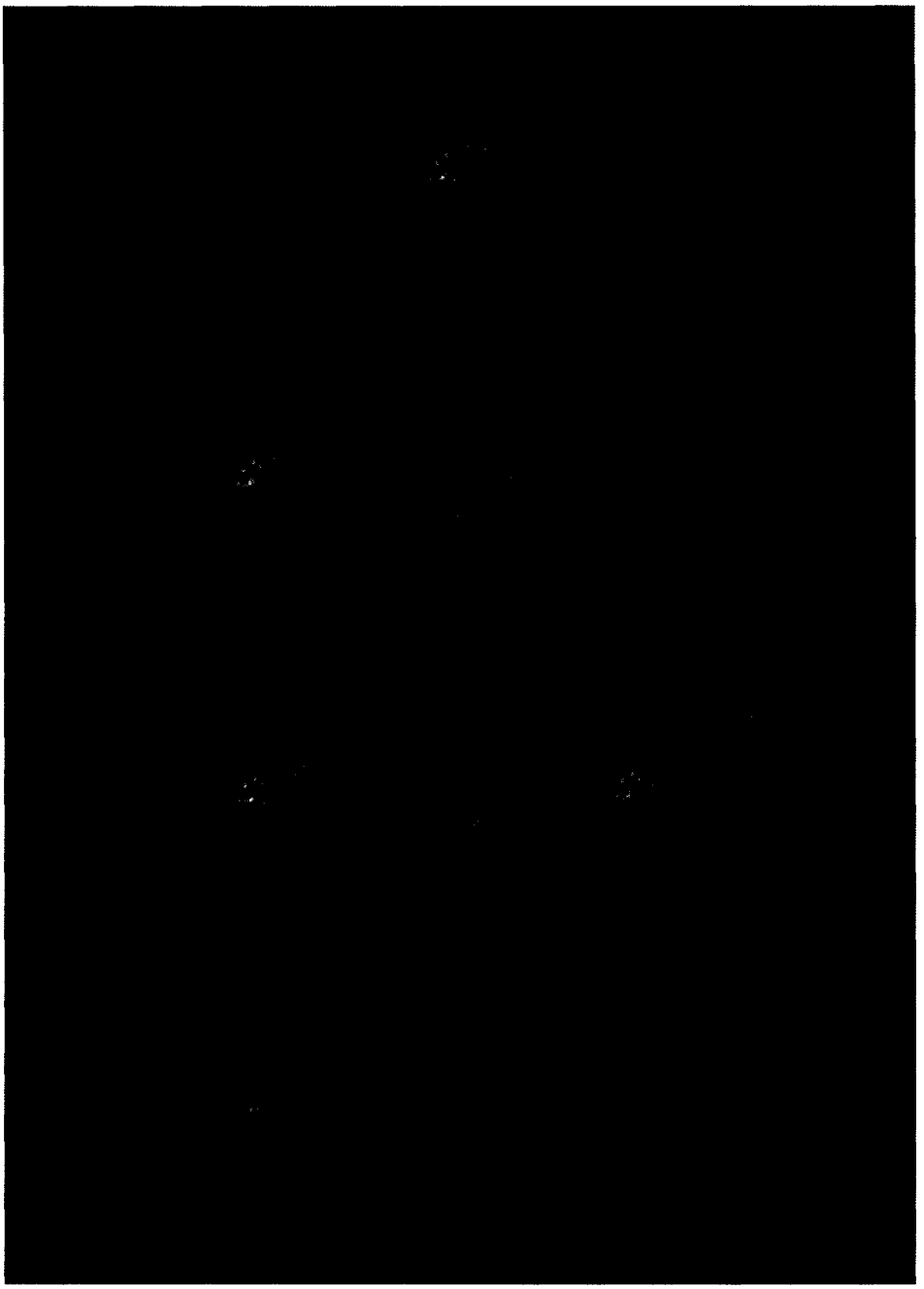


Figure 6 – Visual key for QDM protocol (Part I). To be used as a guide for the written protocol in Box 1. Keep in mind that the steps described are completed for each *z*-stack in order to encapsulate the entire cell volume. Step number is denoted in red, while image reference is blue.

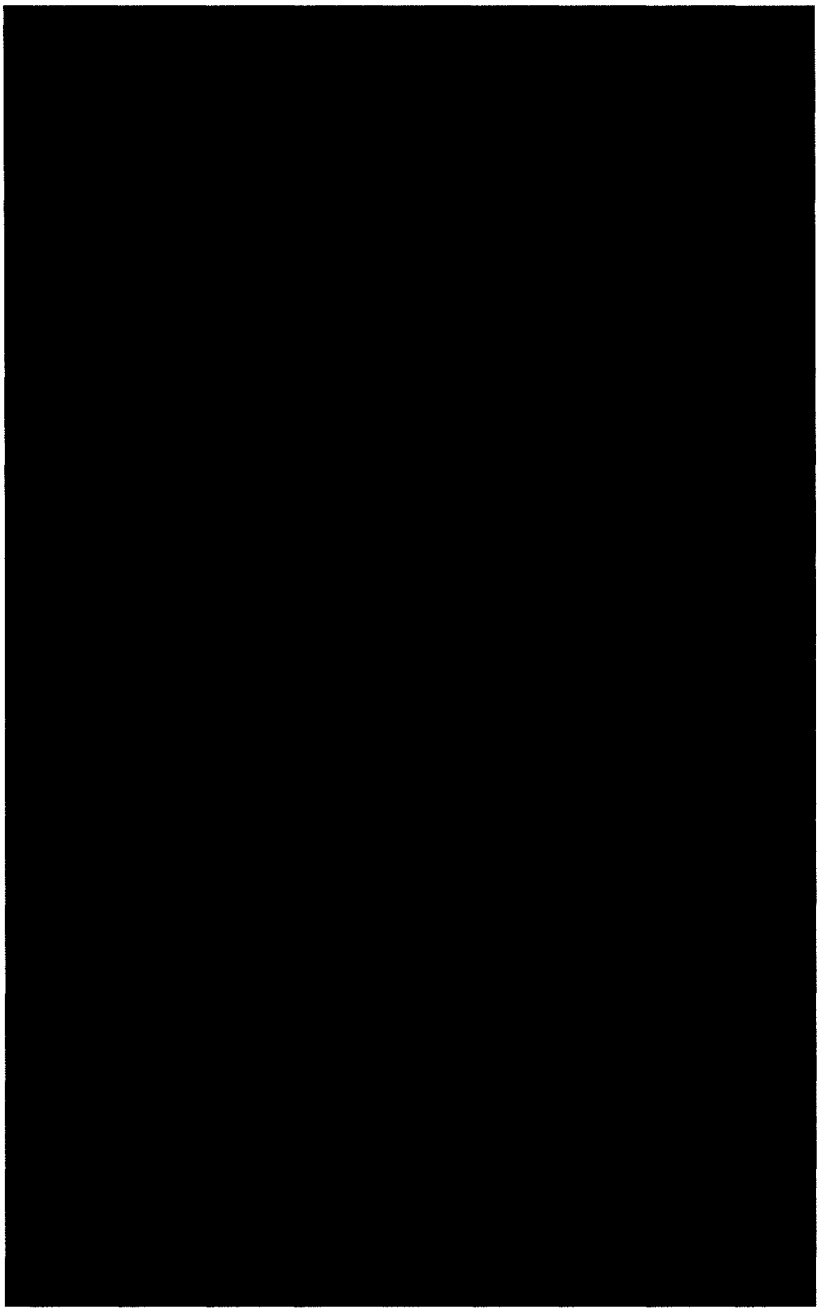


Figure 7 - Visual key for QDM protocol (Part II). To be used as a guide for the written protocol in Box 1. Keep in mind that the steps described are completed for each *z*-stack in order to encapsulate the entire cell volume. Step number is denoted in red, while image reference is blue.

strength of the fluorescent signal. F442 cells were not analyzed by this method because they are not capable of being infected by baculovirus, the DNA delivery vector used in Organelle Lights technology. Because HepGR is a different cell line than HepGP, the negative control PM:ES ratios are different. In order to compare the HepGR and HepGP PM:ES ratios, the HepGR PM:ES mean ratio was normalized to the serum free mean PM:ES ratio of HepGP samples.

2.1.8 Molecular biology

The pEYFP-N1 expression plasmids containing subcloned wt-PDGFR or wt-EGFR were used as a template for the construction of mutants employed in this study. For truncation mutants, polymerase chain reaction (PCR) primers were designed complementary to the 5' end of PDGFR template as well as the 3' end at the designated truncation point. PCR was used to amplify the coding region beginning at the 5' end of PDGFR coding sequence to the 3' end of PDGFR coding region as dictated by the complementation point of the PCR primer. For the construction of Δ952-965-PDGFR, Pe, and Ep mutants, primers were designed so that they would anneal at the junction of interest (952-965 or 1005-1017) and in place of the wild type junction of interest sequence, one-half of the exogenous sequence was tagged onto each primer. Following PCR with these primers, the resultant product was the entire vector excluding the junction of interest, which was now exchanged for the desired sequence. To allow for blunt end ligation of PCR products, 5' phosphorylation modification was incorporated into the oligonucleotides. The deletion mutant oligonucleotides simply lacked an exogenous

sequence. The methylated template strands were degraded using DpnI, and XL-10 Gold competent bacterial cells were transformed.

2.1.9 Whole cell lysate

Protein lysates from 293T, HepG2 or F442 cells were obtained by lysing cells with M-PER Mammalian Protein Extraction Reagent and NP40 with protease and phosphatase inhibitor cocktail. The lysates were centrifuged at 4° C for 30 minutes at 17, 000 x *g*. Following protein quantification using the Bradford protein dye assay (Bradford, 1976) and a Beckman DU 640 spectrophotometer, the supernatant was boiled in SDS-loading buffer for 5 minutes.

2.1.10 PAGE and Western blot

SDS-polyacrylamide gels ranging from 6-8% were used depending on the size of the protein being studied. In order to maintain a constant total mass of protein added, in each experiment 10-25 μL of protein lysate was inserted into each well. Gels were electrophoresed at 179 V. Proteins in the SDS-polyacrylamide gels were transferred onto Transblot nitrocellulose membranes at 18 V for 50 minutes using a semi-dry blotting apparatus (Model SD transfer cell, BioRad). Nitrocellulose membranes were blocked with 3% skim milk in 0.05% Tween-PBS for 15 minutes. Membranes were then incubated with primary antibody in blocking buffer overnight at 4° C, briefly washed with ddH_2O (with a pH of 5.5), incubated with appropriate HRP-conjugated IgG

secondary antibody for 1 hour, washed twice with 0.05% Tween-PBS for 5 minutes, and washed with TBS buffer for an additional 5 minutes. HRP-conjugated antibodies were detected utilizing Super Signal ECL Western Blotting Detection Reagents (Pierce) and exposed on SuperRx X-ray film (Fugifilm). Densitometric analysis of the resulting bands was completed using Image J software. All antibodies, including their dilutions, are described in Table 2.2.7.

2.1.11 PDGFR-FKBP internalization in the presence or absence of GM1 or kinase inhibitor AG1296

293T cells were transiently transfected with PDGFR-FKBP construct and serum starved as previously mentioned. Cells either were or were not treated with 100 μM monosialotetrahexosylganglioside (GM1) in serum free medium for 1 hour with subsequent addition of either PDGF (20 ng/mL) or AP20187 (50 nM). Samples were prepared for immunofluorescence using the previously mentioned protocol. If kinase inhibitor AG1296 was utilized in the experiment it was used as previously mentioned.

For GM1-FKBP experiments (Figure 19) at least 50 cells per sample cover slips were analyzed for the presence of substantial receptor internalization. Because this is preliminary data, no quantitative based assay was used. Cells demonstrating a receptor localization phenotype similar to a serum free cell sample are considered non-internalizers, while cells exhibiting a phenotype similar to the 30 minute PDGF/AP20187 treatment are internalizers. In particular, the presence of larger circular endosomal-like structures was an indicator of endocytosis (Figure 8, arrows), although cells exhibiting

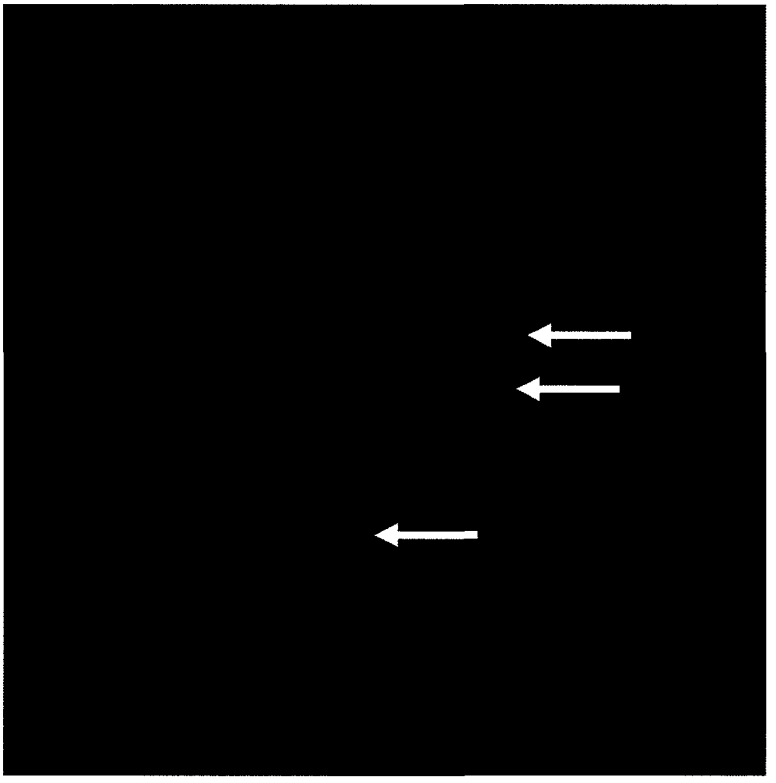


Figure 8 – Immunofluorescently labelled HepGW cell transfected with PDGFR-FKBP. Each cell analyzed in this experiment was either considered internalization positive or internalization negative based on the criteria mentioned in Section 2.1.11. Arrows indicated large circular endosomal-like structures and punctate positively stained structures. This cell is an internalizer, whereas a cell lacking circular endosomal-like and punctate stained structures is considered a non-internalizer.

small punctate positively stained structures were also considered to be internalization competent. For DSS cross-linking, the cells were treated in the same manner as above except gathered from the plates and subjected to DSS (0.05 mM) as previously mentioned, rather than fixed for immunostaining.

2.1.12 Statistics

Two-tailed t-tests with separate variances due to small sample sizes were completed using MYSTAT 12 (student version of SYSTAT 12) with an alpha level of 0.05.

2.2 Materials

All reagents and lab supplies were used in accordance to the manufacturer's specification as well as the protocols set out by the Environmental Health and Safety of the University of Alberta and Workplace Hazardous Materials Information System (WHMIS).

Table 2.2.1 Reagents

Reagent	Vendor
Acetic acid, glacial	BDH
Acrylamide/bis	BioRad
AEBSF (4-[2-aminoethyl]-benzenesulfonyl fluoride)	Sigma
AG1296	Calbiochem

Agar	Gibco
Agarose	Gibco
Alexa-fluor 647-labeled EGF	Mol. Probes
Ammonium persulfate	BDH
Aprotinin	Sigma
AP20187 (Argent Homodimerization Kit)	Ariad
Bromophenol blue	Biorad
Buria-Bertani media, broth base	Gibco
Calcium chloride	Sigma
Camptothecin	Calbiochem
Dimethyl sulfoxide	Fisher
Di-succinimidyl suberate (DSS)	Calbiochem
Dulbecco's modified eagle medium (DMEM)	Gibco
Epidermal growth factor (EGF)	Upstate
Ethanol, 95%	Fisher
Ethidium bromide	OmniPur
Diaminoethanetetraacetic acid (EDTA)	Sigma
Fetal bovine serum (FBS)	Sigma
Glucose	EM science
Glycerol	BDH
Glycine	Biorad
GM1 (monosialotetrahexosylganglioside)	
Hydrochloric acid	Fisher

Isopropanol	Fisher
Kanamycin	Sigma
Lipofectin Reagent	Invitrogen
Magnesium chloride	BDH
Mammalian Protein Extraction Reagent (M-PER)	Pierce
N,N-bis[2hydroxyethyl]-2-aminoethanesulfonic acid (BES)	Sigma
Non-essential amino acids	Gibco
Nonidet P40 (NP-40)	BDH
Organelle Lights (Rab5a-eGFP)	Mol. Probes
PDGF-BB	Biosource
Penicillin/streptomycin (10000U)	Gibco
Pepstatin A	Sigma
Phosphate buffered saline, 10x	OmniPur
Potassium chloride	BDH
Sodium azide	Sigma
Sodium chloride	BDH
Sodium dodecyl sulfate (SDS)	Biorad
Sodium fluoride	Sigma
Sucrose	Biobasic
Tetramethylethylenediamine (TEMED)	Gibco
Tris (hydroxymethyl) aminomethane	Biorad
Triton X-100	BDH
Tween 20	Fisher

Yeast extract, select	Gibco
β-mercaptoethanol	Sigma

Table 2.2.2 Enzymes

DNA ligase, T4	Gibco
dNTPs	Invitrogen
HotStarTaq, DNA polymerase	QIAGEN
Restriction endonucleases	Gibco, NEB
RNase A	Sigma

Table 2.2.3 Experimental Kits

QIAprep Spin Miniprep Kit	QIAGEN
Alexa Fluor 647 Microscale Protein Labelling Kit	Mol. Probes
HiSpeed Plasmid Midi Kit	QIAGEN
QIAquick Gel Extraction Kit	QIAGEN
QuikChange XL Site-Directed Mutagenesis Kit	Stratagene
Super signal ECL Western Blotting Detection Reagents	Pierce
Topo-XL PCR cloning kit	Invitrogen
Argent Regulated Homodimerization Kit V 2.0	Ariad

Table 2.2.4 Plasmids

pCR-XL-TOPO	Invitrogen

pEYFP-C1	Clontech
pC4EN-F1	Ariad
pEGFP-C2	Clontech

Table 2.2.5 Molecular Size Markers

100bp DNA ladder	Gibco
1kb DNA ladder	Gibco
Pre-stained markers for SDS-PAGE	Sigma

Table 2.2.6 Other Materials

Medical X-ray Film	Fuji
Transblot Nitrocellulose	Biorad
Whatman Chromatography Paper	Fisher
Dehydrated milk for WB blocking	Pacific

Table 2.2.7 Antibodies and their dilutions used for immunoblotting and immunofluorescence

Primary Antibody	**Dilution**	**Vendor**
Mouse anti-PDGFR-β (sc-6252)	1:100	Santa Cruz
Rabbit anti-GFP/YFP	1:4000	Dr. Luc Berthiaume
Rabbit anti-PDGFR-β (sc-339)	1:200	Santa Cruz

Rabbit anti-phospho-PDGFR-β (sc-12909)	1:200	Santa Cruz
Rabbit anti-phospho-PDGFR-β (sc-12907)	1:200	Santa Cruz

Secondary Antibody	**Dilution**	**Vendor**
HRP-conjugated anti-rabbit	1:1000	BioRad
HRP-conjugated anti-mouse	1:1000	BioRad
HRP-conjugated anti-goat	1:1500	BioRad
FITC-conjugated anti-rabbit	1:250	BioRad
FITC-conjugated anti-goat	1:250	BioRad
Rhodamine-conjugated anti-goat	1:250	BioRad

Table 2.2.8 Buffers and other solutions

Solution	**Composition**
Acidic stripping buffer	100 mM acetic acid, 150 mM NaCl, pH 2.8
BES buffer	50 mM-N,N-bis[2-hydroxyethyl]-2-aminoethane sulfonic acid, 280 mM NaCl, 1.5 μM $Na_2HPO_4 \cdot 2H_2O$, pH 6.96
FACS buffer	2% FBS, 0.01% sodium azide, PBS
FACS fixation solution	4% formaldehyde, PBS
Phosphate-buffered saline (PBS)	137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer
Protease inhibitor cocktail	0.5 mM Na_3VO_4, 0.1 mM 4-[2-aminoethyl]-benzenesulfonyl fluoride, 10 μg/ml aprotinin and 1 μM pepstatin A
SDS-loading buffer	250 mM Tris-Cl, 40% glycerol, 8% sodium dodecyl sulfate, 20% β-mercaptoethanol, 2% bromophenol blue

SOC medium	2% bactotyptone, 0.5% yeast extract, 10mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose
Tris-acetate EDTA buffer	2 M Tris , 1 M glacial acetic acid, 300 mM ethylenediaminetetraacetic acid, pH 8.0
Tris-buffered saline	NaCl 150 mM, Tris-HCl 50 mM, pH 7.5
Transfer buffer	48 mM Tris-Cl, 39 mM Glycine, 20% methanol, 0.03% sodium dodecyl sulphate
Triton X-100 lysis buffer	0.4% triton X-100, 140 mM NaCl, 50 mM Tris-Cl, pH 7.2, 1 mM ethylene glycol tetra acetic acid

Table 2.2.9 Oligonucleotides used in this thesis

Name	**Sequence (5'-3')**
PDGFR-lead	GCTAGCATGCGGCTTCCGGGTG
CT155-lag	GGTACCGAAGGGGGGCCGAATCTC
CT141-lag	GGTACCACCTTCGCCCAACAGTC
PDGFR 952-65 deletion-lead	p-TACAAAAAGAAGTACCAGGTGG
PDGFR 952-65 deletion-lag	p-GAAGGGGGGCCGAATCTC
PDGFR truncation sequencing primer	CCATGCCATCAAACGGGGTTACC
Pe-lead	p-AGCTCTCTGAGTGCAACCAGCTACAA AAAGAAGTACCAGCAGGTGG
Pe-lag	p-CAGGAGGGGAGTCCGTGACGTGAAGGG IGGCCGAATCTC

Ep-lead	p-GAGAGACTGTTGGGCGAAGGTAACAATT CCACCGTGGCTTGC
Ep-lag	p-GAGAAGCAGCACCAGCTGGGAGGAGGGGC TGCTGAAGAAGC

Chapter Three:

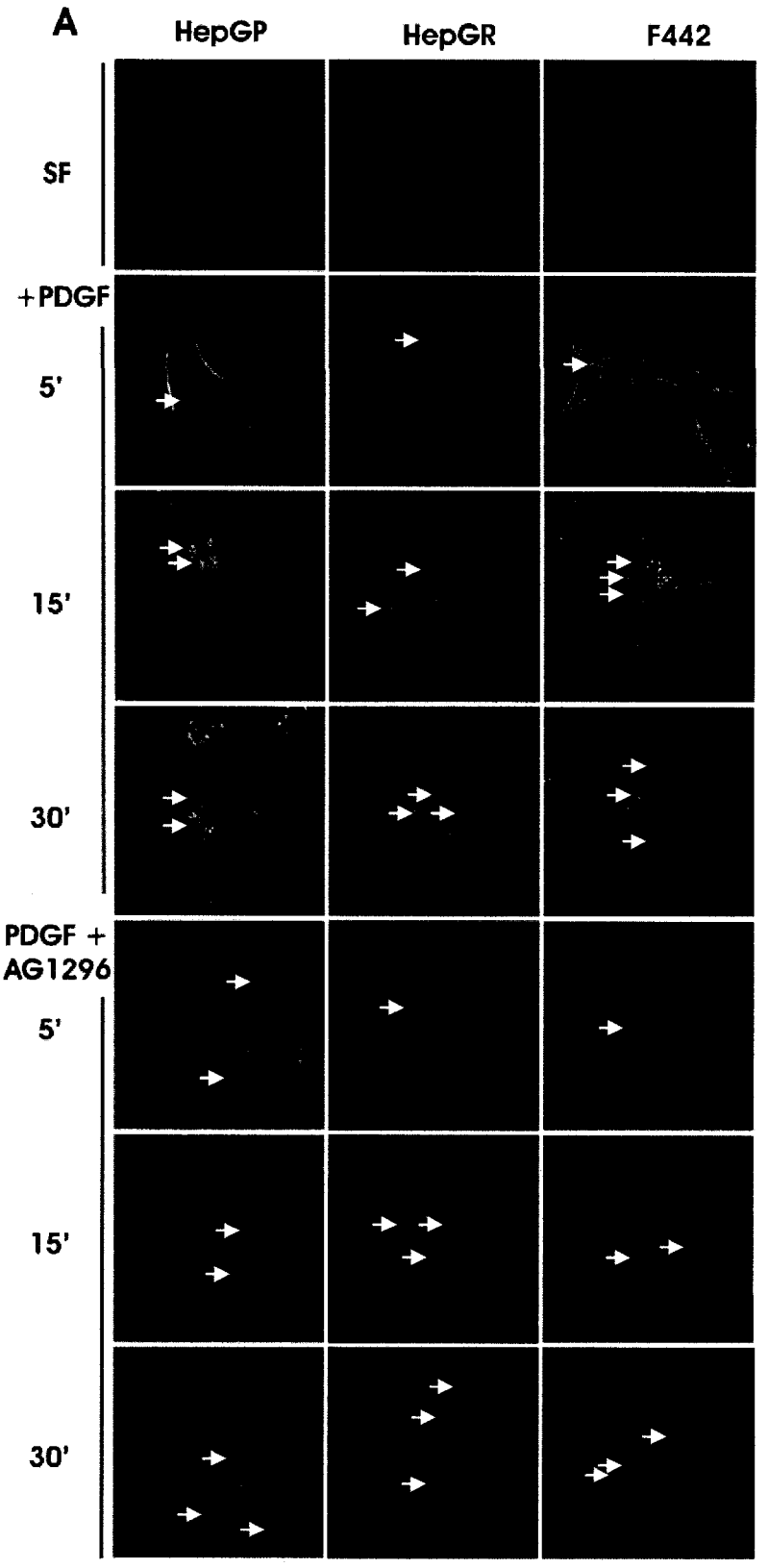
Results - Mechanisms of PDGFR Endocytosis

3.1 Kinase activity is not necessary for PDGFR internalization

To determine whether PDGF receptor kinase activity is required for PDGF-BB induced PDGFR internalization, we used immunofluorescence microscopy (IF) to visualize the location of PDGFRs before and after PDGF-BB treatments (Figure 9). HepGP and F442 cells were used as indicators for kinase-active PDGFR internalization. HepGR cells as well as HepGP and F442 cells treated with PDGFR kinase inhibitor AG1296 were used to visualize PDGFR internalization in absence of kinase activity. Serum starved cells without PDGF-BB treatment were used as negative controls.

In the serum free (SF) samples of each cell type, the majority of PDGFR is localized to the plasma membrane and has a very low level of kinase activity which is reflected experimentally by virtually undetectable phospho-PDGFR (pPDGFR) staining (Figure 9). The PDGFR stained puncta in serum starved samples may be present partially due to basal receptor internalization, but these puncta do not colocalize with endosomal markers. This suggests that the cytoplasmic localized PDGFRs in SF samples internalized before ligand treatment and are likely in the latter stages of trafficking (possibly in lysosomes). In kinase active HepGP and F442 cells, after 5 minutes treatment with PDGF-BB, extensive PDGFR phosphorylation is detected and PDGFR begins to traffic into punctate structures in the cytoplasm while the majority remains on the plasma membrane (Figure 9, arrows). At 15 and 30 minutes post-stimulation, an increasing amount of the PDGFR is localized into punctate cytosolic structures and is extensively phosphorylated (Figure 9, arrows). In HepGR, as well as AG1296 treated F442 and HepGP, phosphorylation is undetectable in the 5, 15 and 30 minute time points while

Figure 9 - Immunofluorescence images of PDGFR-β internalization in the presence or absence of PDGFR kinase activity. This figure displays immunostaining of PDGFR-β (green) and their phosphorylation state (red) after PDGF-BB treatment in the presence or absence of kinase inhibitor AG1296. In the presence of PDGF-BB and absence of AG1296, PDGFRs expressed in HepGP and F442 cell lines exhibited phosphorylation after 5' with marked internalization and accumulation of PDGFR phosphorylated punctate structures at 15' and 30' (arrows). In the presence of AG1296, PDGFR phosphorylation was not detected in HepGP, HepGR and F442 cells while detection of PDGFR accumulation in punctate cytosolic structure still occurred (arrows). PDGFR phosphorylation was not detected in HepGR cells, but PDGFR still internalized into punctate cytosolic structures (arrows).


A
HepGP
HepGR
F442
SF
+PDGF
5'
15'
30'
PDGF +
AG1296
5'
15'
30'

internalization continues to occur. The internalization in the HepGR cells with and without AG1296 is qualitatively similar suggesting that AG1296 elicits few non-specific effects on PDGFR internalization (Figure 9, arrows). We can conclude from these data that PDGFR kinase activity is not necessary for PDGF-BB induced PDGFR internalization because we indirectly detected translocation and accumulation of PDGFR in punctate structures in the cytoplasm of all three cell types in both the presence and absence of PDGFR kinase activity.

Western blot analysis showing the overall phosphorylation status of PDGFR clearly indicates a loss of kinase activity in the kinase-dead HepGR and AG1296 treated F442 and HepGP cell lines (Figure 10). In kinase-active PDGFR expressing cells (AG1296 (-) lanes), pPDGFR bands are detected which indirectly shows that PDGFR phosphorylation is occurring after PDGF-BB treatment (Figure 10). Analysis of protein from kinase-inactive PDGFR expressing cells (AG1296 (+) lanes and HepG R634 sample), however, resulted in no detection of phosphorylated PDGFR (Figure 10). These data indicate that an sufficient experimental concentration of AG1296 was used, and also that PDGFRs in HepGR cells do not become phosphorylated after PDGF-BB treatment.

3.2 Kinase inactivation attenuates PDGFR internalization

The qualitative data in Figure 9 indicated that the intrinsic kinase activity of PDGFR is not required for endocytosis to occur, but left open the question of whether a discernable quantitative difference in the rate of PDGFR internalization occurs in kinase-

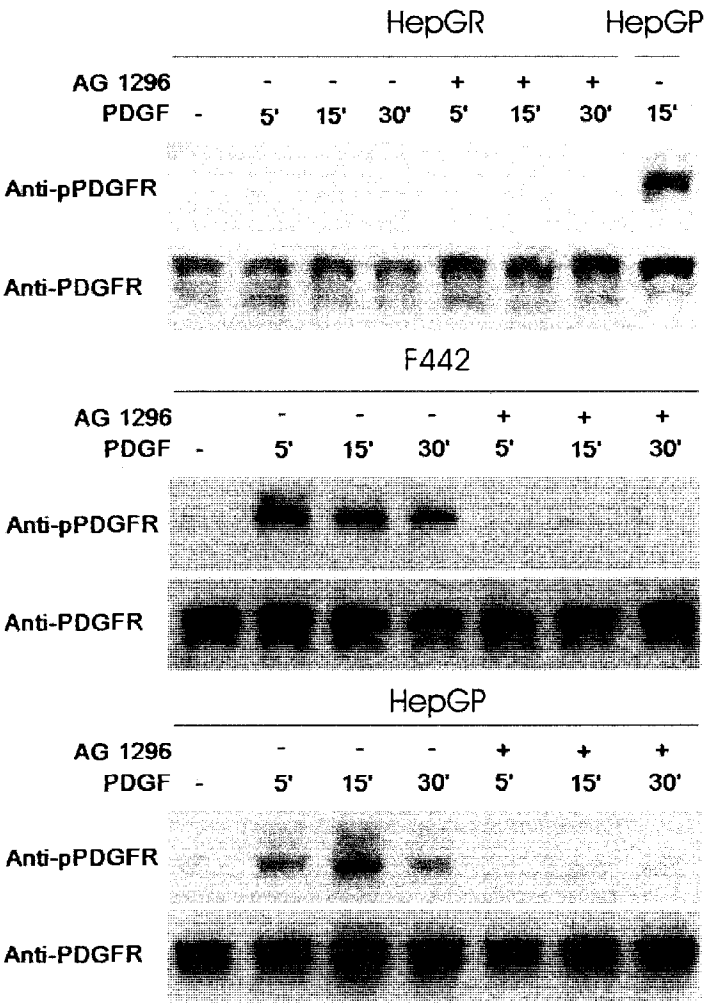


Figure 10 – Phosphorylation status of kinase-active or kinase-inactive PDGFR expressing cell extract. PDGFR phosphorylation is detected in WCL from PDGF-BB (15 ng/ml) treated HepGP and F442 cells expressing kinase-active PDGFR (AG1296 (-) lanes). WCL from each cell line treated with both the PDGFR kinase inhibitor AG1296 (50 μM) and PDGF-BB (15 ng/ml) was analyzed and little PDGFR phosphorylation was detected. Only low levels of PDGFR phosphorylation was detected in HepGR cells after PDGF-BB treatment in the presence or absence of AG1296. Little PDGFR phosphorylation was detected in negative control WCL from all PDGFR-expressing cells that were not treated with PDGF-BB.

dead receptors. To obtain a quantitative depiction of PDGFR's rate of internalization, quantitative deconvolution microscopy (QDM) and flow cytometry (FC) were used (Figures 11, 12). HepGP, HepGR and F442 cells were treated in a similar manner as the immunofluorescence experiments with the exception that HepGR was not treated with AG1296. For FC experiments, Alexa 647 labelled PDGF-BB was used as the ligand.

Using intrinsic fluorescence from the fluorescent Rab5a-GFP marker in QDM, we created 2D masks for each z-plane of the acquired images. With these masks, we were able to cut out and quantify the fluorescence from immunofluorescently labelled PDGFR that was either in the plasma membrane or in endosomes. Summing the fluorescence intensities of each PDGFR mask population enabled us to derive a ratio of plasma membrane to internalized receptors. This protocol enabled us to intrinsically compare kinase active and kinase dead populations of cells to see whether the resulting internalization into Rab5a containing endosomes is similar or different.

A drawback of using Rab5a as a fluorescent marker in these experiments is that Rab5a is associated with the internalization pathway (Schmidlin *et al.*, 2001). When expressing exogenous Rab5a, the pathway being analyzed becomes altered. There is no easy way to control for this, but one can assume that all cells analyzed in the QDM experiments expressed Rab5a and thus the effects of heightened protein expression are experienced by all samples including controls. Schmidlin *et al.* (2001) used exogenous Rab5a-GFP expression and noted that internalization of the studied ligand was similarly internalized in both Rab5a-GFP transfected and non-transfected cells (Schmidlin *et al.*,

2001). Furthermore, as you will see this experiment is completed in conjunction with FC, a protocol that does not employ exogenous expression of proteins. Lastly, this protocol is not designed to derive absolute RTK internalization values. It is simply used to address whether there is a difference between sample populations.

Using QDM we determined a total plasma membrane to endosome fluorescence ratio (PM:ES) in serum starved HepGP cells to be 11 ± 2.9. In kinase-active PDGFR expressing cells after 30 minutes PDGF stimulation, the PM:ES ratio dropped to 2.1 ± 0.11 (Figure 11). The drop in the PM:ES ratio from 11 to 2.1 over a period of 30 minutes suggests that PDGFR internalization is occurring in kinase-active cells. In AG1296 treated HepGP (HepGAP) cells after 30 minutes PDGF stimulation, we saw a reduction in the PM:ES ratio from the SF value of 11 ± 2.9 to 3.9 ± 0.85 (Figure 11). These data suggest that kinase-inactive PDGFR internalize after PDGF-BB treatment. Furthermore, HepGR was analyzed and the resulting values were normalized to the SF value of HepGP samples. Over a period of one hour, the PM:ES in HepGR cells decreased from 11 ± 0.55 to 3.8 ± 0.050, further suggesting that kinase-dead PDGFRs internalize after PDGF-BB treatment (Figure 11).

Interestingly, when comparing PM:ES ratios from kinase-active and kinase-inactive samples, a difference in the kinetics reduction was noted. Kinase-active cells exhibited a lower PM:ES than kinase-dead cells at all time points after addition of PDGF-BB. Notably, at the 5 minute time point, while kinase-active PM:ES drops to 5.1 ± 0.32, the HepGAP PM:ES drops to 7.2 ± 0.73 and no reduction is detected in kinase dead HepGR as the PM:ES value was 11 ± 0.31 (Figure 11). These data suggest that while

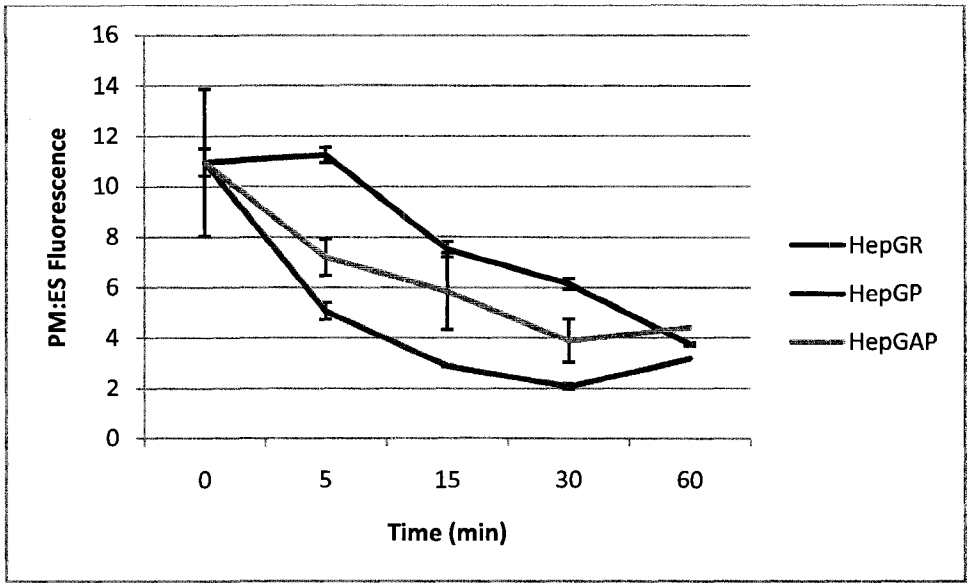


Figure 11 – Quantitative deconvolution microscopy (QDM) analysis of PDGF receptor endocytosis. Sub-cellular locations of the plasma membrane (PM) and endosome (ES) were used as masks that were applied to immuno-labelled PDGF receptor fluorescence. The total fluorescence in the PM and ES were made into a ratio of fluorescence PM/ES. A drop in the ratio value indicates translocation of PDGF receptors from the PM into ESs. (n=6)x2 independent experiments. Quantitative analysis has yet to be replicated.

PDGFR intrinsic kinase activity is not required for PDGFR internalization, kinase activity is likely involved. Additionally, the discrete difference between kinase-active and kinase-inactive PM:ES values at the 5 minute time point allude to the possibility that the 0 to 5 minute time interval is quite important with respect to kinase activity and PDGFR internalization.

To obtain a more accurate description of PDGFR internalization, we examined the fluorescence intensity of internalized fluorescently labelled PDGF-BB in single cells using flow cytometry. In cell lines expressing kinase-active receptors, 80% of PDGF-BB internalized at 30-60 minutes ligand treatment compared to 60% PDGF-BB internalization in kinase-inactive receptor expressing cells (Figure 12). The kinase-inactive receptor expressing cells exhibit internalization similar to kinase-active receptors at 2 hours. Assuming that the indirect signal detected from the PDGF-BB is representative of the internalized PDGFR population, this suggests that kinase activity is not required for complete internalization of PDGFR because the kinase-dead receptors reached similar maxima as kinase-active receptors, albeit at a later time point. Additionally, a delay in internalization occurs from time 0 minutes to 5 minutes in the kinase-inactive samples, allowing us to conclude that PDGFR employs kinase activity in the early time period of PDGFR internalization (Figure 12).

The slope of the FC internalization curves for each receptor subgroup is statistically different between the kinase-active and kinase-dead receptors in the 0 to 5 minute time period (Figure 13). Notably, the mean slope of internalization for kinase-active PDGF receptors in F442 and HepGP are 8.1 and 4.0, respectively, while kinase-

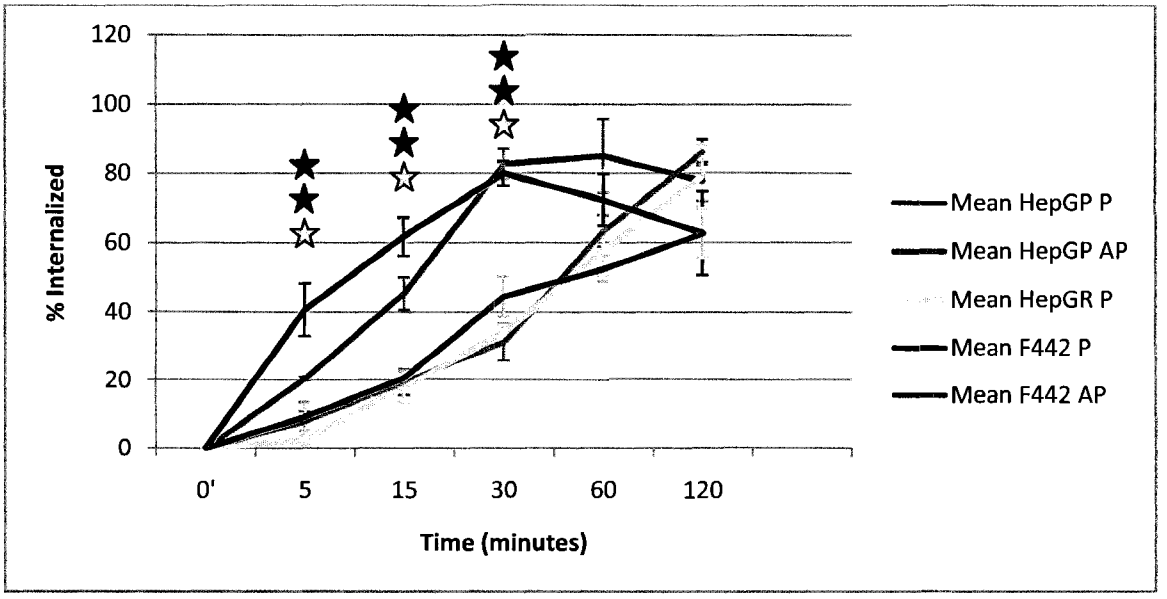


Figure 12 – Flow cytometry internalization kinetics of PDGF receptor with and without kinase activity. Using flow cytometry, endogenous and exogenous receptors were analyzed for rate of internalization with or without the PDGFR kinase inhibitor AG1296 (50 μM) in the presence of PDGF-BB. Furthermore, a kinase-dead PDGF receptor mutant (K634R) was also analyzed for internalization kinetics in the presence of PDGF-BB. These data indicate that kinase activity plays a role in the initiation of receptor internalization. Stars indicate significant difference comparing kinase-active and kinase dead samples of same cell type. HepGR was grouped with kinase-active HepGP. ($p \leq 0.05$)

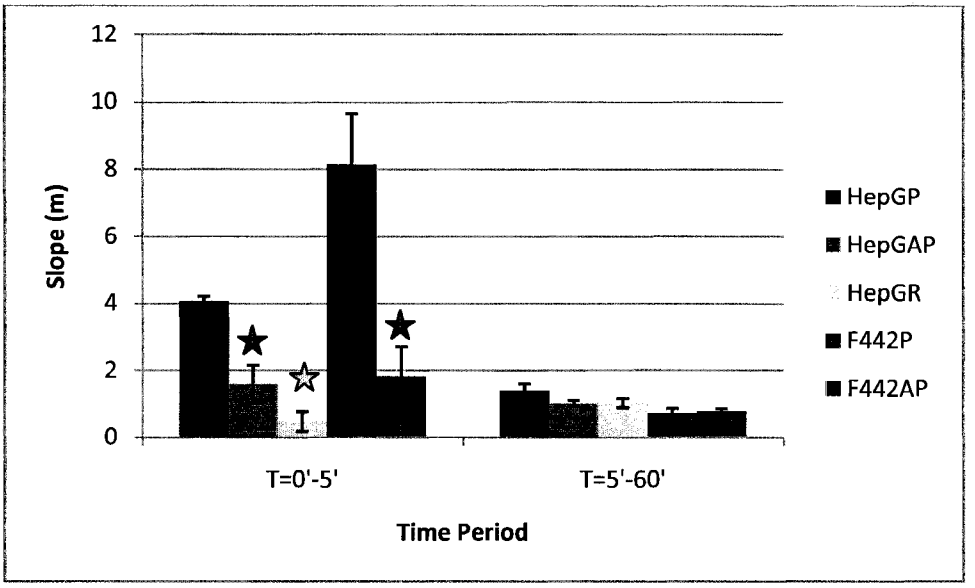


Figure 13 - Computing the slope of time periods from flow cytometry data. The mean internalization values of at least three independent experiments were used to determine the slope of PDGF receptor internalization curves. P: PDGF treatment, AP: AG1296 + PDGF treatment, R: K634R kinase dead mutant. Stars indicate significant difference comparing kinase-active and kinase-dead samples of same cell type. HepGR was grouped with kinase-active HepGP. ($p \leq 0.05$)

dead F442, HepGP, and HepGR have slopes of 1.8, 1.6, and 0.47, respectively (Figure 13). Statistical analysis of the mean internalization slopes from 5 to 60 minutes suggests the rates of internalization were not significantly different. The computed internalization slopes of kinase active F442 and HepGP are 0.73 and 1.4, respectively, and kinase-dead F442, HepGP, and HepGR show slopes of 0.79, 1.0, and 1.0, respectively (Figure 13). These data suggest that kinase-employed PDGF receptor internalization occurs directly after stimulation, while subsequent PDGF receptor internalization does not appear to employ kinase activity.

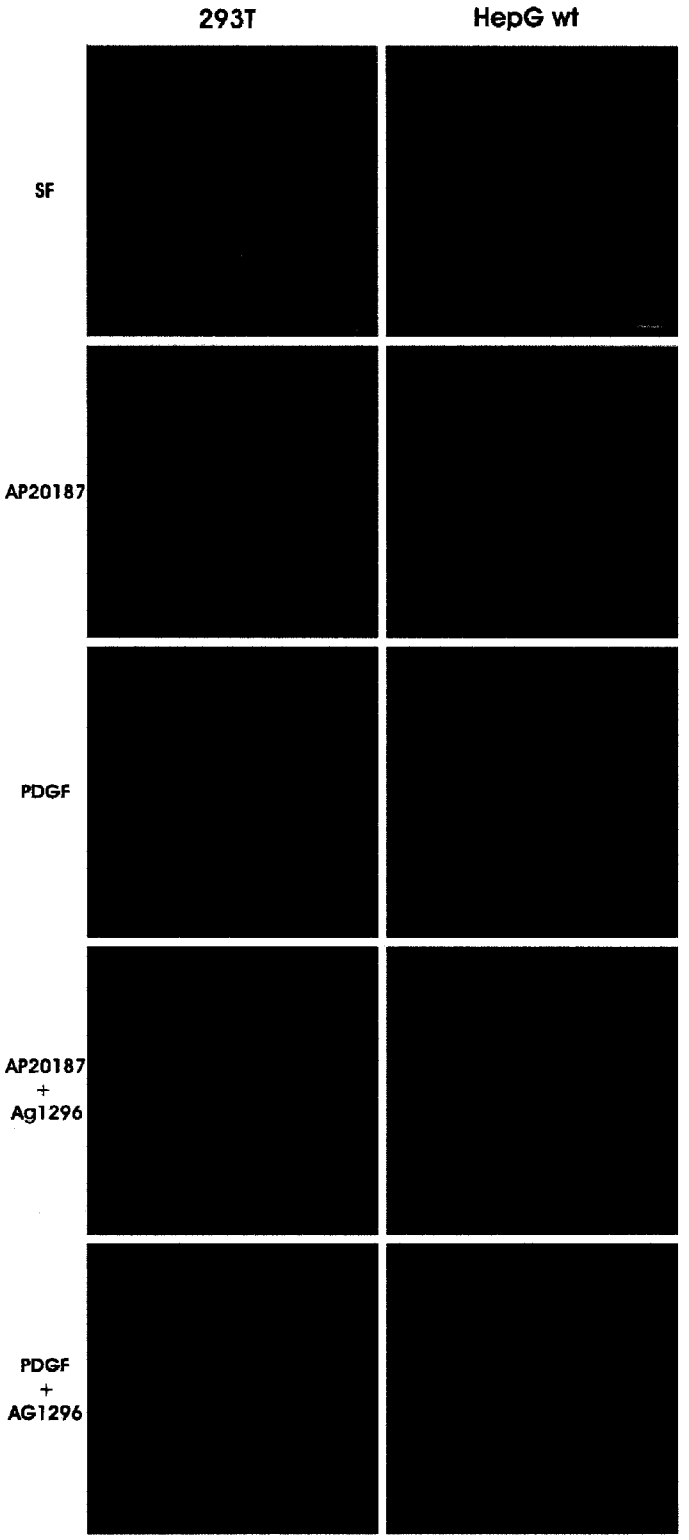
These data suggest that kinase activity is somehow involved in increasing initial rates of PDGF receptor endocytosis compared to kinase dead receptors. Additionally, these data support the hypothesis that kinase activity is not essential for PDGF receptor internalization. These results contrast with EGF receptor in which kinase activity has no significant effect on the rate of internalization (Wang *et al.*, 2005). These data corroborate earlier studies where PDGFR mutations resulted in dimished phosphorylation of the receptor and reduced the rate of PDGFR internalization (Joly *et al.,* 1994; Mori *et al.,* 1993; Sorkin *et al.,* 1991). Strikingly, the quantitative data suggest that a difference in rate of internalization occurs in kinase-active and kinase-dead PDGFR (Figure 11, 12), whereas visually analyzing the qualitative immunofluorescence data (Figure 9) does not. With this in mind, it is important to note that there are two conclusions that can be realized when an experiment shows a negative result. The first is that the negative result is in fact valid, and the second is that the experimental protocol is not sensitive enough to properly address the question. In this instance it appears the latter is the case.

3.3 PDGFR internalization and dimerization are not directly dependent on PDGF binding

It has not been clear up to now whether the binding of PDGF to PDGFR is required for PDGFR internalization. In the case of EGF receptor, EGF exists as an extracellular monomer that binds to the extracellular domain of EGFR and in turn elicits a conformational change in the dimerization loop of EGFR enabling it to dimerize (Garret *et al.*, 2002; Wang *et al.,* 2005). In contrast, the mechanisms involved in PDGF-BB binding to PDGFR have not been as well defined. We aimed to ask whether ligand binding is necessary for PDGFR-β dimerization and internalization by probing the mechanisms/events involved in PDGFR internalization.

To address whether PDGF-BB binding is necessary for PDGFR internalization, we engineered a construct in which the domain of the FKBP protein was fused to the carboxyl terminus of PDGFR. This enabled us to artificially dimerize PDGFR with the artificial ligand AP20187. Immunofluorescence images demonstrate that treatment of PDGFR-FKBP with PDGF-BB or AP20187 stimulates PDGFR internalization (Figure 14). This is indirectly shown by accumulation of punctate immuno-labelled PDGFR-FKBP in the cytoplasm of cells. These data indicate that PDGF-BB binding is not directly necessary for the initiation of PDGFR internalization. As previously suggested, the role of dimeric PDGF-BB appears to be exclusively to bring PDGF receptors into close proximity forcing receptor dimerization and does not extend to eliciting structural changes in PDGFR that enable the internalization of the receptor (Heldin, 1995; Schlessinger, 2000). If this is the case, the physical act of receptor dimerization is an

Figure 14 - PDGFR-β internalization can occur independently of ligand binding. 293T and HepG2 wild type cells (no stable transfection) were transiently transfected with PDGFR-FKBP vectors and internalization was induced with treatment of either PDGF-BB or AP20187. After a 30 minute treatment of either AP20187 or PDGF-BB, PDGFR-β-FKBP is internalized in both cell types. Cells were immunostained with α-PDGFR and labelled with FITC secondary antibodies.


293T
HepG wt
SF
AP20187
PDGF
AP20187
+
Ag1296
PDGF
+
AG1296

essential event in mediating receptor internalization. To further test this hypothesis, we analyzed the PDGFR-FKBP dimerization using di-succinimidyl suberate (DSS) cross-linking.

Using DSS to analyze PDGFR-FKBP oligomeric interactions in 293T cells expressing PDGFR-FKBP after PDGF-BB or AP20187 treatment, we confirmed that cytoplasmic cross-linking of PDGFR-FKBP with AP20187 was able to induce dimerization of the receptor (Figure 15). Compared to the non-stimulated samples, treatment with AP20187 or PDGF-BB in the presence or absence of AG1296 increased the intensity of the dimer band (Figure 15, lanes 2-5). These data indicate that receptor dimerization is occurring during both ligand-dependent and ligand-independent stimulation; consistent with the hypothesis that dimerization is involved in receptor internalization.

3.4 PDGFR dimerization is independent of PDGFR kinase activity

Receptor dimerization is a well described event that occurs during PDGF receptor mediated endocytosis (Brocklyn *et al.,* 1993), but it remains unclear whether receptor dimerization is a necessary event for the process of internalization in PDGFR. A previous study on the EGF receptor suggests that dimerization is the driving force of internalization (Wang *et al.*, 2005); this remains to be analyzed in other receptor tyrosine kinases. Furthermore, although kinase activity is proposed to follow receptor dimerization, it has not been confirmed whether kinase activity affects the duration or

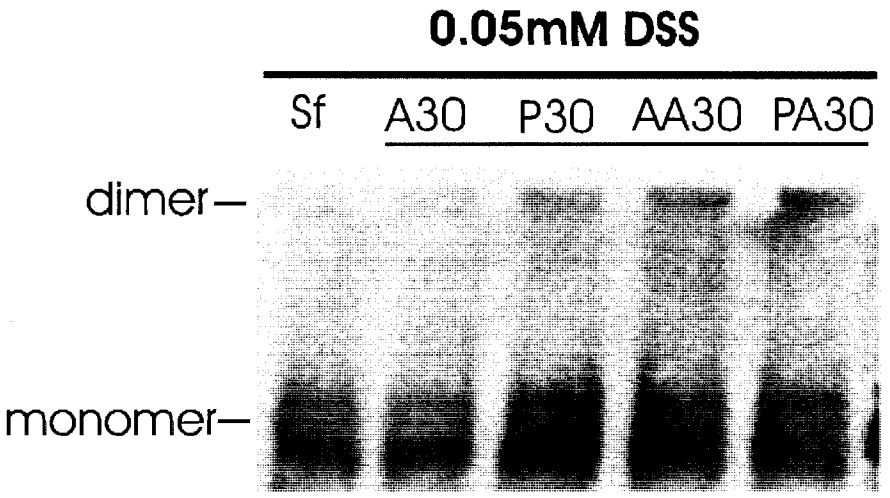


Figure 15 – DSS cross-linking of PDGFR-FKBP transfected 293T cells. Cells were treated in the absence of AG1296 kinase inhibitor with FKBP cross-linker AP20187 (A30) or PDGF (P30), or in the presence of AG1296 (AA30) and (PA30) for 30 minutes prior to addition of DSS to isolated cells. Upper band indicates PDGF receptor dimer while lower bands represent PDGF receptor monomer. Monomer doublet band consists of a non-glycosylated 160 kDa PDGFR-β (lower band), and a mature 180 kDa PDGFR-β (upper band).

magnitude of receptor dimerization (Bishayee *et al.*, 1989). My results in Section 3.3 suggested that an absence of kinase activity in PDGFR is accompanied by delay in internalization (Figures 11, 12). It is possible that the noted decrease in kinase-dead receptor internalization is due to a decrease or delay in dimerization.

We began to address these questions by studying the effect of kinase activity on ligand-dependent receptor dimerization. This was tested in F442, HepGP and HepGR cells that were treated with PDGF-BB (10 ng/mL) for various periods of time followed by protein cross-linking with DSS (Figure 16). After 5 minutes of PDGF-BB stimulation, the level of dimerization increased compared to the non-treated cells, suggesting that the process leading up to dimerization occurs in a short period of time (Figure 16). A significant difference in magnitude of receptor dimerization was not detected between the kinase-active and kinase-dead receptors (Figure 16b). Based on the kinetic data in Figure 11 and 12, the defect in kinase-dead internalization presents itself as a delay in internalization from 0 to 5 minutes. If kinase activity is affecting the degree of PDGFR dimerization, during this time period we should have likely detected a difference in PDGFR dimerization. We therefore ruled out the possibility that kinase activity directly elicits an effect on PDGFR dimerization and the mechanisms causing the aforementioned delay must be from a different source.

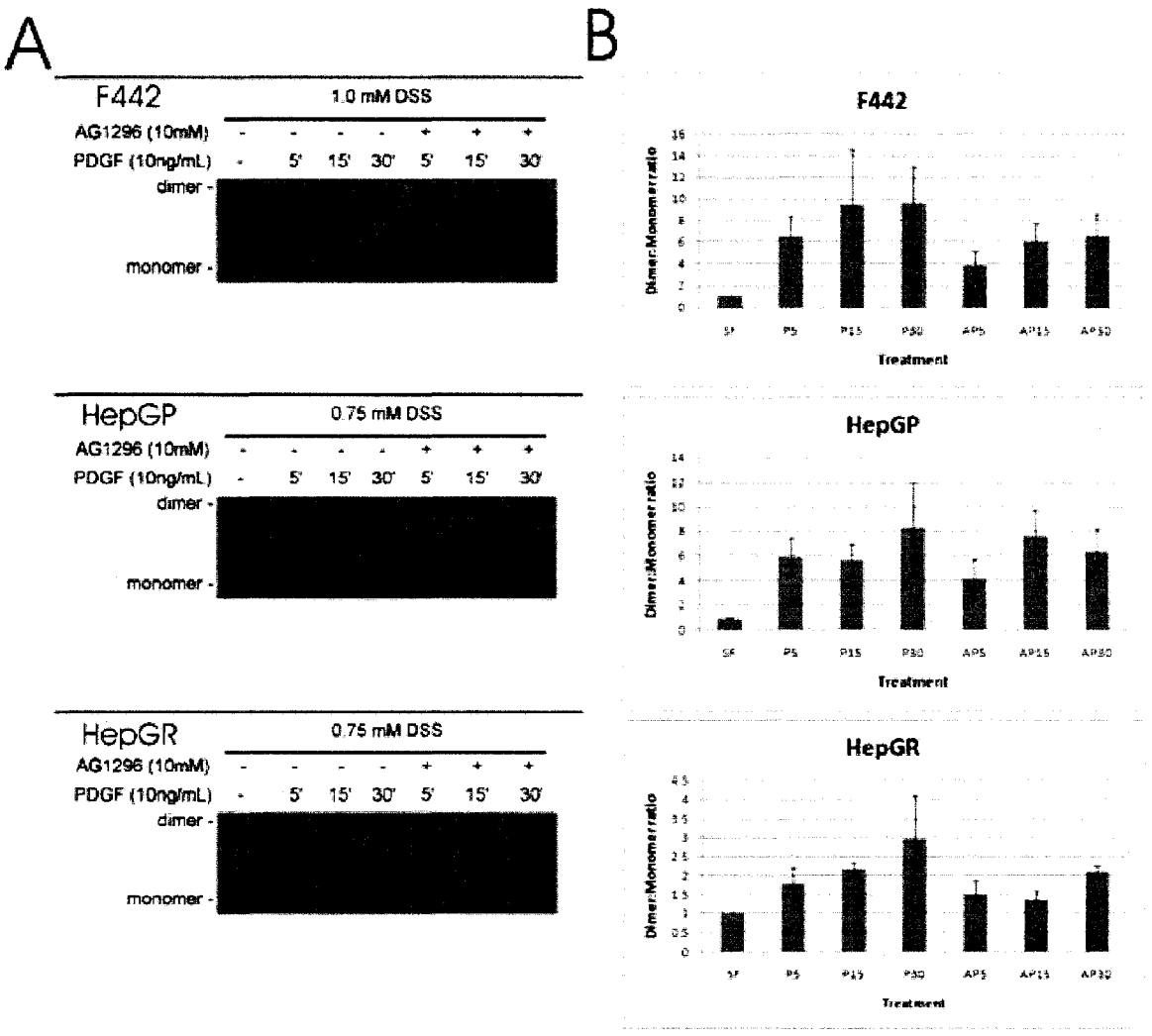


Figure 16 - PDGF receptors dimerize indifferently upon PDGF-BB stimulation in the presence or absence of kinase activity. A) Receptors were treated with PDGF-BB (10 ng/mL) for the designated duration of time and the dependency of dimerization on kinase activity was assayed using DSS cross-linking. B) Protein band densitometric quantification considering at least three independent experiments. Data are illustrated as ratios of dimer band intensity to monomer band intensity. Bars indicate standard deviation of the data. All samples are normalized to the serum free samples before calculating statistics. Statistical analysis computed no significant difference in dimerization between kinase-active and kinase-dead samples across all time points and in each cell line ($p \leq 0.05$).

3.5 PDGFR dimerization is necessary for PDGF-dependent PDGFR internalization

To directly answer the question of whether dimerization is necessary for PDGFR internalization, PDGF receptor expressing cells were treated with ganglioside GM1. It has been previously shown by several groups that ganglioside GM1 inhibits PDGFR dimerization (Brocklyn *et al.*, 1993; Mitsuda *et al.*, 2002) which we also observed (Figure 17). The current understanding for ganglioside GM1 inhibition of PDGFR dimerization involves only the transmembrane domain or the extracellular domain of PDGFR (Oblinger *et al.*, 2003). The cytoplasmic domain has been ruled out due to the creation of GM1 insensitive TrkA/PDGFR chimeric receptors where TrkA extracellular and transmembrane domains were fused with PDGFR intracellular domain (Oblinger *et al.*, 2003). The precise mechanism of GM1 inhibition of PDGFR dimerization is currently unknown.

In comparison to the normal physiology that occurs over the course of PDGFR exposure to PDGF-BB (Figure 9), treatment of PDGFR expressing cells with GM1 greatly inhibited the internalization of PDGF receptors (Figure 18). Although in these experiments, HepGP and HepGR cells cannot be serum starved to the point where PDGFR is absent from punctate structures, PDGFR remains on the plasma membrane after PDGF-BB treatment. Additionally, at all PDGF-BB treatment time points in the presence and absence of AG1296, further accumulation of PDGFR in punctate or sorting endosomal structures is absent (compared to serum free samples) (Figure 18). This

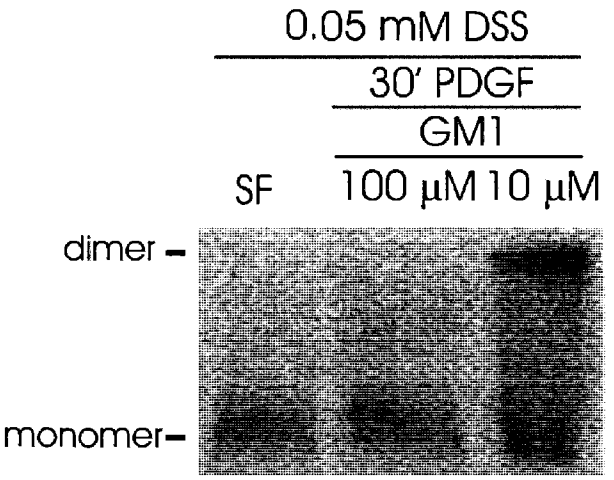
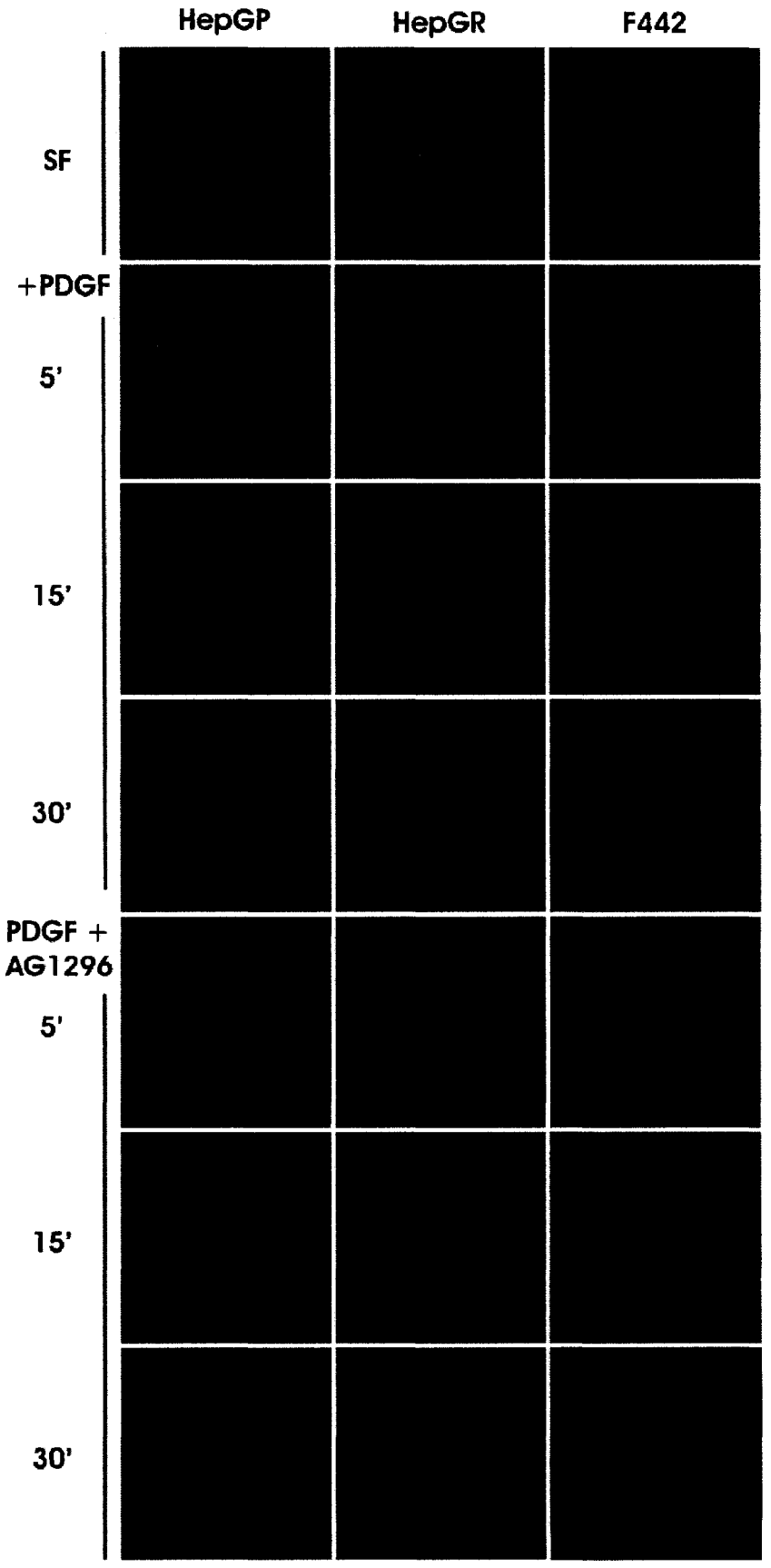


Figure 17 – Effect of GM1 on dimerization of PDGFR-β in F442 cells. F442 cells pre-treated with varying concentrations of GM1 for 1 hour and then treated with standard PDGF treatment for 30 minutes. Top band indicates the dimer band while lower band indicates PDGF receptor monomer band. SF and 100 μM GM1 treated samples exhibit virtually undetectable levels dimerization. PDGFR dimerization is detected in 10 μM treated samples.

Figure 18 – Dimerization drives PDGFR-β internalization. Immunofluorescence images of HepGP, HepGR, and F442 cells treated with 100 μM GM1 for 1 hour prior to treatment with PDGF-BB were obtained (FITC α-PDGFR, rhodamine α-pPDGFR). All samples in these experiments were treated with GM1. During the 30 minutes of PDGF-BB treatment, PDGF receptors remain on the surface. In all ligand-treated samples, presence of GM1 results in an absence of receptor phosphorylation. Compare these data to those in Figure 9.


HepGP
HepGR
F442
SF
+PDGF
5'
15'
30'
PDGF +
AG1296
5'
15'
30'

phenomenon is also seen in F442 cells expressing endogenous PDGFR (Figure 18). When juxtaposed to the internalization that occurs in the absence of GM1 (Figure 9), PDGF-BB-mediated PDGFR internalization in all three cell types is reduced in the presence of GM1 (Figure 18). Additionally, PDGF receptors were not phosphorylated in response to PDGF-BB treatment. This further confirms that PDGFR does not undergo PDGF-induced dimerization in the presence of GM1 because receptor phosphorylation is a direct result of receptor dimerization. In conclusion, these data suggest that inhibition of PDGFR dimerization results in inhibition of PDGF-mediated PDGFR endocytosis. This is strong evidence that dimerization is a foundational event that must occur for PDGFR internalization to take place. It is important to note that Brocklyn *et al.* (1993) state that the inhibition of PDGFR dimerization in the presence of GM1 is not due to inhibition of PDGF-BB binding to the receptor, ruling out the possibility that the absence of receptor dimerization, phosphorylation and internalization were due to a lack of ligand binding.

3.6 Cytoplasmic dimerization of PDGFR in the presence of GM1 elicits PDGFR endocytosis

The Oblinger *et al.* (2003) data allowed us to ask: If GM1 prevents dimerization of PDGFR in a cytoplasmic domain independent mechanism, will artificially dimerizing PDGFR in the cytoplasmic domain in the presence of GM1 elicit PDGFR dimerization and subsequent internalization? DSS cross-linking from the PDGFR-FKBP studies demonstrated that artificial cross-linking of PDGFR-FKBP results in the production of PDGFR dimers (Figure 15). We therefore tested whether GM1 inhibition can be applied

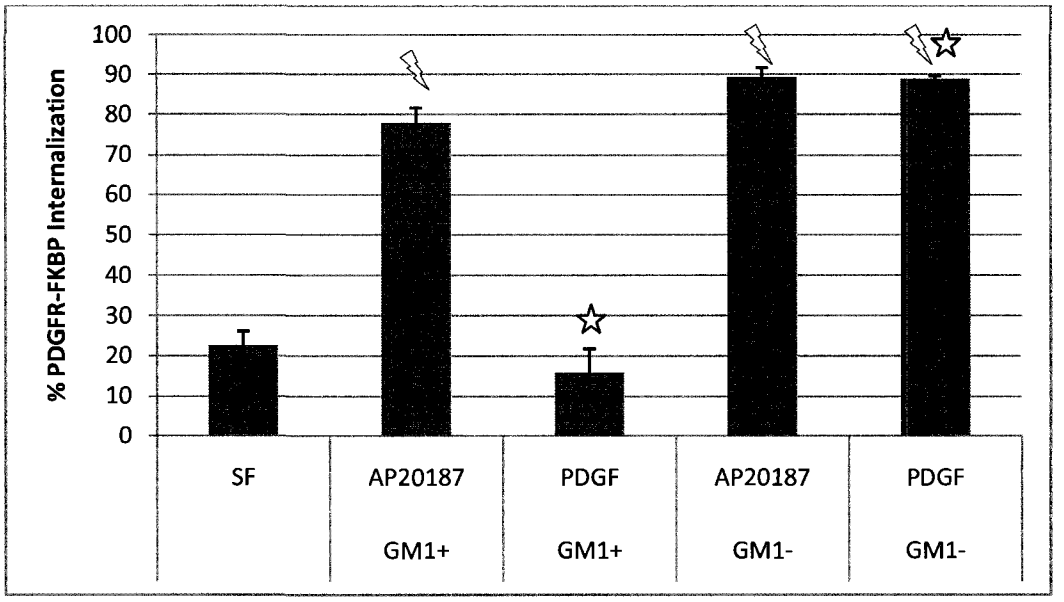


Figure 19 – Receptor dimerization of the cytosolic domain of PDGFR reconstitutes internalization in the presence of GM1. 293T cells expressing FKBP-PDGFR were pre-incubated with GM1 (100 μM) for 1 hour and then treated for 30 minutes with either PDGF-BB (20 ng/ml) or AP20187 (50 nM). Confirming data from Figure 6, PDGF does not drive PDGFR internalization while AP20187 treatment does elicit internalization. Data is a result of three independent experiments. Bolts indicate significant difference compared to SF sample, stars indicate significant difference comparing GM1+ to GM1- samples of the same ligand treatment. ($p \leq 0.001$)

to cytoplasmic dimerization of PDGFR-FKBP. As we will see, preliminary results further confirm the hypothesis that dimerization drives receptor internalization.

Immunofluorescent samples were prepared in order to visualize and record whether this cytoplasmic domain-mediated dimerization of PDGFR drives receptor internalization. Cells treated with or without GM1 were stimulated with PDGF-BB or AP20187 for 30 minutes and compared to non-stimulated controls. Cells were scored based on whether they displayed receptor internalization (see Chapter 2 for scoring method). PDGFR-FKBP expressing cell populations treated with either PDGF or AP20187 in the absence of GM1 exhibited strong internalization phenotypes (Figure 19). Cells that were treated with GM1 and stimulated with PDGF-BB exhibited very low internalization, and stimulation with AP20187 exhibited strong internalization (Figure 19). While we were unable to concretely differentiate between cytoplasmic and extracellular dimerization and their ability to drive receptor internalization, these data suggest that targeted dimerization of PDGFR cytoplasmic domain does in fact drive receptor internalization. The mechanism for this phenomenon remains unknown, but we hypothesize that because GM1 is thought to exert its effect on the transmembrane or extracellular domains of PDGFR, GM1 inhibition would also result in the inhibition of cytoplasmic domain dimerization. These data suggest that the central element in PDGFR internalization is the dimerization of PDGFR's cytoplasmic domain. Cross-linking and quantification experiments should be completed in order to accurately confirm this interpretation.

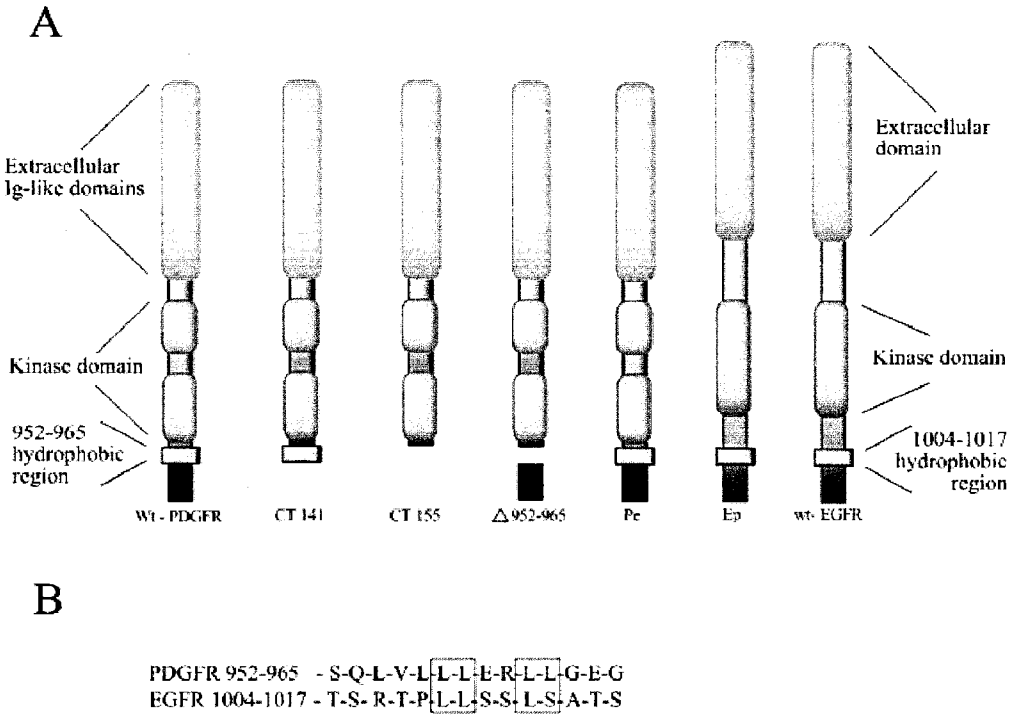


Figure 20 – PDGFR and EGFR mutants. A) In order to identify and characterize putative internalization motifs on PDGF receptor, several mutants were made. Centered on amino acids 952-965, these mutants include a truncation including the 952-965 region (CT141), a truncation excluding the 952-965 region (CT155), and a mutant with only the 952-965 region deleted (Δ952-965). The annotation corresponds to the *Homo sapien*s PDGFR amino acid sequence. A recently characterized internalization motif in EGF receptor, amino acids 1004-1017, was swapped into PDGFR in place of 952-965 (PDGFR with EGFR hydrophobic region (Pe)). EGFR region 1004-1017 was replaced byPDGFR region 952-965 (EGFR with PDGFR hydrophobic region (Ep)). B) A schematic of the putative internalization motif in PDGFR is juxtaposed to EGFR's recently characterized internalization motif.

3.7 Hydrophobic amino acids 952-965 are necessary for PDGFR internalization

Our data suggest that the dimerization of the cytoplasmic domain of PDGFR is required for endocytosis. Our next aim was to identify and characterize cytosolic segments of PDGFR that are responsible, in association with cytoplasmic PDGFR dimerization, for internalization of PDGFR. Mori *et al.* (1991) suggested that a portion of the carboxyl terminus of PDGFR is essential for the internalization of PDGFR. Specifically, they made several truncation mutants, which include CT141 and CT155, that lead to the suggestion that amino acids 952-965 in PDGFR are important for PDGFR internalization (Figure 20a) (Mori *et al.*, 1991). We too constructed eGFP/eYFP vectors where two PDGFR-β truncation mutants, CT141 and CT155, were inserted. The CT141 and CT155 mutants are PDGFR with 141 and 155 amino acids removed from the carboxyl terminus, respectively (Figure 20a). In addition to these mutants, we generated the PDGFR deletion mutant Δ952-965 which has the amino acids between CT141 and CT155 removed (Figure 20a). Each construct was transfected into 293T cells, treated with PDGF-BB (10 ng/mL) for up to 60 minutes and the cells were fixed as described in Chapter 2 (Figure 21).

Untreated serum-free cells exhibited little endocytosis in each of the full-length PDGFR, CT141, CT155, and Δ952-65 PDGFR expressing cells. At 5 minutes post-PDGF-BB stimulation, PDGFR internalization was visually detected in the WT and CT141 expressing cells (arrows), while the CT155 and Δ952-65 expressing cells appeared devoid of PDGFR endocytosis (Figure 21). The trend continued as punctate

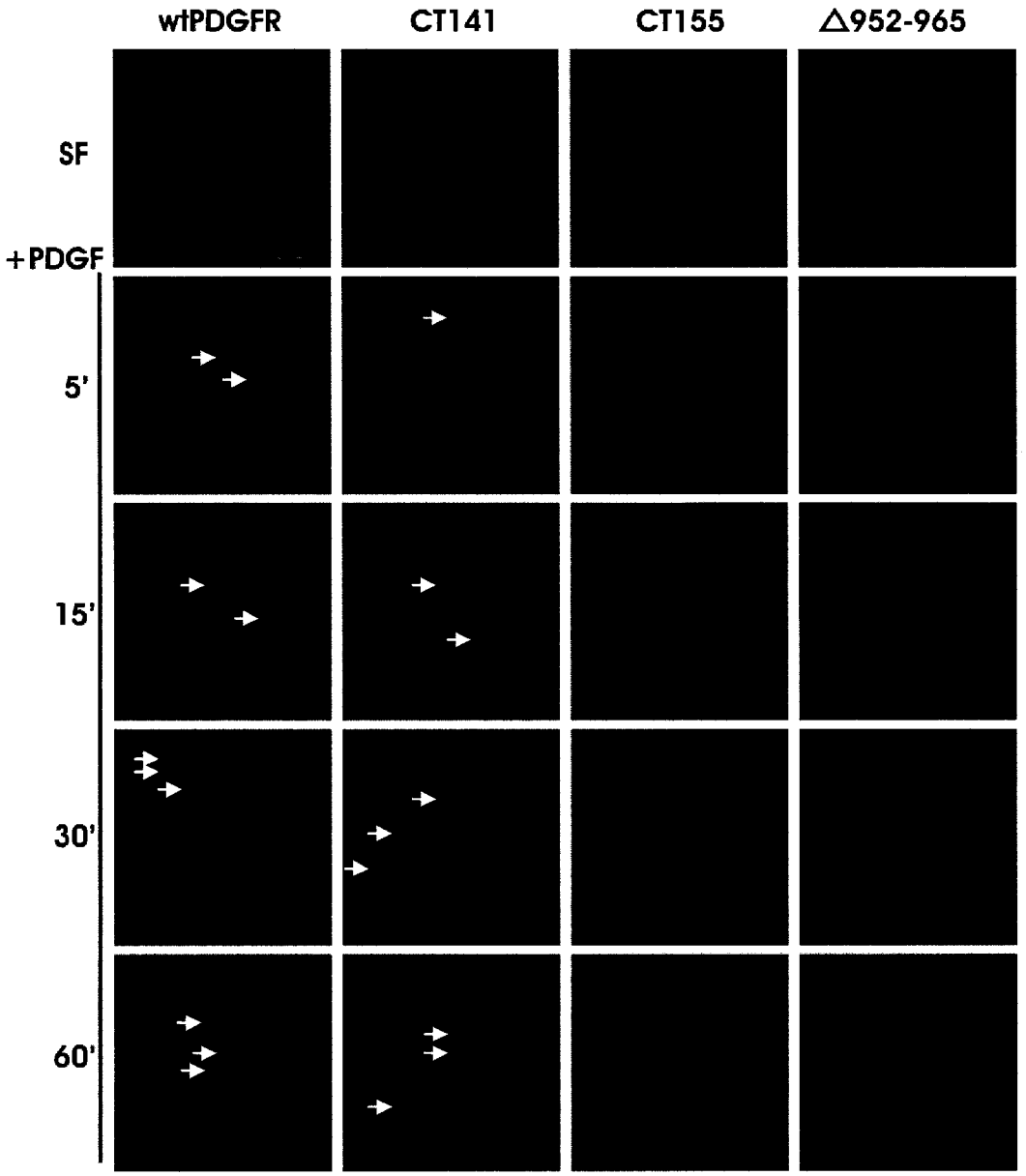


Figure 21 – Fluorescence microscopy of transiently transfected mutant PDGF receptors. The cells were serum starved and then treated with PDGF-BB (10 ng/mL) for the designated duration of time. Wild-type and CT141 receptors visibly endocytose into punctate structures (arrows) while CT155 and Δ952-65 remain on the plasma membrane.

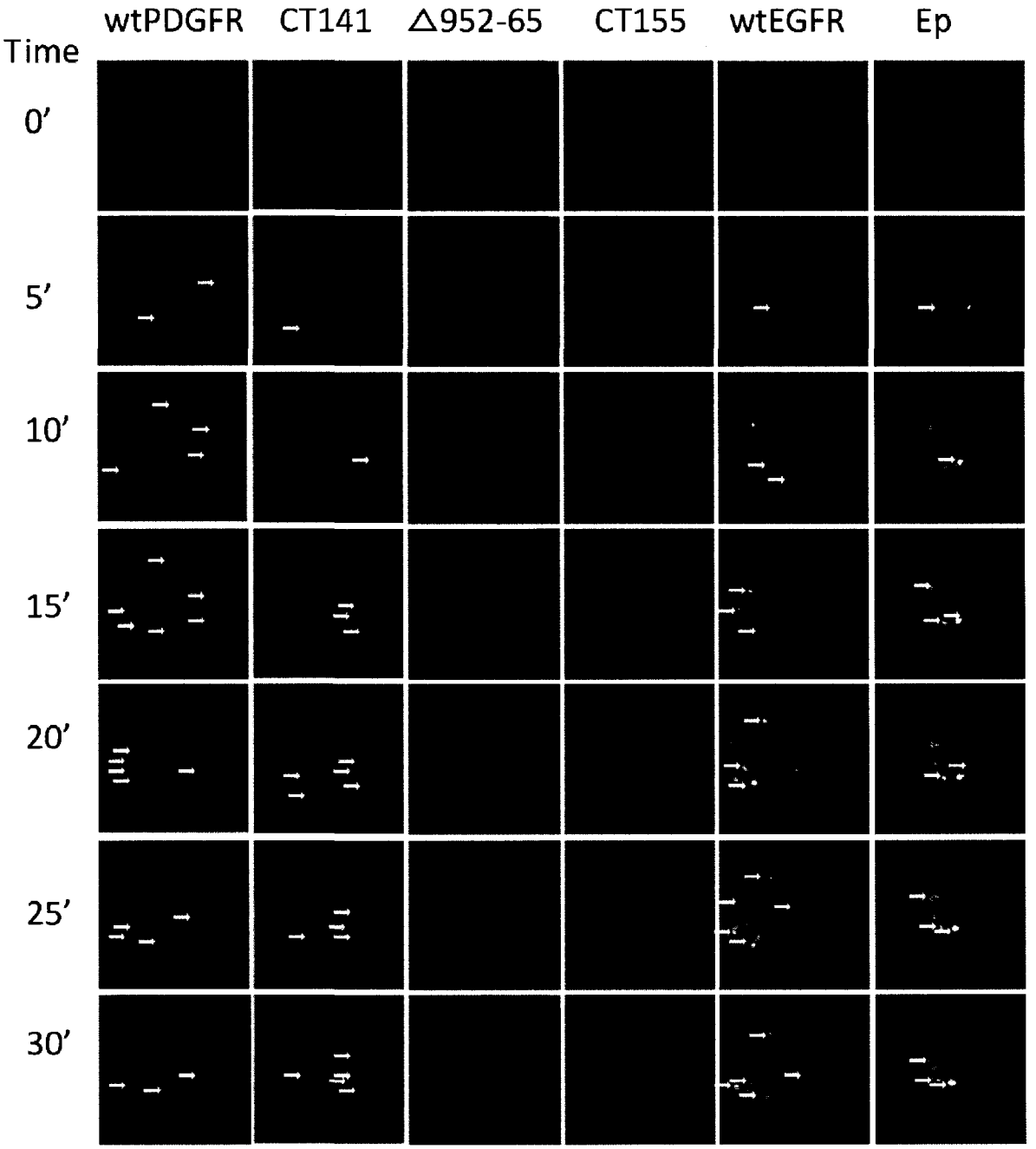


Figure 22 - Live cell fluorescence microscopy of PDGFR and EGFR mutants. Wild type and mutant eYFP-tagged PDGFR-expressing cells were treated with PDGF-BB (10 ng/ml) and analyzed without fixation. Wild type and mutant eYFP tagged EGFR expressing cells were treated with Texas Red labelled EGF (TR-EGF). While wtPDGFR and CT141 exhibited receptor translocation into endosomes (arrows), CT155 and Δ952-65 did not. Both wtEGFR and Ep swap internalized into punctate cytosolic structures upon treatment with TR-EGF (arrows).

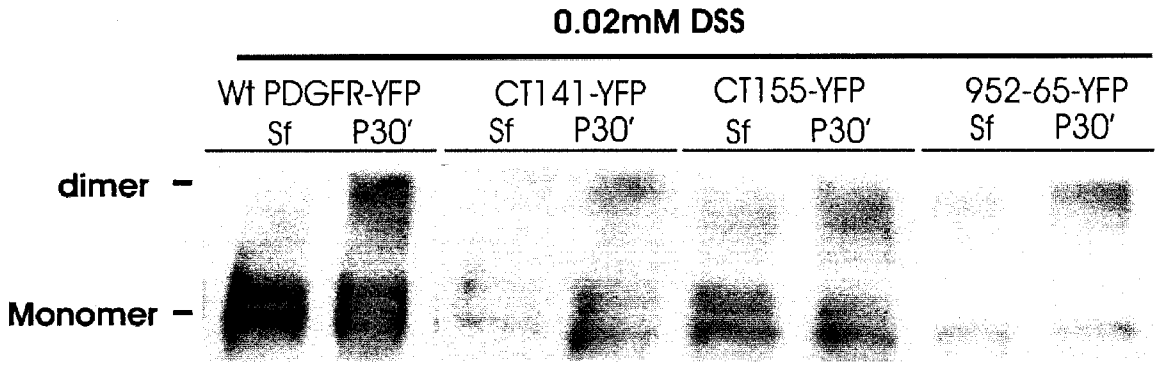


Figure 23 – DSS cross-linking of PDGF receptor mutants. Following a 30 minute addition of PDGF-BB to the cell culture (P30'), a marked increase in receptor dimerization for all samples was detected.

intracellular accumulation of PDGFR occurred in WT and CT141 PDGFR expressing cells up to the 60 minute time point (arrows), but not in the CT155 and Δ952-65 expressing cells treated with PDGF-BB for 15, 30, and 60 minutes (Figure 21). These data suggest that the 952-965 amino acid region of PDGFR is required for receptor internalization.

Additional support for this hypothesis comes from fluorescence live cell imaging of 293T cells transfected with each construct (Figure 22). Similar to fixed cell imaging, wt PDGFR readily internalized upon subjection to PDGF-BB (arrows). Truncation mutant CT141 also internalized into punctuate cytosolic endosomes, although at a slower rate than WT PDGFR. Truncation mutant CT155 and Δ952-965 PDGFR failed to internalize as indicated by positioning of PDGFRs on the plasma membrane. Moreover, upon PDGF-BB stimulation, both CT155 and Δ952-965 mutant receptors failed to aggregate into PDGFR-YFP containing endosomes; a hallmark of endocytosis.

An interesting phenotype of the CT155 and Δ952-965 mutants is sub-cellular mis-localization of the receptors following transient gene expression. Only a minority of YFP emitting cells - those with relatively low YFP emission and hence, low receptor expression - exhibited proper membrane localization. During data capture, all cells exhibiting a mis-localization phenotype were disregarded while cells expressing PDGFRs in membranous structures (PM or ES) were considered. It will be useful in the future to characterize these mutants in low-expression plasmids to analyze whether the PDGFR plasma membrane mis-localization phenotype is lost in the majority of cells.

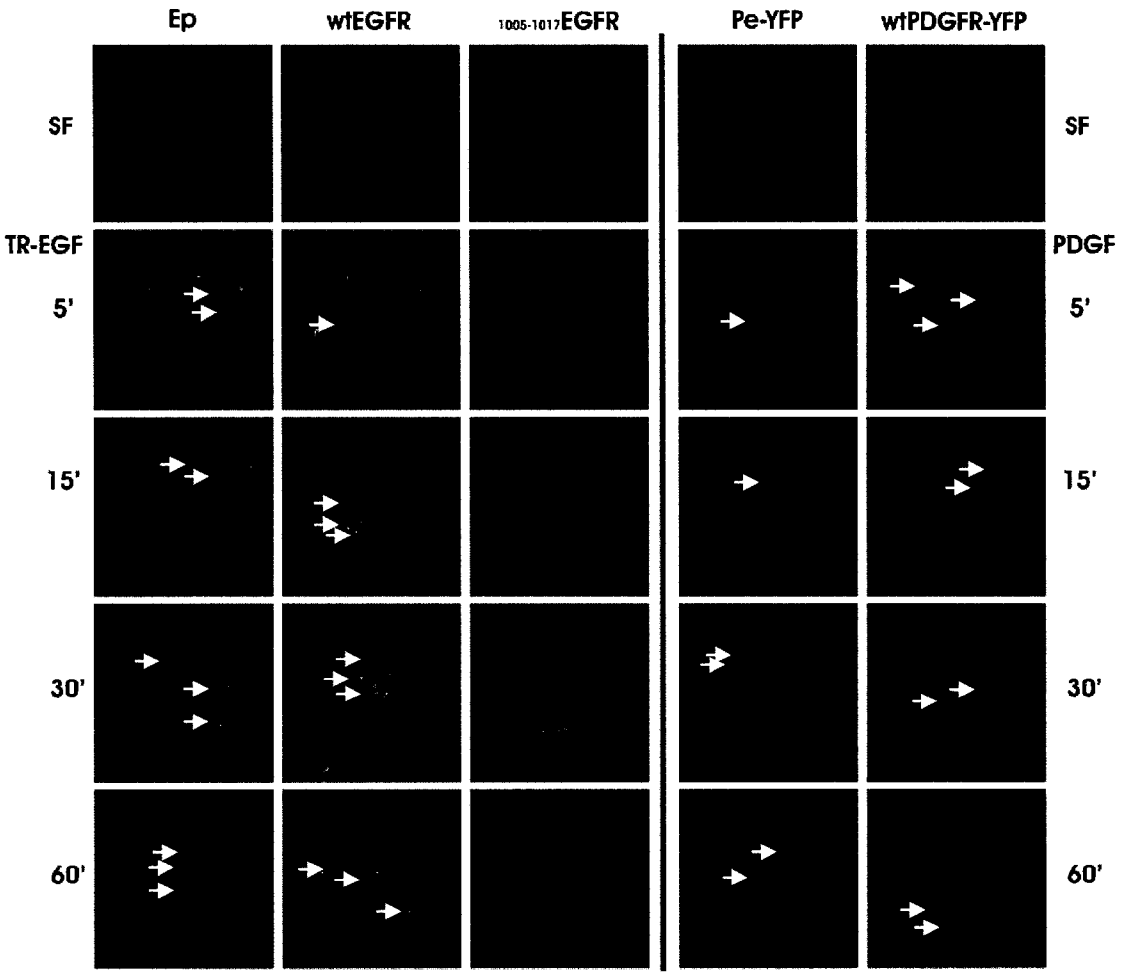


Figure 24 – EGF receptor swap reconstitutes receptor internalization. While wtEGFR-YFP exhibits receptor internalization upon EGF treatment and the Δ1005-1017EGFR-YFP mutant remains on the plasma membrane upon EGF treatment, EGFR containing the PDGFR 952-65 hydrophobic region in place of the 1005-1017 segment exhibits marked internalization. In 293T cells exhibiting low Pe expression, internalization is detectable after treatment of cells with PDGF-BB.

Analysis of the dimerization potential for the hydrophobic region of mutant PDGF receptors has provided further insight on the mechanism of PDGFR internalization. In the case of the non-internalizing mutants, CT155 and Δ952-965, the most likely conclusion is that the receptors are no longer dimerizing and, hence, do not internalize. The extent of PDGFR mutant dimerization using DSS cross-linking was evaluated (Figure 23). After 30 minutes of PDGF-BB stimulation, increased receptor dimerization was detected in all PDGFR mutants (Figure 23). PDGFR dimer band intensity increased in the CT155 and Δ952-965 mutant, illustrating that even though the receptors are able to dimerize, they are defective for internalization. This suggests that while dimerization of the cytoplasmic domain is likely a driving force for PDGF receptor internalization, it is equally important that the cytoplasmic domain retains an internalization motif. PDGFR kinase activity was not assayed for potential involvement with the internalization regions investigated. This is an interesting topic that remains to be investigated.

3.8 Internalization of RTKs is reconstituted with exogenous internalization motifs

Deletion of a short region in EGF receptor has recently been shown to prevent EGF receptor endocytosis (Wang *et al.*, 2007). Amino acids 1005-1017 in EGFR and amino acids 952-965 in PDGFR are similar in that there are a high number of leucine residues (Figure 20b). This gave us the idea to generate PDGFR (Pe) and EGFR (Ep) mutants where these short regions were swapped in order to study whether internalization could be rescued (Figure 20a). Termed Ep and Pe swaps, the capitalized letter refers to

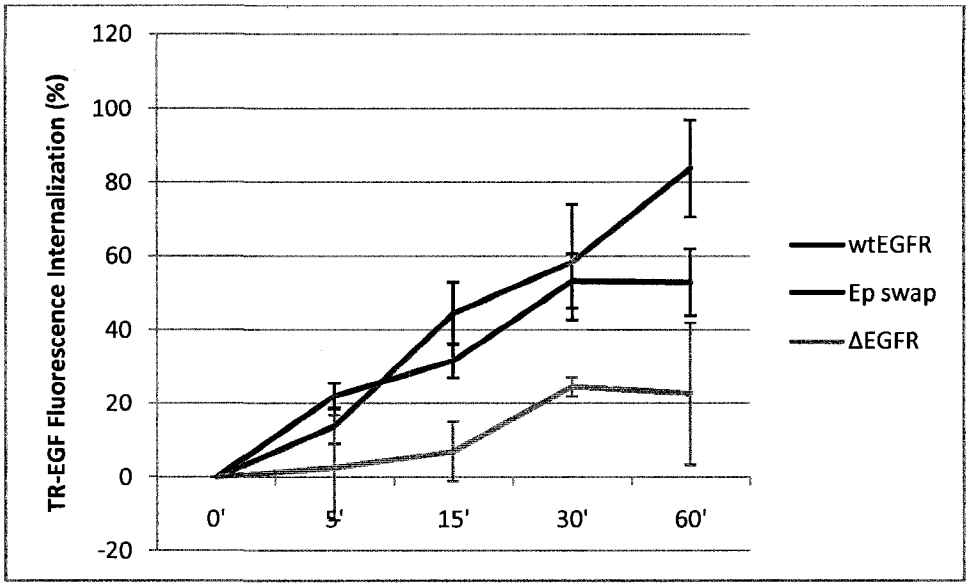


Figure 25 – Flow cytometry data indirectly illustrating internalization kinetics of Ep. Wild-type EGFR is used as a positive control while internalization defective Δ1005-1017 (ΔEGFR) is used as a negative control. After Alexa 647-EGF treatment begins, wtEGFR and Ep continue to internalize while the ΔEGFR internalization is less. Data are representative of at least three independent experiments. Bars indicate standard deviation of data.

the main part of the receptor and the lowercase letter refers to the source of the hydrophobic region (E(e) for EGFR and P(p) for PDGFR).

Analysis of images from fixed cells showed that EGFR containing the PDGFR 952-965 region (Ep) internalized upon treatment with Texas Red-labelled EGF (TR-EGF) (Figure 24, 22). At all TR-EGF treatment time points, TR-EGF/Ep-eYFP cytosolic puncta were present (Figure 24, arrows). A control panel exhibiting the EGFR Δ1005-17 internalization defective mutant highlights the functionality of the PDGFR internalization motif with EGFR (Figure 24).

Analysis of the Pe swap indicates phenotypes similar to the Ep swap. A small population of cells expressing low levels of membrane localized Pe-YFP retained the ability to internalize upon PDGF treatment (Figure 24, arrows). This indicates that a universal internalization motif has been recognized in the carboxyl terminus of two receptors. The sequence similarity is not striking (Figure 20b), but the hydrophobic nature of this region may be fundamental for internalization driving protein:protein interactions. It is important to note that as with other PDGFR constructs, Pe mutants exhibited sensitivity to over-expression of Pe-eYFP, and the resulting phenotype was global mis-localization of the receptor.

Quantitative FC analysis of Ep, using wild-type EGFR and internalization defective mutant Δ1005-1017 as controls, clearly illustrates that introduction of PDGFR's 952-965 region into EGFR reconstitutes EGFR internalization (Figure 25). Ep internalizes with similar kinetics as wtEGFR (Figure 25). The mechanisms differentiating

these wild-type and Ep mutants are not explored in this thesis, but may provide further insights into the mechanisms of EGFR and PDGFR receptor-mediated endocytosis.

Chapter Four:

Discussion, Future Direction and Conclusions

4.1.1 Discussion Overview

A decade old debate about whether RTK endocytosis requires intrinsic kinase activity remains unresolved. Classical biochemical experiments and standard light microscopy have provided many valuable clues regarding the mechanism of RTK endocytosis, but contemporary methods enabled us to provide further in-depth analysis of RTK kinase/endocytosis.

RTK dimerizaton is well known, but the research focus typically settles on the correlation between dimerization and kinase activation. Recent evidence suggests that dimerization is capable of driving RTK internalization, and there may be more functions yet to be discovered. Dimerization might elicit novel molecular interactions and/or structural alterations leading to the presently known cellular effects. In this thesis we provide evidence that dimerization is involved in molecular events other than kinase activation, notably, PDGFR internalization.

Cellular trafficking and the presence of internalization codes have been studied for over a decade. Many RTKs have been shown to contain short amino acid sequences that are required for receptor endocytosis to occur (Welsh *et al.*, 1994; Chang *et al.*, 1993; Backer *et al.,* 1990). As an extension of this previous work, we assessed whether this is happening in PDGFR. We confirmed that PDGFR houses endocytic sequences essential for internalization. Further refining the sequence down to the single amino acid level is required to understand the full scope of these findings.

4.1.2 Kinase activity and PDGFR internalization

With the data presented in this thesis, I was able to address the three hypotheses stated in Chapter 1. The first hypothesis stated that, like EGFR internalization, PDGFR internalization is not dependent on intrinsic kinase activity. The data indicated that kinase activity is not required for PDGFR internalization, however, the rate of PDGFR internalization is slower without kinase activity (Figures 11, 12, 13). These data do not completely support the original hypothesis because EGFR internalization was shown to be independent of kinase activity (Wang *et al.*, 2005). I conclude that PDGFR and EGFR do not have identical mechanisms governing their internalization.

The first set of experiments we completed made use of immunofluorescence protocols where the qualitative data suggested that intrinsic kinase activity plays a minor role, if any role at all, in PDGFR internalization (Figure 9). These data matched those of Sorkin *et al.* (1991). We decided to probe this question further by utilizing a new quantitative method we called quantitative deconvolution microscopy (QDM). The QDM data supported the flow cytometry data showing that kinase-active PDGFR expressing cells exhibited a lower PM:ES ratio than kinase-dead receptor expressing cells (Figure 11). The FC data suggested a lower overall internalization in kinase-dead receptor expressing cells during the 60 minute PDGF-BB time treatment. Moreover, both the QDM and FC data revealed a delay in PDGFR internalization within the 0-5 minute time interval when comparing kinase-active and kinase-dead samples (Figures 11, 12). In fact, HepGR cells exhibited no detectable reduction in PM:ES ratio at the 5 minute time point in the QDM experiments which were mimicked by the FC data 5 minute time point. It is

important to note that although the 60 minute time point in the flow cytometry data displayed a distinct difference in total internalization when comparing kinase-active samples to kinase-dead samples, the 120 minute time point illustrated that kinase-dead PDGFR internalization continued to rise to maxima similar to kinase-active PDGFR.

In conclusion it seems as though intrinsic PDGFR kinase inactivity results in the drastic reduction in initial rate of PDGFR internalization. While previous groups have shown that kinase activity is involved in PDGFR internalization, we have shown that intrinsic kinase activity is involved in PDGFR internalization in the 0-5 minute time period after PDGF-BB stimulation.

4.1.3 Dimerization in association with PDGFR internalization

The second hypothesis that we wanted to test was whether dimerization as observed with EGFR, is a driving force for PDGFR internalization. The data presented in this thesis strongly support this hypothesis. Inhibition of PDGFR dimerization using GM1 provided direct evidence that dimerization is a key player in PDGFR internalization (Figure 18). Further evidence supporting this hypothesis included the use of GM1 inhibition in the presence of PDGFR carboxyl terminus cross-linking, which resulted in PDGFR internalization (Figure 19). These data suggest that similar to EGFR, PDGFR internalization is dependent on receptor dimerization (Wang *et al.*, 2005).

Regarding the early delay of PDGF receptor endocytosis in a kinase dead setting, we initially attributed this observation to the possibility that dimerization may require a

kinase-active mechanism to stabilize the dimerization complex. Analysis of receptor dimerization using DSS mediated cross-linking coupled with western blot densitometric band quantification suggested that this is not the case (Figure 16). These results confirm previous PDGFR studies completed by Bishayee *et al.* (1989). The cause of the internalization delay in kinase-dead PDGF receptor remains elusive.

4.1.4 Endocytic amino acid codes in the carboxyl-terminus of PDGFR

The third hypothesis we set out to test was whether internalization of PDGFR, like EGFR, is dependent on the presence of internalization codes in the carboxyl terminus of each receptor. The generation and analysis of PDGFR truncation and deletion mutants provided evidence in support of this hypothesis (Figures 20, 21, 22). PDGFR and EGFR mutants lacking the internalization motifs do not efficiently endocytose. Furthermore, swapping the internalization codes between PDGFR and EGFR resulted in reconstitution of EGFR and PDGFR internalization (Figures 21, 22).

Although PDGFR was mis-localized in a large proportion of the CT155 and Δ952-965 expressing cells, transfectants expressing low levels of exogenous receptors showed proper membranous PDGFR localization and lacked the ability to internalize the receptors (Figure 21). Similarly, PDGFR was largely mis-localized in 293T cells expressing Pe, but proper membranous localization and receptor internalization was detected in cells expressing low levels of receptor. This raises the question of whether the 952-65 deletion alters the structure of PDGFR enough to non-specifically inhibit receptor internalization. Completing site-directed mutagenesis of this region to identify single

amino acids required for internalization of PDGFR will limit the possibility of eliciting profound structural changes that could affect receptor internalization.

These data suggest that like other RTKs, PDGFR has endocytic codes in its carboxyl terminus (Welsh *et al.*, 1994; Chang *et al.*, 1993; Backer *et al.,* 1990; Mori *et al.*, 1991). Other RTKs such as IR and EGFR have been scrutinized for internalization codes and have been shown to house more than one sequence capable of mediating internalization (Welsh *et al.*, 1994; Chang *et al.*, 1993; Backer *et al.,* 1990). It is possible that PDGFR also has multiple internalization motifs, and it is likely that future studies will confirm this speculation.

4.1.5 Receptor internalization hypotheses in relation to PDGFR kinase activity

The data presented in this thesis fortify the notion that PDGF receptor kinase activity is involved in PDGFR's endocytic process. When kinase activity is blocked by a chemical inhibitor or through site-directed mutagenesis, the internalization process is attenuated in comparison to wild-type receptors. Why does this occur in PDGFR and not in EGFR? What could the mechanism of action be? Why did we detect a delay in internalization during the early onset of internalization?

In order to answer why PDGFR internalization relies on kinase activity while EGFR endocytosis does not, I will use a different PDGFR signalling pathway as an example. PDGFR-β has been shown to mediate the process of cell motility in what is called a chemotactic response (Allen and Bayraktutan, 2008). The molecular mechanism

of cell migration mediated by PDGF-BB has been shown to occur independently of other cell migration activated pathways (Allen and Bayaktutan, 2008). This suggests that PDGFR specific cytosolic mediators of the cell migration response exist. This allows for the possibility that distinct cytosolic molecules can exist in the PDGFR internalization pathway, and that the cytosolic RTK internalization machinery is not universal, but specific to each receptor. PDGFR internalization can be coupled to PDGFR-specific effectors, while EGFR internalization is brought about by EGFR endocytic effectors. Universal internalization factors likely exist, but the cytosolic factors involved in nucleating endocytic events may be RTK specific.

Keeping the cell motility response in mind, let us now address the question of how PDGFR kinase activation could enhance the rate of PDGFR internalization. As mentioned in the introduction, PDGFR initiates phosphorylation and activation of many signal transducing molecules, many of which are intimately tied to cellular migration (Heldin *et al.*, 1998). PDGFR kinase activation results in the accumulation of molecules such as PI3K, PLC-γ, and Grb2 at the cell surface (Heldin *et al.*, 1998). Upon recruitment of these factors to the plasma membrane, accompanying cascades become activated, and cell migration is enhanced. It is possible that PDGFR kinase activation could also be actively attracting the PDGFR-specific internalization machinery such as adaptor molecules or scaffolding proteins into its proximity. But what constitutes the attractive force?

Protein structural changes due to post-translational events are common amongst cellular proteins and may be the driving recruitment factor (Harrison, 2003; Galinska-

Rakoczy *et al.*, 2008). For example, STAT proteins are well characterized transcription factors that dimerize upon phosphorylation (Decker and Kovarik, 2000). This dimerization event enables them to enter the nucleus and interact with DNA (Decker and Kovarik, 2000). Similarly, structural changes may occur in PDGFR or other proteins upon PDGFR kinase activation and may reduce the dissociation constant between internalization factors and PDGFR. Because there have been, to the best of my knowledge, no RTK specific internalization factors characterized, I can only mention general examples regarding enhanced protein binding affinity. One example involves casitas B-lineage lymphoma (Cbl) protein that interacts with PDGFR. c-Cbl has been shown to be involved in PDGFR down-regulation, and thus, enhanced association with PDGFR due to kinase activation should theoretically enhance PDGFR's rate of endocytosis (Lennartsson *et al.*, 2006). Importantly, there is not a complete loss of binding to PDGFR when PDGFR kinase is inactive, the probability of association is simply lower.

A second possibility that I will briefly mention is that PDGFR kinase activity is involved in nucleating and modulating structures involved in protein movement (Figure 26). Actin cytoskeleton rearrangements are a common result of PDGFR activation (Heldin *et al.*, 1998). Furthermore, is has been shown that endocytic site-specific actin polymerization occurs during clathrin-coated pit formation (Merrifield *et al.*, 2002). Actin has been shown to potently and suddenly accumulate at endocytic sites immediately preceding dynamin fission events (Lanzetti, 2007). A large body of evidence from studying endocytosis in yeast suggests that actin is required for all

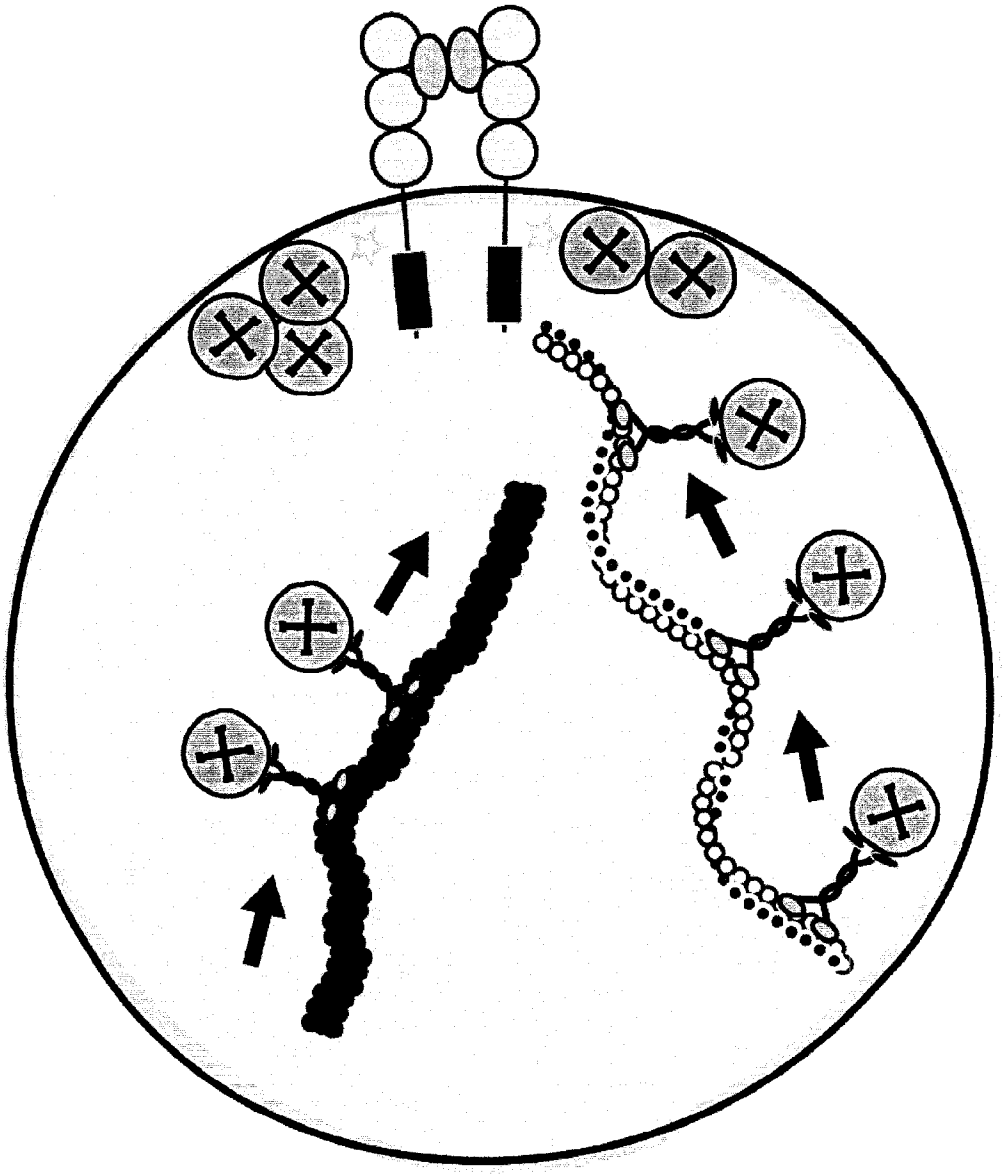


Figure 26 – PDGFR kinase activity may enhance internalization via microtubule or actin cable rearrangements. The structure and/or localization of microtubules (blue) and actin cables (red) may be directly or indirectly affected by PDGFR kinase activity (stars) and result in enhanced transport of internalization factors (X) to sites of PDGFR activation. This in turn enhances the process of PDGF-BB-mediated PDGFR internalization.

endocytic events in yeast (Reviewed in Engqvist-Goldstein and Drubin, 2003). Altering actin cable structure and position via PDGFR kinase activation may therefore facilitate translocation of internalization factors into the proximity of PDGFR (Figure 26). Similarly, the 5 minute delay could be due to a reduction or elimination of actin-dependent dynamin fission events and thus coated pits would not progress to coated vesicles. The FC and QDM protocols used in this thesis are not capable of resolving the difference between coated pits and non-internalization. Transmission electron micrographs of membrane cross sections of ligand treated kinase-active and kinase-inactive PDGFR expressing cells may confirm this speculation. A higher number of PDGFR containing coated pits in kinase-inactive PDGFR internalizing cells would be the expected observation.

Formation of structures called circular dorsal ruffles (CDRs), are a common result of PDGF and EGF stimulation (Orth and McNiven, 2006). It has been suggested that large amounts of EGFRs are internalized through CDRs, a process that is independent of clathrin and caveolin (Orth *et al.*, 2006). CDRs are composed of F-actin, cortactin, dynamin 2, Arp2/3 complex, and multiple kinases (Orth *et al.,* 2006). Of particular interest to this topic, kinases (other than RTKs) shown to be involved in CDR formation and regulation include c-Src and c-Abl (Orth and McNiven, 2006). The dynamic involvement of c-Src and c-Abl kinases in PDGFR signalling has been characterized (Heldin *et al.*, 1998; Plattner *et al.*, 2004). It is therefore interesting to consider that the enhanced rate of PDGFR kinase-active internalization during the early period of PDGFR endocytosis could be due to CDR formation. The kinetics of CDR formation and

dissociation mimic the time period in which we found kinase activity to be involved in PDGFR internalization. CDRs form soon after ligand stimulation and disappear within 10 to 20 minutes (Orth *et al.*, 2006). PDGFR internalization was found to be delayed up to the 5 minute mark (Figures 11, 12). It is possible that eliminating kinase activity during the process of PDGFR endocytosis reduces the formation of CDRs which in turn lowers the rate of PDGFR internalization. The complete role of CDRs has yet to be completely worked out. Research tying PDGFR kinase activity and internalization to CDRs is required to provide evidence for the validity of this suggestion.

Microtubules have been shown to be required for RTK internalization and trafficking; two distinct populations of endosomes rely on microtubule transport during RTK internalization (Lakadamyali *et al.,* 2006). PDGFR kinase activation may also play a role in microtubule dynamics. In neurons, phosphorylation of specific proteins have been shown to modulate their ability to stabilize microtubules (Genningloh *et al.*, 2004). To the best of my knowledge, it has not been unambiguously shown whether RTKs are responsible for initiating signalling cascades that affect microtubule dynamics (Figure 26), and thus these suggestions are only speculative and must of course be addressed with rigorous experimentation.

To address the delay in PDGFR endocytosis and specifically why kinase activity seems to be involved in PDGFR internalization during the 0-5 minute time period, I will further discuss the translocation of signalling molecules into the vicinity of PDGFR. I will address this question by presenting a hypothetical situation that includes a PDGFR activated phosphatase. Phosphatases such as a SHP-2 are known to directly interact with

PDGFR (Lechleider *et al.,* 1993; Kazlauskas *et al.,* 1993). SHP-2 becomes phosphorylated 5 minutes after PDGF-BB stimulation in the presence of kinase-active PDGF receptors, while the kinase-inactive PDGFR is unable to activate SHP-2 after PDGF-BB stimulation (Kazlauskas *et al.*, 1993). SHP-2 phosphorylation coincides with phosphatase activity on PDGFR-β tyrosine residues which in turn modulate the activity of PDGFR and its substrates (Klinghoffer and Kazlauskas, 1995). Therefore, it is possible that phosphatase activity may be involved in limiting the recruitment or preventing the stabilization of PDGFR proximal internalization factors after the 5 minute mark. Data in Section 3.3 show that the slope of PDGFR internalization in kinase-active and kinase-inactive samples in the 0-5 minute time period are significantly different while the internalization slopes after the 5 minute time point are not significantly different (Figure 13). This supports the suggestion that kinase activity participates in PDGFR internalization in the 0-5 minute time interval and that PDGFR internalization factors might be modulated by phosphatase activity. Furthermore, phosphatase activity may reduce actin remodelling in the PDGFR vicinity at the 5 minute time point. This is only one possible solution addressing the problem at hand. Dozens of other signalling molecules, RTKs, and G-protein coupled receptors (GCPRs) may also be involved in mediating this process, so it is likely that the solution is difficult and will require extensive analysis to completely resolve.

4.1.6 Receptor internalization hypotheses in relation to RTK dimerization

Corroborating the data presented in this thesis, recent data suggest that kinase activation in EGF receptor is not required for receptor endocytosis and that dimerization of the receptors is the deciding factor in receptor internalization (Wang *et al.*, 2005). With these data in mind, there are several potential mechanisms where dimerization may drive receptor internalization. The first is that hypothetical primary internalization associated protein (PIAP) monomers can bind to single non-oligomerized receptors, but cannot further recruit internalization machinery (Figure 27a). Upon ligand binding, two receptor monomers dimerize causing the bound PIAPs to also dimerize. This enables further internalization machinery binding and drives internalization (Figure 27a).

A second possibility is that PIAPs can only associate with receptors when they are dimerized (Figure 27b). In this case, both RTK monomers must dimerize to create a recognizable binding motif. This mechanism is promising because internalization machinery interaction domains have not yet been identified using bio-informatics data from monomeric receptors. Short endocytic codes have been identified, but no complete binding domains. Protein structural prediction of dimerized receptors would provide insight as to the viability of this hypothesis, but this is technologically not possible at this time. Ultimately, RTK dimer crystal structures may provide the key insights as to whether this is the mechanism governing RTK internalization.

A third possible mechanism involves the occurrence of dimerization induced structural alterations of RTKs that expose a hidden binding motif. In this instance, two

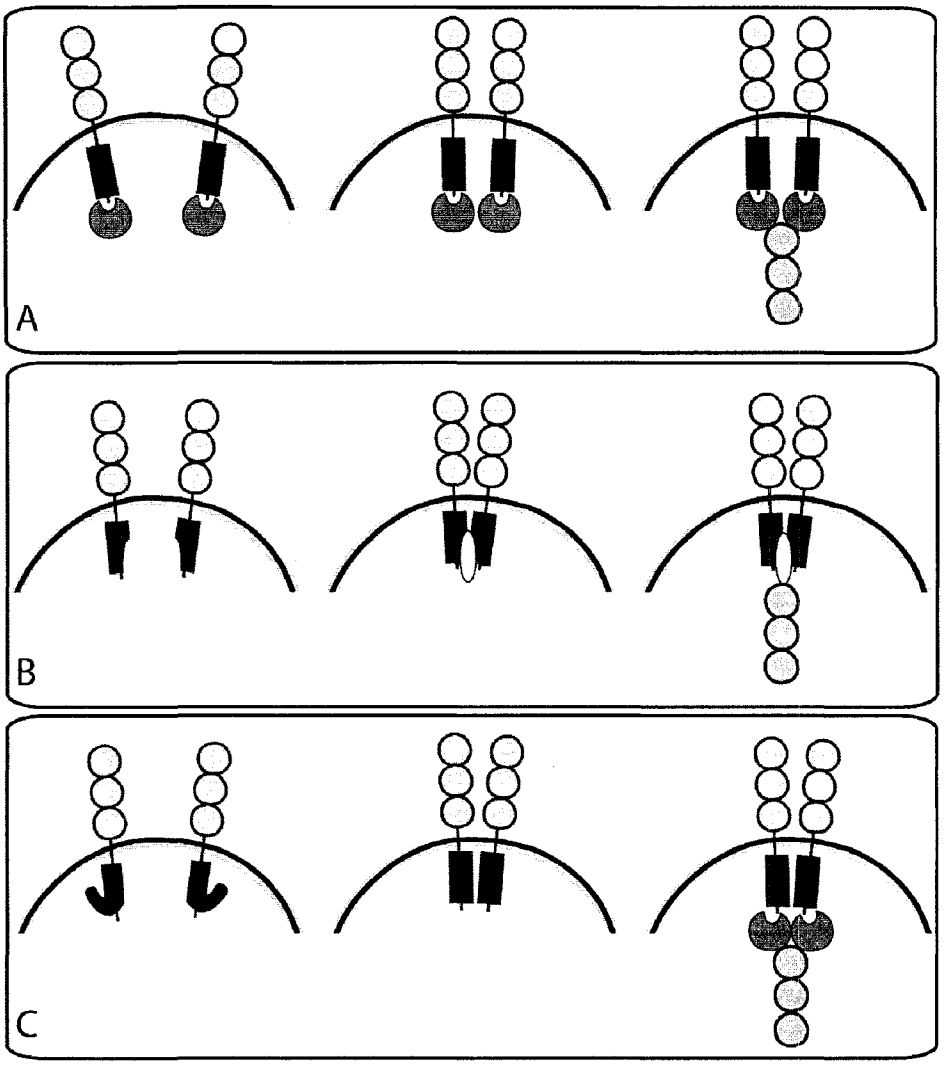


Figure 27 - Possible mechanisms of PDGFR internalization. A) Hypothetically, monomeric primary internalization associated proteins (PIAP) can bind to single PDGFRs but are unable to recruit additional internalization-associated proteins. Upon dimerization of PIAPs, nucleation of internalization complexes occurs. B) Monomeric (or dimeric) PIAPs cannot bind monomeric PDGFRs. Dimerization of PDGFRs results in the emergence of a complete and sufficient PIAP binding motif. This binding event enables the nucleation of internalization complexes. C) Monomeric PDGFR internalization motif(s) are concealed by tertiary or quaternary protein structure preventing PIAP binding. Dimerization of PDGFRs instigates a structural change revealing the PIAP binding motif.

two RTK monomers may or may not contribute to generate a complete binding domain, but the act of dimerization creates a structural conformational change in the RTK revealing the binding motif(s) (Figure 27c). There is limited evidence to support this in RTKs, however, extensive structural changes due to steric protein: protein interference or post-translational modifications have been recorded in other cellular systems (Harrison, 2003; Galinska-Rakoczy *et al.*, 2008).

The three described dimerization-associated internalization mechanisms that enable PDGFR internalization are consistent with our dimerization hypothesis. The critical data that are now direly needed to confirm these hypotheses are the crystal structures of dimerized and un-dimerized PDGF receptors, and the isolation of key internalization machinery that bind to PDGFR and drive its internalization.

4.2 Concluding thoughts

Data reported in this thesis supports the conclusion that dimerization is the central driving force of RTK endocytosis. The data further show that PDGFR kinase activity is not necessary for PDGFR endocytosis, but it acts to increase the rate of PDGFR ligand-mediated endocytosis compared to kinase-dead internalized receptors. This suggests that the EGFR kinase-independent internalization detected by Wang *et al.* (2005) is different from that of PDGFR. Lastly, the involvement of endocytic codes in PDGFR was confirmed. These data corroborate present day dogma which suggests that RTK internalization requires endocytic codes.

While we made progress in elucidating the principles of RTK endocytosis, the complete characterization of RTK internalization requires the development of new, high resolution technologies. The short time interval under investigation and the ambiguous nature of plasma membranes make the quantification and analysis of RTK endocytosis very difficult. The elusive nature of RTK internalization is further complicated because the crystal structure of PDGF receptor monomers and dimers have not been obtained. There is no doubt that high resolution structures of PDGFRs in active and inactive states and monomeric and dimeric conformations would provide extensive insight into the mechanisms involved in receptor internalization.

4.3 Future Direction

4.3.1 Fluorescently labelled PDGF-BB

While the Alexa Fluor 647-labelled PDGF used in the experiments presented in this thesis provided clues into the mechanism of PDGF receptor internalization and trafficking, the stability and efficiency of the conjugation reaction needs to be improved. Although the labelled ligand could be detected using flow cytometry, compared to other commercially available labelled ligands we used in the field, the emission signal was much weaker. Exacerbating the problem, when fluorescence microscopy was used to examine the manually labelled PDGF, little fluorescence could be visualized. These observations reinforce the requirement for more efficient labelling of PDGF.

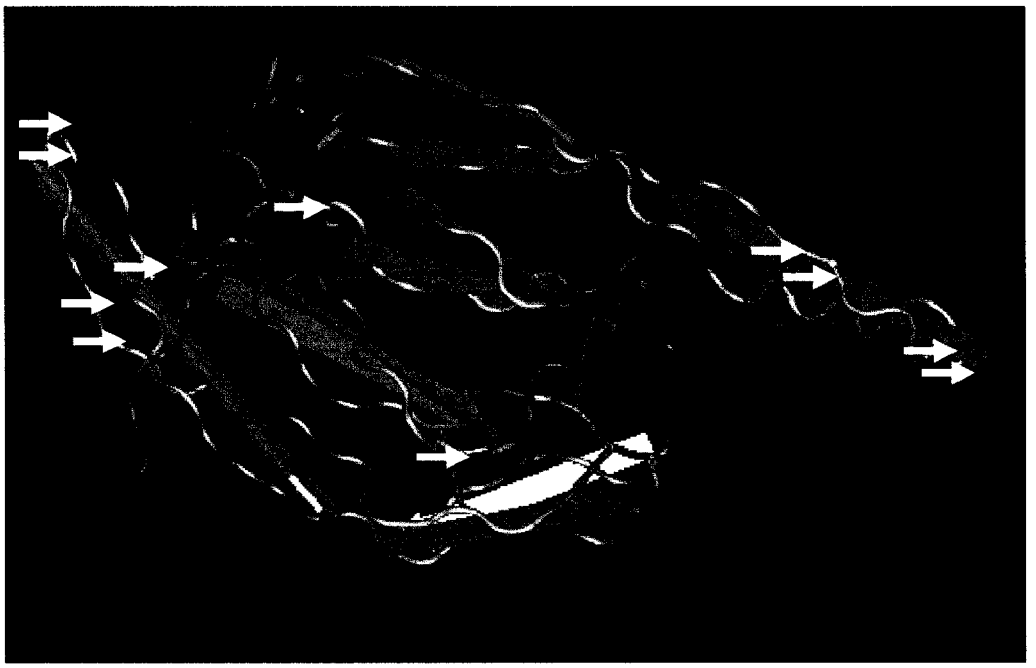


Figure 28 – 3.0Å resolution PDGF-BB crystal structure. This solved crystal structure of PDGF allows for educated engineering of a recombinant PDGF-BB anti-parallel homodimer that can be properly labelled with a fluorophore. Lysine residues displaying primary amine-containing R-groups are yellow and further enhanced with white arrows. Red arrows indicate the positions of the amino-termini of the dimer, while green arrows point out the carboxyl termini of the homodimer. (Oefner, *et al.,* 1992)

In order to obtain an extensively labelled form of PDGF, it will have to be designed using the original structure as a starting block. No corporation has successfully mass produced a labelled PDGF molecule, and this is why I completed the reaction manually. Because the Alexa Fluor 647 protein labelling kit relies on the presence of primary amine groups, it is likely that the nature of PDGF folding restricts the availability of these residues. Figure 28 partially confirms this suggestion as the resident lysine residues are either located in the adjoining space between the anti-parallel PDGF-B monomers or within organized secondary structure (beta-sheets). This may dramatically reduce the availability of these residues for fluorophore labelling. The organized beta sheet structure of PDGF is likely the point of interaction with PDGF receptor and thus addition of a lysine tag cannot be in close proximity to areas of organized structure. Situating a lysine peptide onto the amino terminus is ideal as it would be more distant from the organized structures of the PDGF homodimer than the carboxyl termini (green arrows). This lysine peptide containing PDGF-BB dimer will likely be chemically tagged with much higher efficiency than wild-type PDGF-BB, but the consequence of this protein alteration in a physiological setting will have to be analyzed. The utility of efficiently labelled PDGF-BB dimer with new technologies such as quantum dots will be great as there are many experiments where it can be applied.

4.3.2 Quantitative deconvolution microscopy (QDM)

Fluorescence microscopy has become one of the most widely used technologies in cell biology. Although biochemical protocols for quantification of cellular processes are

no doubt sensitive and reproducible, virtually all approaches have their drawbacks. Investigating hypotheses using more than just biochemical means is now not only possible, but also affordable, which has resulted in an increase of the quality of research currently underway. Furthermore, Moore's law has held true since its debut in 1965, and as it predicts, computational power has become extremely cheap and powerful (Moore, 1965) and has resulted in the possibility of overseeing billions of computations in short intervals of time. This has opened up a new realm of microscopy – quantitative microscopy.

Quantitative microscopy (QM) involves the digital capture of cellular images which can be subsequently analyzed in fine detail. The use of imaging for qualitative purposes is dependent on the sensitivity of one's eye to visually confirm or reject a hypothesis. This can generate ambiguities between self and groups when analyzing data. Using software to analyze light intensities provides a researcher with a precise numeric representation of the image and thus when a population of pictures is scrutinized, unbiased conclusions can be drawn from the data.

Data sets obtained using QM can be quite extensive and diverse. Spatial and temporal relative location can be analyzed in images, including not only the capability to measure distances, but also the capacity to analyze the extent of molecular co-localization. Specifically pertaining to this thesis was the analysis of pixel intensities of entire or subsections of images in two-, three-, and four-dimensions. Protocols such as fluorescence resonance energy transfer (FRET) or fluorescence recovery after photo-

bleaching (FRAP) are examples of protocols that can now utilize the quantitative foundation in QM to yield precise dynamic data.

Although the QDM protocol presented in this thesis was recently developed and therefore has much room for improvement, it was my intent to present this protocol as a starting point for software run data capture development. A large proportion of the QDM process requires human input and, while we aim for consistency in image processing, no one is as consistent as a computer algorithm that is governed by strict rules of conduct. This is therefore a call out to designers of computer code to generate versatile automated software for quantitative image processing.

4.3.3 Glycosylation

Receptors tyrosine kinases are transmembrane proteins that are extensively glycosylated (Contessa *et al.*, 2008). Current evidence suggests that inhibition of N-linked glycosylation in cells expressing EGFR, attenuates downstream signalling (Contessa *et al.*, 2008). Because initiation and duration of receptor signalling are intimately associated with mechanisms of receptor activation and internalization, an interesting suggestion to propose is the involvement of glycosylation in the process of receptor internalization. Glycosylation is a ubiquitous post-translational modification that occurs on many proteins within eukaryotic cells. Two forms of glycosylation exist: N-linked and O-linked, where N-linked refers to the covalent linkage of a sugar structure to an asparagine amino acid residue, and O-linked is the joining of a sugar group to either threonine or serine amino acid residues. Only O-linked glycosylation, however, have

been shown to occur in the cytosol. Upon analyzing the amino acid content of the both the PDGFR and EGFR internalization deficient associated regions, I noted a high concentration of serine and threonine residues (Figure 20b). PDGFR exhibits a serine residue in the midst of many other hydrophobic amino acids, allowing for potential O-linked glycosylation. EGFR 1004-1017 is composed of serine and threonine amino acids, potentiating the presence of both O-linked glycosylations. While I did not assay for the change in glycosylation in either the PDGFR or EGFR deletion mutants, I feel this may be an exciting area to explore.

Further evidence for involvement of glycosylation in receptor internalization has been observed. For example, upon expressing the PDGFR 952-965 deletion mutant, CT155 or Pe, mis-localization to the endoplasmic reticulum or Golgi was observed, notably in higher expressing cells. Co-localization studies must be completed to confirm this observation. Is this mis-localization due to a lack of O-linked glycosylation of PDGFR? Serine 952 exists within the hydrophobic region of PDGFR and by deleting this region, this potential glycosylation site is removed (Figure 20b). Although the EGFR mutants do not mis-localize in the same way PDGFR mutants do, EGFR 1005-1020 region contains several serine and threonine residues - S1005, S1012, S1013, S1016, S1019, T1005, T1008, T1015, T1020, respectively, that may be involved in glycosylation. Logically, because these amino acid regions of PDGFR and EGFR are necessary for receptor internalization, and if a glycosylation event is in fact occurring within this domain, it is possible that glycosylation is somehow involved in receptor internalization. Glycosylation would likely be involved in protein folding which in turn

could allow for required internalization dependent protein:protein interaction. Alternatively, glycosylation could also be involved in direct interactions with cytosolic internalization factors, an area of research that is virtually un-explored. While I provided almost no evidence for the existence of this glycosylation model, I feel it is worth pursuing and may open up a brand new area of research involving post-translational modification in RTK regulation.

Chapter Five:

References

Allen C. L., and U. Bayraktutan. 2008. Differential mechanisms of angiotensin II and PDGF-BB on migration and proliferation of coronary artery smooth muscle cells. Journal of Molecular and Cellular Cardiology. 45(2): 198-208.

Alvarez R.H., H. M. Kantarjian, and J. E. Cortes. 2006. Biology of platelet-derived growth factor and its involvement in disease. Mayo Clinic Proceedings. 81(9):1241-57.

Amit I., R. Wides, and Y. Yarden. 2007. Evolvable signalling networks of receptor tyrosine kinases: relevance of robustness to malignancy and to cancer therapy. Molecular Systems Biology. 3(151): 1-14.

Apperley J. F., M. Gardembas, J. V. Melo, R. Russell-Jones, B. J. Bain, E. J. Baxter, A. Chase, J. M. Chessells, M. Colombat, C. E. Dearden, S. Dimitrijevic, F. X. Mahon, D. Marin, Z. Nikolova, E. Olavarria, S. Silberman, B. Schultheis, N. C. Cross, and J. M. Goldman. 2002. Response to imatinib mesylate in patients with chronic myeloproliferative disease with rearrangements of the PDGFR-β. New England Journal of Medicine. 347(7):481-7.

Backer J. M., C. R. Kahn, G. A. Cahill, A. Ullrich, and M. F. White. 1990. Receptor-mediated internalization of insulin requires a 12- amino acid sequence in the juxtamembrane region of the insulin receptor β-subunit. Journal of Biological Chemistry. 265 (27): 16450-54.

Backer J. M., S. E. Shoelson, M. A. Weiss, Q. X. Hua, R. B. Cheatham, E. Haring, D. C. Cahill, and M. F. White. 1992. The insulin receptor juxtamembrane region contains two independent tyrosine/beta-turn internalization signals. Journal of Biological Chemistry. 118:381-39.

Bansal A., and L. M. Gierasch. 1991. NPXY internalization signal of the LDL receptor adopts a reverse-turn conformation. Cell. 67(6): 1195-201.

Baxter R. M., J. P. Secrist, R. R. Vaillancourt, and A. Kazlauskas. 1998. Full activation of the platelet-derived growth factor β-receptor kinase involves multiple events. Journal of Biological Chemistry. 273(27): 17050-55.

Baulida J., M. H. Kraus, M. Alimandi, P. P. Di Fiore, and G. Carpenter. 1996. All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. Journal of Biological Chemistry. 271(9):5251-7.

Baroni, S. S., M. Santillo, F. Bevilacqui, M. Luchetti, T. Spadoni, M. Mancini, P. Fraticelli, P. Sambo, A. Funaro, A. Kazlauskas, E. V. Avedimento, and A. Gabrielli. 2006. Stimulatory auto-antibodies to the PDGF receptor in systemic sclerosis. New England Journal of Medicine. 354:2667-76.

Bishayee S., S. Majumdar, J. Khire, and M. Das. 1989. Ligand-induced dimerization of the platelet-derived growth factor receptor. Monomer-dimer interconversion occurs independent of receptor phosphorylation. Journal of Biological Chemistry. 264(20):11699-11705.

Bradford M. M.. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72: 248-254.

Brocklyn J. V., E. G. Bremer, and A. J. Yates. 1993. Gangliosides inhibit platelet-derived growth factor stimulated receptor dimerization in human glioma U-124MG and Swiss 3T3 cells. Journal of Neurochemistry. 61:371-374.

Burke P., K. Schooler, and H. S. Wiley. 2001. Regulation of epidermal growth factor receptor signalling by endocytosis and intracellular trafficking. Molecular Biology of the Cell. 12(6): 1897-1910.

Carlos C., P. Tsai, K. Okada, V. A. Briner, and R. W. Schrier. 1990. Mechanisms of rapid desensitization to arginine vasopressin in vascular smooth muscle cells. American Journal of Physiology. Renal Physiology. 260(1):46-52.

Chang C. P., C. S. Lazar, B. J. Walsh, M. Komuro, F. F Collawn, L. A. Kuhn, J. A. Tainer, I. S. Trowbridge, M. G. Farquhar, M. G. Rosenfeld, H. S. Wiley, and G. N. Gill. 1993. Ligand-induced internalization of the epidermal growth factor receptor is mediated by multiple endocytic codes analogous to the tyrosine motif found in constitutively internalized receptors. Journal of Biological Chemistry. 268: 19312-19320

Cirri P., M. L. Taddei, P. Chiarugi, F. Buricchi, A. Caselli, P. Paoli, E. Giannoni, G. Camici, G. Manao, G. Raugei, and G. Ramponi. 2005. Insulin inhibits platelet-derived growth factor-induced cell proliferation. Molecular Biology of the Cell. 16:73-83.

Contessa J. N., M. S. Bhojani, H. H. Freeze, A. Rehemtulla, and T. S. Lawrence. 2008. Inhibition of N-linked glycosylation disrupts receptor tyrosine kinase signalling in tumour cells. Cancer Research. 68(10): 3803-9.

Cools J., D. J. DeAngelo, J. Gotib, E. H. Stover, R. D. Legare, J. Cortes, J. Kutok, J. Clark, I. Galinsky, J. D. Griffin, N. C. Cross, A. Tefferi, J. Malone, R. Alam, S. L. Schrier, J. Schmid, M. Rose, P. Vandenberghe, G. Verhoef, M. Boogaerts, I. Wlodarska, H. Kantarjian, P. Marynen, S. E. Coutre, R. Stone, and D. G. Gilliland. 2003. A tyrosine kinase created by fusion of the PDGFRA and FIP1L1 genes as a therapeutic target of imatinib in idiopathic hypereosinophilic syndrome. New England Journal of Medicine. 348(13):1201-14.

Decker T., and P. Kovarik. 2000. Serine phosphorylation of STATS. Oncogene. 19:2628-37.

Dougher M., B. I. Terman. 1999. Autophosphorylation of KDR in the kinase domain is required for maximal VEGF-stimulated kinase activity and receptor internalization. Oncogene. 18:1619-27.

Ercolani L., H. L. Lin, and B. H. Ginsberg. 1985. Insulin-induced desensitization at the receptor and post receptor level in mitogen-activated human T-lymphocytes. Diabetes. 34(9):931-7.

Felder, S.,K. Miller, G. Moehren, A. Ullrich, J. Schlessinger, and C. R. Hopkins. 1990. Kinase activity controls the sorting of the epidermal growth factor receptor within the multi-vesicular body. Cell 61:623-34.

Fleming T. P., A. Saxena, W. C. Clark, J. T. Robertson, E. H. Oldfield, S. A. Aaronson, and I. U. Ali. 1992. Amplification and/or over-expression of platelet-derived growth factor receptors and epidermal growth factor receptor in human glial tumours. Cancer Research. 52:4550-3.

Galinska-Rakoczy A., P. Engel, C. Xu, H. Jung, R. Craig, L. S. Tobacman, and W. Lehman. 2008. Structural basis for the regulation of muscle contraction by troponin and tropomyosin. Journal of Molecular Biology. 379(5): 929-35.

Garrett P. J., N. M. Mckern, M. Lour, T. C. Elleman, T. E. Adams, G. O. Lovrecz, H-J Zhu, F. Walker, M.J. Frenkel, P. A. Hoyne, R. N. Jorissen, E. C. Nice, A. W. Burgess, and C. W. Ward. 2002. Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor α. Cell. 110:763-773.

Glenney, J. R. Jr., W. S. Chen, C. S. Lazar, G.M. Walton, L. M. Zokas, M. G. Rosenfeld and G. N. Gill. 1988. Ligand-induced endocytosis of EGFR is blocked by mutation inactivation and by microinjection of anti-phosphotyrosine antibodies. Cell. 52:675-84.

Grandal M. V., R. Zandi, M. W. Pedersen, B. M. Willumsen, B. Van Deurs, and H. S. Poulsen. 2007. EGFRvIII escapes down-regulation due to impaired internalization and sorting to lysosomes. Carcinogenesis. 28(7):1408-17.

Grenningloh G., S. Soehrman, P. Bondallaz, E. Ruchti, and H. Cadas. 2004. Role of the microtubule destabilizing proteins SCG10 and strathmin in neuronal growth. Journal of Neurobiology. 58(1): 60-9.

Harrison S.C.. 2003. Variation on Src-like theme. Cell. 112:737-40.

Heldin C.H, B. Westermark, and A. Wasteson. 1981. Specific receptors for platelet-derived growth factor on cells derived from connective tissue and glia. Proceedings of the National Academy of Sciences. 78(6):3664-8.

Heldin C. H., A Ernlund, C. Rorsman, and L. Rönnstrand. 1989. Dimerization of B type PDGF receptors occurs after ligand binding and is closely associated with receptor kinase activation. Journal of Biological Chemistry. 264:8905-12.

Heldin C. H.. 1995. Dimerization of the cell surface receptors in signal transduction. Cell. 80: 213-23.

Heldin C. H., and B. Westermark. 1999. Mechanism of action and *in vivo* role of platelet-derived growth factor. Physical Reviews. 79(4):1283-1316.

Heldin C. H., A. Östman, and L. Rönnstrand. 1998. Signal transduction via platelet-derived growth factor receptors. Biochimica et Biophysica Acta. 1378: 79-113.

Holegoua S., R. C. Armstrong, and N. E. Kremer. 1991. Dissecting the mode of action of neuronal growth factor. Current Topics in Microbiology and Immunology. 165:119-170.

Joly M., A. Kazlauskas, F. S. Fay, and S. Corvera. 1994. Disruption of PDGF receptor trafficking by mutation of its PI-3 kinase binding sites. Science. 263:684-87.

Kapeller R., R. Chakrabarti, L. Cantley, F. Fay, and S. Corvera. 1993. Internalization of activated platelet-derived growth factor receptor phosphatidylinositol-3' kinase complexes: potential interactions with the microtubule cytoskeleton. Molecular and Cell Biology. 13(10):6052-63.

Kazlauskas A., G. S. Feng, T. Pawson, and M. Valius. 1993. The 64 kDa protein that associates with the PDGFR beta subunit via Tyr-1009 is the SH2-containing phosphotyrosine phosphatase Syp. Proceedings of the National Academy of Sciences. 90(15): 6939-43.

Kelly J. D., B. A. Haldeman, F. J. Grant, M. J. Murray, R. A. Seifert, D. F. Bowen-Pope, J. A. Cooper, A. Kazlauskas. 1991. PDGF stimulates PDGF receptor subunit dimerization and inter-subunit trans-phosphorylation. Journal of Biological Chemistry. 266:8987-92.

Klinghoffer R. A., and A. Kazlauskas. 1995. Identification of a putative Syp substrate, the PDGFR-β. Journal of Biological Chemistry. 270 (38): 22208-22217.

Klinghoffer R. A., B. Duckworth, M. Valius, L. Cantley, A. Kazlauskas. 1996. Platelet-derived growth factor-dependent activation of phosphatidyl inositol 2-kinase is regulated by receptor binding of SH2-domain-containing proteins which influence RAS activity. Molecular and Cell Biology. 16(10):5905-14.

Koff J. L., M. X. G. Shao, I. F. Ueki, J. A. Nadel. 2008. Multiple TLRs activate EGFR via signalling cascade to produce innate immune response in airway epithelium. American Journal of Physiology. Lung, Cellular and Mol Physiology 294: 1068-75.

Lakadamyali M., M. J. Rust, and X. Zhuang. 2006. Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. Cell. 124(5): 997-1009.

Lange A., R. E. Mills, C. J. Lange, M. Stewart, S. E. Devine, and A. H. Corbett. 2007. Classical nuclear localization signals: definition, function, and interaction with importin α. Journal of Biological Chemistry. 282(8):5105-5.

Lanzetti L.. 2007. Actin in membrane trafficking. Current Opinion in Cell Biology. 19: 453-458.

Lechleider R. J., S. Sugimoto, A. M. Bennett, A. S. Kashihian, J. A. Cooper, S. E. Shoelson, C. T. Walsh, B. G. Neel. 1993. Activation of the SH2-containing phosphotyrosine phosphatase SH-PTP2 by its binding site, phosphotyrosine 1009, on the human PDGFR-β. Journal of Biological Chemistry. 268(29): 21478-81.

Lennartsson J., P. Wardega, U. Engström, U. Hellman, and C. H. Heldin. 2006. Alix facilitates the interaction between c-Cbl and PDGFR and thereby modulates receptor down-regulation. Journal of Biological Chemistry. 281(51): 39152-8.

Levitzki A.. 2005. PDGF receptor kinase inhibitors for the treatment of restenosis. Cardiovascular Research. 65:581-86.

Liu P., Y. Ying, Y-G. Ko, and R. G. W. Anderson. 1996. Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae. Journal of Biological Chemistry. 271(17): 10299-10303.

Liu P., Y-Y. Wang, P. Michaely, M. Zhu, and R. G. W. Anderson. 2000. Presence of oxidized cholesterol in caveolae uncouples active platelet-derived growth factor receptors from tyrosine kinase substrates. Journal of Biological Chemistry. 275 (41): 31648-31654.

Lodish H., A. Berk, P. Matudaira, C. A. Kaiser, M. Krieger, M. P. Scott, S. L. Zipursky, and J. Darnell. Molecular Cell Biology. Fifth Edition. W. H. Freeman and Company: New York , NY, 2004, pp 534.

Lokker N. A., J. P. O'Hare, A. Barsoumian, J. E. Tomlinson, V. Ramakrishnan, L. J. Fretto, and N. A. Giese. 1997. Functional importance of platelet-derived growth factor (PDGF) receptor extracellular immunoglobulin-like domains. Journal of Biological Chemistry. 272(52):33037-33044.

Lytton J.. 2007. Na+/Ca2+ exchangers: three mammalian gene families control Ca2+ transport. Biochemical Journal. 406(3):365-82.

Merrifield C. J., M. E. Feldman, L. Wang, and W. Almers. 2002. Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. Nature Cell Biology. 4: 691-698.

Millette E., B. H. Rauch, O. Defawe, R. D. Kenagy, G. Daum, and A. W. Clowes. 2005. PDGF-BB-induced human smooth muscle cell proliferation depends on basic FGF release and FGFR-1 activation. Circulation Research. 96: 172-79.

McDonald N. Q., and W. A. Hendrickson. 1993. A structural superfamily of growth factors containing a cysteine knot motif. Cell. 73: 421-24.

McKay M. M., D. K. Morrison. 2007. Integrating signals from RTKs to ERK/MAPK. Oncogene. 26: 3113-21.

Montmayeur J. P., M. Valius, J. Vandenheede, and A. Kazlauskas. 1997. The platelet-derived growth factor beta receptor triggers multiple cytoplasmic signalling cascades that arrive at the nucleus as distinguishable inputs. Journal of Biological Chemistry. 272(51): 32670-8.

Moore, G. E.. 1965. Cramming more components onto integrated circuits. Electronics Magazine. 38(8).

Mori S., L. Claesson-Welsh, and C. H. Heldin. 1991. Identification of a hydrophobic region in the carboxyl terminus of the PDGFR-β which is important for ligand-mediated endocytosis. Journal of Biological Chemistry. 266(31):21158-64.

Mori S., C. H. Heldin, and L. Claesson-Welsh. 1992. Ligand induced polyubiquitination of the platelet-derived growth factor β-receptor. Journal of Biological Chemistry. 267:6429-34.

Mori S., L. Rönnstrand, L. Claesson-Welsh, and C. H. Heldin. 1993. A tyrosine residue in the juxtamembrane segment of the platelet-derived growth factor β-receptor is critical for ligand mediated endocytosis. Journal of Biological Chemistry. 269(7):4917-21.

Mori S., K. Tanaka, S. Omura, and Y. Saito. 1995. Degradation process of ligand-stimulated platelet-derived growth factor β-receptor involves ubiquitin-proteasome proteolytic pathway. Journal of Biological Chemistry. 270:29447-52.

Murshid A., J. F. Presley. 2004. ER-to-Golgi transport and cytoskeletal interactions in animal cells. Cellular and Molecular Life Sciences. 61(2):133-45.

Oblinger J. L., C. L. Boardman, A. J. Yates, and R. W. Burry. 2003. Domain-dependent modulation of PDGFR-β by ganglioside GM1. Journal of Molecular Neuroscience. 20: 103-113.

Oefner C., A. D'Arcy, F. K. Winkler, B. Eggimann, and M. Hosang. 1992. Crystal structure of human platelet-derived growth factor BB. EMBO J. 11(11): 3921-6.

Olivier L. M., and S. K. Krisans. 2000. Peroxisomal protein targeting and identification of peroxisomal targeting signals in cholesterol biosynthetic enzymes. Biochimica et Biophysica Acta. 1529:89-102.

Orth J. D., and M. A. McNiven. 2006. Get off my back! Rapid receptor internalization through circular dorsal ruffles. 66(23): 11094-6.

Orth J. D., E. W. Krueger, S. G. Weller, and M. A. McNiven. 2006. A novel endocytic mechanism of epidermal growth factor receptor sequestration and internalization. Cancer Research. 66(7): 3603-10.

Pawson T.. 2002. Regulation and targets of receptor tyrosine kinases. European Journal of Cancer. 38(5): s3-10.

Pawson T., P. Nash. 2000. Protein-protein interactions define specificity in signal transduction. Genes and Development. 14:1027-47.

Pennock S., and Z. Wang. 2003. Stimulation of cell proliferation by endosomal epidermal growth factor receptor as revealed through two distinct phases of signalling. Molecular and Cell Biology. 23(16):5803-15.

Plattner R., A. J. Lokeske, A. Kazluaskas, and A. M. Pendergast. Bidirectional signalling links the Abelson kinases to the platelet-derived growth factor receptor. Molecular and Cell Biology. 24(6): 2573-2583.

Rajaopalan M., J. L. Neigigh, and D. A. McClain. 1991. Amino acid sequences Gly-Pro-Leu-Tyr in the sub-membranous domain of the insulin receptor are required for normal endocytosis. Journal of Biological Chemistry. 266: 23068-23073.

Rappoport J. Z.. 2008. Focusing on clathrin-mediated endocytosis. Biochemical Journal. 414: 415-423.

Reigstad L. J., J. E. Varhaug, and J. R. Lillehaug. 2005. Structural and functional specificities of PDGF-C and PDGF-D, the novel members of the platelet-derived growth factors family. FEBS Journal. 272: 5723-5741.

Robertson S. C., J. A. Tynan, D. J. Donoghue. 2000. RTK mutations and syndromes: when good receptors turn bad. Trends in Genetics. 16(6): 265-71.

Robinson D. R., Y-M. Wu, S-F. Lin. 2000. The protein tyrosine kinase family of the human genome. Oncogene. 19:5548-57.

Rocconi, R. P., K. S. Matthews, K. J. Kimball, M. G. Conner, A. C. Baker, and M. N. Barnes. 2008. Expression of C-kit and platelet-derived growth factor receptors in ovarian granulosa cell tumours. Reproductive Sciences. Ahead of print.

Ross R., J. Glomset, B. Kariya, and L. Harker. 1974. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proceedings of the National Academy of Sciences. 71(4):1207-10.

Samanta A. K., J. J. Oppenheim, and K. Matsuchima. 1990. IL-8 (monocyte-derived neutrophil chemotactic factor) dynamically regulates its own receptor expression on human neutrophils. Journal of Biological Chemistry. 265:183-87.

Schechter Y., L. Hernaez, J. Schlessinger, and P. Cuatrecasas. 1979. Local aggregation of hormone-receptor complexes is required for activation by epidermal growth factor. Nature 278:835-838.

Schlessinger J.. 2000. Cell signalling by receptor tyrosine kinases. Cell. 103: 211-25.

Schmidlin F., O. Dery, K. O. DeFea, L. Slice, S. Patierno, C. Sternini, E. F. Grady, and N. W. Bunnett. 2001. Dynamin and Rab5a-dependent trafficking and signalling of the neurokinin 1 receptor. Journal of Biological Chemistry. 278(27): 25427-37.

Shao Y., W. Akmentin, J. J. Toledo-Aral, J. Rosenbaum, G. Valdez, J. B. Cabot, B. S. Hilbush, and S. Haleguoa. 2002. Pincher, a pinocytic chaperone for nerve growth factor/TrkA signalling endosomes. Journal of Biological Chemistry. 157(4):679-91.

Sorkin A., B. Westermark , C. H. Heldin, L. Claesson-Welsh. 1991. Effect of receptor kinase inactivation on the rate of internalization and degradation of PDGF and the PDGFR-β receptor. Journal of Biological Chemistry. 112(3):469-78.

Stumvoll M., B. J. Goldstein, and T. W. Van Haeften. 2007. Pathogenesis of type 2 diabetes. Endocrine Research. 32(1-2): 19-37.

Sun P. D., and D. R. Davies. 1995. The cysteine-knot growth-factor superfamily. Annual. Review of Biophysical and Biomolecular Structure. 24: 269-291.

Szabo A., G. Horvath, J. Szollosi, and P. Nagy. 2008. Quantitative characterization of the large-scale association of ErbB1 and ErbB2 by flow cytometric homo-FRET measurements. Biophysical Journal. Epub ahead of print.

Tan F. K.. 2006. Autoantibodies against PDGF receptor in scleroderma. New England Journal of Medicine. 354(25): 2709-11

Ullrich A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. Cell. 61: 203-12.

Vaillancourt R. R., A. M. Gardner, A. Kazlauskas, and G. L. Johnson. 1996. The kinase-inactive PDGF beta-receptor mediates activation of the MAP kinase cascade via the endogenous PDGF alpha-receptor in HepG2 cells. Oncogene. 13(1):151-9.

Van Brocklyn J., E. G. Bremer, and A. J. Yates. 1993. Gangliosides inhibit PDGF stimulated receptor dimerization in human glioma U-1242MG and Swiss 3T3 cells. Journal of Neurochemistry. 61:371-374.

Wang Y., S. D. Pennock, X. Chen, A. Kazlauskas, and Z. Wang. 2004. Platelet-derived growth factor receptor mediated signal transduction from endosomes. Journal of Biological Chemistry. 279(9):8038-46

Wang Q., G. Villeneuve, and Z. Wang. 2005. Control of epidermal growth factor receptor endocytosis by receptor dimerization, rather than receptor kinase activation. EMBO. 6(10): 942-948.

Welsh J. B., R. Worthylake, H. S. Wiley, and G. N. Gill. 1994. Specific factors are required for kinase-dependent endocytosis of insulin receptors. Molecular Biology of the Cell. 5: 539-47.

Yarden Y.. 2001. The EGFR family and its ligands in human cancer signalling mechanisms and therapeutic opportunities. European Journal of Cancer. 37(4): 3-8.