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Analysis of the Role of SHIP2 in the Regulation of Preadipocyte Proliferation and Differentiation by PDGF

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Analysis of the Role of SHIP2 in the Regulation of Preadipocyte Proliferation and Differentiation by PDGF

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Thesis submitted to the Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the Ph.D. degree in Biochemistry

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ABSTRACT

Obesity, coupled with inadequate proliferation and/or differentiation of preadipocytes, favours development of adipose tissue dysfunction, characterized by hypertrophied, inflamed, and insulin-resistant adipocytes that predispose to type 2 diabetes and cardiovascular disease. Platelet-derived growth factor (PDGF) promotes proliferation and inhibits differentiation of preadipocytes, and thus might be relevant to preadipocyte fate and adipose tissue function. I hypothesized that SH2 domain-containing inositol 5-phosphatase 2 (SHIP2) modulates both proliferation and differentiation of preadipocytes by mediating PDGF signalling in these cells.

Two principal pathways regulating cell proliferation are PI3K/PI(3,4,5)P3/Akt and Shc/Ras/ERK1/2. SHIP2 is important for proliferation likely due to its ability to dephosphorylate PI(3,4,5)P3 and associate with Shc in response to PDGF. Indeed, overexpression of wild-type SHIP2 inhibits 3T3-L1 preadipocyte proliferation; however, the underlying mechanisms are unclear. My first objective was to determine the role of regulatory regions of SHIP2 for its anti-proliferative action. PKC inhibition attenuated PDGF-stimulated SHIP2 tyrosine phosphorylation and Shc association. In addition, disruption of the SH2 domain, the NPAYY motif, or a novel PDGF-responsive phosphorylation site, Thr958, reduced SHIP2 tyrosine phosphorylation and/or Shc association, but neither altered the anti-proliferative effect of SHIP2. In contrast, inactivation of the 5-phosphatase domain potentiated the ability of SHIP2 to inhibit preadipocyte proliferation. This effect was explained by attenuated PDGF signalling caused by a decrease in receptor levels.

My second objective was to characterize the negative effect of PDGF on adipocyte differentiation, and to determine the role of SHIP2 in this process. In 3T3-L1 preadipocytes, pro-adipogenic insulin stimulates production of PI(3,4,5)P3 only, whereas PDGF generates PI(3,4,5)P3 and PI(3,4)P2, suggesting activation of a 5-phosphatase. PDGF, but not insulin, leads to SHIP2 tyrosine phosphorylation and Shc association, suggesting that SHIP2 might mediate the anti-adipogenic action of PDGF. I established that PDGF impaired late markers of adipogenesis, and did not inhibit the required exit from the mitotic clonal expansion phase. Overexpression of catalytically inactive, dominant-negative SHIP2 accelerated differentiation and attenuated the anti-adipogenic effect of PDGF.

Overall, this study suggests that SHIP2 mediates PDGF signalling in preadipocytes, thereby affecting both proliferation and differentiation, two distinct processes modulated by PDGF in opposite directions.

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LIST OF ABBREVIATIONS

Standard one and/or three letter codes were used for amino acids.
Non-standard abbreviations are as follows:

BisI – bisindolylmaleimide I
BMI – body mass index
BSA – bovine serum albumin
cAMP – cyclic AMP
CDK – cyclin-dependent kinase
C/EBP – CCAAT enhancer-binding protein
CS – calf serum
CSF-1 – colony stimulating factor-1
DAG – diacylglycerol
DMEM – Dulbecco's modified Eagle's medium
DMSO – dimethyl sulfoxide
EGF – epidermal growth factor
ERK1/2 – extracellular signal-regulated kinase 1/2
FAS – fatty acid synthase
FBS – fetal bovine serum
FFA – free fatty acid
GPDH – glycerol-3-phosphate dehydrogenase
Grb2 – growth factor receptor-bound protein 2
GSK3β – glycogen synthase kinase 3β
HIAP2 – human inhibitor of apoptosis protein 2
IBMX – isobutylmethylxanthine
IGF-I – insulin-like growth factor I
IGFR – insulin-like growth factor receptor
IL – interleukin
IOD – integrated optical density
IR – insulin receptor
IRS – insulin receptor substrate
KRH – Krebs-Ringer-Hepes
MAPK – mitogen-activated protein kinase
MCE – mitotic clonal expansion
MCP-1 – macrophage chemoattractant protein-1
MCS – mesenchymal stem cell
PBS – phosphate-buffered saline
PDGF – platelet-derived growth factor
PDGFR – platelet-derived growth factor receptor
PDK – phosphoinositide-dependent kinase
PH – pleckstrin homology
PI – phosphatidylinositol
PI3K – phosphoinositide 3-kinase
PI3K-C2 – class II PI3K

PI(3,4)P2 – phosphatidylinositol-3,4-bisphosphate
PI(3,4,5)P3 – phosphatidylinositol-3,4,5-trisphosphate
PI4P – phosphatidylinositol-4-phosphate
PI(4,5)P2 – phosphatidylinositol-4,5-bisphosphate
PKC – protein kinase C
PPAR – peroxisome proliferator-activated receptor
PS – phosphatidylserine
PTB – phosphotyrosine binding
pTyr – phosphotyrosine
Rb – retinoblastoma
SAM – sterile alpha motif
SH2 – Src homology 2
SHIP2 – SH2 domain-containing inositol 5-phosphatase 2
SNP – single nucleotide polymorphism
Sos – son of sevenless
SREBP-1 – sterol regulatory element binding protein 1
STAT – signal transducer and activator of transcription
TAPP – tandem-PH-domain-containing protein
TGFβ – transforming growth factor β
TNFα – tumour necrosis factor α
TPA – 12-O-tetradecanoylphorbol-13-acetate
WHO – World Health Organization
WT – wild-type

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INTRODUCTION

Obesity

Definition of the problem

The overwhelming increase in the prevalence of obesity in recent years has caught the attention of the medical and scientific communities. Obesity is defined as excess accumulation of white adipose tissue, and is commonly assessed using the body mass index (BMI), which is calculated as the weight in kilograms divided by the square of the height in meters (WHO Expert Consultation, 2004). Individuals with BMI above 25 kg/m^2 are classified as overweight, while those with BMI above 30 kg/m^2 are obese. Even though BMI is a convenient measure that is roughly proportional to body fat mass, it is also influenced by lean body mass and does not reflect the anatomical distribution of fat. For this reason, other measurements, including waist circumference and waist to hip ratio, are sometimes used together with BMI to assess the extent of adiposity (Lau et al., 2007).

Although usually thought of as a problem of industrialized countries, obesity is also on the rise in developing and under-developed countries. The World Health Organization (WHO) actually coined the term 'globesity' to reflect the world-wide prevalence of this disease. The latest estimates from the WHO state that almost one tenth of the world population is obese, and a third is overweight (WHO, 2006). In Canada, the prevalence is even higher than the global average, with over 20% of adults classified as obese, and over 55% as overweight (Lau et al., 2007).

Obesity does not only affect adults. In fact, childhood obesity is also at an all-time high. In 2004, over one quarter of Canadian children and adolescents aged 2-17 were

overweight (Lau et al., 2007). This is particularly disturbing, considering that over 75% of overweight children become obese in adulthood (Freedman et al., 2001).

Potential causes

Excess deposition of adipose tissue occurs when energy intake is greater than energy expenditure. Over millenia, humans evolved efficient ways of storing energy in order to survive during times of food scarcity (Bellisari, 2008). However, the over-abundance of calorie-dense food combined with the sedentary lifestyle of today's population creates an 'obesogenic' environment which promotes excess accumulation of fat. In addition to environmental influences, obesity has a large genetic component (Barsh et al., 2000). Mutations in several genes, including leptin (Montague et al., 1997) and melanocortin 4 receptor (Vaisse et al., 1998; Yeo et al., 1998), have been linked to severe childhood-onset obesity. However, these rare monogenic causes of obesity account for a very small percentage of cases. Instead, obesity is usually referred to as a polygenic disorder, with a combination of susceptibility genes, as well as environmental factors determining adiposity (Farooqi and O'Rahilly, 2006; Mutch and Clément, 2006).

Associated comorbidities

The increasing prevalence of obesity is particularly disturbing due to its association with a number of deleterious health consequences, including type 2 diabetes, cardiovascular disease, hypertension, dyslipidaemia, osteoarthritis, polycystic ovarian syndrome, and some types of cancer (Conway and Rene, 2004). The precipitating condition for many of the above complications is insulin resistance, which refers to the inability of muscle, liver, and

adipose tissue to respond to insulin (Kopelman, 2000). Ineffective suppression of gluconeogenesis in the liver combined with reduced glucose uptake in muscle and adipose tissue lead to increased glucose levels in the blood. In addition to its role in glucose homeostasis, insulin also modulates lipid metabolism. In a state of insulin resistance, impaired suppression of lipolysis in the adipose tissue results in increased release of free fatty acids (FFA) that further contribute to diminished insulin sensitivity in muscle, liver, and adipose tissue itself. To compensate for the reduced insulin action, pancreatic β-cells initially enhance insulin secretion. However, when β-cells can no longer compensate, an individual develops overt type 2 diabetes, characterized by hyperglycaemia.

Thus, excess accumulation of adipose tissue can lead to insulin resistance in the whole organism, highlighting the importance of this tissue to whole-body metabolism. However, excess fat accumulation per se cannot fully explain obesity-associated complications, since only some obese individuals develop insulin resistance, while others remain metabolically healthy (Sims, 2001). Clearly, although measures are needed to reduce the prevalence of obesity in general, the immediate focus should be on the individuals at highest risk for metabolic complications.

Current treatment options

Since obesity is caused by chronic positive caloric balance, the straightforward approach to treatment of this condition is reduction in caloric intake and increase in physical activity. Unfortunately, long-term successful adherence to this weight loss approach is poor. Limited pharmacological therapies for obesity treatment are available (Eckel, 2008). However, these medications, which work by reducing either appetite or dietary fat

absorption, are often associated with multiple side effects and result in only modest weight reduction with no confirmed long-term mortality benefits (Lau et al., 2007). Finally, bariatric surgery is the most drastic approach reserved for morbidly obese patients (BMI $\geq$ 40 kg/m^2 or BMI $\geq$ 35 kg/m^2 with severe comorbidities), who require rapid weight loss to avoid imminent health problems (Lau et al., 2007). Accumulating evidence shows long-term cardiovascular protection and reduced mortality rates from obesity-related complications with this approach, but the procedures are technically demanding and can lead to serious side effects (Adams et al., 2007; Sjöström et al., 2007).

Overall, existing treatment options for obesity are very limited. However, even more importantly, current treatment is often missing an assessment of whether an obese individual is metabolically healthy or not. Unfortunately, it is still unclear why some obese individuals develop metabolic complications and some do not. A better understanding of adipose tissue expansion might reveal the molecular basis for the differences between healthy and dysfunctional fat.

White adipose tissue

Adipose tissue as an endocrine organ

Recent advances in the field of of adipose tissue biology have led to the use of the term "adipose organ", which reflects both structural and functional complexity of this tissue. The adipose organ, which consists primarily of white adipose tissue, is located in a variety of sites throughout the human body, with the majority being in the subcutaneous and some in the intra-abdominal depots (Gesta et al., 2007). Brown adipose tissue, which is mainly involved in non-shivering thermogenesis (Cannon and Nedergaard, 2004), is another

component of the adipose organ. Unlike unilocular white adipocytes, which store large amounts of triglycerides in a single lipid droplet, brown adipocytes are characterized by the presence of multiple lipid droplets, increased mitochondrial content, and the presence of uncoupling proteins. In humans, levels of brown adipose tissue decline shortly after birth, and hence do not contribute to the accumulation of adipose tissue seen in obesity. Small amounts of brown fat present in the adult may potentially be important for whole-body metabolism (Costford et al., 2007). The research presented here focuses on white adipose tissue.

White adipose tissue is a heterogeneous organ. In addition to adipocytes, which account for approximately 50% of the cell population, the stromovascular fraction of adipose tissue contains preadipocytes, endothelial cells, fibroblasts, macrophages, and other cell types (Bays et al., 2008). Adipose tissue is also innervated and receives input from the sympathetic, and possibly parasympathetic, nervous system (Bartness and Song, 2007).

In addition to its complex role in storing and releasing energy, adipose tissue is now also recognized to be a dynamic endocrine organ with important roles in the regulation of whole-body metabolism. Aside from storing triglycerides in their lipid droplets, adipocytes secrete numerous factors, termed adipokines, which can act either locally or enter the circulation to elicit effects at distal sites.

One of the best known adipokines is leptin, a hormone released almost exclusively from adipocytes (Rosen and Spiegelman, 2006). Leptin inhibits orexigenic and stimulates anorexigenic pathways in the hypothalamus leading to a reduction in food intake and an increase in energy expenditure. Adiponectin (also known as AdipoQ, ACRP30) is a well-known adipokine with insulin-sensititizing properties. Adiponectin appears to act primarily

via stimulation of AMP kinase activity in liver and muscle, leading to enhanced fatty acid oxidation in these tissues (Rosen and Spiegelman, 2006).

Aside from leptin and adiponectin, adipose tissue is a source of over 100 adipokines with diverse roles, including lipid and glucose homeostasis, angiogenesis, and regulation of blood pressure (Hauner, 2005; Trayhurn and Wood, 2004). The release of a wide variety of adipose tissue-derived factors into the circulation, together with the sensory innervation of this organ (Bartness and Song, 2007), allows adipose tissue not only to convey the state of adiposity to the brain, but also to modulate the metabolic state of the whole organism.

Adipose tissue expansion

Adipose tissue expansion is a balance between two distinct processes. The first is hypertrophy, which occurs via accumulation of triglycerides in the lipid droplets of existing adipocytes. The second is hyperplasia, characterized by an increased number of adipocytes due to proliferation and subsequent differentiation of preadipocytes into adipocytes. During early childhood, while adipose tissue is still forming, both processes are thought to occur (Knittle et al., 1979). Hypertrophy appears to always precede hyperplasia, suggesting that adipocytes enlarge until a certain critical volume is reached, at which point they may signal recruitment of preadipocytes. For the purposes of this thesis, the term "preadipocyte recruitment" is used to describe preadipocyte proliferation and differentiation. This is supported by the observation that conditioned medium from enlarged adipocytes induces preadipocyte proliferation to a greater extent than medium conditioned by smaller adipocytes (Marques et al., 1998).

In a lean individual, the total adipocyte number appears to be set during the initial adipose tissue expansion in childhood and adolescence, and remains constant throughout adulthood (Spalding et al., 2008). However, even in adults, adipose tissue undergoes continuous remodeling, with almost 10% of existing adipocytes dying and new adipocytes forming every year (Spalding et al., 2008).

The process of adipose tissue expansion is not as well understood in obesity. During chronic positive caloric balance, adipose tissue accommodates excess calories as triglycerides in the lipid droplets of adipocytes. The enlargement of fat stores requires an increase in adipocyte size, number, or both. It has been generally believed that childhood-onset obesity is associated with both hypertrophy and hyperplasia (Knittle et al., 1979). Indeed, compared to lean subjects, individuals with early-onset obesity appear to have a greater number of adipocytes starting in childhood, with this number gradually leveling off and remaining constant in adults (Spalding et al., 2008). The situation is less clear in adult-onset obesity. Several reports suggested that hypertrophy is the predominant route to fat gain in late-onset obesity (Brook et al., 1972; Hirsch and Batchelor, 1976; Salans et al., 1973); however, this might not be the case in all subjects (van Harmelen et al., 2003).

Adipose tissue dysfunction

Even though adipose tissue is well suited for the storage of excess energy, in many individuals, fat accumulation leads to insulin resistance and other metabolic complications. The inability of such dysfunctional adipose tissue to properly accommodate excess lipid results in ectopic fat storage in liver and muscle, which promotes insulin resistance in these organs and eventually leads to overt hyperglycaemia (Ravussin and Smith, 2002). The

development of insulin resistance and chronic low-grade inflammation in the adipose tissue itself further perpetuates accumulation of dysfunctional fat. Several factors are thought to contribute to the dysfunction of adipose tissue, including 1) distribution, 2) inflammatory state, and 3) the size of individual adipocytes in the tissue.

Contribution of adipose tissue distribution

Distribution of fat tissue in the body might partially explain differences in susceptibility to metabolic dysfunction. Central obesity, characterized by intra-abdominal or visceral adipose tissue accumulation, is associated with the development of insulin resistance and other metabolic complications much more commonly than peripheral obesity, i.e. fat accumulation in the femoral and gluteal subcutaneous regions (Després and Lemieux, 2006).

Adipocytes from visceral compared to subcutaneous depots have distinct metabolic characteristics. For example, compared to subcutaneous adipocytes, visceral adipocytes are less sensitive to the anti-lipolytic actions of insulin (Bolinder et al., 1983; Zierath et al., 1998). This leads to the release of increased amounts of non-esterified FFA, which promote development of insulin resistance in muscle, liver, as well as adipose tissue itself (Boden, 2002). Moreover, the physical location of the visceral depot might allow for the products of these adipocytes to drain directly to the liver via the portal vein, contributing directly to insulin resistance in the liver (Kabir et al., 2005). However, Nielsen and colleagues demonstrated that even in obese individuals only 20% of FFA in the portal vein are derived from visceral adipose tissue, while the rest come from subcutaneous fat (Nielsen et al., 2004).

In addition to increased FFA release, visceral adipose tissue also secretes an altered adipokine profile compared to subcutaneous fat. In particular, excess accumulation of visceral adipose tissue in obese subjects is associated with reduced levels of adiponectin, as well as increased amounts of a pro-inflammatory cytokine interleukin (IL) 6 (Cartier et al., 2008; Côté et al., 2005; Fontana et al., 2007). However, given that visceral adipose tissue constitutes less than 20% of the total body fat (Lafontan and Berlan, 2003), the relative contribution of adipocyte-derived metabolites from this depot to the overall metabolic health of the organism remains unclear.

Contribution of adipose tissue inflammation

Mounting evidence suggests that obesity is a state of chronic low-grade inflammation. Levels of C reactive protein have been found to be elevated in obese individuals with metabolic complications (Forouhi et al., 2001; Visser et al., 1999), suggesting that the pro-inflammatory state found in some obese subjects contributes to the development of insulin resistance. Adipose tissue of obese rodents and humans has been shown to release greater amounts of tumour necrosis factor α (TNFα), an inflammatory cytokine, than the lean counterparts (Hotamisligil et al., 1995; Hotamisligil et al., 1993). In addition to TNFα, adipose tissue of obese subjects has been shown to secrete an abundance of other pro-inflammatory cytokines, including IL-6 and IL-8 (Straczkowski et al., 2002; Vozarova et al., 2001).

Although adipocytes themselves are capable of secreting numerous cytokines, the majority of these molecules appear to come from the stromovascular cells, which include macrophages (Fain et al., 2004a; Fain et al., 2004b). Indeed, adipose tissue of obese mice

and humans exhibits dramatically enhanced macrophage infiltration, with macrophages constituting up to 50% of the total number of cells in adipose tissue of obese subjects compared to 5-10% in lean ones (Weisberg et al., 2003; Xu et al., 2003).

The mechanism of increased macrophage recruitment is not entirely clear. The majority of macrophages in adipose tissue appear to be localized to dying adipocytes (Cinti et al., 2005), suggesting that macrophage infiltration might be a protective mechanism. Perhaps, dying adipocytes signal recruitment of scavenging macrophages to minimize release of adipocyte debris (mainly lipid) into circulation. Recent evidence suggests that rapid accumulation of adipose tissue in *ob/ob* mice or mice on a high-fat diet is associated with local hypoxia (Rausch et al., 2008). Inadequate oxygen supply in turn promotes infiltration of macrophages and cytotoxic T-cells (Rausch et al., 2008), possibly accounting for adipose tissue inflammation characteristic of these mouse models of obesity. Furthermore, mature adipocytes secrete factors, including leptin and macrophage chemoattractant protein-1 (MCP-1), that directly signal recruitment of macrophages into adipose tissue (Curat et al., 2004; Kanda et al., 2006). Although all of the above possibilities are attractive, further testing is necessary to determine the initial events leading to macrophage recruitment.

Contribution of adipocyte hypertrophy vs. hyperplasia

As mentioned above, normal adipose tissue expansion involves both adipocyte hypertrophy and hyperplasia. Once the capacity of existing adipocytes is reached, preadipocyte recruitment is initiated, allowing for accommodation of fat in new adipocytes. However, if preadipocyte recruitment is somehow impaired, either at the stage of

preadipocyte proliferation or differentiation, excess lipid is forced to accumulate in the existing adipocytes as a compensatory measure. These hypertrophied adipocytes are metabolically unhealthy, due to development of insulin resistance and production of increased amounts of FFA, TNFα and IL-6, as well as reduced amounts of adiponectin (Skurk et al., 2006). Reduced plasma membrane cholesterol content observed in hypertrophied adipocytes and related inflammatory gene expression profiles might account for the development of insulin resistance associated with enlarged adipocytes (Le Lay et al., 2001).

Adipocyte hypertrophy was found to predict insulin resistance and type 2 diabetes, independently of other risk factors (Weyer et al., 2000). Moreover, the effectiveness of thiazolidinediones (TZD), which are insulin-sensitizing anti-diabetic agents, is in part attributed to their ability to stimulate adipocyte differentiation both *in vitro* and *in vivo*, i.e. promote hyperplasia (Adams et al., 1997; Hallakou et al., 1997; Kletzien et al., 1992). Thus, treatment with TZDs is often accompanied by accumulation of metabolically healthy adipose tissue that contributes to improved insulin sensitivity in the whole organism (Okuno et al., 1998).

Several lines of evidence support the notion of impaired preadipocyte recruitment in obesity with metabolic complications. Non-diabetic insulin-resistant relatives of type 2 diabetic subjects, as well as obese individuals with type 2 diabetes, demonstrate reduced expression of adipogenic genes and a predominance of enlarged adipocytes in their adipose tissue (Dubois et al., 2006; Yang et al., 2004). Tchoukalova et al. reported that subcutaneous preadipocytes committed to the adipocyte lineage were reduced in obese compared to lean women (Tchoukalova et al., 2007), suggesting a deficit in preadipocyte proliferation.

Furthermore, preadipocytes isolated from individuals with upper body (or central) obesity demonstrated reduced capacity for differentiation compared to subjects with lower body (or peripheral) adiposity (Tchoukalova et al., 2007). In addition, adipocytes isolated from obese patients with insulin resistance have reduced expression of differentiation markers compared to insulin-sensitive, but equally obese subjects (McLaughlin et al., 2007). Hence, adipose tissue dysfunction appears to be associated with reduced recruitment of preadipocytes and their subsequent differentiation to adipocytes.

Integrated view of adipose tissue dysfunction

Although adipose tissue distribution, macrophage infiltration, and adipocyte hypertrophy might serve as independent explanations for why some obese individuals develop insulin resistance whereas others do not, these factors might well be interconnected. For example, the increased susceptibility of visceral adipocytes to apoptosis (Prins et al., 1994) might explain why visceral adipose tissue of genetically obese mice (*ob/ob* and *db/db*) has more crown-like structures, composed of dead adipocytes surrounded by macrophages, than subcutaneous fat (Murano et al., 2008). In addition, Drolet et al. demonstrated that only the subcutaneous depot showed adipocyte hyperplasia, whereas hypertrophy was observed in both subcutaneous and visceral regions (Drolet et al., 2008). Hence, the ability of subcutaneous but not visceral adipose tissue to expand via new adipocyte formation might explain why central but not peripheral obesity is associated with insulin resistance.

In addition to secreting increased levels of various pro-inflammatory cytokines, hypertrophied adipocytes also release greater amounts of MCP-1 (Skurk et al., 2006), and hence might recruit more macrophages compared to small adipocytes. In turn, macrophages

have been shown to impair adipocyte differentiation (Constant et al., 2006; Lacasa et al., 2007). This might lead to an even greater predominance of inflamed hypertrophied adipocytes, further exacerbating the state of insulin resistance.

All of the above factors likely contribute to dysfunctional adipose tissue, and adipocyte hypertrophy appears to be critical to the development of insulin resistance. Thus, it is necessary to understand the molecular mechanisms of preadipocyte recruitment and to explore potential mediators of this process.

Preadipocyte models

The adipocyte lineage can be traced back to a multipotent mesenchymal stem cell (MSC) with the capacity to also form bone, cartilage and muscle (Gesta et al., 2007). MSC gives rise to a precursor (adipoblast) cell that becomes a committed preadipocyte that can differentiate into a mature adipocyte. Knowledge of the adipocyte lineage allowed development of several models for the study of adipocyte formation *in vitro*.

3T3 and C3H10T1/2 are two multipotent cell lines that mimic MSC in that they can be differentiated into adipocytes, chondrocytes, or osteocytes with appropriate stimulation (Taylor and Jones, 1979). The 3T3 cell line was derived from 17 to 19 day-old disaggregated mouse embryos (Todaro and Green, 1963). The rare spontaneous conversion of these cells to adipocytes allowed establishment of 3T3-L1 and 3T3-F442A preadipocyte cell lines by clonal isolation (Green and Kehinde, 1974, 1976). Ob1771 is another preadipocyte cell line derived from the epididymal fat pads of an adult *ob/ob* mouse (Négrel et al., 1978). Although both differentiated 3T3-L1 and 3T3-F442A adipocytes possess all of the morphological features of a typical adipocyte (Green and Meuth, 1974), implantation of

only 3T3-F442A preadipocytes in mice leads to formation of normal fat pads (Green and Kehinde, 1979). This is in part due to reduced expression of anti-adipogenic Wnt-10b in 3T3-F442A compared to 3T3-L1 preadipocytes (Ross et al., 2000). Both 3T3-L1 and 3T3-F442A are fibroblast-like cells that are thought to be committed to the adipocyte lineage, although some plasticity might still be possible for 3T3-L1 cells (Charrière et al., 2003). However, 3T3-F442A preadipocytes appear to be further along the differentiation pathway than 3T3-L1 cells, making them less useful for the study of proximal events in preadipocyte recruitment.

The use of these immortalized mouse cell lines has several limitations, including species difference, their embryonic origin, as well as aneuploidy (Green and Kehinde, 1974; Todaro and Green, 1963). The utilization of primary preadipocytes isolated from the stromovascular fraction of human adipose tissue circumvents these issues. However, although primary preadipocytes can be successfully differentiated in culture, this process is longer than for 3T3-L1 preadipocytes (14-21 vs. 6 days). In addition, the limited availability of the starting material, the finite ability to passage isolated cells, as well as significant variation in the differentiating potential between donors hinders the use of this model system for the study of molecular mechanisms. Hence, the 3T3-L1 model system, used by many in the field of adipogenesis, was chosen to analyze the mechanisms of preadipocyte recruitment and factors regulating this process.

PDGF as a physiologically relevant regulator of preadipocyte recruitment

Both proliferation and/or differentiation of preadipocytes can be modulated by numerous molecules *in vitro*. However, the physiological role of these factors in adipose

tissue function is often unclear. Several lines of evidence suggest that platelet-derived growth factor (PDGF) is potentially a physiologically relevant regulator of preadipocyte recruitment.

PDGF is one of the most potent mitogenic components of the serum, and is known to stimulate proliferation of mesenchymal cells, including fibroblasts and preadipocytes (Bachmeier and Löffler, 1995; Heldin and Westermark, 1999). Moreover, PDGF inhibits differentiation of 3T3-L1 as well as primary human preadipocytes (Hauner et al., 1995; Hayashi et al., 1981). The importance of this growth factor in the regulation of preadipocyte fate is suggested by the finding that mRNA and protein levels of the PDGF receptor (PDGFR) decline following induction of adipocyte differentiation (Summers et al., 1999; Vaziri and Faller, 1996; Whiteman et al., 2003). In addition, PDGFR expression correlates with adipocyte size (Blüher et al., 2004).

PDGF was originally discovered as a component of serum that was absent in plasma, pointing to platelets as the storage site for this growth factor (Ross and Vogel, 1978). However, since the initial isolation of PDGF from platelets, it has become clear that many other cell types, including fibroblasts and macrophages, also secrete this growth factor (Heldin and Westermark, 1999). Since macrophage infiltration is enhanced in metabolically unhealthy obese individuals, these macrophages could provide a local source of PDGF. Indeed, a recent study by Pang et al. indicated that PDGF levels are elevated in the plasma and adipose tissue of obese mice, and that macrophages are the main source of PDGF in adipose tissue (Pang et al., 2008). Moreover, Nishimura et al. demonstrated the presence of activated platelets in the adipose tissue of obese mice (Nishimura et al., 2008), once again pointing to a potential local source of PDGF.

When PDGF was systemically administered to estrogen-deficient rats to analyze its effects on bone formation, PDGF-treated rats displayed dramatically diminished percentage of body fat compared to controls, despite similar weight gain (Mitlak et al., 1996). Although the mechanisms of adipose tissue reduction were not examined, these findings provide a further indication that PDGF is a physiologically relevant regulator of adipose tissue growth.

Preadipocyte proliferation

PDGF signalling

Prior to their differentiation to lipid-laden adipocytes, preadipocytes possess all of the features of a typical fibroblast (Green and Meuth, 1974). Treatment of fibroblasts with PDGF or serum initiates a series of intracellular signal transduction cascades that lead to the progression from the G_1 to the S phase of the cell cycle, resulting in DNA synthesis (Jones and Kazlauskas, 2001b). Moreover, PDGF prevents growth arrest in the G_0 phase. Once the cells are past the restriction point, which occurs in late G_1 phase, they are committed to complete the cell cycle and do not require further addition of the growth factor until the next G_1 phase. In addition to proliferation, PDGF might also exert other effects, such as chemotaxis and prevention of apoptosis, although these will not be addressed here (Staiger and Löffler, 1998; Vaziri and Faller, 1996).

PDGFR activation in preadipocytes

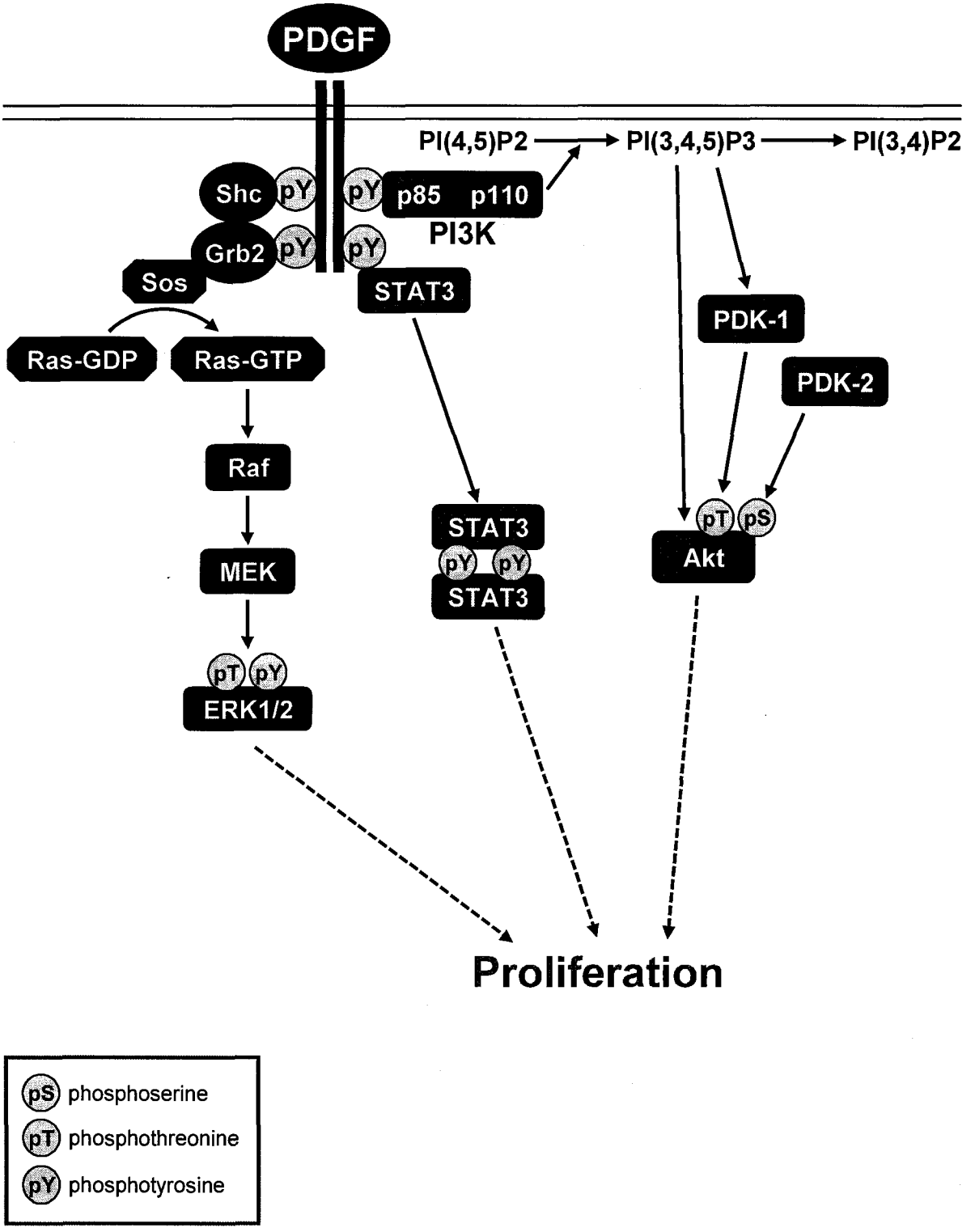
PDGF exists as homo- or heterodimers of disulfide-linked polypeptide chains (Heldin and Westermark, 1999). Although five isoforms (AA, AB, BB, CC, DD) of this growth factor are currently known, only the classical isoforms (PDGF-AA, -AB, and -BB)

have been studied in detail (Li and Eriksson, 2003). PDGF dimers bind to two structurally-related Tyr kinase receptors, α and β, resulting in their homo- or heterodimerization. PDGFRβ can only bind the B chain of PDGF, whereas PDGFRα can bind either PDGF-A or PDGF-B.

Similarly to other murine fibroblast cell lines, 3T3-L1 preadipocytes express predominantly PDGFRβ, with α receptors constituting less than 20% of total PDGFR levels (Vaziri and Faller, 1996). Since PDGF-BB is the only universal ligand for all PDGFR combinations, this is the isoform that was used to study preadipocyte recruitment.

PDGFR is a membrane-spanning protein with an extracellular ligand-binding domain, and an intracellular Tyr kinase domain (Heldin and Westermark, 1999). Ligand-induced PDGFR dimerization stimulates the Tyr kinase activity of the receptor, leading to autophosphorylation of the two receptors in *trans*. Phosphorylation of Tyr residues potentiates receptor kinase activity. In addition, Tyr phosphorylation creates multiple docking sites for proteins with Src homology 2 (SH2) domains, which are conserved stretches of approximately 100 amino acid residues that recognize phosphotyrosine residues in sequence-specific context (Machida and Mayer, 2005). Recruitment of SH2 domain-containing proteins leads to activation of distinct as well as overlapping downstream events. In particular, activation of the phosphoinositide 3-kinase (PI3K), extracellular signal-regulated kinase 1/2 (ERK1/2), and signal transducers and activators of transcription (STAT) family of proteins is thought to be important for the mitogenic effects of PDGF (Fig. 1).

Figure 1. Mitogenic PDGF signalling in preadipocytes. PDGF binding to PDGFR induces dimerization and trans-autophosphorylation of the two receptors. This is followed by recruitment of SH2 domain-containing proteins that initiate a series of signal transduction cascades resulting in proliferation. The PI3K/PI(3,4,5)P3/Akt cascade starts with recruitment of the p85 regulatory subunit of PI3K, which stimulates activation of the p110 catalytic subunit, resulting in the production of PI(3,4,5)P3 and its by-product PI(3,4)P2. PI(3,4,5)P3 recruits Akt and kinases PDK-1 and PDK-2, that phosphorylate Akt onThr308 and Ser473, respectively, thereby activating it. The ERK1/2 pathway is initiated when Grb2 binds to PDGFR, either directly or via Shc, which allows the nucleotide exchange factor Sos to activate Ras by promoting exchange of GDP for GTP. Activated Ras initiates a kinase cascade that ultimately leads to activation of ERK1/2 by phosphorylation of Thr202 and Tyr204. Finally, recruitment of STAT3 allows for Tyr phosphorylation of this protein either directly by PDGFR or via another kinase. This leads to STAT3 dimerization and activation. Ultimately, activated Akt, ERK1/2 and STAT3 modulate expression and/or stability of a variety of proteins involved in G_1/S progression. See text for further details and abbreviations.


PDGF
PI(4,5)P2
PI(3,4,5)P3
PI(3,4)P2
Shc
pY
pY
p85
p110
PI3K
Grb2
pY
pY
Sos
STAT3
PDK-1
PDK-2
Ras-GDP
Ras-GTP
Raf
MEK
pT
pY
ERK1/2
STAT3
pY
pY
STAT3
pT
pS
Akt
Proliferation
pS phosphoserine
pT phosphothreonine
pY phosphotyrosine

PI3K pathway

In mammalian cells PI3K is subdivided into three classes, some of which have multiple isoforms (Vanhaesebroeck et al., 2001). However, class IA PI3K (from now on referred to as PI3K) is the enzyme with an established role in cell proliferation, and will be the focus of this study. PI3K is a dimer of a p110 (α, β, or δ) catalytic subunit and a regulatory adaptor subunit (Hiles et al., 1992; Vanhaesebroeck et al., 1997). At least seven adaptor proteins generated by alternative splicing of three different genes (p85α, p85β, and p55γ) are known to date, with p85α being the most common regulatory subunit (Vanhaesebroeck et al., 2001). p85 dimerizes and thereby inhibits the p110 catalytic subunit. Following PDGFR activation, p85 is recruited to phosphotyrosine residues of the receptor via its SH2 domain. This leads to a conformational change allowing for activation of p110. Mutation of the Tyr residues responsible for PI3K recruitment abolished PDGF-induced DNA synthesis, confirming the importance of this pathway for the mitogenic effects of PDGF (Fantl et al., 1992).

Recruitment of PI3K to the receptor places this enzyme in close proximity to its substrate, phosphatidylinositol-4,5-bisphosphate, PI(4,5)P2, and allows production of phosphatidylinositol-3,4,5-trisphosphate, PI(3,4,5)P3, as well as its by-product phosphatidylinositol-3,4-bisphosphate, PI(3,4)P2. PI(3,4,5)P3 is a key second messenger that recruits downstream targets via pleckstrin homology (PH) domains (Lemmon, 2008). One of the key molecules recruited by PI(3,4,5)P3 is a Ser/Thr kinase Akt (also known as protein kinase B). Full activation of Akt requires phosphorylation of Thr308 and Ser473 residues by phosphoinositide-dependent kinase (PDK) 1 and PDK-2, respectively (Bellacosa

et al., 1998). The identity of PDK-2 remains unclear, although integrin-linked kinase and mammalian target of rapamycin complex 2 are possible candidates (Sale and Sale, 2008).

Akt is thought to promote cell proliferation by acting on several players during the G_1 phase of the cell cycle. In response to growth factor treatment, cyclin D1 assembles with and thereby activates cyclin-dependent kinases (CDK) 4 and 6 (Sherr, 1994). Active cyclin D1/CDK4 or 6 complexes partially phosphorylate retinoblastoma (Rb) protein, which is a member of the pocket protein family, allowing for the release of E2F family transcription factors. This promotes transcription and accumulation of cyclin E, which works with CDK2 to further phosphorylate Rb and release more E2F. Subsequently, E2F drives transcription of genes necessary for entry into the S phase of the cell cycle, which is characterized by DNA replication.

Glycogen synthase kinase 3β (GSK3β)-dependent phosphorylation of cyclin D1 promotes its proteasomal degradation (Diehl et al., 1998). Akt phosphorylates and thereby inhibits GSK3β (Cross et al., 1995), which results in stabilization of cyclin D1. In addition, Akt phosphorylates members of the FoxO family of transcription factors, which normally drive transcription of a CDK inhibitor $p27^{Kip1}$ (Medema et al., 2000). Akt-dependent phosphorylation sequesters these transcription factors outside of the nucleus, leading to reduced $p27^{Kip1}$ levels. Thus, PDGF-induced activation of the PI3K/PI(3,4,5)P3/Akt pathway promotes the G_1 to S phase transition by affecting both cyclin and CDK inhibitor levels.

ERK1/2 pathway

Activation of ERK1/2 (also known as p42/44 mitogen-activated protein kinase, MAPK) begins with the recruitment of the adaptor protein growth factor receptor-bound protein 2 (Grb2) via its SH2 domain either directly to the PDGFR, or via another SH2-domain containing adaptor protein Shc (Heldin and Westermark, 1999). Grb2 is associated with a nucleotide exchange factor, son of sevenless (Sos), that activates Ras by promoting exchange of GDP for GTP. The active Ras-GTP recruits and activates Raf (MAPK kinase kinase). This initiates a series of phosphorylation events, leading to activation of MEK (MAPK kinase), and ultimately, ERK1/2. Both Thr and Tyr phosphorylation is necessary for full activation of ERK1/2 (Payne et al., 1991). Active ERK1/2 then Ser/Thr phosphorylates various targets both in the cytoplasm and in the nucleus.

ERK1/2 is necessary for growth factor-induced proliferation (Pagès et al., 1993). ERK1/2 promotes progression through the G_1 phase of the cell cycle in part by increasing expression of cyclin D1 mRNA (Lavoie et al., 1996). ERK1/2 also promotes the assembly of cyclin D1/CDK4 complexes by phosphorylating Hsc70, a chaperone mediating this process (Diehl et al., 2003). In addition, ERK1/2 stabilizes the c-Myc protein that drives transcription of many genes necessary for proliferation (Sears et al., 2000).

STAT pathway

3T3-L1 preadipocytes express several STAT isoforms, including STATs 1, 3, 5 and 6 (Harp et al., 2001). Although usually considered mediators of cytokine signalling, the STAT family of transcription factors can also be activated by receptor Tyr kinases, including PDGFR. Various STATs have been shown to be important for proliferation in a

cell-type dependent manner. In particular, STAT3 has been implicated in preadipocyte proliferation (Deng et al., 2000).

Activated PDGFR can directly recruit STAT proteins via their SH2 domains (Heldin and Westermark, 1999). STAT transcription factors are activated by phosphorylation, which induces their dimerization and translocation to the nucleus. PDGFR can phosphorylate and thereby activate STATs directly or via another Tyr kinase Src (Wang et al., 2000). Once in the nucleus, STAT proteins can directly drive transcription of their target genes. With respect to proliferation, Bowman et al. demonstrated that PDGF-stimulated STAT3 activation induced expression of c-Myc, which was necessary for cell cycle progression from G_1 to S phase (Bowman et al., 2001).

PDGFR inactivation

The most fascinating feature of cellular signal transduction mechanisms is the built-in ability to control the duration and the intensity of the signalling event. For example, along with Tyr phosphorylation of various downstream targets, activated PDGFR also recruits Tyr phosphatases, such as SH2 domain-containing tyrosine phosphatase-2, which can dephosphorylate and hence inactivate the receptor and its substrates (Markova et al., 2003). In addition, following ligand binding, PDGFR is rapidly internalized. The endosomal PDGFR continues to be active (Sorkin et al., 1993; Wang et al., 2004), but eventually is degraded in the lysosomes. Following PDGF binding, PDGFR is also ubiquitinated (Mori et al., 1993). This promotes efficient internalization and degradation of the receptor, and negatively regulates the mitogenic effects of PDGF (Mori et al., 1993).

Compartmentalization of PDGFR in different membrane subdomains could be another potential mechanism for attenuation of PDGFR signalling (Matveev and Smart, 2002).

Aside from these common inhibitory pathways, not a lot is known about regulation of PDGF signalling in preadipocytes. PDGF-mediated proliferation of 3T3-L1 preadipocytes was inhibited by IL-4 and TNFα, but the molecular mechanisms of these events are unclear (Hua et al., 2004; Molander et al., 2000). Unfortunately, preadipocyte proliferation is often neglected despite its clear importance to adipose tissue biology. Hence, further understanding of the factors that can modulate PDGF signalling in preadipocytes is necessary.

SHIP2 as a mediator of PDGF signalling

SH2 domain-containing inositol 5-phosphatase 2 (SHIP2) is a ubiquitously expressed member of the inositol polyphosphate 5-phosphatase family that dephosphorylates PI(3,4,5)P3 to produce PI(3,4)P2 (Pesesse et al., 1998). Studies examining the physiological role of SHIP2, as well as its role as a signalling intermediate provide reasons to consider the potential involvement of SHIP2 as a mediator of PDGF signalling in preadipocytes.

Physiological role of SHIP2

SHIP2-null mice are viable, survive into adulthood, and do not develop any gross abnormalities (Sleeman et al., 2005). However, these animals grow more slowly than the wild-type littermates, and display reduced levels of serum triglycerides, non-esterified FFA, and cholesterol, without significant alterations in glucose or insulin levels. Hence, under normal conditions, these mice appear to be metabolically healthier than controls. However,

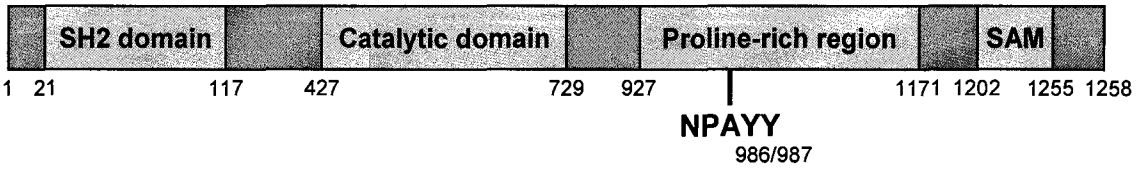
the most striking phenotype of SHIP2-null mice is that these animals are resistant to diet-induced obesity. Unlike wild-type littermates, SHIP2-null mice do not gain fat mass and remain insulin-sensitive following 6 weeks on a high fat diet. Clearly, the absence of SHIP2 influences adipose tissue expansion, although the molecular mechanisms of this effect were not addressed.

Several studies also found association between SHIP2 gene polymorphisms and the metabolic syndrome, type 2 diabetes, or impaired fasted glycaemia in human subjects (Ishida et al., 2006; Kagawa et al., 2005; Kaisaki et al., 2004; Marion et al., 2002). Moreover, increased SHIP2 expression in epididymal fat tissue and skeletal muscle was associated with insulin resistance of *db/db* mice, which are characterized by development of type 2 diabetes due to mutation of the leptin receptor (Hori et al., 2002). Mice with transgenic overexpression of wild-type SHIP2 (WT) develop mild glucose intolerance and insulin resistance, as well as gain slightly more weight compared to controls on the normal chow diet (Kagawa et al., 2008). Overall, this is consistent with a role for SHIP2 in the regulation of insulin sensitivity and adipose tissue biology.

SHIP2 structure and regulation

Human SHIP2 consists of 1258 amino acids, and shares approximately 95% identity with mouse SHIP2 (Pesesse et al., 1997; Schurmans et al., 1999). In addition to its central 5-phosphatase domain, SHIP2 possesses several regions that could potentially mediate protein-protein interactions (Fig. 2). In fact, it has been demonstrated that SHIP2 can associate with $p130^{Cas}$ adaptor protein via its N-terminal SH2 domain (Prasad et al., 2001). A proline-rich region, which is recognized by Src homology 3 (SH3) domain-containing

Figure 2. Structural organization of human wild-type SHIP2. Numbers refer to amino acid residues.


SH2 domain
Catalytic domain
Proline-rich region
SAM
1
21
117
427
729
927
1171
1202
1255
1258
NPAYY
986/987

proteins, participates in binding to filamin (Dyson et al., 2001), Abl (Wisniewski et al., 1999), and c-Cbl associated protein (Vandenbroere et al., 2003). Within the proline-rich region, SHIP2 possesses an NPAYY motif, which when phosphorylated, interacts with phosphotyrosine binding (PTB) domain-containing proteins (Uhlik et al., 2005). This consensus sequence was important for the interaction of SHIP2 with Shc (Prasad et al., 2002). Sterile alpha motif (SAM), which allows oligomerization with other SAM-containing proteins, is another domain present in SHIP2 (Schurmans et al., 1999). This domain was necessary for the interaction between SHIP2 and a GTPase activating protein Arap3 (Raaijmakers et al., 2007), as well as the EphA2 receptor (Zhuang et al., 2007).

The precise role or cellular consequences of the above interactions is unclear, although they might be involved in the appropriate localization of SHIP2 and/or in modulation of its activity. For example, the association between SHIP2 and proteins involved in cytoskeleton organization, such as $p130^{Cas}$, filamin, and vinexin, might explain the localization of SHIP2 to focal contacts and lamellipodia following cell adhesion (Dyson et al., 2001; Paternotte et al., 2005; Prasad et al., 2001). In turn, the localization of SHIP2 to these areas of actin remodelling, together with its 5-phosphatase activity, are important forthe ability of SHIP2 to potentiate cell spreading (Dyson et al., 2001; Koch et al., 2005; Paternotte et al., 2005; Prasad et al., 2001).

A number of studies demonstrated that various cytokines and growth factors, as well as cell adhesion, stimulate SHIP2 Tyr phosphorylation (Habib et al., 1998; Prasad et al., 2001; Wisniewski et al., 1999). Although the identity of the kinase(s) responsible for this effect remains unclear, the Tyr kinase Src was implicated in mediating SHIP2 phosphorylation *in vitro* and under certain conditions *in vivo* (Prasad et al., 2002; Taylor et

al., 2000). Whether SHIP2 Tyr phosphorylation is important for its function as a 5-phosphatase is a subject of debate, and likely depends on the cell type and stimulus used (Batty et al., 2007; Blero et al., 2001; Giuriato et al., 2002; Pesesse et al., 2001; Taylor et al., 2000).

The effect of SHIP2 on mitogenic signalling

Aside from its role in cell adhesion, SHIP2 is ideally suited to regulate growth factor signalling. First of all, SHIP2 dephosphorylates PI(3,4,5)P3 to produce PI(3,4)P2, and thereby fits into the PI3K/PI(3,4,5)P3/Akt signalling cascade. Secondly, SHIP2, by virtue of its association with Shc, might modulate the ERK1/2 pathway. In fact, numerous studies demonstrated effects of SHIP2 on the activation of Akt, ERK1/2, or both. For example, overexpression of SHIP2 inhibits Akt phosphorylation in response to epidermal growth factor (EGF) and PDGF in COS-7 cells and primary astrocytes, respectively (Pesesse et al., 2001; Taylor et al., 2000; Wada et al., 2001). Choi et al. demonstrated an inhibition of ERK1/2 phosphorylation basally and in response to IL-6 treatment of SHIP2-overexpressing OPM2 cells (Choi et al., 2002). In addition, both Akt and ERK1/2 phosphorylation was reduced following stimulation by insulin in CHO-IR cells (Blero et al., 2001; Kagawa et al., 2005), and by PDGF or insulin-like growth factor I (IGF-I) in rat vascular smooth muscle cells overexpressing SHIP2 (Sasaoka et al., 2003).

Several studies have implicated SHIP2 in the regulation of mitogenesis. SHIP2 overexpression inhibited proliferation of glioblastoma and chronic myeloid leukemia cell lines (Giuriato et al., 2002; Taylor et al., 2000), whereas overexpression of a catalytically inactive, dominant-negative SHIP2 enhanced proliferation of the rat insulin-producing cell

line INS1E (Grempler et al., 2007). The anti-proliferative action of SHIP2 observed in these studies is not surprising given the effects of this protein on the two major mitogenic pathways.

Role of SHIP2 in preadipocyte proliferation

SHIP2 regulation and its role in 3T3-L1 preadipocytes have not been extensively studied. We have previously demonstrated that PDGF stimulates SHIP2 Tyr phosphorylation and association with Shc in these cells (Gagnon et al., 2003). Hence, SHIP2 could influence PDGF-stimulated activation of the ERK1/2 pathway. PDGF treatment of 3T3-L1 preadipocytes also leads to accumulation of PI(3,4)P2, in addition to PI(3,4,5)P3 (Gagnon et al., 1999; Sorisky et al., 1996), suggesting that SHIP2 might be activated in this context. Thus, SHIP2 could also affect PDGF-stimulated activation of the Akt pathway by modulating phosphoinositide levels.

Given the potential role of SHIP2 in both the PI3K/PI(3,4,5)P3/Akt and the Shc/Ras/ERK1/2 pathways, this 5-phosphatase might be a negative regulator of PDGF-mediated preadipocyte proliferation. In fact, we have previously demonstrated that overexpression of SHIP2 WT in 3T3-L1 preadipocytes consistently reduces serum-based proliferation by approximately 20-30% compared to empty vector (Gagnon et al., 2003), although the mechanism of this effect remains unknown.

Objective 1

My first objective was to determine the role of the regulatory regions of SHIP2 in its anti-proliferative action. To address this question, I assessed the contribution of SHIP2

Ser/Thr phosphorylation, its protein-protein interaction motifs, as well as its catalytic activity. These studies will be presented in Chapter I and were in part published in (Artemenko et al., 2005), (Artemenko et al., 2007), and (Artemenko et al., 2008).

Adipocyte differentiation

Adipogenic program

Preadipocyte recruitment, necessary for adipocyte hyperplasia, encompasses proliferation of preadipocytes followed by their differentiation into mature lipid-laden adipocytes. The process of adipocyte differentiation, referred to as adipogenesis, has been extensively studied in immortalized preadipocyte cell lines. The cascade of events taking place during 3T3-L1 adipogenesis closely approximates the events that occur during adipogenesis *in vivo*.

Transcriptional control of adipogenesis

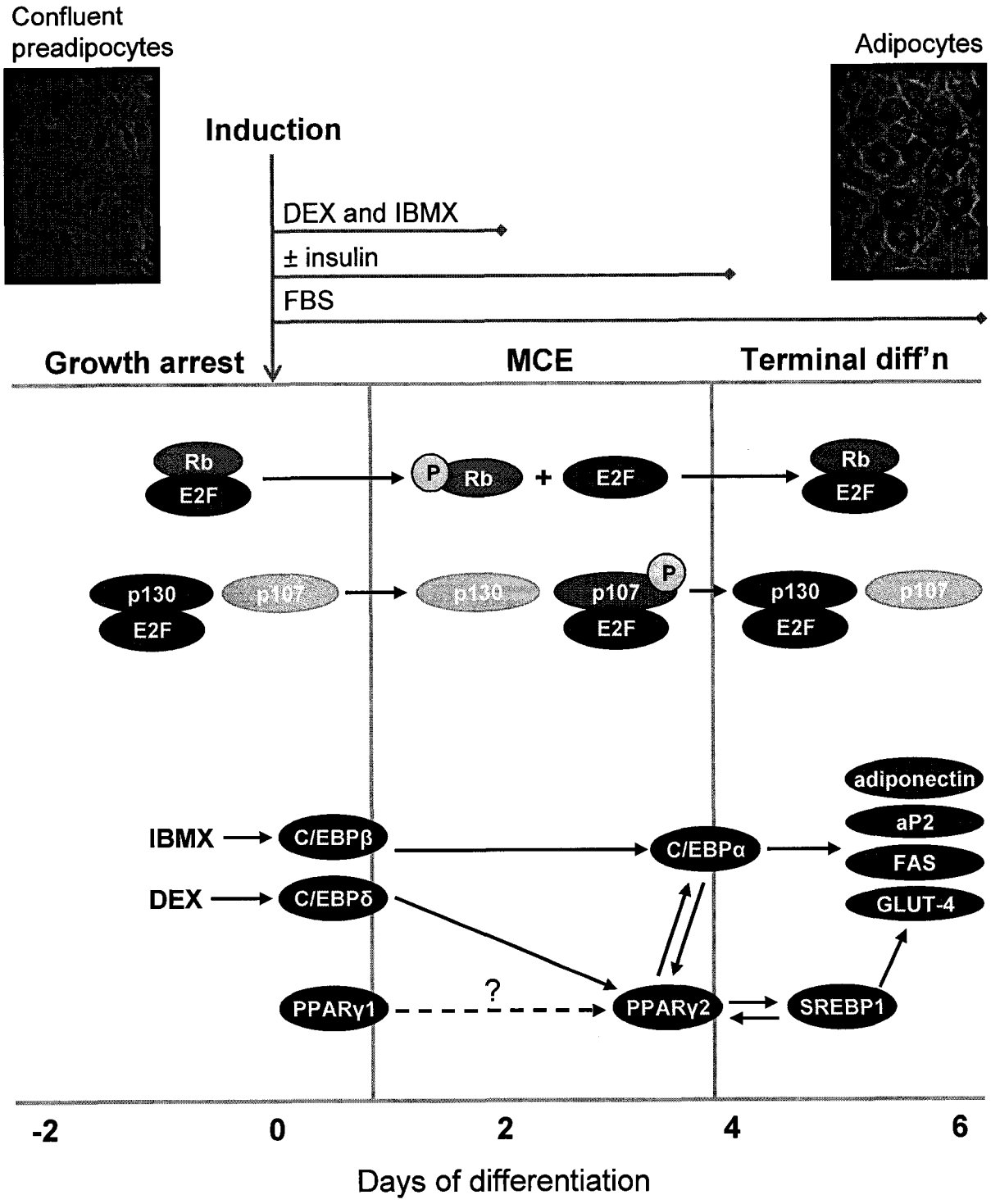
In culture, preadipocytes proliferate exponentially until they reach confluence and become growth-arrested due to contact inhibition. Both growth arrest in the G_0 phase of the cell cycle and cell-to-cell contact have been shown to be required for adipogenesis (Garcés et al., 1997; Pairault and Green, 1979). At this point, treatment with a hormonal cocktail initiates the adipogenic program, leading to the formation of mature lipid-filled adipocytes in 6 to 8 days. For 3T3-L1 preadipocytes, the induction cocktail consists of a synthetic glucocorticoid (dexamethasone) and a cyclic AMP (cAMP) elevating agent (isobutylmethylxanthine, IBMX) in the presence of fetal bovine serum (FBS), which provides the required IGF-I (Smith et al., 1988). Insulin can also be added at very high

concentrations to potentiate lipid accumulation, although it is not required for the differentiation process itself (Green and Kehinde, 1975). The main steps involved in adipogenesis are summarized in Figure 3.

Following induction (day 0), preadipocytes synchronously re-enter the cell cycle and undergo 1 to 2 rounds of mitotic cell division, known as the mitotic clonal expansion (MCE) phase. By day 4, the cells once again exit the cell cycle, become growth arrested in the G_D (terminal differentiation) stage, and begin acquiring characteristics of mature adipocytes. MCE is essential for differentiation of 3T3-L1 preadipocytes (Tang et al., 2003b). Changes in chromatin structure taking place during DNA replication are thought to allow access to promoters to initiate transcription of genes characteristic of mature adipocytes (MacDougald and Lane, 1995). In contrast, primary human preadipocytes do not require MCE during *in vitro* differentiation (Entenmann and Hauner, 1996), suggesting that these cells might have undergone clonal expansion *in vivo* prior to isolation.

The re-entry of preadipocytes into the cell cycle is similar, although not identical, to PDGF-stimulated progression through the G_1/S phase described earlier for subconfluent proliferation. For both cases, one of the key events is Rb phosphorylation by active cyclin D1/CDK4 or 6 complexes (Tang et al., 2003b). This releases E2F, which promotes transcription of genes necessary for the progression from the G_1 to the S phase of the cell cycle. However, one of the distinguishing features of MCE is the involvement of two other pocket protein family members: p107 and p130 (Richon et al., 1997). Before the onset of MCE, p130 associates with E2F. During the MCE, p130 is downregulated, whereas p107 levels and its phosphorylation are increased. At this time, E2F exists either in the free form, or bound to p107. After exit from MCE, the pattern of expression and phosphorylation of

Figure 3. Overview of 3T3-L1 adipocyte differentiation. Two-day post-confluent preadipocytes can be induced to differentiate to mature lipid-containing adipocytes with a mixture of dexamethasone (DEX) and IBMX in FBS-containing medium with or without high concentration of insulin over the course of 6 days. Following induction (day 0), preadipocytes undergo 1-2 rounds of mitotic cell division during the MCE phase. After day 4, the cells exit the cell cycle and undergo terminal differentiation. MCE is accompanied by Rb phosphorylation and release of E2F. Additionally, downregulation of p130 and upregulation and phosphorylation of p107 during the MCE phase leads to E2F release from p130 and association with p107. E2F initiates transcription of genes necessary for the G_1/S progression. E2F1, in particular, also induces transcription of PPARγ. After exit from MCE, the pattern of expression and phosphorylation of Rb, p107 and p130 returns back to the levels observed in growth-arrested preadipocytes. The early induction of C/EBPβ and δ, important for MCE progression, leads to upregulation of C/EBPα and PPARγ2. Together with SREBP-1, these transcription factors lead to acquisition of the mature adipocyte phenotype, characterized by the expression of proteins such as adiponectin, aP2, FAS and GLUT-4. See text for further details and abbreviations. Representative photographs showing preadipocytes and adipocytes are taken from (Artemenko et al., 2005). Triglycerides are stained with Oil Red O dye.


Confluent preadipocytes
Adipocytes
Induction
DEX and IBMX
± insulin
FBS
Growth arrest
MCE
Terminal diff'n
Rb
E2F
P
Rb
+
E2F
Rb
E2F
p130
E2F
p107
p130
p107
P
E2F
p130
E2F
p107
IBMX
C/EBPβ
C/EBPα
adiponectin
aP2
FAS
GLUT-4
DEX
C/EBPδ
PPARγ1
?
PPARγ2
SREBP1
-2
0
2
4
6
Days of differentiation

Rb, p107 and p130 returns back to the levels observed in confluent preadipocytes. The p130:p107 switch is specific to MCE, since p107 expression is not modulated by serum stimulation of growth-arrested subconfluent preadipocytes (Richon et al., 1997).

The initiation of MCE is coordinated with the expression of the immediate early genes, including members of the CCAAT enhancer-binding proteins (C/EBP) β and δ. Both C/EBPβ and C/EBPδ have been shown to be essential for adipocyte differentiation *in vivo* (Tanaka et al., 1997). In particular, C/EBPβ is required for the progression through the MCE phase (Tang et al., 2003a; Zhang et al., 2004b). The expression of C/EBPβ and C/EBPδ is induced directly in response to IBMX and dexamethasone, respectively (Cao et al., 1991). The elevated cAMP levels resulting from the IBMX treatment are thought to drive C/EBPβ expression by activating a panel of other transcription factors, including cAMP response element-binding protein, Krox20, and Krüppel-like factor 4 (Birsoy et al., 2008; Chen et al., 2005; Zhang et al., 2004a).

Together, C/EBPβ and C/EBPδ induce expression of two master regulators of adipogenesis, C/EBPα and peroxisome proliferator-activated receptor (PPAR) γ, both of which possess C/EBP regulatory elements (Christy et al., 1991; Zhu et al., 1995). In particular, the PPARγ2 isoform, which is expressed almost exclusively in adipocytes, has been shown to be necessary and sufficient for the induction of adipogenesis, even in the absence of C/EBPα (Wu et al., 1999). On the other hand, C/EBPα cannot drive differentiation without PPARγ, but is important for maintaining PPARγ expression in mature adipocytes (Rosen et al., 2002). In addition, C/EBPα is essential for coupling glucose transport to insulin signalling in adipocytes, a process which cannot be established by PPARγ alone (Hamm et al., 1999). Both C/EBPα and PPARγ possess anti-mitotic activity,

and hence contribute to the exit from MCE and the irreversible commitment to terminal differentiation (Altiok et al., 1997; Timchenko et al., 1996). Sterol regulatory element binding protein 1 (SREBP-1) / adipocyte determination and differentiation-dependent factor 1 is another transcription factor important for adipogenesis due to its ability to potentiate PPARγ expression (Kim and Spiegelman, 1996). PPARγ expression is also regulated by E2F family members, further establishing the importance of MCE in the differentiation process (Fajas et al., 2002).

Ultimately, C/EBPα, PPARγ, and SREBP-1 drive expression of various genes characteristic of mature adipocytes. Enhanced levels of fatty acid synthase (FAS), glycerol-3-phosphate dehydrogenase (GPDH) and hormone sensitive lipase, among others, confer the increased ability of adipocytes to perform *de novo* lipogenesis, triglyceride synthesis, and lipolysis (Kawamura et al., 1981; Mackall et al., 1976; Spiegelman et al., 1983). Adipocytes also acquire sensitivity to insulin due to increased expression of insulin receptor and insulin-dependent glucose transporter GLUT-4 (Kaestner et al., 1989; Rubin et al., 1977). Numerous other adipocyte-specific proteins are expressed, including leptin, adiponectin, fatty acid binding protein aP2, and perilipin (Gregoire et al., 1998).

Insulin/IGF-I signalling in adipogenesis

IGF-I, or insulin, acting through the IGF-I receptor (IGFR), is an essential component of the differentiation cocktail (Smith et al., 1988). In fact, neither dexamethasone nor IBMX is capable of inducing adipogenesis on its own. IGFR is thought to be the primary receptor for the insulin/IGF-I signal in preadipocytes, since insulin receptor (IR) expression is very low in preadipocytes and increases with adipogenesis (Rubin et al., 1978; Smith et

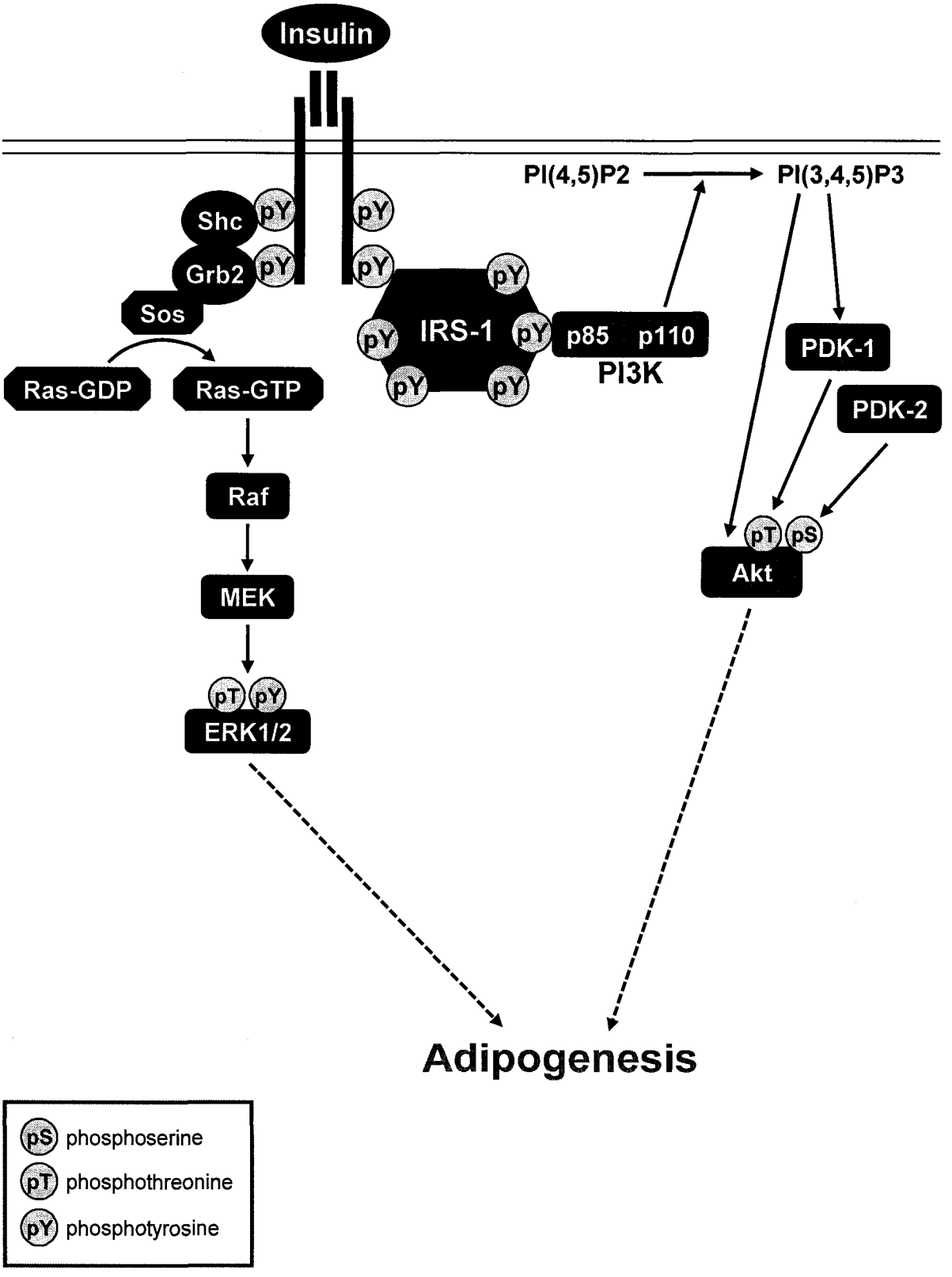
al., 1988). However, inactivation of the IR gene by homologous recombination, as well as stimulation of a colony stimulating factor-1 (CSF-1)/IR chimera by CSF-1 provided evidence for a direct role of IR in adipogenesis (Accili and Taylor, 1991; Chaika et al., 1997). Since both IR and IGFR function similarly, IR signalling is discussed below and is summarized in Figure 4.

Unlike other receptor Tyr kinases, the insulin receptor already exists as an $\alpha_2\beta_2$ tetramer on the plasma membrane (Saltiel and Kahn, 2001). Insulin binding to the α subunits induces a conformational change and stimulates the autophosphorylation of the transmembrane β subunits. The activated IR recruits and phosphorylates insulin receptor substrate (IRS) 1 protein. The recruitment of IRS-1 occurs via its PTB domain, which interacts with phosphotyrosine residues on IR, as well as its PH domain, which recognizes plasma membrane phosphoinositides. The phosphorylated IRS-1 serves as a docking protein, allowing recruitment of other proteins via their SH2 domains. Binding of PI3K and Grb2 to IRS-1 leads to activation of Akt and ERK1/2, respectively. Insulin can also stimulate the ERK1/2 pathway by direct recruitment of Shc to IR (Saltiel and Pessin, 2002).

The initiation of the PI3K/PI(3,4,5)P3/Akt signalling cascade is one the key events necessary for adipogenesis. In fact, Akt was shown to be both necessary and sufficient for adipogenesis (Kohn et al., 1996; Magun et al., 1996; Peng et al., 2003). The precise targets of Akt that drive differentiation are not clear, but likely involve modulation of cell cycle proteins leading to stimulation of MCE.

Overall, it appears that insulin triggers initiation of signalling cascades similarly to other growth factors, such as PDGF. Both insulin and PDGF induce expression of the PI3K/PI(3,4,5)P3/Akt and the ERK1/2 pathways, yet only insulin is adipogenic.

Figure 4. Adipogenic insulin signalling in preadipocytes. Insulin binding to IR induces receptor autophosphorylation and recruitment of IRS-1 via the IRS-1 PTB domain. The PI3K/PI(3,4,5)P3/Akt cascade is initiated by recruitment of the p85 regulatory subunit of PI3K to IRS-1, resulting in the activation of the p110 catalytic subunit and the production of PI(3,4,5)P3. PI(3,4,5)P3 recruits Akt and its kinases PDK-1 and PDK-2, which phosphorylate Akt on Thr308 and Ser473, respectively, thereby activating it. The ERK1/2 pathway is initiated when Grb2 binds to IRS-1 directly, or via Shc interaction with IR. This allows the nucleotide exchange factor Sos to activate Ras by promoting exchange of GDP for GTP. Activated Ras initiates a kinase cascade, ultimately leading to activation of ERK1/2 by phosphorylation of Thr202 and Tyr204. The activation of Akt is both necessary and sufficient for adipogenesis. The role of ERK1/2 activation in adipogenesis is complex and might depend on the stage of differentiation. See text for further details and abbreviations.


Insulin
Shc
Grb2
Sos
pY
Ras-GDP
Ras-GTP
Raf
MEK
pT
ERK1/2
IRS-1
p85
p110
PI3K
PI(4,5)P2
PI(3,4,5)P3
PDK-1
PDK-2
pS
Akt
Adipogenesis
phosphoserine
phosphothreonine
phosphotyrosine

Understanding the difference between pro- and anti-adipogenic signals might be important for explaining the lack of differentiation seen in metabolically unhealthy obese individuals.

SHIP2 as a mediator of the anti-adipogenic effect of PDGF

Although the anti-adipogenic effects of PDGF are well-established (Hauner et al., 1995; Hayashi et al., 1981), the molecular mechanisms of these effects are not clear. The inhibitory effects of PDGF on adipocyte differentiation are also not well-characterized. In fact, previous reports used only a single late marker of adipogenesis, GPDH, to demonstrate the inhibitory action of PDGF on differentiation (Hauner et al., 1995; Hayashi et al., 1981; Krieger-Brauer and Kather, 1995). Clearly, further understanding of PDGF action during adipogenesis is warranted.

Several attempts have been made to determine the molecular mechanisms of the anti-adipogenic effects of PDGF. One group reported that PDGF induces ERK1/2-dependent phosphorylation of PPARγ, which reduces its transcriptional activity (Camp and Tafuri, 1997). A less well-understood method involves NADPH-dependent H_2O_2 generation in response to anti-adipogenic PDGF signalling (Krieger-Brauer and Kather, 1995). In addition, we have demonstrated that inhibition of protein kinase C (PKC) with bisindolylmaleimide I (BisI) partially alleviates the negative effects of PDGF on 3T3-L1 and primary human preadipocyte differentiation (Artemenko et al., 2005).

Our laboratory previously reported that one of the differences between preadipocytes treated with pro-adipogenic insulin vs. anti-adipogenic PDGF is the phospholipid profile they generate (Gagnon et al., 1999; Sorisky et al., 1996). Insulin induces accumulation of

PI(3,4,5)P3 only, whereas PDGF leads to accumulation of PI(3,4)P2 in addition to PI(3,4,5)P3. This suggests that a lipid 5-phosphatase responsible for the conversion of PI(3,4,5)P3 into PI(3,4)P2 could be involved in the anti-adipogenic effects of PDGF. As described earlier, the 5-phosphatase SHIP2 is specific for PI(3,4,5)P3. Further evidence potentially implicating this phosphatase as a mediator of PDGF action is that only PDGF, but not insulin, stimulates Tyr phosphorylation of SHIP2 in growth-arrested preadipocytes (Gagnon et al., 2003). The differential regulation of SHIP2 Tyr phosphorylation by insulin vs. PDGF disappears as the cells differentiate. We and others demonstrated that insulin treatment does lead to SHIP2 Tyr phosphorylation in mature 3T3-L1 adipocytes (Gagnon et al., 2003; Habib et al., 1998).

Objective 2

My second objective was to characterize the negative effect of PDGF on adipocyte differentiation, and to determine the role of SHIP2 in this process. These findings are presented in Chapter II and were in part published in (Artemenko et al., 2005).

Hypothesis

The overall hypothesis of this research is that SHIP2 modulates both proliferation and differentiation of preadipocytes by mediating PDGF signalling in these cells.

CHAPTER I:

Structure-Function Analysis of SHIP2 Regulation by PDGF – Implications for Preadipocyte Proliferation

MATERIALS AND METHODS

Cell culture of 3T3-L1 preadipocytes

Murine 3T3-L1 preadipocytes (American Tissue Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS) and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin).

Constructs

SHIP2 WT cDNA was generated by mutating Ala 690 to Asp in human C-terminally FLAG-tagged SHIP2 D690A cDNA in the pcDNA3 vector (from S. Decker, University of Michigan, Ann Arbor, MI). SHIP2 D690A cDNA was isolated from pcDNA3 by EcoRV digest. The following primers were used for site-directed mutagenesis:

5'-CCTCATGGTGTGACCGGATTCTGTGGAAATCC-3' (primer 1)

5'-CCACAGAATCCGGTCACACCATGAGGGCACATT-3' (primer 2)

5'-TTTGGGGACCTCAACTACCGCCTGG-3' (primer 3)

5'-ACTGTGAGCAGGAGGTGCTGGTCCT-3' (primer 4).

PCR with primers 1 and 4, as well as primers 2 and 3 was performed to incorporate the A690D mutation into two fragments of SHIP2 cDNA; this was followed by PCR with primers 3 and 4 to generate full length SHIP2 WT cDNA that was subcloned into the EcoRV site of pcDNA3. The correct orientation of the insert was verified by BglII restriction digest analysis. To subclone SHIP2 WT cDNA into the retroviral vector pLXSN,

cDNA was first isolated from pcDNA3 by HindIII and XbaI restriction digest followed by Klenow treatment, and ligated into the HpaI site of pLXSN. SstII restriction digest was used to verify proper insert orientation. All subsequent SHIP2 mutants were generated from the above SHIP2 WT construct using a QuikChange kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All of the mutations were verified by sequencing at the Ottawa Genomics Innovation Centre DNA Sequencing Facility (University of Ottawa, Ottawa, ON, Canada).

For SHIP2 T958A, in which the Thr phosphorylation site is eliminated by substitution of this residue to Ala, the following primers were used:

5'-GAGGAGCCCTTGGCCCCCAGGTTGAAG-3'

5'-CTTCAACCTGGGGGCCAAGGGCTCCTC-3'.

SHIP2 R/Q mutant, in which the SH2 domain is disrupted by changing Arg47 to Gln (Ishihara et al., 2002), was generated using the following primers:

5'-CAGCTTCCTGGTCCAGGACAGCGAGAGCG-3'

5'-CGCTCTCGCTGTCCTGGACCAGGAAGCTG-3'.

SHIP2 YY/FF mutant with a disrupted NPAYY motif was made by changing Tyr986 and Tyr987 to Phe using the following primers:

5'-GCTTCAATAACCCTGCCTTCTTCGTCCTTGAAGGGGTC-3'

5'-GACCCCTTCAAGGACGAAGAAGGCAGGGTTATTGAAGC-3'.

Catalytically inactive, dominant-negative SHIP2 (referred to as PDR/AAA) was generated according to Wada et al. (Wada et al., 2001). The three conserved residues (Pro686, Asp690, Arg691) in the 5-phosphatase domain of human SHIP2 were mutated to Ala. First, SHIP2 D690A R691A was generated using the following primers:

5'-GTGCCCTCATGGTGTGCCGCGATTCTGTGGAAATCCTA-3'

5'-TAGGATTTCCACAGAATCGCGGCACACCATGAGGGCAC-3'.

This mutant was used to make SHIP2 PDR/AAA using the following primers:

5'-CGGACCAATGTGGCCTCATGGTGTGCC-3'

5'-GGCACACCATGAGGCCACATTGGTCCG-3'.

Retroviral transduction

All of the above constructs were transfected into BOSC23 cells by the calcium phosphate method as previously described (Gagnon et al., 2005; Pear et al., 1993). Viral supernatants were collected between 48 and 72 hours post-transfection. Subconfluent 3T3-L1 preadipocytes were transduced with the viral supernatants in the presence of 4 μg/ml polybrene. Stable overexpressors were selected with 400 μg/ml G418.

Phosphoinositide analysis

Confluent 3T3-L1 preadipocytes were maintained in inositol-free DMEM supplemented with 10% CS and antibiotics for 24 hours, and then labelled with 100 μCi/ml [^{3}H]-myoinositol (American Radiolabeled Chemicals, Inc., St. Louis, MO) in serum-reduced medium (inositol-free DMEM supplemented with 0.5% CS and antibiotics) (Gagnon et al., 1999). Following cell stimulation (as described below), lipids were extracted, deacylated, and separated on a Whatman Partisphere 5 SAX column as previously described (Gagnon et al., 1999). Peaks representing the deacylated products of [^{3}H]-PI(3,4)P2, [^{3}H]-PI(3,4,5)P3, and [^{3}H]-PI(4,5)P2 were identified by radioactive content of ^{3}H in 0.5 or 1 ml fractions mixed with Ultima-Flo^{TM} AP scintillation fluid (PerkinElmer, Boston, MA) using a

Beckman LS 3801 or LS 6500 scintillation counter. To account for variation in labelling between experiments, [^{3}H]-PI(3,4)P2 and [^{3}H]-PI(3,4,5)P3 levels were normalized for the [^{3}H]-PI(4,5)P2 content for each treatment, since PI(4,5)P2 levels do not change in response to stimulation (unpublished observations).

Stimulation

Prior to acute stimulation, confluent preadipocytes were maintained in DMEM supplemented with 0.5% CS and antibiotics for 16-20 hours. For experiments where PDGF treatment lasted up to 60 min, cells were kept in DMEM supplemented with 10% CS and antibiotics before stimulation. Cells were then switched to Krebs-Ringer-Hepes (KRH) buffer, and stimulated with 10 ng/ml PDGF-BB (referred to as PDGF; Calbiochem, San Diego, CA) or vehicle (0.02 mg/ml bovine serum albumin, BSA, in KRH buffer) for the indicated time at 37°C (Gagnon et al., 1999). For some experiments, cells were treated with 1 μM BisI (Sigma, Oakville, ON, Canada) or vehicle (0.1% dimethyl sulfoxide, DMSO) for 15 min prior to stimulation with PDGF. Alternatively, cells were treated with 100 nM 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma) or vehicle (0.1% DMSO) for 5 min. Pre-treatment with 10 μM imatinib (provided by Novartis, Basel, Switzerland) or vehicle (0.1% DMSO) was performed for 90 min prior to PDGF stimulation in DMEM supplemented with 0.1% BSA. Total cell lysates were generated in Laemmli buffer (Laemmli, 1970) containing 1 mM sodium orthovanadate, 5 mM EGTA, pH 8.0, 50 mM sodium fluoride, and 5 mM sodium pyrophosphate. Resulting cell lysates were subjected to immunoblotting, or to immunoprecipitation prior to either immunoblotting or PI3K assay.

Immunoblotting

Following cell lysis, protein concentration was determined using a modified Lowry reaction with BSA as a standard (D_C Protein Assay, Bio-Rad, Mississauga, ON, Canada). Equal amounts of protein (10-70 μg, depending on the experiment) were separated by 7.5% SDS-PAGE and transferred onto a nitrocellulose membrane. Non-specific binding sites were blocked with phosphate buffered saline (PBS) containing 0.1% Tween 20, and 5% skim milk powder or 3% BSA (for PY20 immunoblots only). Membranes were incubated overnight in PBS containing 3% BSA, 0.02% sodium azide, and the relevant primary antibodies to detect the following: Shc (mouse monoclonal; 1.25 μg/ml; BD Biosciences, Mississauga, ON, Canada); phospho-Akt (pAkt S473; rabbit polyclonal; 1:1000), phospho-ERK1/2 (pERK1/2; T202/Y204; rabbit polyclonal; 1:1000), phosphoserine PKC substrate (rabbit polyclonal; 1:500), phospho-Src family (pSrc Y416; rabbit polyclonal; 1:1000), phospho-Src (pSrc Y527; rabbit polyclonal; 1:1000), phospho-STAT3 (pSTAT3; S727; rabbit polyclonal; 1:1000), phosphotyrosine (pTyr; PY20; mouse monoclonal; 1 μg/ml; or PY100; mouse monoclonal; 1:1000), Src (rabbit polyclonal; 1:1000), STAT3 (rabbit polyclonal; 1:1000), all from Cell Signaling, Pickering, ON, Canada; phospho-GSK-3β (pGSK-3β; rabbit polyclonal; 1:1000; New England Biolabs, Mississauga, ON, Canada); Akt1 (C-20; goat polyclonal; 1 μg/ml), PDGFRβ (#958; rabbit polyclonal; 1 μg/ml), SHIP2 (I-20; goat polyclonal; 2 μg/ml), all from Santa Cruz Biotechnology, Santa Cruz, CA; FLAG (rabbit polyclonal; 2.5 μg/ml; Sigma); ERK1/2 (rabbit polyclonal; 1 μg/ml; Upstate, Charlottesville, VA). The Akt antibody (rabbit polyclonal; 1:9000) was generously provided by B.M. Burgering, University Medical Centre Utrecht, the Netherlands. The antibody for GSK-3β (rabbit polyclonal; 1:1000) was kindly provided by J.R. Woodgett, Ontario Cancer

Institute, ON, Canada. Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies in PBS with 5% skim milk powder or 3% BSA (for PY20 immunoblots only), immunoreactivity was detected by enhanced chemiluminescence (Millipore, Nepean, ON, Canada) using a Kodak M35A X-OMAT Processor. Relative band intensity was determined with AlphaEaseFC$_{TM}$ Software (version 4.0.0) and expressed as integrated optical density (IOD) units. For the analysis of phospho-proteins from total cell lysates absolute densitometric values are given. Corresponding total protein levels are also shown to indicate that changes in the phosphorylation status are not due to alterations in protein expression or inconsistencies in loading. Therefore, normalization of phospho-protein to total protein levels was not routinely performed.

Immunoprecipitation

Cells were lysed in PBS, pH 7.4, 1% Nonidet P-40, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.1 mg/ml phenylmethylsulfonylfluoride, 200 μM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 4 μg/ml benzamidine, 1 mM β-glycerophosphate. Soluble proteins were quantified with a commercial kit (Micro BCA^{TM} Protein Assay, Pierce, Rockford, IL), and pre-cleared with protein G-agarose (for anti-phosphotyrosine immunoprecipitation) or protein A-Sepharose (for anti-FLAG and anti-phosphoserine PKC substrate immunoprecipitation). Lysates containing equal amounts of protein (0.5-3.2 mg, depending on the experiment) were incubated with 5 μg anti-phosphotyrosine (PY20) antibody adsorbed to protein G-agarose, or 2.5 μg anti-FLAG or 1.5 μl anti-phosphoserine PKC substrate antibodies adsorbed to protein A-Sepharose. Immunoprecipitated proteins were washed, resuspended in Laemmli buffer containing 1 mM

sodium orthovanadate, and processed for immunoblot analysis as described below. Specificity of immunoprecipitated proteins was verified using non-immune control antibodies: 1 μg ChromPure mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or 2.5 μg normal rabbit IgG (Santa Cruz Biotechnology). Due to differences in the overexpression levels between SHIP2 R/Q and SHIP2 WT, four plates of SHIP2 R/Q cells were pooled for the anti-FLAG immunoprecipitation to obtain the same amount of SHIP2 as from one plate of SHIP2 WT cells.

PI3K assay

Preadipocytes were lysed in PBS, pH 7.4, 1% Triton X-100, 50 mM sodium fluoride, 0.1 mg/ml phenylmethylsulfonylfluoride, 200 μM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 4 μg/ml benzamidine, 1 mM β-glycerophosphate. Anti-phosphotyrosine immunoprecipitates were prepared as described above, washed, resuspended in assay buffer (20 mM Tris, 0.1 M NaCl, 0.5 mM EGTA, 0.2 mg/ml PI from Sigma), and PI3K activity was then measured as previously described (Bell et al., 2002; Liu et al., 1995). The reaction was started by the addition of Mg-ATP cocktail (10 mM $MgCl_2$, 10 μM cold ATP, 20 μCi [γ-^{32}P]ATP (GE Healthcare)), and terminated after 3 minutes by adding chloroform-methanol-HCl (50:100:1, v:v:v). The lipid product was extracted with chloroform, and resolved by thin-layer chromatography with methanol-chloroform-NH_4OH-HCl (100:70:15:25, v:v:v:v). 1 mg/ml phosphatidylinositol 4-phosphate (PI4P; Sigma) visualized by iodine staining was used as a standard. Following autoradiographic detection with Kodak M35A X-OMAT Processor, the relative intensity of the band was measured with AlphaEaseFC™ Software (version 4.0.0) and expressed as IOD units.

Phosphopeptide mapping

Confluent 3T3-L1 preadipocytes stably overexpressing SHIP2 WT were maintained in DMEM supplemented with 0.5% CS and antibiotics overnight and stimulated with 10 ng/ml PDGF or vehicle as described above. Following immunoprecipitation of cell lysates with an anti-FLAG antibody, proteins were separated by 7.5% SDS-PAGE, and stained with Coomassie Brilliant Blue. Bands corresponding to SHIP2 protein were excised, and analyzed for the presence of phosphorylated residues by Protana Analytical Services Inc. (Toronto, Ontario, Canada) using ABI-Sciex QSTAR-Pulsar QqTOF mass spectrometry. The report from Protana Analytical Services is included in the Appendix.

Cell enumeration

For assessment of cell number, 3T3-L1 preadipocytes were seeded at $2.5x10^3$ cells/cm^2 in 35 mm culture dishes. Cells were detached with trypsin-EDTA on the indicated days. Trypsin was inactivated with an equal volume of DMEM supplemented with 20% FBS. Cells were counted with a Neubauer hemacytometer in duplicate according to the manufacturer's instructions. For some experiments, 10 μM imatinib or vehicle (0.1% DMSO) was added at the time of plating, and again on the two following days. Cells were enumerated in duplicate on the third day as described above.

For assessment of attachment, cells were seeded at 10^4 cells/cm^2. Attached cells were trypsinized and counted in duplicates three hours after plating as described above.

[^{3}H]-thymidine incorporation assay

3T3-L1 preadipocytes were seeded at 2.5x10^3 cells/cm^2 in 35 mm culture dishes. On day 3, cells were incubated with 1 μCi/ml [^{3}H]-thymidine (GE Healthcare, Baie D'Urfe, QC, Canada) for 1 hour. Following cell solubilization with 0.5% SDS, DNA was precipitated with 10% trichloroacetic acid onto GF/C filters. Dried filters were placed in EcoLume™ scintillation fluid (MP Biomedicals, Irvine, CA), and radioactivity was quantified with a Beckman LS 6500 counter. The resulting counts were normalized for cell number from replicate plates. Both radioactivity counts and cell numbers were obtained in triplicates.

Cell death

3T3-L1 preadipocytes were seeded at 2.5x10^3 cells/cm^2 in 35 mm culture dishes. On day 3, cells were fixed in 10% formalin and stained with Hoechst 33248. Nuclei were visualized and photographed at 200X magnification by a Zeiss Axioplan 2 imaging microscope equipped with an Axiocam digital camera. The number of apoptotic (i.e. bright, condensed) nuclei was expressed as a percentage of the total number of nuclei to estimate % apoptosis. 20 random fields were scored for each condition. To obtain a positive control, confluent preadipocytes were placed in serum-free DMEM for 3 hours, and then processed as described above.

Statistical analysis

ANOVA with the Newman–Keuls post-hoc test for paired values was used to assess differences between multiple means, and a paired t-test was used to assess the difference

between two means (GraphPad Instat, version 3.05). A P value <0.05 was considered significant.

RESULTS

Analysis of the role of SHIP2 Ser/Thr phosphorylation

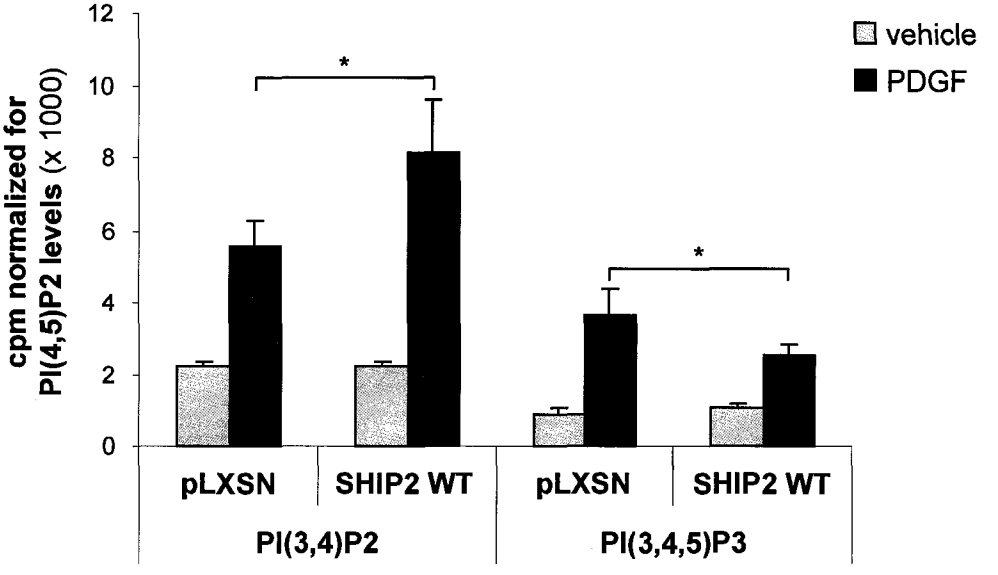
SHIP2 is an active 5-phosphatase in 3T3-L1 preadipocytes

To facilitate the analysis of SHIP2 regulation in 3T3-L1 preadipocytes, I stably overexpressed human FLAG-tagged SHIP2 WT or empty vector (pLXSN) by retroviral transduction followed by antibiotic selection. SHIP2 dephosphorylates PI(3,4,5)P3 to produce PI(3,4)P2. Hence, I examined the phospholipid profile of cells expressing SHIP2 WT compared to cells expressing empty vector alone to confirm that overexpressed SHIP2 WT is active. Acute stimulation of 3T3-L1 preadipocytes with 10 ng/ml PDGF led to accumulation of both PI(3,4,5)P3 and PI(3,4)P2 (n=4; Fig. 5). SHIP2 WT expressing cells exhibited a 1.4±0.1 fold increase in the levels of PI(3,4)P2 (mean±SE; n=4; $P<0.05$), and a 1.4±0.2 fold decrease in the levels of PI(3,4,5)P3 (mean±SE; n=4; $P<0.05$) compared to pLXSN. SHIP2 WT had no effect on the basal levels of PI(3,4,5)P3 or PI(3,4)P2 (n=4; $P>0.05$). The data are consistent with the ability of SHIP2 WT to dephosphorylate PI(3,4,5)P3 following PDGF stimulation in preadipocytes.

BisI attenuates SHIP2 WT Tyr phosphorylation and association with Shc

In 3T3-L1 preadipocytes, PDGF treatment leads to Tyr phosphorylation of SHIP2 (Gagnon et al., 2003). I have previously observed that an inhibitor of PKC, BisI, reduces PDGF-stimulated Tyr phosphorylation of endogenous SHIP2 (Artemenko et al., 2007). To establish that the overexpressed FLAG-tagged SHIP2 WT construct behaves similarly to endogenous SHIP2, I examined its PDGF-stimulated state of Tyr phosphorylation following

Figure 5. Wild-type SHIP2 increases PI(3,4)P2 and reduces PI(3,4,5)P3 in PDGF-stimulated cells. Confluent 3T3-L1 preadipocytes expressing SHIP2 WT or empty vector (pLXSN) were maintained in inositol-free growth medium for 24 hours, and labelled with 100 μCi/ml [^{3}H]-myoinositol overnight. Following a 5 min stimulation with 10 ng/ml PDGF or vehicle, phosphoinositides were extracted, separated by HPLC, and quantified. Levels of PI(3,4)P2 and PI(3,4,5)P3 were normalized to PI(4,5)P2 levels (x1000). Data representative of 4 independent experiments, each performed in duplicate, are expressed as means±SE. *$P<0.05$ compared to the same condition for pLXSN.


12
10
8
6
4
2
0
cpm normalized for PI(4,5)P2 levels (x 1000)
vehicle
PDGF
*
*
pLXSN
SHIP2 WT
pLXSN
SHIP2 WT
PI(3,4)P2
PI(3,4,5)P3

pre-treatment with BisI. Using anti-FLAG immunoprecipitation to specifically analyze overexpressed SHIP2, followed by immunoblotting with anti-phosphotyrosine antibody, I determined that BisI reduced PDGF-stimulated SHIP2 WT Tyr phosphorylation by 49±11% (mean±SE; n=5; $P<0.01$; Fig. 6). To account for variation in the amounts of immunoprecipitated SHIP2, phosphotyrosine signal was normalized for FLAG levels for the corresponding treatments.

Endogenous SHIP2 also associates with an adaptor protein Shc in response to PDGF (Gagnon et al., 2003). SHIP2 WT also associated with Shc following stimulation with PDGF; moreover, this interaction was inhibited by BisI (n=3; Fig. 6).

BisI is a selective inhibitor of PKC

I confirmed that PDGF can activate PKC in 3T3-L1 preadipocytes, and that BisI can inhibit this response. Immunoprecipitation of proteins recognized by a phosphoserine PKC substrate antibody revealed that PDGF stimulation leads to accumulation of several phosphorylated proteins with approximate molecular weights of 80, 145, 155, and 180 kDa (n=3; Fig. 7A). BisI reduced the appearance of these phosphorylated PKC substrates, indicating that it inhibits PDGF-stimulated PKC activation.

One concern with the use of pharmacological inhibitors is potential lack of specificity (Davies et al., 2000). BisI did not inhibit phosphorylation of PDGFR itself (n=3; Fig. 7B). Phosphorylation of a panel of PDGF targets, including Akt, ERK1/2, STAT3 and GSK-3β, was also not affected by BisI (n=3; Fig. 7C). Densitometric analysis of PDGFR, Akt, ERK1/2, STAT3, and GSK-3β phosphorylation following stimulation with PDGF revealed no statistically significant differences between treatments in the presence vs. in the

Figure 6. BisI reduces PDGF-stimulated SHIP2 Tyr phosphorylation and association with Shc. Confluent 3T3-L1 preadipocytes overexpressing FLAG-tagged SHIP2 WT were pre-treated with 1 μM BisI or vehicle for 15 minutes, and stimulated with 10 ng/ml PDGF or vehicle for 5 minutes. Cell lysates were incubated with anti-FLAG antibodies. Immunoprecipitated proteins were immunoblotted with antibodies against phosphotyrosine, Shc, or FLAG. An immunoblot representative of 5 (phosphotyrosine) or 3 (Shc) separate experiments is shown. Densitometric data were normalized for FLAG levels and expressed as means±SE. **P<0.01.

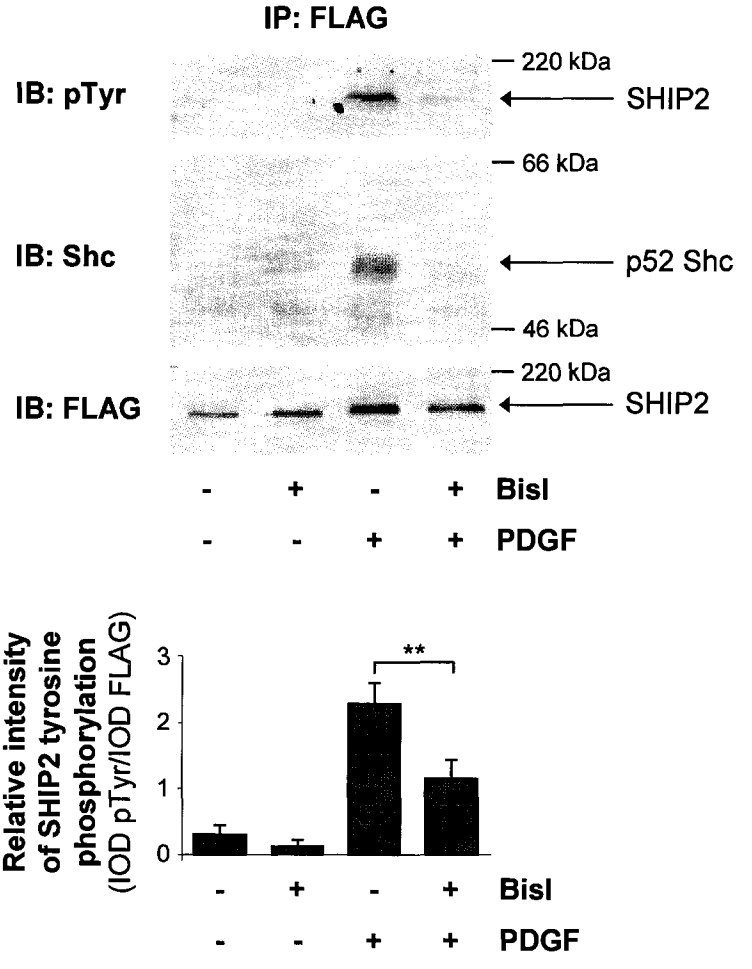
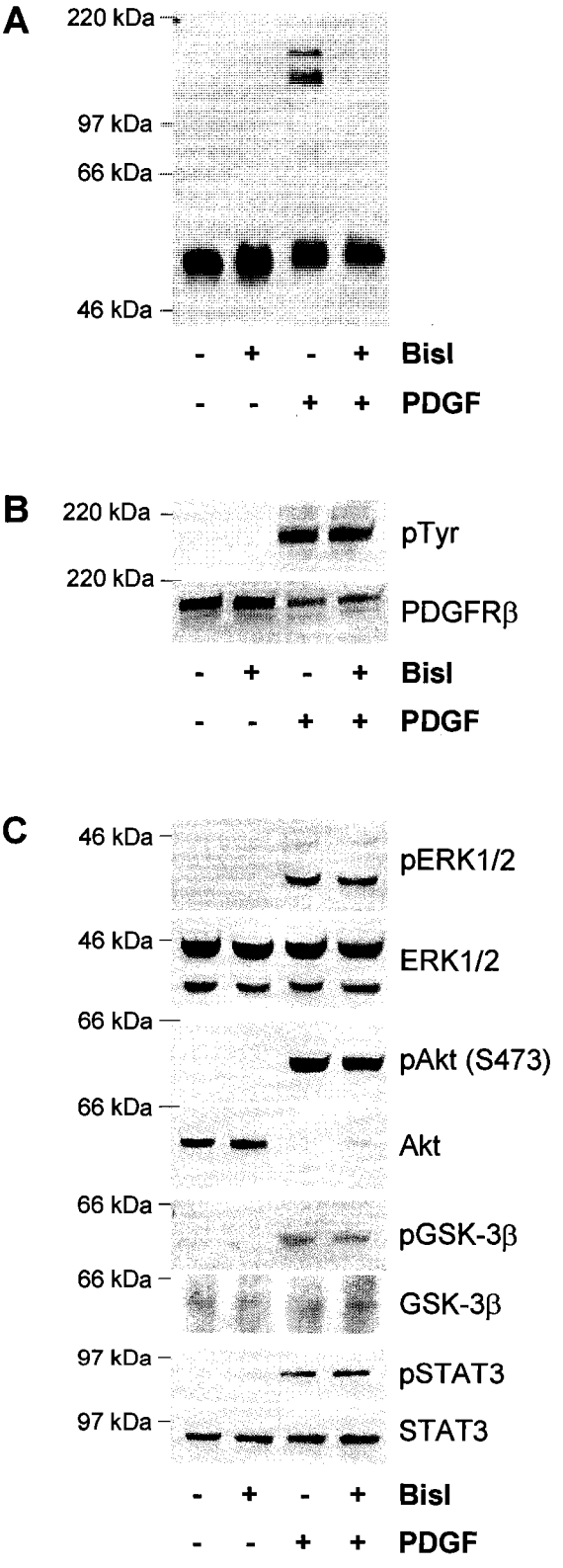

IP: FLAG
IB: pTyr
220 kDa
SHIP2
66 kDa
IB: Shc
p52 Shc
46 kDa
IB: FLAG
220 kDa
SHIP2
- + - + BisI
- - + + PDGF
Relative intensity of SHIP2 tyrosine phosphorylation (IOD pTyr/IOD FLAG)
3
2
1
0
**
- + - + BisI
- - + + PDGF

Figure 7. BisI selectively inhibits PDGF-stimulated activation of PKC. Confluent 3T3-L1 preadipocytes were pre-treated with 1 μM BisI or vehicle for 15 min, and stimulated with 20 ng/ml PDGF (A), 10 ng/ml PDGF (B, C), or vehicle for 5 min. **(A)** Cell lysates were incubated with anti-phosphoserine PKC substrate antibody. Immunoprecipitated proteins were immunoblotted with anti-phosphoserine PKC substrate antibody. **(B, C)** Equal amounts of solubilized protein were immunoblotted with antibodies against phosphotyrosine and PDGFRβ, or antibodies against ERK1/2, Akt, GSK-3β, STAT3 and their phosphorylated forms. Immunoblots representative of 3 separate experiments are shown.


A
220 kDa
97 kDa
66 kDa
46 kDa
- + - + Bisl
- - + + PDGF
B
220 kDa
pTyr
220 kDa
PDGFRβ
- + - + Bisl
- - + + PDGF
C
46 kDa
pERK1/2
46 kDa
ERK1/2
66 kDa
pAkt (S473)
66 kDa
Akt
66 kDa
pGSK-3β
66 kDa
GSK-3β
97 kDa
pSTAT3
97 kDa
STAT3
- + - + Bisl
- - + + PDGF

absence of BisI (n=3; P>0.05). In addition, BisI did not affect PDGF-stimulated PI3K activity observed in anti-phosphotyrosine immunoprecipitates (n=3; P>0.05; Fig. 8). Based on this survey of kinases, it appears that BisI is a selective inhibitor of PKC in 3T3-L1 preadipocytes.

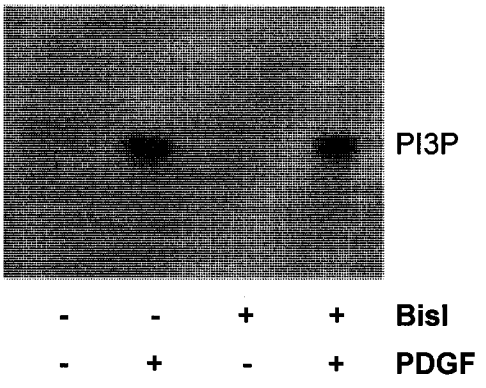
BisI does not modulate Src activation

Src has been implicated in Tyr phosphorylation of SHIP2 in several cell lines (Prasad et al., 2002; Taylor et al., 2000). Hence, the observed effect of BisI on SHIP2 Tyr phosphorylation might be mediated by inhibition of Src. Activation of Src occurs by dephosphorylation of Tyr527 and phosphorylation of Tyr416 (Roskoski, 2005), and this response was observed following PDGF stimulation in 3T3-L1 preadipocytes (Fig. 9). BisI did not inhibit PDGF-stimulated dephosphorylation of Tyr527 (n=3; P>0.05), and actually enhanced Tyr416 phosphorylation by 41±2% (mean±SE; n=3; P<0.01). Thus, BisI does not attenuate SHIP2 Tyr phosphorylation by inhibiting Src.

BisI does not alter the PDGF-stimulated phosphoinositide profile

It remains unclear whether SHIP2 Tyr phosphorylation is necessary for its activity. We tested whether BisI modulates the phosphoinositide profile generated in response to PDGF. A 5 min stimulation with PDGF led to an increase in both PI(3,4)P2 and PI(3,4,5)P3 levels by 4.4±0.5 and 8.2±0.5 fold, respectively (mean±SE; n=6; P<0.001; Fig. 10). BisI did not alter basal or PDGF-stimulated levels of phosphoinositides significantly (n=6; P>0.05). Based on these findings, BisI does not appear to have a major effect on the activity of endogenous SHIP2 in preadipocytes.

Figure 8. BisI does not affect PDGF-stimulated phosphoinositide 3-kinase activity. Confluent 3T3-L1 preadipocytes were pre-treated with 1 μM BisI or vehicle for 15 min, and stimulated with 10 ng/ml PDGF or vehicle for 5 min. Cells were lysed and the anti-phosphotyrosine immunoprecipitates were assayed for PI3K activity. Densitometric data were obtained from 3 separate experiments, each performed in duplicate, and expressed as means±SE. A representative thin-layer chromatography exposure, indicating the *in vitro* PI3P product, is shown.


PI3P
- - + + BisI
- + - + PDGF

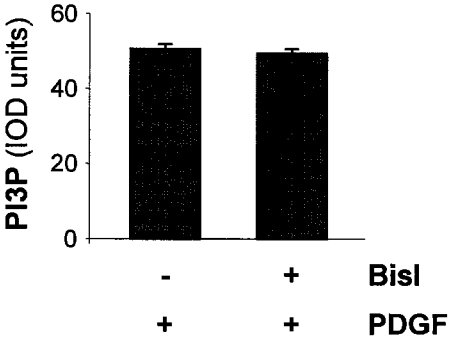
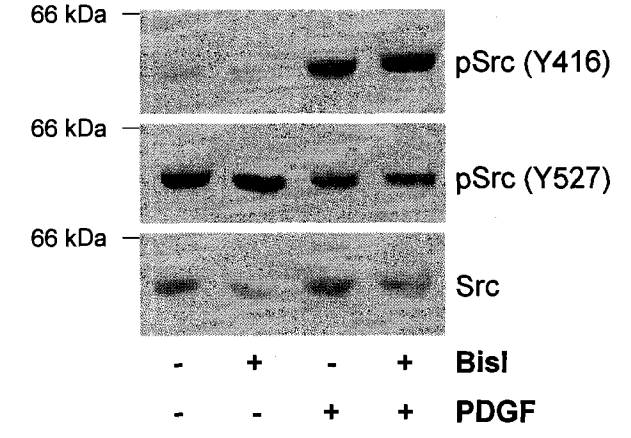

PI3P (IOD units)
60
40
20
0
- + BisI
+ + PDGF

Figure 9. BisI does not affect PDGF-stimulated Src activation. Confluent 3T3-L1 preadipocytes were pre-treated with 1 μM BisI or vehicle for 15 min, and stimulated with 10 ng/ml PDGF or vehicle for 5 min. Equal amounts of solubilized protein were immunoblotted with antibodies against phospho-Src family (Y416), phospho-Src (Y527), or Src. Densitometric data were obtained from 3 separate experiments and expressed as means±SE. A representative immunoblot is shown. **P<0.01.


66 kDa
pSrc (Y416)
66 kDa
pSrc (Y527)
66 kDa
Src
- + - + Bisl
- - + + PDGF

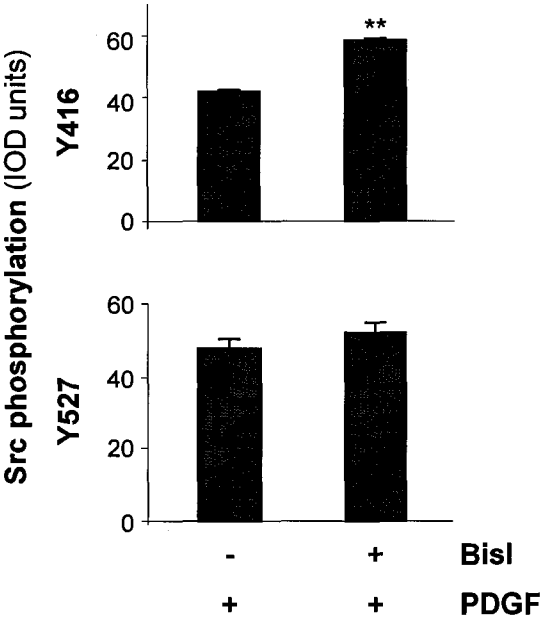
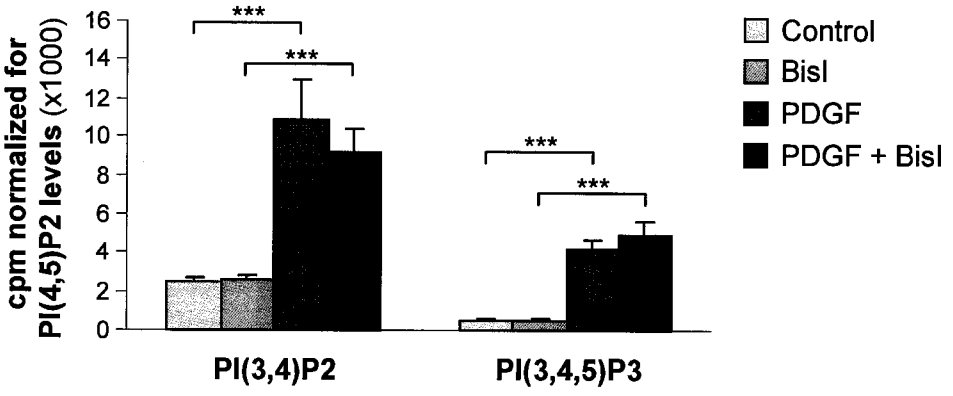

Src phosphorylation (IOD units)
Y416
Y527
60
40
20
0
**
- + Bisl
+ + PDGF

Figure 10. BisI does not alter the phosphoinositide profile generated in response to PDGF. Confluent 3T3-L1 preadipocytes were maintained in inositol-free growth medium for 24 hours, labelled with 100 μCi/ml [^{3}H]-myoinositol overnight, and pre-treated with 1 μM BisI or vehicle for 15 min. Following a 5 min stimulation with 10 ng/ml PDGF or vehicle (control), phosphoinositides were extracted, separated by HPLC, and quantified. Levels of PI(3,4)P2 and PI(3,4,5)P3 were normalized to PI(4,5)P2 levels (x1000). Data representative of 6 separate experiments are expressed as means±SE. ***P<0.001.


cpm normalized for PI(4,5)P2 levels (x1000)
16
14
12
10
8
6
4
2
0
***
***
***
***
PI(3,4)P2
PI(3,4,5)P3
Control
Bisl
PDGF
PDGF + Bisl

Activation of PKC does not modulate SHIP2 Tyr phosphorylation

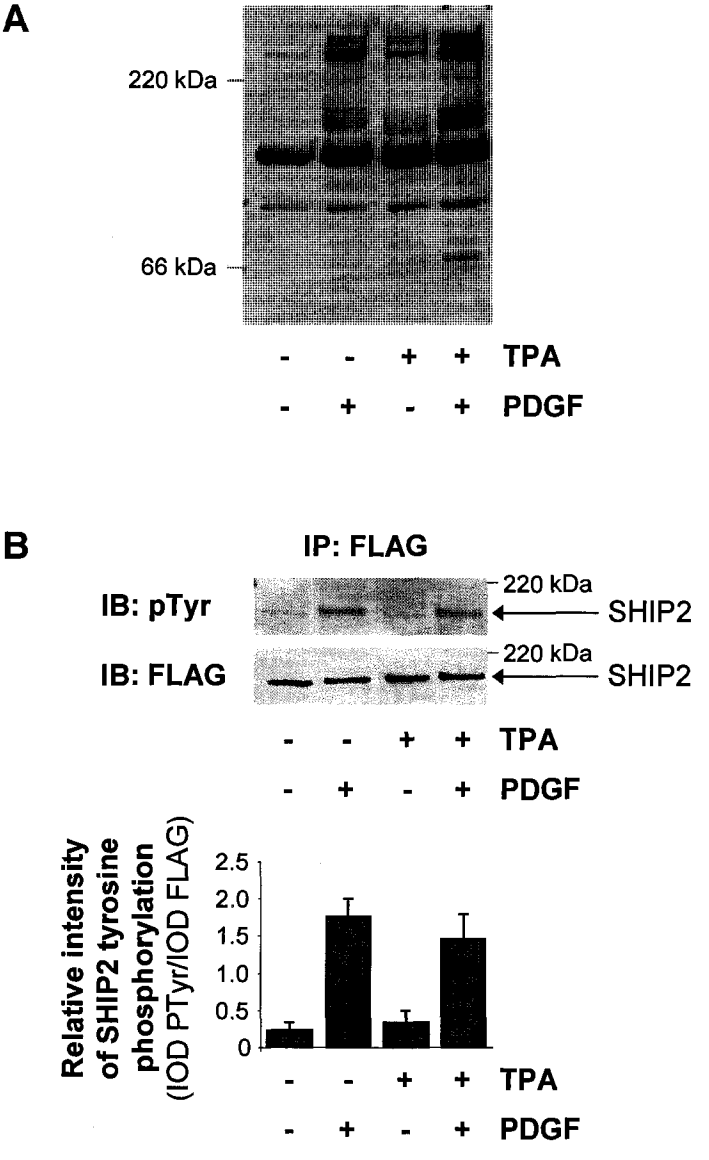
Since inhibition of PKC with BisI reduces PDGF-stimulated SHIP2 Tyr phosphorylation, I tested whether activation of PKC would enhance this response. A 5 min treatment of preadipocytes with TPA, a known activator of PKC, led to accumulation of phosphoserine PKC substrates comparable to that observed with PDGF stimulation (n=3; Fig. 11A). The presence of both TPA and PDGF together slightly potentiated PKC activation compared to either agent alone.

Despite the robust activation of PKC, TPA treatment was not sufficient to induce SHIP2 WT Tyr phosphorylation, as assessed by anti-FLAG immunoprecipitates (n=3; Fig. 11B). TPA also failed to enhance PDGF-stimulated SHIP2 WT Tyr phosphorylation (n=3; $P>0.05$). Thus, PKC activation does not affect SHIP2 Tyr phosphorylation.

SHIP2 is phosphorylated at Thr958 in response to PDGF

Since BisI inhibits PKC, a Ser/Thr kinase, we assessed whether SHIP2 might be directly Ser/Thr phosphorylated. SHIP2 WT was isolated from preadipocytes by anti-FLAG immunoprecipitation and subjected to phosphopeptide mapping. This analysis, which involves tryptic digest of a protein of interest followed by mass spectrometry, was performed by Protana Analytical Services Inc. (Toronto, Ontario, Canada). A 5 min stimulation with PDGF resulted in the phosphorylation of SHIP2 Thr958, which was not observed following stimulation with vehicle. Hence, we have identified a novel PDGF-responsive phosphorylation site in SHIP2. This residue is conserved in human, chimpanzee and dog SHIP2. SHIP2, in mouse and rat, has an asparagine in this position.

Figure 11. TPA treatment does not affect PDGF-stimulated SHIP2 Tyr phosphorylation. Confluent 3T3-L1 preadipocytes overexpressing FLAG-tagged SHIP2 WT or empty vector (pLXSN) were pre-treated with 100 nM TPA or vehicle for 5 min, and stimulated with 10 ng/ml PDGF or vehicle for 5 min. **(A)** Equal amounts of solubilized protein were immunoblotted with anti-phosphoserine PKC substrate antibody. An immunoblot representative of 3 separate experiments is shown. **(B)** Cell lysates were incubated with anti-FLAG antibodies. Immunoprecipitated proteins were immunoblotted with antibodies against phosphotyrosine or FLAG. A representative immunoblot is shown. Densitometric data from 3 separate experiments were normalized for FLAG levels and expressed as means±SE.

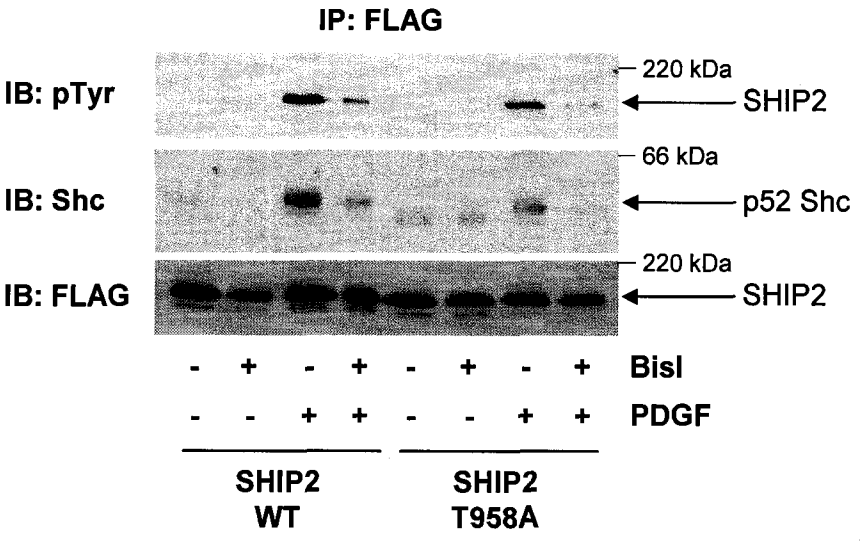

A
220 kDa
66 kDa
- - + + TPA
- + - + PDGF
B
IP: FLAG
IB: pTyr
220 kDa
SHIP2
IB: FLAG
220 kDa
SHIP2
- - + + TPA
- + - + PDGF
Relative intensity of SHIP2 tyrosine phosphorylation (IOD PTyr/IOD FLAG)
2.5
2.0
1.5
1.0
0.5
0
- - + + TPA
- + - + PDGF

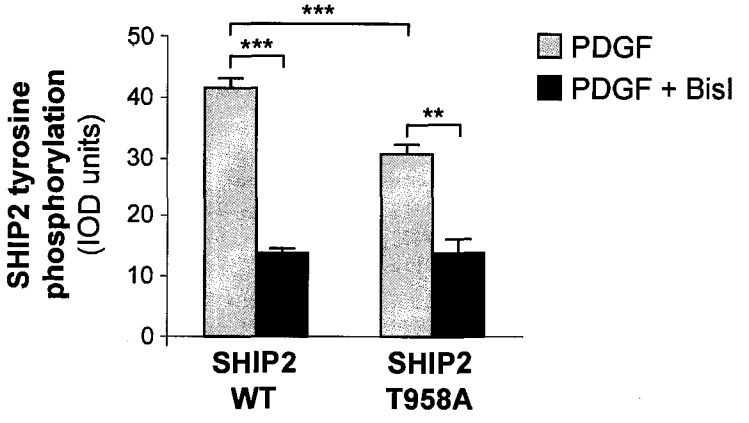
Thr958 is a PDGF-responsive regulatory residue in SHIP2

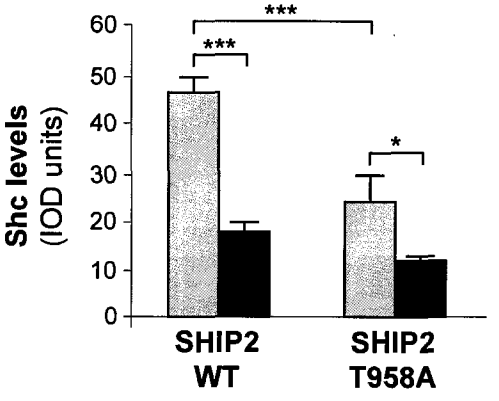
To assess the importance of Thr958 with respect to SHIP2 regulation by PDGF, we changed this residue to Ala in human FLAG-tagged SHIP2 (SHIP2 T958A). We then stably overexpressed this construct, SHIP2 WT, or empty vector pLXSN in 3T3-L1 preadipocytes by retroviral transduction followed by G418 selection. Both SHIP2 WT and T958A were expressed at similar levels (see Fig. 14 for a SHIP2 immunoblot). Using anti-FLAG immunoprecipitation followed by immunoblotting with an anti-phosphotyrosine antibody, we determined that PDGF-stimulated Tyr phosphorylation of SHIP2 T958A was reduced by 27±5% compared to SHIP2 WT (mean±SE; n=5; $P<0.001$; Fig. 12). As previously observed, addition of BisI diminished Tyr phosphorylation of SHIP2 WT seen in response to PDGF by 67±2% (mean±SE; n=5; $P<0.001$). Although BisI reduced PDGF-stimulated SHIP2 T958A phosphorylation (51±7% decrease; mean±SE; n=5; $P<0.001$), the effect was not as pronounced as for SHIP2 WT (n=5; $P<0.05$ between the two decreases).

PDGF-stimulated Shc association with SHIP2 T958A was 43±14% lower than with SHIP2 WT (mean±SE; n=4; $P<0.001$; Fig. 12). Consistent with previous data, BisI attenuated PDGF-induced SHIP2 WT interaction with Shc by 61±1% (mean±SE; n=4; $P<0.001$). Although the inhibitory effect of BisI on SHIP2 T958A association with Shc in response to PDGF tended be slightly reduced compared to SHIP2 WT (48±13% decrease; mean±SE; n=4; $P<0.05$), there was no significant difference between the two effects (n=4; $P>0.05$). It should be noted that the total amount of SHIP2 immunoprecipitated with a FLAG antibody was not significantly different between any of the treatments (n=5 for phosphotyrosine, n=4 for Shc; $P>0.05$). Normalization of the phosphotyrosine or Shc bands for the levels of total SHIP2 protein in the immunoprecipitates confirmed that the inhibitory

Figure 12. Mutation of Thr958 to Ala reduces PDGF-stimulated SHIP2 Tyr phosphorylation and Shc association independently of BisI action. Confluent 3T3-L1 preadipocytes overexpressing FLAG-tagged SHIP2 WT or SHIP2 T958A were pre-treated with 1 μM BisI or vehicle for 15 min, and stimulated with 10 ng/ml PDGF or vehicle for 5 min. Cell lysates were incubated with anti-FLAG antibodies. Immunoprecipitated proteins were immunoblotted with antibodies against phosphotyrosine, Shc, or FLAG. Densitometric data were obtained from 5 (phosphotyrosine) or 4 (Shc) separate experiments and are expressed as means±SE. A representative immunoblot is shown. *P<0.05, **P<0.01, ***P<0.001.


IP: FLAG
IB: pTyr
220 kDa
SHIP2
IB: Shc
66 kDa
p52 Shc
IB: FLAG
220 kDa
SHIP2
- + - + - + - + BisI
- - + + - - + + PDGF
SHIP2 WT
SHIP2 T958A


SHIP2 tyrosine phosphorylation (IOD units)
PDGF
PDGF + BisI
***
***
**
50
40
30
20
10
0
SHIP2 WT
SHIP2 T958A


Shc levels (IOD units)
***
***
*
60
50
40
30
20
10
0
SHIP2 WT
SHIP2 T958A

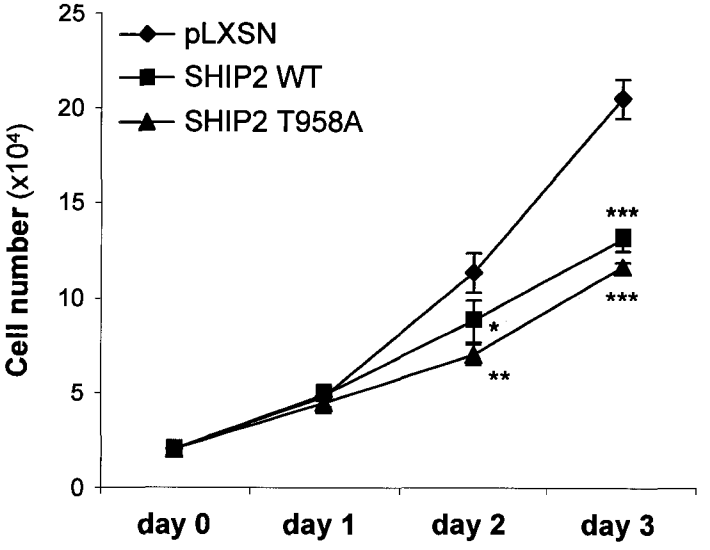
effect of BisI on SHIP2 Tyr phosphorylation, but not Shc association, was significantly reduced for SHIP2 T958A mutant compared to SHIP2 WT.

Overall, phosphorylation of Thr958 appears to be important for PDGF-stimulated SHIP2 Tyr phosphorylation and Shc association. The inhibitory effects of BisI on the Tyr phosphorylation of SHIP2, but not on SHIP2-Shc association, in response to PDGF might be mediated in part through this novel regulatory residue.

T958A does not alter the anti-proliferative effects of SHIP2

We have previously reported that overexpression of SHIP2 WT inhibits proliferation of 3T3-L1 preadipocytes compared to empty vector (Gagnon et al., 2003). To examine if there was a role of Thr958 phosphorylation in this process, we assessesed proliferation in cells overexpressing SHIP2 T958A, SHIP2 WT, or empty vector (pLXSN). Cells were trypsinized and counted for 3 consecutive days after seeding. As expected, SHIP2 WT reduced the cell number by 23±2% on day 2 (mean±SE; n=3; $P<0.05$; Fig. 13) and 36.2±0.5% on day 3 (mean±SE; n=3; $P<0.001$) compared to pLXSN. SHIP2 T958A similarly reduced the cell number by 37±9% on day 2 (mean±SE; n=3; $P<0.01$) and 43±3% on day 3 (mean±SE; n=3; $P<0.001$) compared to empty vector. In fact, there were no significant differences in the number of preadipocytes expressing SHIP2 WT and T958A on any of the days analyzed (n=3; $P>0.05$). Hence, despite the ability of T958A mutation to reduce PDGF-stimulated SHIP2 Tyr phosphorylation and Shc association, this mutation did not alter the anti-proliferative effects of SHIP2.

Figure 13. Mutation of Thr958 to Ala does not alter the anti-proliferative effects of SHIP2. 3T3-L1 preadipocytes overexpressing SHIP2 WT, SHIP2 T958A, or empty vector (pLXSN) were seeded at $2.5x10^3$ cells/cm^2. Cells were trypsinized and counted on the indicated days after plating. Data was obtained from 3 separate experiments, each performed in duplicate, and expressed as means±SE. *P<0.05, **P<0.01, ***P<0.001 compared to pLXSN.


25
20
15
10
5
0
Cell number (x10⁴)
pLXSN
SHIP2 WT
SHIP2 T958A
***
*
**
***
day 0
day 1
day 2
day 3

SHIP2 WT or T958A does not modulate PDGF signalling in preadipocytes

PDGF is an important mitogen found in serum (Heldin and Westermark, 1999; Ross and Vogel, 1978). To understand the mechanism of the anti-proliferative action of SHIP2, we examined PDGF-mediated signalling in preadipocytes. Following acute stimulation with PDGF, PDGFR and its downstream targets Akt and ERK1/2 were all phosphorylated in empty vector-expressing cells (Fig. 14). Overexpression of SHIP2 WT or T958A did not alter PDGF-stimulated phosphorylation of PDGFR, Akt, or ERK1/2 (n=4; $P>0.05$).

Analysis of the role of SHIP2 protein-protein interaction motifs

Mutation of the SH2 domain or NPAYY motif alters SHIP2 Tyr phosphorylation and association with Shc

To examine the role of SHIP2 protein-protein interaction regions in its regulation by PDGF, I changed key residues in the SH2 domain (Arg47 to Gln; R/Q) and in the NPAYY motif (Tyr986 and Tyr987 to Phe; YY/FF) of human FLAG-tagged SHIP2 by site-directed mutagenesis. These constructs, along with SHIP2 WT and empty vector (pLXSN), were stably overexpressed in 3T3-L1 preadipocytes by retroviral transduction followed by antibiotic selection. SHIP2 WT and YY/FF protein levels were comparable; SHIP2 R/Q was expressed at lower levels than SHIP2 WT (see Fig. 17 for a SHIP2 immunoblot).

Analysis of SHIP2 Tyr phosphorylation by anti-FLAG immunoprecipitation followed by anti-phosphotyrosine immunoblotting revealed that PDGF-stimulated phosphorylation of both SHIP2 R/Q and YY/FF was much lower than SHIP2 WT (70±10 and 80±3% decrease, respectively; mean±SE; n=3; $P<0.001$; Fig. 15).

Figure 14. SHIP2 T958A does not affect activation of PDGFR, Akt, or ERK1/2 in response to acute stimulation with PDGF. Confluent 3T3-L1 preadipocytes overexpressing SHIP2 WT, SHIP2 T958A, or empty vector (pLXSN) were stimulated with 10 ng/ml PDGF or vehicle for 5 min. Cells were lysed, and equal amounts of protein were immunoblotted with antibodies against phosphotyrosine, PDGFRβ, phospho-Akt (Ser473), Akt, phospho-ERK1/2, or ERK1/2. Densitometric data analyzing phosphorylated proteins were obtained from 4 separate experiments and expressed as means±SE. A representative immunoblot is shown.

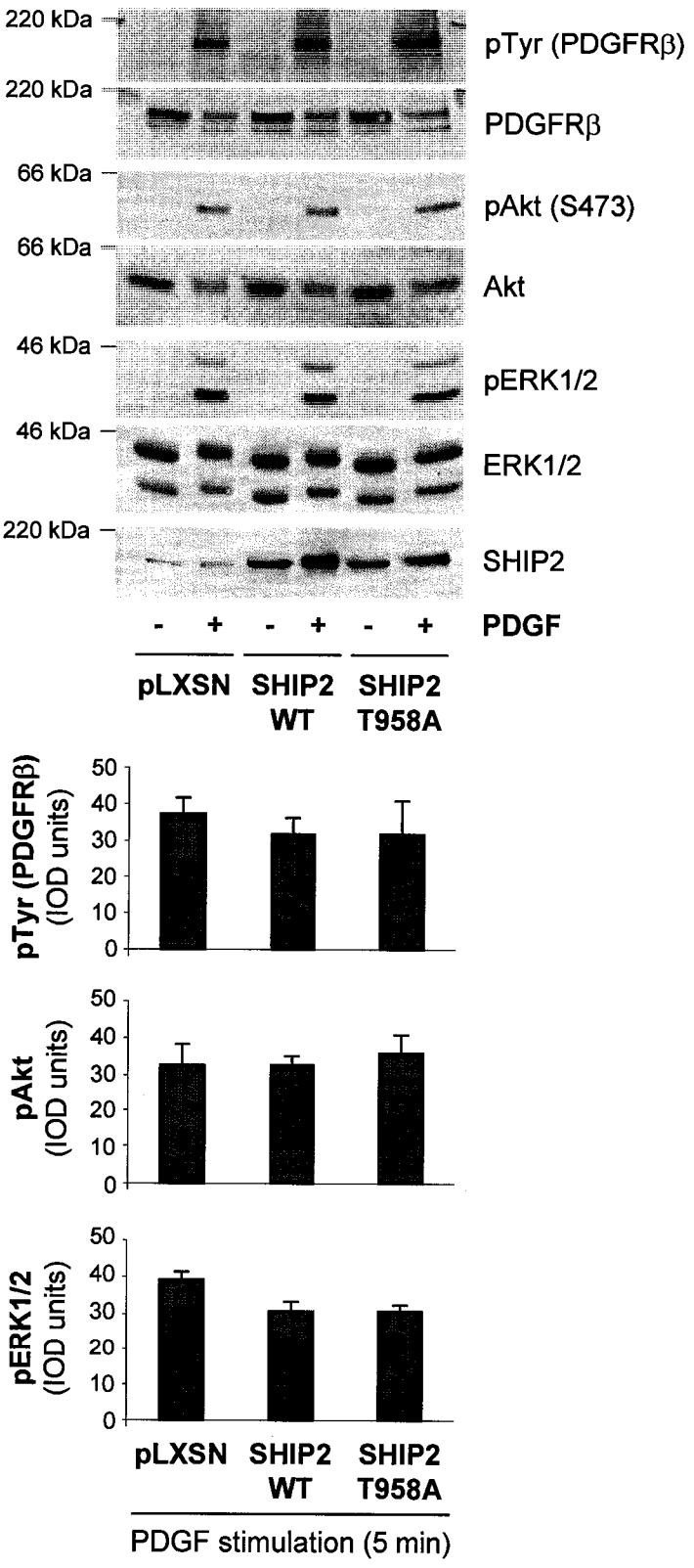
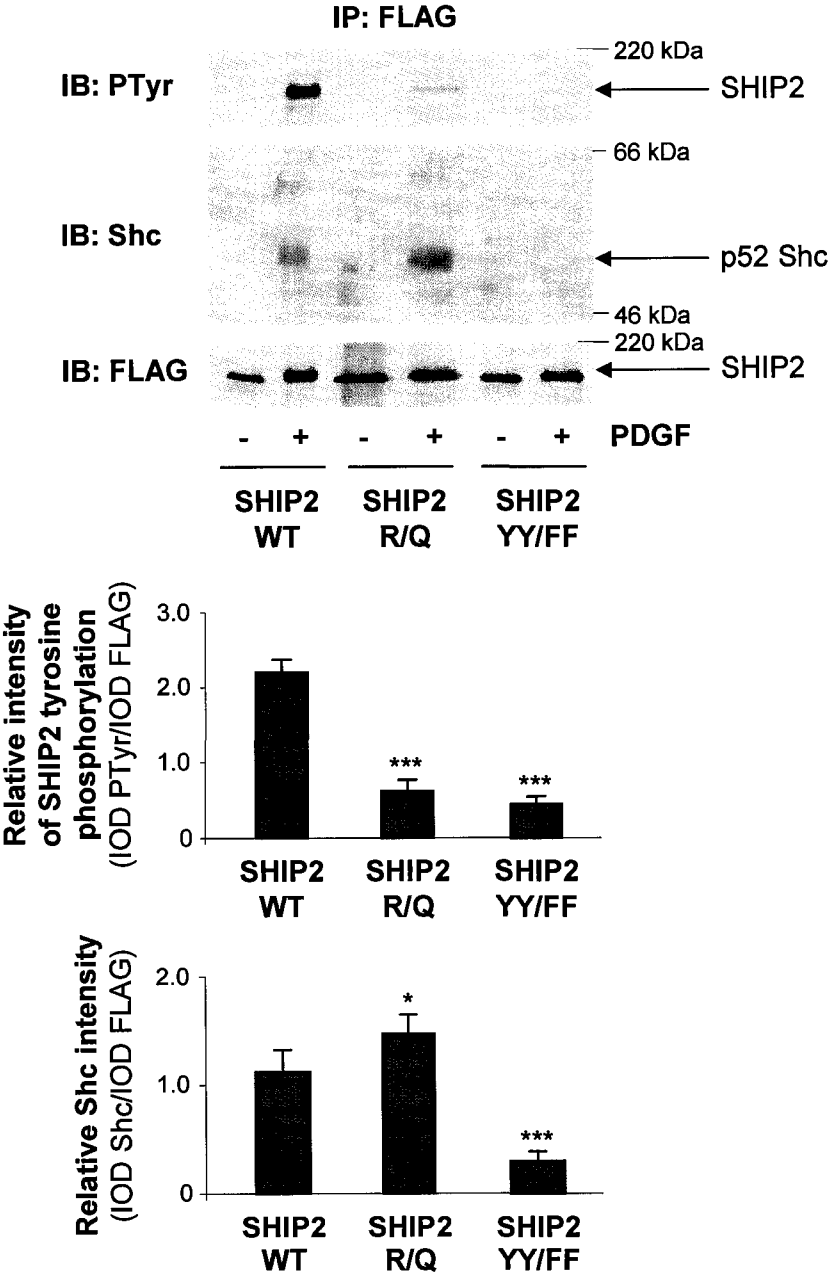

220 kDa
pTyr (PDGFRβ)
220 kDa
PDGFRβ
66 kDa
pAkt (S473)
66 kDa
Akt
46 kDa
pERK1/2
46 kDa
ERK1/2
220 kDa
SHIP2
- + - + - +
PDGF
pLXSN
SHIP2 WT
SHIP2 T958A
pTyr (PDGFRβ) (IOD units)
pAkt (IOD units)
pERK1/2 (IOD units)
50
40
30
20
10
0
pLXSN
SHIP2 WT
SHIP2 T958A
PDGF stimulation (5 min)

Figure 15. Mutation of the SH2 domain or the NPAYY motif alters PDGF-stimulated SHIP2 Tyr phosphorylation and association with Shc. Confluent 3T3-L1 preadipocytes overexpressing FLAG-tagged SHIP2 WT, SHIP2 R/Q, or SHIP2 YY/FF were stimulated with 10 ng/ml PDGF or vehicle for 5 min. Cell lysates were incubated with anti-FLAG antibodies. Immunoprecipitated proteins were immunoblotted with antibodies against phosphotyrosine, Shc, or FLAG. Densitometric data were obtained from 3 separate experiments, normalized for FLAG levels, and expressed as means±SE. A representative immunoblot is shown. *P<0.05, ***P<0.001 compared to the same condition for SHIP2 WT.


IP: FLAG
IB: PTyr
220 kDa
SHIP2
IB: Shc
66 kDa
p52 Shc
46 kDa
IB: FLAG
220 kDa
SHIP2
- + - + - + PDGF
SHIP2 WT
SHIP2 R/Q
SHIP2 YY/FF
Relative intensity of SHIP2 tyrosine phosphorylation (IOD PTyr/IOD FLAG)
3.0
2.0
1.0
0
***
***
SHIP2 WT
SHIP2 R/Q
SHIP2 YY/FF
Relative Shc intensity (IOD Shc/IOD FLAG)
2.0
1.0
0
*
***
SHIP2 WT
SHIP2 R/Q
SHIP2 YY/FF

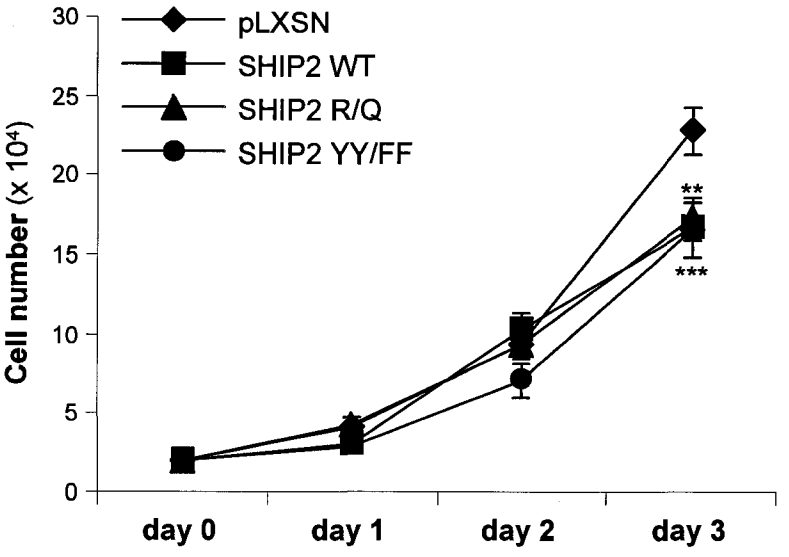
Based on the earlier findings with BisI and the T958A mutation, the association of SHIP2 with Shc appears to correlate with the extent of SHIP2 Tyr phosphorylation. Hence, the 75±3% reduction (mean±SE; n=3; $P<0.001$) in PDGF-stimulated association between Shc and SHIP2 YY/FF compared to SHIP2 WT was not surprising. In contrast, SHIP2 R/Q did not demonstrate such a reduction in PDGF-induced Shc association; in fact, this mutant showed a 23±11% increase compared to SHIP2 WT (mean±SE; n=3; $P<0.05$).

Overall, it appears that SH2 domain and NPAYY motif are important for PDGF-stimulated SHIP2 Tyr phosphorylation, yet only NPAYY motif is necessary for association with Shc.

Mutation of SH2 domain or NPAYY motif does not alter the anti-proliferative effects of SHIP2 or PDGF signalling in preadipocytes

To understand physiological implications of the mutated SH2 domain or NPAYY motif, I assessed proliferation of preadipocytes overexpressing SHIP2 WT, SHIP2 R/Q, SHIP2 YY/FF, or empty vector. On day 3 after seeding, the number of preadipocytes overexpressing SHIP2 WT, SHIP2 R/Q, or SHIP2 YY/FF was reduced by 18±5%, 26±2%, and 27±7%, respectively (mean±SE; n=4; $P<0.01$ for SHIP2 WT, $P<0.001$ for SHIP2 R/Q and YY/FF, all compared to pLXSN; Fig. 16). Despite the ability of R/Q and YY/FF mutations to alter SHIP2 Tyr phosphorylation and/or Shc association, these mutants did not behave any differently from SHIP2 WT with respect to inhibition of proliferation (n=4; $P>0.05$; Fig. 16). Similarly to SHIP2 WT, both SHIP2 R/Q and YY/FF did not affect PDGF-stimulated phosphorylation of PDGFR, Akt, or ERK1/2 compared to empty vector (n=5;

Figure 16. Mutation of the SH2 domain or the NPAYY motif does not alter the anti-proliferative effects of SHIP2. 3T3-L1 preadipocytes overexpressing SHIP2 WT, SHIP2 R/Q, SHIP2 YY/FF, or empty vector (pLXSN) were seeded at $2.5x10^3$ cells/cm^2. Cells were trypsinized and counted on the indicated days after plating. Data were obtained from 4 separate experiments, each performed in duplicate, and expressed as means±SE. **P<0.01, ***P<0.001 compared to pLXSN. ** refer to SHIP2 WT, whereas *** refer to R/Q and YY/FF.


pLXSN
SHIP2 WT
SHIP2 R/Q
SHIP2 YY/FF
Cell number (x 10⁴)
30
25
20
15
10
5
0
day 0
day 1
day 2
day 3
**
***

P>0.05; Fig. 17). A small difference in PDGFR phosphorylation was noted between SHIP2 WT and SHIP2 R/Q, but the significance of this difference is unclear.

Analysis of the role of SHIP2 catalytic domain

Catalytically inactive SHIP2 inhibits preadipocyte proliferation

To assess the role of the 5-phosphatase domain of SHIP2 in its anti-proliferative action, I changed Pro686, Asp690, and Arg691, three conserved residues previously shown to be critical for the 5-phosphatase domain (Ono et al., 1997), to corresponding Ala residues in human FLAG-tagged SHIP2 (SHIP2 PDR/AAA). An equivalent mutation of rat SHIP2 has been demonstrated to render it catalytically inactive and dominant-negative (Wada et al., 2001). SHIP2 WT, SHIP2 PDR/AAA, or empty vector pLXSN were stably overexpressed in 3T3-L1 preadipocytes by retroviral transduction followed by G418 selection. SHIP2 PDR/AAA levels were higher than the levels of endogenous SHIP2, although lower than SHIP2 WT levels (see Fig. 21 for a SHIP2 immunoblot).

Unexpectedly, mutating the catalytic domain enhanced the anti-proliferative effects of SHIP2. On day 3 after plating, cell number was reduced by 53±5% (mean±SE; n=8; P<0.001; Fig. 18A) for SHIP2 PDR/AAA vs. 15±10% (mean±SE; n=8; P<0.01) for SHIP2 WT cells compared to empty vector control preadipocytes. On day 3, [^{3}H]-thymidine incorporation normalized for cell number was inhibited by 35±9% in the presence of SHIP2 PDR/AAA compared to empty vector (mean±SE; n=4; P<0.05; Fig. 18B). Thymidine incorporation for SHIP2 WT expressing cells was reduced by 15±7% compared to pLXSN, but did not reach statistical significance (n=4; P>0.05).

Figure 17. SHIP2 R/Q or YY/FF does not affect activation of PDGFR, Akt, or ERK1/2 in response to acute stimulation with PDGF. Confluent 3T3-L1 preadipocytes overexpressing SHIP2 WT, SHIP2 R/Q, SHIP2 YY/FF, or empty vector (pLXSN) were stimulated with 10 ng/ml PDGF or vehicle for 5 min. Cells were lysed, and equal amounts of solubilized protein were immunoblotted with antibodies against phosphotyrosine, PDGFRβ, phospho-Akt (Ser473), Akt, phospho-ERK1/2, or ERK1/2. A representative immunoblot is shown. Densitometric data analyzing phosphorylated proteins were obtained from 5 separate experiments and expressed as means±SE.

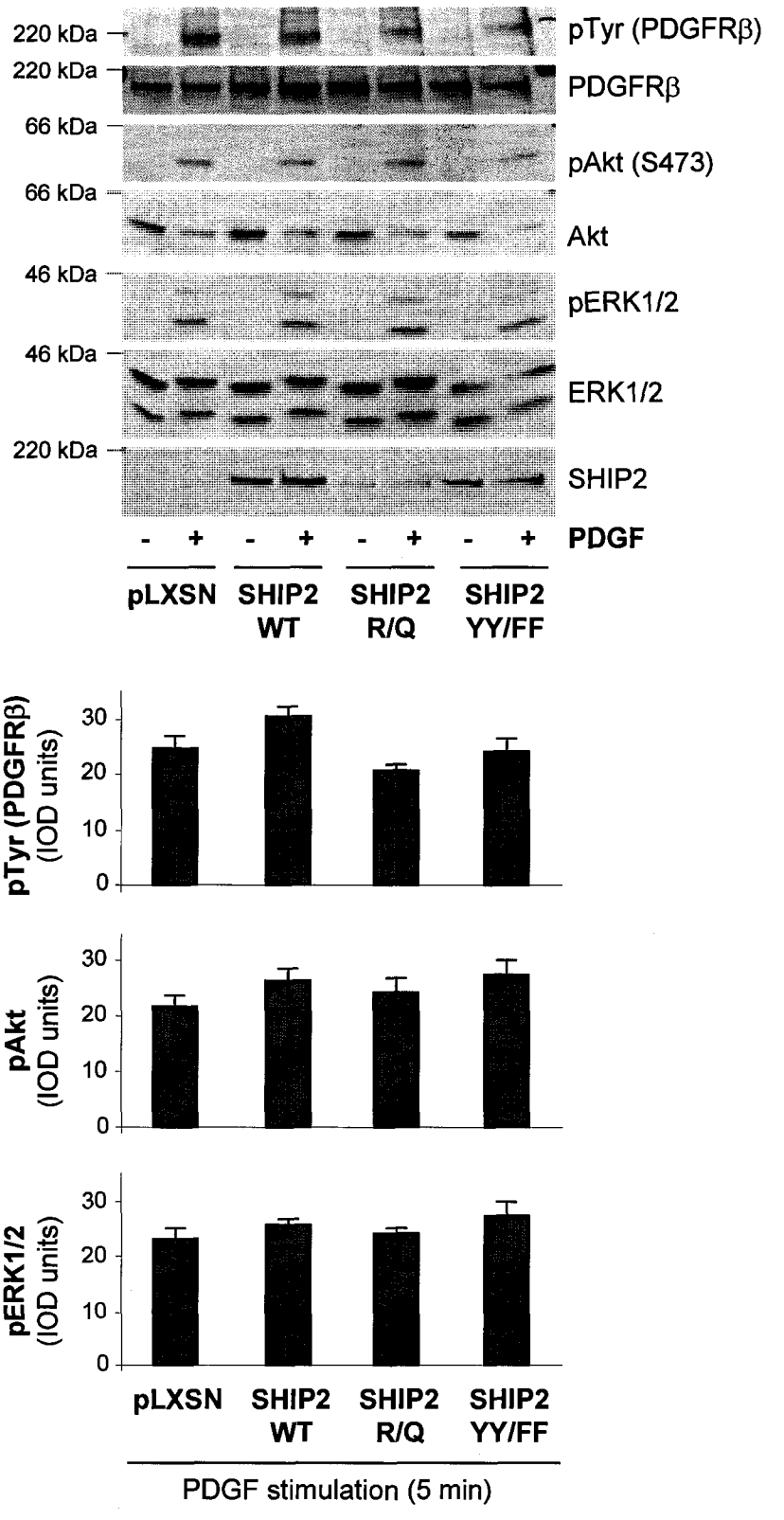
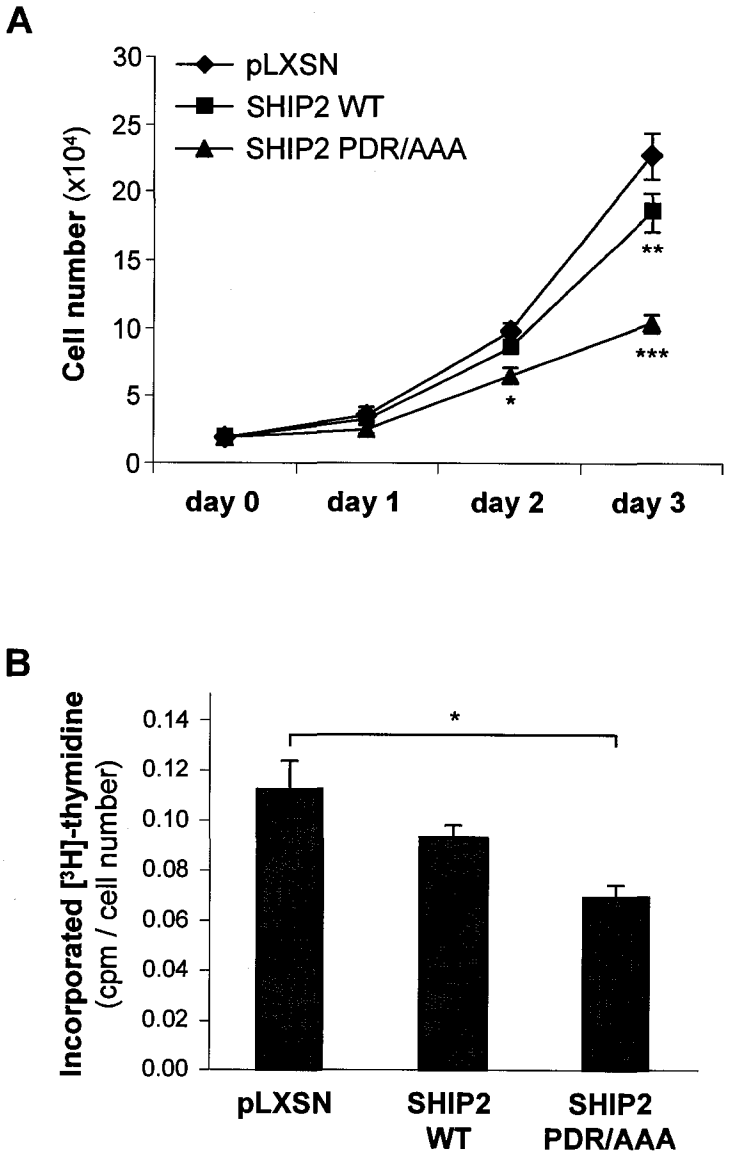

220 kDa
220 kDa
66 kDa
66 kDa
46 kDa
46 kDa
220 kDa
pTyr (PDGFRβ)
PDGFRβ
pAkt (S473)
Akt
pERK1/2
ERK1/2
SHIP2
- + - + - + - +
PDGF
pLXSN
SHIP2 WT
SHIP2 R/Q
SHIP2 YY/FF
pTyr (PDGFRβ) (IOD units)
pAkt (IOD units)
pERK1/2 (IOD units)
0
10
20
30
PDGF stimulation (5 min)

Figure 18. Mutation of the 5-phosphatase domain enhances the anti-proliferative effects of SHIP2 in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes overexpressing SHIP2 WT, SHIP2 PDR/AAA, or empty vector (pLXSN) were seeded at $2.5x10^3$ cells/cm^2. **(A)** Cells were trypsinized and counted on the indicated days after plating. Data were obtained from 8 separate experiments, each performed in duplicate, and expressed as means±SE. **(B)** On day 3, cells were incubated with 1 μCi/ml [^{3}H]-thymidine for 1 hour. Following cell solubilization, DNA was precipitated, and radioactivity was quantified by scintillation spectroscopy. The resulting counts were normalized for cell number from replicate plates. Data were obtained from 4 separate experiments, each performed in triplicate, and expressed as means±SE. *P<0.05, **P<0.01, ***P<0.001 compared to pLXSN.


A
pLXSN
SHIP2 WT
SHIP2 PDR/AAA
Cell number (x10⁴)
30
25
20
15
10
5
0
day 0
day 1
day 2
day 3
*
**
***
B
Incorporated [³H]-thymidine
(cpm / cell number)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
pLXSN
SHIP2 WT
SHIP2 PDR/AAA

To exclude enhanced cell death as an explanation for reduced cell number, I stained preadipocytes with Hoechst 33248 on the third day after seeding. No significant differences in the percentage of apoptotic cells were found between the constructs (n=5; P>0.05; Fig. 19A).

To confirm that the observed reduction in cell number was not due to attenuated attachment, I trypsinized and counted preadipocytes expressing pLXSN, SHIP2 WT, or SHIP2 PDR/AAA 3 hours after seeding and attachment. No significant differences were found between the three constructs (n=6; P>0.05; Fig. 19B).

Catalytically inactive SHIP2 reduces both Akt and ERK1/2 signalling, and PDGFR expression in proliferating preadipocytes

To determine which mitogenic pathway was affected by catalytically inactive SHIP2 during proliferation, I lysed proliferating preadipocytes overexpressing SHIP2 WT, SHIP2 PDR/AAA, or empty vector on day 3 after seeding. Compared to pLXSN, SHIP2 PDR/AAA reduced phosphorylation of both Akt and ERK1/2 by 36±10% and 27±5%, respectively (mean±SE; n=8; P<0.01; Fig. 20). There was also a trend toward reduced Akt, but not ERK1/2, phosphorylation in SHIP2 WT preadipocytes, although this did not reach statistical significance (n=8; P>0.05).

Since catalytically inactive SHIP2 reduced both Akt and ERK1/2 phosphorylation, I considered that this mutant might affect an upstream regulator of both of these pathways. As noted earlier, one important growth factor that is found in serum used for proliferation studies is PDGF, so I assessed PDGFR cellular expression. Indeed, the levels of PDGFRβ

Figure 19. SHIP2 WT or PDR/AAA does not enhance cell death or reduce cell attachment. 3T3-L1 preadipocytes overexpressing SHIP2 WT, SHIP2 PDR/AAA, or empty vector (pLXSN) were seeded at $2.5x10^3$ cells/cm^2 (A) or 10^4 cells/cm^2 (B). **(A)** On day 3, cells were fixed, stained with Hoechst 33248, and visualized by fluorescence microscopy. Representative images with no apoptotic nuclei are shown for each construct. Positive control refers to confluent 3T3-L1 preadipocytes that have been placed in serum-free medium for 3 hours. Representative apoptotic nuclei are marked by white arrowheads. Data were obtained from 5 separate experiments, with 20 random fields scored for each construct, and expressed as means±SE. **(B)** Cells were trypsinized and counted 3 hours after plating to assess attachment. Data were obtained from 6 separate experiments, each performed in duplicate, and expressed as means±SE.

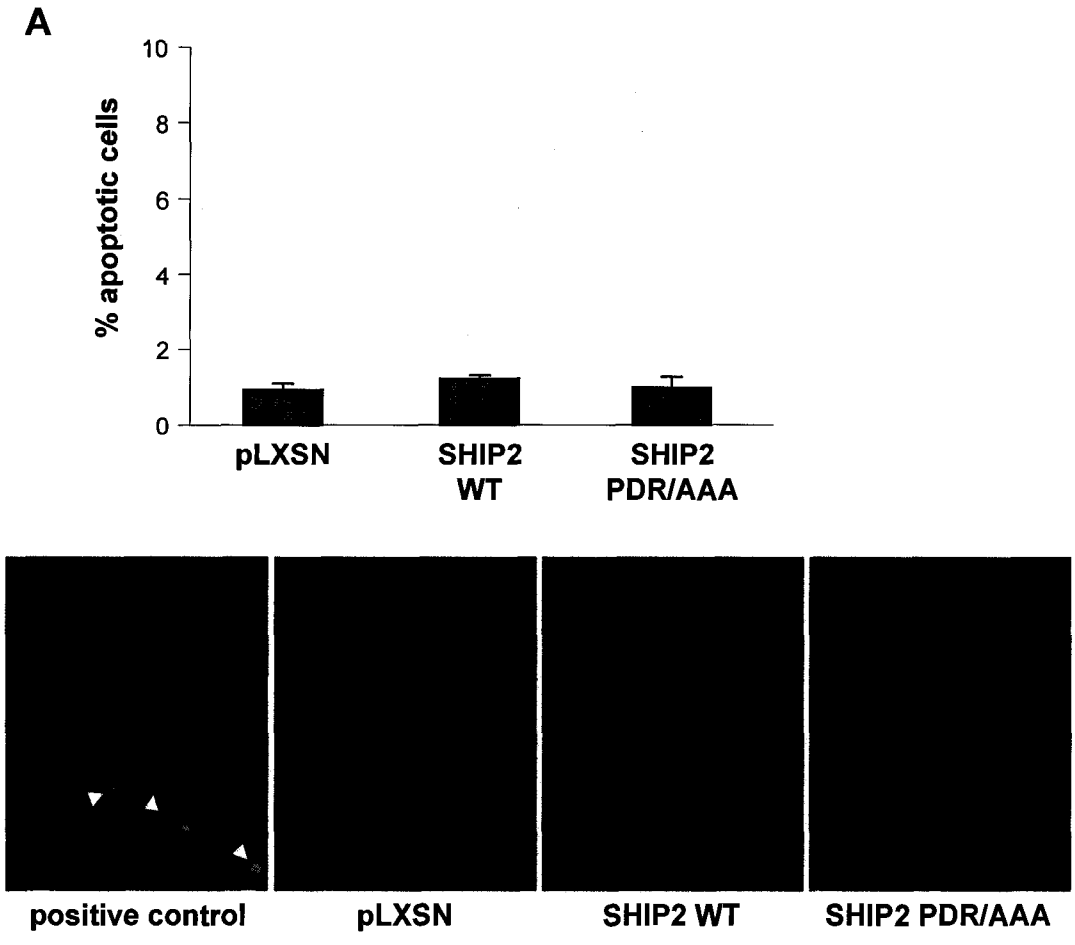
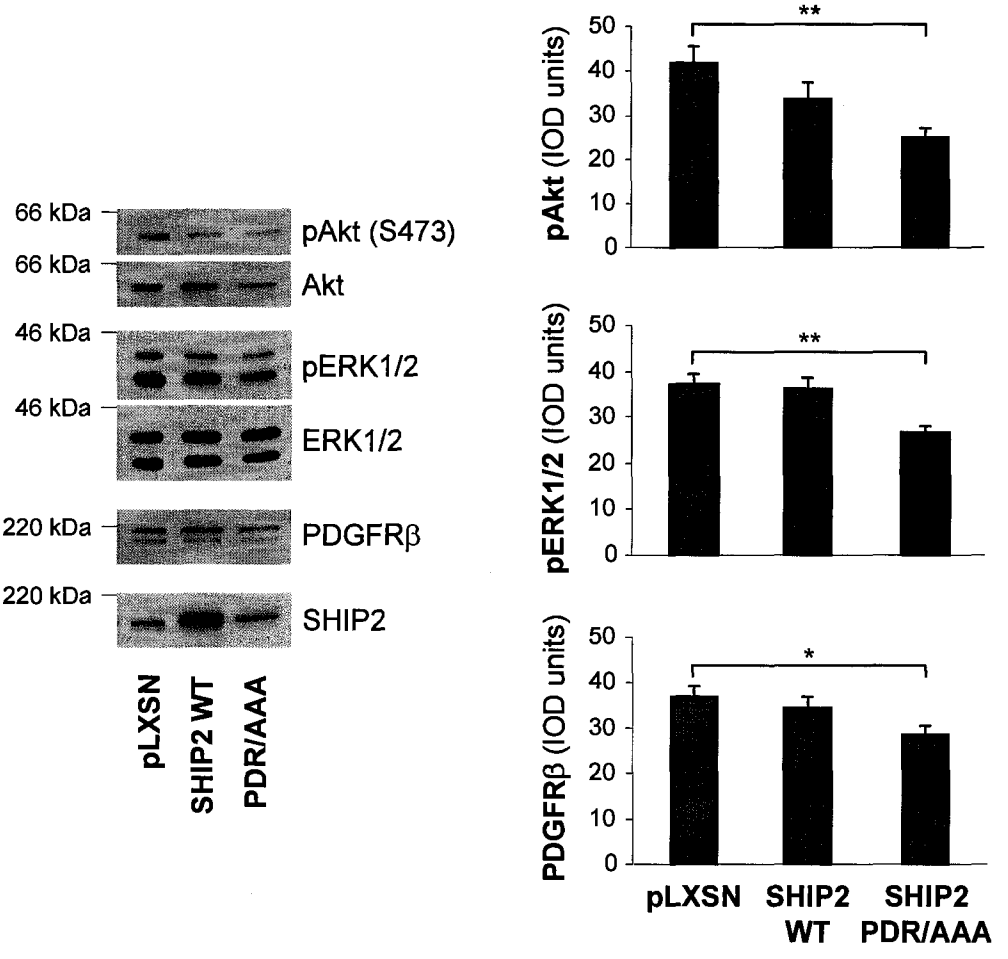

A
% apoptotic cells
10
8
6
4
2
0
pLXSN
SHIP2 WT
SHIP2 PDR/AAA
positive control
pLXSN
SHIP2 WT
SHIP2 PDR/AAA
B
Number of attached cells (X10⁴)
10
8
6
4
2
0
pLXSN
SHIP2 WT
SHIP2 PDR/AAA

Figure 20. Catalytically inactive, dominant-negative SHIP2 reduces Akt and ERK1/2 phosphorylation as well as PDGFR expression in proliferating 3T3-L1 preadipocytes. 3T3-L1 preadipocytes expressing SHIP2 WT, SHIP2 PDR/AAA, or empty vector (pLXSN) were seeded at $2.5x10^3$ cells/cm^2. Cells were lysed on day 3 after plating. Equal amounts of solubilized protein were immunoblotted with antibodies against phospho-Akt (Ser473), Akt, phospho-ERK1/2, ERK1/2, PDGFRβ, or SHIP2. A representative immunoblot is shown. Data were obtained from 8 separate experiments and expressed as means±SE. *P<0.05, **P<0.01 compared to pLXSN.


66 kDa
pAkt (S473)
66 kDa
Akt
46 kDa
pERK1/2
46 kDa
ERK1/2
220 kDa
PDGFRβ
220 kDa
SHIP2
pLXSN
SHIP2 WT
PDR/AAA
pAkt (IOD units)
pERK1/2 (IOD units)
PDGFRβ (IOD units)
0
10
20
30
40
50
**
**
*
pLXSN
SHIP2 WT
SHIP2 PDR/AAA

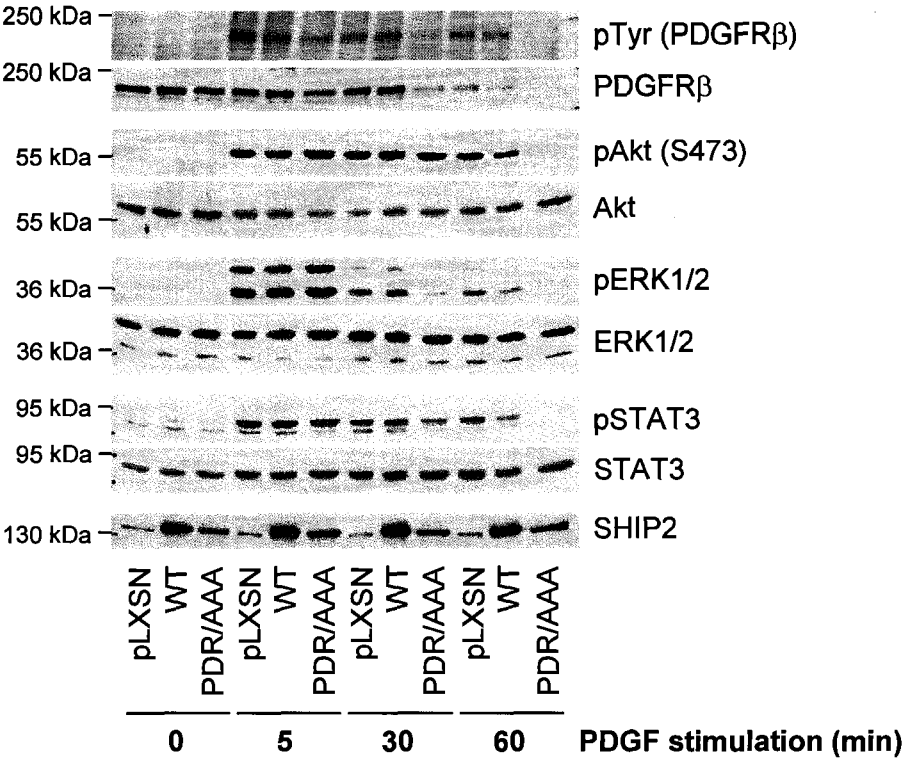
were reduced by 20±9% for preadipocytes overexpressing SHIP2 PDR/AAA compared to pLXSN (mean±SE; n=8; P<0.05).

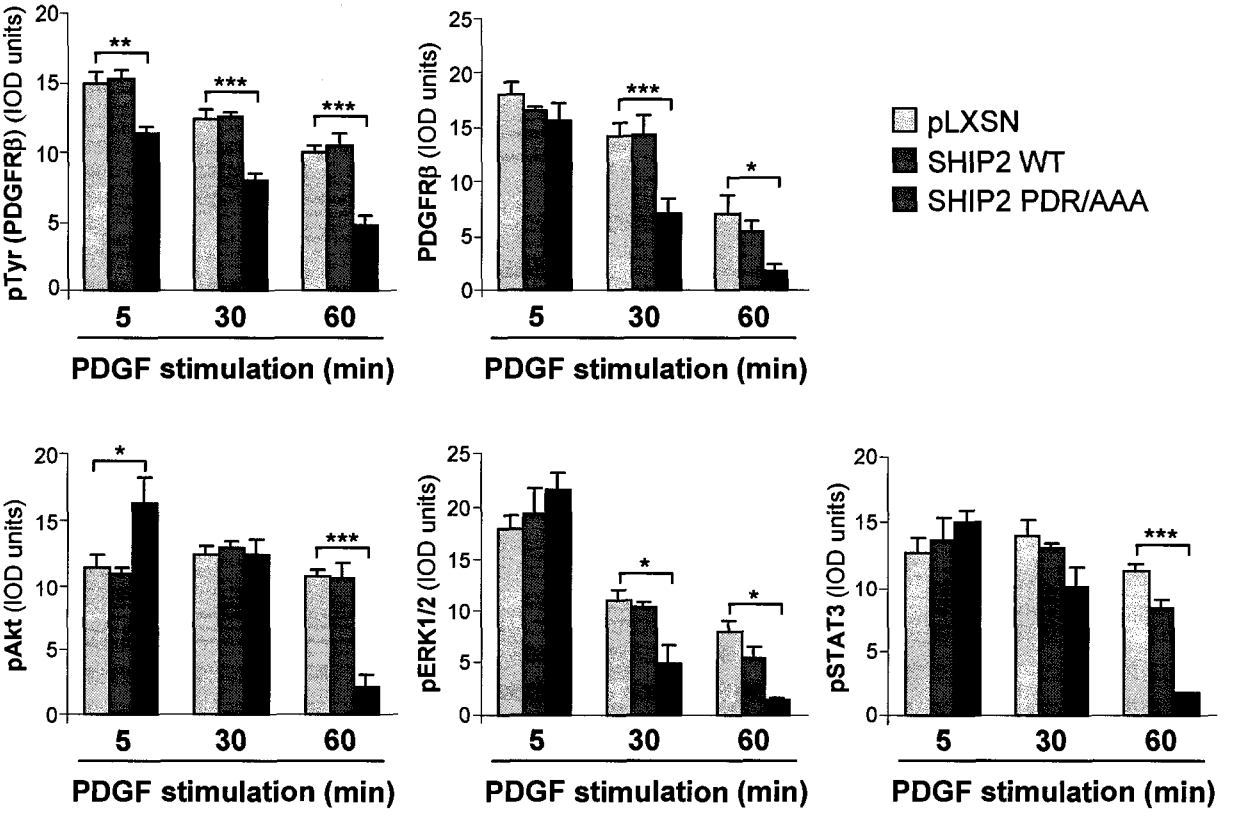
Catalytically inactive SHIP2 reduces PDGFR signalling following acute stimulation with PDGF

To specifically analyze the effects of catalytically inactive SHIP2 on PDGF signalling, I stimulated preadipocytes expressing pLXSN, SHIP2 WT, or SHIP2 PDR/AAA with 10 ng/ml PDGF-BB for up to 60 min. Compared to pLXSN, SHIP2 PDR/AAA significantly reduced the phosphorylation of the PDGFR at 5, 30 and 60 min, reaching a 52±7% decrease by 60 min (mean±SE; n=4; P<0.001; Fig. 21). This attenuation of PDGFR activation correlated with a reduction in the total levels of PDGFRβ. By 60 min the level of the receptor was 71±9% lower in SHIP2 PDR/AAA compared to pLXSN-expressing cells (mean±SE; n=4; P<0.05).

Activation of several mitogenic pathways downstream of PDGFR was greatly diminished in the presence of SHIP2 PDR/AAA. Phosphorylation of Akt, ERK1/2, and STAT3 was reduced by 81±7%, 82±3%, and 84±1%, respectively, by 60 min of stimulation with PDGF for SHIP2 PDR/AAA compared to empty vector cells (mean±SE; n=4; P<0.001 for Akt and STAT3, P<0.05 for ERK1/2). Prior to the robust inhibition observed at 60 min, phosphorylation of Akt was significantly elevated after 5 min stimulation with PDGF in SHIP2 PDR/AAA compared to pLXSN-expressing preadipocytes (42±8% increase; mean±SE; n=4; P<0.05).

Figure 21. Catalytically inactive, dominant-negative SHIP2 reduces PDGFR signalling following acute stimulation with PDGF. Confluent 3T3-L1 preadipocytes overexpressing SHIP2 WT, SHIP2 PDR/AAA or empty vector (pLXSN) were stimulated with 10 ng/ml PDGF for the indicated time. Following cell lysis, equal amounts of solubilized protein were immunoblotted with antibodies against phosphotyrosine, PDGFRβ, phospho-Akt (Ser473), Akt, phospho-ERK1/2, ERK1/2, phospho-STAT3, STAT3, or SHIP2. A representative immunoblot is shown. Densitometric data were obtained from 4 separate experiments and expressed as means±SE. *P<0.05, **P<0.01, ***P<0.001 compared to pLXSN.


250 kDa
250 kDa
pTyr (PDGFRβ)
PDGFRβ
55 kDa
55 kDa
pAkt (S473)
Akt
36 kDa
36 kDa
pERK1/2
ERK1/2
95 kDa
95 kDa
pSTAT3
STAT3
130 kDa
SHIP2
pLXSN
WT
PDR/AAA
0
5
30
60
PDGF stimulation (min)


pTyr (PDGFRβ) (IOD units)
PDGFRβ (IOD units)
pAkt (IOD units)
pERK1/2 (IOD units)
pSTAT3 (IOD units)
pLXSN
SHIP2 WT
SHIP2 PDR/AAA
5
30
60
PDGF stimulation (min)

In contrast to SHIP2 PDR/AAA, no significant changes in the activation of the PDGFR or its downstream targets were observed for SHIP2 WT compared to empty vector (n=4; P>0.05).

Anti-proliferative effect of imatinib is reduced by catalytically inactive SHIP2

To evaluate whether the anti-proliferative effect of SHIP2 PDR/AAA was due to attenuation of PDGFR signalling, I utilized imatinib, which inhibits the Tyr kinase activity of the PDGFR, as well as proto-oncogenes c-kit and c-Abl (Buchdunger et al., 2000). Pretreatment of 3T3-L1 preadipocytes with 10 μM imatinib for 90 minutes, followed by a 5 minute stimulation with 10 ng/ml PDGF, abolished PDGF-induced phosphorylation, without affecting expression of the PDGFRβ (n=1; Fig. 22).

Addition of 10 μM imatinib during subconfluent preadipocyte proliferation (i.e. at seeding, and once daily on the two following days), resulted in a 48±5% reduction in cell number compared to cells grown in the presence of vehicle alone (mean±SE; n=3; P<0.05; Fig. 23A). The difference in cell number was not due to reduced attachment of cells (n=3; P>0.05 compared to vehicle; Fig. 23B). These observations confirmed that PDGF is a major mitogenic component of the serum used for the predipocyte proliferation studies.

Consistent with the above observations with non-transduced 3T3-L1 preadipocytes, the number of pLXSN-expressing preadipocytes on day 3 after seeding was reduced by 41±7% in the presence of imatinib compared to vehicle (mean±SE; n=3; P<0.01; Fig. 23C). Cells overexpressing SHIP2 WT exhibited a similar 43±9% decrease with imatinib (mean±SE; n=3; P<0.05). However, in SHIP2 PDR/AAA-overexpressing cells, the effect of imatinib was reduced to 33±10%, and in fact no longer reached statistical significance

Figure 22. Imatinib inhibits PDGFR activation. Confluent 3T3-L1 preadipocytes were kept in serum-reduced medium overnight prior to pretreatment with 10 μM imatinib or DMSO (vehicle) for 90 min, followed by stimulation with 10 ng/ml PDGF or vehicle for 5 min. Following cell lysis, equal amounts of proteins were immunoblotted with antibodies against phosphotyrosine, PDGFRβ, or ERK1/2. A single immunoblot is shown.

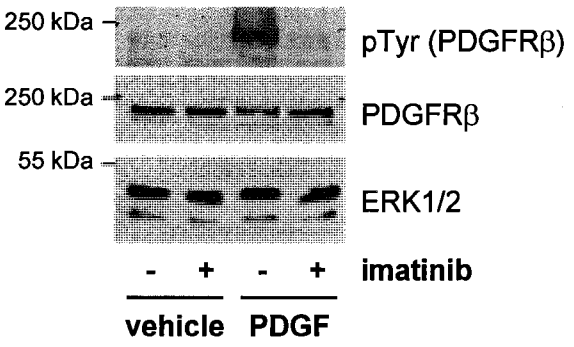
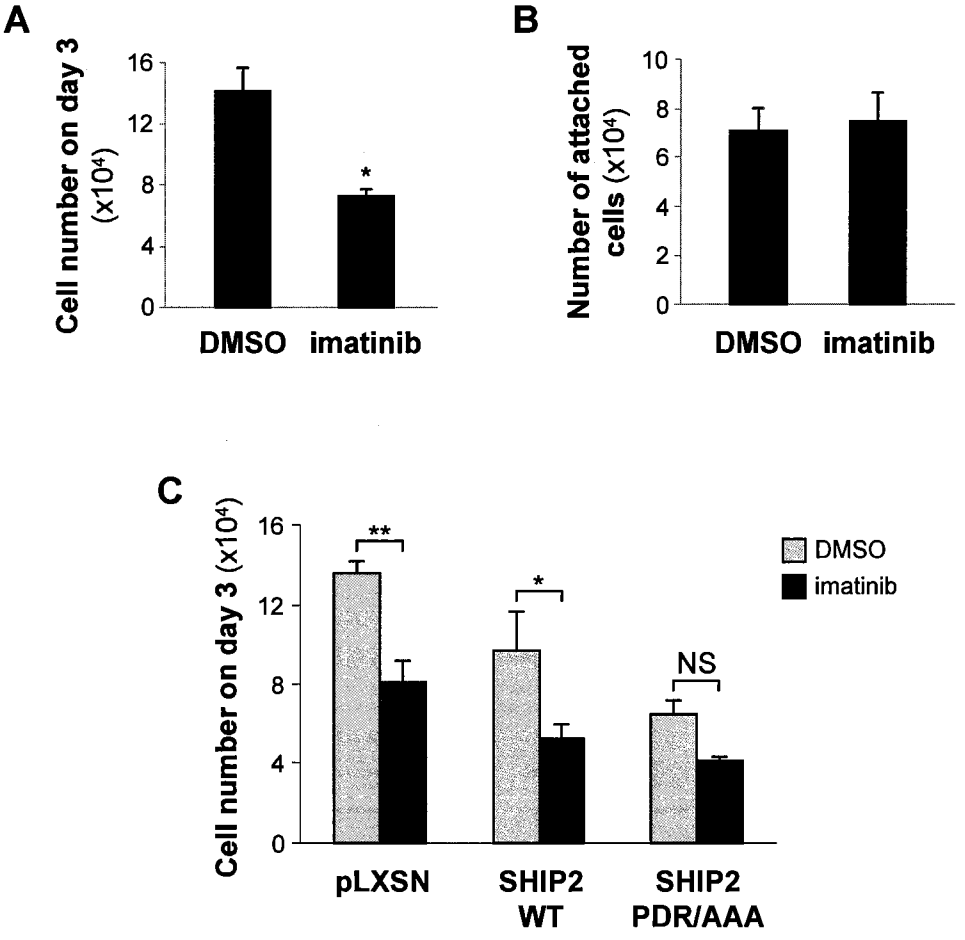

250 kDa
pTyr (PDGFRβ)
250 kDa
PDGFRβ
55 kDa
ERK1/2
- + - +
imatinib
vehicle
PDGF

Figure 23. Catalytically inactive, dominant-negative SHIP2 attenuates the anti-proliferative effects of imatinib. (A, B) 3T3-L1 preadipocytes were seeded at $2.5x10^3$ cells/cm^2 (A) or 10^4 cells/cm^2 (B) in the presence of 10 μM imatinib or DMSO (vehicle). **(A)** Fresh 10 μM imatinib or DMSO was also added on days 1 and 2 after seeding. Cells were trypsinized and counted on day 3. Data were obtained from 3 separate experiments, each performed in duplicate. **(B)** Cells were trypsinized and counted 3 hours after plating. Data were obtained from 3 separate experiments, each performed in duplicate. **(C)** 3T3-L1 preadipocytes overexpressing SHIP2 WT, SHIP2 PDR/AAA, or empty vector (pLXSN) were seeded at $2.5x10^3$ cells/cm^2 in the presence of 10 μM imatinib or DMSO, and subjected to the same treatment as in (A). Data were obtained from 3 separate experiments, each performed in duplicate. All values are expressed as means±SE. *P<0.05, **P<0.01 compared to DMSO. NS – not significant.


A
Cell number on day 3 (x10⁴)
16
12
8
4
0
*
DMSO
imatinib
B
Number of attached cells (x10⁴)
10
8
6
4
2
0
DMSO
imatinib
C
Cell number on day 3 (x10⁴)
16
12
8
4
0
**
*
NS
DMSO
imatinib
pLXSN
SHIP2 WT
SHIP2 PDR/AAA

(mean±SE; n=3; P>0.05). These findings suggest that the inhibition of proliferation observed in the presence of SHIP2 PDR/AAA is due to impaired PDGFR signalling, so that further inhibition with imatinib had minimal impact.

CHAPTER II:

Evaluation of SHIP2 as a Mediator of the Anti-adipogenic Effects of PDGF

MATERIALS AND METHODS

In addition to the materials and methods described in Chapter I, the following procedures are pertinent to Chapter II.

Differentiation of 3T3-L1 preadipocytes

Two-day post-confluent 3T3-L1 preadipocytes were induced to differentiate in DMEM supplemented with 10% FBS and antibiotics with or without 10 ng/ml PDGF for six days. 0.25 μM dexamethasone and 0.5 mM IBMX were present for the first two days (Rubin et al., 1978). 1 μM insulin (Roche; Indianapolis, IN) was also present during the first four days of differentiation of 3T3-L1 preadipocytes stably expressing SHIP2 WT, SHIP2 PDR/AAA, or empty vector (pLXSN). Control cells were maintained in DMEM supplemented with 10% CS and antibiotics. On the indicated days following induction of differentiation, cells were enumerated, or analyzed for triglyceride accumulation. Alternatively, cells were lysed in Laemmli buffer and subjected to immunoblotting as described below.

Triglyceride accumulation

On day 6 after induction of differentiation, cells were rinsed twice with PBS. Triglycerides were extracted with an isopropanol:heptane (2:3, v:v) mixture and quantified by a colourimetric method with a triolein standard as previously described (Gagnon et al.,

1999). Briefly, triglycerides were saponified with potassium hydroxide, and resulting glycerol was oxidized by sodium metaperiodate. Following addition of acetylacetone, the final chromogen was quantified at 410 nm on Ultrospec 3000 spectrophotometer (Pharmacia Biotech).

Immunoblotting

Following cell lysis, protein concentration was determined using a modified Lowry reaction with BSA as a standard (D_C Protein Assay). Equal amounts of protein (10-70 μg, depending on the experiment) were separated by 7.5% or 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Non-specific binding sites were blocked with PBS containing 0.1% Tween 20 and 5% skim milk powder. Membranes were incubated overnight in PBS containing 3% BSA, 0.02% sodium azide, and the relevant primary antibodies to detect the following: FAS (mouse monoclonal; 1.0 μg/ml; BD Biosciences); phospho-Rb (rabbit polyclonal; 1:500; Cell Signaling); human inhibitor of apoptosis protein 2 (HIAP2; rabbit polyclonal; 1.0 μg/ml; R&D systems, Minneapolis, MN); actin (rabbit polyclonal; 0.4 μg/ml), C/EBPα (rabbit polyclonal; 1.0 μg/ml), C/EBPβ (rabbit polyclonal; 1.0 μg/ml), PPARγ (mouse monoclonal; 2.0 μg/ml), SHIP2 (I-20; goat polyclonal; 2 μg/ml), all from Santa Cruz Biotechnology; ERK1/2 (rabbit polyclonal; 1 μg/ml; Upstate). The antibody for adiponectin (rabbit polyclonal; 1:1000) was a gift from P. Scherer, Albert Einstein College of Medicine, NY. The aP2 antibody (rabbit polyclonal; 1:250) was a gift from D. Bernlohr, University of Minnesota, MN. Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies in PBS with 5% skim milk powder, immunoreactivity was detected by enhanced chemiluminescence (Millipore, Nepean, ON,

Canada) using a Kodak M35A X-OMAT Processor. Relative band intensity was determined with AlphaEaseFC$_{TM}$ Software (version 4.0.0) and expressed as IOD units.

PI3K assay

Following stimulation with 10 ng/ml PDGF, 1 μM insulin, or vehicle (0.02 mg/ml BSA in KRH), preadipocytes were lysed in PBS, pH 7.4, 1% Triton X-100, 50 mM sodium fluoride, 0.1 mg/ml phenylmethylsulfonylfluoride, 200 μM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 4 μg/ml benzamidine, 1 mM β-glycerophosphate. Anti-phosphotyrosine immunoprecipitates were prepared as described above, washed, resuspended in assay buffer (20 mM Tris, 0.1 M NaCl, 0.5 mM EGTA, 0.5 mM EDTA, 0.2 mg/ml PI from Sigma), and PI3K activity was then measured as previously described (Bell et al., 2002; Liu et al., 1995). The reaction was started by the addition of either Mg-ATP or Ca-ATP cocktail (10 mM $MgCl_2$ or 10 mM $CaCl_2$, 10 μM cold ATP, 20 μCi [γ-^{32}P]ATP), and terminated after 3 minutes by adding chloroform-methanol-HCl (50:100:1, v:v:v). The lipid product was extracted with chloroform, and resolved by thin-layer chromatography with methanol-chloroform-NH_4OH-HCl (100:70:15:25, v:v:v:v). 1 mg/ml PI4P (Sigma) visualized by iodine staining was used as a standard. Following autoradiographic detection with Kodak M35A X-OMAT Processor, the relative intensity of the band was measured with AlphaEaseFC^{TM} Software (version 4.0.0) and expressed as IOD units.

Cell enumeration

For assessment of cell number during differentiation, cells were detached with trypsin-EDTA on the indicated days after induction of differentiation. Trypsin was

inactivated with an equal volume of DMEM supplemented with 20% FBS. Cells were counted with a Neubauer hemacytometer according to the manufacturer's instructions.

Statistical analysis

ANOVA with the Newman–Keuls post-hoc test for paired values was used to assess differences between multiple means (GraphPad Instat, version 3.05). A P value <0.05 was considered significant.

RESULTS

Characterization of the anti-adipogenic effects of PDGF

PDGF inhibits late stages of adipogenesis

Exposure of 3T3-L1 preadipocytes to the standard adipogenic medium for 6 days resulted in a robust differentiation response, characterized by triglyceride accumulation (6±2 fold increase; mean±range; n=2; Fig. 24), as well as expression of several markers of adipogenesis (Fig. 25). Addition of 10 ng/ml PDGF to the differentiation medium led to a pronounced inhibition of triglyceride accumulation (77±1%; mean±range; n=2; Fig. 24). Expression of C/EBPα, PPARγ, aP2, and adiponectin was also attenuated by 57±10%, 79±1%, 79±7%, and 64±10%, respectively (mean±SE; n=3; $P<0.001$; Fig. 25). Actin and HIAP2 immunoblots were used to confirm equal loading of the protein on the gel. Photomicrographs that visually demonstrate the anti-adipogenic effects of PDGF have been previously published in (Artemenko et al., 2005).

PDGF does not modulate C/EBPβ expression

To determine the time point at which PDGF exerts its effects, I examined expression of adipogenic markers on days 2, 4 and 6 following induction of differentiation. Both C/EBPα and PPARγ were expressed by day 2, and their levels continued to increase, reaching the highest point at day 4 of differentiation (n=3; Fig. 26). Addition of PDGF to the differentiation medium strongly attenuated the induction of both C/EBPα and PPARγ expression at all time points.

C/EBPβ expression peaked at day 2 and returned to basal levels by day 4 of differentiation, consistent with its role in the early stages of adipogenesis (n=3; Fig. 26). In

Figure 24. PDGF reduces triglyceride accumulation in differentiating 3T3-L1 preadipocytes. Two-day post-confluent 3T3-L1 preadipocytes were induced to differentiate or kept in control medium with or without 10 ng/ml PDGF. On day 6 of differentiation, triglycerides were extracted, quantified, and normalized for the protein content of the cells. Results represent 2 separate experiments and are expressed as means±range.

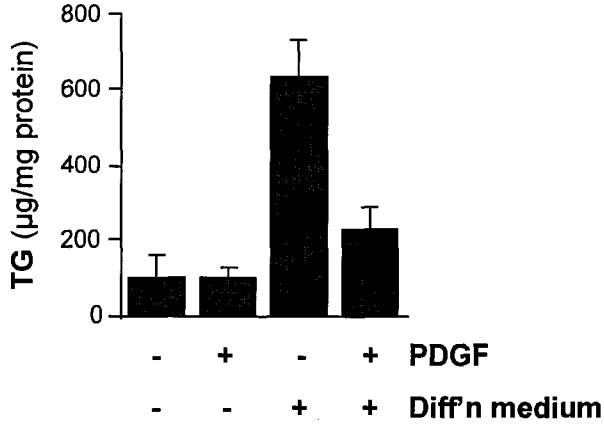
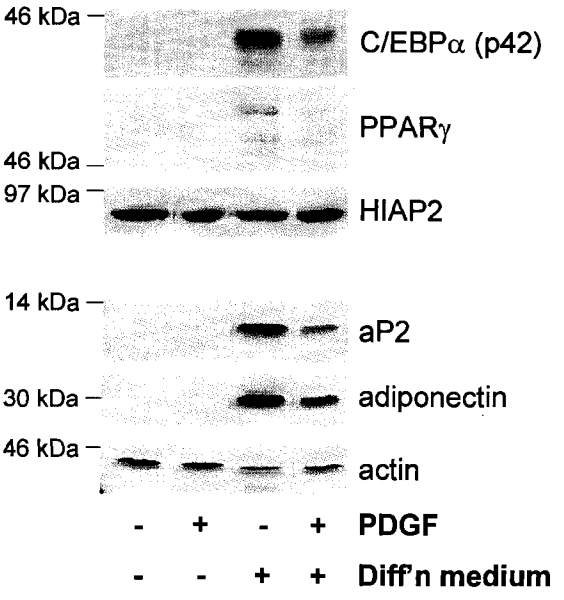

800
600
400
200
0
TG (µg/mg protein)
- + - + PDGF
- - + + Diff'n medium

Figure 25. PDGF suppresses expression of adipogenic markers in differentiating 3T3-L1 preadipocytes. Two-day post-confluent 3T3-L1 preadipocytes were induced to differentiate or kept in control medium with or without 10 ng/ml PDGF. On day 6 of differentiation, cells were lysed and equal amounts of solubilized protein were immunoblotted with antibodies against C/EBPα, PPARγ, HIAP2, aP2, adiponectin, or actin. Representative immunoblots are shown. Densitometric data from 3 separate experiments are expressed as means±SE. ***P<0.001.


46 kDa
C/EBPα (p42)
PPARγ
46 kDa
97 kDa
HIAP2
14 kDa
aP2
30 kDa
adiponectin
46 kDa
actin
- + - + PDGF
- - + + Diff'n medium

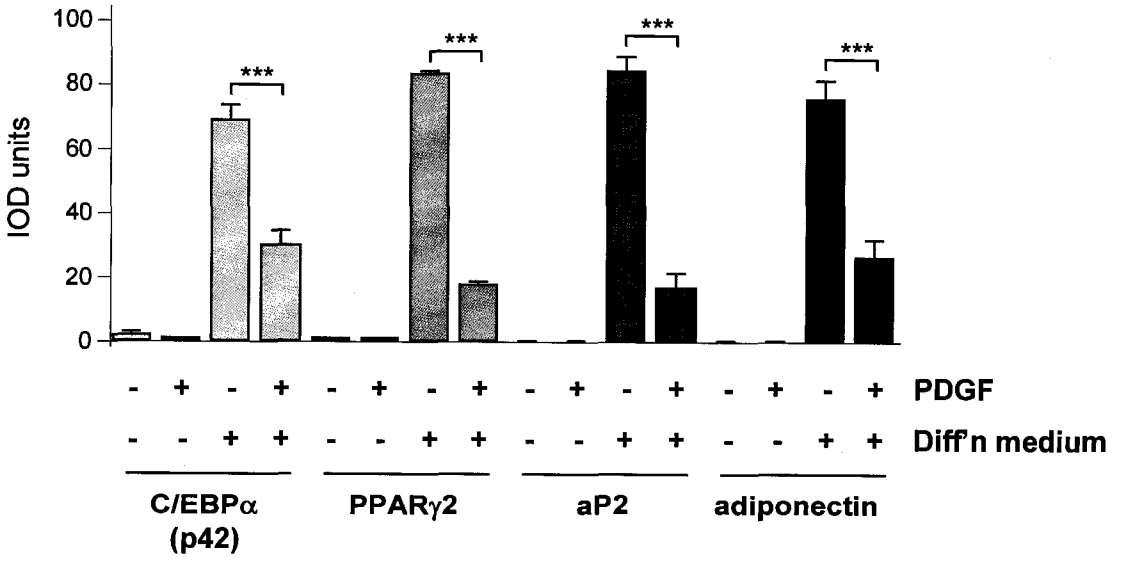
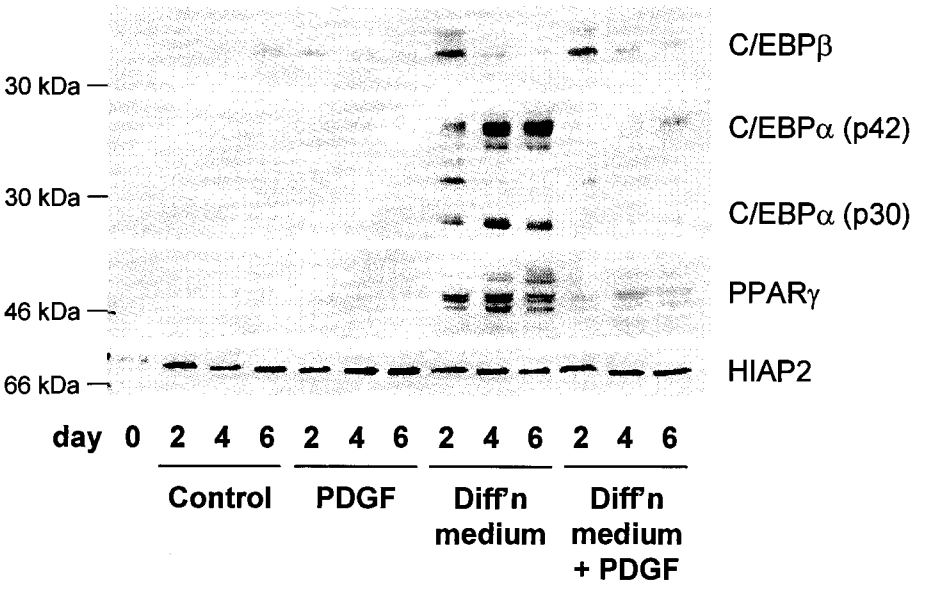

IOD units
100
80
60
40
20
0
***
***
***
***
- + - + - + - + - + - + - + - + PDGF
- - + + - - + + - - + + - - + + Diff'n medium
C/EBPα (p42)
PPARγ2
aP2
adiponectin

Figure 26. PDGF inhibits PPARγ and C/EBPα, but not C/EBPβ, expression in differentiating 3T3-L1 preadipocytes. Two-day post-confluent 3T3-L1 preadipocytes were induced to differentiate or kept in control medium with or without 10 ng/ml PDGF. Cells were lysed on the indicated days after induction of differentiation (day 0) and equal amounts of solubilized protein were immunoblotted with antibodies against C/EBPβ, C/EBPα, PPARγ, or HIAP2. An immunoblot representative of 3 separate experiments is shown.


C/EBPβ
30 kDa
C/EBPα (p42)
30 kDa
C/EBPα (p30)
PPARγ
46 kDa
HIAP2
66 kDa
day 0 2 4 6 2 4 6 2 4 6 2 4 6
Control
PDGF
Diff'n medium
Diff'n medium + PDGF

contrast to the findings with C/EBPα and PPARγ, PDGF had no effect on C/EBPβ expression.

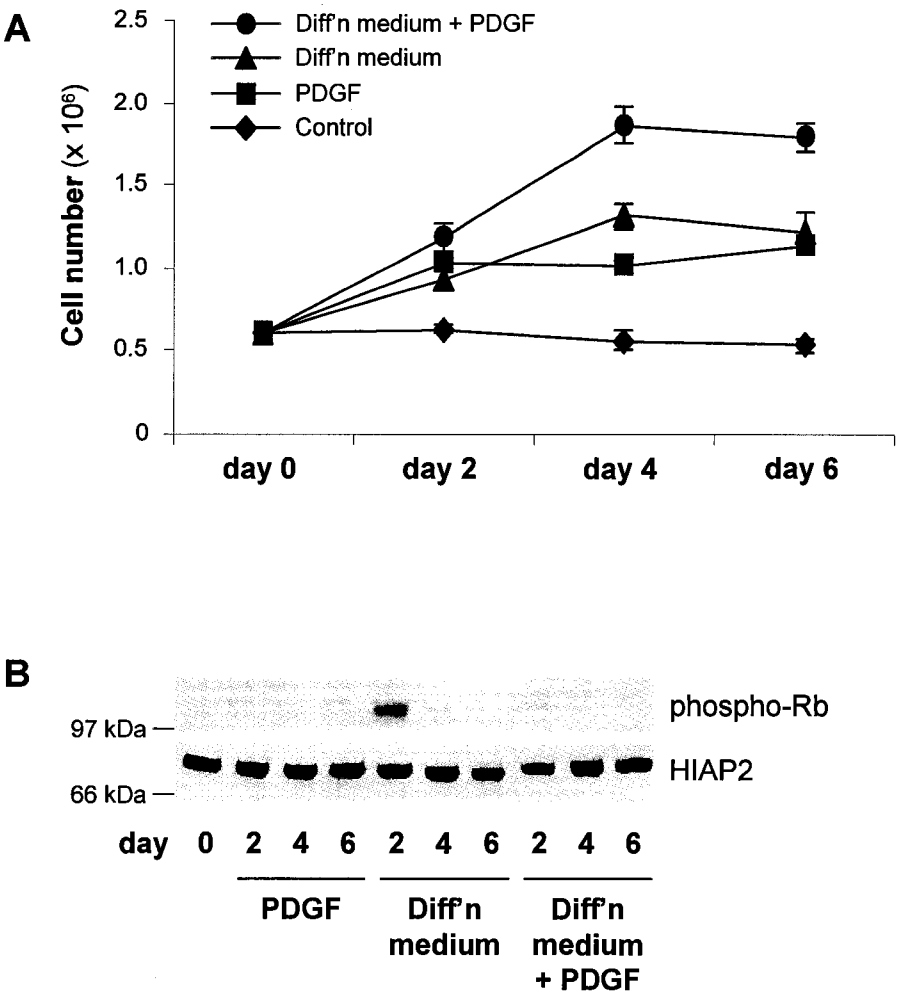
PDGF does not prevent exit from the MCE phase

Since PDGF is a known mitogen, it might affect the exit from the MCE phase required for successful adipogenesis. To examine this possibility, cells were counted throughout the differentiation program. As expected, the number of preadipocytes subjected to the regular differentiation protocol increased by 2.1±0.1 fold on day 4 of differentiation compared to day 0 (mean±SE; n=3; $P<0.001$; Fig. 27A); there was no further increase from day 4 to day 6 (n=3; $P>0.05$). In the presence of PDGF in the differentiation cocktail, the number of cells increased to an even greater extent than without PDGF (3.1±0.1 fold increase; mean±SE; n=3; $P<0.001$); however, as for the standard differentiation, no additional increase was observed between days 4 and 6 (n=3; $P>0.05$). Thus, PDGF appears to promote cell division to a greater extent than differentiation medium alone, but it does not block exit from the cell cycle at the end of MCE.

These findings were corroborated by the expression pattern of phospho-Rb. The levels of phospho-Rb sharply rose on day 2, coinciding with the MCE phase, and fell by day 4 in preadipocytes subjected to regular differentiation (n=3; Fig. 27B). Although the overall pattern was not altered in the presence of PDGF in the differentiation cocktail (Fig. 27B), the levels observed on day 2 were not as high as those observed without PDGF (73±4% decrease; mean±SE; n=3; $P<0.01$).

Overall, the data indicate that PDGF does not prevent the exit from the cell cycle that is required for successful differentiation.

Figure 27. PDGF does not prevent the exit from the MCE phase in differentiating 3T3-L1 preadipocytes. Two-day post-confluent 3T3-L1 preadipocytes were induced to differentiate or kept in control medium with or without 10 ng/ml PDGF. Analysis was performed on the indicated days after induction of differentiation (day 0). **(A)** Cells were trypsinized and counted. Results representative of 3 separate experiments are expressed as means±SE. **(B)** Cells were lysed and equal amounts of proteins were immunoblotted with antibodies against phospho-Rb or HIAP2. An immunoblot representative of 3 separate experiments is shown.


A
Diff'n medium + PDGF
Diff'n medium
PDGF
Control
Cell number (x 10⁶)
2.5
2.0
1.5
1.0
0.5
0
day 0
day 2
day 4
day 6
B
97 kDa
66 kDa
phospho-Rb
HIAP2
day 0 2 4 6 2 4 6 2 4 6
PDGF
Diff'n medium
Diff'n medium + PDGF

Analysis of the role of SHIP2 in adipogenesis

Differences between PDGF- and insulin-generated phosphoinositide profiles are not due to activation of class II PI3K

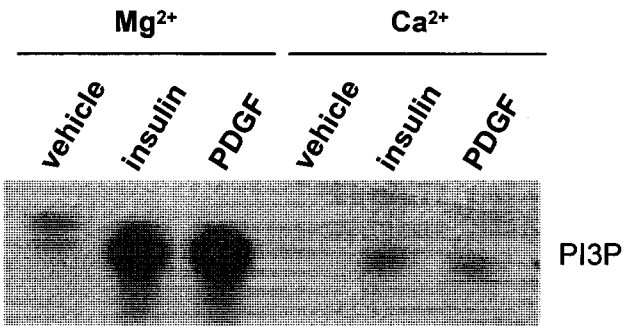
Stimulation of 3T3-L1 preadipocytes with pro-adipogenic insulin leads to production of PI(3,4,5)P3. On the other hand, treatment with anti-adipogenic PDGF leads to elevation of PI(3,4)P2 in addition to PI(3,4,5)P3. The observed accumulation of PI(3,4)P2 might be responsible for the negative effects of PDGF on differentiation; however, the origin of this phosphoinositide following PDGF stimulation is not known. In response to agonists, PI(3,4)P2 can be generated either by 1) by dephosphorylation of PI(3,4,5)P3 by SHIP2 or 2) by another 5-phosphatase, or 3) by phosphorylation of PI4P by the class II PI3K (PI3K-C2).

It is possible to distinguish activation of PI3K-C2 from other classes of PI3K, including the common class I PI3K. Other classes of PI3K are active only in the presence of Mg^{2+} as a co-factor, but PI3K-C2 can utilize Ca^{2+} for this purpose (Arcaro et al., 2000). Following a 5 minute stimulation with insulin, PDGF, or vehicle, I performed a PI3K assay in the presence of either Mg^{2+} or Ca^{2+}. Although PI3K-C2 constituted only a small part of total PI3K activity in 3T3-L1 preadipocytes, it was activated equally well with insulin or PDGF (n=1 in duplicates; Fig. 28). Thus, the production of PI(3,4)P2 in response to PDGF but not insulin is likely not due to differential activation of PI3K-C2 by these factors, but instead might be due to specific activation of SHIP2 (or another 5-phosphatase) by PDGF.

Catalytically inactive, dominant-negative SHIP2 accelerates adipogenesis

The ability of PDGF, but not insulin, to stimulate production of PI(3,4)P2, as well as induce Tyr phosphorylation of SHIP2, suggests that this lipid 5-phosphatase might mediate

Figure 28. Both PDGF and insulin can activate class II PI3K. Confluent 3T3-L1 preadipocytes were stimulated with 1 μM insulin, 10 ng/ml PDGF or vehicle for 5 min. Cells were lysed and the anti-phosphotyrosine immunoprecipitates were assayed for PI3K activity with Mg^{2+} or Ca^{2+} as the co-factor. A representative thin-layer chromatography exposure indicating the in vitro PI3P product is shown for one of two duplicates.


Mg2+
Ca2+
vehicle
insulin
PDGF
vehicle
insulin
PDGF
PI3P

the anti-adipogenic effects of PDGF. To explore this possibility, I examined differentiation of preadipocytes expressing SHIP2 WT, SHIP2 PDR/AAA, or empty vector in two different ways. Since the presence of PDGF in the serum used for differentiation studies (Hanai et al., 1987) might prevent maximal adipogenic responses, I first subjected preadipocytes to the standard differentiation protocol. Analysis was performed four and six days after induction of differentiation. Overexpression of SHIP2 WT did not alter the expression of differentiation markers compared to cells expressing empty vector ($P>0.05$; Fig. 29-31). In contrast, after four days of differentiation, SHIP2 PDR/AAA-expressing cells demonstrated a 1.51±0.05 and 2.3±0.6 fold increase in the levels of PPARγ and C/EBPα, respectively, compared to empty vector (mean±SE; n=3; $P<0.001$; Fig. 29). This difference was no longer observed after six days of differentiation (n=4; $P>0.05$; Fig. 30). However, at this time point, expression of a later marker of differentiation, FAS, as well as triglyceride levels were increased by 1.21±0.04 and 1.4±0.1 fold, respectively, in SHIP2 PDR/AAA vs. pLXSN preadipocytes (mean±SE; n=7 for FAS, n=4 for triglycerides; $P<0.001$; Fig. 30, 31).

As a second approach to assess the ability of SHIP2 to mediate the anti-adipogenic effects of PDGF, I examined adipogenesis following addition of PDGF to the differentiation cocktail. SHIP2 PDR/AAA enhanced PPARγ and C/EBPα expression on day 4 of differentiation even in the presence of PDGF (2.5±0.8 and 7±2 fold increase, respectively; mean±SE; n=3; $P<0.001$; Fig. 29). On day 6, only FAS expression and triglyceride levels were increased in SHIP2 PDR/AAA compared to empty vector-expressing cells differentiated in the presence of PDGF (1.5±0.2 and 1.6±0.1 fold increase, respectively; mean±SE; n=7, $P<0.001$ for FAS; n=4, $P<0.01$ for triglycerides; Fig. 30, 31). Similarly to findings observed with differentiation medium alone, expression of PPARγ and C/EBPα

Figure 29. Catalytically inactive, dominant-negative SHIP2 enhances expression of PPARγ and C/EBPα on day 4 of differentiation. Two-day post-confluent 3T3-L1 preadipocytes expressing SHIP2 WT, SHIP2 PDR/AAA, or empty vector (pLXSN) were induced to differentiate or kept in control media. Cells were lysed on day 4 of differentiation. Equal amounts of protein were immunoblotted with antibodies against PPARγ, C/EBPα, ERK1/2, and SHIP2 antibodies. A representative immunoblot is shown. Densitometric data was obtained from 3 separate experiments and expressed as means±SE. **P<0.01, ***P<0.001 compared to pLXSN.

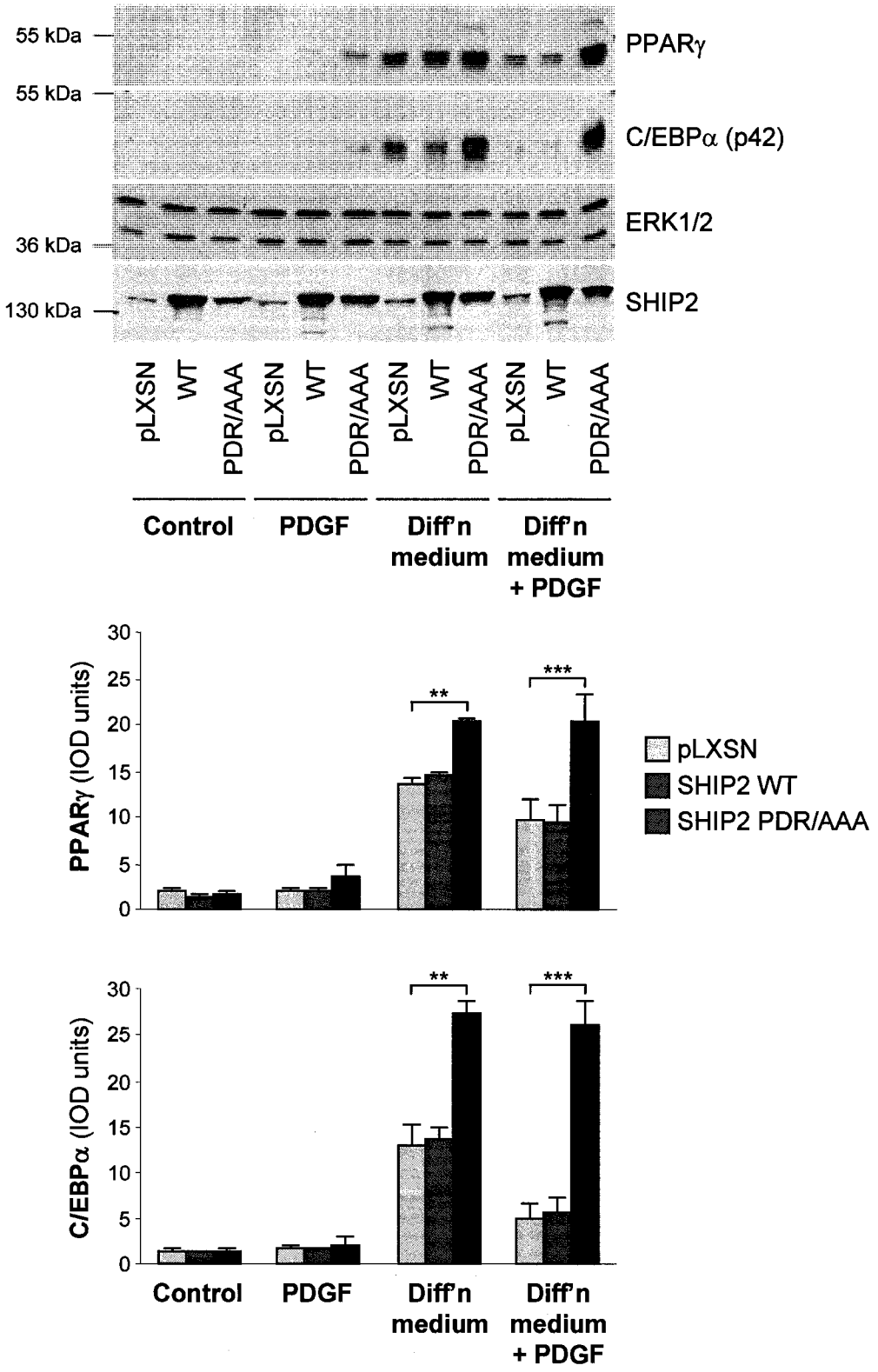

55 kDa
PPARγ
55 kDa
C/EBPα (p42)
ERK1/2
36 kDa
130 kDa
SHIP2
pLXSN
WT
PDR/AAA
Control
PDGF
Diff'n medium
Diff'n medium + PDGF
PPARγ (IOD units)
C/EBPα (IOD units)
**
***
pLXSN
SHIP2 WT
SHIP2 PDR/AAA

Figure 30. Catalytically inactive, dominant-negative SHIP2 enhances expression of FAS, but not PPARγ or C/EBPα, on day 6 of differentiation. Two-day post-confluent 3T3-L1 preadipocytes expressing SHIP2 WT, SHIP2 PDR/AAA, or empty vector (pLXSN) were induced to differentiate or kept in control media. Cells were lysed on day 6 of differentiation. Equal amounts of protein were immunoblotted with antibodies against PPARγ, C/EBPα, FAS, ERK1/2, and SHIP2 antibodies. A representative immunoblot is shown. Densitometric data was obtained from 7 separate experiments and expressed as means±SE. *P<0.05, ***P<0.001 compared to pLXSN.

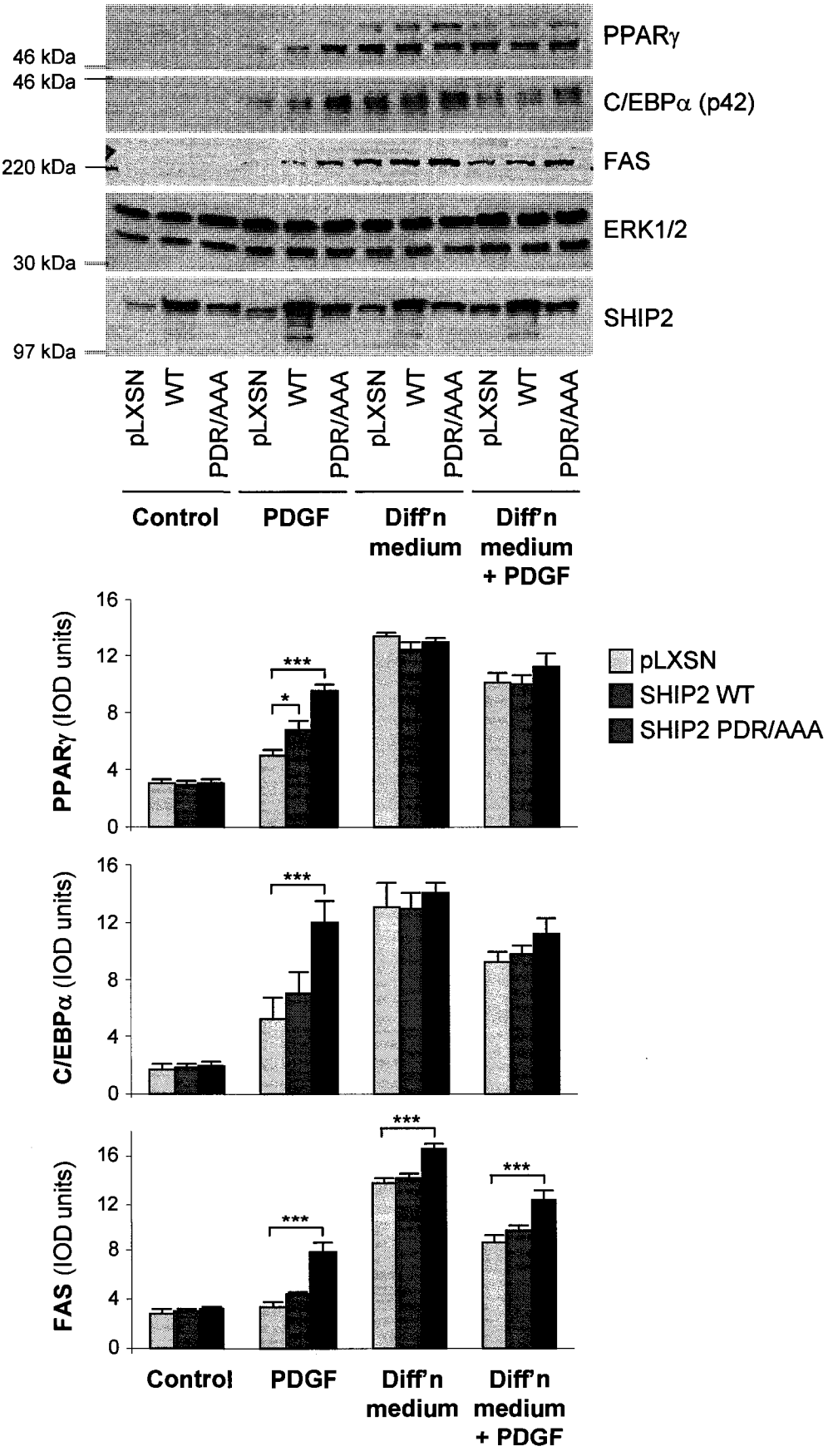
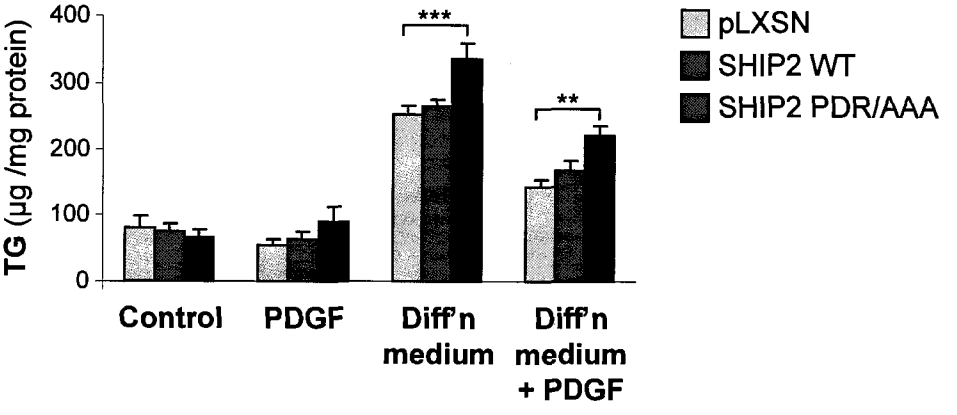

46 kDa
46 kDa
220 kDa
30 kDa
97 kDa
PPARγ
C/EBPα (p42)
FAS
ERK1/2
SHIP2
pLXSN
WT
PDR/AAA
Control
PDGF
Diff'n medium
Diff'n medium + PDGF
PPARγ (IOD units)
C/EBPα (IOD units)
FAS (IOD units)
pLXSN
SHIP2 WT
SHIP2 PDR/AAA
***
*

Figure 31. Catalytically inactive, dominant-negative SHIP2 increases triglyceride accumulation on day 6 of differentiation. Two-day post-confluent 3T3-L1 preadipocytes expressing SHIP2 WT, SHIP2 PDR/AAA, or empty vector (pLXSN) were induced to differentiate or kept in control media. Triglycerides were extracted, quantified, and normalized for the protein content of the cells on day 6 of differentiation. Data was obtained from 4 separate experiments and expressed as means±SE. **P<0.01, ***P<0.001 compared to pLXSN.


TG (µg /mg protein)
400
300
200
100
0
***
**
Control
PDGF
Diff'n medium
Diff'n medium + PDGF
pLXSN
SHIP2 WT
SHIP2 PDR/AAA

was not altered by SHIP2 PDR/AAA on day 6 of differentiation in the presence of PDGF ($n=7$; $P>0.05$; Fig. 30).

Thus, SHIP2 PDR/AAA appears to accelerate 3T3-L1 preadipocyte differentiation, and might alleviate the anti-adipogenic effects of PDGF.

DISCUSSION

My research implicates SHIP2 as a novel participant of PDGF-mediated processes in 3T3-L1 preadipocytes, and advances our understanding of the molecular mechanisms of SHIP2 regulation. PDGF-stimulated SHIP2 Tyr phosphorylation and association with Shc is regulated via Ser/Thr phosphorylation, demonstrated by inhibition of the Ser/Thr kinase PKC, as well as by mutation of a novel PDGF-responsive phosphorylation site, Thr958. However, SHIP2 Tyr phosphorylation and/or association with Shc do not appear to be important for its ability to inhibit preadipocyte proliferation. On the other hand, mutation of the 5-phosphatase domain attenuates PDGF signalling by reducing PDGFR levels and potentiates the anti-proliferative effects of SHIP2. Catalytically inactive SHIP2 not only inhibits preadipocyte proliferation, but also accelerates adipocyte differentiation, and thereby affects two distinct processes modulated by PDGF in opposite directions.

Regulation of SHIP2 by Ser/Thr phosphorylation

The first indication that SHIP2 might be regulated by Ser/Thr phosphorylation came from the observation that BisI, an inhibitor of a Ser/Thr kinase PKC, attenuates PDGF-stimulated SHIP2 Tyr phosphorylation. PKC is a large family of kinases that is divided into three classes based on differences in structure and mechanisms of activation (Mackay and Twelves, 2007). Conventional or classical PKC isoforms (α, βI, βII, γ) are activated by phosphatidylserine (PS) and diacylglycerol (DAG), and are calcium-dependent. Novel PKCs (δ, ε, η, θ) depend on PS and DAG, but not calcium, for activation. Atypical PKCs (λ/ι, ζ) are both calcium- and DAG-independent. Since BisI inhibits all conventional isoforms, as well as PKCδ and ε (Martiny-Baron et al., 1993; Toullec et al., 1991), and all of these PKCs

are expressed in preadipocytes (Fleming et al., 1998; McGowan et al., 1996; Zhou et al., 2006), the specific isoform involved in regulation of SHIP2 Tyr phosphorylation cannot be identified at this time.

Although BisI is widely used to selectively inhibit PKC, the specificity of pharmacological inhibitors cannot be confirmed with complete certainty. In particular, BisI was previously shown to be a potent inhibitor of GSK-3β at concentrations higher than those for PKC, but still in the range used for this study (Hers et al., 1999). PDGF-stimulated GSK3β phosphorylation, which is one way of assessing its state of activation, was not affected by the presence of BisI. Further experiments looking at the phosphorylation state of GSK-3β substrates need to be conducted to completely rule out the potential involvement of GSK3β in processes affected by BisI. Furthermore, the specific involvement of PKC in SHIP2 regulation should be verified using a structurally unrelated PKC inhibitor or genetic approaches to negate PKC expression or function.

Given that inhibition of PKC by BisI reduces PDGF-stimulated SHIP2 Tyr phosphorylation, I explored whether activation of PKC might enhance this response. However, TPA treatment did not increase either basal or PDGF-stimulated Tyr phosphorylation of SHIP2. The latter finding might be because PDGF-stimulated PKC activation was only slightly enhanced by TPA. Hence, it is possible that PDGF induces maximal Tyr phosphorylation of SHIP2 that cannot be further improved. A reduced concentration of PDGF could have been used to detect a potential enhancement of SHIP2 tyrosine phosphorylation in the presence of TPA. Furthermore, PKC is likely not the only mediator of PDGF-induced SHIP2 phosphorylation, and might only facilitate the phosphorylation of SHIP2 by an upstream kinase. For example, PKC might either directly,

or via an intermediate, alter SHIP2 conformation to allow its association with a Tyr kinase that is activated by PDGF. In this scenario, activation of PKC by TPA would prime SHIP2; however, without PDGF, actual phosphorylation would not occur. This area requires further investigation.

Given that PKC is a Ser/Thr kinase, it most likely affects SHIP2 Tyr phosphorylation indirectly. For example, PKC might activate a Tyr kinase or inhibit a Tyr phosphatase. Since the only Tyr kinase implicated in SHIP2 phosphorylation is Src (Prasad et al., 2002; Taylor et al., 2000), I examined whether BisI affects its activation. Numerous studies demonstrated the ability of PKC to activate Src (Brandt et al., 2002; Bruce-Staskal and Bouton, 2001; Gatesman et al., 2004; Harper and Sage, 2006; Hodges et al., 2007). Although the mechanism of this activation is unclear, Brandt et al. reported that PKCδ phosphorylates and activates protein Tyr phosphatase α, which dephosphorylates and thereby activates Src in A7r5 rat vascular smooth muscle cells (Brandt et al., 2003). In contrast to the above studies, pre-treatment of 3T3-L1 preadipocytes with BisI led to enhanced phosphorylation of the activating Tyr416 site in Src, suggesting that inhibition of PKC potentiates PDGF-stimulated Src activation. This is consistent with another report showing that Src is negatively regulated by PKCα in human platelets (Pula et al., 2005). Hence, BisI does not appear to reduce PDGF-stimulated SHIP2 Tyr phosphorylation by inhibiting Src.

Another possibility for how PKC can modulate PDGF-stimulated SHIP2 Tyr phosphorylation is by Ser/Thr phosphorylation of SHIP2, either directly by PKC or via another Ser/Thr kinase, which then enhances SHIP2 Tyr phosphorylation. Phosphopeptide mapping revealed that indeed PDGF stimulates phosphorylation of SHIP2 on a Thr residue (Thr958). The identity of the kinase responsible for Thr958 phosphorylation is not known.

Conventional or novel PKC might be involved since BisI was slightly less potent at reducing PDGF-stimulated Tyr phosphorylation of SHIP2 T958A compared to SHIP2 WT. However, since the difference between the two effects was very small (~15%), other kinases might be involved as well. So far, there are no reports that address the ability of PKC to phosphorylate SHIP2 directly.

Analysis of SHIP2 sequence by NetPhosK 1.0 predicted that Thr958 is phosphorylated by p38MAPK or GSK-3β (Blom et al., 2004). It is unlikely that p38MAPK is involved, since its inhibitor (SB203580) did not have an effect on PDGF-induced Tyr phosphorylation of endogenous SHIP2 (Artemenko et al., 2007). GSK-3β is phosphorylated and inactivated by Akt, which is a downstream effector of PI3K (Cross et al., 1995). However, an inhibitor of PI3K (LY294002) also failed to alter endogenous SHIP2 Tyr phosphorylation in response to PDGF (Artemenko et al., 2007), suggesting that GSK-3β is not responsible for phosphorylating SHIP2. Further studies are necessary to elucidate the identity of the Ser/Thr kinase responsible for Thr958 phosphorylation.

Inhibition of PKC by BisI might also affect a residue other than Thr958 directly. A wide-scale analysis of the Tyr phosphorylation events in chronic myelogenous leukemia cells treated with imatinib identified phosphorylation of Ser1003 in the same peptide as Tyr986 of SHIP2 (Salomon et al., 2003). However, the role of this residue in SHIP2 function or regulation, as well as its ability to be phosphorylated by different growth factors, has not been investigated. Interestingly, Kagawa et al., in searching for single nucleotide polymorphisms (SNP) associated with type 2 diabetes in a Japanese population, identified SNP5 with an Asn to Ser substitution at position 982 in one diabetic patient (Kagawa et al., 2005). When SHIP2 N982S was overexpressed in CHO-IR cells, its insulin-stimulated Tyr

phosphorylation and association with Shc was reduced compared to SHIP2 WT. That study did not examine whether Ser982 in SNP5 SHIP2 is actually phosphorylated.

All three Ser/Thr residues mentioned above (Thr958, Ser982, Ser1003), are located in the vicinity of Tyr986 in the NPAYY motif. This is a known Tyr phosphorylation site of SHIP2 (Blero et al., 2001; Ishihara et al., 2002; Pesesse et al., 2001), and it is possible that modulation of neighbouring residues affects SHIP2 conformation or affinity for its potential Tyr kinase. However, at this point it is unclear whether Thr958 is close to Tyr986 in a three-dimensional context because the crystal structure of SHIP2 has not been solved.

Role of SHIP2 Tyr phosphorylation in SHIP2-Shc interaction

SHIP2 Tyr phosphorylation might be necessary for the interaction between SHIP2 and its binding partners. Indeed, both BisI treatment and the T958A substitution led to decreased association with Shc, in agreement with reduced SHIP2 Tyr phosphorylation. This is consistent with the observation that SHIP2 N982S (SNP5) also has both diminished Tyr phosphorylation and Shc association (Kagawa et al., 2005).

The identity of SHIP2 Tyr phosphorylation sites is not entirely clear. NPAYY is known to be important for SHIP2 Tyr phosphorylation by various growth factors in a number of cell lines (Blero et al., 2001; Ishihara et al., 1999; Ishihara et al., 2002; Pesesse et al., 2001; Prasad et al., 2002; Taylor et al., 2000). Indeed, my data indicate that the SHIP2 YY/FF mutant exhibited dramatically reduced phosphorylation compared to SHIP2 WT. However, some residual Tyr phosphorylation indicates that SHIP2 likely possesses other phosphorylation sites. The finding that SHIP2 R/Q also displayed significantly lower Tyr phosphorylation compared to SHIP2 WT suggests that the SH2 domain is important for

PDGF-stimulated SHIP2 Tyr phosphorylation, but does not identify which Tyr residues are phosphorylated. Other investigators also reported the reduction of SHIP2 Tyr phosphorylation following disruption of the SH2 domain (Pengal et al., 2003; Pesesse et al., 2001; Taylor et al., 2000).

Given that both SHIP2 YY/FF and R/Q mutants display reduced Tyr phosphorylation, their Shc association is also expected to be impaired. In fact, several groups reported that mutation of the NPAYY motif (Blero et al., 2001; Prasad et al., 2002), as well as the SH2 domain (Ishihara et al., 2002; Pesesse et al., 2001) impairs SHIP2-Shc interaction. This is consistent with the presence of a PTB domain in Shc, which can interact with the phosphorylated NPAYY motif (Prigent et al., 1995). In addition, Shc has several Tyr residues, which can become phosphorylated, and then interact with SH2 domain-containing proteins, such as Grb2/Sos (Ravichandran, 2001). Surprisingly, in this study, only SHIP2 YY/FF mutant showed reduced Shc binding, whereas the R/Q mutation actually potentiated SHIP2-Shc interaction, despite a very strong inhibition of Tyr phosphorylation. Perhaps, the SH2 domain is necessary for phosphorylation of Tyr residues other than those in the NPAYY motif. Following mutation of the SH2 domain, the residual phosphorylation of the NPAYY motif would still allow for Shc binding. Furthermore, the disruption of the SH2 domain might also prevent binding to other partners, which reduces steric hindrance, thus allowing easier access for Shc to the NPAYY motif of SHIP2. However, at this point, these ideas are speculative, and further experimental testing is necessary to confirm the exact role of the SH2 domain in SHIP2-Shc interaction.

Role of SHIP2 Tyr phosphorylation and Shc association in SHIP2 function

Ser/Thr phosphorylation often either negatively or positively regulates protein function (Gual et al., 2005). Thr958 appears to be a positive regulator of SHIP2 Tyr phosphorylation, but whether this translates into increased activity is unclear. SHIP2 Tyr phosphorylation is sometimes thought to correlate with its activity (Benkirane et al., 2006; Habib et al., 1998; Ishihara et al., 1999; Pengal et al., 2003). Batty et al. used protein Tyr phosphatase inhibitors to increase SHIP2 Tyr phosphorylation and demonstrated that this led to a dramatic enhancement of SHIP2 5-phosphatase activity in 1321N1 cells (Batty et al., 2007). However, inhibition of BCR/ABL did not alter SHIP2 activity *in vitro* despite reducing SHIP2 Tyr phosphorylation (Giuriato et al., 2002). In addition, mutation of the NPAYY motif, which diminishes SHIP2 Tyr phosphorylation, did not prevent the dephosphorylation of PI(3,4,5)P3 in COS-7 and Glioblastoma cells (Pesesse et al., 2001; Taylor et al., 2000). Similarly, measurement of the catalytic activity of SHIP2 *in vitro* yielded comparable findings whether SHIP2 was Tyr phosphorylated by c-Src or insulin stimulation, or not (Blero et al., 2001; Taylor et al., 2000).

Further evidence suggesting that SHIP2 Tyr phosphorylation might not directly reflect its activation comes from the data showing the effect of BisI on the phosphoinositide profile in 3T3-L1 preadipocytes. Despite its ability to reduce Tyr phosphorylation of SHIP2 in response to an acute stimulation with PDGF, BisI did not affect the generation of PI(3,4,5)P3 and PI(3,4)P2. Since SHIP2 is the 5-phosphatase implicated in dephosphorylating PI(3,4,5)P3, this suggests that modulation of SHIP2 Tyr phosphorylation does not have an impact on its activity. It is possible that alteration in phosphoinositide levels requires a time period longer than the 5 min interval used in my study. While this

remains to be formally tested, it should be noted that a 5 min duration of PDGF stimulation was sufficient to observe an increase in PI(3,4)P2 and a decrease in PI(3,4,5)P3 levels in SHIP2 WT compared to empty vector expressing cells. It should also be mentioned that the the approach used in this study assessed total amounts of specific phosphoinositides in the cells. Hence, the possibility of local changes in the levels of PI(3,4,5)P3 and PI(3,4)P2, without effects on the total levels of these phosphoinositides, cannot be excluded.

The finding that SHIP2 T958A, R/Q, and YY/FF behaved similarly to SHIP2 WT in functional assays despite their ability to diminish SHIP2 Tyr phosphorylation and/or Shc association is very intriguing. All of these mutants had the same effect on PDGFR, Akt, and ERK1/2 phosphorylation as SHIP2 WT. Furthermore, SHIP2 T958A, R/Q, and YY/FF exhibited the same anti-proliferative effects as SHIP2 WT, supporting the notion that SHIP2 Tyr phosphorylation and/or Shc association is not important for its function in this context. It is possible that the SH2 domain actually counteracts the anti-proliferative effects of SHIP2, since SHIP2 R/Q was as potent as SHIP2 WT at inhibiting proliferation despite reduced expression levels of this mutant compared to SHIP2 WT. It should be noted that the expression levels of SHIP2 constructs were only ~2-4 fold higher compared to endogenous SHIP2. Hence, it is unlikely that the findings are due to an artifact of excessive overproduction.

Role of PDGF in preadipocyte proliferation

Activation of the PI3K/PI(3,4,5)P3/Akt and Shc/Ras/ERK1/2 signalling pathways by PDGF is consistent with its mitogenic activity in 3T3-L1 preadipocytes. Indeed, the ability

of the PDGFR inhibitor imatinib to reduce proliferation of 3T3-L1 preadipocytes grown in serum-containing medium confirms that PDGF is a major mitogen in the serum.

Early studies indicated that PDGF was not sufficient to drive Balb-c/3T3 cells through the entire cell cycle (Stiles et al., 1980). Instead, PDGF served as the competence factor capable of bringing growth-arrested cells out of the G_0 and into the G_1 phase. PDGF alone was not sufficient for the G_1/S transition, and other growth factors present in the plasma, such as IGF-I or EGF, were necessary to complete the G_1/S progression. However, recent evidence indicates that these early findings might have been cell type specific. Quiescent NIH-3T3 and HepG2 cells progress through the entire cell cycle with PDGF alone (Jones and Kazlauskas, 2001a). However, similarly to the competence/progression model, Jones and Kazlauskas demonstrated that continuous exposure to PDGF can be mimicked by two PDGF pulses during the G_1 phase. The first early pulse of PDGF provides the necessary activation of ERK1/2 and induction of c-Myc. After 8 hours, the second pulse mainly depends on the activation of PI3K, which promotes activation of cell cycle machinery. Ultimately, phosphorylation of Rb marks the restriction point, after which the cells are committed to go through the S phase.

PDGF is not the only serum growth factor with mitogenic effects. Fibroblast growth factor and lysophosphatidic acid are similar to PDGF in their ability to promote mitogenesis, suggesting that these growth factors might activate redundant pathways (Jones and Kazlauskas, 2001a). On the other hand, despite their ability to activate ERK1/2 and induce c-Myc, insulin and EGF can only substitute for PDGF during the second pulse (Jones and Kazlauskas, 2001a). It is unclear whether PDGF is sufficient for the entire cell cycle progression of 3T3-L1 preadipocytes, or whether it cooperates with other growth factors to

promote the G_1/S transition in these cells. However, since the anti-proliferative effect of imatinib was not complete, as assessed in my studies, other factors present in the serum likely contribute to the full mitogenic response in preadipocytes.

Mechanism of the anti-proliferative effects of SHIP2 WT

Since PDGF constitutes a major part of the mitogenic activity of the serum, impairment of PDGF-stimulated mitogenic cascades might explain the anti-proliferative effect of SHIP2 WT. Surprisingly, despite the reproducible anti-proliferative effect, SHIP2 WT overexpression did not modify PDGFR phosphorylation, or the phosphorylation of two established PDGF targets, Akt and ERK1/2. It is unclear whether the responses observed following acute stimulation with PDGF (up to 60 min) can be extended to long-term events, such as proliferation. SHIP2 WT, as well as the R/Q and YY/FF mutants, might have effects on PDGF-stimulated responses following even longer stimulation times (several hours). This possibility should be assessed in the future.

The lack of an effect of SHIP2 WT overexpression on Akt and ERK1/2 phosphorylation was unexpected given the number of reports demonstrating negative effects of SHIP2 expression on Akt and ERK1/2 phosphorylation and activity. In particular, insulin-stimulated activation of Akt was reduced in 3T3-L1 adipocytes and L6 myotubes overexpressing SHIP2 (Ishihara et al., 2002; Sasaoka et al., 2001; Wada et al., 2001). A reduction in both Akt and ERK1/2 phosphorylation in response to insulin was also observed in SHIP2-overexpressing CHO-IR and HIR cells (Blero et al., 2001; Ishihara et al., 1999). Analogous results were obtained in rat aortic smooth muscle cells stimulated with PDGF or IGF-I (Sasaoka et al., 2003).

However, the effects of SHIP2 on Akt and ERK1/2 phosphorylation might be cell type and growth factor dependent. For example, SHIP2 WT did not alter Akt phosphorylation in OPM2 cells stimulated with IGF-I or IL-6, even though basal and IL-6 stimulated ERK1/2 phosphorylation was reduced (Choi et al., 2002). In addition, Zhou et al. demonstrated that reduction in SHIP2 levels by RNAi did not affect Akt phosphorylation in 3T3-L1 adipocytes stimulated with insulin (Zhou et al., 2004). On the other hand, serum-stimulated mouse embryonic fibroblasts lacking SHIP2 displayed enhanced Akt but unaltered ERK1/2 phosphorylation and activity (Blero et al., 2005).

The effects of SHIP2 on ERK1/2 are usually related to the increased association between SHIP2 and Shc, which leads to dampened activation of ERK1/2 via reduced Shc/Grb2 association (Ishihara et al., 1999; Sasaoka et al., 2003). However, ERK1/2 activity in my study appears to be independent of SHIP2/Shc association. PDGF stimulation of SHIP2 T958A or YY/FF expressing preadipocytes led to reduced Tyr phosphorylation of SHIP2. Despite the reduced association of these mutants with Shc, no effects on ERK1/2 activation in response to PDGF were observed. Several explanations are possible for the lack of an effect of SHIP2 on ERK1/2 in 3T3-L1 preadipocytes. First, it is possible that Grb2/Sos is recruited directly to PDGFR (Heldin and Westermark, 1999). Subsequent recruitment and activation of Ras leads to phosphorylation and activation of ERK1/2 via several intermediates. Alternatively, the sequestration of Shc by SHIP2 does not diminish ERK1/2 activation because a sufficient number of Shc molecules remain unbound and can be recruited by the PDGFR. A third possibility is that SHIP2 itself serves as a platform for recruitment of Shc, Grb2/Sos, etc., leading to activation of ERK1/2.

A possible explanation for the variable effects of SHIP2 on Akt observed in the above studies is the existence of 3 different isoforms of this molecule (Cheng et al., 1992; Jones et al., 1991a; Jones et al., 1991b; Masure et al., 1999; Staal, 1987). In particular, Akt1 and 2 are ubiquitously expressed, even though their relative levels vary depending on the cell type (Cheng et al., 1992; Coffer and Woodgett, 1991; Jones et al., 1991a; Jones et al., 1991b; Masure et al., 1999; Staal, 1987). Akt2 appears to be expressed at higher levels, and may be of more importance than Akt1, in insulin-responsive tissues (Altomare et al., 1995; Altomare et al., 1998). In fact, it appears that the reduced Akt phosphorylation observed in 3T3-L1 adipocytes or CHO-IR cells overexpressing SHIP2 WT in response to insulin stimulation is due exclusively to Akt2 (Kagawa et al., 2005; Sasaoka et al., 2004). This is consistent with Akt2 being the predominant isoform in 3T3-L1 adipocytes. On the other hand, Akt1 has been suggested to be the major isoform in 3T3-L1 preadipocytes (Altomare et al., 1998; Xu and Liao, 2004).

The switch in isoform expression with 3T3-L1 differentiation might be a consequence of the distinct regulation of Akt1 and Akt2. In fact, the dependence of Akt activation on the presence of PI(3,4,5)P3 and PI(3,4)P2 is still controversial. Overexpression of SHIP1, which is a SHIP2- related 5-phosphatase expressed only in hematopoietic cells, was used to show that PI(3,4)P2 is required for Akt phosphorylation on Ser473, although PI(3,4,5)P3 is also necessary for activation (Ma et al., 2008; Scheid et al., 2002). The PH domain of Akt can bind both PI(3,4)P2 and PI(3,4,5)P3 *in vitro* (Frech et al., 1997; Gray et al., 1999), although some investigators report higher affinity for PI(3,4,5)P3 than for PI(3,4)P2 (James et al., 1996). *In vitro* studies suggest that PI(3,4)P2 activates Akt, and PI(3,4,5)P3 might actually inhibit this process (Franke et al., 1997; Frech et al., 1997). On

the other hand, PDK1, which phosphorylates Akt on Thr308, can be recruited to the plasma membrane by both PI(3,4,5)P3 and PI(3,4)P2 (Stephens et al., 1998), although PI(3,4,5)P3 might be necessary for activation of PDK (Stokoe et al., 1997). This might explain why PI(3,4,5)P3 appears to be important only for the activation of membrane-associated Akt, whereas PI(3,4)P2 levels correlate with the overall Akt activity, primarily consisting of the cytosolic and not membrane-associated enzyme (Ma et al., 2008).

In 3T3-L1 preadipocytes, I observed that overexpression of SHIP2 WT elevates PI(3,4)P2 and diminishes PI(3,4,5)P3 levels in response to a 5 min PDGF stimulation. These data suggest that in an acute context, both PI(3,4,5)P3 and PI(3,4)P2 can promote Akt activation in 3T3-L1 preadipocytes, since the presence of SHIP2 WT did not alter Akt phosphorylation. However, in proliferating preadipocytes, Akt phosphorylation was slightly, albeit not significantly, reduced. Hence, one possible explanation for the anti-proliferative effect of SHIP2 WT is the small inhibition of Akt phosphorylation, perhaps due to a reduction in PI(3,4,5)P3 levels.

Another possibility is that the anti-proliferative effects of SHIP2 WT are due to the production of PI(3,4)P2, which might activate downstream targets leading to inhibition of proliferation. Putz et al. found that PI(3,4)P2 synergizes with secretory phospholipase A2 to inhibit proliferation of various cancer cell lines, and exerts minor anti-proliferative effects on its own (Putz et al., 2006). Studies of the isolated effects of PI(3,4)P2 on cellular responses are lacking because most proteins with PH domains respond to both PI(3,4,5)P3 and PI(3,4)P2. However, Dowler et al. demonstrated that tandem-PH-domain-containing protein (TAPP) 1 and 2 specifically binds PI(3,4)P2 and not PI(3,4,5)P3 *in vitro* (Dowler et al., 2000). The preferential binding of TAPP1 to PI(3,4)P2 in response to PDGF or H_2O_2

stimulation has also been confirmed *in vivo* (Kimber et al., 2002). Interestingly, in B cells, TAPP2 is involved in responses mediated by SHIP1 (Krahn et al., 2004). TAPP1 appears to serve as an adaptor protein, which can recruit other downstream molecules to the plasma membrane in response to PI(3,4)P2 production. In fact, TAPP1 has been shown to interact with a multi-PDZ-domain protein-1, as well as with a protein Tyr phosphatase PTPL1 (Kimber et al., 2003; Kimber et al., 2002). A role of TAPP1 in actin remodelling in response to PDGF has also been suggested (Hogan et al., 2004). The involvement of TAPP1, or its downstream targets, in the anti-proliferative effects of SHIP2 in preadipocytes needs further investigation.

Mechanism of the anti-proliferative effects of SHIP2 PDR/AAA

The observation that SHIP2 WT inhibits preadipocyte proliferation is consistent with several other studies which also demonstrated the inhibitory role of SHIP2 in proliferation (Giuriato et al., 2002; Grempler et al., 2007; Taylor et al., 2000). On the other hand, the profound anti-proliferative effect of the catalytically inactive SHIP2 PDR/AAA construct is unexpected given its dominant-negative nature (Wada et al., 2001). The reduced expression of SHIP2 PDR/AAA compared to SHIP2 WT makes the anti-proliferative effect of this 5-phosphatase defective mutant even more impressive. Overexpression of a similar catalytically inactive, dominant-negative human SHIP2 in the rat insulin-producing cell line INS1E actually led to increased proliferation (Grempler et al., 2007). This discrepancy might simply be due to the differences in the model systems used. In addition, I assessed serum-induced proliferation and the involvement of PDGF in this process, whereas Grempler and colleagues analyzed insulin-induced responses. It is possible that SHIP2 is

regulated differently depending on the growth factor used. In fact, in 3T3-L1 preadipocytes we have previously shown that only PDGF, and not insulin, induces SHIP2 Tyr phosphorylation and its association with Shc (Gagnon et al., 2003).

Support for the anti-proliferative effect of SHIP2 PDR/AAA comes from the studies demonstrating that overexpression of catalytically inactive SHIP1 inhibits erythropoietin-mediated proliferation of erythroid AS-E2 cells, as well as IL-4 mediated proliferation of myeloid 32D/IRS-2 cells (Boer et al., 2001; Giallourakis et al., 2000). Whether catalytically inactive SHIP1 and SHIP2 exert their anti-proliferative effects via similar mechanisms is unknown.

Unlike SHIP2 WT, catalytically inactive SHIP2 clearly inhibited mitogenic signalling both in proliferating preadipocytes, as well as in preadipocytes subjected to acute stimulation with PDGF. Both of these effects can be potentially explained by enhanced downregulation of the PDGFR observed in SHIP2 PDR/AAA-expressing preadipocytes. Enhanced PDGF-stimulated receptor degradation, rather than reduced initial receptor expression is likely involved, since PDGFR levels were equal in quiescent preadipocytes expressing SHIP2 PDR/AAA or empty vector prior to stimulation with PDGF. Importantly, Prasad et al. have recently shown that knock-down of SHIP2 by siRNA in a breast cancer cell line slows down proliferation by increasing EGF receptor (EGFR) turnover (Prasad et al., 2008). The same group previously implicated an E3 ubiquitin ligase Cbl, a known binding partner for SHIP2, in the enhanced EGFR degradation observed with SHIP2 knock-down (Prasad and Decker, 2005). Whether the same mechanism is responsible for the accelerated PDGFR degradation in the presence of the catalytically inactive, dominant-negative SHIP2 in 3T3-L1 preadipocytes is unclear. However, this mechanism is plausible

since PDGF-stimulated phosphorylation of Cbl induces its interaction with PDGFR (Miyake et al., 1999). Furthermore, Cbl binding promotes ubiquitination and degradation of the PDGFR, as well as the inhibition of proliferation of NIH-3T3 cells.

Another possible explanation for attenuated PDGFR signalling observed in SHIP2 PDR/AAA expressing preadipocytes might be enhanced activation of Rac1 GTPase downstream of PI(3,4,5)P3. PI(3,4,5)P3 has been shown to not only specifically interact with Rac1, but also to promote GDP dissociation from this small GTPase *in vitro* (Missy et al., 1998). Evidence is accumulating to suggest that Rac1 is involved in clathrin-, dynamin-, and caveolin-independent receptor endocytosis (Grassart et al., 2008; Kumari et al., 2008). Notably, Rac1 was implicated in the increased EphA2 receptor endocytosis and degradation in mammary carcinoma cells with reduced SHIP2 levels (Zhuang et al., 2007). The timing of PDGF-induced Akt activation that I observed in preadipocytes makes this possibility especially appealing. If Akt becomes phosphorylated and activated in response to elevated PI(3,4,5)P3 levels, then the increased Akt phosphorylation that was observed in SHIP2 PDR/AAA expressing cells after 5 min stimulation with PDGF likely reflects an initial accumulation of PI(3,4,5)P3. This is expected, since SHIP2 PDR/AAA is a catalytically inactive construct acting in a dominant-negative fashion. Indeed, overexpression of a dominant-negative 5-phosphatase deficient SHIP2 (rat equivalent of SHIP2 PDR/AAA) was shown to lead to increased PI(3,4,5)P3 accumulation and Akt activation in insulin-stimulated 3T3-L1 adipocytes and PDGF-stimulated vascular smooth muscle cells (Sasaoka et al., 2005; Sasaoka et al., 2003; Wada et al., 2001). Hence, in preadipocytes overexpressing SHIP2 PDR/AAA, the initial accumulation of PI(3,4,5)P3 could lead to Rac1 activation and subsequently affect PDGFR turnover.

Imatinib was used to confirm that impaired PDGFR signalling in SHIP2 PDR/AAA preadipocytes is responsible for the reduced serum-based proliferation of these cells. The anti-mitotic effect of imatinib relies on its ability to reduce PDGFR signalling. Hence, imatinib should not act as potently on cells whose PDGFR function is already compromised. The diminution of the anti-proliferative action of imatinib in SHIP2 PDR/AAA expressing cells supported the notion that catalytically inactive SHIP2 inhibits preadipocyte proliferation in serum by attenuating PDGFR signalling.

My results show that the reduction in cell number observed in SHIP2 PDR/AAA-expressing preadipocytes was not due to enhanced cell death. However, given the established anti-apoptotic effects of PDGF (Staiger and Löffler, 1998; Yao and Cooper, 1995), as well as the robust downregulation of PDGF signalling in preadipocytes expressing catalytically inactive SHIP2, the lack of a difference in cell death between pLXSN and SHIP2 PDR/AAA expressing cells is interesting. The assessment of cell death was performed in preadipocytes proliferating in serum to verify that the difference in cell number that I observed was not due to differences in cell death. It is possible that in an entirely different context, e.g. if cell death was actively triggered with nutrient deprivation or other stressors, PDGF would not be as potent of an anti-apoptotic agent in SHIP2 PDR/AAA compared to empty vector expressing preadipocytes.

Differences in cell number can also be caused by impaired or enhanced attachment of cells to the substrate. This is particularly relevant to this study considering SHIP2 WT has been shown to promote adhesion of HeLa and COS-7 cells to collagen I-coated plates, dependent in part on SH2 and 5-phosphatase domains of SHIP2 (Paternotte et al., 2005; Prasad et al., 2001). In contrast, SHIP2 knock-down with siRNA did not affect the number

of HeLa cells attached to the collagen I-coated surface (Prasad and Decker, 2005). Assessment of the number of cells attached to the plate 3 hours after seeding revealed no differences between empty vector, SHIP2 WT, and SHIP2 PDR/AAA expressing cells. It should be noted that 3 hours is sufficient for the attachment of the majority of 3T3-L1 preadipocytes.

Anti-adipogenic effects of PDGF

In addition to examining the mitogenic effects of PDGF on preadipocytes, the current study also addressed the anti-adipogenic action of this growth factor. Although the ability of PDGF to inhibit adipocyte differentiation has been reported (Hauner et al., 1995; Hayashi et al., 1981), the timing and the mechanism of this effect is not clear. Since previous studies demonstrated PDGF-mediated inhibition of adipogenesis using only a single late differentiation marker GPDH (Hauner et al., 1995; Hayashi et al., 1981; Krieger-Brauer and Kather, 1995), a more careful analysis of PDGF on the adipogenic program was necessary. Examination of a panel of differentiation markers revealed that PDGF inhibited not only accumulation of lipid and proteins characteristic of mature adipocytes (aP2 and adiponectin), but also expression of master regulators of adipogenesis, C/EBPα and PPARγ. This suggests that PDGF acts early during the adipogenic program.

C/EBPα and PPARγ are anti-mitotic and are thought to contribute to the exit from MCE, which is required for successful differentiation (Altiok et al., 1997; Umek et al., 1991). The impaired expression of both C/EBPα and PPARγ in the presence of PDGF in the differentiation cocktail would suggest that cells might not exit MCE, leading to inhibition of adipogenesis. However, this was not the case, since the duration of the MCE that I observed

for preadipocytes differentiated in the presence of PDGF (up to day 4) was similar to cells differentiated without this growth factor. Due to the mitogenic effects of PDGF, the total number of cells on day 4 was greater for cells differentiated with vs. without added PDGF.

Even though PDGF does not appear to inhibit adipogenesis by preventing exit from MCE, it might affect the nature of the growth arrest. Following exit from the MCE, cells enter permanent growth arrest, known as the G_D phase of the cell cycle. In contrast, confluent preadipocytes, which are growth-arrested due to contact inhibition, retain their ability to proliferate, for example after plating at lower density. In fact, another inhibitor of adipogenesis, retinoic acid, leads to growth arrest in the G_0 instead of the G_D phase of the cell cycle following clonal expansion (Shao and Lazar, 1997). Whether PDGF exerts its anti-adipogenic effects by a similar mechanism is unclear.

PDGF might also attenuate C/EBPα and PPARγ expression by interfering with C/EBPβ function. C/EBPβ expression coincides with the MCE phase of differentiation. Moreover, this transcription factor is necessary not only for the progression through MCE (Tang et al., 2003a; Zhang et al., 2004b), but also for inducing the expression of C/EBPα and PPARγ (Christy et al., 1991; Zhu et al., 1995). However, PDGF did not affect the pattern or intensity of C/EBPβ expression. Interestingly, many other inhibitors of adipogenesis, including TNFα, ceramide, retinoic acid, octanoate, activin A, and transforming growth factor β (TGFβ), impair differentiation without altering C/EBPβ expression (Choy et al., 2000; Han et al., 2002; Hirai et al., 2005; Kurebayashi et al., 2001; Schwarz et al., 1997; Sprott et al., 2002). It should be noted that C/EBPβ expression does not always correlate with its activity. C/EBPβ appears as early as 4 hours after induction of differentiation, however, it does not acquire DNA binding activity until 8 hours later (Tang

and Lane, 1999). Consistent with this notion, TGFβ, retinoic acid and ceramide reduce transcriptional activity of C/EBPβ without altering its expression (Choy et al., 2000; Schwarz et al., 1997; Sprott et al., 2002). Hence, even though PDGF does not modulate C/EBPβ expression, it might still affect its function.

The acquisition of DNA-binding activity for C/EBPβ is linked with the progression through the cell cycle during the MCE phase. Prior to MCE initiation, C/EBPβ appears to be associated with hypophosphorylated Rb, which inhibits the DNA-binding activity of C/EBPβ (Cole et al., 2004). Following the re-entry into the cell cycle, Rb becomes phosphorylated and dissociates from C/EBPβ, allowing it to bind DNA and transactivate appropriate genes. Since, in my studies, cells differentiated in the presence of PDGF demonstrated reduced Rb phosphorylation, it is plausible that despite appropriate expression, C/EBPβ DNA-binding activity and its ability to induce transcription of target genes was impaired in this setting.

Alternatively, diminished Rb phosphorylation might reflect earlier dephosphorylation of this protein in the presence of PDGF. Rb phosphorylation in a cell population increases and decreases gradually with time as cells enter and then exit the cell cycle, respectively. Since analysis was performed every two days, maximal Rb phosphorylation for cells differentiated in the presence of PDGF might have been missed. In this scenario, anti-adipogenic effects of PDGF might not depend on Rb phosphorylation. Further testing is required to establish if PDGF modulates C/EBPβ function, and whether Rb is involved in this process.

Effects of SHIP2 PDR/AAA on adipogenesis

The ability of PDGF to elicit anti-adipogenic effects is very intriguing because this growth factor activates the same cellular signalling cascades as the pro-adipogenic insulin/IGF-I. In particular, both insulin and PDGF stimulate activation of Akt, which is necessary and sufficient for induction of adipogenesis. Thus, PDGF might engage additional molecules, which are not activated by insulin, to elicit its anti-adipogenic effects. Treatment of preadipocytes with PDGF leads to production of both PI(3,4,5)P3 and PI(3,4)P2, whereas insulin results in accumulation of PI(3,4,5)P3 only (Gagnon et al., 1999; Sorisky et al., 1996). PI(3,4)P2 might be responsible for the inhibitory effects of PDGF on adipocyte differentiation, perhaps by recruitment of molecules with PH domains specific for this phosphoinositide, such as TAPP1 discussed earlier (see "Mechanism of the anti-proliferative effects of SHIP2 WT" section). Consistent with the potential involvement of a 5-phosphatase in PDGF signalling, PDGF induces SHIP2 Tyr phosphorylation and association with Shc, whereas insulin does not (Gagnon et al., 2003).

Dephosphorylation of PI(3,4,5)P3 by a 5-phosphatase is not the only means to produce PI(3,4)P2. For example, PI3K-C2 can phosphorylate PI4P to produce PI(3,4)P2 (Arcaro et al., 1998; Domin et al., 1997). Evidence is accumulating to suggest that PI3K-C2 can be activated by growth factor stimulation (Arcaro et al., 2000; Brown et al., 1999). Indeed, based on my *in vitro* assay, both insulin and PDGF stimulated PI3K-C2 activity in 3T3-L1 preadipocytes. However, both insulin and PDGF appear to activate PI3K-C2 equally well, suggesting that the differences in the phosphoinositide profile observed with insulin vs. PDGF are likely not due to PI3K-C2 activation.

Another way to produce PI(3,4)P2 is by the phosphorylation of phosphatidylinositol-3-phosphate (PI3P) by a PI3P 4-kinase. Yamamoto et al. demonstrated the presence of PI3P 4-kinase activity in platelets, erythroleukemia and red blood cells (Yamamoto et al., 1990), although the identity of the kinase was not known. PI4P 5-kinases might be responsible for the observed PI3P 4-kinase activity, since they can phosphorylate PI3P to produce PI(3,4)P2 *in vitro* (Zhang et al., 1997); however, the relevance of this pathway *in vivo* is not clear.

Finally, a 5-phosphatase other than SHIP2 might dephosphorylate PI(3,4,5)P3 to make PI(3,4)P2. Several other 5-phosphatases, including oculocerebrorenal syndrome of lowe, skeletal muscle and kidney enriched inositol phosphatase, and proline-rich inositol polyphosphate 5-phosphatase, are ubiquitously expressed and might participate in PDGF signalling (Astle et al., 2006). While these possibilities have not been excluded, the differential regulation of SHIP2 by insulin compared to PDGF makes this candidate very appealing.

Overexpression of SHIP2 WT failed to alter the differentiation response either in the presence or absence of PDGF. It should be noted that PDGF is present in FBS used in the differentiation medium (Hanai et al., 1987), and its presence likely prevents maximal differentiation. Hence, any alteration of the anti-adipogenic PDGF signalling would be expected to affect both basal differentiation, as well as differentiation in the presence of additional PDGF. To enhance the adipogenic response, differentiation of preadipocytes expressing SHIP2 constructs or empty vector was performed in the presence of supraphysiological concentrations of insulin. Unfortunately, under these conditions the inhibitory effects of PDGF were not as pronounced as they were during differentiation with FBS as the only source of insulin/IGF-I. The reduced anti-adipogenic effects of PDGF in the

presence of high concentration of insulin/IGF-I might be due to the ability of insulin to attenuate PDGF signalling in certain cell types (Cirri et al., 2005). Perhaps, a higher concentration of PDGF should have been used in these experiments to counteract insulin action.

The reason for a lack of an effect of SHIP2 WT on adipogenesis is unclear. Overexpressed SHIP2 WT is likely regulated similarly to endogenous SHIP2, and might not lead to drastic changes in downstream effects. Consistent with my results, transgenic overexpression of SHIP2 WT in mice failed to alter epididymal fat pad mass, as well as the average size of individual adipocytes in the this depot (Kagawa et al., 2008). Overexpression of constitutively-active SHIP2 in future studies might circumvent this issue, although such a construct has not been described to date. Membrane-targeting of SHIP1, either full-length protein without the SH2 domain or the 5-phosphatase domain only, by fusion with the extracellular/transmembrane portions of FcγRIIB or CD2 has been shown to produce a constitutively-active enzyme (Bolland et al., 1998; Freeburn et al., 2002). Similarly, myristoylated SHIP2, which is constitutively targeted to the plasma membrane, is more efficient at inhibiting insulin-induced Akt phosphorylation than wild-type SHIP2 in rat1 fibroblasts overexpressing insulin receptors (Ishihara et al., 2002). However, in 3T3-L1 preadipocytes SHIP2 translocates to the plasma membrane in response to both insulin and PDGF, even though only PDGF treatment increases PI(3,4)P2 levels (Gagnon et al., 2003). This suggests that membrane-targeting of SHIP2 might not be sufficient to generate a constitutively-active enzyme.

Another explanation for the lack of effect of SHIP2 WT on adipogenesis might be that SHIP2 does not play a role in PDGF signalling during adipogenesis. However, this

possibility is unlikely since overexpression of the catalytically inactive, dominant-negative SHIP2 accelerated basal differentiation, and attenuated the anti-adipogenic effects of PDGF. Whether this was due to the downregulation of PDGF signalling via enhanced PDGFR degradation, as I found for subconfluent proliferating preadipocytes, is unknown at this point.

Since SHIP2 clearly modulates preadipocyte proliferation, it would have been interesting to examine MCE in cells overexpressing SHIP2 constructs. Given the pronounced anti-proliferative effect of SHIP2 PDR/AAA, this mutant might be expected to impair the MCE phase of adipogenesis. This is counterintuitive, since MCE is thought to be required for successful differentiation. Since in preadipocytes SHIP2 appears to be regulated only by PDGF, and not by insulin, perhaps SHIP2 PDR/AAA inhibits PDGF-driven cell cycle, allowing for insulin/IGF-I to promote MCE more efficiently. If so, this would be a very interesting avenue for future research.

In addition to the potential alleviation of the PDGF signal by SHIP2 PDR/AAA, this mutant is also likely to increase insulin sensitivity as the cells acquire the mature adipocyte phenotype. This would be consistent with studies demonstrating that overexpression of the rat equivalent of SHIP2 PDR/AAA in 3T3-L1 adipocytes leads to improvement of insulin signalling, including Akt phosphorylation and activation (Sasaoka et al., 2005; Wada et al., 2001). The development of insulin sensitivity with differentiation would also be consistent with our previous finding that insulin induces SHIP2 Tyr phosphorylation and association with Shc only in 3T3-L1 adipocytes, but not in preadipocytes (Gagnon et al., 2003). The differential sensitivity to insulin is not a general phenomenon, since other responses, such as insulin-stimulated Akt phosphorylation, occur normally in preadipocytes (Gagnon et al.,

1999). On the other hand, it is possible that SHIP2 PDR/AAA actually inhibits insulin signalling by enhancing IR degradation, similarly to PDGFR. However, the enhanced insulin sensitivity in the presence of catalytically inactive, dominant-negative SHIP2 argues against this point.

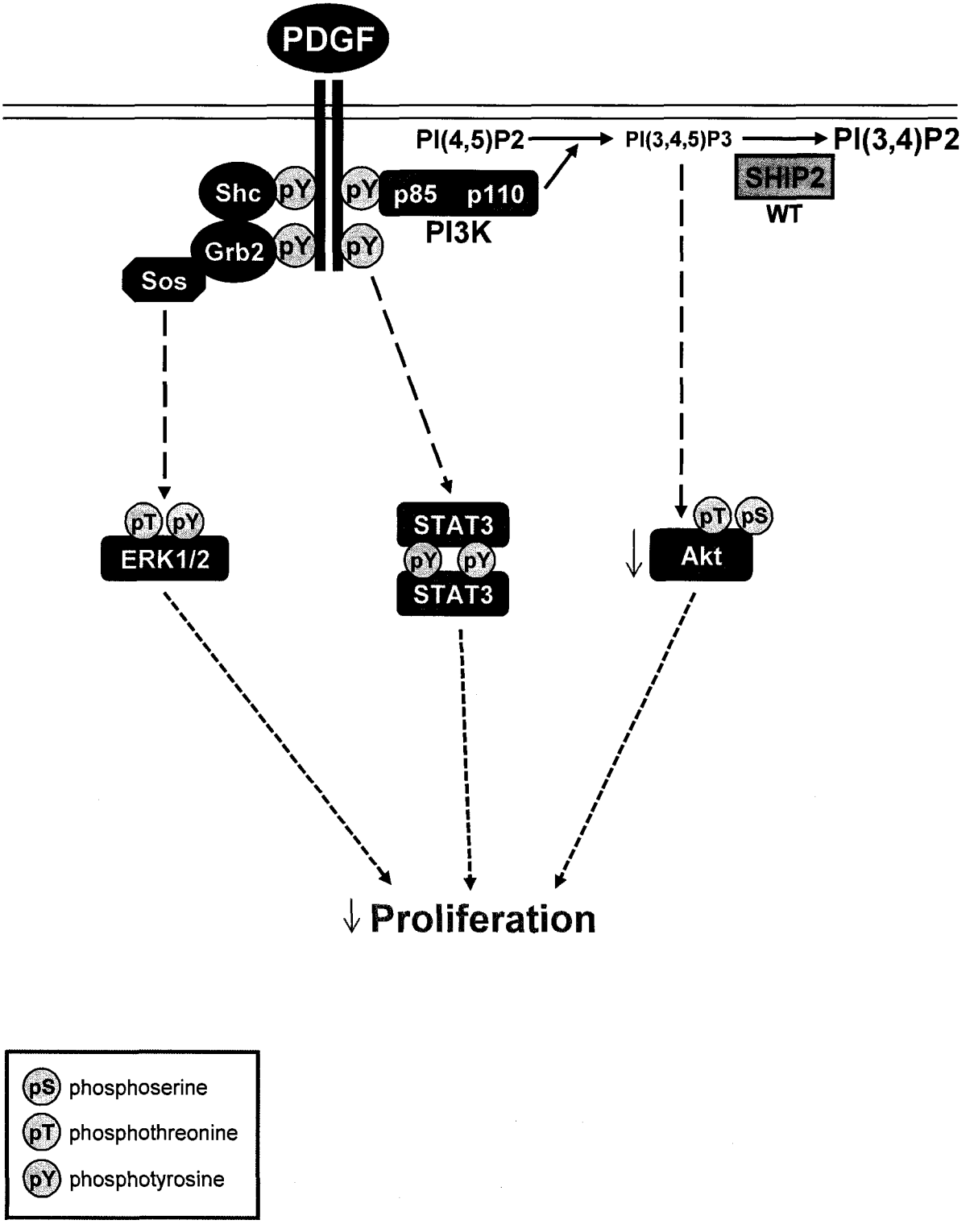
The potential effects of SHIP2 PDR/AAA on PDGF and insulin signalling might be related. For example, enhanced degradation of PDGFR and reduced activation of its downstream targets observed with SHIP2 PDR/AAA might free up substrates, such as Akt, for the insulin signalling pathway. Furthermore, increased insulin signalling might not only accelerate the acquisition of the mature adipocyte phenotype directly, but might also impair PDGF-mediated signalling (Cirri et al., 2005), further contributing to improved differentiation responses. Thus, the presence of SHIP2 PDR/AAA might shift the balance toward the pro-adipogenic response, leading to enhanced expression of transcription factors PPARγ and C/EBPα, and subsequent activation of lipogenesis. This area needs to be further investigated.

Potential model and physiological implications

Based on the findings presented in this study I propose the following model. In proliferating 3T3-L1 preadipocytes, SHIP2 WT converts PI(3,4,5)P3 into PI(3,4)P2, thereby reducing Akt activation, and subsequently has a mild inhibitory effect on proliferation. This process does not depend on the state of SHIP2 Tyr phosphorylation or association with Shc (Fig. 32).

In contrast, catalytically inactive, dominant-negative SHIP2 initially prevents generation of PI(3,4)P2, leading to accumulation of PI(3,4,5)P3 and enhanced activation of

Figure 32. Proposed model of the anti-proliferative effects of SHIP2 WT. In proliferating 3T3-L1 preadipocytes, SHIP2 WT converts PI(3,4,5)P3 into PI(3,4)P2, leading to slightly reduced Akt activation, and a mild inhibitory effect on proliferation. This process does not depend on the state of SHIP2 Tyr phosphorylation or association with Shc.

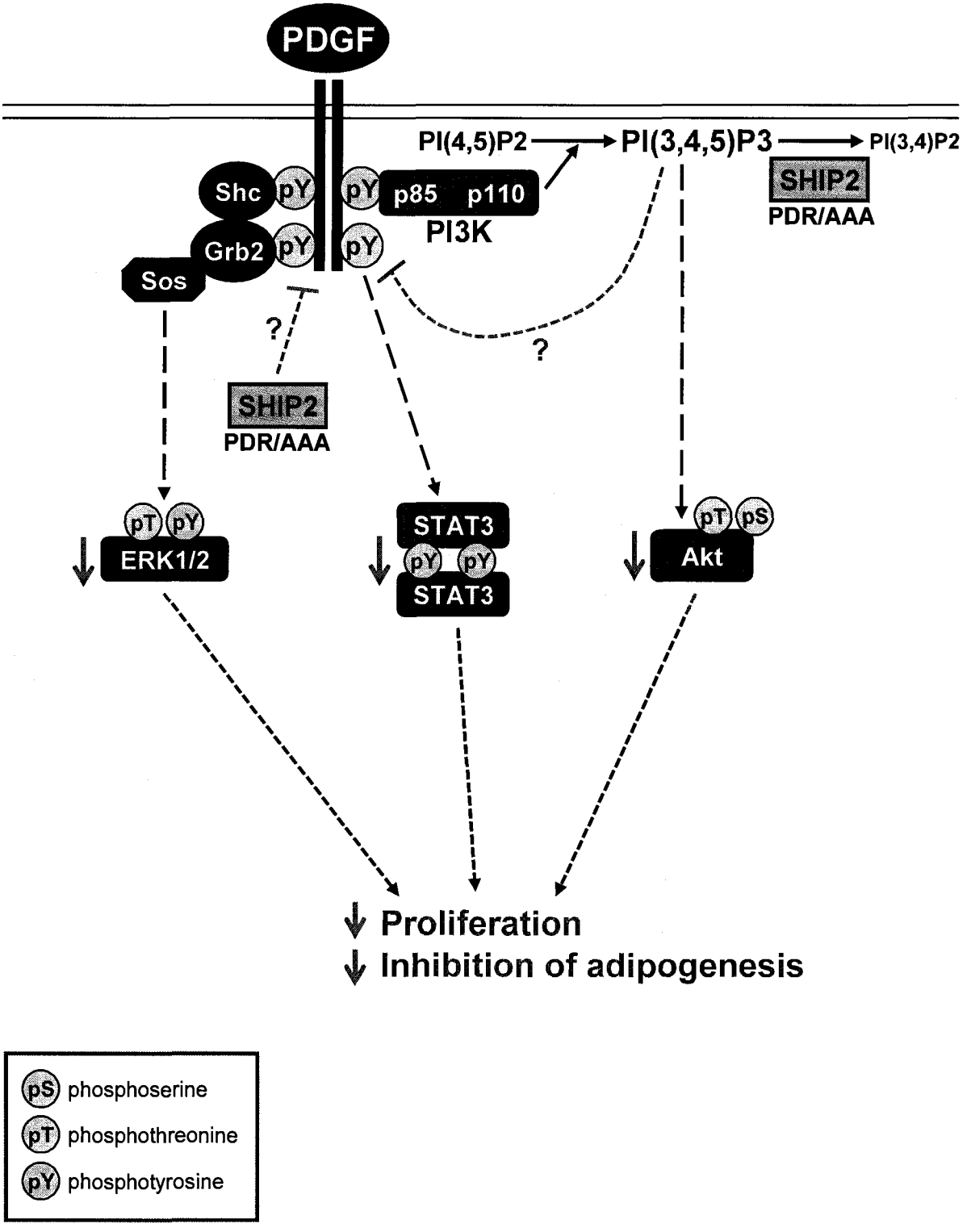

PDGF
PI(4,5)P2
PI(3,4,5)P3
PI(3,4)P2
SHIP2
WT
Shc
pY
pY
p85
p110
PI3K
Grb2
pY
pY
Sos
pT
pY
ERK1/2
STAT3
pY
pY
STAT3
pT
pS
Akt
↓ Proliferation
pS phosphoserine
pT phosphothreonine
pY phosphotyrosine

Akt. However, with longer exposure to PDGF, SHIP2 PDR/AAA accelerates the degradation of the PDGFR, and consequently inhibits all of its downstream signals, leading to a very pronounced anti-proliferative effect. In differentiating preadipocytes, SHIP2 PDR/AAA alleviates the inhibitory signal of PDGF, allowing for a more robust differentiation response (Fig. 33).

The apparent inconsistency between SHIP2 WT and SHIP2 PDR/AAA data is intriguing. The finding that catalytically inactive, dominant-negative SHIP2 promotes PDGFR degradation and slows down preadipocyte proliferation suggests that endogenous SHIP2 plays a positive role in PDGF signalling; however, SHIP2 WT data suggests that SHIP2 is a negative regulator of preadipocyte proliferation. Since SHIP2 PDR/AAA only lacks catalytic activity, it is likely capable of interacting with SHIP2 binding partners. Hence, it is possible that SHIP2 PDR/AAA is dominant-negative with respect to the 5-phosphatase activity of endogenous SHIP2, but might be equivalent to SHIP2 WT overexpression in terms of protein-protein interactions that do not depend on a subsequent catalytic event. This possibility can be addressed in the future by the examination of PDGF signalling and preadipocyte proliferation in cells with reduced SHIP2 levels, for example, due to RNA interference.

SHIP2 might be a positive regulator of growth factor signalling in general, since in addition to enhanced PDGFR degradation observed in the presence of SHIP2 PDR/AAA, knock-down of SHIP2 also leads to increased degradation of EGF and EphA2 receptors (Prasad and Decker, 2005; Zhuang et al., 2007). Hence, the lack of SHIP2 might result not only in reduced proliferation of preadipocytes, but also of other cell types, potentially explaining the slower growth of SHIP2 knock-out mice compared to controls

Figure 33. Proposed model of the anti-proliferative and pro-adipogenic effects of catalytically inactive, dominant-negative SHIP2. Following acute stimulation with PDGF, SHIP2 PDR/AAA initially prevents generation of PI(3,4)P2, leading to accumulation of PI(3,4,5)P3 and enhanced activation of Akt. However, with longer exposure to PDGF, SHIP2 PDR/AAA accelerates the degradation of the PDGFR, and consequently inhibits all of its downstream signals, including phosphorylation of Akt, ERK1/2 and STAT3. This results in a very pronounced inhibition of proliferation. In differentiating preadipocytes, the reduction of the anti-adipogenic effects of PDGF by SHIP2 PDR/AAA allows for a more robust differentiation response. Note that for the purpose of clarity, the initial enhancement of Akt phosphorylation is not shown. For details of Akt, ERK1/2 and STAT3 pathways refer to Figure 1.


PDGF
PI(4,5)P2
PI(3,4,5)P3
PI(3,4)P2
Shc
pY
pY
p85
p110
PI3K
Grb2
pY
pY
Sos
SHIP2
PDR/AAA
?
?
SHIP2
PDR/AAA
pT
pY
ERK1/2
STAT3
pY
pY
STAT3
pT
pS
Akt
Proliferation
Inhibition of adipogenesis
pS phosphoserine
pT phosphothreonine
pY phosphotyrosine

(Sleeman et al., 2005). Whether this is indeed the case is unclear at the moment, since the findings in this thesis have not been tested *in vivo*.

The improved metabolic profile of SHIP2 knock-out mice might also be in part due to the ability of SHIP2 to positively regulate PDGF signalling in preadipocytes. Mice lacking SHIP2 would be expected to have enhanced differentiation of preadipocytes. This should lead to adipose tissue that is composed of smaller, more insulin-sensitive adipocytes compared to controls, and might explain reduced serum triglycerides and FFA observed in SHIP2 knock-out animals.

Overall, my studies have identified SHIP2 as a novel regulator of PDGF signalling in preadipocytes. The ability of SHIP2 to modulate both preadipocyte proliferation and differentiation suggests that this lipid 5-phosphatase is an important player in adipose tissue dynamics.

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CONTRIBUTIONS OF COLLABORATORS

Dr. AnneMarie Gagnon performed some of the replicate experiments looking at effects of BisI on PDGF-stimulated phosphoinositide profile of 3T3-L1 preadipocytes. An undergraduate student Dan Sumarto was involved in the site-directed mutagenesis of SHIP2 WT to produce SHIP2 T958A. An Honours undergraduate student Sandro Ibrahim stably overexpressed SHIP2 T958A, SHIP2 WT, and empty vector pLXSN in 3T3-L1 preadipocytes and performed some of the replicate experiments involving these constructs.

APPENDIX

Report from Protana Analytical Services

The following report from Protana Analytical Services was directly copied from the e-mail sent by Paul Taylor to Dr. Sorisky.

Protana Analytical Services

Report on Project 4956419

Prepared by Paul Taylor

March 3, 2005

Samples

2 gel bands were received, one labeled S for sample and the other labeled C for control.

Method

The gel bands were washed, the proteins were reduced with DTT and the free sulfhydryl groups were alkylated with iodoacetamide. The proteins were then digested with trypsin and the peptides produced were extracted from the gel matrix. The peptides were separated by reverse phase chromatography on C-8 resin directly into an ABI-Sciex QSTAR-Pulsar QqTOF mass spectrometer. The resulting raw spectra were searched with the Mascot search engine to identify the fragmented peptides. The data was examined manually to determine if there was any evidence for phosphorylation.

Results

The protein was identified as inositol polyphosphate phosphatase-like 1 gi|4755142. The peptides identifies are shown highlighted in red in figure 1.

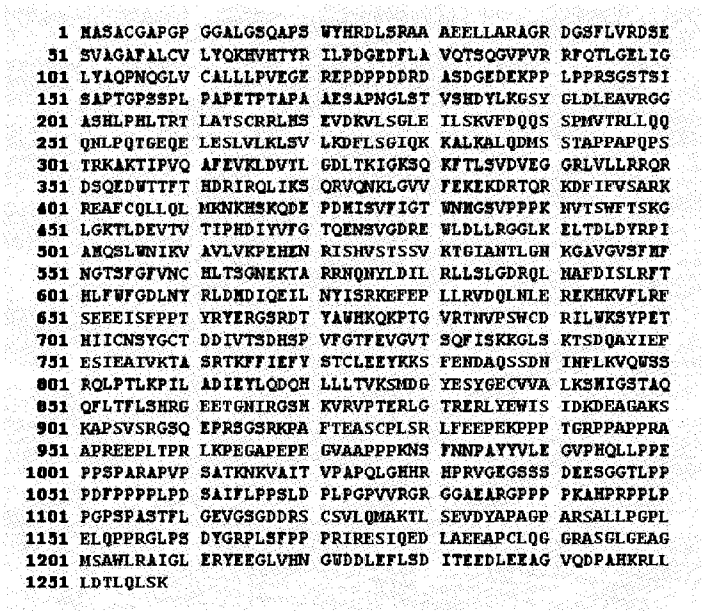

Figure 1

The spectra were examined manually for evidence of phosphorylation and the following phosphorylation event was noted.

The peptide AAPREEPLTPR was found to have the T9 residue phosphorylated in the treated sample (this corresponds to threonine 958 in the supplied sequence). This phosphorylated ion was absent in the control sample.

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Data presented in Fig. 7, 24, 25 has been published in:

Artemenko, Y., Gagnon, A., Aubin, D., and Sorisky, A. (2005) Anti-adipogenic effect of PDGF is reversed by PKC inhibition. J Cell Physiol *204*, 646-53. © 2005 Wiley-Liss, Inc.

Data presented in Fig. 6, 8-14 has been published in:

Artemenko, Y., Gagnon, A., Ibrahim, S., and Sorisky, A. (2007) Regulation of PDGF-stimulated SHIP2 tyrosine phosphorylation and association with Shc in 3T3-L1 preadipocytes. J Cell Physiol *211*, 598-607. © 2007 Wiley-Liss, Inc.

Data presented in Fig. 18-23 has been published in:

Artemenko, Y., Gagnon, A., and Sorisky, A. (2008) Catalytically inactive SHIP2 inhibits proliferation by attenuating PDGF signaling in 3T3-L1 preadipocytes. J Cell Physiol *Sep 22 [Epub ahead of print]* © 2008 Wiley-Liss, Inc.

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- University of Ottawa National Excellence Scholarship
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04/2004
- Top M.Sc. poster at the Graduate Student Poster Day

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ABSTRACTS:

Artemenko, Y., Gagnon, A., Sorisky, A. (2008) Catalytically inactive SH2 Domain-Containing Inositol 5-Phosphatase 2 (SHIP2) Attenuates PDGF-Regulated Processes in 3T3-L1 Preadipocytes. Endocrine Society's 90th Annual Meeting (June 15-18, 2008; San Francisco, CA, USA). *(poster)*

Artemenko, Y., Gagnon, A., Sorisky, A. (2007) Catalytically Inactive SHIP2 Inhibits Preadipocyte Proliferation and PDGF Signaling. 47th Annual Meeting of the American Society for Cell Biology (December 1-5, 2007; Washington, DC, USA). *(poster)*

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