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IGF TYPE I RECEPTOR LOCALIZATION AND TRAFFICKING IN Oligodendrocyte Progenitor Cells: Regulation of the PI-3 Kinase/Akt Pathway

A Thesis in

Cellular and Molecular Biology

by

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ABSTRACT

Insulin-like growth factor (IGF)-I is required for the survival of cells within the central nervous system (CNS), including oligodendrocytes (OLs) and their progenitors (OPs). Previously we demonstrated that IGF-I mediates long-term survival of OPs via sustained phosphorylation and activity of Akt, with concomitant stability and activity of the IGF type-I receptor (IGF-IR). The mechanism by which IGF-I regulates sustained Akt phosphorylation, however, is currently undefined. In this dissertation, we investigate the role of IGF-IR trafficking and subcellular localization in sustained Akt phosphorylation in OPs.

We report that there are no change in total IGF-IR protein levels during IGF-I stimulation; however, there is maximal loss of surface receptors at 30 min and recovery of receptors at the cell surface by 120 min. To determine the role of IGF-IR surface loss we used dansylcadaverine, an inhibitor of receptor clustering and internalization. Dansylcadaverine blocked the clustering of biotin-conjugated IGF-I at the cell surface and prevented the internalization of the IGF-IR. In addition, dansylcadaverine blocked IGF-I mediated Akt phosphorylation. To determine the source of IGF-IR surface recovery at 120 min we used monensin, an inhibitor of endosomal trafficking, and cycloheximide, an inhibitor of protein translation. Whereas monensin attenuated IGF-I mediated Akt phosphorylation and blocked surface recovery of the IGF-IR, cycloheximide had no effect on Akt phosphorylation or on total IGF-IR protein levels. We also show that the IGF-IR co-localized with markers for the recycling endosome, including the transferrin receptor (TfR) and Rab11. Together, the results of these studies suggest that IGF-IR clustering and internalization are required to promote Akt
phosphorylation and that recycling of the IGF-IR is required to sustain Akt phosphorylation. Mathematical analyses of our empirical data predicted a model of IGF-IR trafficking that is consistent with receptor surface loss and recovery as well as sustained Akt phosphorylation through 120 min.

We also report that the cholesterol chelating agent, MβCD, altered OP membrane integrity and blocked IGF-I mediated Akt phosphorylation, which were both reversed by cholesterol repletion. Notably, the extent of Akt phosphorylation positively correlated with the dose of cholesterol. These data suggest that cholesterol-membrane integrity is required for IGF-I mediated Akt phosphorylation. Based on these findings, we propose that the IGF-IR and its signaling proteins are localized within cholesterol/glycosphingolipid-enriched membrane microdomains (CEMs), also known as lipid rafts. Extraction with triton or sodium carbonate, following fractionation on a sucrose gradient, yielded CEMs from OPs that contained the classical rafting proteins caveolin-1 and flotillin-1. Furthermore, the IGF-IR and PI-3K/Akt signaling proteins co-localized in these microdomains. Together the results of these studies suggest that IGF-I mediated Akt phosphorylation requires CEMs in OPs.

We were further interested in determining the role of caveolae, a subset of lipid rafts that contain the protein caveolin-1, in OL biology. In these studies, we report that caveolin-1 protein expression increased during OL maturation. In addition, caveolin-1 localization within CEMs correlated with IGF-I stimulation. We also show that the IGF-IR co-localized with caveolin-1 at a stage of OL maturation when caveolin-1 is maximally expressed, and that the IGF-IR contains a caveolin binding motif that is conserved through evolution. To determine the function of caveolin-1 in OLs, we
examined the expression of myelin- and OL-specific proteins from the brains of caveolin-1 null mice versus wild type animals. We report that loss of caveolin-1 resulted in aberrant expression of myelin- and OL-specific proteins. Together, these data suggest that caveolin-1 plays a role in OL biology.

The results of this dissertation support our hypothesis that IGF-IR trafficking and localization mediate the sustained phosphorylation of Akt in OPs. Furthermore, a better understanding of the regulation of OL and their progenitors will contribute to the design of novel therapeutics to promote survival in diseases of demyelination, such as multiple sclerosis (MS).
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LIST OF ABBREVIATIONS

BMP: bone morphogenic protein
CCV: clatherin coated vesicles
CEM: cholesterol-enriched membrane microdomains
CG-4: central glial-4
CBM: caveolin binding motif
CNS: central nervous system
CNP: cyclic-2’, 3’ nucleotide phosphodiesterase
CNTF: ciliary neurotrophic factor
EAE: experimental autoimmune encephalomyelitis
EE: early endosome
ERC: endocytic recycling compartment
ERK: extracellular regulated kinase
EGFR: epithelial growth factor receptor
FGF: fibroblast growth factor
GLUT: glucose transporter
GPCR: G-protein coupled receptor
GSK: glycogen synthase kinase
IGF: insulin-like growth factor
IGF-IR: IGF type-I receptor
IR: insulin receptor
LE: late endosome
MAPK: mitogen activated protein kinase
MBP: myelin basic protein
MS: multiple sclerosis
MVB: multivesicular body
NCAM: neural cell adhesion molecule
NGF: nerve growth factor
NT: neurotrophin
ODE: ordinary differential equation
OL: oligodendrocyte
OP: oligodendrocyte progenitor
PI-3K: phosphotidylinositol-3 kinase
PDGF: platelet-derived growth factor
PKC: protein kinase C
PLC: phospholipase C
PLP: proteolipid protein
PTP: protein tyrosine phosphatase
PNS: peripheral nervous system
RTK: receptor tyrosine kinase
Tg: transgenic
SE: sorting endosome
Shh: sonic hedgehog
SVZ: subventricular zone
TfN: transferrin
TfR: transferrin receptor
TGN: trans-golgi network

TNF: tumor necrosis factor

Trk: tyrosine receptor kinase
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CHAPTER ONE

Literature Review
Oligodendrocytes and Myelination

Oligodendrocytes (OLs) are the myelin producing cells of the vertebrate central nervous system (CNS). Myelin, specialized OL plasma membrane, is comprised of cholesterol, galactosylceramides, sulfatides, and contain myelin-specific proteins, including proteolipid and myelin-basic proteins (Simons et al., 2000). This specialized membrane ensheathes nerve axons and provides insulation for the fast, saltatory conduction of electrical impulses. Notably, each OL in the CNS can provide myelination for up to 60 nerve axons (Butt and Ransom, 1989). This is in contrast to the peripheral nervous system, where multiple Schwann cells are required to myelinate one axon (Mirsky and Jessen, 1999). Accordingly, the loss of a single OL leads to the demyelination of a large number of neurons within the CNS.

Several neurological conditions have demyelination as their pathological hallmark, including multiple sclerosis (MS) and amyotrophic lateral sclerosis (Armani et al., 1987; Bruck, 2005). In MS, white matter plaques are formed in the brain and spinal cord (Wingerchuk et al., 2001), which result from the loss of OLs and myelination, culminating in severe sensory and motor dysfunction. In the acute stages of MS there is partial regeneration of lost myelin; however, regeneration is not observed as the disease progresses (Wingerchuk, et al., 2001).

OL progenitor cells (OPs) are found in the adult brain and in white matter plaques (Wolswijk and Noble, 1989). These adult progenitors have the potential to replace lost OLs; however, over time these cells fail to repair demyelinated regions of the CNS (Scolding et al., 1998). The lack of regeneration may be attributed to several factors, including failure of OPs to proliferate, migrate, or differentiate into mature OLs.
(Franklin, 2002). Therefore, it is important to understand mechanisms that dictate cellular decisions in the OL lineage in order to promote proper myelination during development and remyelination during pathological conditions.

**Oligodendrocytes and Their Progenitors**

Mature, myelinating OLs arise from OPs, which originate from the proliferating neuro-epithelium of the neural tube and migrate throughout the developing brain and spinal cord to form the white matter tracts of the CNS (Doetsch et al., 1997; Frost et al., 1996). In general, the appearance of OPs and OLs in the brain occurs subsequent to the production of neurons and astrocytes (Goldman and Vaysse, 1991). In fact, it has been hypothesized that neurons provide a pathway along which OPs migrate. This is the case for OPs that myelinate the optic nerve, which migrate down the optic chiasm along the nerve axon (Small et al., 1987). Additionally, the migration of OPs is influenced by the neural cell adhesion molecule, NCAM (Barral-Moran et al., 2003), which is expressed by progenitor cells (Grinspan and Franceschini, 1995).

The initial production of OPs is controlled by local factors within the neural tube. In multiple studies it was reported that the neural tube is competent to produce OPs after ectopic transplantation (Orentas and Miller, 1996; Yamada et al., 1991). Likewise, OLs fail to develop within spinal cord regions of embryos lacking a notochord (Maier and Miller, 1997). Sonic hedgehog (shh), a developmental transcription factor endogenous to the notochord, is instructive for the production of OPs in the chick spinal chord, whereas inhibition of shh blocks this production (Orentas et al., 1999). In contrast, bone morphogenic proteins (BMPs)-2 and -4 inhibit the development of OLs in vitro and
instead, promote an astroglial fate (Mabie et al., 1997; Mehler et al., 2000). Taken together, these data suggest that multiple factors —inductive and inhibitory— contribute to the production of OPs in the developing CNS.

OPs, also termed early progenitors, are characterized by a bipolar morphology and by expression of specific surface antigens, including the proteoglycan NG2 and A2B5-reactive gangliosides (Nishiyama et al., 1996; Raff, 1989). Early progenitors are migratory, highly proliferative, and have the ability to differentiate into both type-2 astrocytes and OLs (Raff et al., 1984). Early progenitors proceed through several stages of development, including the late progenitor, immature OL, and finally, the mature OL stage. Similar to early progenitors, each subsequent stage of OL maturation is characterized by cell morphology and the expression of specific cellular antigens (see Figure 1.1). Additionally, the proliferative and migratory properties of OLs decrease during maturation, whereas their cellular processes become further branched and more elaborate (Zhang et al., 2000a). Terminally differentiated, mature OLs are characterized, moreover, by the expression of proteins that are essential for myelination, including myelin basic protein (MBP) and proteolipid protein (Timsit et al., 1995; Yu et al., 1994).

**Regulation of the Oligodendrocyte Lineage**

Various transcription factors are well characterized in OL lineage progression. The basic helix-loop-helix (bHLH) proteins Olig-1 and Olig-2 and the high-motility-group protein Sox10 are expressed early during OL development. The former two are regulated by shh expression in the notochord and commit neuroepithelial cells to an oligodendrogial fate (Lu et al., 2000; Zhou et al., 2000). In addition, myelin
transcription factor1 (Myt1) was recently shown to modulate the proliferation and differentiation of the OL lineage by influencing the transcription of myelin-specific genes (Nielsen et al., 2004). Along with aforementioned surface antigens and cellular markers, these transcription factors serve to identify specific stages of the OL lineage.

Multiple trophic factors contribute to the survival, proliferation, migration and differentiation of OL progenitors, including fibroblast growth factor (FGF)-2, platelet derived growth factor (PDGF)-AA, neurotrophin (NT)-3, cilliary neurotrophic factor (CNTF) and insulin-like growth factor (IGF)-I (Miller, 2002; Orentas and Miller, 1998). PDGF-AA and FGF-2 are well characterized mitogens for OL progenitors both in vitro and in vivo (Eccleston and Silberberg, 1984; Fruttiger et al., 1999; Giordano et al., 1992). In combination, these trophic factors promote proliferation and block differentiation of early progenitor cells (Bogler et al., 1990; Goddard et al., 2001). FGF-2 sensitizes these cells to PDGF-AA by up-regulating the PDGF-α receptor (McKinnon et al., 1990); however, OL progenitors lose expression of the PDGF-α receptor at the late progenitor state, which is identified by the expression of O4 antigen (Ellison and de Vellis, 1995). Alone, FGF-2 permits progression from the early to the late progenitor stage but blocks further differentiation into immature OLs (McKinnon et al., 1991). NT-3 also promotes proliferation and cell survival of OPs both in vitro and in vivo (Kumar et al., 1998). Mice lacking NT-3 or its receptor, TrkC, exhibit deficiencies in OPs, as well as a reduction in spinal cord diameter (Kahn et al., 1999). In addition, CNTF promotes oligodendrocyte survival in vivo (Barres et al., 1993).

IGF-I is a potent survival factor for OPs both in vivo and in vitro (Barres et al., 1993; D'Ercole et al., 1996; McMorris and Dubois-Dalcq, 1988). This trophic factor will
be discussed at greater length in the following section of this chapter. However, it can be mentioned here that previous studies demonstrated that IGF-I synergizes with FGF-2 and PDGF to promote proliferation of early progenitors (Jiang et al., 2001). Furthermore, IGF-I and FGF-2 synergism is mediated through the activation of the PI3-K/Akt and MAPK signaling pathways, respectively, and results in the modulation of important regulators of cell cycle kinetics, including cyclin D1 and the retinoblastoma (rb) protein (Frederick and Wood, 2004). Taken together, these trophic factors, alone and in combination, control the biological fate of OLs and their progenitors both in vivo and in vitro.

**IGF-I Expression and Function in the CNS**

IGF-I, originally termed somatomedin, is a 70 amino acid peptide with a molecular weight of 7.5 kD and shares approximately 70% and 50% homology with IGF-II and pro-insulin, respectively (Webster, 1997). The main source of circulating IGF-I is the liver (Daughaday and Rotwein, 1989); however, IGF-I is also expressed in the CNS, primarily in neurons (for review see D’Ercole, et al., 1996). IGF-I is detected in the subventricular zone (SVZ) at a time when OLs are generated (Bartlett et al., 1992). Moreover, the expression of IGF-I mRNA transcripts have been localized in OPs and O4+ late progenitors, and more weakly in O1+ immature OLs, in primary rat cultures (Shinar and McMorris, 1995). In contrast, the IGF type-I receptor (IGF-IR), the primary signaling receptor for IGF-I, is ubiquitously expressed in the CNS (D’Ercole, et al., 1996). IGF-IR mRNA is found in the neuroepithelium as early as embryonic day 11.
(Bondy and Lee, 1993) and in all stages of OL development (Baron-Van Evercooren et al., 1991).

During development IGF-I functions as a neurotrophic agent, mediating survival and proliferation of neurons (Torres-Aleman et al., 1994; Zheng and Quirion, 2004). Relevant to this thesis, IGF-I has multiple effects on cells of the OL lineage. IGF-I mediates proliferation and commitment of rat neural progenitors to an oligodendroglial fate in vitro (McMorris and Dubois-Dalcq, 1988). IGF-I also promotes the differentiation of adult neural progenitor cells to mature OLs through the inhibition of BMP signaling (Hsieh et al., 2004), a negative regulator of OL production in the CNS. As discussed previously, IGF-I cooperates with other mitogens to enhance OP proliferation (Jiang et al., 2001) and promotes the expression of myelin-specific genes in differentiated OLs (Saneto et al., 1988).

In vivo studies demonstrate that IGFs regulate OL development and myelination in the CNS (McMorris et al., 1993). IGF-IR (-/-) null mice invariably die at birth of respiratory failure. However, embryonic and post-natal analyses of these mice reveal a neurological phenotype (Liu et al., 1993). Most notably, embryonic forebrain cultures from IGF-IR (-/-) null mice have substantially fewer OPs compared to wild-type cultures (Sommer and Schachner, 1981). Whereas IGF-I (-/-) null mice have limited viability, those animals that survive have a marked decrease in total brain size, as well as a hypomyelinated phenotype, with a reduced number of OLs, OPs, and neurons (Ye et al., 2002b). This also includes a significant reduction in myelinating proteins, MBP and PLP. Conversely, IGF-I over expression in transgenic (tg) mice results in brain overgrowth characterized by an increase in the number of neurons and OLs, as well as a
marked increase in MBP, PLP, and myelination (Carson et al., 1993; Ye et al., 1995a). Additionally, IGF-I tg mice have an increase in the anti-apoptotic protein Bcl-2 in distinct brain regions, primarily the cerebral cortex and cerebellum, and are protected from demyelination in under-nutritional insults compared to wild-type mice (Chrysis et al., 2001; Ye et al., 2000). Likewise, in experimental models of hypoxia-ischemia, which specifically target the periventricular white matter, exogenous IGF-I treatment reduces post-ischemic injury in fetal sheep (Guan, et al., 2001). When given to rats after experimental autoimmune encephalomyelitis (EAE), an in vivo model of MS, IGF-I promotes remyelination (Guan et al., 1996). Lastly, IGF-I is expressed during recovery from EAE, as well as recovery from cerebral ischemia and other forms of neurological trauma that particularly target OLs and the white matter of the CNS (Komoly et al., 1992; Lee et al., 1992; Gehrmann et al., 1994). These data demonstrate that IGF-I is vital for the survival of OLs and their progenitors, contributes to proper myelination, and is protective during experimental models of injury in vivo. Accordingly, a better understanding of the mechanisms by which IGF-I promotes the survival and maintenance of OLs will significantly contribute to the design of therapeutic interventions in demyelinating diseases.
The IGF type-I Receptor

The IGF type-I receptor (IGF-IR) is a member of the receptor tyrosine kinase (RTK) family, which shares a high degree of homology with the insulin receptor (Ullrich et al., 1986). The IGF-IR is the primary signaling receptor for IGF-I and IGF-II. Unlike other RTKs, which undergo dimerization in the presence of ligand, the IGF-IR exists as a heterotetramer, consisting of: (1) two extracellular alpha subunits that bind ligand at cysteine rich domains; and (2) two transmembrane beta subunits, which possess tyrosine kinase domains. The IGF-IR is transcribed as a single polypeptide, which is then glycosylated and cleaved to form the alpha and beta subunits (for review see Rubin and Baserga 1995). Subsequently, these subunits are reassembled in the trans-golgi network (TGN) as a tetrameric unit connected by disulfide bridges, followed by transport to the plasma membrane (Ullrich et al., 1986; for review see DeMetys et al., 1994; see figure 1.2).

Upon ligand binding, the IGF-IR undergoes specific conformational changes that induce autophosphorylation of the beta subunits at multiple tyrosine residues and results in an increase in tyrosine kinase activity (Gronborg et al., 1993; Kato et al., 1993). The activation of the receptor is followed by the recruitment of multiple adaptor proteins, including the insulin receptor substrate (IRS)-1 or -2 (Sun et al., 1991) and Shc (Yamauchi and Pessin, 1994), as well as a variety of signaling molecules, including the PI-3K subunits p85 and p110 (Giorgetti et al., 1993), mitogen activated protein kinase (Natalicchio et al., 2004), and protein kinase C (PKC; Heidenreich et al., 1993).
**The PI-3K/Akt Pathway in Oligodendrocytes**

The phosphatidylinositol-3 kinase (PI-3K)/Akt signaling pathway is a potent mediator of survival in many cell types (Lee et al., 2005; Stam et al., 2001). These effects are primarily regulated by direct downstream targets of the serine/threonine kinase Akt (also termed protein kinase B), including the forkhead (FKHR) family of transcription factors, the pro-apoptotic protein Bad, and glycogen synthase kinase (GSK)-3β (Stoica, et al., 2003; for review see Scheid and Woodgett, 2003).

Trophic factors, including members of the neurotrophin family and the IGFs, induce phosphorylation and subsequent activity of Akt in many cell types, including neural and glial cells of the CNS (Zheng and Quirion, 2004; Bhave et al., 1999). Disruption of Akt phosphorylation in vitro, via inhibitors of PI-3K, results in the impaired ability of IGF-I to promote survival of primary neuronal cultures (Zheng et al., 2002) and of oligodendrocytes as discussed below.

Relevant to this thesis, IGF-I is a potent mediator of the PI-3K/Akt pathway in cells of the OL lineage. As aforementioned, IGF-I synergizes with FGF-2 to induce proliferation of OPs (Jiang et al., 2001). The mitogenic effects of IGF-I is primarily mediated through the PI-3K/Akt pathway and results in stability of cyclin D1, an important regulator of cell cycle entry (T.J. Frederick and T.L. Wood, unpublished data). IGF-I also protects late OL progenitors from glutamate-mediated apoptosis (Ness and Wood, 2002). These protective effects correlate with a decrease in Bax translocation to the mitochondria and a decrease in cytochrome C release (Ness et al., 2004), as well as with sustained Akt and IGF-IR phosphorylation (Ness et al., 2002). Furthermore, the PI-
3K inhibitor, LY294002, blocks IGF-I mediated phosphorylation of Akt and prevents protection of late progenitors from glutamate (Ness and Wood, 2002).

In support of these previous findings in rat OPs, it was recently reported that the protective effects of IGF-I are mediated primarily through the sustained activation of Akt in mouse OPs (Zaka et al., 2005). These data have been also corroborated by multiple studies that demonstrate the importance of the PI-3K/Akt signal pathway in the survival of OPS. The transfection of OPs with a mutated PDGF-αR results in the attenuation of Akt phosphorylation and a decrease in proliferation (Ebner, et al., 2000). Additionally, expression of a dominant/negative Akt in OLs prevents neuregulin-mediated survival (Flores et al., 2000). Similarly, PI-3K inhibitors, wortmannin and LY294002, block the protective effects of multiple survival factors, including IGF-I, in OLs (Vemuri and McMorriss, 1996). Moreover, in these cells nerve growth factor (NGF) inhibits TNF-α mediated apoptosis via activation of the PI-3K/Akt pathway (Takano et al., 2000). Taken together, these studies clearly demonstrate the importance of the PI-3K/Akt pathway in the survival of OLs.

Other trophic factors, including CNTF and NT-3, are unable to mediate long-term survival of OPs. In the case of NT-3, this trophic factor induces a transient phosphorylation of Akt, followed by down-regulation of its cognate receptor tyrosine kinase, TrkC (Ness et al., 2002). The ability of IGF-I to promote survival of OPs through the sustained activation of the PI-3K/Akt pathway appears to be a unique property of this trophic factor in these cells. IGF-I mediated MAPK pathway activation has been observed, however this pathway does not appear to enhance the survival effects of IGF-I in the OL lineage (Cui et al., 2005). We and others have shown the sustained
phosphorylation of Akt mediated by IGF-I in OPs and smooth muscle cells (Allen et al., 2005; Cui et al., 2005; Ness and Wood, 2002); however, the mechanism by which this occurs is undefined. Accordingly, it is of great interest to delineate the mechanisms by which IGF-I mediates the sustained phosphorylation of Akt.

**Receptor Trafficking**

Subsequent to activation and autophosphorylation, RTKs are internalized within distinct regions of the plasma membrane, forming endocytic vesicles or endosomes (Willingham and Pastan, 1984). This process was first described as a mechanism to desensitize receptors, and consequently to attenuate signal transduction since receptors within endosomes clear ligand from the extracellular environment and are unavailable to bind new ligand (Dunn et al., 1986).

Endocytosis requires the recruitment of RTKs into invaginated regions of the plasma membrane, which become coated with the scaffolding protein clathrin after receptor activation. Whereas receptor-mediated endocytosis is most often clathrin-dependent (Brodsky et al., 2001), endocytosis may occur via caveolae, which is discussed in the following section. In addition to clathrin, the scaffolding/adaptor proteins AP-2 and Eps15 are necessary for the proper formation of the clathrin lattice (Benmerah et al., 1995; Santolini et al., 1999). This is followed by the pinching off of the plasma membrane, a process regulated by the dynamin family of small GTPases (Damke, 1996), and the formation of endosomes.

Endosomes represent a variety of intracellular compartments classified by morphology, subcellular location, pH, and associated proteins (Bishop, 2003; Cavalli et
such as the Rab family of small GTPases, which are involved in the fusion and budding of distinct endosomal compartments (Christoforidis et al., 1999; McLauchlan et al., 1998). After internalization, clathrin-coated vesicles (CCVs) shed their clathrin-coat marking the formation of early endosomes (EEs). EEs are localized near the plasma membrane and are associated with the early endosomal antigen (EEA) 1 and Rab5, both of which contribute to EE formation (Gorvel et al., 1991). Rab5 also is intimately involved in the internalization process (Bucci et al., 1992). Endosomal maturation of the EE requires the acidification of endosomal vesicles (pH ~ 6.0) via an H⁺ ATPase, which results in the dissociation of ligand from receptor (Rybak and Murphy, 1998). Numerous studies report, however, that ligands can remain bound to their receptor in this acidic environment. Epithelial growth factor (EGF) remains bound to the EGF receptor (EGFR) in the EE (Maeda et al., 2002). However, transforming growth factor (TGF)-α rapidly dissociates from the EGFR (Maeda et al., 2002). Therefore, the stability of ligand:receptor complexes in the endosome is likely based on the individual affinity of each ligand:receptor pair.

Ligand:receptor dissociation leads to the formation of sorting endosomes (SEs), which are associated with similar adaptor proteins to the EE. However, sorting endosomes are classified as such because they are formed from coalesced EEs and are the proverbial cross-roads of intracellular trafficking. It is from this compartment that receptors are targeted either for degradation or for recycling to the plasma membrane.

The degradation pathway leads to the budding of vesicles from the SEs and fusion of these vesicles with the increasingly more acidic (~pH 5.0) late endosome (LE), also termed multi-vesicular body (Mellman et al., 1986). The formation of the LE is, in part,
regulated by Rabs 7 and 9 (Papini et al., 1997). Moreover, the majority of mannose-6-phosphate/IGF type II receptors (M-6-P/IGF-IIR) at steady-state are localized in the LE (Matovcik et al., 1990). Accordingly, the M-6-P/IGF-IIR is a marker for this endosomal compartment (see table 1.1). Fusion of the LE with the lysosomal compartment (~ pH 4.0) results in a further decrease in endosomal pH, as well as an increase in the activation of acidic enzymes required for protein degradation (Hirota et al., 2004; Tjelle et al., 1998).

In contrast to lysosomal degradation, various cellular proteins are recycled to the plasma membrane. The recycling pathway can be divided into: 1) the fast recycling of material to the plasma membrane directly from the SE (Baravalle et al., 2005), which is regulated by Rab4 (Peters et al., 2001) and 2) the slow recycling of material to the plasma membrane, which requires transit through an additional, tubular and peri-nuclear endosomal recycling compartment (Sakai et al., 1998). Trafficking through the ERC, which is regulated by Rab11 (Pasqualato et al., 2004), has been extensively described for transferrin (TfN) and the transferrin receptor (Bartz et al., 2005; Touret et al., 2003), and is also known as the constitutive recycling pathway (Ren et al., 1998; Ullrich et al., 1996).

The distinction between endosomal compartments is seemingly clear; however, there is overlap between these vesicles. Variability exists between endosomal morphology and localization in different cell types. Furthermore, there is evidence that endosomal compartments traffic between and each other as well as exchange cargo proteins with the trans-golgi network (TGN) and endoplasmic reticulum (Seto et al., 2002). Lastly, whereas LEs are defined as part of the degradation pathway, signaling
molecules associate with this endosomal compartment, and in some cases contain active receptors (Gaudriault et al., 1997; Law et al., 1984). Based on these data, our understanding of endosomal trafficking is incomplete and requires further investigation.

As previously mentioned, the internalization of RTKs after ligand binding was originally hypothesized to desensitize receptors and attenuate signal transduction. However, the evidence that certain receptors remain bound to ligand within endosomes and that signaling molecules associate with endosomal compartments lends support to the hypothesis that signal transduction is not terminated at the cell surface but rather continues throughout multiple endosomal compartments. In fact, a strong correlation has been established between the localization of RTKs and the activation of distinct signaling pathways (Wiley and Burke, 2001). In CHO cells blocking internalization of the IGF-IR results in an attenuation of ERK activation, without affecting PI-3K/Akt activation (Chow et al., 1998). Similarly, when the internalization of the EGFR is blocked, a decrease in ERK activity and an increase in PKC activity are observed (Veirra et al., 1996). Altering the trafficking of RTKs not only affects signal transduction but also alters biological responses. In PC12 cells nerve growth factor (NGF)-mediated survival is associated with surface localized TrkA receptors via the activation of the PI-3K/Akt pathway, whereas internalized TrkA receptors induce NGF-mediated differentiation via the ERK pathway (Zhang et al., 2000b). Together, these studies suggest that PI-3K/Akt activation is mediated at the cell surface and ERK activation is mediated within the endosome, which is attributed to the spatial organization of signaling machinery into distinct subcellular compartmentalization. This is supported by the observation that the integral membrane phosphatidylinositol bisphosphate (PI-4,5-P₂), which is required for
the activation of the PI-3K/Akt and PKC pathways, is exclusively localized to the plasma membrane. Accordingly, PI(4,5)P$_2$ is only accessible to receptors at the cell surface (Haugh, 2002). Multiple studies have shown that plasma membrane localization is required for Ser473 phosphorylation of Akt (Scheid et al., 2002). Furthermore, to date there is no evidence that Akt is activated in endosomes, although the catalytic subunit of PI-3K (p110) has been localized to these vesicles (Burke et al., 2001; Kelly and Ruderman, 1993). In contrast, upstream components of mitogen activated protein kinase (MAPK)/ERK, pathway, including Ras and Shc, are globally accessible since they have been localized both at the plasma membrane and EE (Di Guglielmo et al., 1994; Haugh, 2002).

Various studies support the idea that signaling can occur exclusively within the endosome. This was shown first for the EGFR and insulin receptor (Baass et al., 1995) and later was reported for the PDGFR (Sorkin et al., 1993) and Trk (Grimes et al., 1996). In one study, the intrinsic kinase activity of the EGFR was blocked with a specific tyrosine kinase inhibitor; however, the receptor still internalized after EGF stimulation. Subsequently, the inhibitor was washed away, inducing EGFR autophosphorylation and normal signaling through the ERK pathway (Vierra et al., 1996). Others also have demonstrated that phosphorylation levels of the EGFR and Shc are higher at the endosome than at the plasma membrane (Di Guglielmo et al., 1994). In addition, endosomal EGFRs are phosphorylated at distinct tyrosine residues compared with surface receptors (Nesterov et al., 1994). EGFRs trapped at the cell surface are rapidly dephosphorylated, whereas receptors in endosomes retain maximal activity (Kay et al., 1986; Lai et al., 1989; Wada et al., 1992). As previously discussed, receptor
internalization was previously thought to attenuate signal transduction. To the contrary, emerging evidence suggests that receptor internalization and trafficking fine tune signaling pathways and give rise to a repertoire of biological responses.

To date, EGFR trafficking is the most understood as this receptor has been studied as a model for RTKs (Sorkin and Von Zastrow, 2002). Nevertheless, multiple studies demonstrate that not all RTKs are regulated by the same internalization machinery and that not all receptors are targeted to the same subcellular locations. The processes that regulate receptor trafficking are not fully understood; however, the affinity of ligand:receptor complexes, as well as the associated degree of phosphorylation at each autophosphorylation site likely influences subcellular targeting. In a human carcinoma cell line, EGFRs within early endosomes remain bound to EGF ligand, with significant EGFR tyrosine autophosphorylation (Cohen and Fava, 1985; Nesterov et al., 1990). TGFα internalization via the EGFR results, however, in rapid dephosphorylation of this receptor (Haugh et al., 1999). Interestingly, EGF binding results in EGFR degradation, whereas TGFα binding leads to EGFR recycling (Ebner and Derynck, 1991; French et al., 1995; Korc and Finman, 1989).

In addition to phosphorylation, ubiquitylation is a post-translational modification that also influences the subcellular targeting of receptors (Marmor and Yarden, 2004). Receptor ubiquitylation involves the addition of ubiquitin, a 76 amino acid protein, to lysine residues at the cell surface and promotes RTK internalization (Jekely et al., 2005). Several associated proteins are required for the recruitment of ubiquitin and the addition of ubiquitin polymers to activated receptors at the plasma membrane. In particular, NEDD4, an ubiquitin ligase, is involved in the initial sorting of
receptors for clathrin coated pits (for review see Marmar and Yarden, 2004). Cbl, another ubiquitin ligase, negatively regulates RTK signaling by promoting internalization and lysosomal degradation (Joazeiro et al., 1999; Levkowitz et al., 1998). Mono-ubiquitinylation regulates receptor internalization and targeting to the lysosome, whereas poly-ubiquitinylation regulates receptor targeting to the proteosome (Fuchs and Neuwirtova, 2006). Taken together, these studies clearly demonstrate that post-translational modification and the consequent association of adaptor and signaling molecules regulates the trafficking and subcellular fate of RTKs.

** Trafficking of the Insulin and IGF Receptors **

As previously mentioned, the IR and IGF-IR are homologous RTKs that exist as constitutive heterotetramers, consisting of two alpha and two beta subunits. The latter subunits possess intrinsic kinase activity that are activated upon ligand binding (Nissley et al., 1985). Subsequently, the IR and IGF-IR are internalized by clathrin-coated pits (Carpentier et al., 1996). Internalization of these receptors is regulated by specific tyrosine residues within the cytoplasmic region of the beta subunit, which are phosphorylated and lead to the anchoring of specific adaptor proteins for signal transduction and the internalization process (Morrione, 2003). Amino acid mutation in both the extracellular juxta-membrane and intracellular c-terminal regions of the beta subunit of the IGF-IR has been shown to disrupt internalization (Chow et al., 1998; Condorelli et al., 1994). As described for other RTKs, the internalization of the IR and IGF-IR does not simply terminate signal transduction. Instead, internalization of these
receptors leads to the activation of specific signaling pathways and diverse biological responses (Ceresa et al., 1998; Chow et al., 1998).

Although homologous, the IGF-IR and IR have different internalization kinetics and intracellular itineraries in various cell types. At concentrations for IGF-IR binding, 45% of $^{125}\text{I}$ IGF-I internalizes maximally at 20 minutes in hippocampal neurons (Dore et al., 1997). Similarly, in rat fibroblasts approximately 40% of radio-labeled IGF-I internalizes maximally in 25 minutes (Zapf et al., 1994). In these cells, the internalization rate of IGF-I is 3 times slower than insulin (Zapf et al., 1994). Furthermore, insulin, compared to IGF-I, more readily dissociates from its receptor and is subsequently targeted for degradation. Nearly 60% of IGF-I returned to the cell surface after 120 minutes, whereas only 20% of insulin retroendocytosed in these cells (Zapf et al., 1994). Taken together, these data suggest that IGF-I/IGF-IR complexes are predominantly targeted for recycling, whereas insulin/IR complexes are predominantly targeted for degradation.

The differential trafficking and stability of the IR and IGF-IR are likely attributed to the factors previously discussed (see above section on Receptor Trafficking), including ligand:receptor complex affinity, degree of receptor autophosphorylation, and ubiquitinylation (Ebner and Derynck, 1991; French et al., 1995; Korc and Finman, 1989). In terms of the IGF-IR, stability is regulated by association with Grb10, which promotes the recruitment of an E3 ubiquitin ligase and culminates in receptor ubiquitinylation and degradation (Vecchione et al., 2003).
Lipid Rafts and Signal Transduction

The traditional view of the plasma membrane as a random assortment of proteins and lipids has been challenged by the discovery of structured microdomains, termed lipid rafts. Lipid rafts are biochemically distinct from the surrounding plasma membrane since they are enriched in glycosphingolipids and saturated phospholipids whose straight, saturated fatty-acid side chains permit close packing with cholesterol (Brown and London, 1988). Consequently, the hydrophobic interactions of cholesterol/glycosphingolipid-enriched membrane microdomains (CEMs) form a liquid-ordered that is distinct from the surrounding plasma membrane.

A subset of lipid rafts, termed caveolae, specifically contain the protein caveolin and form 50-100 nm flask-like invaginations, which distinguish them from non-caveolae lipid rafts (Rothberg et al., 1992). Caveolin, a 21-24 kD cholesterol-binding protein, is integrated within the plasma membrane with both N- and C- termini exposed to the cytoplasm (Lisanti et al., 1993). Caveolins are highly conserved through evolution from humans to C.elegans (Bickel, 2002). Three isoforms of caveolin are expressed in mammalian cells. Caveolin-1 is ubiquitously expressed and often coincides with expression of caveolin-2. Caveolin-3 is found almost exclusively in muscle cells (Scherer and Lisanti, 1997; Tang et al., 1996; Way and Parton, 1995). Caveolin isoforms oligomerize, first as homo-oligomers and then as hetero-oligomers (Okamoto et al., 1998; Scherer et al., 1997) and provide the structural framework for caveolae.

Whereas lipid rafts are found in most of mammalian cells, caveolae are absent from erythrocytes, lymphocytes, and most neurons (Fra et al., 1994; Fra et al., 1995; Parton and Simons, 1995). Nevertheless, caveolae are abundant in many differentiated
cell types, including adipocytes (Couet et al., 1997a), endothelial cells (Lisanti et al., 1994b), and smooth, skeletal, and cardiac muscle cells (Anderson, 1993). The abundance of caveolae in these cell types correlates with an increase in caveolin expression during differentiation (Mikol et al., 2002; Scherer et al., 1994; Song et al., 1996). Caveolin-1 null (-/-) mice, which have no visible caveolae structures, are viable (Drab et al., 2001); however, these animals result in a variety of phenotypes, such as insulin resistance (Cohen et al., 2003), shorter lifespan (Park et al., 2003), hyperproliferation of fibroblasts and epithelial cells (Razani et al., 2001), as well as cardiac abnormalities (Park et al., 2002), suggesting an important in vivo role of caveolae rafts.

The isolation of caveolae and non-caveolae raft-like microdomains, which are consistent with lipid rafts from intact cellular membranes, involves the use of non-ionic detergents (e.g. Triton X-100, CHAPS) at 4°C or sodium carbonate buffer (pH 11), in which rafts and their proteins are insoluble due to their relatively high lipid content. These insoluble membranes can be separated from the bulk plasma membrane by sucrose density centrifugation, since these microdomains are buoyant in low density sucrose.

Although lipid rafts have been well-studied and raft-like microdomains have been isolated from a variety of cell types, the existence of these membranes in intact cells is still a contentious issue. Due to their small size, lipid rafts are well below the resolution of conventional microscopy and therefore only have been identified by indirect methods (Kusumi and Suzuki, 2005; Prior et al., 2003). It is also believed that lipid rafts are artifacts of the detergent and non-detergent isolation methods commonly used for their study. Nevertheless, recent biophysical, computational, and membrane modeling studies provide evidence for the presence of lipid raft microdomains in living cells (Hancock,
2006; Kusumi et al., 2005; Silvius, 2003). From here on, the term CEMs will be used in place of lipid rafts. Those rafts that contain caveolins and form membrane invaginations will be referred to as caveolae.

Whereas more ordered than the surrounding plasma membrane, CEMs are far from static. To the contrary, these dynamic structures spatially organize, and are host to, a variety of proteins, whereas they exclude other proteins (e.g. transferrin receptor and clathrin). Several factors influence protein sequestration within caveolae and CEMs: 1) proteins that bind cholesterol, such as caveolin; 2) a caveolin binding motif-CBM-\((\Phi\Phi\Phi\Phi\Phi)\), where \(\Phi\) are aromatic amino acids (Tryptophan, Phenylalanine, or Tyrosine); 3) post-translational modification involving the addition of lipid groups, such as N-linked myristoylation or palmitoylation (Hiol et al., 2003; Huang et al., 1997; Song et al., 1997); and 4) strong hydrophobic residues that cause preferential co-localization with glycosphingolipids and cholesterol (Foster et al., 2003); 5) glycosylphosphatidylinositol (GPI)–anchored proteins.

A CBM has been identified for multiple RTKs, including the IGF-IR, insulin receptor (IR), EGFR, and Trk (Couet et al., 1997b; Nystrom et al., 1999), and is found within their highly conserved tyrosine kinase domain. The binding of the N-terminal region of caveolin to the EGFR kinase domain inhibits autophosphorylation of this RTK (Couet et al., 1997b). Moreover, IRs with a disruption in the CBM do not undergo autophosphorylation (Nystrom et al., 1999). This mutation is clinically relevant as patients with severe forms of insulin resistance have two distinct point mutations in their IR caveolin binding motif, W1193L and W1200S, which result in impaired autophosphorylation (Imamura et al., 1994; Iwanishi et al., 1993; Moller et al., 1991).
Accumulating data suggest a role for CEMs in the regulation of signal transduction (Nabi and Le, 2003). Various signaling proteins, including heterotrimeric G proteins, RTKs, Src family kinases, and calmodulin-binding proteins have been extracted localized within CEMs (Lisanti et al., 1994a; Maekawa et al., 1997; Masserini et al., 1999). Furthermore, the cholesterol-depleting agent methyl-β-cyclodextrin (MβCD), flattens caveolae, dissociates CEM-associated proteins, and alters signal transduction pathways (Buk et al., 2004; Holleran et al., 2003). Proteomic analysis of CEMs reveals the identification of 241 authentic “rafting” proteins, including multiple kinases, phosphatases, and heterotrimeric G proteins (Foster et al., 2003). Interestingly, this proteomic study demonstrated that these signaling molecules are enriched by greater than 10 fold within these regions of the plasma membrane (Foster et al., 2003). The concentration of effector molecules within CEMs permits the sharing of limited proteins, overcoming issues of stoichiometry. This is particularly the case for GPCRs that are in abundance compared to their downstream effector adenylyl cyclase (Insel et al., 2005). That signal transduction pathways are rapidly activated with high-fidelity and that signaling proteins and receptors have been localized to CEMs, lends support to the hypothesis that these microdomains are platforms for signal transduction (Pierchala et al., 2006).

Several studies report that caveolae inhibit RTK-mediated signaling pathways. The EGFR translocates out of caveolae upon ligand binding (Couet et al., 1997b). In addition, caveolae negatively regulate IR signaling in undifferentiated pre-adipocytes but positively regulate IR signaling in differentiated adipocytes (Nystrom et al., 1999).
Therefore, the role of caveolae in the regulation of RTK-mediated signaling is dependent upon the specific RTK, as well as the stage of cellular development.

Relevant to this dissertation, CEMs are involved in OL biology. In OLs, CEMs play an important role in cell shape through interactions with actin-binding proteins at the onset of myelination (Taguchi et al., 2005). The GPI-anchored cell adhesion molecules, NCAM and F3, which regulate axonal-glial interactions and myelination, increasingly associate with CEMs during OL differentiation. In addition, the association of Src kinase, Fyn, with NCAM and F3 in CEMs is requisite for Fyn kinase activity (Kramer et al., 1999) and essential for myelination (Umemori et al., 1994). These data suggest that Fyn activity within these microdomains may contribute to normal myelination in the CNS. CEMs have been also shown to regulate signaling within OLs through the co-localization of the PDGFα receptor and αβ1 integrins, which enhance PDGF-mediated Akt phosphorylation and OL survival (Decker and ffrench-Constant, 2004).

Myelin is concentrated with CEMs since these membranes contain ~70% of brain cholesterol (Dietschy and Turley, 2001) and are enriched with glycosphingolipids, including galactosylceramides (GalCer) and sulfatides (Marcus and Popko, 2002). Myelin-specific proteins, including MBP and PLP, increasingly localize within CEMs during OL development (DeBruin et al., 2005). Furthermore, CEMs are hypothesized to be a vehicle for the delivery of proteins to myelin (Lee, 2001) and provide structural organization for protein and lipid organization within these specialized membranes (Kramer et al., 2001). Mice, genetically altered to prevent cholesterol biosynthesis under the OL specific promoter, cyclic- 2’, 3’ nucleotide phosphodiesterase (CNPase), result in hypomyelination, ataxia, and tremors (Saher et al., 2005). In these animals, however,
OLs appear morphologically normal and obtain minimal lipids and cholesterol from neighboring cells. Taken together, these data suggest that CEMs may play an important role in OL biology and myelination.

**Statement of Thesis**

IGF-I is required for the survival and maintenance of oligodendrocytes (OLs) and their progenitors (OPs), in vitro and for normal development in vivo. The actions of IGF-I are primarily mediated through its cognate receptor tyrosine kinase (RTK), the IGF-IR, via sustained activity of the PI-3K/Akt signaling pathway. Few laboratories have reported the sustained activity of signal transduction for the IGF-IR or other RTKs; fewer have addressed the mechanism by which sustained signal transduction occurs. Accordingly, it is of great interest to delineate the mechanism by which IGF-I mediates sustained Akt phosphorylation.

The temporal and spatial regulation of signal transduction has been well established to depend upon the subcellular localization and trafficking of RTKs. RTKs localized at the plasma membrane, when compared to internalized RTKs, regulate distinct signal transduction cascades and biological outcomes. Furthermore, cholesterol/glycosphingolipid-enriched membrane microdomains (CEMs) have been implicated in the regulation of signal transduction as these microdomains compartmentalize various RTK, as well as their downstream signaling partners, at the plasma membrane. Disruption of CEMs by cholesterol depletion results in changes in RTK-mediated signal transduction, as well as alterations in downstream biological
outcomes. In OLs the organization of proteins within CEMs is also important for the production of myelin membranes and myelination.

Based on the evidence from the literature, as outlined in this chapter, the goal of this thesis is to delineate the mechanisms by which IGF-I sustains the phosphorylation of Akt in OPs. The central hypotheses of this thesis are: (1) IGF-IR internalization and recycling are required for sustained phosphorylation of Akt, (2) CEM integrity is required for IGF-I mediated Akt phosphorylation, and (3) caveolae/CEMs are important for IGF-I signaling and OL biology. Toward these ends, the central hypotheses are tested in the following chapters:

• In chapter one, we test the role of IGF-IR internalization and recycling in sustained Akt phosphorylation.

• In chapter two, we test the role of cholesterol-membrane integrity and CEMs in IGF-I mediated Akt phosphorylation.

• In chapter three, we investigate the expression of caveolin-1 and its role in the OL lineage, as well as the role of IGF-I in regulating caveolin-1 localization.
Figure 1.1 Oligodendrocyte lineage progression. The lineage progression of OLs is controlled by multiple trophic factors in vitro. FGF-2 promotes proliferation and partial differentiation of progenitors to late progenitors. PDGF, in combination with FGF-2, promotes proliferation of early progenitors. IGF-I synergizes with FGF-2 to promote cell cycle entry. Differentiation is promoted by thyroid hormone (T3) and is associated with decreases in proliferation and migration but increases in process branching and the expression of myelin basic protein (MBP) and proteolipid protein (PLP).
Figure 1.2 The IGF type-I Receptor and PI-3K/Akt Signaling Pathway. The IGF-IR consists of two alpha extracellular subunits and two beta transmembrane subunits, connected by multiple disulfide bridges. Cysteine rich regions within the alpha subunit are required for ligand binding. Autophosphorylation, subsequent to ligand binding, occurs within the tyrosine kinase domain of the beta subunit at multiple tyrosine residues, and results in the recruitment of adaptor proteins (IRS-1/2) and the phosphorylation and activity of PI-3K, PDK1, and Akt (PKB). Akt mediates the phosphorylation and consequent inhibition of its downstream substrates, promoting cell survival.
Figure 1.3 Endosomal Trafficking. The schematic diagram illustrates the differential fate of internalized receptor tyrosine kinases (RTKs). Targeting of RTKs to the lysosome or to the plasma membrane is regulated by the Rab family of small GTPases. Invagination and cleavage from the plasma membrane yields an initial sorting endosome (SE), also termed the early endosome, which is positive for early endosomal antigen (EEA)1 and is associated with Rab5. Formation of the late endosome (LE), which ultimately targets proteins for lysosomal degradation, is regulated by Rab7 and 9. Alternatively, the formation of the endosomal recycling compartment (ERC) involves association with Rab11. The transferrin receptor (TfR) is a marker for the recycling compartment as it is constitutively recycled to the plasma membrane. The glucose transporter (GLUT1) is a marker for the plasma membrane since, at steady-state- the majority of GLUT1 protein is localized at the cell surface.
Figure 1.4 The Dynamic Structure of Lipid Rafts. (A) non-caveolae (left) and caveolae (right) raft. (B) The ability to reside within lipid raft microdomains is dependent on the property of the individual protein. Transmembrane domains with extremely hydrophobic residues, cholesterol binding proteins, or post-translational modifications, including myristoylation or palmitoylation (A), as well as interactions with GPI-anchored proteins (B), and association with raft-interacting proteins (C) mediate the localization within lipid raft microdomains. Proteins that do not fit these criteria will likely reside outside of lipid rafts (D).
Table 1.1 Endosome Associated Proteins

<table>
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<tr>
<th>Early Endosome/Sorting Endosome</th>
<th>Endosomal Recycling Compartment</th>
<th>Late Endosome/Multi-Vesicular Bodies</th>
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<tr>
<td>Rab5</td>
<td>Rab11</td>
<td>Rab7</td>
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<td>EEA1</td>
<td>Transferrin/Transferrin Receptor</td>
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<td>Mannose-6-Phosphate Receptor</td>
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CHAPTER TWO

Materials and Methods
**Materials**

Cell culture medium (MEM, DMEMF-12) and fetal bovine serum (FBS) were purchased from Gibco-BRL (Long Island, NY). Additional cell culture media supplements were purchased from Sigma (St. Louis, MO). Recombinant human IGF-I and biotin-conjugated IGF-I were purchased from Upstate Biochemicals (Lake Placid, NY). Antibodies to Akt, phospho (Ser473)-Akt, phospho(Ser9)-GSK3β were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to Cyclin D1, EEA1, and IGF type-I Receptor β and α subunits were purchased from Santa Cruz (Santa Cruz, CA). The β-Actin antibody, streptavidin-conjugated TRITC antibody, and goat-anti rabbit and mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson Labs (West Grove, PA). Antibodies to Rab11, flotillin-1, and total GSK-3β were purchased from Transduction Laboratories (San Diego, CA). The Antibody to phospho-specific IGF-IR was purchased from Biosource (Camarillo, CA). The glucose transporter (GLUT) 1 antibody was a generous gift from Dr. Ian Simpson (Penn State College of Medicine). Sulfo-NHS-Biotin and Immunopure Streptavidin were purchased from Pierce. Chemical inhibitors including, dansylcadaverine, monensin, and cycloheximide were purchased from Sigma (St. Louis, MO). Polyallomer ultracentrifuge tubes were purchased from Beckman Instruments (Palo Alto, CA). Dynabeads M-500 and the antibody to the transferrin receptor were purchased from Invitrogen (Carlsbad, CA).
Preparation of Primary Oligodendrocyte Progenitor Cultures

Oligodendrocyte progenitor cells were harvested from P0-P2 day Sprague Dawley rats (Charles River Laboratories) as previously described (Levison and McCarthy, 1991). Cortices were dissected and menninges were surgically removed in PBS-glucose. Cortices were enzymatically dissociated with 2.5% trypsin and DNaseI (Sigma) and mechanically dissociated with trituration. Cells were passed through a 15 micron filter and centrifuged at 800 rpm to achieve a mixed glial cell pellet. Cells were resuspended in MEM-C media and passed through a 40 micron filter. Mixed glia were seeded onto T75 flasks at a density of 1.5 x 10^7 cells/flask. Cells spontaneously form a mono-layer of astrocytes upon which oligodendrocytes and microglia attach. Mixed glia were grown for 12 days. Afterwards, cells were shaken for 1.5 hours to remove most of the microglial. Media was replaced and cells were shaken overnight for 18.5 hours to remove oligodendrocyte progenitors. Media from each T75 flask was passed through a 130 micron filter. Cells were centrifuged, resuspended in N2B2, and seeded onto 100 mm petri dishes to remove residual microglia. Remaining cells in media were centrifuged and resuspended in N2S and seeded in T75 flasks. Oligodendrocyte progenitors were split after two days of amplification and then seeded for experimental conditions at a density of 2.5 x10^4/cm^2.
**Cell Culture and Treatment Conditions**

The CG-4 oligodendrocyte progenitor cell line was a generous gift from Dr. Lynn Hudson (NIH). CG-4 and primary cells were maintained in chemically defined N2S media composed of: (1) 66% N2B2 media [DMEM/F-12 supplemented with 0.6mg/ml BSA, 10ng/ml d-biotin, 20 nM progesterone, 100 μM putrescine, 5 ng/ml selenium, 50 μg/ml apo-transferrin, 100 U/ml penicillin, and 100 μg/ml streptomycin, (2) 34% B104 conditioning medium, (3) 5 ng/ml FGF-2, and (4) 0.5% fetal bovine serum. CG-4 cells were plated on poly-d-lysine coated dishes at a density of 2.0 x 10^4 cells/cm^2 in N2S media. Prior to all experiments cells were serum starved in N1-A (N2B2 media without insulin) for 2 hours and subsequently treated with 10 ng/ml IGF-I with and without pharmacological inhibitors at the following concentrations: 100 μM dansylcadaverine, 10 μM monensin, 10 μg/ml cycloheximide. For all inhibitor groups, cells were pre-treated for 15 minutes.

Primary OP cultures were differentiated with N2B2 media supplemented with 1% serum and thyroid hormone. Cells were harvested for analysis at 24, 48, 72, and 96 hours.

**Western Blot Analysis**

Following treatments, CG-4 or primary progenitor cells were washed in ice-cold PBS and total cell lysates were isolated in SDS sample buffer (62.5 mM Tris-HCl, 2% SDS, 10 % glycerol, 50 mM DTT) containing 1:100 protease inhibitor cocktail, 1 mM sodium orthovanadate, and 1 mM sodium fluoride (Sigma). Lysates were briefly sonicated on ice and quantified by the RC/DC protein assay (BioRad Laboratories, Hercules, CA).
Approximately 20 µg of protein from each sample was boiled for 5 minutes, cooled, and separated by SDS-polyachrylamide gel electrophoresis (PAGE) on 4-12% mini gels (Invitrogen; Carlsbad, CA) and subsequently transferred to nitrocellulose membranes. Membranes were blocked in 5% milk in TBS-0.05% or 0.1% Tween for 1 hour and incubated with primary antibodies overnight (1:1000; except cyclin D1, which was incubated at 1:250), at 4º C in blocking buffer with gentle rocking. Appropriate goat anti-rabbit or goat anti-mouse HRP-conjugated secondary antibodies were used at a dilution of 1:5000 in blocking buffer for 1 hour at room temperature. The detection of HRP-conjugated secondary antibodies was performed with enhanced chemiluminescence (ECL; New England Nuclear, Boston, MA). Membranes were exposed on film (Kodak) and digitally captured and quantified by NIH Image 1.62.

**Cell Surface Biotinylation Assay**

After IGF-I treatments (+/- pharmacological inhibitors), cells were rinsed with PBS (pH 7.4) and incubated with 0.3 mg/ml of sulfo-NHS-biotin for 60 minutes at 4º C with end-over rocking. Reactions were quenched with 100 mM glycine for 10 minutes to remove unreacted biotin. Cells were then scraped in ice-cold PBS (plus protease inhibitors) and the volume of each sample was adjusted to 500 µL. Triton X-100 was added to a final concentration of 1% (v/v). Cells were passed through a 23 gauge needle 10 times and incubated on ice for 30 minutes with constant rocking. Lysates were briefly sonicated and an aliquot was taken for protein normalization. Samples were next incubated with Immunopure streptavidin for 1 hour at 4º C with end-over rocking. Streptavidin-precipitated complexes were rinsed three times with PBS+ 1% Triton X-100 and once
with PBS. After rinsing, SDS buffer (+ DTT) was added and samples were boiled for 5 minutes to elute biotinylated proteins. Protein samples were separated by SDS-PAGE and subjected to Western blot analysis (as described above) for surface IGF-IRβ subunit immunoreactivity. In parallel, control experiments were performed in which cells were untreated with sulfo-NHS-biotin. Protein lysates from untreated cells were then subjected to the streptavidin-pull down (as described above) to assess non-specific binding.

**Immunocytochemistry and Confocal Microscopy**

CG-4 progenitors were seeded onto poly-d-lysine/laminin coated cover slips at a density of 2x10^4/cm^2 in 24 well plates. Cells were serum starved in basal media and subsequently treated with 10 ng/ml biotin-labeled IGF-I (IGF-I_b) for 2 minutes. After treatments, cells were rinsed in PBS, fixed in 2% paraformaldehyde for 10 minutes, and blocked in diluent (10% newborn calf serum, 10% lamb serum in PBS) for 15 minutes. Cells were incubated with streptavidin-conjugated TRITC (1:200) and DAPI (1:1000) for 1 hour at room temperature, and thoroughly rinsed with PBS. Confocal Imaging was performed at the JDRF Imaging Core Facility (Penn State College of Medicine) with a Leica TCS SP2 AOBS microscope and software (Heidelberg, Germany). A 63X objective was used to capture images. TRITC Immunofluorescence was detected at a wavelength of 514nm with an Ar/Kr laser.
Subcellular Fractionation

Subcellular fractionation of endosomal vesicles was performed as previously described (Melikian and Buckley, 1999) with slight modification. Briefly, four 100 mm dishes were combined and resuspended in 0.5 ml of homogenization buffer [150 mm NaCl, 10 mM HEPES, 1 mM EDTA, 0.1 mM MgCl$_2$, 250 mM Sucrose]. Cells were homogenized and centrifuged to separate nuclei (800 x g for 10 minutes) and subsequently centrifuged to separate large membranes (10,000 x g for 10 minutes). Clarified cell homogenates were loaded onto a linear 10-50% sucrose gradient (4.5 mls) preformed in polyallomer centrifuge tubes (Beckman Instruments). Samples were fractionated by centrifugation in a SW55Ti rotor at 48,000 rpm for 16 hours at 4° C. Eight (0.6 ml) fractions were taken from the top of each gradient and were ethanol precipitated overnight at -20° C. Protein was resuspended in 20 μl of 3X SDS buffer, boiled, and loaded onto 4-12% gels for PAGE and Western Blot analysis.

Organelle Immunoisolation

Cells were homogenized (as described above) and subjected to immunoisolation with magnetic Dynabeads (Invitrogen) according to the manufacture’s protocol. Briefly, magnetic beads were coated with a linker goat anti-mouse IgG and subsequently conjugated with a mouse anti-human TfR antibody. Crude cell homogenates were pre-incubated with mouse IgG linker antibody to eliminate non-specific binding. Afterwards, cleared homogenates were incubated with TfR antibody conjugated magnetic beads for 2 hours at 4° C with end-over rocking. Beads were washed thoroughly and protein was eluted with SDS buffer.
Cholesterol-enriched Membrane Microdomain Isolation

Detergent Extraction: OPs from three 100 mm dishes were collected. Cells were resuspended in TNE homogenization buffer [25 mM Tris, 150 mM NaCl, 5 mM EDTA] and lysed by passage through a 23 gauge needle fixed to a plastic syringe 10 times. Nuclei were pelleted at 800 x g for 10 minutes and 10% Triton X-100 was added to the post-nuclear supernatants (PNS) to a final concentration of 1%. The PNS was centrifuged at 17,000 x g for 20 minutes to separate detergent resistant membranes (DRMs). Supernatants were removed and DRMs were resuspended in 0.5 ml of TNE buffer + 1% Triton X-100. Samples were mixed with 1 ml of 2 M (~68.5% w/v) sucrose and loaded into the bottom of a polyallomer ultracentrifuge tube. Samples were overlaid with 2 ml of 1 M (~35% w/v) sucrose and 1.5 ml of 0.2 M (~7% w/v) sucrose. Sucrose gradients were fractionated by centrifugation in a SW55Ti at 45,000 rpm for 16.5 hours. Fractions (0.5 ml) were taken from the top of each gradient and were ethanol precipitated overnight at -20° C. Protein was resuspended in SDS buffer and loaded onto 4-12% gels for PAGE and Western blot analysis.

Sodium Carbonate Extraction: Alternatively, cholesterol-enriched membrane microdomains were isolated using a detergent free method as previously described by Song et al. (1996) with few modifications. Briefly, cells were scraped in 500 mM sodium carbonate buffer (pH 11) and lysed by passage through a 23 gauge needle fixed to a syringe. Lysates were sonicated 3 times for 15 seconds. Subsequently, lysates were brought to a final volume of 1 ml and mixed with 80% sucrose in MES-buffered saline (MBS: 25 mM MES, pH 6.5; 150 mM NaCl, 1 mM EDTA; 250 mM sodium carbonate).
to yield a final sucrose concentration of 45% (1.67 mls). The mixture was placed at the bottom of a polyallomer centrifuge tube and carefully layered with 1.67 ml of 35% sucrose and 1.67 ml of 5% sucrose. Gradient was fractionated in a SW55Ti rotor for 16 hours at 45,000 rpm at 4° C. Ten fractions (0.5 ml) were taken from the top of the sucrose gradient and were processed for SDS-PAGE and Western blot analysis.

**Cholesterol Depletion and Replacement**

Primary OL progenitor cultures were treated with 5 mM MβCD for 30 minutes at 37° C prior to trophic factor stimulation. Cells were rinsed twice with basal media and were subsequently treated with 10 ng/ml IGF-I for 30 minutes. Cholesterol was replaced as described by (Klein et al., 1995). Briefly, 6 mg of cholesterol (Sigma) in 0.2 mls of isopropyl alcohol was added to 100 mg/ml MβCD at 80° C, yielding a 6.8 mM cholesterol solution. Cholesterol-complexes were diluted into treatment media to a final concentration of 0.2 mM and were incubated for 30 minutes at 37° C.

**Isolation of Protein from Murine Brain**

Caveolin heterozygous mice from Jackson Laboratory (Bar Harbor, MA) were bred and genotyped by the JDRF animal core facility at the Penn State College of Medicine and were a generous gift from Dr. Mark Kester. Caveolin-1 null and wild type mouse brains were isolated from 14-17 week old animals. Frozen brains were pulverized and resuspended in 1X SDS buffer in phosphate-buffered saline (pH 7.0). Samples were briefly sonicated and centrifuged at 10,000 x g for 10 minutes at 4° C to clear debris.
Lysates were processed for SDS-PAGE and Western blot analysis (as previously described).

**Statistical Analyses**

Analyses were performed using Statview statistical software program. For all experiments, one way ANOVA followed by a Fisher’s posthoc test was used to assess the statistical significance between treatment groups. All experiments were performed with an n of 3 and repeated at least three times.
**Figure 2.1 Sucrose Refractometry.** 10% to 50% linear sucrose gradients were made by layering 1.125 ml of 50% with equal volumes of 40%, 30% and 10% sucrose in homogenization buffer as described in materials and methods. Sucrose gradients were incubated overnight at 4° C. Fractions (0.5 ml) were taken from the top of the gradient and density was determined with a refractometer. Fraction (x-axis) versus % sucrose (y-axis) was plotted and $R^2$ value was determined (0.9882).
CHAPTER THREE

IGF type-I Receptor Internalization and Recycling mediates Sustained Akt Phosphorylation
Introduction

Receptor tyrosine kinases (RTKs) mediate the activity of various signal transduction pathways, which instruct a cell to survive, proliferate, migrate, or differentiate. Classically, the activity of these signaling pathways is transient since RTKs often are internalized, followed by ligand/receptor dissociation and degradation. This paradigm of transient RTK signaling leading to ligand and receptor degradation has been well studied, particularly for the epidermal growth factor receptor (EGFR) and is thought to be a major mechanism for the termination of signal transduction (Levkowitz et al., 1998).

In contrast to transient signal transduction, less attention has been given to RTKs and mechanisms that mediate the long-term activation of signal transduction. We previously reported that stimulation of the insulin-like growth factor type-I receptor (IGF-IR), mediates sustained activation of the phosphatidylinositol-3 Kinase (PI-3K)/Akt pathway in oligodendrocyte progenitors (Ness et al., 2002; Ness et al., 2004; Ness and Wood, 2002). We further demonstrated that sustained Akt phosphorylation, and consequent activity, is essential for long-term survival and IGF-I mediated protection of these cells from excitotoxicity (Ness and Wood, 2002; Ness et al., 2004). In contrast, neurotrophin (NT)-3 promotes only short-term survival of OPs, which correlated with a transient Akt phosphorylation and a down-regulation of the TrkC receptor (Knusel et al., 1997; Ness and Wood, 2002); however, we observed sustained phosphorylation and stability of the IGF-IR when continuously stimulated by ligand (Ness and Wood, 2002).

IGF-I/IGF-IR signaling is important for the survival and maintenance of multiple cell types within the central nervous system (D'Ercole et al., 1996). Loss of IGF-I results
in a decrease in the number of neurons and oligodendrocytes (Beck et al., 1995; Cheng et al., 1998; Ye et al., 2002a), whereas overexpression of IGF-I results in brain overgrowth and hypermyelination (D'Ercole et al., 2002; Popken et al., 2004; Ye et al., 1995b; Ye et al., 1995c; Ye et al., 2004; Ye et al., 1996). Similarly, severe neurological deficits have been reported in embryonic brains of IGF-IR null mice, which invariably die at birth (Liu et al., 1993). While the downstream properties of IGF/IGF-IR signaling have been well studied, less is known about the subcellular trafficking of the IGF-IR and its regulation of signaling pathway activation in neural cells. Few studies have reported long-term receptor stability and downstream activity for the IGF-IR or other RTKs (Allen et al., 2005; Cui et al., 2005). Thus, it is of considerable interest to understand the mechanisms by which the IGF-IR maintains the phosphorylation of Akt and promotes long-term survival of progenitor cells.

The temporal and spatial regulation of signal transduction has been attributed to the subcellular localization of receptors and their downstream effectors (Haugh, 2002). That signal transduction occurs rapidly and with high fidelity lends support to the hypothesis that receptors and signaling molecules are organized into specific subcellular compartments. It has been well established that surface-localized RTKs activate distinct signaling pathways compared to internalized, endosome-associated RTKs, and lead to specific biological responses. (Jullien et al., 2002). Chow and colleagues demonstrated that blocking internalization of the IGF-IR inhibits ERK activation without affecting IGF-I-mediated Akt activation (Chow et al., 1998). Likewise, blocking EGFR internalization results in a decrease in ERK activation, but an increase in PKC activation (Vieira et al., 1996). Furthermore, nerve-growth factor (NGF)-mediated survival of
PC12 cells is associated with surface localized TrkA via the activation of the PI-3K/Akt pathway, whereas internalized, endosome-associated TrkA induces NGF-mediated differentiation of these cells via the ERK pathway (Zhang et al., 2000b). Together, these studies suggest that the activation of specific signal transduction pathways and consequent biological responses are mediated through the subcellular compartmentalization of RTKs.

In the present study, we combine empirical and mathematical analyses to investigate how IGF-IR trafficking promotes and sustains phosphorylation of Akt in central glial (CG)-4 progenitor cells. We test the hypothesis that internalization and recycling of the IGF-IR is required to sustain Akt phosphorylation. Our results show that clustering and internalization of the IGF-IR promotes Akt phosphorylation; however, recycling, but not de novo synthesis, is necessary to sustain this phosphorylation. Furthermore, IGF-IRs co-localize with established markers for the endosomal recycling compartment (ERC), including Rab11 and the transferrin receptor (TfR). Mathematical analyses of these data predict a model of IGF-IR internalization and recycling that recapitulates our empirical data. Interestingly, the model reveals an additional kinetic state of the receptor, not elucidated by our empirical studies.
Results

IGF-I is required for sustained IGF-IR and Akt phosphorylation

Previously, we demonstrated that IGF-I promotes the survival and long-term protection of primary rat OPs from glutamate-mediated toxicity via sustained phosphorylation of Akt. The pro-survival effects of IGF-I correlated with prolonged IGF-IR activity and stability (Ness et al., 2002; Ness et al., 2004; Ness and Wood, 2002). In the present study, we utilized the CG-4 cell line of rat OPs to better understand the mechanisms by which the IGF-IR mediates sustained Akt phosphorylation (Ranjan and Hudson, 1996). To verify that IGF-I activates its receptor similarly in primary and CG-4 progenitors, we treated CG-4 cells with IGF-I through 24 hours. Similar to our previous results in primary OPs, the IGF-IRβ subunit was phosphorylated in IGF-I treated cells through 24 hours compared to t0 and untreated control cells (P = 0.01; Figure 3.1A, B), without changes in total IGF-IR protein levels (Figure 3.1 A, C).

To determine if IGF-I sustains Akt phosphorylation in CG-4 progenitors, and to determine further whether the presence of IGF-I is required for the sustained phosphorylation of Akt, cells were stimulated with IGF-I through 24 hours or for 30 min and then incubated in basal media without IGF-I through 24 hours. In the continual presence of IGF-I, Akt phosphorylation was sustained through 24 hours (P < 0.009; Figure 3.2A, B), consistent with our previous results in primary OPs (Ness et al., 2002). The removal of IGF-I after 30 min of stimulation resulted in decreased phosphorylation of Akt to control (t0) levels within 30 min (Figure 3.2A, B). Similarly, the phosphorylation of GSK-3β, a direct downstream substrate of Akt, was sustained also through 24 hours in the presence of IGF-I (P < 0.001) and was markedly decreased after
IGF-I removal (P < 0.03; Figure 3.2A, C). In comparison, the dephosphorylation of GSK-3β occurred at a slower rate than Akt dephosphorylation (compare Figure 3.2B and 3.2C). Together, these experiments demonstrate that the kinetics of IGF-I stimulation of its receptor and downstream targets are similar in CG-4 and primary OPs. Accordingly, we utilized the CG-4 progenitor cell line in the subsequent experiments to elucidate the mechanisms of IGF-IR mediated sustained Akt phosphorylation.

**IGF-I stimulation alters the surface availability of the IGF-IR**

Whereas total levels of IGF-IR protein remained unchanged during IGF-I treatment in these and our previous studies (Ness and Wood, 2002; Ness et al., 2002), we were interested in determining whether surface localization of the IGF-IR was altered by IGF-I stimulation. Experiments were designed to measure IGF-IR availability at the cell surface following IGF-I stimulation using a biotinylation assay. Progenitor cells were serum starved for 120 min and then treated with IGF-I or basal media (N1A), free of serum and growth factors, from 0 to 120 min, and surface IGF-IRs were quantified following cell surface biotinylation and streptavidin pull-down. The IGF-IR was constitutively present at the cell surface in control cells in N1A media through 60 min; however, prolonged absence of IGF-I (4 hrs) induced an approximate 80% increase in surface IGF-IR expression. (P<0.005; Figure 3.3A, B). In contrast, IGF-I stimulation decreased IGF-IR surface availability by 32% after 15 minutes (P < 0.04; Figure 3.3A, B) and 86 % after 30 min (P = 0.006; Figure 3.3A, B). The decrease in surface IGF-IR was followed by recovery to 36% after 60 minutes (P < 0.002; Figure 3.3A, B) and 90% after 120 minutes (Figure 3.3A, B). Cells treated with IGF-I, without prior NHS-sulfo biotin
incubation, were negative for surface IGF-IRβ subunit immunoreactivity (data not shown). Together, these data suggest that IGF-I mediates IGF-IR internalization and subsequent recovery at the cell surface.

**Dansylcadaverine blocks IGF-IR internalization and Akt phosphorylation**

To examine the relationship between Akt phosphorylation and surface availability and internalization of the IGF-IR, we used a pharmacological inhibitor of receptor internalization, dansylcadaverine, which was previously used to block IGF-IR internalization in CHO cells (Chow et al., 1998) and in mouse embryo fibroblasts (Vecchione et al., 2003). CG-4 progenitors were treated with IGF-I with or without dansylcadaverine for 30 min, the time when we observed maximal loss of surface IGF-IR following IGF-I stimulation (see Figure 3.3). As in previous experiments, IGF-IR surface availability was markedly decreased after 30 min of IGF-I stimulation (P = 0.03; Figure 3.4A, B). In the presence of dansylcadaverine, however, receptor surface availability remained at the level of the untreated control cells (Figure 3.4A, B). IGF-IR phosphorylation was unchanged in cells treated with IGF-I plus dansylcadaverine compared with cells treated alone with IGF-I (P < 0.005; Figure 3.4A, C).

To determine whether dansylcadaverine altered IGF-IR mediated Akt phosphorylation, progenitors were treated with IGF-I plus or minus dansylcadaverine for 5, 20, and 30 min. The presence of dansylcadaverine decreased Akt phosphorylation compared to cells treated with IGF-I alone at all time points (P < 0.003; Figure 3.4D, E). These experiments demonstrate that dansylcadaverine blocks internalization of the IGF-IR, which is consistent with previously published studies (Chow et al., 1998; Vecchione...
et al., 2003). Additionally, dansylcadaverine blocks IGF-I mediated Akt phosphorylation, without affecting IGF-I-mediated phosphorylation of the IGF-IR.

**Dansylcadaverine blocks biotin-conjugated IGF-I clustering**

Previous studies demonstrated that dansylcadaverine blocks receptor internalization by inhibiting receptor clustering, a step preceding receptor-mediated endocytosis (Haigler et al., 1980). Specifically, dansylcadaverine blocked clustering of rhodamine-conjugated alpha2-macroglobulin at the surface of normal rat kidney cells (Levitzki et al., 1980). Thus, we investigated whether the inhibition of IGF-IR internalization, mediated by dansylcadaverine, also disrupted clustering of the IGF-IR at the plasma membrane in IGF-I-treated cells. CG-4 progenitors were stimulated with biotin-conjugated IGF-I (IGF-I$^b$) for 2 min plus or minus dansylcadaverine. IGF-I$^b$, bound to the IGF-IR, was labeled with streptavidin-conjugated TRITC and visualized by confocal microscopy. Untreated cells showed relatively low immunofluorescence (Figure 3.5A, D), whereas cells treated with IGF-I$^b$ for 2 min showed strong punctate immunofluorescence staining (Figure 3.5B, E). In contrast, cells co-treated with dansylcadaverine and IGF-I$^b$ had a diffuse staining pattern (Figure 3.5C, F). The dose of IGF-I used for these experiments (10 ng/ml = 1.3 nM) is slightly below the $K_d$ of the IGF-IR (1.6 nM) and well below the $K_d$ of the insulin receptor (> 30 nM). Accordingly, the binding of IGF-I$^b$ to the surface of these cells is mediated through the IGF-IR. These data suggest that dansylcadaverine blocks IGF-I mediated clustering of the IGF-IR.
Recycling is required for IGF-IR surface recovery and sustained Akt phosphorylation

Our biotinylation data showed that IGF-IRs recover at the cell surface after 120 min of IGF-I stimulation (see Figure 3.2); although, it is unknown if these receptors originate from a recycling or from a de novo pool of IGF-IRs. To investigate the role of recycling in surface recovery of the IGF-IR and sustained phosphorylation of Akt, we utilized the pharmacological inhibitor monensin, which blocks acidification of endosomal vesicles and consequently prevents trafficking to the lysosome and the recycling endosome (Yamashiro and Maxfield, 1984). Previously, monensin was shown to block IGF-IR and IR endosomal trafficking (Dore et al., 1997). Using the surface biotinylation assay, we again observed loss and recovery of surface IGF-IR at 30 min (P<0.02) and 120 min of IGF-I treatment, respectively (Figure 3.6A, B). The presence of monensin prevented, however, receptor recovery at 120 min (P < 0.03; Figure 3.6A, B). To determine the effects of monensin on Akt phosphorylation, progenitors were treated with IGF-I plus or minus monensin for 15 and 120 min. Monensin significantly inhibited IGF-I mediated Akt phosphorylation compared to cells treated alone with IGF-I for 120 min (P=0.0003; Figure 3.6C, D). In contrast, there was no change in Akt phosphorylation at 15 min of IGF-I stimulation plus or minus monensin (Figure 3.6C, D).

The previous data suggest that IGF-IR recycling contributes to recovery of the receptor at the cell surface after IGF-I stimulation; however, it does not preclude an additional contribution from synthesis of new receptors. Total IGF-IR levels appear stable through 24 hours (see Figure 1); however, this can be due to steady-state kinetics of receptor synthesis and degradation. To examine if de novo synthesis of the IGF-IR
contributes to the IGF-I mediated phosphorylation of Akt, cells were treated with IGF-I plus or minus cycloheximide to block protein synthesis. Cycloheximide had no effect on IGF-I-induced phosphorylation of Akt (P < 0.0001; Figure 3.6E, F) or on total IGF-IR protein levels (Figure 3.6E, H) compared to cells treated with IGF-I alone for 120 min. To confirm that cycloheximide was used at an effective dose during these experiments, we investigated cyclin D1 expression levels after treatment with this pharmacological inhibitor. Cycloheximide decreased cyclin D1 protein levels in the same cells (P < 0.0006; Figure 3.6E, G).

The IGF-IR co-localizes with markers for the endosomal recycling compartment

To determine further if IGF-IRs follow a recycling pathway, we analyzed whether the IGF-IR co-localizes with markers of the endosomal recycling compartment (ERC). Progenitor cell homogenates were fractionated on a 10-50% linear sucrose gradient and subsequently were analyzed for markers of distinct endosomal vesicles as described in Materials and Methods. Early endosome antigen (EEA) 1, a marker for the early endosome, and glucose transporter (GLUT) 1, a marker for the plasma membrane (Czech et al., 1993), localized predominantly in fraction #3 (Figure 3.7A, B). The transferrin receptor (TfR), which at steady-state is predominantly localized to recycling endosomes (Touret et al., 2003) was distributed in fractions #2-#6 (Figure 3.7A, C). The distribution of the IGF-IR was similar to that of the TfR, localizing in fractions #3-#5 (Figure 3.7A, C).
To determine if there are specific interactions between the IGF-IR and the TfR, crude cell homogenates were incubated with Dynal magnetic beads coated with mouse IgG plus or minus TfR antibody. The IGF-IR co-precipitated with TfR only in lysates that were incubated with TfR antibody (Figure 3.8). Moreover, lysates incubated with TfR antibody were also positive for the small GTPase Rab11 (Pasqualato et al., 2004), which is involved in the formation of the ERC (Figure 3.8). Cell lysates incubated with only mouse IgG coated magnetic beads were negative for IGF-IR, TfR, and Rab11 (Figure 3.8). Together, these data suggest that IGF-IRs co-localize with TfR and Rab11, markers for the ERC, and likely follow a recycling pathway.

A four-state mathematical model for IGF-IR trafficking is inconsistent with empirical data

Based on our data from the present study, we propose that the surface loss and recovery of IGF-IR in OPs is due to receptor internalization followed by receptor recycling. Our data support the hypothesis that receptor recycling is essential for the ability of IGF-I to sustain Akt phosphorylation and promote long-term survival of OPs (see Figure 3.6). Moreover, the IGF-IR co-localizes with markers of the ERC, including the TfR and Rab11 (see Figure 3.7 and 3.8). To elucidate further the complex behavior of receptor trafficking, we performed mathematical analyses to construct a model of IGF-IR trafficking. These analyses were based on the time-course of IGF-IR surface availability provided by our biotinylation assay (see Figure 3.3). The simplest model, based on these data, includes four receptor states: 1) RM1 (unbound receptor at the plasma membrane), 2) RM2 (receptor-ligand complex at the plasma membrane), 3) RM3
(clustered IGF-IR), and 4) RI1 (internalized receptor; Figure 3.9A). In this model, Akt is activated by receptors in the RM3 state since we observe that dansylcadaverine inhibited IGF-IR mediated Akt phosphorylation and blocked IGF-I\textsuperscript{b} clustering (see Figures 3.4 and 3.5). The fractional occupancy of the IGF-IR in each state was coded as a set of ordinary differential equations (ODEs) over time and solved for the predicted Akt phosphorylation and IGF-IR surface occupancy (Figure 3.9B). Whereas sustained phosphorylation of Akt was predicted by this model, there was negligible loss of receptors at the cell surface and lacked a trend of surface recovery (Figure 3.9C, D). Thus, the four-state receptor model is inconsistent with our empirical data for receptor trafficking.

A five-state mathematical model of IGF-IR trafficking recapitulates empirical data

To account for the inconsistencies between the four-state model and our empirical data, five-state receptor trafficking models were constructed with two potential receptor states. The first potential receptor state, designated RI2, is localized internally and occurs after RI1. A second potential receptor state, designated RM4, is localized at the cell surface and also occurs after RI1. Testing RI2 and RM4 individually, the fractional occupancy of the IGF-IR was coded as a set of ODEs over time and solved for the predicted Akt phosphorylation and IGF-IR surface occupancy. Similar to the four-state model, a five-state model with the inclusion of RI2, showed negligible surface loss of receptors with no surface recovery at 120 min (data not shown). When RI2 was replace with RM4 in the five-state model (Figure 3.10A, B), the IGF-IR surface occupancy curve was strikingly similar to our observations for IGF-IR surface availability, with significant surface loss of the receptor followed by receptor recovery at 120 min (Figure 3.10C, D).
Taken together, mathematical analyses predict a model of IGF-IR trafficking, consistent with our empirical data, in which the IGF-IR is internalized and then recovers to the plasma membrane for further activation. The five-state model also predicts an additional kinetic state of the receptor at the cell surface that was not elucidated by empirical analyses, which was required after surface recovery and prior to further ligand binding.
Figure 3.1 IGF-I sustains IGF-IR phosphorylation through 24 hours with no change total IGF-IR levels. Cells were serum starved and treated with 10 ng/ml IGF-I (I) or basal media (C) for 24 hours. Cell lysates were isolated and protein was processed for SDS-PAGE and Western blot analysis for phospho- and total IGF-IR β subunit immunoreactivity. Band densities are represented as a ratio of phospho- to total IGF-IR β normalized to β-Actin. Data represent the mean ± SEM (n=3). Phosphorylation of IGF-IR β subunit was observed in the presence of IGF-I (p=0.01) but not in basal media through 24 hours or t0 control (A, B). Total IGF-IR β subunit protein levels remained unchanged during these time points (A, C).
Figure 3.2 IGF-I is required for sustained Akt phosphorylation. Cells were serum starved and treated with 10 ng/ml IGF-I for indicated times. Cell lysates were isolated and protein was processed for SDS-PAGE and Western blot analysis for phospho-Akt and phospho-GSK-3β immunoreactivity. Band densities were normalized to β-Actin. Data represent the mean ± SEM (n=3). In the presence of IGF-I, the phosphorylation of Akt (**p<0.009; A, B) and GSK-3β (**p<0.001; A, C) was sustained through 24 hours. The removal of IGF-I after 30 minutes resulted in the dephosphorylation of Akt and GSK-3β. Although, GSK-3β phosphorylation was still significant over control groups at 1 and 2 hrs (*p<0.03).
Figure 3.3 IGF-I alters IGF-IR surface availability. Cells were serum starved and treated with 10 ng/ml IGF-I or N1A basal media for indicated times. Cells were labeled with 0.3 mg/ml NHS-sulfo biotin for 1 hour. Unreacted biotin was quenched with 100 mM glycine. Total and surface precipitated lysates were processed for SDS-PAGE and Western blot analysis for IGF-IRβ subunit immunoreactivity. Data represent the mean ± SEM (n=3). Representative Western blot from the biotinylation assay (A). Surface availability was represented as a ratio of surface to total IGF-IR and as a percentage of t0. Stimulation with IGF-I resulted in a decrease in IGF-IR β subunit surface expression at 15 min (*p<0.04) and 30 min (*p=0.006) and recovery of receptors at the cell surface was observed from 60 to 120 minutes (B). At 60 minutes receptor loss at surface was still significantly lower than t0 (*p<0.002). In cells incubated in N1A basal media, an increase in surface receptors was observed at 120 minutes (*p=0.0005; B).
Figure 3.4 Dansylcadaverine blocks IGF-IR internalization and phosphorylation of Akt. Cells were serum starved and treated with 10 ng/ml IGF-I for 30 minutes +/- 100 μM dansylcadaverine. After biotinylation, total and surface precipitated lysates were processed for SDS-PAGE and Western blot analysis for phospho- and total IGF-IRβ subunit immunoreactivity. Data represent the mean ± SEM (n=3). At 30 min, dansylcadaverine significantly blocked the decrease in IGF-IR surface availability (p = 0.03; A, B) with no effect on phosphorylation of the IGF-IR β subunit (p < 0.05, A, C). GG-4 progenitors were also treated with IGF-I for 5, 20, and 30 minutes +/- dansylcadaverine. Total lysates were subjected to SDS-PAGE and Western blot analysis for phospho-Akt immunoreactivity and were normalized to β-Actin. Dansylcadaverine blocked the phosphorylation of Akt at 5, 20, and 30 minutes (p < 0.0003; D, E).
Figure 3.5 Dansylcadaverine blocks the clustering of biotin-conjugated IGF-I. Cells were treated with 10 ng/ml biotin-conjugated IGF-I for 0 or 2 minutes on poly-d-lysine/laminin coated glass coverslips. Cells were fixed in 2% paraformaldehyde and blocked in diluent for 15 minutes as described in materials and methods. Streptavidin-conjugated TRITC (1:200) and DAPI (1:1000) were incubated on coverslips for 60 minutes. Cells were thoroughly washed with PBS and mounted onto glass microscope slides. Confocal microscopy was used to visualize cells with a 63X objective. (B, E) Cells treated with IGF-I for 2 minutes exhibited a punctate staining. Arrowheads indicate areas of interest. (C, F) Cells co-treated with IGF-I and dansylcadaverine exhibit diffuse staining. (A, D) Cells untreated with biotin-conjugated IGF-I were negative for TRITC immunofluorescence.
Figure 3.6 Recycling is required for IGF-IR surface recovery and sustained Akt phosphorylation. Cells were serum-starved and treated with 10 ng/ml IGF-I for 30 and 120 minutes +/- 10 µM monensin. After biotinylation, total and surface precipitated lysates subjected to PAGE and Western blot analysis for IGF-IRβ immunoreactivity. Data represent the mean ± SEM (n=3). Monensin significantly blocked recovery of the IGF-IR at 120 minutes (p< 0.01; A,B). Monensin also blocked IGF-I mediated Akt phosphorylation at 120 minutes (P=0.0003; C, D), but not at 15 min(C, D). CG-4 progenitors were treated with IGF-I +/- 20 ng/ml cycloheximide for 120 minutes. Cycloheximide had no effect on IGF-I mediated Akt phosphorylation (p<0.0001; E, F), or on total IGF-IR levels (E, H), but significantly decreased Cyclin D1 protein levels (p< 0.0006; E, G).
Figure 3.7 IGF-IR co-fractionates with the transferrin receptor. (A) Cell homogenates were fractionated on a 10-50% linear sucrose gradient as described in Materials and Methods. Fractions were taken from the top of the gradients and were processed for SDS-PAGE and Western blot analysis. (B) EEA1 and GLUT1 predominantly localized to fraction #3, markers for of the early endosome and plasma membrane, respectively. (C) The TfR was distributed within fractions #2-#6. The distribution of the IGF-IR overlapped with the TfR, which localized within fractions #3-#5. (D) Merge.
Figure 3.8 IGF-IRs co-precipitate with transferrin receptor and Rab11 positive endosomes. Crude CG-4 progenitor homogenates were incubated with TIR antibody conjugated magnetic beads as described in Materials and Methods. Total protein (T) is shown next to pulldown (IP) samples incubated +/- TIR antibody. IGF-IR co-precipitated with the TIR and Rab11 in the presence of TIR antibody (+), but not in the absence of TIR antibody (-).
Figure 3.9 Four-state mathematical model for IGF-IR trafficking is inconsistent with empirical data. The time course of IGF-IR surface availability during IGF-I stimulation was used as the basis for the mathematical modeling. The simplest model based on these data include four receptor states. (A) RM1 (unbound receptor at the cell surface); RM2 (ligand:receptor complex at the cell surface); RM3 (clustered ligand:receptor complex), which leads to Akt activation; RI1 (internalized receptor). (B) The fractional occupancy of the IGF-IR in each receptor state was coded as a set of ordinary differential equations (ODEs), and solved for the theoretical IGF-IR surface occupancy and Akt activation over time. (C) Model predicted negligible receptor surface loss, without a trend of recovery. (D) Akt activation was sustained over time. ***Mathematical analyses performed by Andrew LeBeau.
Figure 3.10 Five-state mathematical model of IGF-IR trafficking recapitulates empirical data. For this model an additional receptor state (RM4) was added to the original four-state model (A). The fractional occupancy of the IGF-IR in each state was coded as a set of ODEs and solved for the theoretical IGF-IR surface occupancy and Akt activation (B). The five-state model predicted surface loss maximal between 30-60 min, and surface recovery through 120 minutes (C), as well as sustained Akt activation (D). *** Mathematical analyses performed by Andrew Lebeau.
**Discussion**

Previously, we demonstrated that IGF-I sustains phosphorylation of Akt in primary OPs and promotes long-term survival of these cells (Ness and Wood, 2002). The pro-survival effects of IGF-I are mediated through the IGF-IR (Ness et al., 2002). In the present study, we investigated the role of IGF-IR trafficking in the sustained phosphorylation of Akt. We provide evidence that IGF-IR internalization and recycling are required for sustained Akt phosphorylation in OPs. Furthermore, mathematical analyses predict a model of IGF-IR trafficking that is consistent with our empirical data.

**Sustained phosphorylation of Akt requires IGF-IR clustering and internalization**

We report that IGF-I mediates IGF-IR phosphorylation through 24 hours and that the continual presence of IGF-I is requisite to sustain the phosphorylation of Akt and its downstream substrate, GSK-3β. Whereas total IGF-IR protein remained unchanged, we observed that the localization of the IGF-IR is altered during IGF-I stimulation. Maximal loss of the IGF-IR from the cell surface occurred within 30 minutes, which is consistent with other studies that have shown maximal internalization of RTKs, including the IGF-IR, between 15 to 30 minutes after ligand stimulation in various cell types, including primary hippocampal neurons, rat fibroblasts, and mouse embryonic fibroblasts (Dore et al., 1997; Haugh, 2002; Haugsten et al., 2005).

To understand the significance of IGF-IR internalization we utilized dansylcadaverine, a pharmacological inhibitor that blocks receptor clustering and internalization. In a study by Levitzki and colleagues, dansylcadaverine was used to block clustering and internalization of surface bound rhodamine-conjugated alpha2-
macroglobulin in normal rat kidney cells (Levitzki et al., 1980), which correlated with a
decrease in transglutaminase activity. Dansylcadaverine also blocked internalization of
the IGF-IR in CHO cells and in mouse and human fibroblasts (Baldwin et al., 1980;
Chow et al., 1998; Vecchione et al., 2003). In the present study, we used
dansylcadaverine to block internalization of the IGF-IR, due to its well-characterized
ability to block enzymatic activity of transglutaminase (Levitzki et al., 1980).

Chow and colleagues showed that IGF-IR internalization is blocked by
dansylcadaverine without affecting IGF-I mediated Akt phosphorylation (Chow et al.,
1998). In another study, over expression of EDH1, an Eps15 homology domain
containing protein, which is involved in the formation of clathrin coated vesicles and
promotes internalization, resulted in an attenuated IGF-I mediated Akt phosphorylation
(Rotem-Yehudar et al., 2001). Together, these studies support the hypothesis that Akt is
activated at the cell surface and that internalization decreases Akt phosphorylation.
Whereas these data are contrary to our findings, which demonstrated that
dansylcadaverine blocks IGF-IR internalization and IGF-I mediated Akt phosphorylation
in CG-4 progenitors, we further showed that dansylcadaverine blocks the clustering of
IGF-I bound to surface IGF-IRs, a step preceding internalization. Therefore, it is
possible that clustering is not required for IGF-IR signaling in all cell types.

Our data are consistent with previously published studies, which demonstrate that
clustering is required for receptor-mediated signal transduction. This is primarily
attributed to the localization of the signaling machinery into discrete regions of the
plasma membrane, which become activated upon receptor translocation (Ichinose et al.,
2004) and spatially organized for internalization (Schreiber et al., 1983). Recently, it was
reported in a computational study that receptor clustering decreases the dissociation kinetics of ligand binding (Gopalakrishnan et al., 2005). Collectively, these data suggest that receptor clustering co-localizes receptors with signaling proteins, as well as stabilizes ligand:receptor interactions.

**The IGF-IR follows a recycling pathway**

The internalization of ligand:receptor complexes desensitizes receptors and is a common mechanism to attenuate signal transduction (Brismar et al., 2005; Penengo et al., 2003). However, receptor can also recycle to the cell surface for further ligand binding. In CG-4 progenitors, we observed the recovery of IGF-IRs at the cell surface between 60 and 120 minutes of IGF-I stimulation, which correlated with sustained Akt phosphorylation. Similarly, recycling of the G-protein coupled receptor (GPCR), neurotensin (NT)-1 receptor, led to prolonged activation of ERK in neuroblastoboma cells (Toy-Miou-Leong et al., 2004). Taken together, these published data demonstrate that the recovery of receptors at the cell surface can lead to long-term activation of signaling pathways.

Subsequent to clustering, receptors internalize within distinct regions of the plasma membrane and are targeted for transport to various subcellular compartments, including the lysosome, trans-golgi network, or plasma membrane (Clague, 1998; Sorkin and Von Zastrow, 2002). Previously, we have reported that TrkC, a member of the RTK family, is degraded in response to NT-3 in primary OPs (Ness and Wood, 2002). We also have shown this in CG-4 progenitors (see appendix, Figure A.1, pg. 125).
contrast, in these and our previous studies IGF-IR protein levels did not change in response to ligand in primary or CG-4 progenitors (Ness and Wood, 2002).

The mechanisms that control the differential subcellular fates of TrkC and the IGF-IR in OPs are currently unknown. Generally, the regulation of receptor sorting involves several molecules, including cbl, an E3 ubiquitin ligase, which is involved in targeting proteins to the lysosome, as well as members of the Rab protein family of small GTPases that are involved in budding and fusion of various endosomal compartments (Clague, 1998). Recently, it was demonstrated that IGF-IR stability is regulated by association with Grb10, which promotes the recruitment of an E3 ubiquitin ligase and culminates in receptor ubiquitinylation and receptor down regulation (Vecchione et al., 2003).

The recycling of cellular proteins to the plasma membrane is a common mechanism that is more energy efficient compared to de novo synthesis (Maxfield and McGraw, 2004). We observed surface recovery of the IGF-IR after 120 minutes of IGF-I stimulation and determined that these receptors originate from a recycling, rather than a de novo pool since monensin, an inhibitor of endosomal trafficking, blocked recovery of IGF-IRs at the cell surface. Additionally, monensin, but not cycloheximide – an inhibitor of protein translation - decreased IGF-I mediated Akt phosphorylation. Lastly, we demonstrated that the IGF-IR and TfR are similarly distributed on a linear 10-50% sucrose gradient. The presence of these proteins in fraction #4, in which early endosome and plasma membrane markers were relatively low, may be indicative of a recycling endosome. We further showed that the IGF-IR and Rab11, a maker for the recycling endosome, co-precipitate with the TfR. Previously, precipitation of the TfR has been
used to identify proteins that localize within the recycling endosome (Melikian and Buckley, 1999). Our data are also supported by previous studies performed in rat fibroblasts, which show that approximately sixty percent of IGF-I/IGF-IR complexes recycle to the cell surface after 120 minutes of ligand stimulation (Zapf et al., 1994). Furthermore, IGF-IR recovery at the cell surface at 120 minutes is consistent with the peri-nuclear recycling endosome, which is positive for Rab11, and is responsible for the constitutive trafficking of the TfR (Ren et al., 1998). Together, these data strongly suggest that IGF-IRs follow a recycling pathway in the presence of IGF-I. Interestingly, in the absence of ligand we observed an increase in IGF-IR surface expression after 120 minutes. This can be likely attributed to the mobilization of internal receptor pools and not to de novo synthesis, since we observe no changes in total IGF-IR levels during this time course. Since IGF-I is an important survival factor for oligodendrocytes and their progenitors, the up-regulation of surface IGF-IR in its absence suggests this may be due to sensitization in response to trophic factor and serum deprivation.

Mathematical modeling is consistent with empirical data

To elucidate the complex behavior of IGF-IR trafficking, mathematical modeling of our empirical data was performed. Mathematical studies were used previously to delineate the dynamic interaction of EGFR/HER2 dimerization and ERK activation (Hendriks et al., 2005). We report that the initial four state model of receptor trafficking was insufficient to simulate our empirical observations. This model predicted negligible surface loss and lacked a trend of recovery. Conversely, a five-state receptor model with an additional receptor state (RM4) at the cell surface, following receptor recovery but
preceding further ligand binding, was sufficient to recapitulate our empirical data. The role of RM4 is currently unknown. We hypothesize that the transition from RM4 to RM1 may represent a putative dephosphorylation of the IGF-IR, leading to resensitization and further ligand binding. Whereas receptor dephosphorylation is classically regarded as an endosomal event (Romsicki et al., 2004; Zhang et al., 1997), there is evidence for the dephosphorylation of the IGF-IR at the cell surface. Specifically, Maile and Clemmons have shown that IGF-IR dephosphorylation is promoted by recruitment of the tyrosine phosphatase, SHP-2 to the plasma membrane in an IGF-dependent manner in smooth muscle cells (Maile and Clemmons, 2002). Thus, the transition of RM4 to RM1 as a putative dephosphorylation step is supported by previously published work.

In conclusion, our empirical and computation data support our hypothesis that internalization and recycling of the IGF-IR is required for sustained Akt phosphorylation. Importantly, both our empirical and mathematical findings are well supported by previous work on receptor trafficking and signal transduction. Future studies are designed to understand the molecular machinery involved in IGF-IR internalization and recycling in OPs, as well as to clarify the role of the putative RM4 state.
CHAPTER FOUR

IGF-I mediated Akt Phosphorylation is Associated with Cholesterol-enriched Membrane Microdomains
Introduction

The regulation of signal transduction has been attributed, in part, to the subcellular compartmentalization of receptors and their downstream effector molecules. This has been demonstrated for RTKs localized at the plasma membrane compared to those localized at the endosome, which activate distinct signaling cascades and lead to diverse biological outcomes (Chow et al., 1998; Haugh, 2002; Zhang et al., 2000b).

In the previous chapter, we demonstrated that IGF-I mediated Akt phosphorylation requires IGF-IR internalization and recycling (see Chapter 3). Furthermore, we showed that dansylcadaverine, an inhibitor of receptor clustering and internalization, blocks IGF-I mediated Akt phosphorylation. The role of IGF-IR clustering in these cells is undefined. It has been reported, however, that clustering spatially organizes receptors with signaling molecules at the plasma membrane and stabilizes ligand:receptor interactions (Gopalakrishnan et al., 2005; Ichinose et al., 2004; Schreiber et al., 1983). Furthermore, the spatial organization of receptors and signaling proteins into discrete microdomains of the plasma membrane, including lipid rafts, has been implicated in the specificity and fidelity of signal transduction (Matthews et al., 2005; Remacle-Bonnet et al., 2005). Lipid rafts microdomains are enriched in cholesterol and glycosphingolipids, which form a liquid order distinct from the surrounding plasma membrane. Multiple RTKs localize within cholesterol/glycosphingolipid-enriched membrane microdomains (CEMs), and move in or out of these microdomains in a ligand-dependent manner (Mineo et al., 1999). The disruption of CEMs with the cholesterol chelating agent, methyl-β-cyclodextrin (MβCD), often results in the displacement of receptors from CEMs and alters RTK mediated signal
transduction (Karlsson et al., 2004; Parpal et al., 2001). CEMs are well established to regulate insulin receptor (IR) and IGF type-I receptor (IGF-IR) signaling, most notably in adipocytes and pre-adipocytes (Bartz et al., 2005; Baumann et al., 2001; Huo et al., 2003). In 3T3L-1 fibroblasts, IGF-I mediated adipocyte differentiation requires CEMs (Hong et al., 2004); however, disruption of CEMs in these cells results in an increase in IGF-I mediated proliferation (Matthews et al., 2005). In addition, the disruption of CEMs resulted in the displacement of IGF-IRs and consequently inhibited IGF-I mediated Akt activity and apoptosis in colon carcinoma cells (Remacle-Bonnet et al., 2005). Together, these studies provide evidence that the spatial organization of IGF-IRs within or outside of CEMs confers the activity of distinct signal pathways and leads to specific biological outcomes. Thus, it is of considerable interest to understand the role of CEMs in IGF-I/IGF-IR signaling in oligodendrocyte progenitors (OPs).

CEMs have been isolated from mature oligodendrocyte (OL) plasma membranes, including myelin membranes, and have been implicated signal transduction and myelination (Arvanitis et al., 2005). Platelet-derived growth factor (PDGF)-mediated PI-3K/Akt activation is augmented by integrin activity within CEMs of newly differentiated OLs (Baron et al., 2003). The disruption of CEMs results in a decrease in PDGF-mediated OL survival (Decker and ffrench-Constant, 2004). Furthermore, myelin-specific proteins, including myelin basic protein (MBP; DeBruin et al., 2005) and proteolipid protein (Simons et al., 2000) localize within CEMs during OL maturation. The Src kinase Fyn also localizes within these microdomains, which confers an increase in its kinase activity and promotes OLs differentiation (Kramer et al., 1999). Lastly, neural cell adhesion molecule (NCAM) 120 and F3, which facilitate neuronal:glial
interactions, are localized within CEMs from OLs (Arvanitis et al., 2005; Kramer et al., 1999). Taken together, these data suggest that CEMs within OLs are required for signal transduction, as well as for the spatial organization of proteins for appropriate axonal-glial interactions and myelination. Whereas much attention has been given to CEMs in mature OLs, few studies have focused on the function of these microdomains in OPs. Thus, it is of considerable interest to understand the role of CEMs in these cells.

In the present study we investigate the role of CEMs in the regulation of IGF-I mediated PI-3K/Akt pathway activation in OPs. We test the hypothesis that cholesterol-membrane integrity is required for IGF-I mediated Akt phosphorylation and that the IGF-IR and its signaling partners localize within CEMs. We report that cholesterol depletion of OPs had no effect on IGF-I mediated IGF-IR phosphorylation but blocked short-term Akt phosphorylation and altered OP membrane morphology. Furthermore, cholesterol repletion reversed the morphological and biochemical effects of MβCD. We also demonstrated that p85 (the regulatory subunit of the PI-3K), Akt, and the IGF-IR localized within CEMs isolated from OPs.
Results

Cholesterol depletion alters OL progenitor membrane integrity

Disruption of CEMs is classically performed by treatment with the cholesterol chelating agent MβCD. To determine that the effects of MβCD are specific to cholesterol depletion in OPs, we replaced cholesterol after drug treatment and examined cellular morphology. Whereas untreated cells had normal and continuous bipolar processes extending from their cell bodies (Figure 4.1 A), treatment with 5 μM MβCD for 30 min resulted in punctate and discontinuous processes (Figure 4.1 B). Subsequently, cholesterol was replaced as previously described by Klein et al., 1995 (see Material and Methods). OPs were treated with 200 μM cholesterol-MβCD complexes. Alone, MβCD strips membranes of cholesterol; however, when complexed with cholesterol, MβCD serves as a vehicle for cholesterol replacement (Klein et al., 1995). In these experiments, cholesterol treatment reversed the morphological effects of MβCD. The processes of cholesterol treated cells were indistinguishable from untreated control cells (Figure 4.1 A, C). These results suggest that MβCD induces morphological changes in OP membranes that are specific to cholesterol depletion.

Cholesterol depletion alters IGF-I mediated Akt Phosphorylation

To investigate the role of cholesterol depletion on IGF-I mediated signal transduction OPs were stimulated with IGF-I for 30 min, plus or minus pre-treatment with MβCD. To determine that the biochemical effects of MβCD were specifically due to loss of cholesterol, cholesterol was replaced following MβCD treatment as previously described. Treatment with IGF-I significantly increased Akt phosphorylation at 30 min
compared to untreated cells (P < 0.0001; Figure 4.2A, B). Pre-treatment with MβCD, however, blocked IGF-I mediated Akt phosphorylation (Figure 4.2A, B). We also show that cholesterol replacement rescued IGF-I mediated Akt phosphorylation (P < 0.0001; Figure 4.2A, B). No significant changes were observed for IGF-I mediated ERK phosphorylation (data not show). Pre-treatment with MβCD also had no significant effects on IGF-I mediated IGF-IR phosphorylation (P < 0.003; Figure 4.2C, D).

To confirm that the effects on IGF-I signaling were cholesterol-specific, OPs were depleted of cholesterol with MβCD as previously described and were subsequently treated with 100 or 200 μM cholesterol for 30 min. After cholesterol treatment, OPs were stimulated with IGF-I for 30 min. We observed a dose-dependent increase in IGF-I mediated Akt phosphorylation in the presence of cholesterol. The treatment of OPs with 100 μM cholesterol rescued IGF-I mediated phosphorylation of Akt significantly versus cells not treated with cholesterol (P < 0.008; Figure 4.2E, F). However, in OPs treated with 200 μM cholesterol there was a 3 fold increase in IGF-I mediated Akt phosphorylation compared to cells treated with 100 μM cholesterol (P < 0.04; Figure 4.2E, F). These results suggest that cholesterol membrane integrity is required for short-term IGF-I mediated Akt phosphorylation.

**Acute cholesterol depletion does not affect long-term IGF-I signal transduction**

The disruption of cholesterol-membrane integrity with MβCD alters biological responses to extracellular ligands in multiple cell types (Buk et al., 2004; Huo et al., 2003; Matthews et al., 2005). Therefore, we were interested in determining whether acute cholesterol depletion had long-term effects on IGF-I mediated signal transduction
and biological responses in OPs. For these studies, we investigated PI-3K/Akt and ERK pathway activation after 6 hours of IGF-I stimulation (plus and minus pre-treatment with MβCD). Cells were also treated with FGF-2 as a positive control for long-term ERK phosphorylation since we previously have demonstrated that FGF-2 is a potent activator of the ERK pathway (Frederick and Wood, 2004). IGF-I stimulation induced the phosphorylation of Akt compared to control groups at 6 hours both alone (P = 0.017) and more significantly after MβCD treatment (P < 0.0001; Figure 4.3A, B). No significant increase in FGF-2 mediated Akt phosphorylation was observed compared to control groups at 6 hours (Figure 4.3A, B). FGF-2, however, induced ERK phosphorylation through 6 hours compared to all treatment groups (P < 0.0001; Figure 4.3A, C). IGF-I mediated ERK phosphorylation, plus or minus MβCD pretreatment, was not significant versus control groups (Figure 4.3A, C).

We previously have shown that FGF-2, but not IGF-I, markedly enhances cyclin D1 expression and consequently promotes cell cycle progression via the ERK signaling pathway (Frederick and Wood, 2004). We were interested in determining, however, the effects of MβCD on IGF-I mediated cyclin D1 expression since previous studies have demonstrated that cholesterol depletion augments IGF-I mediated cell cycle progression (Matthews et al., 2005). IGF-I stimulation, plus or minus MβCD pretreatment, induced a modest but significant increase in cyclin D1 protein expression (P < 0.02; Figure 4.3A, D). FGF-2 markedly increased cyclin D1 protein levels above all other treatment groups (P < 0.0001; Figure 4.3A, D). The results of these studies show that acute cholesterol depletion does not attenuate, but actually enhances, long-term IGF-I mediated Akt
phosphorylation. Furthermore, cholesterol depletion has no effect on IGF-I mediated ERK phosphorylation or cyclin D1 protein expression.

**Isolation of cholesterol-enriched membrane microdomains from OPs**

Since we observe that cholesterol depletion alters short-term IGF-I mediated Akt phosphorylation, we were interested in determining whether CEMs are present in OPs. The isolation of these membranes from intact cells involves extraction in non-ionic detergents, such as Triton X-100 at 4°C, or sodium carbonate buffer (pH 11), in which CEMs are insoluble due to their high lipid content. Insoluble membranes are separated from bulk plasma membrane by density centrifugation on a sucrose gradient since CEMs are buoyant in low density sucrose fractions (Locke et al., 2005). These methods commonly yield CEMs that are consistent with lipid raft microdomains from intact cells.

We first examined the CEMs from OPs by using the sodium carbonate method (as described in Materials and Methods). Membranes were fractionated on a discontinuous sucrose gradient and analyzed for the presence of caveolin-1 and flotillin-1, since these are well characterized rafting proteins (Bickel, 2002; Evans et al., 2003). Both caveolin-1 and flotillin-1 are present within low density sucrose fractions (3-5) (Figure 4.5A). In addition, pre-treatment with MβCD resulted in the dissociation of caveolin-1 and flotillin-1 from low density fractions (Figure 4.5A). To determine whether IGF-IR and its signaling partners localize within CEMs, we took advantage of both the Triton X-100 and sodium carbonate methods. The IGF-IR, p85 (the regulatory subunit of PI-3K), and Akt co-localized with caveolin-1 and flotillin-1 in low density sucrose fractions (#3-5; Figure 4.5B, C) under conditions of chronic serum and growth factor stimulation.
However, the majority of these signaling proteins is localized within the high density fractions.
Figure 4.1 Cholesterol depletion alters OP morphology. OPs were treated with 5 μM MβCD for 30 minutes and visualized by phase contrast microscopy. Magnified insets highlight areas of interest (A) Untreated cells exhibited normal, contiguous processes that extended from the cell body. (B) Treatment with MβCD resulted in discontinuous, punctuated processes (as indicated by arrowheads). (C) Post-treatment with 200 μM cholesterol- MβCD complexes reversed the effects of cholesterol depletion.
Figure 4.2 Acute cholesterol depletion alters IGF-I mediated signal transduction but not IGF-IR phosphorylation. OPs were treated for 30 minutes with 10 ng/ml IGF-I (+/- pretreatment with MβCD). Cell lysates were processed for SDS-PAGE and Western blot analysis. Data represent the mean ± SEM (n=3). Treatment with IGF-I induced phosphorylation of Akt (A, B; p<0.0001). Pre-treatment with MβCD blocked IGF-I mediated Akt phosphorylation (A, B). Cholesterol repletion reversed the effects of MβCD (A, B; p<0.0001). MβCD pretreatment had no effect on IGF-I mediated IGF-IR phosphorylation compared to untreated control cells (C, D; p<0.003). The recovery of Akt phosphorylation was dependent upon the dose of cholesterol. The presence of 200 μM cholesterol caused an approximately 3 fold increase in IGF-I mediated Akt phosphorylation (p<0.008) compared to 100 μM cholesterol (E, F; p<0.04).
Figure 4.3 Acute cholesterol depletion does not alter long-term IGF-I signaling. OPs were treated for 6 hours with basal media (con), 10 ng/mL IGF (+/- pre-treatment with 5 μM MβCD; IGF+M and IGF-I, respectively) or 10 ng/ml FGF-2. Cell lysates were processed for SDS-PAGE, and Western blot analysis was performed for phospho- and total Akt and ERK, cyclin D1, and β-Actin. Data represent the mean ± SEM (n=3). (A) Representative Western blot. (B) IGF-I induced Akt phosphorylation above con and FGF-2 treated groups (p=0.017), IGF+M phosphorylation of Akt was significant above all other groups (p<0.01). (C) FGF-2 induced ERK phosphorylation above all treatment groups (p<0.0001). (D) IGF-I and IGF+M treatment resulted in a small but significant increase in cyclin D1 protein levels over con (p<0.02). FGF-2 treatment resulted in large increase in cyclin D1 protein levels over all other groups (p<0.0001).
Figure 4.4 Extraction of CEMs from intact cells. The isolation of CEMs classically involves extraction in the non-ionic detergent, Triton X-100 at 4°C, or sodium carbonate (pH 11), which leads to the solubilization of most cellular membranes except for regions enriched with cholesterol and glycosphingolipids. Soluble and insoluble membranes are subsequently separated by density gradient centrifugation. Due to their relatively high lipid to protein ratio, lipid raft membranes will float towards the top of the gradient, whereas soluble membranes will remain at the bottom of the gradient.
Figure 4.5 IGF-IR, p85, and Akt co-localize with markers for lipid raft microdomains. OPs under conditions of chronic serum and growth factor treatment were harvested via the triton or sodium carbonate method (see Materials and Methods). (A) Isolation of CEMs with sodium carbonate +/- MβCD. (B) Triton insoluble membranes were fractionated on a discontinuous sucrose gradient. (C) Lysates in sodium carbonate buffer were sonicated and subsequently fractioned on a discontinuous sucrose gradient. IGF-IR, Akt, and p85 co-localized with caveolin-1 and flotillin-1 in buoyant fractions (3-5).
Discussion

Established and emerging evidence from the literature implicate cholesterol/glycosphingolipid-enriched membrane microdomains (CEMS; i.e. lipid rafts) in the regulation of signal transduction (Decker and ffrench-Constant, 2004; Hong et al., 2004; Remacle-Bonnet et al., 2005). In the present study we sought to investigate the role of CEMs in IGF-I mediated Akt phosphorylation. We provide evidence that cholesterol membrane integrity is required for short-term IGF-I mediated Akt phosphorylation in OPs. Moreover, we demonstrate that CEMs isolated from OPs and contain IGF-IRs and PI-3K/Akt signaling proteins.

Cholesterol integrity and IGF-I signal transduction

We show that cholesterol depletion with MβCD altered OP membrane integrity and blocked short-term IGF-I mediated Akt phosphorylation but had no effect on the phosphorylation of the IGF-IR. Cholesterol replacement rescued the morphological and biochemical effects of MβCD. Moreover, the level of IGF-I mediated Akt phosphorylation positively correlated with the dose of cholesterol (see Figure 4.2). Together, these data suggest that the effects of MβCD on Akt phosphorylation were due specifically to cholesterol depletion and not secondary effects of this pharmacological agent. Our findings are also consistent with previously published studies, which have shown that cholesterol depletion blocks Akt phosphorylation but not receptor activity (Remacle-Bonnet et al., 2005). Thus, IGF-I mediated Akt phosphorylation, but not ligand binding or IGF-IR autophosphorylation, requires cholesterol-membrane integrity.
Whereas acute cholesterol depletion inhibited short-term IGF-I mediated Akt phosphorylation, cholesterol depletion augmented long-term IGF-I mediated Akt phosphorylation (see Figure 4.3). However, we observed no changes in IGF-I mediated ERK phosphorylation or cyclin D1 expression. This is contrary to previously published studies in pre-adipocytes, in which acute cholesterol depletion results in an increase in IGF-I mediated ERK phosphorylation but a decrease in IGF-I mediated Akt phosphorylation (Matthews et al., 2005). Furthermore, these changes in signal transduction correlated with an increase in IGF-I mediated mitogenesis and a decrease in protection from toxic insults (Matthews et al., 2005). The disparity between our results and previously published studies may be due to cell-specific differences in the rate of cholesterol biosynthesis. OLs and their progenitors may have a higher rate of cholesterol biosynthesis compared to other cell types since cholesterol is abundant in myelin and is essential for proper myelination (Saher et al., 2005). It is also possible that our culture media contains trace amounts of lipids that can contribute to the cholesterol biosynthesis pathway. Accordingly, it is important to prevent cholesterol biosynthesis in these cells by using lipid depleted serum and/or pharmacological inhibitors to assess the long-term effects of cholesterol depletion on IGF-I mediated signal transduction and biological outcomes accurately.
Isolation and characterization of cholesterol-enriched microdomains from OPs

In the present study, we demonstrated that cholesterol-membrane integrity is required for IGF-I mediated Akt phosphorylation, which suggest that CEMs likely play a role in IGF-I signaling. We successfully isolated CEMs from OP membranes, which contain the classical “rafting” proteins, caveolin-1 and flotillin-1. In both the triton and sodium carbonate method of CEM isolation, we observed that IGF-IR, Akt, and p85 (the regulatory subunit of PI-3K) co-localize with caveolin-1 and flotillin-1 in buoyant sucrose fractions. These data suggest that the IGF-IR and PI-3K/Akt signaling proteins are present within CEMs. It is undetermined, however, if localization of PI-3K/Akt pathway components within CEMs is mediated by IGF-I stimulation. Therefore, it is of great interest to determine if IGF-I stimulation induces the sequestration of receptors and signaling proteins into these microdomains.

Whereas CEMs have been studied in mature OLs, few studies have investigated these microdomains in OPs. We provide evidence for cholesterol-membrane integrity and CEMs in the regulation of IGF-I mediated Akt phosphorylation. Nevertheless, the function of protein localization within CEMs and signal transduction is still elusive. It has been hypothesized that these microdomains not only spatially organize proteins, but also enrich signaling molecules to overcome problems of stoichiometry. This has been demonstrated for heterotrimeric G-proteins, which are less abundant than their transmembrane G-protein coupled receptors (Insel et al., 2005). Proteomic analysis of CEMs demonstrated that signaling molecules are concentrated in these microdomains by > 10 fold compared to the bulk plasma membrane (Foster, et al., 2003). Thus, although
only a small portion of the total IGF-IR, p85, and Akt pools localize within CEMs from OPs it is likely that this fraction remains functionally important.

In conclusion, we provide evidence, supporting our hypothesis that cholesterol membrane integrity is required for IGF-I mediated Akt phosphorylation. Furthermore, we demonstrated that CEMs are present in OPs and that the IGF-IR and its signaling partners localize within these microdomains. Future studies are designed to elucidate the role of CEMs in long-term IGF-I/IGF-IR signal transduction. Toward these ends, specific inhibitors will be used to prevent cholesterol biosynthesis following cholesterol depletion. Additional studies are designed to examine the role of IGF-I in mediating IGF-IR and signaling protein localization within CEMs.
CHAPTER FIVE

Caveolin-1 Expression, IGF-I mediated Localization, and Function in Oligodendrocytes
**Introduction**

Caveolae are a subset of cholesterol/glycosphingolipid-enriched membrane microdomains (CEMs), which form flask-like invaginations of the plasma membrane. Caveolae have been implicated in various processes, including cell survival, proliferation, and growth (Chun et al., 2005; Kawabe et al., 2006). These invaginated structures are abundant in differentiated cell types and contain caveolin, a 21-24 kD cholesterol-binding protein (Lisanti et al., 1993), which is integrated in the membrane and is required for the formation of caveolae (Couet et al., 1997a; Lisanti et al., 1994b). Three isoforms of caveolin are expressed in mammalian cells. Caveolin-1 is ubiquitously expressed and often coincides with expression of caveolin-2, whereas caveolin-3 is found almost exclusively in muscle cells (Tang et al., 1996; Scherer and Lisanti, 1997; Way and Parton, 1995). Caveolin-1, however, appears to be the most essential for caveolae structure and function, since mice with deletions in caveolin-2 or -3 still contain caveolae (Woodman et al., 2004).

The role of caveolins in the central nervous system (CNS) is still elusive. However, all caveolin isoforms are expressed in the brain (Cameron et al., 1997), most notably in astrocytes and oligodendrocytes (Cameron et al., 2002; Silva et al., 2005). Whereas CEMs have been implicated in OL survival and myelination, caveolae have not been specifically investigated in these cells. Myelin-specific proteins, including myelin basic protein (MBP) and proteolipid protein (PLP), localize within CEMs during OL development (DeBruin et al., 2005). In addition, it has been demonstrated that the platelet-derived growth factor (PDGF) α receptor and integrins interact within CEMs to promote OL survival (Decker and ffrench-Constant, 2004). Together, these data suggest
the importance of CEMs in the development and maintenance of OLs. Therefore, it is likely that caveolin enriched microdomains (i.e. caveolae) are also involved in OL survival and myelination. Interestingly, caveolin-1 expression increases in both glioblastoma cell lines and primary tumors (Abulrob et al., 2004). Accordingly, it is important to elucidate the role of caveolin-1 in both normal neural cells and tumors.

In the previous chapters, we demonstrated that IGF-IR internalization and recycling are required for sustained Akt phosphorylation (see Chapter 3). We also demonstrated that cholesterol-membrane integrity is required for IGF-I mediated Akt phosphorylation and that the IGF-IR as well as PI-3K/Akt signaling proteins co-localize with caveolin-1 in CEMs isolated from OPs (see Chapter 4). It is unknown, however, if the IGF-IR and its signaling partners are associated with caveolae or non-caveolae CEMs in these cells. Since a role for caveolae in OL biological is unknown, it is of considerable interest to define IGF-IR and caveolin-1 interactions, as well as the function of caveolins in OL lineage.

It is well established that caveolae regulate RTK mediated signal transduction (Okamoto et al., 1998). It was previously shown that RTKs localizes within caveolae via a direct interaction with caveolins through a putative caveolin binding motif (CBM) within their tyrosine kinase domain (Couet et al., 1997b; Nystrom et al., 1999). RTKs, including the epithelial growth factor receptor (EGFR), insulin receptor (IR), and insulin-like growth factor (IGF)-type-I receptor (IGF-IR), localize within caveolae (Biedi et al., 2003; Matthews et al., 2005). The EGFR translocates out of caveolae upon EGF stimulation in mouse fibroblasts (Mineo et al., 1999). Furthermore, disruption of caveolae structures augments EGFR mediated signal transduction (Couet et al., 1997b;
Engelman et al., 1998). In adipocytes, IRs and IGF-IRs move into caveolae upon ligand stimulation. The disruption of caveolae in these cells inhibits IR and IGF-IR mediated signal transduction (Parpal et al., 2001). In pre-adipocytes, however, disruption of caveolae augments IGF-IR mediated Erk activation and results in an increase in pre-adipocyte proliferation (Matthews et al., 2005). Taken together, these data suggest that the role of caveolae in RTK mediated signal transduction and downstream biological outcomes is dependent upon cell type and developmental stage.

In the present study, we investigated the expression of caveolin-1 in the oligodendrocyte (OL) lineage and its role in the regulation of OL- and myelin-specific proteins. We test the hypothesis that caveolin-1 and the IGF-IR interact and that caveolin-1 has a functional role in OL biology. We report a marked increase in total caveolin-1 expression during OL differentiation. Furthermore, we demonstrate that caveolin-1 localization within CEMs correlates with IGF-I stimulation, and that cholesterol depletion blocks this localization. We also show that the IGF-IR and Akt co-localize with caveolin-1 in CEMs from immature OLs, a developmental stage when caveolin-1 is maximally expressed. Lastly, we report that caveolin-1 null mice have aberrant expression of OL- and myelin-specific proteins, including cyclic-2’, 3’ nucleotide phosphodiesterase (CNPase) and myelin basic protein (MBP).
Results

Caveolin-1 expression increases during oligodendrocyte differentiation

Previous studies demonstrated that caveolin-1 increases during differentiation in multiple cell types, including Schwann cells, the myelinating cells of the peripheral nervous system (Mikol, et al., 2003). To investigate the expression of caveolin-1 in the OL lineage, OPs were differentiated in defined media for 0, 24, 48, 72, and 96 hours. Caveolin-1 protein was detected at all stages of OL development. Caveolin-1 expression, however, increased as these cells differentiated, with maximal expression 2 fold of t0 at 72 hours (p<0.01; Fig 5.1A, B). At this time of differentiation, in vitro, these cells are consistent with immature OLs both morphologically and biochemically (previously established by ours and other laboratories; see Figure 1.1).

IGF-I mediates caveolin-1 localization in cholesterol-enriched microdomains

It was previously reported that IGF-IR localizes within CEMs and that IGF-I mediates the transient phosphorylation of caveolin-1 and augments is localization with CEMs in fibroblasts (Maggi et al., 2002; Panetta et al., 2004). In chapter 4, we also demonstrated that IGF-IRs localize within CEMs isolated from OPs. Thus, we were interested in determining the role of IGF-I in regulating caveolin-1 localization in OPs. Cells were stimulated with IGF-I for 30 min and CEMs were subsequently isolated. We observed caveolin-1 localization within CEMs in response to IGF-I (Figure 5.2). Caveolin-1 localization within CEMs, however, was ablated in cells that were treated with MβCD prior to IGF-I stimulation. We also investigated whether IGF-I mediated the
phosphorylation of caveolin-1. IGF-I stimulation for 30 min had no effect on caveolin-1 phosphorylation (data not shown).

**IGF-IR and Akt co-localize with caveolin in CEMs in immature OLs**

In the previous chapter, we demonstrated that the IGF-IR co-localizes with caveolin-1 in CEMs in OPs (see Figure 4.5). In the present chapter, we show that caveolin-1 increases during OL differentiation (see Figure 5.1) and that IGF-I mediates the localization of caveolin-1 to CEMs (see Figure 5.2). Thus, we were interested in determining whether IGF-IRs co-localize with caveolin-1 in CEMs at a later stage of OL development. We chose to investigate the co-localization of these proteins in immature OL, a developmental stage when caveolin-1 is maximally expressed. Primary OPs were differentiated in defined media for 72 hours and subjected to Triton X-100 extraction and sucrose density centrifugation as described in Materials and Methods. The majority of caveolin-1 and IGF-IR co-localize within low density buoyant sucrose fractions (Figure 5.3). We also investigated the localization of Akt in CEMs since we previously demonstrated that this protein localizes to CEMs in OPs. Akt co-localizes with cav-1 and the IGF-IR in low density, buoyant sucrose fractions (figure 5.3).

**The caveolin-1 binding motif of the IGF-IR is conserved through evolution**

It is well established that RTKs, including the IGF-IR, contain a putative CBM in their tyrosine kinase domain (Couet et al., 1997a; Couet et al., 1997b). The human sequence of this binding motif is conserved across various RTKs (Couet et al., 1997b). However, no studies to date have reported the degree of conservation for a particular
RTK across species. A BLAST search was performed for the amino acid sequence of the IGF-IR across multiple species (Figure 5.4). The CBM sequence and juxta-CBM (JM) sequence were compared. The CBM was conserved 100% from human to zebrafish, with the exception of the nematode (*C. elegans*) sequence, which only shared 87.5% identity with the human CBM sequence. In comparison, for all species the JM sequence of the tyrosine kinase domain of the IGF-IR shared 90% identity with the human sequence, except for the nematode, whose sequence only had 60% identity. The results of this informatic analysis, demonstrating high conservation of CBM, suggest that this amino acid sequence is functionally important.

**Caveolin-1 null mice have aberrant expression of OL- and myelin-specific proteins**

To elucidate a role for caveolin-1 in oligodendrocyte biology in vivo, we analyzed the expression levels of the OL- and myelin-specific proteins, cyclic-2’, 3’ nucleotide phosphodiesterase (CNPase), myelin basic protein (MBP), and proteolipid protein (PLP), in caveolin-1 null mice via Western blot analysis. MBP and PLP are myelin-specific proteins that are upregulated during OL differentiation (Timsit et al., 1995; Yu et al., 1994), whereas CNPase is expressed early in the OL lineage (Knapp et al., 1988). CNPase is expressed exclusively in OLs within the CNS and in Schwann cells within the peripheral nervous system. Total brain lysates from caveolin-1 null mice have a significant increase in CNPase (*P* < 0.01; Figure 5.5C), and a modest increase in MBP (*P* < 0.09; Figure 5.5B) compared to wild-type mice. No significant changes, however, were observed for PLP (Figure 5.5A).
Figure 5.1 Caveolin-1 expression in the oligodendrocyte lineage. OPs were differentiated for 0, 24, 48, 72, and 96 hours in chemically defined media. Cell lysates were harvested and subjected to SDS-PAGE and Western blot analysis for caveolin (cav)-1 immunoreactivity. (A) Representative Western blot. (B) Caveolin-1 protein levels were adjusted to β-Actin and represented as a percent of t0 (N2S). Data represents the mean ± SEM (n=3). Caveolin-1 protein increases during OL differentiation, with maximal expression at 72 hrs (B; p<0.01).
Figure 5.2 IGF-I mediates caveolin-1 localization into CEMs. OPs were treated with 10 ng/ml IGF-I for 30 minutes (+/- pre-treatment with MβCD). Cholesterol-enriched microdomains were isolated in 1% Triton X-100 at 4°C and were subjected to sucrose density centrifugation. Fractions were taken from the top (1) to bottom (10) of the sucrose gradient. Samples were processed for SDS-PAGE and Western blot analysis for cav-1 immunoreactivity. Untreated cells (N1A) were negative for cav-1 in buoyant fractions (3-5), which are consistent with lipid raft microdomains. IGF-I induced cav-1 localization within buoyant fractions (3) and pre-treatment with MβCD prevented this localization (I+MβCD).
**Figure 5.3** IGF-IR and Akt co-localize with caveolin in CEMs from immature OLs. OPs were harvested after 72hr differentiation in defined media. Cholesterol-enriched microdomains were isolated in 1% Triton X-100 at 4° C and were subjected to sucrose density centrifugation. Fractions were taken from the top (1) to bottom (9) of the sucrose gradient. Samples were processed for SDS-PAGE and Western blot analysis. IGF-IR, Akt1, and caveolin-1 are enriched in the low density sucrose fractions, which are consistent with cholesterol-enriched membrane microdomains.
**Figure 5.4 IGF type-I receptor caveolin binding motif.** The caveolin binding motif (CBM) of the IGF-IR amino acid sequence was compared from human to nematode (*C. elegans*) and expressed as a percentage of identity to the human sequence. The amino acid sequence flanking the CBM, termed the juxta-motif (JM) sequence was also analyzed to compare the relative identity. Red type indicates amino acid changes from the human sequence. Bold type indicates aromatic amino acids (Φ) of the CBM.

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Figure 5.5 Caveolin-1 null mice have aberrant expression of OL- and myelin-specific proteins. Total protein homogenates were isolated from adult cav-1 null (−/−) and wt (+/+ ) brains as described in Materials and Methods. Homogenates were processed for SDS-PAGE and Western blot analysis for CNPase, MBP, and PLP immunoreactivity. Densitometry values were adjusted to β-Actin. Data represent the mean ± SEM (n=3). (A) No significant changes were observed in PLP expression levels between wt and null mice. (B) MBP expression was moderately increased (p<0.09) and (C) CNPase expression was significantly increased (p<0.02) in null mice compared to wild type mice.
Discussion

In the present study, we investigated the expression and localization of caveolin-1 in the OL lineage, as well as its role in OL biology. We provide evidence that caveolin-1 increases during OL differentiation and that caveolin-1 localization with CEMs correlates with IGF-I stimulation. Furthermore, caveolin-1 null mice have aberrant expression of myelin- and OL-specific proteins.

Caveolin-1 expression and localization in OPs

Consistent with previously published studies in other cell types, caveolin-1 expression increases during OL differentiation (Mikol et al., 2002). It is likely that the increase in caveolin-1 expression correlates with an increase in the formation of caveolae, since caveolin-1 is required for this process and that caveolae are most abundant in differentiated cell types (Couet et al., 1997a). Caveolae formation during cellular development likely plays an important role in biological responses to extracellular ligand since caveolae are well known to regulate RTK-mediated signal transduction in various cell types. For example, insulin signaling is negatively regulated by caveolae in undifferentiated fibroblasts, whereas it is positively regulated by caveolae in mature adipocytes (Nystrom et al., 1999).

We report that IGF-I increases the localization of caveolin-1 in CEMs in OPs. Moreover, IGF-I mediated caveolin-1 localization is ablated by pre-treatment with MβCD. Previously, it was demonstrated that both IGF-I and insulin mediate the phosphorylation of caveolin-1, but only IGF-I augments caveolin-1 localization within CEMs in fibroblasts (Kimura et al., 2002; Maggi et al., 2002). Interestingly, we observed
that caveolin-1 localization within these microdomains is nearly undetectable after serum and growth factor deprivation. Therefore, the ability of caveolin-1 to localize within CEMs may be dependent on serum and/or growth factor stimulation in OPs. Furthermore, since caveolin localization within CEMs correlates with IGF-I stimulation, we hypothesize that IGF-I plays a role in the formation of caveolae invaginations in these cells.

We did not observe IGF-I mediated caveolin-1 phosphorylation at 30 minutes in OPs. Previous studies, however, reported that this growth factor induces a transient phosphorylation of caveolin-1 (Maggi et al., 2002). Therefore, it is important to investigate caveolin-1 phosphorylation at earlier time points in OPs.

**IGF-IR co-localizes with caveolin-1**

We report that the IGF-IR co-localizes with caveolin-1 in CEMs from immature OLs, a developmental stage when caveolin-1 is maximally expressed. Interestingly, the majority of IGF-IR and caveolin-1 present in triton-insoluble membranes are localized within CEMs in immature OLs. This is in contrast to their localization in CEMs isolated from early OL progenitors (OPs), which appears to be only a small portion of proteins within triton-insoluble membranes (see Figure 4.5 and 5.3 for comparison). These differences in localization are likely attributed to an increase in cholesterol and glycosphingolipid (i.e. galactosylceramide) production during OL maturation, since these lipids are enriched within myelin and are required for myelination (Saher et al., 2005; Simons et al., 2000). We predict that protein localization within caveolae increases
during OL differentiation, and therefore, caveolae may differentially modulate responses to extracellular ligands during OL development.

**RTK and Caveolin-1 Interactions**

It is hypothesized that RTKs directly interact with caveolin via CBMs, which are located within the tyrosine kinase domain of these receptors (Nystrom et al., 1999; Couet et al., 1997b). The CBM is conserved across various human RTK sequences, including the EGFR, FGFR, IR, and IGF-IR (Couet et al., 1997b). In the present study, we demonstrated that the CBM of the IGF-IR is conserved through evolution, from humans to C.elegans. Relatively, these amino acid residues are more conserved than the flanking juxta-CBM sequence, which is indicative of the functional importance of this binding domain for RTKs. Nevertheless, there is still controversy regarding the sequence as a true CBM since these amino acids are hydrophobic and are likely buried within the 3-D structure of the protein. It can be argued, however, that the hydrophobicity of these amino acid residues provides the appropriate structural conformation required for RTK/caveolin interactions. Mutations within the CBM of the IR result in impaired autophosphorylation in vitro (Nystrom et al., 1999). Furthermore, two point mutations have been found in the CBM of the IR of several patients with severe forms of insulin resistance (Imamura et al., 1994; Iwanishi et al., 1993; Moller et al., 1991). Accordingly, these experimental and clinical data lend support to the hypothesis that RTK/caveolin interactions are functionally relevant, particularly for the IR.

We did not validate an interaction between caveolin-1 and the IGF-IR in OLs; however, we showed that IGF-IRs co-localize with caveolin-1 in low density sucrose
fractions in both OPs and immature OLs. It was previously demonstrated that the IGF-IR co-precipitates with caveolin-1 in both adipocytes and pre-adipocytes (Maggi et al., 2002; Panetta et al., 2004). Based on ours and previously published data, we propose that IGF-IRs interact with caveolin-1. The direct role of caveolae in IGF-IR signaling remains to be elucidated for OLs and their progenitors.

The role of caveolin-1 in OL biology

Caveolin-1 null mice were generated by Lisanti and colleagues using standard homologous recombination techniques on a C56BL/6 background (Razani et al., 2001). These mice have no visible caveolae (Drab et al., 2000) and result in a variety of phenotypes including insulin resistance (Cohen et al., 2003), shorter lifespan (Park et al., 2003), fibroblast and endothelial cell hyperproliferation (Razani et al., 2001), as well as cardiac abnormalities (Park et al., 2002). A neurological phenotype of these mice has not been reported. This is likely due to the absence of caveolin-1 protein and caveolae structures in most neuronal cell types. Interestingly, we found that caveolin-1 null mice have aberrant expression of OL- and myelin-specific proteins. Whereas CNPase is significantly increased in null versus wild type animals, there was only a marginal increase in MBP. It is unknown how caveolin-1 regulates OL- and myelin-specific protein expression. Thus, it will be important to determine how loss of caveolin-1 affects the number of OLs and their progenitors in vivo.

There is little experimental evidence for a role of caveolin-1 and/or caveolae in OL biology. It is known, however, that caveolin-1 expression is elevated in glial tumors of the CNS (Abulrob et al., 2004). Whether this increase in caveolin-1 expression is
causal or a result of cellular transformation remains to be determined. It has been reported that in Schwann cells, the myelinating cells of the peripheral nervous system, there is an increase in caveolin-1 protein expression during differentiation and a decrease after axonal injury (Mikol, 2002). Interestingly, after experimental autoimmune encephalomyelitis (EAE) caveolins-1, 2 and 3 are upregulated in spinal cord homogenates from mice (Shin et al., 2005). Thus, caveolin-1 likely plays an important role in Schwann cell myelination and may also play a similar role in OLs within the CNS.

In conclusion, the results of these studies suggest that caveolin-1 is important for the expression of OL- and myelin-specific proteins. That IGF-I induces caveolin-1 localization with CEMs and that the IGF-IR has a highly conserved CBM suggests a functional interaction between caveolae and the IGF-IR, which remains to be elucidated. Using siRNA strategy, future studies are designed to down regulate caveolin-1 protein levels to better define its role in IGF-I/IGF-IR signaling. Additional studies are designed to examine the effects of caveolin-1 knockdown on the expression of myelin- and OL-specific proteins and the number of OLs and their progenitors from the brains of these animals.
CHAPTER SIX

General Discussion
Summary of Thesis

In this dissertation we investigated the mechanisms whereby IGF-I mediates the sustained phosphorylation of Akt in oligodendrocyte progenitors (OPs). We demonstrated that clustering and internalization of the IGF-IR is required to promote Akt phosphorylation, whereas recycling is required to sustain Akt phosphorylation. Furthermore, mathematical analyses of our empirical data predicted a model of IGF-IR surface loss, followed by recovery, consistent with the sustained phosphorylation of Akt. Interestingly, this model revealed an additional kinetic state of the IGF-IR that was required after surface recovery but before further ligand binding. We also showed that the cholesterol chelating agent, MβCD, altered OP membrane integrity and blocked IGF-I mediated Akt phosphorylation, which were both reversed by cholesterol repletion. In addition, the isolation of cholesterol/glycosphingolipid-enriched membrane microdomains (CEMs) by two different methods demonstrated that PI-3K/Akt signaling proteins, as well as a small portion of the total IGF-IR pool, localized within these microdomains. To determine a role of caveolae lipid rafts in OL biology, we showed that caveolin-1 protein expression increases during OL maturation and that localization of caveolin-1 within CEMs correlated with IGF-I stimulation. Furthermore, the IGF-IR co-localized with caveolin-1 at a stage of OL maturation when caveolin-1 is maximally expressed, and contains a caveolin binding motif, which is evolutionarily conserved. Lastly, we observed an aberrant expression of myelin- and OL-specific proteins in caveolin-1 null mice.

The results of this dissertation support our hypothesis that IGF-IR trafficking and localization are required for IGF-I mediated sustained Akt phosphorylation in OPs. We
also provide evidence for an interaction between IGF-I signaling and caveolins. In what follows, we will further discuss the role of subcellular targeting and compartmentalization in the regulation of IGF-IR stability, phosphorylation, and the initiation of various biological responses through the PI-3K/Akt signaling pathway. Furthermore, we will discuss the implication of these findings on the development of therapies for demyelinating disorders, such as multiple sclerosis (MS).

**Role of Compartmentalization in long-term IGF-IR signaling and stability**

We previously demonstrated that IGF-I promotes long-term survival and protection of OPs from serum deprivation and glutamate toxicity via sustained Akt phosphorylation (Ness and Wood, 2002). These actions of IGF-I correlate with prolonged activity and stability of the IGF-IR (Ness et al., 2002). In contrast, neurotrophin (NT)-3 is unable to mediate long-term OP survival since ligand stimulation results in a transient phosphorylation of Akt and a down regulation of its cognate RTK, TrkC (Ness et al., 2002). We have shown similar effects of NT-3 in CG-4 progenitors (see appendix, figure A.1, pg. 125).

The ability of IGF-I to promote long-term activation of signal transduction pathways has been recently validated in a mouse-model of OPs (Cui et al., 2005). Sustained signal transduction, however, has not been reported for IGF-I in other cell types or for other growth factors. Therefore, IGF-I appears to have the unique ability to promote long-term signal transduction and survival in OLs.

The mechanisms that regulate the differential subcellular fates of the IGF-IR and TrkC are currently undefined. As previously discussed, the sorting of RTKs is generally
associated with several proteins including cbl, an E3 ubiquitin ligase, which is involved in targeting proteins to the lysosome, as well as members of the Rab protein family of small GTPase, which are involved in the budding and fusion of various endosomal compartments (Clague, 1998). In a recent study, Vecchione et al. demonstrated that the stability of the IGF-IR is regulated by association with Grb10, which promotes the recruitment of cbl to the plasma membrane, culminating in receptor ubiquitinylation and a four-fold decrease in receptor protein levels (Vecchione et al., 2003). Accordingly, we propose that the stability of IGF-IR during ligand stimulation in OPs is due to receptor compartmentalization, which restricts Grb10/cbl interactions and consequent ubiquitinylation. In contrast, RTKs that undergo down regulation after ligand stimulation, such as TrkC in response to NT-3, localize within regions of the plasma membrane where they are available for ubiquitinylation. This hypothesis is supported by our preliminary data, which demonstrates that the IGF-IR and TrkC are differentially compartmentalized in detergent resistant cell extracts (see appendix, figure A.2, pg 126). The IGF-IR localizes in both soluble and triton-insoluble fractions (TIF), whereas TrkC exclusively localizes in soluble fractions that contains the cytosolic protein tyrosine phosphatase (PTP) 1D. These data suggest that a portion of the total IGF-IR pool associates with membranes consistent with CEMs/lipid rafts.

In this dissertation we provided evidence for a role of CEMs in IGF-I mediated Akt phosphorylation, and also showed that PI-3K/Akt signaling proteins and a portion of IGF-IRs localize within these microdomains (see Chapter 4). Thus, we hypothesize that localization within these hydrophobic regions of the plasma membrane protects the IGF-IR from ubiquitinylation and consequently promotes IGF-I signaling. Our hypothesis is
further supported by a previously published study, which showed that the RTK Ret translocates into CEMs upon activation, and is protected from ubiquitinylation and degradation in sympathetic neurons (Pierchala et al., 2006). To test our hypothesis, we plan to: 1) validate Grb10/cbl interactions with Trk, 2) demonstrate that these interactions are exclusively in non-raft fractions, and 3) show that long-term disruption of IGF-IR from CEMs results in Grb/cbl interactions and receptor degradation.

The role of compartmentalization in signal transduction is also suggested by our mathematical analyses, which predicted an additional kinetic state of the IGF-IR at the cell surface following internalization and recycling (see Chapter 3, Figures 3.9-3.10). In chapter 3, we hypothesized that this transition from receptor recovery to ligand binding (RM4 → RM1) represents a putative tyrosine dephosphorylation of the IGF-IR. Whereas dephosphorylation is classically regarded as an endosomal event, there is evidence for the dephosphorylation of the IGF-IR at the cell surface via the SHP-2 tyrosine phosphatase (Maile and Clemmons, 2002). Alternatively, the transition from RM4 to RM1 may represent a movement of receptors from one microdomain to another at the cell surface. These hypotheses are not mutually exclusive, however, and may both contribute to the kinetics of receptor trafficking and sustained Akt phosphorylation. It was previously shown in Xenopus muscle cells that PTP activity is required for the dispersal of acetylcholine receptors at the cell surface (Dai and Peng, 1998). Based on these findings, we propose that the IGF-IR is recycled to the plasma membrane to a specific microdomain, in which SHP-2 or another putative PTP is localized. Here, IGF-IR clusters are dephosphorylated and redistributed at the plasma membrane for further ligand binding. The validation of this hypothesis first will require the identification of
the PTP that mediates IGF-IR dephosphorylation in OPs. Once identified, a dominant negative PTP may be transfected into OPs and the effects on IGF-IR cluster dispersal observed by confocal microscopy.

In summary, the differential trafficking of TrkC and the IGF-IR may contribute to mechanisms that regulate receptor degradation and stability. The stability of the IGF-IR and its continual ability to bind new ligand at the cell surface is an important mechanism that likely promotes the sustained phosphorylation of Akt, and thereby contributes to the survival of OPs.

**Role of Compartmentalization in the specificity of IGF-I signaling**

IGF-I is primarily a survival factor for OLs and their progenitors; however in combination with fibroblast growth factor (FGF)-2, IGF-I has a synergistic effect on the proliferation of these cells (Jiang et al., 2001). Current studies in our laboratory are also underway to understand the role of IGF-I in the differentiation of OPs to mature OLs. The fundamental goal of these research activities is to delineate the molecular mechanisms by which IGF-I promotes various biological responses through the activity of the PI-3K/Akt signaling pathway.

It is well characterized that subcellular compartmentalization of RTKs and their downstream effectors regulates the differential activity of signal transduction cascades and leads to diverse biological outcomes. The majority of studies report that the PI-3K/Akt and PKC pathways are exclusively activated at the plasma membrane due to the restricted availability of their upstream signaling partner, phosphatidylinositol bisphosphate (PI-4, 5-P₂), whereas the ERK pathway is activated both at the plasma
membrane and the endosome due to global accessibility of its upstream signaling partners, Shc, Ras, and Raf (Haugh, 2002; Figure 6.1). Much attention has been given to the localization of RTKs and their signaling proteins at the plasma membrane versus the endosome; however, less attention has been given to the spatial organization of these signaling proteins at the plasma membrane within specific microdomains.

The plasma membrane is comprised of various microdomains involved in signal transduction (i.e. caveolae), cell adhesion (i.e. gap junctions), and protein trafficking (i.e. clathrin-coated pits), which are morphologically distinct from the surrounding membrane (Baass et al., 1995; Blonder et al., 2004; Shigematsu et al., 2003). In addition to the identification of these microdomains, the use of biochemical methods and high resolution microscopy has facilitated the identification of smaller and morphologically featureless regions of the plasma membrane, including non-caveolae lipid rafts (termed CEMs in this dissertation).

The emerging and established literature has implicated CEMs in the organization of proteins for the specificity and fidelity of cellular responses to extracellular ligands by spatially organizing signaling machinery. Various isoforms of the signaling protein Ras, for example, have been localized to distinct membrane microdomains, including CEMs, via electron microscopy (Hancock and Prior, 2005). Similarly, in NIH3T3 cells, two pools of Src kinases were discovered after cholesterol depletion and CEM disruption (Veracini et al., 2005). In this study, Src proteins localized within CEMs were involved in PDGF mediated mitogenesis, whereas Srcs localized outside of these microdomains were involved in G-protein coupled receptor-mediated dorsal ruffle formation (Veracini et al., 2005). In these cells, the spatial organization of Src at the plasma membrane
determined receptor interactions and the specific biological responses mediated by this kinase. In this dissertation, we report that Akt localizes both to buoyant and non-buoyant sucrose fractions (see Chapter 4), likely representing two distinct pools of Akt. Based on our own and previously published studies, we propose that spatial organization of Akt into distinct plasma membrane microdomains may contribute to the various actions of IGF-I.

We further hypothesize that Akts partition into microdomains of the plasma membrane in an isoform-specific manner. Three isoforms of Akt have been identified in mammalian cells (Datta et al., 1999), which are differentially expressed during development. Knockout mice of each isoform have specific deficits in survival and development, suggesting that these kinases are not redundant (Cho et al., 2001; Yang et al., 2005). The paradigm of compartmentalization at the plasma membrane may determine the upstream transmembrane receptors that target Akt and specify the downstream substrates of this kinase, resulting in distinct biological outcomes.

Targeting to the plasma membrane is requisite for the phosphorylation and activity of Akt (Scheid et al., 2002). Thus, it is of considerable interest to understand the mechanisms that regulate the localization and activity of distinct Akt isoforms at the plasma membrane, particularly in terms of the survival and maintenance of cells within the CNS. It has been previously demonstrated that an increase in Akt-1 expression levels in cortical precursor cells enhances Akt activation after growth factor stimulation, leading to an increase in survival and proliferation (Sinor and Lillien, 2004). It remains to be seen how increases in Akt-2 and/or -3 affect responses of these precursor cells to growth factors.
Model of IGF-IR Internalization and Trafficking

Our model of IGF-IR internalization and trafficking is illustrated in figure 6.2. In this model, we propose that clustering of IGF-IRs is required for Akt activation, which is supported by our data demonstrating that dansylcadaverine blocked IGF-I mediated Akt phosphorylation (see Chapter 3). In addition, we propose that the activity of Akt is initiated within CEMs, since we observed that cholesterol depletion via MβCD also blocked IGF-I mediated Akt phosphorylation (see Chapter 4). This is supported by previous studies in various cell types, which report that CEM integrity is required for activation of the PI-3K/Akt pathway (Baron et al., 2003; Remacle-Bonnet et al., 2005).

In a recent study, ffrench-Constant and colleagues showed that PDGF-mediated PI-3K/Akt pathway activation occurs within CEMs of newly differentiated OLs, and that co-localization of PDGFα receptors and α6β1 integrins within CEMs amplifies the activity of this survival pathway (Decker and ffrench-Constant, 2004). Furthermore, in this study, disruption of CEMs with the sphingolipid synthesis inhibitor fumonisin B1 (FB1) blocked PDGFα receptor and integrin co-localization, and resulted in a decrease in PDGF-mediated survival of these cells (Decker and ffrench-Constant, 2004). Thus, these findings support the hypothesis that compartmentalization within CEMs is required for the specificity of signal transduction and biological outcomes in the OL lineage. In this dissertation, we showed that acute disruption of cholesterol-membrane integrity alters short-term IGF-I mediated signal transduction. Future studies are designed block cholesterol and/or sphingolipid biosynthesis, and determine its effects on long-term IGF-I signaling and the survival of OPs.
Our preliminary data in supplemental figure A.2 (see appendix) suggest that the IGF-IR and TrkC are differentially localized within membrane microdomains. As previously discussed, we propose that the segregation of these RTKs plays a role in their accessibility for ubiquitinylation and their differential subcellular fates. It is possible, however, that their segregation does not prevent the IGF-IR and TrkC from mediating the activity of the PI-3K/Akt pathway. It was previously determined that two phospholipase C (PLC)-coupled receptors, neurokinin A and endothelin, share the same pool of PI-4, 5-P$_2$ for PLC activation, although the neurokinin A receptor, but not the endothelin receptor, localized within CEMs (van Rheenen et al., 2005). Therefore, the differential compartmentalization of the IGF-IR and TrkC does not necessarily restrict their ability to utilize a homogenous pool of PI-4, 5-P$_2$ for the activity of the PI-3K/Akt pathway.

We demonstrated a loss of IGF-IRs from the cell surface during ligand stimulation; however, we did not identify the mechanism by which the IGF-IR internalizes. Whereas previously published studies have reported that IGF-IRs internalize through a clathrin-dependent mechanism in other cell types (Foti et al., 2004), our observation that the IGF-IR localizes within CEMs supports a role for these microdomains in the internalization and trafficking of the receptor in OPs. Nevertheless, we observed co-localization of the IGF-IR with transferrin receptor (TfR) and Rab11 positive endosomes, which is indicative of clathrin-mediated internalization (Touret et al., 2003). Although seemingly contradictory, these results are not mutually exclusive. Recent evidence indicates that there is overlap between CEM and clathrin-mediated internalization and trafficking (Shogomori and Futerman, 2001). Alternatively, it is
possible that CEMs are platforms for IGF-I mediated signal transduction but that internalization of the IGF-IR occurs via a clathrin-dependent mechanism. To test these hypotheses, we will measure IGF-IR surface availability and/or internalization after treating OPs with MβCD (causing lipid raft disruption) or treating OPs with hyperosmotic concentrations of sucrose (blocking receptor clustering into clathrin-coated pits; Veyrat-Durebex et al., 2005). To determine further if a clathrin-dependent mechanism is required for IGF-IR internalization, we will use siRNA strategies to down regulate clathrin and measure IGF-IR surface availability and internalization.

**Compartmentalization of Caveolin in OLs**

The expression of caveolin-1 and the formation of caveolae correlate with cellular maturation in various cell types. Furthermore, caveolae differentially regulate cellular responses to extracellular ligand during development (Nystrom et al., 1999). In Chapter 5, we showed that caveolin-1 increases in protein expression during OL maturation. We propose that this increase in protein corresponds to an increase in the formation of caveolae in OLs. Caveolin-1 null mice have no reported neurological phenotype, however our data suggest that these animals have aberrant expression of OL- and myelin-specific proteins (see Chapter 5). Notably, mice genetically engineered to inhibit cholesterol biosynthesis specifically in OLs have dysmyelination, tremors, and ataxia (Saher et al., 2005). Along with various other dysfunctions, cells from these mice are also likely deficient in caveolae, since cholesterol depletion results in the flattening of these membrane structures (Holleran et al., 2003). Thus, it is of considerable interest to determine if loss of caveolae plays at least a partial role in the neurological phenotypes of
these animals. Our observations that the IGF-IR co-localizes with caveolin-1, and that the IGF-IR has a highly conserved caveolin binding motif suggests a role of caveolae in the regulation of IGF-I signaling in OPs. This hypothesis, however, requires further validation.

**Implications for demyelinating disorders**

Multiple Sclerosis (MS) is a demyelinating disorder, characterized by the formation of white matter plaques in the CNS, and results in severe sensory and motor dysfunction (Wingerchuk et al., 2001). The direct cause is currently unknown; however, MS is traditionally regarded as an autoimmune disorder, which is associated with the loss of OLs and culminates in demyelination. At present, therapeutic strategies have focused on blocking the immune response and inflammation in patients with MS, using drugs such as corticosteroids, statins, and interferons (Fox and Ransohoff, 2004). Recent evidence, however, suggests that the immune response in MS is secondary to the loss of OLs (Chaudhuri and Behan, 2004). Based on these findings, immune-suppressive and anti-inflammatory therapeutics may not be fully effective in treating this disorder. Instead, the design of new therapies that prevent the loss of OLs may have better clinical success.

During early stages of MS, partial regeneration of myelin is observed; however, myelination fails over time (Franklin, 2002). The inability of myelination to proceed in the later stages of MS may be attributed to several factors, including failure of OPs to proliferate, migrate, or differentiate into mature OLs. Interestingly, OPs are found in the adult brain and in white matter plaques (Wolswijk and Noble, 1989). Theoretically, these progenitors have the potential to replace lost OLs. Thus, a better understanding of
mechanisms that regulate the survival and maintenance of OLs within the CNS will provide critical insight toward the development of novel therapeutics for the treatment of MS and other demyelinating disorders. As discussed throughout this dissertation, IGF-I has the unique ability to promote long-term survival of OPs through the sustained activation of the PI-3K/Akt pathway. Therefore, IGF-I/IGF-IR mediated signal transduction may be an important pharmacological target for intervention in MS.

Few therapies, if any, have been designed to promote signal transduction due to the high risk of cellular transformation. The refinement of signal transduction by the attenuation or augmentation of specific steps in receptor trafficking may be, however, a valid target for the design of novel therapeutics. As we have shown in this dissertation, clustering and internalization of the IGF-IR is required to promote Akt phosphorylation, whereas recycling of the IGF-IR is important to sustain the phosphorylation of Akt. Accordingly, in MS it may be important to promote IGF-I mediated signaling by augmenting the recycling of these receptors to the cell surface, resulting in OL survival. In addition, mechanisms of receptor sequestration into specific membrane microdomains, such as lipid rafts, may be a valid target for pharmacological intervention.

As previously mentioned, statins, a family of cholesterol biosynthesis inhibitors, have been used to treat the early stages of MS as these drugs are effective in decreasing MRI-detectable inflammation (Fox and Ransohoff, 2004). It has been demonstrated, however, that mice deficient in OL-cholesterol biosynthesis have dysmyelination, tremors, and ataxia (Sahe et al., 2005). Thus, the prolonged use of statins over time may be more detrimental than useful in the treatment of MS. Furthermore, it is likely that the inhibition of cholesterol has negative effects on the formation of caveolae structures,
which are required for a variety of cellular functions. Several studies have shown that caveolins, integral-membrane proteins required for the formation of caveolae, are up-regulated in crude spinal cord homogenates from mice after experimental autoimmune encephalomyelitis (Shin et al., 2005). Additionally, an increase in caveolin-1 phosphorylation, which is required for caveolae partitioning, was also shown in the spinal cords of mice after EAE (Kim et al., 2006). It can be speculated that an increase in caveolin expression and phosphorylation is compensatory, promoting caveolae signaling and cellular survival. Based on these data, the design of future therapies to treat MS may involve drugs that promote the formation of caveolae and caveolae-mediated signal transduction.

**Conclusions**

In the present chapter we have developed further our model of IGF-I mediated Akt phosphorylation by proposing that compartmentalization of the IGF-IR contributes to its long-term stability and activity, as well as to its ability to promote survival of OPs. We have hypothesized that the spatial organization of Akt isoforms at the plasma membrane determines the upstream transmembrane receptors that activate this kinase and specifies its downstream substrates, and is responsible, in part, for the diversity of signal transduction through the PI-3K/Akt pathway. We also have suggested that increases in caveolin-1 protein expression contribute to the formation of caveolae during OL development, which alter signaling responses to extracellular ligands during cellular development. Taken together, we propose that the subcellular targeting and compartmentalization of the IGF-IR, its downstream effectors, and caveolin-1 likely play
a role in the regulation of IGF-IR signal transduction, as well as the maintenance and survival of OLs and their progenitors. Furthermore, we propose that a better understanding of the mechanism by which IGF-I mediates the phosphorylation and activity of Akt in OPs may lend critical insight to the development of novel therapeutics used to treat MS and other demyelinating disorders.
Figure 6.1 Compartmentalization of Signal Transduction. (A) Limited accessibility of PIP2 and DAG result in the activation of the PI-3K/Akt and PKC pathways exclusively at the cell surface. (B) In contrast, the global accessibility of Shc, Ras, and Raf results in the activation of the ERK pathway at both the cell surface and plasma membrane. Adapted from Haugh, J.M. 2002. Localization of receptor-mediated signal transduction pathways: The inside story. *Mol Interv.*
Figure 6.2 Model of IGF-IR and TrkC Localization and Trafficking. IGF-IR internalization and recycling mediates sustained Akt phosphorylation. Stimulation of the TrkC leads to a transient phosphorylation of Akt and subsequent receptor degradation. We hypothesize that the differences in IGF-IR and TrkC subcellular fates is attributed to the differential compartmentalization of these RTKs, as well as to their ability to promote Akt phosphorylation and concomitant survival of OPs.
Appendix
Figure A.1 Stimulation of Trk results in receptor down regulation. CG-4 progenitors were stimulated with 5 ng/ml NT-3 for times indicated. Cells were harvested and lysates were processed for SDS-PAGE and Western blot analysis. Band densities for Trk were normalized to β-Actin. Data represent the mean ± SEM (n=3). A decrease in Trk protein levels was observed at 2hrs (p=0.003) and more significantly at 10hrs (p<0.0001). At 24hr, Trk levels begin to recover, but remain significantly lower than in control cells (p=0.013).
Figure A.2 Differential compartmentalization of TrkC and IGF-IR. Cells were harvested and lysates were incubated with 1% Triton X-100 as described in Materials and Methods. Triton insoluble fractions (TIFs) were separated from soluble material by centrifugation. TIF and soluble material were processed for SDS-PAGE and Western blot analysis for TrkC, IGF-IRβ, and protein tyrosine phosphatases (PTP)1D (a cytosolic protein).
References


Curriculum Vitae
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Education

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Research Publications


Romanelli RJ, Wood TL. Cholesterol-enriched membrane microdomain integrity is associated with IGF type-I receptor mediated Akt phosphorylation in oligodendrocyte progenitors. (in preparation)

Selected Abstracts


Honors & Awards

Young Investigator Educational Enhancement Award, ASN 37th annual meeting (2006)
Graduate Fellowship, Pennsylvania State University (2001)
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Professional Societies

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American Society for Neurochemistry (ASN)
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