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MAIJA PÄIVÄRINTA

# Phosphatidylinositol 3-kinase and type 2 diabetes

## Catalytic subunit p110β as a candidate gene for type 2 diabetes and *in vitro* modelling of the insulin signalling pathway

Doctoral dissertation

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## ABSTRACT

Type 2 diabetes is a new global epidemic. The prevalence of type 2 diabetes is increasing in all age groups and in addition to human suffering, the future is threatened by the heavy economic burden caused by increased morbidity associated with type 2 diabetes.

Activity of phosphatidylinositol (PI) 3-kinase is required for many of the effects of insulin, including glucose uptake. Since impaired insulin-stimulated glucose uptake is a fundamental defect in insulin resistance and type 2 diabetes, the primary aim of our study was to investigate the gene encoding the catalytic subunit, p110β, of human PI 3-kinase as a candidate gene for insulin resistance and type 2 diabetes. Furthermore, we aimed to establish an *in vitro* model to study the insulin signalling pathways.

The gene encoding human p110β was cloned, sequenced and its genomic structure was determined. All exons and 1.5 kb of the promoter region were screened in non-diabetic and type 2 diabetic subjects using the single-strand conformation polymorphism analysis. Glucose metabolism was assessed by oral and intravenous glucose tolerance tests and the euglycemic hyperinsulinemic clamp study. To model the insulin signal pathways *in vitro*, we differentiated commercial 3T3-L1 cells into adipocytes using a cocktail of differentiation-promoting agents. In addition, we optimized an adenovirus-mediated gene transfer protocol by examining the effects of preincubation of viral constructs at 0°C, +20°C and +37°C and the presence of various sera on the viral transduction efficiency.

Ultimately, we did not detect any polymorphisms in exons of the p110β gene. In the promoter region of the p110β gene, we identified two polymorphisms, –359T/C and –303A/G. The allele frequencies of the polymorphisms were similar in non-diabetic and type 2 diabetic subjects and these polymorphisms were not associated with insulin secretion or insulin sensitivity in two normoglycemic study groups.

3T3-L1 cells were readily differentiated into adipocytes. In response to insulin, the major pathways of insulin signal transduction, PI 3-kinase/Akt and mitogen-activated protein kinase pathways, were activated. Insulin also stimulated 2-deoxyglucose uptake by 13-fold in these cells. This effect was abolished by the PI 3-kinase inhibitors, Wortmannin and LY294002.

The transduction efficiency of recombinant adenovirus was improved in coxsackie B virus and adenovirus type 2 and 5 receptor-deficient cells *in vitro* after a 20-30 min preincubation at +37°C. Similar heat activation of the adenoviral construct was observed *in vivo* in rat brain tissue. The infectivity of adenovirus was rapidly abolished in the presence of human serum while bovine serum retained the viral infectivity.

This study showed that variants in the p110β gene are not a major risk factor for type 2 diabetes in the Finnish population. In addition, our results indicate that differentiated 3T3-L1 cells are a potential cell model to investigate insulin signal transduction *in vitro* and that it is important and worthwhile to optimize the adenoviral transduction protocol to achieve maximal gene transfer efficiency.

National Library of Medicine Classification: WK 810, QZ 50
Medical Subject Headings: diabetes mellitus, type 2/genetics; diabetes mellitus, type 2/enzymology; genotype; insulin resistance/genetics; 1-phosphatidylinositol 3-kinase/genetics; insulin/metabolism; signal transduction; catalytic domain; 3T3 cells; human; Finland; adenoviridae/genetics; gene transfer techniques

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Kuopio, April 2005

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**ABBREVIATIONS**

| | |
|---|---|
| Ad | adenovirus |
| aPKC | atypical protein kinase C |
| APS | adapter protein with PH and SH2 domain |
| Arg | arginine |
| BAD | Bcl-2/Bcl-$X_L$-antagonist, causing cell death |
| BMI | body mass index |
| bp | base pair |
| CAP | Cbl-associated protein |
| CAR | coxsackie B virus and adenovirus type 2 and 5 receptor |
| C/EBP | CCAAT/enhancer binding protein |
| CMV | cytomegalovirus |
| EGF | epidermal growth factor |
| eIF | eukaryotic initiation factor |
| ERK1/2 | extracellular signal-regulated kinase 1 and 2 |
| FBS | fetal bovine serum |
| FFA | free fatty acid |
| G-6-Pase | glucose-6-phosphatase |
| GFP | green fluorescent protein |
| GPCR | G protein coupled receptor |
| Grb2 | growth factor receptor-bound protein 2 |
| GS | glycogen synthase |
| GSK3 | glycogen synthase kinase-3 |
| HSL | hormone-sensitive lipase |
| $IC_{50}$ | inhibitor concentration that decreases the enzyme activity by 50% |
| IRE | insulin-responsive element |
| IRS | insulin receptor substrate |
| IVGTT | intravenous glucose tolerance test |
| JNK | NH2-terminal Jun kinase |
| kb | kilobase |
| kDa | kilodalton |
| Lys | lysine |
| MAPK | mitogen-activated protein kinase |
| MODY | maturity onset diabetes of the young |
| mTOR | mammalian target of rapamycin |
| Nab | neutralizing antibody |
| p70S6k | p70 ribosomal protein S6 kinase |
| PDGF | platelet-derived growth factor |
| PDK1 | PI(3,4,5)$P_3$-dependent protein kinase-1 |
| PEPCK | phosphoenolpyruvate carboxykinase |
| PGC-1 | peroxisome proliferator-activated receptor-γ coactivator-1 |
| PI | phosphatidylinositol |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PP1 | protein phosphatase-1 |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| Ser/Thr | serine/threonine |
| SH2 | Src homology 2 domain |
| SH3 | Src homology 3 domain |
| SREBP | sterol response element-binding protein |
| SSCP | single-strand conformation polymorphism |
| SUR | sulfonylurea receptor |
| TNFα | tumor necrosis factor α |
| Vps34p | vesicular protein sorting 34p |
| WBGU | whole body glucose uptake |

**ORIGINAL PUBLICATIONS**

I Kossila M, Sinkovic M, Kärkkäinen P, Laukkanen M O, Miettinen R, Rissanen J, Kekäläinen P, Kuusisto J, Ylä-Herttuala S, Laakso M. Gene encoding the catalytic subunit p110β of human phosphatidylinositol 3-kinase: cloning, genomic structure and screening for variants in patients with type 2 diabetes. Diabetes 49:1740-1743, 2000

II Kossila M, Pihlajamäki J, Kärkkäinen P, Miettinen R, Kekäläinen P, Vauhkonen I, Ylä-Herttuala S, Laakso M. Promoter polymorphisms −359T/C and −303A/G of the catalytic subunit p110β gene of human phosphatidylinositol 3-kinase are not associated with insulin secretion or insulin sensitivity in Finnish subjects. Diabetes Care 26:179-182, 2003

III Päivärinta M, Levonen A-L, Ylä-Herttuala S. Differentiated 3T3-L1 cells – a potential tool to study insulin signal transduction *in vitro*. Manuscript

IV Kossila M, Jauhiainen S, Laukkanen M O, Lehtolainen P, Jääskeläinen M, Turunen P, Loimas S, Wahlfors J, Ylä-Herttuala S. Improvement in adenoviral gene transfer efficiency after preincubation at +37°C *in vitro* and *in vivo*. Mol Ther 5:87-93, 2002

# CONTENTS

# 1 INTRODUCTION

Type 2 diabetes is an increasing health problem worldwide. It has been estimated that in the year 2025 there will be 300 million adul individuals with type 2 diabetes (King et al., 1998). During recent years, reports of increased childhood obesity and type 2 diabetes have created a totally new viewpoint into the epidemic of type 2 diabetes (Zimmet et al., 2001; Saha et al., 2003). Therefore, it is important that we understand the mechanisms leading to type 2 diabetes if we are to find preventive treatments to avoid the future epidemic of this disease.

Type 2 diabetes is a slowly progressing, lethal disease characterized by peripheral insulin resistance and inadequate insulin secretion by pancreatic β-cells (DeFronzo et al., 1992). In addition, this disease leads to micro- and macrovascular complications (Tooke, 1995; Pyorala et al., 1987). Although the pathophysiology of type 2 diabetes is not fully understood, it is believed that both genetic and acquired factors contribute to the development of type 2 diabetes (Newman et al., 1987; Kaprio et al., 1992; Hu et al., 2001). Genetic predisposition to type 2 diabetes can be detected early in life as impaired insulin action (Rothman et al., 1995). Type 2 diabetes is a polygenic disease with an unknown mode of inheritance. The pathophysiology of several monogenic forms of type 2 diabetes, including subtypes of maturity onset diabetes of the young (MODY), have been clarified (Shih and Stoffel, 2002) and the information provided by these studies can be exploited in the investigation of the polygenic forms of type 2 diabetes. One commonly used method to investigate both polygenic and monogenic forms of type 2 diabetes is the candidate gene approach. Although important information has been obtained using this approach, no major breakthroughs in the understanding of the genetics of type 2 diabetes have been made. This stresses the importance of using a multidisciplinary approach in diabetes research, including *in vitro* models, if we want to clarify the pathological mechanisms leading to insulin resistance and type 2 diabetes.

In this study, our aim was to investigate the gene encoding the catalytic subunit, p110β, of human phosphatidylinositol (PI) 3-kinase as a candidate gene for insulin resistance and type 2 diabetes. In addition, we aimed to establish an *in vitro* model to investigate insulin signal transduction. We differentiated commercial 3T3-L1 cells into adipocytes and studied the effects of insulin stimulation on known insulin signal transduction pathways. Furthermore, we optimized the utilization of recombinant adenoviral vectors, which are widely used tools in studies of insulin signalling. To optimize the adenoviral transduction efficiency, we tested how preincubation at various temperatures and in the presence of different sera affects the adenoviral gene transfer efficiency.

# 2 REVIEW OF THE LITERATURE

## 2.1 Type 2 diabetes

Type 2 diabetes has been designated as the epidemic of the 21st century. Type 2 diabetes represents a highly heterogenous group of conditions all of which are characterized by disturbed glucose homeostasis (Alberti and Zimmet, 1998). The most severe clinical problem of type 2 diabetes is the increased risk of the patient to develop cardiovascular disease, particularly coronary heart disease, which is the most common cause of death of type 2 diabetic patients (Laakso, 2001). Type 2 diabetes is also associated with microvascular complications i.e. nephropathy, neuropathy and retinopathy (Koivisto and Sipilä, 2000). There are many mechanisms involved in the pathogenesis of type 2 diabetes but for the most part their actual roles are unknown. This emphasizes the importance of the research aiming to solve the mechanisms leading to type 2 diabetes.

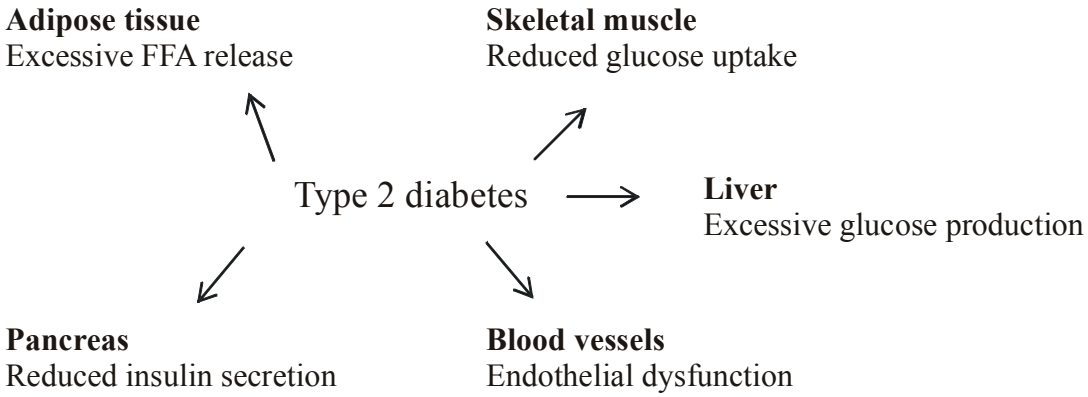


**Figure 1.** Characteristics of type 2 diabetes in various tissues.

### 2.1.1 Pathophysiology

Type 2 diabetes is caused by two abnormalities in glucose metabolism, peripheral insulin resistance in skeletal muscle, adipose tissue and liver and impaired insulin secretion in β-cells of pancreatic islets of Langerhans. Peripheral insulin resistance is characterized by impaired insulin action in the target tissues which means that a higher concentration of insulin in the bloodstream is needed to achieve proper insulin action (DeFronzo et al., 1992). Prospective studies indicate that insulin resistance is the most important predictor for the development of type 2 diabetes (Warram et al., 1990). Peripheral insulin resistance can be present even a decade before the development of type 2 diabetes but impaired insulin action is compensated

by enhanced insulin secretion. Type 2 diabetes is manifested when β-cells are no longer able to secrete sufficient amounts of insulin to compensate for the impaired insulin action (DeFronzo et al., 1992). Pancreatic β-cell failure in type 2 diabetic patients is characterized by decreased β-cell mass due to an increased rate of apoptosis (Butler et al., 2003). The characteristics of type 2 diabetes in various tissues are summarized in Figure 1.

The pathophysiology of insulin resistance and type 2 diabetes is complex and involves both genetic and acquired factors (Kaprio et al., 1992; Hu et al., 2001). Many monogenic forms of type 2 diabetes have been identified. Defects in the genes encoding glucokinase (Froguel et al., 1992), hepatocyte nuclear factor-1α (Yamagata et al., 1996b), -4α (Yamagata et al., 1996a), -1β (Horikawa et al., 1997), insulin promoter factor-1 (Stoffers et al., 1997), NeuroD1 (Malecki et al., 1999) and sulphonylurea receptor 1 (SUR1) (Huopio et al., 2003) have been identified to cause autosomally dominantly inherited MODY. In addition, mutations in maternally inherited mitochondrial DNA have been shown to lead to type 2 diabetes (van den Ouweland et al., 1992). Although these monogenic forms of type 2 diabetes account only for a minor fraction (approximately 5%) of the total type 2 diabetes cases (Alcolado et al., 2002; Elbein, 2002), the decreased insulin secretion involved in all of these conditions has provided essential information that can be utilized in the investigation of the polygenic forms of diabetes. The mode of inheritance of polygenic type 2 diabetes is unknown. However, a genetic predisposition to the polygenic form of type 2 diabetes can be demonstrated by the observation that lean and normoglycemic offsprings of parents with type 2 diabetes have impaired whole body glucose uptake (WBGU) and decreased glucose uptake in skeletal muscle after insulin stimulus compared to control subjects (Rothman et al., 1995).

Obesity is the most important acquired factor that predisposes to type 2 diabetes (Hu et al., 2001). The majority (~80%) of type 2 diabetics are obese (Prof. Markku Laakso, personal communication). In particular, the accumulation of visceral and deep subcutaneous fat in the abdominal region is related to insulin resistance (Kelley et al., 2000). Recently, it has been suggested that adipose tissue and altered fatty acid metabolism contribute to the pathogenesis of insulin resistance and type 2 diabetes (Bays et al., 2004). Insulin resistant states, such as obesity and type 2 diabetes, are characterized by an elevated circulating free fatty acid (FFA) levels (Reaven et al., 1988; Groop et al., 1991). In skeletal muscle, the elevated FFA level impairs insulin signal transduction which leads to inhibition of glucose uptake in response to insulin stimulation (Roden et al., 1996; Dresner et al., 1999; Kruszynska et al., 2002). In liver, the increased FFA concentration abolishes the insulin-mediated suppression of glycogenolysis

(Boden et al., 2002) and/or gluconeogenesis (Saloranta et al., 1993). In pancreas, prolonged elevation in the FFA level is associated with β-cell apoptosis via the caspase-9 and ceramide pathways *in vitro* (Lingohr et al., 2003; Lupi et al., 2002) and impaired insulin secretion *in vivo* (Kashyap et al., 2003). In addition to an increment in circulating FFA levels, insulin resistance has been associated with accumulation of triglycerides in skeletal muscle (Jacob et al., 1999) and liver (Seppala-Lindroos et al., 2002). It has been shown that intramyocellular lipid is linked with impaired insulin signal transduction (Virkamaki et al., 2001).

Adipose tissue is a dynamic endocrine organ which, in addition to storing triglycerides, secretes several adipokines into the circulation. In obesity and type 2 diabetes, their secretion profile is altered. The secretion of factors that are normally produced, i.e. adiponectin (acrp 30 or adipoQ), is reduced (Arita et al., 1999; Hotta et al., 2000). Adiponectin is exclusively produced by adipocytes (Maeda et al., 1996) and a reduction in its circulating level is associated with insulin resistance (Weyer et al., 2001). On the contrary, secretion of other adipokines, i.e. resistin, tumor necrosis factor α (TNFα), plasminogen activator inhibitor-1, angiotensinogen, interleukin 6 and leptin becomes elevated (Bays et al., 2004). These proinflammatory factors induce insulin resistance and also contribute to the pathogenesis of atherosclerosis (Lyon et al., 2003).

Hyperglycemia is a fundamental feature of type 2 diabetes (DeFronzo et al., 1992). Chronic hyperglycemia contributes to the development of insulin resistance (Yki-Järvinen, 1998). In mice that have undergone a partial pancreatectomy, chronic hyperglycemia downregulates the expression of the insulin gene in β-cells (Jonas et al., 1999) and furthermore, hyperglycemia results in β-cell exhaustion and desensitization to glucose stimulation (Robertson et al., 2003). At first, β-cell function is normalized after the restoration of normoglycemia but over time, the β-cell dysfunction becomes irreversible (Robertson et al., 2003).

Hyperglycemia and an elevated FFA level result in the generation of mitochondrial reactive oxygen species (ROS) and subsequently the formation of oxidative stress. Proinflammatory cytokines and oxidative stress stimulate multiple stress-activated signalling pathways which contribute to a number of cellular processes including insulin resistance, inflammation, apoptosis and gene expression (Evans et al., 2002; Ceriello and Motz, 2004). It has also been proposed that oxidative stress contributes to the formation of micro- and macrovascular complications of type 2 diabetes (Endemann and Schiffrin, 2004; Dandona et al., 2004).

## 2.2 Insulin signal transduction

### 2.2.1 Insulin receptor

Insulin is an anabolic hormone (Zubay et al., 1995b). The physiological effects of insulin are mediated through the insulin receptor which was discovered in 1971 (Freychet et al., 1971). Subsequently, the insulin receptor has been characterized as a transmembrane glycoprotein containing intrinsic tyrosine kinase activity (Ullrich et al., 1985; Ebina et al., 1985). The human insulin receptor gene is located on chromosome 19 (Ebina et al., 1985). The gene encodes a proreceptor polypeptide which is proteolytically cleaved into α- and β-subunits (Ronnett et al., 1984). Mature insulin receptor is a heterotetramer, $\alpha_2\beta_2$, containing two α- and two β-subunits connected to each other by disulfide bonds (Sparrow et al., 1997). The α-subunits are entirely extracellular while β-subunits contain both extracellular and intracellular domains (Ebina et al., 1985; Ullrich et al., 1985). The intracellular part of the β-subunit is divided into the juxtamembrane domain, tyrosine kinase domain and C-terminal domain (Ebina et al., 1985). Insulin binds to the α-subunit of the receptor (Ebina et al., 1985). This leads to autophosphorylation of specific tyrosine residues of the β-subunit (Tornqvist et al., 1987; White et al., 1988; Feener et al., 1993; Kohanski, 1993) and a conformational change in the activation loop of the kinase domain (Hubbard, 1997). These changes enable the binding of ATP and protein substrate to the catalytic site of the insulin receptor and subsequent tyrosine kinase activity of the β-subunit of insulin receptor (Hubbard, 1997).

The insulin receptor tyrosine kinase has several substrates including members of the insulin receptor substrate (IRS) protein family (Sun et al., 1992; White, 2002), Shc (Pelicci et al., 1992), adapter protein with PH and SH2 domains (APS) (Moodie et al., 1999) and Cbl (Ribon and Saltiel, 1997). In response to insulin stimulation, these proteins bind to the β-subunit of the insulin receptor and specific tyrosine residues become phosphorylated (Sun et al., 1993; Ahmed et al., 1999). To date, four members of IRS family (IRS 1-4) have been characterized (Sun et al., 1991; Sun et al., 1995; Lavan et al., 1997b; Lavan et al., 1997a). Downstream effectors of IRS proteins, e.g. PI 3-kinase and growth factor receptor-bound protein 2 (Grb2), bind to the phosphorylated tyrosine residues of IRS proteins via the Src homology 2 (SH2) domains (White, 1994). Insulin signal transduction via IRS proteins is inhibited by serine/threonine (Ser/Thr) kinases which phosphorylate the serine residues of IRS proteins (Sun et al., 1992; Zick, 2003). Serine phosphorylation of IRS-1 and IRS-2 has been shown to contribute to the pathogenesis of insulin resistance (Aguirre et al., 2000; de Alvaro et al., 2004).

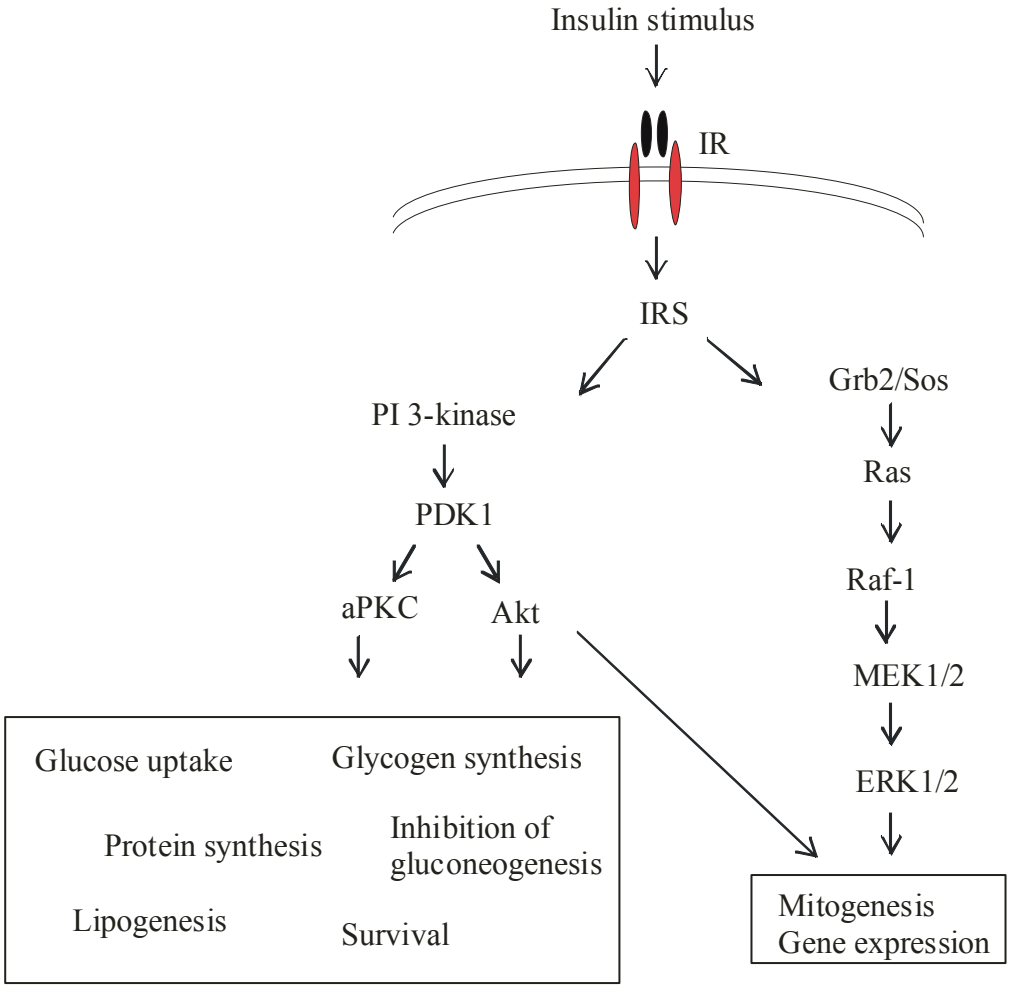


**Figure 2**. Main signalling pathways of insulin. Abbreviations used: IR, insulin receptor; IRS, insulin receptor substrate; PI, phosphatidylinositol; PDK1, PI(3,4,5)$P_3$-dependent protein kinase-1; aPKC, atypical protein kinase C; Grb2, growth factor receptor-bound protein 2; MEK1/2, MAP/ERK kinase 1 and 2; ERK1/2, extracellular signal-regulated kinase 1 and 2

### 2.2.2 Phosphatidylinositol 3-kinase pathway

PI 3-kinases are intracellular lipid kinases which phosphorylate membrane-bound PI, PI(4)P and PI(4,5)$P_2$ at the 3$^{rd}$ position of the inositol ring resulting in the formation of PI(3)P, PI(3,4)$P_2$ and PI(3,4,5)$P_3$ (Whitman et al., 1988; Auger et al., 1989). The association of PI 3-kinase in insulin signal transduction was discovered in 1990 (Ruderman et al., 1990). In response to insulin stimulation, PI 3-kinase binds to tyrosine phosphorylated IRS proteins which leads to formation of 3'-PI-lipids (Backer et al., 1992; Vanhaesebroeck et al., 2001). These lipids function as signalling molecules to mediate the multiple actions of insulin (Fig. 2). Akt and isoforms of atypical protein kinase C (aPKC) have been shown to be the major downstream effectors of PI 3-kinase in insulin signal transduction (Whiteman et al., 2002).

***PI 3-kinase/Akt pathway.*** Akt, which is also known as protein kinase B, is a cellular Ser/Thr kinase containing a C-terminal pleckstrin homology (PH) domain (Konishi et al., 1994). Three isoforms of Akt (Akt1-3) have been characterized (Jones et al., 1991; Meier et al., 1997; Nakatani et al., 1999). Insulin activates Akt in a PI 3-kinase-dependent manner (Alessi et al., 1996). Phosphorylation of Thr308 and Ser473 (in Akt1) residues in Akt is a prerequisite for full activation of Akt (Alessi et al., 1996). Insulin stimulation leads to the binding of IRS to activated insulin receptor, recruitment of PI 3-kinase activity to plasma membrane and formation of $PI(3,4,5)P_3$ (Backer et al., 1992; Vanhaesebroeck et al., 2001). Akt is translocated from cytoplasm to plasma membrane after binding of its PH domain to $PI(3,4,5)P_3$ (James et al., 1996; Andjelkovic et al., 1997). After membrane recruitment, Thr308 and Ser473 of Akt are phosphorylated by a co-localized $PI(3,4,5)P_3$-dependent protein kinase-1 (PDK1) (Alessi et al., 1997) and DNA-dependent protein kinase, respectively (Feng et al., 2004). The PI 3-kinase/Akt pathway participates in mediating many of the metabolic effects of insulin (Whiteman et al., 2002) (Fig. 2). In addition, activated Akt is translocated to the nucleus where it participates in the regulation of gene expression (Andjelkovic et al., 1997; Kido et al., 2001; Puigserver et al., 2003).

In skeletal muscle of patients with type 2 diabetes, the increased FFA level induces decreased tyrosine phosphorylation of IRS-1 and impaired IRS-1 associated PI 3-kinase activity (Roden et al., 1996; Dresner et al., 1999). However, the phosphorylation of Akt in response to insulin stimulation is reported to be unaltered (Kruszynska et al., 2002).

***PI 3-kinase/protein kinase C pathway.*** The family of protein kinase C (PKC) contains 11 Ser/Thr kinases which are subdivided into typical ($\alpha$, $\beta_1$, $\beta_2$, $\gamma$), novel ($\delta$, $\varepsilon$, $\eta$, $\theta$, $\mu$) and atypical ($\zeta$, $\lambda$) PKCs based on their molecular structure, activation mechanism and enzymatic properties (Gschwendt, 1999). Typical and novel PKCs are thought to have an inhibitory effect on insulin signalling (Standaert et al., 1999; Leitges et al., 2002; Griffin et al., 1999) while aPKCs are considered as mediators of insulin signal transduction (Farese, 2002). PKC$\zeta$ and PKC$\lambda$ share considerable amino acid homology and thereby it appears that they are able to function interchangeably (Bandyopadhyay et al., 1999). Insulin activates PKC$\zeta/\lambda$ via PI 3-kinase (Bandyopadhyay et al., 1997b), subsequent formation of $PI(3,4,5)P_3$ and activation of PDK1. Activation of PKC$\zeta/\lambda$ is a multistep process including phosphorylation of Thr410 by PDK1 (Le Good et al., 1998), autophosphorylation of Tyr560 and a conformational change leading to release of the enzyme from pseudosubstrate autoinhibition (Standaert et al., 2001).

In type 2 diabetes, an increased FFA level promotes insulin resistance in skeletal muscle (Griffin et al., 1999), liver (Lam et al., 2002) and pancreas (Wrede et al., 2003) through activation of serine kinase activities of typical and novel PKCs. In addition, the contribution of hyperglycemia to insulin resistance involves activation of typical and novel PKCs (Berti et al., 1994).

### 2.2.3 MAPK pathway

Members of the mitogen-activated protein kinase (MAPK) family are Ser/Thr kinases which regulate cellular proliferation, growth, differentiation and death. The main members of the MAPK family are extracellular signal-regulated kinase 1 and 2 (ERK1/2), NH2-terminal Jun kinase (JNK) and p38 (Pearson et al., 2001). ERK1/2 are mainly activated by various mitogens while JNK and p38 are regarded as stress-activated MAPKs (Evans et al., 2002). In type 2 diabetes, proinflammatory cytokines and oxidative stress stimulate JNK and p38 MAPKs and nuclear factor-κB (Evans et al., 2002; Ceriello and Motz, 2004).

The mitogenic effects of insulin are mediated by Ras and the MAPK pathway (Fig. 2) (Skolnik et al., 1993a; Virkamaki et al., 1999). In response to insulin stimulation, Grb2 containing two SH2 and SH3 domains binds to IRS-1 and Shc (Lowenstein et al., 1992; Skolnik et al., 1993b). Grb2 associates with a guanine nucleotide exchange factor Son of Sevenless (Sos) through SH3 domains (Egan et al., 1993). Sos stimulates the interaction of Ras and GTP, which activates Ras to mediate the stimulation of the MAPK phosphorylation cascade (Alberts et al., 1994a). The first member and the initiator of the MAPK phosphorylation cascade is a ubiquitously expressed Raf-1 which is activated as a result of binding to Ras-GTP (Dhillon and Kolch, 2002). Raf-1 phosphorylates and thereby activates MAP/ERK kinase 1 and 2 (MEK1/2) which in turn activates ERK1/2 by phosphorylating the Thr202 and Tyr204 (Payne et al., 1991). Activated ERK1/2 are translocated to the nucleus where they modulate gene expression by phosphorylating transcription factors and other protein kinases which are involved in the regulation of gene expression. In addition, ERK1/2 have several cytoplasmic substrates (Pearson et al., 2001).

### 2.2.4 Metabolic effects

***Glucose uptake.*** In the postprandial state, an elevated blood glucose level induces pancreatic β-cells to secrete insulin (Zubay et al., 1995b). Insulin stimulation leads to the translocation of insulin-sensitive glucose transporters, GLUT4, from intracellular storage vesicles to plasma membrane and the stimulation of cellular glucose uptake to normalize the elevated blood

glucose level (Saltiel and Kahn, 2001). Skeletal muscle is the major tissue which takes up glucose upon insulin stimulation (Shulman et al., 1990). According to our current understanding, two signalling pathways, PI 3-kinase dependent and PI 3-kinase independent, mediate the effects of insulin on glucose uptake (Khan and Pessin, 2002).

PI 3-kinase has been shown to have a crucial role in mediating the insulin-stimulated glucose uptake (Shepherd et al., 1998). First, wortmannin (Kanai et al., 1993) and LY294002 (Cheatham et al., 1994), which are inhibitors of PI 3-kinase, inhibit the insulin-stimulated GLUT4 translocation to plasma membrane and subsequent glucose uptake in adipocytes (Cheatham et al., 1994), L6 myotubes (Tsakiridis et al., 1995) and isolated muscle (Marchand-Brustel et al., 1995). Second, the inhibitory effect of wortmannin on glucose uptake can be overcome with the use of membrane-permeant $PI(3,4,5)P_3$ (Jiang et al., 1998). Third, the use of dominant negative mutant of PI 3-kinase inhibits the insulin-stimulated glucose uptake (Kotani et al., 1995; Sharma et al., 1998). Fourth, inactivation of certain protein phosphatases, which leads to an increase in the level of $PI(3,4,5)P_3$, results in stimulation of GLUT4 translocation and glucose uptake (Nakashima et al., 2000; Clement et al., 2001). Fifth, overexpression of wild-type or constitutively active form of PI 3-kinase is sufficient to induce the translocation of GLUT4 to plasma membrane (Katagiri et al., 1996; Frevert and Kahn, 1997; Martin et al., 1996; Asano et al., 2000). Downstream effectors of PI 3-kinase, Akt (Kohn et al., 1996; Cong et al., 1997) and PKCζ/λ (Bandyopadhyay et al., 1997b; Bandyopadhyay et al., 1997a), have both been shown to contribute to the insulin-stimulated GLUT4 translocation and glucose uptake.

During recent years, the existence of a second pathway to regulate GLUT4 translocation has been identified (Saltiel and Pessin, 2002). This PI 3-kinase independent pathway is located within caveolin-enriched lipid raft microdomains (Watson et al., 2004). In response to insulin, Cbl becomes tyrosine phosphorylated (Ribon and Saltiel, 1997). The association of Cbl to the β-subunit of the insulin receptor is mediated by APS and Cbl-associated protein (CAP) (Moodie et al., 1999; Ribon et al., 1998). Tyrosine phosphorylation of Cbl leads to the recruitment of the Cbl/CAP complex to the lipid rafts subdomain of plasma membrane (Baumann et al., 2000). The SH2 domain of CrkII mediates the binding of the CrkII/C3G complex to the phosphorylated Cbl in the lipid rafts (Ribon et al., 1996). Subsequently, C3G activates a small GTP-binding protein TC10 (Chiang et al., 2001). The TC10 activity has been associated with the redistribution of GLUT4 from intracellular vesicles to plasma membrane (Watson et al., 2001). However, there are conflicting data about the importance of CAP, Cbl and CrkII in the insulin-stimulated glucose uptake. These proteins can be deleted

using siRNA technology without compromising the insulin-stimulated glucose uptake (Mitra et al., 2004).

The substrates and mechanisms downstream of Akt, PKCζ/λ and TC10 leading to GLUT4 translocation and stimulation of glucose uptake in response of insulin are largely unknown (Watson et al., 2004). However, it has been shown that the remodeling of actin is essential for the insulin-stimulated GLUT4 translocation (Kanzaki and Pessin, 2001).

***Glycogen synthesis.*** Cellular glucose is stored as glycogen. Glycogen synthesis accounts for a major part of whole-body glucose uptake and almost all of the nonoxidative glucose metabolism (Shulman et al., 1990). In response to extracellular signals, glycogen synthesis and glycogenolysis are controlled by several kinases, phosphatases and allosteric regulation. High blood glucose level in the postprandial state stimulates glycogen synthesis while catabolic signals e.g. epinephrine, liberate glucose from glycogen for utilization in energy production (Alberts et al., 1994b). The crucial enzymes in glycogen synthesis and glycogenolysis are glycogen synthase (GS) and glycogen phosphorylase, respectively (Zubay et al., 1995a). The main glycogen containing tissues are skeletal muscle and liver (Zubay et al., 1995b). Insulin stimulates glycogen synthesis by activating GS (Cohen et al., 1978). Already in 1978, Cohen *et al.* suggested that inhibition of glycogen synthase kinase-3 (GSK3) would mediate the insulin-stimulated GS activity and subsequent stimulation of glycogen synthesis (Cohen et al., 1978). To date, two isoforms of GSK3 (GSK3α and β) have been identified and both of them are ubiquitously expressed (Woodgett, 1990). The PI 3-kinase/Akt pathway mediates the insulin-stimulated inhibition of GSK3 (Shepherd et al., 1995; Jiang et al., 2003; Hurel et al., 1996). Akt phosphorylates the N-terminal serine residues of GSK3 (Ser21 in GSK3α, Ser9 in GSK3β) (Cross et al., 1995). The phosphorylated N-terminus functions as a pseudosubstrate which competes with GS for binding to the C-terminal residues of GSK3 (arginine (Arg) 96, Arg180, lysine (Lys) 205, valine 214) leading to the dephosphorylation and activation of GS and subsequent stimulation of glycogen synthesis (Dajani et al., 2001; Frame et al., 2001). In the absence of insulin, these C-terminal residues of GSK3 interact with GS resulting in the phosphorylation and inactivation of GS and a consequental reduction in glycogen synthesis (Frame et al., 2001).

Protein phosphatase-1 (PP1) has a central role in the regulation of glycogen metabolism. PP1 is a Ser/Thr phosphatase which dephosphorylates and thus activates GS and simultaneously inactivates glycogen phosphorylase via dephosphorylation (Ragolia and

Begum, 1998). The phosphatase activity of PP1 is targeted to the glycogen-containing compartment of the cell by a regulatory subunit which is called the glycogen targeting subunit (Stralfors et al., 1985; Newgard et al., 2000). Insulin stimulates the phosphatase activity of PP1 *in vitro* by phosphorylating the glycogen targeting subunit and by promoting the binding of the catalytic subunit of PP1 to its regulatory subunit (Ragolia and Begum, 1998). Glycogenolytic hormones, e.g. epinephrine, induce dissociation of the catalytic and regulatory subunits which leads to inhibition of the phosphatase activity of PP1 and subsequent activation of the glycogen phosphorylase activity (Hubbard and Cohen, 1989). *In vivo* studies have provided convincing evidence of the important role of PP1 in the regulation of glycogen synthesis. Mice lacking the muscle-specific glycogen targeting subunit of PP1 exhibited a decreased glycogen content in muscle (Suzuki et al., 2001; Delibegovic et al., 2003) and the study performed by Delibegovic *et al.* further demonstrated a decreased GS activity after insulin stimulation and the development of obesity, glucose intolerance and insulin resistance in these mice (Delibegovic et al., 2003).

***Inhibition of gluconeogenesis.*** During starvation, the liver releases glucose into the bloodstream through gluconeogenesis. In the postprandial state, the glucose level in the bloodstream increases and gluconeogenesis in liver is suppressed by insulin (Barthel and Schmoll, 2003). Insulin inhibits gluconeogenesis by suppressing the expression of genes encoding the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) (Granner et al., 1983) and glucose-6-phosphatase (G-6-Pase) (Lange et al., 1994). PI 3-kinase has a central role in mediating the suppression of the gluconeogenic enzymes by insulin. Wortmannin and LY294002 abolish the suppression of the PEPCK (Agati et al., 1998) and G-6-Pase (Dickens et al., 1998) gene expression evoked by insulin. The use of dominant negative mutant of PI 3-kinase has a similar effect. Furthermore, overexpression of PI 3-kinase leads to the repression of the PEPCK and G-6-Pase gene expression (Miyake et al., 2002). Possible downstream effectors of PI 3-kinase are Akt and GSK3. Disruption of the Akt2 gene in mouse leads to insulin resistance and hyperglycemia due to the failure of insulin to suppress hepatic glucose production (Cho et al., 2001a) while disruption of Akt1 has no effect on glucose homeostasis (Cho et al., 2001b). Lithium chloride, a relatively specific inhibitor of GSK3, has been shown to suppress the expression of PEPCK and G-6-Pase (Lochhead et al., 2001).

Promoters of the PEPCK and G-6-Pase genes contain an insulin-responsive element (IRE) via which the effects of insulin on gene expression are mediated (O'Brien et al., 1990).

At the transcriptional level, a member of the forkhead transcription factor family, Foxo1, and peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1) have important roles in the suppression of the PEPCK and G-6-Pase gene expression. In starvation, Foxo1 binds to IRE and, in co-operation with PGC-1, induces expression of the PEPCK and G-6-Pase genes (Puigserver et al., 2003). However, insulin stimulation, probably through phosphorylation of Foxo1 by Akt, disrupts the transcriptional activity of PGC-1/Foxo1 complex, resulting in the repression of gluconeogenesis (Puigserver et al., 2003). In addition to Foxo1, other transcription factors including sterol response element-binding protein-1c (SREBP-1c) (Becard et al., 2001) and CCAAT/enhancer binding proteins (C/EBP) (Wang et al., 1995; Arizmendi et al., 1999) are thought to be involved in the regulation of gluconeogenesis.

***Lipogenesis.*** Excess nutritional carbohydrate and fatty acids are stored in the adipose tissue as triglycerides. Insulin promotes lipogenesis i.e. the formation of triglycerides by stimulating the expression of several lipogenic enzymes and by the inhibiting hormone-sensitive lipase (HSL) which is an important lipolytic enzyme (Lafontan et al., 1997). Stimulation of lipogenesis by insulin occurs to a large extent at the transcriptional level through transcription factor SREBP-1 (Shimano, 2001). The mammalian genome contains three isoforms of SREBPs, SREBP-1a, SREBP-1c, and SREBP-2 (Horton et al., 2002). One gene encodes both SREBP-1a and SREBP-1c (Yokoyama et al., 1993). SREBP isoforms enhance fatty acid and triglyceride synthesis (SREBP-1a, -1c) and cholesterol synthesis (SREBP-2) (Shimano, 2001). SREBP isoforms are produced as precursor proteins that are bound to the cytoplasmic membrane. SREBPs are activated via a proteolytic processing after which SREBPs are translocated into the nucleus where they enhance the transcription of more than 30 genes by binding to the sterol response element in the promoter of the target gene (Horton et al., 2002). Insulin induces expression of SREBP-1 (Kim et al., 1998; Fleischmann and Iynedjian, 2000; Guillet-Deniau et al., 2002) through the PI 3-kinase/Akt pathway (Fleischmann and Iynedjian, 2000; Nadeau et al., 2004) and the MAPK pathway (Nadeau et al., 2004). Also the elevated glucose level stimulates SREBP-1 expression (Hasty et al., 2000). SREBP-1c induces the expression of several lipogenic enzymes including ATP-citrate lyase (Sato et al., 2000), acetyl-CoA carboxylase (Magana et al., 1997), fatty acid synthase (Magana and Osborne, 1996), malic enzyme (Shimano et al., 1999) and glycerol-3-phosphate acyltransferase (Ericsson et al., 1997). In addition to regulating the expression of lipogenic enzymes, insulin controls the phosphorylation of lipogenic enzymes e.g. ATP-citrate lyase through the PI 3-kinase/Akt pathway (Hill et al., 2000; Berwick et al., 2002).

In adipocytes, catecholamines induce lipolysis by binding to β-adrenergic receptors, which results in an elevation in the cellular cAMP level (Lafontan et al., 1997). This leads to the activation of protein kinase A (PKA) and subsequent phosphorylation and stimulation of HSL and perilipin (Holm, 2003). The ability of insulin to antagonize lipolysis is mainly accounted for its ability to reduce the cellular cAMP level via phosphodiesterase 3B (Elks and Manganiello, 1985). This lowers PKA activity, HSL phosphorylation and finally, lipolysis (Holm, 2003).

***Protein synthesis***. Protein synthesis is crucial to cell growth and maintenance (Zubay et al., 1995c). Insulin promotes protein synthesis by stimulating multiple pathways leading to increased biosynthesis of cellular proteins (Proud and Denton, 1997). First, insulin stimulates the phosphorylation and activation of the p70 ribosomal protein S6 kinase (p70S6k) in a PI 3-kinase dependent manner (Chung et al., 1994). Downstream effectors of PI 3-kinase in the activation of p70S6k include PDK1 (Pullen et al., 1998) Akt and mammalian target of rapamycin (mTOR) (Chung et al., 1994; Nave et al., 1999). Activated p70S6k phosphorylates the 40S ribosomal protein S6 and thereby facilitates translation of a subset of mRNAs containing a 5'-terminal oligo-pyrimidine tract. These mRNAs encode ribosomal proteins and translational elongation factors. Thus, the activation of p70S6k increases the synthesis of many proteins required in the cellular protein synthesis machinery (Dufner and Thomas, 1999).

Second, insulin stimulates the action of the eukaryotic initiation factor (eIF) 4E and eIF4E-binding protein (E4-BP) (Proud and Denton, 1997). eIF4E has a central role in the initiation of mRNA translation as it interacts with mRNA molecules recruiting them to the ribosome (Rhoads, 1993). In quiescent cells, eIF4E is bound to E4-BP and the complex is translationally inactive (Proud and Denton, 1997). After insulin stimulation, both factors become phosphorylated in a PI 3-kinase dependent manner (Mendez et al., 1996). Phosphorylation leads to the dissociation of the eIF4E/E4-BP complex, the stimulation of eIF4E affinity towards mRNA and finally, to the stimulation of protein synthesis (Whiteman et al., 2002). Downstream effectors of PI 3-kinase in the phosphorylation of eIF4E and E4-BP are Akt (Nave et al., 1999) and mTOR (Mendez et al., 1996; Burnett et al., 1998).

Third, insulin regulates general protein synthesis through the guanine nucleotide exchange factor eIF2B which has a crucial role in recruiting the initiator transfer-RNA containing methionine to the ribosome (Proud and Denton, 1997). In quiescent cells, the function of eIF2B is repressed by phosphorylation via GSK3 (Welsh and Proud, 1993).

Insulin stimulation leads to the inactivation of GSK3 through the PI 3-kinase/Akt pathway, dephosphorylation and activation of eIF2B and subsequent stimulation of the general protein synthesis (Frame and Cohen, 2001). Activation of eIF2B might also involve PKC (Mendez et al., 1997).

Fourth, in addition to stimulation of the initiation of protein synthesis, insulin also promotes the elongation step of protein synthesis by phosphorylating the eukaryotic elongation factor F2 (Proud and Denton, 1997).

### 2.2.5 Other effects

***Mitogenesis and survival.*** Mitogenic effects of insulin are mediated through the MAPK signalling cascade. When compared to other growth factors, insulin has a relatively weak mitogenic effect (Virkamaki et al., 1999).

Insulin possesses a potential anti-apoptotic effect which is mediated by the PI 3-kinase/Akt pathway (Shepherd et al., 1998). In response to insulin, Akt phosphorylates and inhibits several proteins that mediate apoptosis (Lawlor and Alessi, 2001). Under pro-apoptotic conditions, BAD (Bcl-2/Bcl-$X_L$-antagonist, causing cell death) forms a heterodimer with anti-apoptotic Bcl-2 and Bcl-$X_L$ proteins and thus abolishes their survival-promoting action (Yang et al., 1995). In response to insulin and some other survival factors, Akt phosphorylates BAD resulting in its cytosolic sequestration, inhibition of the heterodimer formation with Bcl-2 or Bcl-$X_L$ and ultimately, inhibition of apoptosis (Datta et al., 1997). In addition, insulin affects the function of caspase proteases which are important enzymes in the apoptosis (Lawlor and Alessi, 2001). Akt phosphorylates caspase-9, inhibiting its protease activity (Cardone et al., 1998).

Insulin protects pancreatic β-cells from oxidative stress-induced apoptosis (Maeda et al., 2004). IRS-2 and its downstream effector, Akt, have a crucial role in mediating the β-cell survival (Withers et al., 1998; Lingohr et al., 2003). Similarly, insulin protects cardiomyocytes against oxidative stress (Aikawa et al., 2000) and interestingly, insulin has been reported to reduce the size of a myocardial infarction in rat heart *in vivo* via a mechanism involving Akt and BAD (Jonassen et al., 2001). In endothelial cells, insulin antagonized the apoptotic effect of TNFα by phosphorylation of caspase-9 (Hermann et al., 2000). In addition, insulin activates nitric oxide synthase in endothelial cells by the PI 3-kinase/Akt pathway and thereby promotes angiogenesis (Lawlor and Alessi, 2001). The

increased supply of nutrients and oxygen in tumor cells is reported to promote cellular survival (Snyder and Jaffrey, 1999).

## 2.3 Phosphatidylinositol 3-kinase

PI 3-kinase activity was purified for the first time in 1990 by Carpenter *et al.* (Carpenter et al., 1990). Eucaryotes possess several isoforms of PI 3-kinase. The isoforms are divided into three classes (I - III) on the basis of the structure, regulation and substrate specificity (Table 1) (Vanhaesebroeck et al., 1997a).

**Table 1.** Phosphatidylinositol 3-kinase family in mammals

| Class I | | | | Class II | Class III | |
|---|---|---|---|---|---|---|
| Catalytic | | Regulatory | | | Catalytic | Regulatory |
| A | B | A | B | | | |
| p110α, β, δ | p110γ | p85α, β, p55γ | p101 | PI 3-kinase C2α, β , γ | Vps34p | p150 |

Table modified from (Vanhaesebroeck et al., 2001)

### 2.3.1 Class I

Class I PI 3-kinases are heterodimeric proteins consisting of a 110-kilodalton (kDa) catalytic subunit, p110, and a regulatory subunit which is around 50-100 kDa in size (Carpenter et al., 1990). Class I PI 3-kinases are able to phosphorylate PI, PI(4)P and PI(4,5)$P_2$ in *in vitro* conditions (Whitman et al., 1988; Auger et al., 1989). However, it seems that in intact cells, the preferred substrate of Class I PI 3-kinases is PI(4,5)$P_2$ which is phosphorylated into PI(3,4,5)$P_3$ (Stephens et al., 1991). PI 3-kinases in Class I participate in the fast-acting signalling pathways which are activated by various extracellular signals (Vanhaesebroeck et al., 2001) (Table 2). In unstimulated cells, Class I PI 3-kinases are mainly cytosolic but upon stimulation, PI 3-kinase is recruited to the plasma membrane where its substrates reside (Backer et al., 1992; Brock et al., 2003). Class I PI 3-kinases possess a dual kinase activity. In addition to lipid kinase activity, they have an intrinsic protein kinase activity (Dhand et al., 1994b). Class I is further divided into two subgroups, A and B, based on the differences in the lipid kinase activation process (Table 1) (Vanhaesebroeck et al., 1997a).

**Table 2.** Factors that mediate their effects through PI 3-kinase

| | Activator | Reference | Activator | Reference |
|---|---|---|---|---|
| Hormones | insulin* | (Ruderman et al., 1990) | TSH | (Bell et al., 2002) |
| | leptin | (Cohen et al., 1996) | PTH | (Gentili et al., 2002) |
| | GH | (Ridderstrale et al., 1995) | estradiol | (Richards et al., 1998) |
| | prolactin | (al Sakkaf et al., 1996) | testosterone | (Sharma et al., 2002) |
| | LH | (Carvalho et al., 2003) | aldosterone | (Blazer-Yost et al., 1999) |
| | FSH | (Park et al., 2004) | gastrin | (Ferrand et al., 2004) |
| Growth factors | PDGF* | (Auger et al., 1989) | bFGF | (Raffioni and Bradshaw, 1992) |
| | VEGF | (Guo et al., 1995) | NGF | (Carter and Downes, 1992) |
| | PlGF | (Cai et al., 2003) | erythropoietin | (Miura et al., 1994) |
| | IGF-1 | (Yamamoto et al., 1992) | angiopoietin-1 | (Fujikawa et al., 1999) |
| | EGF | (Carter and Downes, 1992) | TGFα, β | (Sivaprasad et al., 2004) |
| | HGF | (Graziani et al., 1991) | | (Bakin et al., 2000) |
| Platelet activation | vWf | (Jackson et al., 1994) | collagen | (Pasquet et al., 1999) |
| | thrombin | (Gutkind et al., 1990) | fibrinogen | (Zhang et al., 1998) |
| Cytokines, chemokines, inflammation | IL-1 | (Reddy et al., 1997) | INFα, β | (Yang et al., 2001) |
| | IL-2 | (Remillard et al., 1991) | INFγ | (Nguyen et al., 2001) |
| | IL-3 | (Gold et al., 1994) | PAF | (Stephens et al., 1993) |
| | IL-4 | (Gold et al., 1994) | CSFs (1-3)* | (Varticovski et al., 1989) |
| | IL-5 | (Gold et al., 1994) | | (Gold et al., 1994) |
| | IL-6 | (Chen et al., 1999) | | (Hunter and Avalos, 1998) |
| | IL-7 | (Dadi et al., 1993) | MCPs (1-4) | (Turner et al., 1998) |
| | IL-8 | (Knall et al., 1997) | | (Wain et al., 2002) |
| | IL-9 | (Demoulin et al., 2000) | antigen + TcR | (Carrera et al., 1994) |
| | IL-10 | (Crawley et al., 1996) | antigen + CD28 | (Ueda et al., 1995) |
| | IL-11 | (Fuhrer and Yang, 1996) | antigen + BcR | (Gold and Aebersold, 1994) |
| | IL-12 | (Yoo et al., 2002) | antigen + IgE | (Laffargue et al., 2002) |
| | IL-13 | (Dubois et al., 1998) | | |
| | IL-15 | (Yano et al., 2003) | | |
| | IL-18 | (Morel et al., 2001) | | |
| Other factors | cell-cell interaction | (Pece et al., 1999) | cell-matrix interaction | (Khwaja et al., 1997) |
| | NmU | (Johnson et al., 2004) | | |

*Participation of p110β in signal transduction has been demonstrated
Abbreviations used: BcR, B cell receptor; bFGF, basic fibroblast growth factor; FSH, follicle stimulating hormone; CSF, colony-stimulating factor; GH, growth hormone; EFG, epidermal growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; IL, interleukin; INF, interferon; LH, luteinizing hormone; MCP, monocyte chemotactic protein; NmU, neuromedin U; NGF, nerve growth factor; PAF, platelet activating factor; PDGF, platelet-derived growth factor; PlGF, placenta growth factor; PTH, parathyroid hormone; TcR, T cell receptor; TGF, transforming growth factor; TSH, thyroid stimulating hormone; VEGF, vascular endothelial growth factor; vWf, von Willebrand factor

***Class IA.*** The Class IA contains three isoforms of the catalytic subunit, p110α, p110β and p110δ (Table 1, Table 3, references therein) which are encoded by three separate genes. Similarly, three genes encode the regulatory subunits. The p85α gene can generate three proteins through alternative splicing. These are entitled p85α, p55α and p50α (Table 3,

references therein). Of these proteins, p85α and p50α are the most abundantly expressed in human skeletal muscle and adipose tissue (Lefai et al., 2001). The p85β and p55γ/p55$^{PIK}$ genes encode each one protein, called p85β and p55γ/p55$^{PIK}$, respectively (Table 1, Table 3, references therein). In unstimulated cells, p85α stabilizes the catalytic subunit and inhibits its lipid kinase activity (Yu et al., 1998). Class IA PI 3-kinases are acutely activated by receptor tyrosine kinases of e.g. insulin, platelet-derived growth factor (PDGF) and vascular endothelial growth factor receptors (Ruderman et al., 1990; Auger et al., 1989; Guo et al., 1995) (Table 2). SH2 domains of the regulatory subunit bind to the tyrosine phosphorylated YXXM-motifs of the activated receptors or receptor-associated docking proteins e.g. IRS and cbl (Backer et al., 1992; Soltoff and Cantley, 1996; Songyang et al., 1993). This interaction is followed by an increase in the lipid kinase activity of PI 3-kinase (Backer et al., 1992; Shoelson et al., 1993).

**Table 3.** Identified subunits of Class I PI 3-kinases in different organisms

| Catalytic | | | Regulatory | | |
|---|---|---|---|---|---|
| Protein | Organism | Reference | Protein | Organism | Reference |
| p110α | Homo sapiens | (Volinia et al., 1994) | p85α | Homo sapiens | (Skolnik et al., 1991) |
| | Bos taurus | (Hiles et al., 1992) | | Bos Taurus | (Otsu et al., 1991) |
| | Mus musculus | (Klippel et al., 1994) | | Mus musculus | (Escobedo et al., 1991) |
| | Gallus gallus | (Chang et al., 1997) | | Rattus norvegicus | (Inukai et al., 1996) |
| | Rattus norvegicus | AF395897* | p55α | Homo sapiens | (Antonetti et al., 1996) |
| p110β | Homo sapiens | (Hu et al., 1993) | | Rattus norvegicus | (Inukai et al., 1996) |
| | Rattus norvegicus | AJ012482 | p50α | Mus musculus | (Fruman et al., 1996) |
| | | NM_053481* | | Rattus norvegicus | (Fruman et al., 1996) |
| | Mus musculus | AK090116 | | | (Inukai et al., 1997) |
| | | NM_029094* | p85β | Homo sapiens | (Janssen et al., 1998) |
| p110δ | Homo sapiens | (Vanhaesebroeck et al., 1997b) | | Mus musculus | BC006796* |
| | Mus musculus | (Chantry et al., 1997) | | Rattus norvegicus | (Inukai et al., 1996) |
| | Rattus norvegicus | XM_345606* | | Bos taurus | (Otsu et al., 1991) |
| p110γ | Homo Sapiens | (Stoyanov et al., 1995) | p55γ | Homo sapiens | (Dey et al., 1998) |
| | Sus scrofa | (Stephens et al., 1997) | | Rattus norvegicus | (Inukai et al., 1996) |
| | Mus musculus | (Hirsch et al., 2000) | | Mus musculus | (Pons et al., 1995) |
| | Rattus norvegicus | XM_234053* | p101 | Homo sapiens | AF128881* |
| | | | | Sus scrofa | (Stephens et al., 1997) |
| | | | | Mus musculus | AY156924* |

*Accession number for the Entrez Nucleotides database of National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/)

Many observations suggest that p110α and p110β have distinct roles in the cell. First, gene disruption studies provide important information about the unique roles of p110α and p110β in the cell. The lack of functional p110α (Bi et al., 1999) or p110β (Bi et al., 2002) protein in mice results in death during embryogenesis. This indicates that the preserved isoform cannot compensate for the missing isoform. Second, in addition to receptor tyrosine kinases, the lipid kinase activity of p110β is activated by the Gβγ subunit of the heterotrimeric G protein (Kurosu et al., 1997). Acting separately, the stimulating capacity of receptor tyrosine kinase and Gβγ is approximately the same whereas costimulation of p110β/p85α with receptor tyrosine kinase and Gβγ results in a significant synergistic effect (Maier et al., 1999). Third, the lipid kinase activities of p110α and p110β are reported to be different. At high substrate concentrations, p110α is the more efficacious lipid kinase while at low concentration of PI lipids, the lipid kinase activity of p110β becomes more effective (Beeton et al., 2000). Fourth, also the protein kinase activities of p110α and p110β are thought to be different. The intrinsic protein kinase activity of p110α is directed towards p85 (Ser608) while p110β is preferentially autophosphorylated (Ser1070) (Foukas et al., 2004; Czupalla et al., 2003). Phosphorylation of p85 by p110α results in decreased lipid kinase activity of p110α (Dhand et al., 1994b). It is not known how the autophosphorylation of p110β affects the lipid kinase activity of the p110β/p85 heterodimer.

***Class 1B.*** The Class IB contains one isoform of the catalytic subunit, p110γ and one regulatory subunit, p101 (Table 1, Table 3, references therein). There is a conflicting data on the tissue distribution of p110γ. Stoyanov *et al.* demonstrated the presence of p110γ mRNA in various tissues while some reports claim that it has a more restricted expression (Stoyanov et al., 1995; Vanhaesebroeck et al., 2001). The activity of Class 1B PI 3-kinase is not associated with receptor tyrosine kinases. The kinase activities of p110γ/p101 are stimulated by G protein coupled receptors (GPCRs) (Stoyanov et al., 1995). Following the stimulation of GPCR, p110γ/p101 translocates from cytosol to plasma membrane and binds to the Gβγ subunit of the G protein. This interaction stimulates the lipid and protein kinase activities of p110γ/p101 (Brock et al., 2003). The protein kinase activity of p110γ results in autophosphorylation (Ser1101) and phosphorylation of p101 (Stoyanova et al., 1997; Czupalla et al., 2003; Bondev et al., 1999). Gβγ is also able to bind and stimulate the lipid kinase activity of p110γ in the absence of p101 (Leopoldt et al., 1998). However, this does not

lead to the accumulation of p110γ activity in plasma membrane (Brock et al., 2003). Thus, it seems that p101 functions as a targeting molecule to localize p110γ activity to plasma membrane. In addition, the presence of p101 significantly increases autophosphorylation of p110γ (Maier et al., 1999). In the absence of p101, autophosphorylation of p110γ does not significantly impair the lipid kinase activity of p110γ (Bondev et al., 1999). However, it is not known how the phosphorylation of p110γ/p101, as a result of intrinsic protein kinase activity, affects the lipid kinase activity.

### 2.3.2 Class II

Class II contains three isoforms, PI 3-kinase C2α, C2β and C2γ (Domin et al., 1997; Arcaro et al., 1998; Misawa et al., 1998) (Table 1). Proteins in Class II are larger than the other PI 3-kinases, being approximately 180 kDa in size (Arcaro et al., 2000). PI 3-kinase C2α and C2β are ubiquitously expressed while the expression of PI 3-kinase C2γ is restricted to hepatocytes. Class II PI 3-kinases are thought to be monomeric proteins. *In vitro*, they prefer to utilize PI and PI(4)P as substrates (Domin et al., 1997; Arcaro et al., 1998; Misawa et al., 1998) but the substrate specificity *in vivo* has not yet been determined. Class II PI 3-kinases are characterized by a C-terminal C2 domain. The detailed function of the C2 domain is unknown. However, it is possible that the C2 domain participates in the regulation of the lipid kinase activity since the deletion of the C2 domain results in increased lipid kinase activity (Arcaro et al., 1998). In resting cells, the subcellular location of C2-deleted PI 3-kinase C2β mutants is similar to that of the full length protein (Arcaro et al., 1998). Thus, it could be suspected that the C2 domain does not define the subcellular localization Class II PI 3-kinases in unstimulated cells. The role of Class II PI 3-kinases in cellular processes is poorly understood. However, it has been shown that *in vitro* Class II PI 3-kinases participate in the signal transduction of certain growth factors (epidermal growth factor (EGF) and PDGF), insulin (Brown et al., 1999; Arcaro et al., 2000), leptin, TNFα (Ktori et al., 2003) and monocyte chemotactic protein-1 (Turner et al., 1998). Studies in fruit flies have provided the first evidence about the function of Class II PI 3-kinases *in vivo*. Fruit flies lacking the functional Class II PI 3-kinase (PI 3-kinase_68D) show developmental disturbances, due to disrupted EGF signal transduction (MacDougall et al., 2004). This indicates that the Class II PI 3-kinases play an important role, at least in EGF signal transduction *in vivo*.

### 2.3.3 Class III

Class III PI 3-kinase is a complex of the vesicular protein sorting (Vps) 34p protein which acts as a catalytic subunit and the protein kinase p150 (in mammals, Vps15p in yeasts) as the regulatory subunit (Table 1) (Stack and Emr, 1994; Volinia et al., 1995). The sizes of Vps34p and p150 proteins are 100 kDa and 150 kDa, respectively (Volinia et al., 1995). Similar to catalytic subunits in Class I and Class II, Vps34p is a dual kinase possessing both protein and lipid kinase activities. As a result of the intrinsic protein kinase activity, Vps34p undergoes predominantly serine autophosphorylation (Stack and Emr, 1994). Subunits of Class III PI 3-kinases are highly preserved during evolution and human proteins show significant homology to yeast Vps34p and Vps15p (Volinia et al., 1995; Panaretou et al., 1997). Both subunits are ubiquitously expressed (Volinia et al., 1995; Panaretou et al., 1997). In contrast to the other PI 3-kinase classes, the Vps34p/p150 complex utilizes exclusively PI as its substrate, leading to the formation of PI(3)P (Volinia et al., 1995). PI(3)P is the most abundant 3'-PI-lipid in the cell and cellular PI(3)P level is not affected by extracellular stimuli (Vanhaesebroeck et al., 2001). All these above observations support the proposal that the Vps34p/p150 complex has a fundamental housekeeping function in the cell. Indeed, Class III PI 3-kinase and its lipid product PI(3)P have been shown to have specific roles in intracellular trafficking in the endosomes (Roth, 2004). In yeasts, Vps15p is attached to Golgi or endosomal membrane and activated by autophosphorylation. This leads to the formation of the Vps15p/Vps34p complex and subsequent activation of the lipid kinase activity of Vps34p. The formation of PI(3)P is recognized by downstream effectors participating in the membrane traffic signalling (Stack et al., 1993; Stenmark, 2000). In mammals, the Vps34p/p150 complex is assumed to function in a similar manner.

### 2.3.4 Structure of Class I phosphatidylinositol 3-kinases

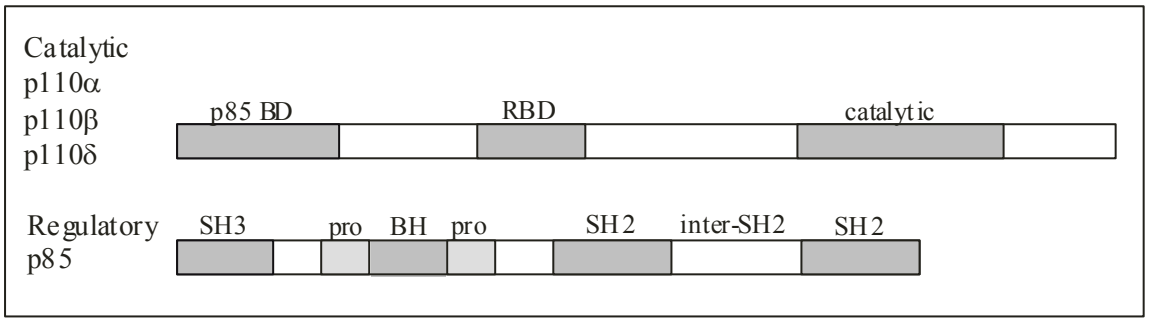


**Figure 3.** Structure of Class IA PI 3-kinases. Abbreviations in the figure are summarized in Table 4. Picture modified from (Stephens et al., 2000).

**Table 4.** Domains of the subunits of Class IA PI 3-kinases

| Protein | Domain | Definition | Function | Reference |
|---|---|---|---|---|
| p110 | p85 BD | p85-binding domain | heterodimerization, increase in kinase activity | (Klippel et al., 1994) |
| | RBD | Ras-binding domain | activation of lipid kinase activity *in vitro*, significance *in vivo* unclear | (Rodriguez-Viciana et al., 1996)<br>(Vanhaesebroeck et al., 2001) |
| | catalytic | domain containing kinase activity | substrate binding<br>ATP-binding | (Walker et al., 1999) |
| p85 | SH3 | Src homology 3 domain | binds to proline-rich proteins, mediates signal transduction | (Soltoff and Cantley, 1996)<br>(Harrison-Findik et al., 2001) |
| | pro | proline-rich domain | binds to proteins containing SH3 domain, mediates signal transduction | (Wu et al., 2003)<br>(Yuan et al., 1997) |
| | BH | breakpoint cluster region-homology domain | possibly binds to Ras | (Musacchio et al., 1996) |
| | SH2 | Src homology 2 domain | binds to tyrosine phosphorylated proteins, mediates signal transduction | (Backer et al., 1992) |
| | inter-SH2 | region between SH2 domains | heterodimerization, increase in kinase activity | (Klippel et al., 1994)<br>(Dhand et al., 1994a) |

### 2.3.5 Inhibitors of phosphatidylinositol 3-kinase

Wortmannin and LY249002 are structurally unrelated, cell-permeable compounds that are widely used PI 3-kinase inhibitors (Davies et al., 2000). Wortmannin is a fungal metabolite with an *in vitro* 50% inhibitory concentration ($IC_{50}$) of around 5 nM (Vanhaesebroeck et al., 2001). Inhibition of PI 3-kinase activity is mediated by a covalent interaction of wortmannin and the ATP-binding site (Lys802) of the catalytic domain of p110α (Wymann et al., 1996). LY294002 is a flavonoid-based synthetic compound with an $IC_{50}$ value of approximately 1 μM (Vlahos et al., 1994). It inhibits PI 3-kinase activity by interfering the binding of ATP to the catalytic domain of p110 (Walker et al., 2000). Wortmannin and LY294002 inhibit Class I, II and III PI 3-kinases with a similar potency with the exception that Class II PI 3-kinase C2α is at least 10-fold less sensitive to the inhibitory effect of wortmannin and LY294002 (Virbasius et al., 1996; Domin et al., 1997; Vanhaesebroeck et al., 2001).

### 2.3.6 Phosphatidylinositol 3-kinase and type 2 diabetes

The potential role of PI 3-kinase in the development of type 2 diabetes has been elucidated by creating knockout animals. Suprisingly, mice lacking the regulatory subunit p85α were hypoglycemic due to increased insulin sensitivity (Terauchi et al., 1999) and further, in an

insulin resistant mouse model, reduction of p85α expression by 50% increased insulin sensitivity and decreased the incidence of type 2 diabetes by 50% (Mauvais-Jarvis et al., 2002). Mice lacking p85β (Ueki et al., 2002) or p55α and p50α (Chen et al., 2004) show enhanced insulin sensitivity. However, the deletion of all splice variants of p85α leads to death during the perinatal period (Fruman et al., 2000). Similarly, the deletion of either p110α or p110β is lethal (Bi et al., 1999; Bi et al., 2002). Thus, knockout technology is not a suitable alternative if one wishes to investigate the role of the catalytic subunits of PI 3-kinase in the pathophysiology of type 2 diabetes. Gene silencing by RNA interference provides a promising method to specifically shut down the expression of a target gene (Hannon and Rossi, 2004). This technology has been utilized to investigate the PI 3-kinase pathway but not in the context of insulin signal transduction (Czauderna et al., 2003).

Several clinical trials have clarified the contribution of PI 3-kinase and other signalling molecules that mediate the effects of insulin in the pathophysiology of type 2 diabetes. These studies demonstrate that in skeletal muscle, IRS-1 and IRS-2 associated PI 3-kinase activity is decreased in type 2 diabetic subjects compared to lean control subjects (Bjornholm et al., 1997; Kim et al., 1999; Beeson et al., 2003; Kim et al., 2003). In addition, insulin-stimulated tyrosine phosphorylation of IRS-1, the activities of PKCλ/ζ and glycogen synthase and glucose uptake are all impaired in muscle biopsies of type 2 diabetics (Bjornholm et al., 1997; Kim et al., 1999; Beeson et al., 2003; Kim et al., 2003). Interestingly, there is no difference in PDK1 or Akt activity between type 2 diabetic and control subjects (Krook et al., 1998; Beeson et al., 2003; Kim et al., 2003). In skeletal muscle of type 2 diabetic subjects, the expression of IRS-1, p85α, Akt, PDK1 and GLUT4 is not changed (Bjornholm et al., 1997; Kim et al., 1999; Krook et al., 1998; Kim et al., 2002; Beeson et al., 2003; Kim et al., 2003). However, PKCλ/ζ represents an exception, because the expression of PKCζ is decreased in type 2 diabetic subjects (Beeson et al., 2003; Kim et al., 2003)

Similar but milder defects in IRS-1 and IRS-2 associated PI 3-kinase activity have been detected in muscle biopsies of obese non-diabetic subjects (Kim et al., 1999). Body weight reduction increased the insulin-stimulated IRS-1 tyrosine phosphorylation, IRS-1 associated PI 3-kinase activity and PKCλ/ζ activity (Kim et al., 2003). In addition, treatment with the thiazolidinediones, troglitazone or rosiglitazone, has been reported to restore IRS-1 associated PI 3-kinase (Kim et al., 2002; Beeson et al., 2003) and aPKC activity (Farese, 2002). Furthermore, troglitazone increases the expression of p110β (Kim et al., 2002).

**Table 5.** Genes encoding the major insulin signalling proteins as candidate genes for type 2 diabetes

| Gene | Polymorphism | Population | n (T2D/control) | Association* (+/-) | Reference |
|---|---|---|---|---|---|
| IRS-1 | Gly971Arg | Caucasian | 86/76 | - | (Almind et al., 1993) |
| | Ala513Pro | | | - | |
| | Gly971Arg | Caucasian | 112/104 | - | (Laakso et al., 1994) |
| | Gly818Arg | | | - | |
| | Ser892Gly | | | - | |
| | Gly971Arg | Caucasian | 233/130 | - | (Hager et al., 1993) |
| | Ala513Pro | | | - | |
| | Gly971Arg | Asian | 197/178 | - | (Shimokawa et al., 1994) |
| | Gly971Arg | Caucasian, Asian | 597/447 | + | (Hitman et al., 1995) |
| | Gly971Arg | Asian | 100/70 | - | (Ura et al., 1996) |
| | Pro170Arg | | 47/47 | - | |
| | Met209Thr | | | - | |
| | Ser809Phe | | | - | |
| | Gly971Arg | Caucasian | 49/164 | + | (Zhang et al., 1996) |
| | Ala513Pro | | | - | |
| | Gly971Arg | Caucasian | 725/742 | - | (van Dam et al., 2004) |
| IRS-2 | Gly879Ser | Caucasian | 252/267 | - | (Bernal et al., 1998) |
| | Gly1057Asp | | | - | |
| | Gly1057Asp | Caucasian | 85/82 | - | (Wang et al., 2001) |
| | | Asian | 100/85 | - | |
| | Gly1057Asp | Caucasian | 186/240 | - | (D'Alfonso et al., 2003) |
| | Gly1057Asp | Pima Indians | cohort of 998 | + | (Stefan et al., 2003) |
| IRS-4 | Leu34Phe | Caucasian | 324/267 | - | (Almind et al., 1998) |
| | Arg411Gly | | | - | |
| | His879Asp | | | - | |
| p85α | Met328Ile | Caucsian | 404/224 | - | (Hansen et al., 1997) |
| | Met328Ile | Asian | 200/260 | - | (Kawanishi et al., 1997) |
| | Met328Ile | Pima Indians | cohort of 950 | - | (Baier et al., 1998) |
| p110α | ND** | | | | |
| PTEN | G → T in intron | Caucasian | 379/224 | - | (Hansen et al., 2001) |
| | C → G in 5'UTR | Asian | 107/100 | + | (Ishihara et al., 2003) |
| PKCζ | G → A in intron 5 | Asian | 192/172 | + | (Li et al., 2003) |

*Association of the polymorphism with type 2 diabetes +, $p<0.05$; -, $p>0.05$

**p110α gene has not been studied as a candidate gene for type 2 diabetes

Abbreviations used: Ala, alanine; Arg, arginine; Asp, aspartic acid; Gly, glycine; IRS, insulin receptor substrate; His, histidine; Ile, isoleucine; Leu, leucine; Met, methionine; ND, not determined; Phe, phenylalanine; PKC, protein kinase C; Pro, proline; PTEN, phosphatase and tensin homolog deleted on chromosome 10; Ser, serine; T2D, type 2 diabetes; Thr, threonine; UTR, untranslated region

## 2.4 Candidate gene studies

Candidate gene approach can be used in studies investigating the genetic background of type 2 diabetes. Genes that encode proteins having an important role in mediating the effects of insulin are potential candidate genes for insulin resistance and type 2 diabetes. Numerous candidate genes have been screened but no major gene defects causing insulin resistance or

type 2 diabetes have been identified (Elbein, 2002). Candidate genes are screened using the single-strand conformation polymorphism (SSCP) analysis (Orita et al., 1989). Several genes that encode proteins participating in insulin signalling cascade have also been studied as susceptibility genes for type 2 diabetes. The major polymorphisms and their association with type 2 diabetes in selected studies are shown in Table 5.

## 2.5 3T3-L1 cells and recombinant adenoviruses as tools in studies of type 2 diabetes

### 2.5.1 3T3-L1 cell line

The 3T3-L1 cell line was established as a clonal subline from the mouse fibroblasts cell line, 3T3. In 1974, Green *et al.* observed that a portion of 3T3 cells were spontaneously able to accumulate cytoplasmic lipid and to differentiate into adipocytes (Green and Kehinde, 1974). The differentiation process of 3T3-L1 cells is characterized by increased triglyceride synthesis (Green and Kehinde, 1975) and coordinated activation of several lipogenic enzymes, i.e. ATP-citrate lyase, acetyl-CoA carboxylase, fatty acid synthase (Mackall et al., 1976), pyryvate carboxylase (Mackall and Lane, 1977), malic enzyme (Wise et al., 1984) and lipoprotein lipase (Wise and Green, 1978). At the transcriptional level, the differentiation process is regulated by the members of C/EBP family (Lane et al., 1999), SREBPs (Fajas et al., 1999) and peroxisome proliferator-activated receptor-γ (Lowell, 1999). The accumulation of triglyceride droplets is inhibited by lipolytic agents e.g. epinephrine (Green and Kehinde, 1974). The first experiments were performed with spontaneously differentiated 3T3-L1 cells but it was soon discovered that several agents i.e. insulin (Green and Kehinde, 1975), dexamethasone (Rubin et al., 1978), 3-isobutyl-1-methylxanthine (IBMX), prostaglandin $F_{2\alpha}$ (Russell and Ho, 1976), serum (Green and Meuth, 1974), biotin (Mackall et al., 1976) and indomethacin (Williams and Polakis, 1977) facilitated the differentiation process. Nowadays, 3T3-L1 cells are routinely differentiated using a cocktail of agents that promote the differentiation process. The cocktail contains insulin, IBMX, dexamethasone and serum (Rubin et al., 1978; Student et al., 1980).

During the course of differentiation, the expression of many genes in 3T3-L1 cells is altered. The expression of insulin receptor is upregulated by 35-fold with a concomitant increase in the affinity of the receptor towards insulin (Rubin et al., 1978). Similarly, the expression of GLUT4, p110β and C/EBPα is upregulated (Asano et al., 2000).

Adipose tissue is one of the major target tissues of insulin. It is also becoming evident that adipose tissue has a central role in the development of insulin resistance and type 2

diabetes (Bays et al., 2004). Therefore, 3T3-L1 adipocytes are a widely used cellular model to investigate the insulin signalling pathways *in vitro*. In addition, differentiating 3T3-L1 cells can be utilized to investigate adipogenesis (Lane et al., 1999).

### 2.5.2 Adenoviruses

***Classification.*** Adenoviruses compose the *Adenoviridae* family of viruses which contains two genera, *Aviadenovirus* and *Mastadenovirus*. *Aviadenovirus* genus includes exclusively viruses of birds while *Mastadenovirus* genus contains viruses of different species e.g. human, bovine and equine (Shenk, 2001). To date, at least 51 human adenoviral serotypes have been identified (De Jong et al., 1999). These have been divided into six subtypes (A – F) based on their hemagglutination properties (Shenk, 2001).

***Structure.*** Adenoviruses are icosahedral particles that are 70-100 nm in diameter. The core of the viral particle contains a linear, double-stranded DNA genome and certain structural proteins. The genome is 36 kilobase (kb) in length and in the literature it has been divided into 100 map units (mu). The genome is organized into five early (E1a, E1b, E2, E3, E4), two delayed early (IX, IVa2) and one major late transcription unit which gives rise to five families of late mRNAs (L1 - L5). The viral core is enclosed in a protein shell called the capsid which is composed of 240 hexons and 12 pentons. These are the most abundant structural proteins present in the capsid. One penton protein is located at each vertex of the viral icosahedron. The penton is composed of a penton base which lies on the surface of the capsid and a fiber extending from the base (Fig. 4) (Shenk, 2001).

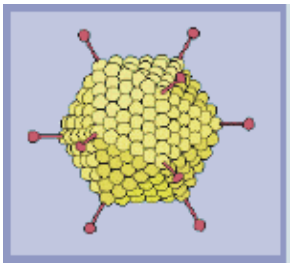

**Figure 4.** Structure of adenovirus.
Figure by L. Stannard, University of Cape Town, South Africa
(http://www.tulane.edu/~dmsander/Big_Virology/BVDNAadeno.html)

***Replicative cycle.*** The replicative cycle of adenovirus is commonly divided into early and late phases. Transition from early to late phase occurs as the replication of the viral genome begins (Shenk, 2001). The attachment of adenovirus to the host cell surface is mediated by the

viral fiber protein which binds to the coxsackie B virus and adenovirus type 2 and 5 receptor (CAR) and the major histocompatibility complex class 1 α-2 domain in the host cell surface (Bergelson et al., 1997; Tomko et al., 1997; Hong et al., 1997). Subsequently, the penton base interacts with cellular $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins to promote viral internalization (Wickham et al., 1993) which occurs by receptor-mediated endocytosis through coated-pit and -vesicle pathways (Chardonnet and Dales, 1970; Varga et al., 1991). The acidic environment in the early endosome activates the penetration of the viral particles into cytoplasm (Seth et al., 1985; Greber et al., 1993). Inside the host cell, the viral genome is released by stepwise disassembly of the protein capsid (Greber et al., 1993) and the viral DNA together with some structural proteins (protein VII, protein V, terminal protein, hexon) are transported into the nucleus through nuclear pore complexes (Greber et al., 1993; Greber et al., 1997; Matthews and Russell, 1998). In the nucleus, the E1A transcription unit is the first viral transcription unit to be transcribed (Nevins et al., 1979; Shenk, 2001). E1A encodes two proteins, 12S and 13S, which interact with several cellular transcription factors including TFIID (Horikoshi et al., 1991; Lee et al., 1991), proteins of retinoblastoma family (pRB, p107, p130) (Harlow et al., 1986; Whyte et al., 1989), SUR2 (Boyer et al., 1999), Dr1 (Kraus et al., 1994), p300/CREB-binding protein (CBP), p300/CBP-associated factor (Whyte et al., 1989; Frisch and Mymryk, 2002) and Yin Yang 1 (Shi et al., 1991) in order to activate cellular genes that induce quiescent host cells to enter the S phase of the cell cycle and thereby provide optimal conditions for the replication of the viral genome. Proteins encoded by E1A also activate transcription of other viral early transcription units (Berk et al., 1979; Jones and Shenk, 1979) in order to synthesize the proteins needed for the viral replication and protection of infected cells from the antiviral actions of the host organism (Shenk, 2001).

When the early phase is completed, the viral genome starts to replicate. Both viral (preterminal protein, DNA-polymerase, DNA-binding protein) and cellular (nuclear factors I-III) proteins participate in the process (Shenk, 2001). The late phase is characterized by the transcription of the major late transcription unit which encodes the viral structural proteins. Viral mRNAs are both transported into cytoplasm and translated more efficiently than host mRNAs (Beltz and Flint, 1979). Structural proteins are transported into the nucleus where the viral capsids are assembled. In the last step of the virion assembly, the viral genome enters the capsid. Progeny viruses are released from the host cell as a consequence of cell lysis (Shenk, 2001).

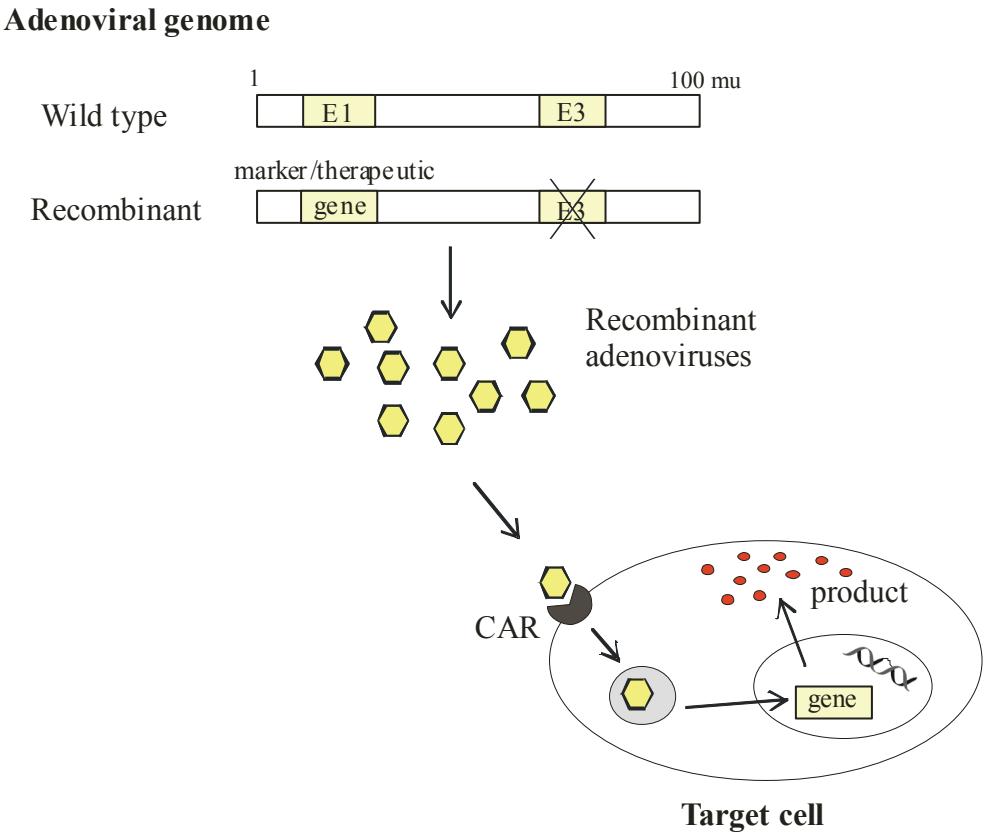


**Figure 5.** Main features of the use of recombinant adenoviruses as gene transfer vectors. Abbreviations used: CAR, coxsackie B virus and adenovirus type 2 and 5 receptor; mu, map unit

### 2.5.3 Recombinant adenoviruses as gene transfer vectors

Adenoviruses are efficient gene transfer vectors due to their natural feature of efficient entry into the host cell. The recombinant adenoviral genome contains a marker gene (e.g. LacZ, green fluorescent protein (GFP)) or a therapeutic gene. The main function of the recombinant adenoviral vectors is to effectively transport the marker gene or the therapeutic gene into the host cells. Recombinant viruses lack some features of the wild type adenoviruses. Recombinant adenoviruses are replication incompetent as a result of deletion of the E1 unit from the viral genome (Fig. 5) (Horwitz, 2001). It is important to prevent the viral propagation as this would lead to lysis and death of the host cell (Shenk, 2001).

Recombinant adenoviruses are widely used as gene transfer vectors. They are able to transduce both dividing and non-dividing cells and thus have a broad spectrum of target cells and tissues. Additionally, recombinant adenoviruses can be produced at high titer. This aspect becomes important especially in *in vivo* experiments. As a result of adenovirus-mediated gene

transfer, a strong extrachromosomal expression of the transferred gene can be achieved. Since the host cell genome remains intact, the possibility of insertional mutagenesis and carcinogenesis, which is associated with vectors that integrate into the host genome, is very small. In addition, an episomally expressed transgene is not inherited to progeny (Amalfitano, 2004).

Recombinant adenoviral vectors are commonly used as gene transfer vectors also in the field of type 2 diabetes research. Adenovirus-mediated gene transfer has been successfully utilized in differentiated 3T3-L1 cells, although some researchers have recommended the use of other vectors e.g. lentiviral vectors (Carlotti et al., 2004). Adenovirus-mediated transduction of a dominant negative mutant of p85α has been used to investigate the effects of acute inhibition of PI 3-kinase signalling in liver (Miyake et al., 2002). In addition, it is possible to restore the expression of deleted gene in knock out animals using an adenovirus-mediated gene transfer and thus to confirm the obtained results. Ueki *et al.* demonstrated a restored insulin sensitivity in IRS-1 deficient mice using adenovirus mediated gene transfer of IRS-1 (Ueki et al., 2000). Furthermore, adenovirus-mediated overexpression of a gene represents a feasible approach to investigate the multiple effects of insulin. Becard *et al.* illustrated that overexpression of SREBP-1c mimicked the effects of insulin on hepatic gene expression in a diabetic mouse model (Becard et al., 2001).

### 2.5.4 Factors affecting the adenoviral gene transfer efficiency

Adenoviral infection of the host cell is a complex series of events and understanding of the factors that affect the viral gene transfer efficiency is crucial if one wishes to achieve maximal gene transfer efficiency. In 1997, Bergelson *et al.* identified CAR as the main receptor for adenoviral serotypes 2 and 5. They also suggested that the expression of CAR is the most important factor that defines the adenoviral gene transduction efficiency in the target cells and tissues (Bergelson et al., 1997), and this conclusion was confirmed by Tomko *et al.* (Tomko et al., 1997).

Cells expressing a low level of CAR are a challenging target for the adenovirus-mediated gene transfer. Several studies have indicated that it is possible to overcome this limitation by genetic manipulation of the fiber protein (Michael et al., 1995; Wickham et al., 1996; Wickham et al., 1997; Dmitriev et al., 1998). The transduction efficiency of the adenoviral vectors in CAR-deficient cells is augmented by a stable introduction of CAR into the target cells (Bergelson et al., 1997; Ross et al., 2003). Worgall *et al.* demonstrated that free cholesterol could enhance the adenoviral gene transfer efficiency in cells expressing low

levels of CAR (Worgall et al., 2000). In addition, the use of polylysine and lipofectamine has been shown to increase the adenoviral gene transfer efficiency (Orlicky and Schaack, 2001). Furthermore, dexamethasone increases the adenoviral gene transfer efficiency into skeletal muscle *in vitro* and *in vivo* (Braun et al., 1999). The maximal effect was obtained by preincubation of the cells in the presence of dexamethasone for 48 hour prior to the gene transfer.

Adenovirus is an immunogenic virus. Adenoviral infection generates serotype-specific neutralizing antibodies (Nab) in the host. The structural proteins of the viral capsid i.e. fiber, hexon and penton base contain most of the epitopes recognized by Nab (Horwitz, 2001). Since adenovirus is a common pathogen, human immunity to adenoviral infection is likely to exist. Nwanegbo *et al.* demonstrated the presence of Nab towards adenovirus serotype 5 in several populations (Nwanegbo et al., 2004). The presence of Nab has been associated with impaired efficacy of the adenovirus-based gene transfer (Wohlfart, 1988). In addition to the structural proteins of the adenoviral capsid, antibodies can be generated towards the product of the therapeutic gene (Molnar-Kimber et al., 1998). In addition to Nab, $CD8^+$ T lymphocytes have been suggested to contribute to the immunity against adenovirus (Sumida et al., 2004).

Storage conditions and transportation might also affect the adenoviral gene transfer efficiency. A decrease in pH of the adenoviral storage buffer during transportation can markedly lower the viral titer (Nyberg-Hoffman and Aguilar-Cordova, 1999). Very little data is available about other factors (e.g. temperature during transportation, the effects of various sera during gene transfer) that might affect the viral infectivity.

# 3 AIMS OF THE STUDY

The aim of the study was to investigate the catalytic subunit p110β of PI 3-kinase as a candidate gene for type 2 diabetes. In addition, we aimed to establish an *in vitro* model in 3T3-L1 adipocytes to investigate the insulin signalling pathways and optimize the gene transfer conditions of recombinant adenoviral vectors. The following questions were addressed:

1. Are mutations in the gene encoding the catalytic subunit p110β of PI 3-kinase associated with type 2 diabetes? (Study I)

2. Are promoter polymorphisms of the p110β gene associated with insulin resistance in healthy normoglycemic subjects? (Study II)

3. Can differentiated 3T3-L1 cells be utilized as an *in vitro* model to study insulin signal transduction? (Study III)

4. What factors affect the adenoviral gene transfer efficiency? (Study IV)

# 4 SUBJECTS AND METHODS

## 4.1 Subjects

### 4.1.1 Subjects in Studies I and II

Clinical characteristics of diabetic and normoglycemic subjects screened in Studies I and II are listed in Table 6 and 7.

**Table 6.** Clinical characteristics of diabetic and control subjects in Study I

| | Diabetic subjects | Control subjects |
|---|---|---|
| Gender (male/female) | 39/40 | 77/0 |
| Age (years) | 63±1 | 54±1 |
| Body mass index (kg/m$^2$) | 30.0±0.6 | 26.4±0.4 |
| Fasting glucose (mmol/l) | 9.6±0.3 | 5.5±0.06 |
| Fasting insulin (pmol/l) | 137.1±10.4 | 55.8±4.1 |
| Reference | (Sarlund et al., 1992) | (Haffner et al., 1994) |

Data are presented as means±SD

**Table 7.** Clinical characteristics of normoglycemic subjects in Study II

| | Group I | Group II |
|---|---|---|
| Gender (male/female) | 150/145 | 82/28 |
| Age (years) | 44±1 | 51±8 |
| Body mass index (kg/m$^2$) | 25.6±0.2 | 26.1±3.6 |
| Metabolic studies | OGTT, IVGTT | OGTT, hyperinsulinemic euglycemic clamp |
| Reference | (Laakso et al., 1988) | (Haffner et al., 1994) (Vauhkonen et al., 1998) (Voutilainen, 1992) |

Data are presented as means±SD.
Abbreviations used: IVGTT, intravenous glucose tolerance test; OGTT, oral glucose tolerance test

### 4.1.2 Approval of the ethics committee

All study subjects participated voluntarily in the study after discussion of the aims and potential risks involved. The study was approved by the Ethics Committee of the University of Kuopio and was in accordance with the Helsinki Declaration.

## 4.2 Methods

The following tables (Table 8-12) contain the summary of methods, primers, cell lines, primary antibodies and adenoviral construct used in Studies I-IV. The methods have been described in detail in Studies I-IV.

**Table 8.** Methods used in Studies I-IV

| | Method | Study |
|---|---|---|
| RNA techniques | RNA isolation | I |
| | Reverse transcriptase polymerase chain reaction (RT-PCR) | I |
| | Northern blot | I |
| DNA techniques | PCR, primer design | I, II |
| | Screening of cDNA and genomic phage libraries | I |
| | Southern blot | I |
| | Subcloning into plasmid | I |
| | DNA isolation | I |
| | Sequencing | I, II |
| | Single-strand conformation polymorphism (SSCP) analysis | I |
| | SNaPshot method | II |
| Metabolic studies | Oral glucose tolerance test (OGTT) | II |
| | Intravenous glucose tolerance test (IVGTT) | II |
| | Hyperinsulinemic euglycemic clamp | II |
| | Indirect calorimetry | II |
| Cell culture | Transduction | IV |
| | Differentiation of 3T3-L1 cells | III |
| | Insulin stimulation, treatment with inhibitors | III |
| | 2-Deoxy-[$^3$H]-glucose uptake | IV |
| *In vitro* procedures | X-gal staining | IV |
| | Oil Red O staining | III |
| | Harris' hematoxylin staining | III |
| | Flow cytometry analysis | IV |
| Protein analysis | Western blot | III |
| Adenoviral studies | Production of recombinant adenoviruses | IV |
| | Preincubation of viral vectors at various temperatures | III, IV |
| | Gene transfer *in vitro* and *in vivo* | III, IV |
| | Neutralization studies | IV |
| Statistical analysis | Mean±SD | I, II |
| | Chi-square test | I |
| | Analysis of variance | II |

***Northern blot.*** To determine the expression of the p110β gene in various tissues Human Multiple Tissue Northern (MTN$^{TM}$) Blot (BD Biosciences Clontech, Palo Alto, CA) containing RNA from various tissues was hybridized with $^{32}$P-labelled p110β probes (Ready-To-Go DNA Labeling Beads, Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. The p110β probes represented base pairs (bp) 1-2169 and 2505-3213 of the p110β cDNA. The signal was detected using Phosphoimager (Storm, Amersham Biosciences). The quality of RNA samples was controlled by hybridization with a β-actin probe which was provided by the manufacturer.

**Table 9.** Primers, sizes of the amplified fragments, restriction enzyme digestions and sizes of the restriction fragments for single-strand conformation polymorphism (SSCP) analysis of the promoter (PR) and exons of the gene encoding the catalytic subunit p110β of human PI 3-kinase (Study I)

| Promoter/ Exon F or R* | Primer sequence 5' → 3' | Size of amplified fragment (bp) | Cleavage enzyme | Restriction fragments (bp) |
|---|---|---|---|---|
| PR1/ F | CCT GTC AAG TGC TGG TTA ACT A | 487 | SmiI | 236, 251 |
| PR1/ R | GAT GTC AAG GAT GTC TGC CAT A | | | |
| PR2/ F | CAT CCT GGC TAA CAC GGT TGA A | 416 | Eco130I | 173, 243 |
| PR2/ R | TGC ATG CTT AAG GAT TAC AGG G | | | |
| PR3/ F | TTA GCG CTC ATG TTC TTC CAA T | 438 | AvaI | 194, 244 |
| PR3/ R | TTC AAC CGT GTT AGC CAG GAT G | | | |
| PR4/ F | GCA GCC TTA GAT TCT TGG ACT C | 315 | Eco31I | 144, 171 |
| PR4/ R | AAT TGG AAG AAC ATG AGC GCT A | | | |
| 1/ F | GTG GTT ATG AAT GTG CTT CAG T | 231 | - | - |
| 1/ R | CCA AGT GAC ACA GTA TGC TAA A | | | |
| 2/ F | TGA GCA AGT GTT TCC ATT CCA GA | 376 | BseNI | 197, 179 |
| 2/ R | CCA TGG ACC ACA CTT TGA AAA GC | | | |
| 3/ F | AGC ATC CAA CAT CCA AGT TAG T | 422 | BseNI | 222, 220 |
| 3/ R | GCA AGC GAC AGA CAC TTC TAA A | | | |
| 4/ F | ACT GCT TTT TTC CCC ATC TCC CT | 324 | MspA1I | 163, 161 |
| 4/ R | TAT TCC AAA TGT TCC AGT TGT GG | | | |
| 5/ F | GGC AGT AAA ATC AAT ACC TTC C | 255 | - | |
| 5/ R | CAC ATG GCT TTT GGG GTT ACT A | | | |
| 6/ F | GCT CTA TTT TCA TAG TTT TGC C | 436 | MbiI | 227, 209 |
| 6/ R | GAA AAA TAA TGT CAA TCT TTC C | | | |
| 7/ F | TTC TTC CAG TAT GTT CCT TCC T | 418 | BseNI | 214, 204 |
| 7/ R | AAA ACA ATC CTC AGA AGT TGG T | | | |
| 8/ F | GGA CAT GTG CAT GTT TAC ACC T | 232 | - | |
| 8/ R | TAT TAC CTA GTC CAC ATG CCA A | | | |
| 9/ F | ATT TGA ATT AAG AGG TAA AGT AG | 431 | BseRI | 190, 241 |
| 9/ R | CAT TCA ATC ATT TCA TGC ATA G | | | |
| 10/ F | CCA TCA TTT CCC TGT TGT CAA GA | 407 | BbvI | 205, 202 |
| 10/ R | TGG GCT GCC ATT TAA CAA AAC AC | | | |
| 11/ F | TGA AAG TTT GCT GTG GTG TTT GC | 302 | Bsp143I | 159, 143 |
| 11/ R | TCC AAC CAA GTA CCA TAC ACC CA | | | |
| 12/ F | GTG AGC TTT GCC TTC TTT TGA CC | 282 | AvaI | 143, 139 |
| 12/ R | CCA AAC CCA CCC AAG TTA TTC CT | | | |
| 13/ F | TCT GGC ACA GGT TGT TTG GTT A | 211 | - | - |
| 13/ R | ACC TGG TGG GCT CAA AGT AAA A | | | |
| 14/ F | CGG TGA TCT GAA GTG TTT GAT A | 216 | - | - |
| 14/ R | CAT GCT TTA AAC GTT GTC TGT C | | | |
| 15/ F | GTG TGG GGA ACT TAT TTT TCA G | 221 | - | - |
| 15/ R | CGC AAA GCA CAG TCA CTT ACT A | | | |
| 16/ F | GGT GAG GAG TTT TCC CAA GCC TA | 324 | BstZ17I | 178, 146 |
| 16/ R | CTC CCT TCC TGG CTG CAA ATT GT | | | |
| 17/ F | GTT ACA GGG CAT AAA AGG AAA AGC | 224 | - | - |
| 17/ R | TGC TAT GGG AAG ACA TTA GAC TGA | | | |
| 18/ F | AGG ATG TTG CCT TAT GGC TGT T | 252 | - | - |
| 18/ R | CAC TGC TGA CTT CTA TTG GGA A | | | |
| 19/ F | CTG TTC TTT TCT CTT GTT CAG G | 190 | - | - |
| 19/ R | AAT AGC ATT ACT AAG GCC CTT G | | | |
| 20/ F | GCC TTT ATA TTT GGA ACC CAC A | 244 | - | - |
| 20/ R | TTA GAA GTG TTC AGC CTT GGC A | | | |
| 21/ F | CTC CCC TCT AAC ACT GTG CTC A | 219 | - | - |
| 21/ R | GCC CAC AAA GTC CAA GAG AGA A | | | |
| 22/ F | CAG CCT CCT GCA GAC TTT GAT A | 366 | HaeIII | 184, 182 |
| 22/ R | TTC TGT GGG ATG CCT TGT TCT T | | | |

*F for forward primer and R for reverse primer

**Table 10.** Cell lines used in Studies III-IV

| Cell line | Definition | Supplier, product number | Study |
|---|---|---|---|
| 3T3-L1 | Mouse embryo fibroblasts, possess capacity to differentiate into adipocytes | ATCC, Manassas, VA CL-173 | III |
| CHO | Chinese hamster ovary cells | ATCC, CCL-61 | IV |
| CHO-CAR | Chinese hamster ovary cells expressing CAR | Kind gift from Dr. Bergelson (Bergelson et al., 1997) | IV |
| A549 | Human lung carcinoma cells | ATCC, CCL-185 | IV |
| BALB/3T3 | Mouse embryo fibroblasts | ATCC, CCL-163 | IV |

**Table 11.** Primary antibodies used in the Study III

| Antibody | Target | IgG concentration/ dilution | Manufacturer |
|---|---|---|---|
| pTyr | Phosphorylated tyrosine | 1.0 μg/ml | Upstate Cell Signaling Solutions, Lake Placid, NY |
| phospho-Akt | Phosphorylated Ser473 in Akt | 1:1000 | Cell Signaling Technology, Beverly, MA |
| Akt | Total Akt | 1:2000 | Cell Signaling Technology |
| phospho-ERK1/2 | Phosphorylated Thr202/Tyr204 in ERK1/2 | 1:2000 | Cell Signaling Technology |
| ERK1/2 | Total ERK1/2 | 1:1000 | Cell Signaling Technology |

**Table 12.** Adenoviral constructs used in Study IV

| Adenoviral construct | Marker gene | Promoter | Detection method |
|---|---|---|---|
| AdLacZ | β-galactosidase | Human β-actin promoter and cytomegalovirus (CMV) enhancer | X-Gal staining |
| AdGFP | Green fluorescent protein (GFP) | Human elongation factor 1α (EF1α) gene promoter | Flow cytometry |

# 5 RESULTS

The essential results of the Studies I-IV are described. Also, some additional results are shown.

## 5.1 Structure and expression pattern of the human p110β gene (Study I)

The gene encoding the catalytic subunit p110β of human PI 3-kinase was cloned from a human genomic phage library. Ten positive phage clones were analyzed and altogether 59 kb of the genomic sequence was analyzed and subsequently saved in the EMBL Nucleotide Sequence Database (accession numbers AJ297549-AJ297560). Figure 6 illustrates the genomic structure of the human p110β gene. The genomic data is in 12 fragments since all introns were not completely sequenced and thus, the total length of these introns is not known. Partially sequenced introns are indicated with dots in Figure 6. The human p110β gene is composed of 22 exons which are 51-252 bp in length. The position in the cDNA and the length of each exon are listed in Table 13. Exon-intron junctions of the p110β gene contain conserved nucleotides AG at 3' splice acceptor and GT at 5' splice donor regions.

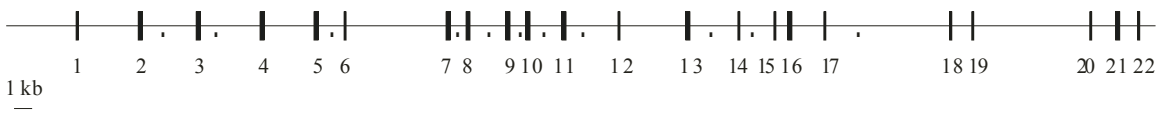


**Figure 6.** Structure of the human p110β gene. Boxes represent the exons and thin line introns. Dots indicate introns that are sequenced only partially.

**Table 13.** Exons of the human p110β gene, position in the cDNA and length

| Exon | Position in cDNA (bp) | Length (bp) | Exon | Position in cDNA (bp) | Length (bp) |
|---|---|---|---|---|---|
| 1 | 1-171 | 171 | 12 | 1771-1892 | 122 |
| 2 | 172-397 | 226 | 13 | 1893-2036 | 144 |
| 3 | 398-621 | 224 | 14 | 2037-2136 | 100 |
| 4 | 622-801 | 180 | 15 | 2137-2315 | 179 |
| 5 | 802-972 | 171 | 16 | 2316-2425 | 110 |
| 6 | 973-1050 | 78 | 17 | 2426-2504 | 79 |
| 7 | 1051-1302 | 252 | 18 | 2505-2672 | 168 |
| 8 | 1303-1399 | 97 | 19 | 2673-2796 | 124 |
| 9 | 1400-1530 | 131 | 20 | 2797-2942 | 146 |
| 10 | 1531-1581 | 51 | 21 | 2943-3075 | 133 |
| 11 | 1582-1770 | 189 | 22 | 3076-2313 | 138 |

Expression of the p110β gene in various human tissues was determined by Northern blot using a commercial RNA membrane. The membrane was first hybridized with $^{32}$P-labelled p110β probes (representing 1-2160 bp and 2505-3213 bp of the p110β cDNA) followed by hybridization with the β-actin probe to determine the amount and quality of RNA in each sample. Signals corresponding to the human p110β mRNA and β-actin mRNA were 4,8 kb and 2,0 kb in length, respectively. The amount of RNA from various tissues was not constant in the membrane, samples from placenta and pancreas contained a higher amount of RNA while there was a lesser amount of RNA from lung (Fig. 7, lower panel). The p110β gene was expressed in heart, brain, placenta, skeletal muscle, kidney and pancreas and to a lesser extent in liver while there was no detectable signal from lung (Fig. 7, upper panel).

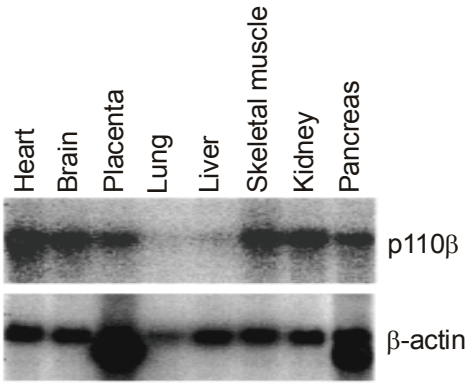


**Figure 7.** Expression of the p110β gene in human tissues. A commercial RNA membrane was hybridized with $^{32}$P-labelled p110β (upper panel) and β-actin probes (lower panel) and the signal was detected using Phosphoimager.

## 5.2 Polymorphisms of the p110β gene (Study I)

All 22 exons, intron areas flanking the exons and 1.5 kb of the promoter region of the p110β gene were screened for variants in 79 subjects with type 2 diabetes. No variants were detected in the exons of the p110β gene. However, two polymorphisms were identified in the promoter area. Polymorphism T→C was identified 359 bp upstream from the first potential ATG initiation codon according to Hu *et al.* (Hu et al., 1993) (–359T/C) and polymorphism A→G 303 bp upstream from the ATG initiation codon (–303A/G). In addition, a 2-bp repeat sequence $(TA)_n$ was detected in intron 4, 44 bp downstream from the 3' end of exon 4. The number of repeats varied between 10 and 13.

The allele frequencies of the promoter polymorphisms –359T/C and –303A/G did not differ between diabetic subjects and normoglycemic control subjects (Table 14). The allele

frequency of the polymorphism T→C was 0.47 and 0.39 in diabetic subjects and in controls, respectively. The allele frequency of the A→G polymorphism was 0.05 and 0.09 in diabetic subjects and in normoglycemic subjects, respectively. Similarly, the length of the $(TA)_n$ repeat sequence in intron 4 did not differ between the study groups (Table 14). Allele frequency of $(TA)_{10}$ was 0.97 vs. 0.95, $(TA)_{11}$ 0.03 vs. 0.03 and $(TA)_{13}$ 0.01 vs. 0.02 in diabetic and control subjects, respectively.

**Table 14.** Allele frequencies of the polymorphisms of the p110β gene in diabetic and control subjects

| Polymorphism | Diabetic subjects (n=79) | Control subjects (n=77) |
|---|---|---|
| Promoter | | |
| –359T/C | 0.47 | 0.39 |
| –303A/G | 0.05 | 0.09 |
| Intron 4 | | |
| $(TA)_{10}$ | 0.97 | 0.95 |
| $(TA)_{11}$ | 0.03 | 0.03 |
| $(TA)_{13}$ | 0.01 | 0.02 |

None of the comparisons between study groups were statistically significant

## 5.3 Effects of the p110β promoter polymorphisms on insulin secretion and insulin sensitivity in normoglycemic subjects (Study II)

The effects of the –359T/C and –303A/G promoter polymorphisms of the p110β gene on insulin secretion and insulin sensitivity were investigated in two study groups of normoglycemic Finnish subjects (Group I and II). In the study groups, the genotype frequencies of –359T/C and –303A/G followed the Hardy-Weinberg equilibrium and were in linkage disequilibrium. The allele frequency of the polymorphism T→C was 0.34 vs. 0.40 in Group I and II, respectively. The allele frequency of the polymorphism A→G was 0.07 vs. 0.09 in Group I and II, respectively. In both study groups, there was no difference in the fasting plasma insulin level or in the area under the insulin curve in the 2-h oral glucose tolerance test, body mass index (BMI) or waist hip ratio between genotypes. In Group I, the –359T/C and –303A/G polymorphisms did not affect the first-phase insulin secretion, insulin sensitivity index, $S_I$, or glucose effectiveness, $S_G$, evaluated by the intravenous glucose tolerance test (IVGTT) (Table 15). In Group II, WBGU, glucose oxidation and nonoxidative glucose disposal evaluated by the hyperinsulinemic euglycemic clamp did not differ between genotypes (Table 16, all p-values >0.1; adjusted for age, sex and BMI).

**Table 15.** Insulin secretion during the first 10 min of IVGTT (insulin AUC (0-10 min)), insulin sensitivity index ($S_I$) and glucose effectiveness ($S_G$) according to the promoter polymorphisms –359T/C and –303A/G of the gene encoding the catalytic subunit p110β of human PI 3-kinase in 295 Finnish nondiabetic subjects

| | –359T/C | | | –303A/G | |
|---|---|---|---|---|---|
| | T/T (n=130) | T/C (n=128) | C/C (n=37) | A/A (n=256) | A/G (n=39) |
| **IVGTT** | | | | | |
| Insulin AUC (0-10 min) (pmol/l ·min) | 2841.1±1947.3 | 2332.9±1315.8 | 2714.4±1509.7 | 2625.3±1614.3 | 2469.5±1939.1 |
| $S_I \cdot 10^{-4}$ ($min^{-1}$/(μU/ml)) | 4.0±2.4 | 4.5±2.4 | 4.5±2.4 | 4.3±2.4 | 4.2±2.3 |
| $S_G \cdot 10^{2}$ (1/min) | 2.1±0.8 | 2.0±0.7 | 2.3±1.0 | 2.1±0.8 | 2.0±0.8 |

Data are presented as means±SD. None of the comparisons between genotypes were statistically significant



**Table 16.** Whole body glucose uptake (WBGU), glucose oxidation and nonoxidative glucose disposal during the hyperinsulinemic euglycemic clamp study according to the promoter polymorphisms –359T/C and –303A/G of the gene encoding the catalytic subunit p110β of human PI 3-kinase in 110 Finnish nondiabetic subjects

| | –359T/C | | | –303A/G | | |
|---|---|---|---|---|---|---|
| | T/T (n=46) | T/C (n=41) | C/C (n=23) | A/A (n=92) | A/G (n=16) | G/G (n=2) |
| **Clamp** | | | | | | |
| WBGU (μmol/kg/min) | 58.5±16.3 | 58.2±14.8 | 57.3±12.6 | 57.9±14.2 | 57.9±19.4 | 70.2±3.9 |
| Glucose oxidation | 19.1±4.7 | 19.4±4.3 | 20.1±5.6 | 19.1±4.6 | 19.9±4.9 | 28.3±4.7 |
| Nonoxidative glucose disposal | 39.4±13.9 | 38.7±13.5 | 37.2±11.4 | 38.7±12.3 | 38.1±18.1 | 42.0±8.6 |

Data are presented as means±SD. None of the comparisons between genotypes were statistically significant

## 5.4 Differentiation of 3T3-L1 fibroblasts into adipocytes (Study III)

The differentiation of 3T3-L1 cells was initiated two days after the cells had reached a confluent state (Day 0). The differentiation was performed according to Student *et al.* (Student et al., 1980). 3T3-L1 cells were differentiated by using a cocktail which contained insulin, dexamethasone, IBMX and fetal bovine serum (FBS). The differentiation protocol is described in Study III. The formation of cytoplasmic triglyceride droplets was detected using Oil Red O staining. Cytoplasmic lipid droplets became visible on Day 3. The size of triglyceride droplets gradually increased and during the differentiation process the nuclei became eccentric. We also detected some spontaneous differentiation of 3T3-L1 cells which occurred in the absence of the differentiation-promoting agents. Figure 8 (Day 0, arrow) shows one typical spontaneously differentiated cell which clearly contained a smaller amount of cytoplasmic lipid than cells differentiated with the differentiation-promoting cocktail. Figure 8 shows 3T3-L1 cells on Day 0 and after a 14-day differentiation (Day 14).

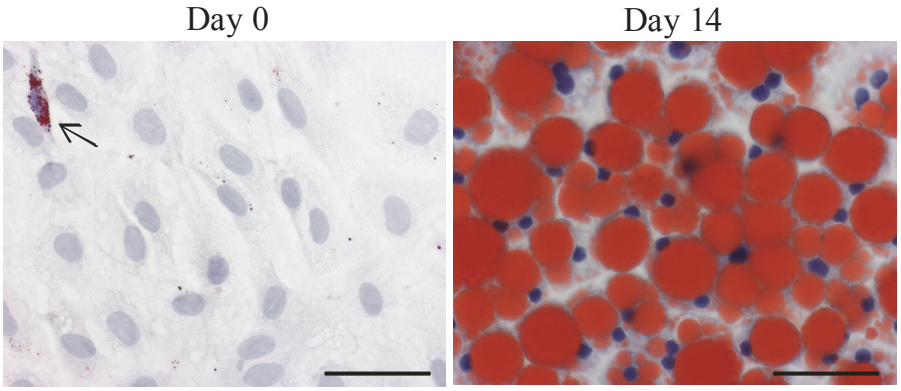


**Figure 8.** 3T3-L1 cells prior to differentiation (Day 0) and 14 days after the initiation of the differentiation (Day 14). The differentiation was performed according to Student *et al.* (Student et al., 1980), the protocol is described in Study III. Cytoplasmic triglyceride droplets and nuclei were detected using Oil Red O staining and Harris' hematoxylin staining, respectively**.** Arrow indicates one spontaneously differentiated cell. Scale bar 50 μm.

## 5.5 Effects of insulin stimulation in differentiated 3T3-L1 cells (Study III)

***Activation of the insulin signalling pathways.*** Differentiated 3T3-L1 cells were stimulated with 100 nM insulin after which cellular proteins were isolated at different time points and analyzed by Western blot. Insulin stimulation caused tyrosine phosphorylation of an approximately 95-kDa protein. This is likely to be the β-subunit of the insulin receptor. Phosphorylation was clearly visible after a 2-min stimulation and it reached its maximum after a 15-min insulin stimulation. At the time point 120 min, phosphorylation was still detectable but clearly diminished (Fig. 2A). Insulin phosphorylated Akt and ERK1/2 in differentiated 3T3-L1 cells. These proteins were phosphorylated after a 2-min insulin stimulation. Phosphorylation of Akt and ERK1/2 reached the maximal level after a 15-min stimulation. Phosphorylation of Akt diminished slowly while phosphorylation of ERK1/2 decreased more rapidly (Fig. 2B and C).

***Glucose uptake.*** Differentiated 3T3-L1 cells were stimulated with 100 nM insulin and glucose uptake was determined by using 2-deoxy-[$^3$H]-glucose. Cytochalasin B was used to indicate the level of the basal glucose uptake since it blocks the GLUT4-mediated glucose uptake by preventing the translocation of GLUT4 molecules from intracellular vesicles to plasma membrane (Lakshmanan et al., 2003). Prior to the insulin stimulation, some samples were treated with the PI 3-kinase inhibitors wortmannin and LY2940002. Insulin stimulated glucose uptake in differentiated 3T3-L1 cells by approximately 13-fold. Treatment with wortmannin or LY294002 prior to insulin stimulation abolished this effect (Table 17). Basal glucose uptake accounted for approximately 5% of the total glucose uptake.

**Table 17.** 2-Deoxyglucose uptake in differentiated 3T3-L1 cells in the basal state and after insulin stimulation

| | 2-Deoxyglucose uptake (CMP/(mg prot*min)) | |
|---|---|---|
| Treatment | Basal | Insulin-stimulation |
| - | 305±15 | 4050±151 |
| Wortmannin | 108±9 | 32±22 |
| LY294002 | 75±15 | 118±17 |

Data are presented as means±SD

## 5.6 Adenoviral transduction efficiency *in vitro* and *in vivo* after preincubation at +37°C, +20°C and 0°C (Study IV)

The purpose of Study IV was to investigate how preincubation at different temperatures affects the adenoviral gene transfer efficiency. Two recombinant adenoviral constructs, AdLacZ and AdGFP, were preincubated for different time periods at +37°C, +20°C and 0°C and subsequently transduced into CAR-deficient (BALB3T3, CHO) and CAR-expressing (A549, CHO-CAR) cells *in vitro*. The main finding was that after a 20-40 min preincubation of AdLacZ and AdGFP at +37°C there was a significant increase in the transduction efficiency of the viral constructs in CAR-deficient cells (Fig. 1C, Fig. 2A and C). If the preincubation time at 37°C was longer, the transduction efficiency started to wane. After preincubation at +20°C, there was a slight improvement in the transduction efficiency of AdGFP at the time point 90-120 min (Fig. 2B). In CAR-expressing cell lines, no heat-activation of adenovirus as described above could be observed. The transduction efficiency of AdGFP was maximal at time point 0 min, i.e. without preincubation (Fig. 2E and G). Preincubation of AdGFP at 0°C had virtually no effect on the transduction efficiency in CAR-deficient or CAR-expressing cells (Fig. 2B, D, F and H).

To investigate the heat activation of adenovirus *in vivo*, AdLacZ was preincubated for 30 min at +37°C and 0°C and subsequently inoculated into corpus callosum of BDIX rats. After 24 h and 72 h, the transduction efficiency of AdLacZ preincubated at +37°C was fourfold compared to the virus preincubated at 0°C (Fig. 3).

## 5.7 Effects of various sera on the adenoviral transduction efficiency (Study IV)

The effects of different sera on the viral transduction efficiency were also studied. AdGFP was incubated with adult human serum (AS), umbilical cord serum (CS) and FBS for different time periods at +37°C and subsequently transduced into BALB3T3 cells. Both active and heat-inactivated sera were used. Incubation of AdGFP with AS neutralized to a great extent the viral infectivity within 30 s (Fig. 4A). Similar results were obtained when AdGFP was incubated with CS (Fig. 4B). Interestingly, FBS had only a minor effect on the viral infectivity (Fig. 4C). In all cases, there was no difference between the effects of active and heat-inactivated serum.

# 6 DISCUSSION

## 6.1 Structure and expression pattern of the human p110β gene (Study I)

Type 2 diabetes is characterized by decreased glucose uptake in skeletal muscle and adipose tissue (Rothman et al., 1992; Cline et al., 1999). PI 3-kinase is an intracellular lipid kinase, which has a crucial role in mediating the insulin-stimulated glucose uptake (Shepherd et al., 1998). This makes PI 3-kinase a promising candidate gene for insulin resistance and type 2 diabetes. We investigated the catalytic subunit p110β of PI 3-kinase as a candidate gene for type 2 diabetes. This approach requires the knowledge of the exon-intron structure of the gene. Therefore, we cloned the human p110β gene from a placental phage library and thereby provided novel information of a gene that encodes an important protein in insulin signal transduction. When the genomic sequence was compared to the cDNA sequence (Hu et al., 1993) it was found that the human p110β gene was composed of 22 exons and that the exon-intron junctions contained typical TG/AG donor/acceptor junctions. These sites are crucial for the proper splicing of the primary RNA transcript (Alberts et al., 1994c).

The expression pattern of p110β in various human tissues was determined by Northern blot. The commercially available blot contained variable amounts of RNA from various tissues which complicated the direct comparison of the expression levels in different tissues. However, the results pointed to a ubiquitous expression pattern of the p110β gene which is consistent with the extensive distribution of PI 3-kinase activity. Our result is also in agreement with data published by other researchers (Vanhaesebroeck et al., 1997b). In mouse, p110β is also widely expressed (Hu et al., 1993). In human, p110α is widely expressed while the third isoform of catalytic subunits of Class IA PI 3-kinases, p110δ, is expressed predominantly in leukocytes (Vanhaesebroeck et al., 1997b).

## 6.2 Screening of the p110β gene (Studies I, II)

### 6.2.1 p110β as a candidate gene for type 2 diabetes (Study I)

All exons, intron areas flanking the exons and 1.5 kb of the promoter region of the p110β gene were screened in samples of subjects with type 2 diabetes. We did not detect any polymorphisms in the exons but we identified two promoter polymorphisms, –359T/C and –303A/G, in diabetic patients. In addition, we identified a variation in the number of TA-repeats in intron 4. The polymorphisms were identified using SSCP, which is a widely used method in screening of candidate genes. The sensitivity of SSCP is approximately 90% (Fan

et al., 1993). This makes the identification of rare mutations more difficult. Our method has been validated against known mutations in the lipoprotein lipase gene (Nevin et al., 1994) and we have successfully identified several variants e.g. in the IRS-1 (Laakso et al., 1994), hexokinase II (Laakso et al., 1995) and GS genes (Rissanen et al., 1997). From this perspective, it is probable that we have not overlooked any notable number of polymorphisms of the p110β gene.

The allele frequencies of the promoter polymorphisms of the p110β gene did not differ between diabetic and control subjects. This implies that the promoter polymorphisms of the p110β gene are not major risk factors for type 2 diabetes in these subjects. Although clinical studies show reduced IRS-associated PI 3-kinase activity in type 2 diabetics (Bjornholm et al., 1997; Kim et al., 1999; Beeson et al., 2003; Kim et al., 2003) our results suggest that this is not due to promoter polymorphisms or variants in the exons of p110β gene. This could indicate that catalytic subunits of PI 3-kinase are both necessary and essential to cellular functions. The results from p110α and p110β knock-out studies support this presumption (Bi et al., 1999; Bi et al., 2002). To our knowledge, p110α has not been studied as a candidate gene for type 2 diabetes. Polymorphisms in the p110α gene could provide one explanation for the reduced IRS-associated PI 3-kinase activity. Interestingly, clinical studies found no difference in PDK1 and Akt activities in type 2 diabetic and control subjects (Krook et al., 1998; Beeson et al., 2003; Kim et al., 2003). This might indicate that in type 2 diabetes factors other than the IRS-1/PI-3 kinase pathway affect the activation of Akt or alternatively, even a diminished PI 3-kinase activity is sufficient to induce normal activation of PDK1 and Akt. However, the activity of another downstream signalling molecule of PI 3-kinase pathway, PCKλ/ζ, has been demonstrated to be decreased in obese subjects and in patients with type 2 diabetes (Beeson et al., 2003; Kim et al., 2003). This finding clarifies, at least in part, the defects that are downstream to PI 3-kinase in insulin resistant states and type 2 diabetes.

### 6.2.2 Normoglycemic subjects (Study II)

In Study II, we analyzed the effects of the promoter polymorphisms of the p110β gene, –359T/C and –303A/G, on insulin secretion and insulin sensitivity in two normoglycemic, Finnish study groups. In both study groups, the promoter polymorphisms did not associate with insulin secretion or insulin sensitivity. Therefore, we presume that these polymorphisms do not have such an effect on the expression of the p110β gene which would lead to changes in insulin secretion or insulin sensitivity. Our result is strengthened by the fact that a similar

result was obtained in two independent study groups and furthermore, that the insulin sensitivity in Group I and Group II was evaluated using two different and independent methods, Bergman Minimal Model and the euglycemic clamp, respectively. To our knowledge, these promoter polymorphisms have not been screened in other populations. The negative result in our study does not exclude the potential relevance of the promoter polymorphisms of the p110β gene in the development of changes in insulin secretion and insulin sensitivity in other populations.

### 6.3 Differentiated 3T3-L1 cells as an *in vitro* model of insulin signal transduction (Study III)

Functional and reliable *in vitro* models are a prerequisite for clarification of cellular defects in insulin resistance and type 2 diabetes. In our study, we investigated whether commercially available 3T3-L1 fibroblasts could be differentiated in our laboratory into adipocytes and subsequently utilized as an *in vitro* model to study the insulin signalling pathway. The 3T3-L1 cell line was chosen since these cells are widely used in the field of diabetes research. In addition, adipose tissue is one of the major target tissues of insulin and recently, adipose tissue has achieved a great deal of attention due to its potential role in contributing to the development insulin resistance and pancreatic β-cell dysfunction (Bays et al., 2004).

3T3-L1 cells were differentiated using a cocktail of differentiation-promoting agents, insulin, dexamethasone, IBMX and FBS. We found that 3T3-L1 fibroblasts could be readily differentiated into adipocytes. The differentiation process was characterized by the accumulation of the cytoplasmic lipid droplets and eccentric location of the nuclei. In addition, spontaneous differentiation of 3T3-L1 cells was observed. It has been shown that insulin promotes lipogenesis by increasing the expression of several lipogenic enzymes via SREBP-1 (Shimano, 2001). Dexamethasone is likely to promote adipogenesis by transcriptional repression of the preadipocyte factor -1 (Smas et al., 1999). IBMX inhibits cAMP phosphodiesterase which leads to an elevation in the cellular cAMP level and thereby activation of the cAMP-dependent protein kinase pathway and adipogenesis (Russell and Ho, 1976).

Upon insulin stimulation, a 95-kDa protein was tyrosine phosphorylated. Based on the molecular size of the phosphoprotein we propose that this protein is the β-subunit of the insulin receptor (Ronnett et al., 1984). However, detailed characterization of the protein would require further experiments with a specific insulin receptor antibody. Similarly to the

β-subunit of the insulin receptor, IRS-1 is tyrosine phosphorylated in response to insulin stimulation. However, there was no signal in the membrane corresponding to the size of tyrosine phosphorylated IRS-1 (molecular size approximately 180 kDa (Sun et al., 1992)). This could be due to technical issues. In Study III, 12% polyacrylamide gel was used. It is possible that during electrophoresis, large proteins are ineffectively separated within such a dense gel. Thereby, the detection of large proteins would perhaps require the use of a less dense polyacrylamide gel. Insulin phosphorylated and thus activated both Akt and ERK1/2. The PI 3-kinase/Akt pathway is the main pathway involved in mediating the metabolic effects of insulin i.e. stimulation of glucose uptake, glycogen synthesis, lipogenesis, protein synthesis and inhibition of gluconeogenesis (Shepherd et al., 1998). The MAPK pathway on the other hand participates in the signalling of the mitogenic effects of insulin (Virkamaki et al., 1999). Insulin also stimulated glucose uptake by 13-fold in differentiated 3T3-L1 cells, which is consistent with earlier studies (Cheatham et al., 1994). The stimulatory effect of insulin on glucose uptake was mediated by GLUT4, since cytochalasin B treatment of differentiated 3T3-L1 cells inhibited the effect of insulin. Similarly, inhibitors of PI 3-kinase, wortmannin and LY294002, abolished the stimulatory effect of insulin on glucose uptake. This observation is also in agreement with previous studies using differentiated 3T3-L1 cells (Evans et al., 1995; Kotani et al., 1995). Our results indicate that the PI 3-kinase/Akt pathway is the major pathway to mediate insulin-stimulated glucose uptake in differentiated 3T3-L1 cells. Taken together, the activation of the insulin receptor and two major insulin signalling pathways and the enhancement of glucose uptake upon insulin stimulation illustrate the usability of differentiated 3T3-L1 cells in studies investigating the insulin signalling pathways *in vitro*.

## 6.4 Factors affecting the adenoviral gene transfer efficiency (Study IV)

Recombinant adenoviruses are widely used gene transfer vectors in the diabetes research (Ali et al., 1994). In order to achieve optimal gene transfer efficiency, the optimization of the gene transfer protocol is of crucial importance. In our study, we determined the effects of preincubation of the viral constructs at various temperatures and the presence of human and bovine sera on the adenoviral transduction efficiency. Interestingly, we found that a 30-min preincubation at +37°C significantly increased the adenoviral transduction efficiency *in vitro* into cells expressing a low level of CAR. The same observation was made in rat brain *in vivo*. Heat activation of the viral constructs seems to be CAR-dependent, since the expression of

CAR in cells abolished the effect of preincubation. Heat activation of adenovirus was detected using two different marker genes, LacZ and GFP, which were under different promoters, CMV and human elongation factor 1α promoters, respectively. Furthermore, similar results were obtained using two CAR-deficient and CAR-expressing cell lines. This rules out the possibility that the heat activation would be associated with a certain adenoviral construct or cell line.

The improvement in the adenoviral gene transfer efficiency into CAR-deficient cells after preincubation at +37°C has not been reported earlier and the mechanisms behind this phenomenon are not known. We suggest that the sequence of events is beneficial to the adenovirus. It is known that adenovirus is a natural cause of respiratory infections, conjunctivitis and gastritis and that the spread of adenovirus occurs as a viral aerosol (Horwitz, 2001). Outside the human body, where the temperature is usually below +37°C it would be beneficial to adenovirus to keep its putative receptor binding sites protected. As the physiological temperature is reached and the viral particles reach the site of infection, it would be advantageous to reveal receptor binding sites that are needed for efficient transduction. Mechanisms leading to the activation of adenovirus at +37°C might involve protease-mediated activation or a conformational change in the viral capsid or alternatively, heat activation might result in the exposure of new receptor binding domains. Several studies have demonstrated the lack of CAR in the luminal surface of airway epithelial cells (Zabner et al., 1997; Walters et al., 1999; Pickles et al., 2000). Since the luminal surface of airway epithelial cells is the primary site of adenoviral infection, this observation points to the existence of novel, still unidentified receptors for adenoviruses.

Valuable information was also obtained about the effects of other temperatures (+20°C, 0°C) on the adenoviral infectivity. Our results show that a 2-hour incubation at 0°C had hardly any effect on the adenoviral gene transfer efficiency. This piece of information can be utilized when the gene transfer experiments are designed. Similarly, it is vital to know that different sera have distinct impacts on the adenoviral gene transfer efficiency. Our results indicate that human serum very rapidly neutralizes the adenoviral infectivity. However, bovine serum had a completely different effect. Incubation of the adenoviral construct in the presence of FBS had only a minor effect on the viral infectivity. These results are understandable since the recombinant adenoviral construct that was used in the study is based on the adenovirus serotype 5 which is a human pathogen (Shenk, 2001). It has been shown that humans have circulating Nab against adenovirus due to naturally acquired infections and

that Nab contribute to the neutralizing of the viral infectivity (Bromberg et al., 1998). Since adenovirus serotype 5 is not a bovine pathogen, bovine serum is not likely to have immunity against the adenoviral construct. The neutralization capacity of human serum did not depend on the heat-inactivation of the serum. This finding supports the important role of Nab and a minor role of complement in the neutralization process. To conclude, adenovirus-mediated gene transfer can be performed in the presence of FBS while the interaction of adenoviral vector with human serum should be avoided.

## 6.5 Concluding remarks

The prevalence of type 2 diabetes is increasing in all age groups and increased morbidity associated with this disease threatens a considerable number of people (King et al., 1998; Saha et al., 2003). During the last decades, the mechanisms leading to the development of insulin resistance and type 2 diabetes have been intensively studied. This is a challenging task since type 2 diabetes is a complex and multifactorial disease, which results from the interaction of genetic predisposition and environmental factors. To date, the mechanisms leading to insulin resistance and type 2 diabetes are still only partly understood.

In this study, we investigated the catalytic subunit p110β of PI 3-kinase which is an important mediator of insulin signalling as a candidate gene for insulin resistance and type 2 diabetes. Our results suggest that the promoter polymorphisms of the p110β gene are not a major risk factor for insulin resistance and type 2 diabetes in Finnish subjects. The candidate gene approach is a valid method if one wishes to investigate the genetic background of insulin resistance and type 2 diabetes. However, due to the multigenic nature of these conditions, also other approaches should be utilized to clarify the mechanisms leading to insulin resistance and type 2 diabetes. In this thesis, we also devised *in vitro* methods that can be utilized in the studies of insulin signal transduction. Differentiated 3T3-L1 cells provide an optimal model to investigate the insulin signalling pathways and recombinant adenoviral vectors can be utilized as efficient gene transfer vectors in various cell types. In the future, studies utilizing RNA interference (Hannon and Rossi, 2004), cDNA microarray technology (Kapranov et al., 2003) and proteomics are likely to provide additional insight into the changes in the gene expression and protein structure that eventually lead to the development of insulin resistance and type 2 diabetes. In addition, animal models are important for testing the novel hypothesis *in vivo*.

During recent years, our understanding of the endocrine function of adipose tissue has increased significantly. It has become evident that adipose tissue has a crucial role in the development of insulin resistance and type diabetes. The future challenges are to obtain a deeper understanding of the relevance of altered secretion of adipokines and fat topography to insulin resistance and β-cell dysfunction.

# 7 SUMMARY

The central purpose of this work was to investigate the catalytic subunit p110β of human PI 3-kinase as a candidate gene for type 2 diabetes. In addition, two important tools in the field of diabetes research, i.e. the 3T3-L1 cell line and recombinant adenoviral vectors were characterized.

In Study I, the genomic structure of the gene encoding the catalytic subunit p110β of PI 3-kinase was determined by cloning the gene from a genomic library. This was followed by the screening of all exons and 1.5 kb of the promoter in samples of subjects with type 2 diabetes. Two promoter polymorphisms, –359T/C and –303A/G, were identified. The allele frequencies of these polymorphisms did not differ between diabetic and control subjects. Thus, the promoter polymorphisms of the p110β gene are not likely to be major risk factors for type 2 diabetes.

In Study II, we showed that the p110β promoter polymorphisms –359T/C and –303A/G were not associated with insulin secretion or insulin sensitivity in normoglycemic Finnish subjects.

In Study III, 3T3-L1 fibroblasts were differentiated into adipocytes. Insulin activated the PI 3-kinase/Akt and MAPK signal pathways and significantly increased cellular 2-deoxyglucose uptake. Therefore, differentiated 3T3-L1 cells can be utilized as an *in vitro* model to investigate insulin signal transduction.

In Study IV, the preincubation of recombinant adenoviruses at +37°C increased significantly the viral transduction efficiency into CAR-deficient cells. Viral constructs maintained their infectivity during a 2-hour incubation at 0°C and in the presence of FBS, whereas human serum inactivated the adenoviral infectivity in 30 s. Therefore, this study provides techniques to optimize the gene transfer protocol to achieve maximal adenoviral transduction efficiency.

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