Anti-Diabetic and Beta-Cell Protective Actions of Imatinib Mesylate

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Dissertation presented at Uppsala University to be publicly examined in B21, BMC, Uppsala, Tuesday, September 26, 2006 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract

Type 1 diabetes is a disease resulting from the progressive immune-mediated destruction of insulin producing β-cells. In order to understand more about diabetes we need to understand the mechanisms governing β-cell death.

The leukemia drug Gleevec is a tyrosine kinase inhibitor that targets c-Abl. Surprisingly, Gleevec also counteracts Type 2 diabetes and acts as a cell death inhibiting agent, via inhibition c-Abl. Since both Type 1 and Type 2 diabetes are characterized by an increased β-cell death, and the role of c-Abl is unknown in β-cells, we wanted to investigate the following:

1. Does Gleevec act via inhibition of c-Abl in β-cells?
2. Can Gleevec treatment prevent beta-cell death and diabetes?
3. Which downstream signaling pathways are affected by Gleevec?

In paper I, in order to determine whether Gleevec acts by inhibiting c-Abl, we used RNA-interference. Interestingly, siRNA against c-Abl produced by recombinant Dicer mediate almost complete and non-toxic silencing of c-Abl mRNA in dispersed islet cells and conferred protection from streptozotocin and cytokines.

In paper II we show that Gleevec protects β-cells from nitric oxide, pro-inflammatory cytokines and streptozotocin in vitro and that Gleevec can prevent diabetes development in the NOD mouse and the streptozotocin-injected mouse. We also present the hypothesis that Gleevec induces a state resembling ischemic preconditioning.

Paper III presents an additional mechanism by which Gleevec might improve β-cell survival, i.e. via the inhibition of the downstream stress-activated protein kinase c-Jun N-terminal kinase (JNK), the activity of which has been implicated in β-cell death signaling pathways.

In paper IV we explore the interactions between the adaptor protein Shb and c-Abl. We presently show an association between Shb-c-Abl and that Shb is a substrate for the c-Abl kinase that might regulate stress-induced c-Abl activity.

Keywords: Diabetes

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ISSN 1651-6206
ISBN 91-554-6615-X
urn:nbn:se:uu:diva-7078 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-7078)
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<th>Full Form</th>
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<tbody>
<tr>
<td>Arg</td>
<td>Abl related gene</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell gene</td>
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<tr>
<td>BCR</td>
<td>Breakpoint cluster region</td>
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<tr>
<td>c-Abl</td>
<td>Cellular Abelson tyrosine kinase</td>
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<td>JNK</td>
<td>C-Jun N-terminal kinase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
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<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>IFN-γ</td>
<td>Interferon-γ</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
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<td>IRS</td>
<td>Insulin receptor substrate</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MLDS</td>
<td>Multiple-low dose model of STZ</td>
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<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>NLS</td>
<td>Nuclear localization signals</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<td>NOD mouse</td>
<td>Non obese diabetic mouse</td>
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<td>NF-kappa B</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>PARP</td>
<td>Poly ADP-ribose polymerase</td>
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<tr>
<td>PDGFR</td>
<td>Platelet derived growth factor receptor</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinases</td>
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<tr>
<td>RISC</td>
<td>RNA induced silencing complex</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>Src homology 2/3 domain</td>
<td>SH2/3</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transduces and activators of transcription</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
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</table>
Introduction

Autoimmunity

Auto-immune disease is characterized by an immune mediated attack on the endogenous tissue. It is still not known why the immune system performs this devastating error of attacking endogenous tissues, which potentially could jeopardize the existence of the whole organism. The prevailing dogma in immunology is that discrimination between self and non-self initiates immune responses to something foreign to the organism. However, the danger model suggests that the immune response is not initiated in response to something foreign to the organism, but in response to something that signals danger (Matzinger P 2002). The danger model is based on the idea that the controlling signals are endogenous alarm signals from stressed cells or injured tissues. This alarm signal in turn switches on the local antigen presenting cells (APCs) in the tissue, so they are now able to offer co-stimulatory signals to lymphocytes and hence initiate an immune response. The difference between the self / non-self model and the danger model is thus that the danger model discriminates between antigens, not between self or non-self, but also between antigens that are associated with danger and those that are not. Auto-immunity might actually be easier to understand using the danger model, since auto-immunity really is an immune response to the self (Matzinger P 2002).

One idea is that auto-immunity is in fact the by-product of an immune response to a viral or bacterial infection. Attempts to explain auto-immunity using this hypothesis falls into two categories; epitope mimicry and bystander activation. Epitope mimicry is defined as: An antigenic determinant on one of the microbe’s proteins is structurally similar to a determinant on one of the proteins made by the host, although different enough to be recognized as foreign by the host’s immune system (Benoist C 2001). This concept has yet not been entirely proven to be true in man; the two most convincing examples of epitope mimicry are the antibiotic resistant Lyme arthritis, which is an inflammatory joint disorder that resembles reumatoid arthritis and the herpetic stromal keratitis (Benoist C 2001). Bystander activation on the other hand, is antigen non-specific and, the endogenous target just happens to stand in harms way when the immune system is attacking a nearby infection of some sort (Benoist C 2001). Destruction of endogenous
cells could then be the result of non-specific mediators, such as cytokines in the pro-inflammatory milieu created by the presence of an infectious agent.

**Diabetes mellitus**

The name diabetes mellitus stems from the Greek words diabetes (passage) and mellitus (honey), thus meaning that diagnosis of this group of diseases can be based on the sweet taste of the urine from the patient. Diabetes is broadly classified in two categories, type 1 and type 2 diabetes. In 1971 came the first demonstration that Type 1 diabetes is an autoimmune disease (Nerup J 1971), in which the insulin producing \( \beta \)-cells of the pancreas are destroyed by the immune system. This results in a complete lack of insulin in the body and as a result hyperglycemia. Insulin injections is the standard treatment of type 1 diabetes, but in severe cases when maintenance of normo-glycemia is difficult, transplantation of islets into the liver is emerging as an alternative method (Shapiro J. 2001). The etiology of type 1 diabetes is presently not known, but is thought to be multifactorial. There is a genetic component that determines the susceptibility to develop type 1 diabetes. The human leukocyte antigen (HLA) class II genes are the genes most closely linked to the disease. However only < 5% of HLA susceptible individuals actually develop the disease (Virtanen S 2003). Furthermore, the concordance rate is only 43% in identical twins (Virtanen S 2003), which suggests that besides having a disease prone phenotype, an environmental signal such as a viral infection or a dietary component may be required for disease development. Evidence that the environment is important for disease development comes in part from the observation that the incidence of type 1 diabetes has increased the last 30-50 years, which indicates an augmented environmental pressure (Honeyman M 2005).

Type 1 diabetes is a slowly developing disease and the first sign of an auto-immune reaction is the occurrence of antibodies against \( \beta \)-cells. These auto-antibodies can function as a predictor of disease development (Lernmark Å 2004). Several types of viruses have been linked to type 1 diabetes development, although evidence for a viral infection during the pre-clinical period is lacking (Honeyman M 2005). It is also not known if there is a sporadic single infection or repeated infections with a ubiquitous virus associated with type 1 diabetes in genetically prone individuals.

An early observation indicating that a dietary factor could be involved in the pathogenesis of type 1 diabetes was reported by (Baum J 1975). They reported that accelerated weight gain during infancy predisposes for type 1 diabetes. The mechanism is not clear, but could involve an increased insulin demand, and it has been shown that \( \beta \)-cells with a high secretory rate are more susceptible to pro-inflammatory cytokines and present antigens more efficiently (Virtanen S, Knip M 2003). A possible link between gut distur-
bances, such as enteroviruses or dietary antigens and βeta–cell autoimmunity, stems from the recent observation that antigens from the gastrointestinal tract can be presented at the pancreatic lymph nodes, hence inflammatory stimuli from the gut could make their way to and activate antigen-presenting cells at the pancreatic lymph nodes (Turley S 2005).

Regardless of why the auto-immune attack is initiated, we must try to elucidate how β−cells respond to stress. It has been proposed that the signaling dynamics of the β−cell must be disturbed for the unknown trigger to result in β−cell destruction (Freiesleben de Blasio, B 1999). Therefore, greater knowledge of damage-induced intracellular pathways in the β-cell might help us to achieve a better understanding and thus opening new options for treatment of type 1 diabetes.

Pro-inflammatory cytokines

In animal models of type 1 diabetes, the immune cell mediated destruction of βeta−cells is thought to be dependent on three factors: Firstly, a feedback circuit of antigen transport / presentation / recognition between APC’s and T-lymphocytes, secondly the amount and type of cytokines produced by these cells, and thirdly the β−cell defense capacity (Freiesleben de Blasio, B 1999). Macrophages and T-cells in the chronic inflammatory lesion around the islet (insulitis) produces the pro-inflammatory cytokines interleukin-1 βeta (IL-1 β), interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). There is extensive evidence that these cytokines cause destruction of human β-cells in vitro (Mandrup-Poulsen T 2001). It is therefore likely that the β−cell, by converting external death signals to internal apoptotic events, participates actively in its own destruction in type 1 diabetes.

Intracellular signaling in response to pro-inflammatory cytokines

The signaling steps elicited by IL-1 β and IFN-γ have been studied in detail. First the cytokine binds to and activates specific receptors, from which the signal transduction pathways involving cytosolic protein kinases (PKC, JNK, p38, ERK, MSK1 and various transcription factors (NF-kappa B, ATF-2, c-Jun, Elk-1, CREB, cEBP-β, IRF-1, STAT-1) (Eizirik D 1996, Welsh N 1996, Larsen C 1998, Heitmeier M 1999, Saldeen J 2001). In the final step these transcription factors will lead to alterations of the β-cell gene transcription network, that has been presented in detail elsewhere (Cardozo A 2001).
One significant pathway inducing rodent β-cell death following cytokine exposure is the NF-kappa B–iNOS-NO pathway leading to an increased NO production. NO in turn, has many detrimental effects on the β-cell, including decreased ATP levels via the inhibition of aconitase, DNA damage induced activation of poly ADP-ribose polymerase (PARP) and the ensuing drop in cellular nicotinamide adenine dinucleotide (NAD) (Mandrup-Poulsen 2001). It has also been proposed that NO can elicit the ER-stress response (Cardozo A 2005). Interestingly, although apoptosis has been described as the main mode of β-cell death in type 1 diabetes, necrosis is also observed and is probably related to the production of NO, because it is absent in iNOS K.O animals. (Eizirik D 2001). Cell death by necrosis may be pathophysiologically relevant as a necrotic cell is a strong inflammatory stimulus.

However, it is also clear that there are NO-independent signaling pathways that are activated in response to cytokines, and these may be the most pertinent to human β-cell death. The NO-independent part relies on the activation of the mitogen activated protein kinase (MAPK) signaling cascade. Members of the MAPK family includes the c-Jun NH2-terminal kinase (JNK) and p38. JNK is known to be activated by IL-1β alone and is further potentiated by IFN-γ and TNF-α (Eizirik D 2001). Interestingly, inhibition of JNK has been shown to decrease cytokine-induced apoptosis in insulin producing cells (Bonny C 2001). JNK is not only induced by cytokines but also by UV-radiation, growth factor deprivation and DNA damaging agents. It should be emphasized that MAPK signaling usually occurs with simultaneous activation of both pro- and anti-apoptotic pathways, which are regulated at multiple levels. Therefore it not surprisingly that JNK is also activated to a lesser extent by survival signals, such as serum and growth factors (Kyriakis J, Avruch J 2001). This strengthens the notion that it is the net balance of the various MAPK family members activation that determines the cellular response to a particular stimulus.

Animal models of type 1 diabetes
The inbred non-obese diabetic mouse (NOD) is the most used animal model for autoimmune type 1 diabetes. The cumulative diabetes incidence in female NOD mice varies between 60-80 % at 30 weeks of age. Also male mice develop the disease to a lesser extent or perhaps more slowly, reaching 10-20 % at 30 weeks of age (Pozzilli P 1993). Insulitis, which is composed mainly of macrophages, CD4+ and CD8+ T-cells is a prominent finding in the prediabetic NOD mouse, and it is thought that these cells are responsible for the killing of β-cells (Jansen A 1994), (Reddy S 1995). Cytokines have been suggested to be important in the NOD model, and it has been shown that IL-1 receptor antagonists can inhibit the recurrence of disease after syngeneic islet transplantation to diabetic NOD mice (Sandberg J 1997). Apoptosis is
considered to be the main mode of β-cell death in the NOD mouse and its onset precedes the insulitis (O’Brien B 1997). Therapies that can prevent or delay diabetes onset are numerous, most of which are targeting the immune system, either being immunosuppressive or aimed at restoring normal immuno-regulation. It should be noted that the NOD mouse is a highly inbred strain and should therefore be viewed as a single case study that does not represent the heterogeneity of the type 1 diabetes development in the human situation (Atkinson M 1999).

Streptozotocin (STZ) is a naturally occurring antibiotic isolated from Streptomyces achromogenes (Vavra J 1959). In 1962 it was discovered that STZ had a diabetogenic effect in rats and dogs. It was also discovered that STZ treatment led to a specific disruption of the islets of Langerhans (Rakieten N 1963). The STZ molecule consists of a glucose moiety and a N-nitrosomethylurea side chain. Due to the former, STZ is rapidly taken up by the glucose transporter-2 on β-cells (Schnedl W 1994). Upon cellular entry, STZ is rapidly decomposed, producing free radicals and DNA alkylations (Ohkuwa T 1995, Murata M 1999). The DNA damage in turn activates PARP, which consumes NAD. The reduction of cellular NAD in islets treated with STZ (Yamamoto H 1981) leads to the impairment of glucoseoxidation and protein synthesis (Gunnarsson R 1974), with ensuing β-cell death, but without any inflammation (Rakieten N 1963). The diabetogenic effect of STZ is reduced by either radical scavengers (Robbins M 1980, Sandler S 1982) or PARP-inhibitors (Schein P 1967). Later it was discovered that multiple low dose injections of STZ in susceptible mouse strains produced a diabetic state with islet inflammation more resembling human type 1 diabetes (Like A, Rossini A 1976). This treatment regimen, with injection of five consecutive low STZ doses results in enough β-cell damage to attract the immune cells and cause insulitis, which then is responsible for the progressive β-cell destruction and diabetes development (Bonnevie-Nielsen V 1981).

Protein tyrosine kinases in cellular signaling

Protein tyrosine kinases (PTK) are enzymes that catalyze the transfer of phosphate groups from ATP to tyrosine residues on substrates. They can be broadly divided into receptor and non-receptor tyrosine kinases. The human genome encodes only 90 PTKs and they participate in the regulation of many functions of a multi-cellular organism, such as growth, differentiation, adhesion, motility and death (Robinson D 2000). The important function of PTKs in the regulation of cellular growth is emphasized by the fact that many of them are oncogenes, and inhibitors of oncogenic forms of PTK are emerging as important drugs for the treatment of human malignancies, recently reviewed by Noble M in 2004.
PTKs are typically not phosphorylated in the resting state and tyrosine phosphorylation of the kinase region helps to activate the kinase. Receptor tyrosine kinases respond to extracellular factors upon which they usually dimerize and auto-phosphorylate several tyrosine residues. Phosphotyrosine residues bind with high affinity to Src homology 2 (SH2) and phospho-tyrosine binding (PTB) domains, thereby creating docking sites for various proteins containing these domains. Signaling complexes are thus formed and we can view the PTK as a platform for recognition and recruitment of signaling proteins (Pawson T 1993). The outcome of a signaling cascade initiated by a growth factor ligand acting on a receptor tyrosine kinase is activation of transcription factors and ultimately alterations in gene expression. In order to keep the activity of PTKs under control, there are multiple levels of signal down-regulation, including receptor antagonists, soluble receptors, receptor endocytosis, ubiquitination and degradation, phosphatases and negative feedback loops.

The importance of PTKs in β-cell survival: The insulin signaling pathway via its PTK receptor and the adaptor protein family IRS (insulin receptor substrates), is both proliferative and anti-apoptotic in β-cells (Kulkarni R 2002). β-cell specific insulin receptor knock-out animals fails to increase their β-cell mass over time (Kulkarni R 1999) and inactivation of the IRS-2 gene reduces the number of β-cells (Withers D 1999), suggesting a significant role for insulin signaling in β-cell survival. Moreover, increasing the IRS-2 expression in β-cells can prevent streptozotocin-induced β-cell apoptosis (Hennig A 2003, Linghor M 2003).

Pro-inflammatory cytokines, such as IFN-γ, signals via JAK, a non-receptor PTK, which in turn activates signal transduces and activators of transcription (STAT). STAT acts on the iNOS gene, which sensitizes the β-cell for IL-1 (Heitmeier M 1999). Moreover, inhibition of the IFN-γ activated JAK/STAT pathway prevents diabetes in an animal model of type 1 diabetes (Flodström-Tullberg M 2003). Another PTK that is implied in β-cell destruction is the Src family member FRK. Overexpression of FRK in β-cells lead to an increased sensitivity to a combination of IL-1 and IFN-γ (Annerén C 2003).

Cellular Abelson tyrosine kinase

The cellular Abelson tyrosine kinase (c-Abl) is a ubiquitously expressed non-receptor tyrosine kinase and its small sub-family consists of only c-Abl and the Abl related gene (Arg). The N-terminal part of c-Abl resembles the Src family of tyrosine kinases and contains an SH3, an SH2 and a kinase domain. Unique to this family of PTK’s is the long carboxy-terminal extension. The complex structure of c-Abl protein comprises interaction domains (SH2, SH3, proline-rich sequences and phosphorylation sites), three nuclear
localization signals (NLS), an nuclear export signal (NES) that allow transport in and out of the nucleus, an actin binding domain for interaction with cytoskeletal components and a DNA-binding domain. Recent data suggest that the equilibrium between nuclear import and F-actin binding domain dependent cytoplasmic retention, decides the subcellular distribution of c-Abl (Hantschel O 2005). It was also recently discovered that acetylation of a lysine residue in a NLS region of c-Abl promotes accumulation of c-Abl in the cytoplasmic compartment (di Bari G 2006).

The structure of c-Abl infers that this protein can sense and integrate information from multiple signaling pathways in different cellular compartments and then interact with downstream effector proteins. It has been suggested that c-Abl best is described as a capable slave that can act upon and regulate a variety of biological responses (Wang J 2004). The outcome of c-Abl activation can be very diverse, depending on initial stimuli, cell type and cellular location of c-Abl. Abl-deficient mice are undersized, have shortened survival, defective spermatogenesis, lymphopenia and show eye malformations (Kharbanda S 1998), while dual c-Abl and Arg knock-outs die on day ten of embryogenesis (Koleske A 1998). This further points to an important role for c-Abl in a variety of cellular processes.

Under normal conditions the kinase activity of c-Abl is under tight control, and like most PTKs c-Abl is activated by phosphorylation at tyrosine 412. It has been shown that Src-family PTKs activate c-Abl (Plattner R 1999). A cytoplasmic adaptor protein PSTPIP1 forms a complex with c-Abl and a phosphatase, a PEST-type PTP that in this complex can dephosphorylate and inactivate c-Abl (Cong F 2000). Besides inactivation by de-phosphorylation, the activated pool of c-Abl is much less stable than the inactive form, due to degradation in the proteasome (Echarri A 2001). However, maintaining c-Abl in an inactive state is an important mechanism to keep c-Abl activity low. It appears that the c-Abl kinase activity is inhibited both through auto-inhibition and via binding of proposed cellular inhibitors such as Rb and Pag in trans (Pluk H 2002, Wang J 2004).

When cells are exposed to different forms of damage, c-Abl becomes highly activated, which leads to cell cycle arrest and apoptosis. Genotoxin-induced apoptosis seem to require nuclear c-Abl (Kharbanda S 1995) whereas apoptosis in response to oxidative stress and endoplasmatic reticulum (ER) stress is mediated by cytosolic c-Abl (Sun X 2000, Ito Y 2001). C-Abl is activated by phosphorylation from Ataxia-telangiectasia mutated (ATM), DNA-dependent protein kinase or PKC-delta, and this leads to activation of the stress-activated protein kinases (JNK and p38 MAP-kinases) and the tumor suppressor p73 (Raina D 2002, Wang J 2001, Choi S 2006). In addition, anti-apoptotic pathways, such as NF-κB, are inactivated (Kawai H 2002). Interestingly, a substantial proportion of c-Abl seems to reside in the ER under normal conditions. It is not until cells are subjected to ER stress
that c-Abl is targeted to the mitochondria and promotes cell death (Ito Y 2001).

Overexpression of c-Abl neither releases its oncogenic potential nor leads to an increased level of activity (Brasher B 2000), but there are two well characterized oncogenic forms of c-Abl that have escaped the auto-inhibition, v-Abl and BCR-Abl. Both forms are strictly located to the cytoplasm.

V-Abl stems from the insertion of the retrovirus Moloney murine leukemia virus into the second exon of the c-Abl gene. This results in the loss of the N-terminal cap and the SH3 domain (Wang J 1984), which are necessary for auto-inhibition. Instead they are replaced with the viral gag peptide whose function is to link v-Abl to the cell membrane via myristoylation (Schultz A 1984), which is essential for transformation. Various signal pathways are hyperactivated by v-Abl, including Ras, JAK / STAT, JNK, ERK and PI3K (Shore S 2002).

BCR-Abl fusion protein is the result of a fusion of the breakpoint cluster region (BCR) and c-Abl gene (termed the Philadelphia chromosome). Dysregulation of BCR-Abl is due to a coiled-coil domain in BCR that leads to oligomerization of BCR-Abl and subsequent inter-autophosphorylation by Abl kinase that disrupts the auto-inhibited conformation (Smith K 2003). BCR-Abl seems to be constrained by the same set of auto-inhibitory functions as the normal c-Abl kinase, but it functions at a higher level of activity (Hantschel O 2004).

Gleevec (Imatinib mesylate, STI571)

Developed by Novartis, Gleevec is a pharmacological PTK inhibitor (Druker B 1996). Gleevec is known to specifically inhibit the PDGF receptor, c-KIT, Arg and c-Abl. PTK inhibitors typically bind to the well-conserved nucleotide-binding pocket of these kinases. Also Gleevec targets this pocket, yet its specificity is high, which can be explained by that it also interacts with the inactive conformation of the activation-loop (Schindler T 2000). In c-Abl the SH2 domain is bound directly to the kinase domain, whereas in Src the SH2 binding is phosphotyrosine dependent, which places the SH2 domain further away from the kinase domain (Nagar B 2006). It appears that the ability of Gleevec to inhibit the Abl family of proteins depends on the activation state of the kinase in a way that is not yet defined (Roumiantsev S 2002).

Gleevec has its greatest use in the treatment of malignancies such as chronic myeloid leukemia, where 95% of all cases are due to the BCR-Abl oncogene (Roumiantsev S 2002), and in intestinal stroma cell cancer, which is caused by a c-KIT mutation. Besides its anti-proliferative effect in cancer treatment, studies using cell lines describe that Gleevec also can inhibit cellular death. It has been shown that Gleevec can protect cells from genotoxic
agents, death receptor activation and oxidative stress (Raina D 2002, Dan S 1999, Kumar S 2003). The protective effect observed in these studies can be explained by inhibition of c-Abl activity. Numerous studies have also explored the potential of Gleevec in other diseases, for example using an animal model of diabetes-induced arteriosclerosis, Gleevec has been shown to be an anti-inflammatory agent, and to prevent β-amyloid production by inhibiting the protease g-secretase (Lassila M 2004, Netzer W 2003). The anti-inflammatory effect of Gleevec in a model of hepatic inflammation could perhaps be explained by inhibition of macrophage-derived TNF-α production (Wolf A 2005). Furthermore, Gleevec attenuated PDGFR-induced phosphorylation of the low density lipoprotein receptor-related protein (LPR) (Loukinova, E. 2002), and improved the lipoprotein metabolism (Gottardi M 2005), which in turn might lead to protection against atherosclerosis (Boucher P 2003).

In the context of diabetes, the most interesting finding using Gleevec was that a small number of patients, suffering from both chronic myeloid leukemia and Type 2 diabetes, were successfully treated for not only their leukemia, but also for diabetes, when given Gleevec (Veneri D 2005, Breccia M 2004).

RNA interference

RNA interference (RNAi) is a biological process leading to the specific post-transcriptional silencing of genes via degradation of their mRNA. A four step model which describes RNAi has been proposed; in step one the enzyme Dicer mediates ATP dependent cleavage of double-stranded RNA (dsRNA) into 21-23 nucleotides long fragments (siRNA) that bind to the RNA induced silencing complex (RISC) in step two. Step three is the ATP dependent unwinding of the siRNA and in step four one of the siRNA strands is incorporated in RISC, which cleaves the targeted mRNA. (Hutvagner G 2002). The phenomenon of RNAi was first observed in C. Elegans (Fire A 1998) and is now commonly used for post-transcriptional silencing of genes both in vitro and in vivo (Elbashir S 2001, Lewis D 2002). RNAi has been shown to play an important function in development, transposon repression and as an anti-viral defense (Hutvagner G 2002).

The most widespread technique to achieve gene silencing in mammalian cells is via pre-designed synthetic siRNAs, homologous to the targeted mRNA, thus bypassing the Dicer-mediated cleavage. The reason for this is that transferring longer dsRNA molecules into mammalian cells activate the interferon response (Caplen N 2002), leading to a halt in protein synthesis and unspecific mRNA degradation. Interestingly, the use of siRNA-targeting BCR-Abl in a BCR-Abl expressing cell line led to silencing of the oncogene.
and a subsequent increase in apoptosis, which was comparable to the effect of Gleevec (Wilda M 2002).

The use of synthetic siRNA molecules is a costly and time consuming technique, because not all siRNAs mediate efficient cleavage of the mRNA. Therefore usually three to four different siRNA sequences needs to be evaluated. What governs if a sequence is efficient or not is poorly understood, but may be related to the thermodynamic features of the siRNA (Khvorova A 2003). Eight characteristics associated with siRNA functionality have recently been described, which will hopefully make design easier in the future (Reynolds A 2004). However, the problem of finding the optimal siRNA sequence can be circumvented using the RNase III like enzyme Dicer. In a recent report, the enzymatic activity of recombinant Dicer was utilized to obtain in vitro cleavage of dsRNA, yielding a pool of Dicer-generated siRNA sequences (d-siRNA), which targets the entire mRNA for degradation (Myers J 2003).

There are, however, some limitations using siRNA: reports hint to off-target (Jackson A 2003) or transient effects (probably due to siRNA degradation). To overcome these obstacles, several groups have used plasmid-based system using RNA polymerase III promoters to drive synthesis of short hairpin RNA molecules (Sui G 2002) that are converted to siRNA in the cell. With the use of viral vectors it is possible to get an even longer effect, for example there are reports of lentiviral mediated RNAi (Rubinson D 2003). This approach has also the advantage of mediating efficient transduction of non-replicating cells, such as islet cells.

The adaptor protein Shb

Adaptor proteins contain only interaction domains and lack intrinsic catalytic activity. The assembly of these interaction domains within one specific protein allows it to bind multiple proteins. Interaction domains can perform various functions; target proteins to specific subcellular locations, recognize post-translational modifications, form signaling complexes and help to control the activity of enzymes (Pawson T 2003). The ubiquitously expressed adaptor protein Shb was originally identified in a β-cell line expressed as two isoforms of 55 and 67 kDa (Welsh M 1994). Shb contains several interaction domains, an N-terminal proline rich region, a central PTB domain, four potential tyrosine phosphorylation sites and a C-terminal SH2 domain (Welsh M 1994). It has been shown that PDGF, FGF-2, NGF, CD3 and IL-2 can induce tyrosine phosphorylation of Shb (Lindholm C 1999, Hooshmand-Rad 2000, Claesson-Welsh 1998, Lindholm C 2002) and the SH2 domain of Shb can bind phosphorylated PDGF receptor, FGF receptor 1, T-cell receptor, GH receptor, IL-2 receptor (LL 64, (Hooshmand-Rad 2000, Lindholm C 2002, Karlsson T 1995, Cross M 2002, Moutoussamy S 1998). Shb has been
shown to be involved in T-cell receptor signaling, via the generation of multi-protein signaling complexes that associate with the TCR (Lindholm 1999). It was recently demonstrated that Shb plays a critical role in VEGF receptor 2 mediated signaling (Holmqvist K 2004). The role of Shb in cell death signaling has also been under investigation; it appears that Shb overexpression increases apoptosis in NIH 3T3 cells cultured in low serum concentration (Karlsson T 1996). Shb overexpression under control of the rat insulin promotor leads to an enhanced β-cell death under stress conditions including an increased sensitivity to MLDS (Welsh M 1999). Since the discovery of Shb, several Shb–like adaptor proteins have been cloned, among them Shd and She. Interestingly, these proteins were found in a yeast two hybrid screening, using the c-Abl kinase domain as a bait, which suggest that the SH2 domain of the adaptor proteins can bind phosphorylated tyrosine residues in the c-Abl kinase domain (Oda T 1997). In addition, the tyrosine phosphorylation sites of both Shb and Shd share the YXXP motif, which is a c-Abl preferred site for phosphorylation. Shd was also found to be tyrosine phosphorylated by c-Abl, but not a kinase dead version of c-Abl, when these proteins were co-overexpressed in COS cells (Oda T 1997).
Methods

The methodology of this thesis is hereby in this section explained in general terms. For more detailed descriptions, see individual papers.

Animals
Male NMRI (Naval Medical Research Institute-established) mice were purchased from Mölle & Bomholt gård, Denmark. Sprague-Dawley rats and NOD mice were from local colonies at Biomedical Center, Uppsala, Sweden. All animals were kept under standard pathogen free condition, with free access to tap water and pelleted food. All experiments were approved by the animal ethics committee at Uppsala University.

Cell culture
C2C12 mouse skeletal myoblasts were cultured in DMEM High Glucose supplemented with 10% FBS. To differentiate C2C12 myoblasts into myotubes, cells were permitted to reach 80% confluence and the serum concentration was reduced to 2% horse serum. Murine βTC-6 cells at passage numbers 20-30, COS and RAW 264.7 cells were maintained in DMEM + 10% FCS and antibiotics. INS-1 832/13 cells (kind gift from Dr. Hindrik Mulder, Lund, Sweden) were grown in RPMI 1640 + 10% FCS, supplemented with 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol and antibiotics. Cells were maintained at 37°C in humidified air + 5% CO₂.

Islet isolation and culture
Isolated human pancreatic islets were kindly provided by Professor Olle Korsgren at the Department of Radiology, Oncology and Clinical Immunology at Uppsala University Hospital, Uppsala, Sweden.

Islets from male NMRI mice and Sprague-Dawley rats were isolated by a collagenase digestion procedure (Sandler S 1987) and pre-cultured free-
floating in the RPMI 1640 culture medium given above. Culture medium was changed three times per week. Local human and animal ethics committees at Uppsala University approved all experiments.

STZ treatment in vivo

Male NMRI mice were divided in groups and in the afternoon injected with 0.2 ml freshly dissolved STZ in 0.9% NaCl into the tail vein. The mice received either gavage with 0.9% NaCl or Gleevec in the morning for three consecutive days starting one day before the STZ injection. Gleevec was administered 2 h before STZ when given the same day. Blood glucose levels were determined by blood samples from the tail tip.

Development of insulitis and diabetes in NOD mice

NOD mice were gavaged with either saline or Gleevec from three weeks of age, i.e. after weaning, to nine weeks of age to study the occurrence of insulitis. To study the development of diabetes, female NOD mice were treated as above between nine and 35 weeks of age. Blood glucose levels were measured every other week until the first sign of hyperglycemia, then weekly. Animals were considered diabetic and sacrificed upon two consecutive days of blood glucose > 12 mM.

Pancreases were fixed, dehydrated and embedded in paraffin. Sections were stained with haematoxylin and eosin or stained for insulin. Insulitis was scored on sections from two different pancreatic locations according to an arbitrary scale. The examiner of the sections was unaware of the origin of the sections.

Evaluation of islet viability:

Islets were vital stained with propidium iodide and bisbenzimide for 15 min at 37°C. After careful washing, islets were placed on coverslips and examined and photographed by fluorescence microscopy. Total number of cells as well as necrotic and apoptotic nuclei were counted.
Analysis of medium nitrite content and determination of insulin release:

Islets were cultured in the presence or absence of Gleevec overnight before addition of cytokines. The Griess reagent was added to aliquots of culture medium. Absorbance was measured in a spectrophotometer at 546 nm. A cytokine combination of IL-1β, + IFN-γ + TNF-α was used to induce NO formation.

Islets in groups of 5 were pre-cultured overnight with 10 μM Gleevec and then incubated for 60 min in Krebs-Ringer Bicarbonate buffer (KRBH) containing 1.67 mM glucose. This was followed by another 60 min incubation in KRBH containing 16.7 mM glucose. Insulin medium concentration was determined using insulin ELISA.

Extraction of nuclear proteins and electromobility shift assay (EMSA).

INS-1 832/13 cells were treated with IL-1β (50 U/ml) for 20 min or 10 μM Gleevec for 180 min, after which the nuclear proteins were extracted. A 100-fold excess of non-labeled oligonucleotide was used as a negative control and p65 antibody was used for the supershift.

Immunoblotting:

INS-1 832/13 cells were washed with ice cold PBS and directly suspended in SDS-sample buffer with 2 mM PMSF, boiled for 5 min and separated on SDS-PAGE. Proteins were electrophoretically transferred to Immobilon filters. Filters were blocked in 5% bovine serum albumin or 2.5 % milk powder for one hour, after which they were probed with antibodies. After a brief wash with PBS + 0,1% Tween 20, membranes were probed with HRP-linked secondary antibodies for one hour and extensively washed. The bound antibodies were visualized using ECL (Amersham) and were quantified by densitometry.

Measurements of ROS and mitochondrial membrane potential:

INS-1 832/13 were treated with Gleevec in culture medium and subsequently labeled with DHCF-DA for 30 minutes. Cells were washed and trypsinized, and kept dispersed in an incubator for the rest of the experiment.
The fluorescent form of DHCF-DA, DCF, was measured using a fluorescence activated cell sorter (FACS) before and every 5 min up to one hour after the addition of STZ (2 mM).

For the analysis of mitochondrial membrane potential, human islets were labeled with 5 μM of the fluorescent probe JC-1 for 30 min at 37°C. Cells were then washed and trypsinized, followed by FACS analysis. JC-1 monomers and J-aggregates fluorescence on the 530 nm and 585 nm were analyzed. The ratio between the 585 and 530 nm signals was calculated to monitor changes in mitochondrial membrane potential.

Isolation of splenocytes and intracellular cytokine staining

After removal from the sacrificed mice, the spleens were punctured repeatedly with a pair of forceps to release the splenocytes. The cell suspension was centrifuged and re-suspended in 1 ml 0.19 M NH₄Cl for 10 min on ice to lyse the erythrocytes. The macrophage content was depleted by incubation of the cells in attachment culture dishes containing full RPMI 1640 culture medium for 1 h. Subsequently, the non-attached splenocytes were either incubated with Brefeldin-A or stimulated with a combination of Brefeldin-A + Phorbol 12-myristate 13-acetate + Ionomycin. The splenocytes were then pelleted, washed and fixed. Splenocytes were then pelleted, washed and permeabilized, followed by incubation with FITC-conjugated antibodies. The splenocytes were analyzed for 530 nm fluorescence using flow cytometry.

Transient transfections

Cultures of COS cells were maintained as described above in culture dishes, washed three times in serum free medium and transfected with 1 μg of each plasmid or empty vector, using Lipofectamine.

Immunoprecipitation and Western blot

COS cells were either left untreated or treated with pervanadate for 15 minutes at 37°C, after which the cells were washed three times with ice-cold PBS and subsequently lysed in lysis buffer. Nuclei were pelleted by centrifugation and extracts were incubated with either Shb or c-Abl rabbit polyclonal antibodies. Immune complexes were pelleted with Protein A Sepharose and washed three times in PBS, 1% Triton X-100 and once with H₂O.
Samples were then resolved by SDS-PAGE and transferred onto Immobilon filters. The blots were blocked and incubated with primary antibodies as indicated. Immunoreactivity was detected using horseradish peroxidase-conjugated secondary antibodies and ECL.

**Pull-down binding experiments**

Cell extracts from COS cells transiently overexpressing wild type Shb or Shb with one tyrosine residue mutated or c-Abl were added to aliquots of GST tagged fusionproteins immobilized on glutathione Sepharose beads. The samples were incubated, washed and resolved on SDS-PAGE as described above. The cells had been pre-treated with Calpain Inhibitor II and some groups also with pervanadate prior to lysis.

**Shb knockdown**

The conditional shRNA knockdown system was used as described (Ventura A 2004) to reduce Shb protein expression and correct insertion of the oligonucleotide was verified by DNA sequencing. Lentiviruses were made as VSV-G pseudotyped viruses as described (Mostoslavsky G 2005). βTC-6 cells were infected with pSico control or pSico-Shb. Analysis for green fluorescence revealed that more than 95% of the cells stably expressed GFP (virus derived). The βTC-6 Sico and Sico-Shb cells were then re-infected with an adenovirus carrying cre-recombinase. Efficient knockdown was verified by a 90 % loss of GFP expression due to cre-deletion of GFP-sequences and simultaneous activation of the U6 promoter (which drives shRNA expression) three to four days later.

**Viability**

COS-7 cells were transfected as indicated and at 32 hours, cells were treated (or not) with 0.25 mM H2O2 for 20 hours and subsequently analyzed for viability as indicated below. Alternatively, pSico or pSico-Shb cells were left untreated or infected with an Adenovirus encoding Cre recombinase. Four days later, groups of cells were pretreated (or not) with 10 μM ST1571 for 12 h, and were subsequently incubated with or without 10 microg/ml tunicamycin for 6 hours. Cells were then stained using propidium iodide (20 μg/ml) and bisbenzimide (5 μg/ml) (Sigma) for 10 min at 37°C to assess viability. Afterwards cells were washed, trypsinized, and cell viability was analyzed by flow cytometry separating for size and propidium iodide fluo-
rescence. Dead cells were mostly smaller than the healthy main cell population of untreated controls.

**Preparation of d-siRNA**

Primers for c-Abl mRNA were used for PCR amplification of mouse c-Abl mRNA rendering a 573 bp long cDNA. The PCR-fragment was purified by gel electrophoresis and subsequently in vitro transcribed as previously described (Myers J 2003). The dsRNA was then subjected to Dicer-mediated cleavage (Myers J 2003).

**Liposome-mediated transfection of free islet cells with d-siRNA**

Free islet cells were prepared by treating islets with trypsin. Trypsination was terminated by the addition of culture medium, and followed by DNAse treatment. Free islet cells were transfected with d-siRNA and Lipofectamine 2000 in Opti-MEM.

**RNA isolation and cDNA synthesis**

Total islet cell RNA was purified using the Ultraspec RNA reagent. cDNA was synthesized using reverse transcriptase M-MuLV and oligo-dT-primers. The obtained cDNA was purified with PCR-cleanup kit.

**Real-time PCR**

PCR reactions were performed using the Lightcycler instrument and the SYBR green Taq Ready Mix using β-actin and c-Abl primers. In some experiments, PCR-amplification was stopped during the exponential phase and PCR-products were analyzed by agarose gel electrophoresis and SYBRgreen staining.
Results

Paper I

In order to achieve a selective knock-down of mRNA molecules encoding proteins involved in β-cell stress responses, we evaluated the ability of different commercial liposome formulations to mediate uptake of FITC-labeled siRNA into βTC-6 cells. In three independent experiments, Lipofectamine mediated a transfection efficiency of 96.2±0.5%, which was the highest of all formulations examined. Second, we evaluated the efficiency of Lipofectamine-mediated transfection of islet cells. Not surprisingly, transfection was much more efficient in dispersed than in intact islets. Thus, in two separate experiments only 10.9±1.5% FITC-positive cells were observed after transfection of intact mouse islets. In contrast, Lipofectamine mediated uptake of siRNA in >90% of dispersed rat and mouse islet cells. We also tested the potentially less toxic Lipofectamine 2000 on human islet cells and found this formulation to be as effective as Lipofectamine.

Having established that Lipofectamine 2000 mediates efficient transport of siRNA into dispersed islet cells, we next attempted to silence the gene coding for the non-receptor tyrosine kinase c-Abl. Following Lipofectamine 2000-mediated transfection, c-Abl mRNA expression was monitored by semi-quantitative real time PCR at 1, 3 and 7 days post transfection. Quantification of c-Abl mRNA revealed that in mouse islet cells c-Abl mRNA expression was decreased at 1 and 3 days after transfection, but no effect of d-siRNA was observed at day 7, probably due to degradation of d-siRNA. Importantly, we did not observe increased cell death in response to d-siRNA as compared to liposomes alone.

Paper II

The recently developed PTK inhibitor compound Gleevec is known to protect against genotoxic agent-, death receptor activation- and hydrogen peroxide-induced apoptosis in various cell types via inhibition of the c-Abl kinase (Raina D 2005; Dan S 1999; Kumar S 2003). Using a screening procedure, we here observe that imatinib protects, at least partially also insulin producing cells, from various inducers of cell death. Importantly, we also observed
that treatment with imatinib potently prevented islet cell death induced by pro-inflammatory cytokines \textit{in vitro}.

In order to evaluate a possible protective effect of imatinib \textit{in vivo}, we injected STZ i.v and found that imatinib treatment p.o protected completely against hyperglycemia induced by the STZ injection. This finding suggests that imatinib prevented STZ induced \(\beta\)-cell apoptosis/necrosis \textit{in vivo}.

Next, we sought to investigate the downstream signaling events affected by imatinib in response to NO production, since NO formation following cytokine stimulation is known to be a major contributor of cytokine-induced \(\beta\)-cell death in rodents (Eizirik and Mandrup-Poulsen, 2001). We tried to determine MAPK activity, a system known to be influenced by NO formation (Welsh, 1996; Makeeva et al., 2006). Islets were analyzed for phosphorylation of p38, JNK2 and ERK1/2 using phosphospecific antibodies and immunoblotting. We observed that imatinib significantly decreased JNK2 phosphorylation in response to NO formation. There was also a trend to a lowered NO-induced activation of p38 and ERK1/2 as a result of the imatinib treatment, but it did not reach statistical significance.

In addition, we found that hydrogen peroxide-induced JNK phosphorylation in myotubes was diminished by imatinib and also that phosphorylation of the JNK substrate c-Jun was decreased.

\textbf{Paper III}

We wanted to investigate if Gleevec can prevent or delay disease development in the widely used NOD mouse model of type 1 diabetes. In order to study the development of auto-immunity during the pre-diabetic period, NOD mice were gavaged with Gleevec or with vehicle alone daily from 3-9 weeks of age. After sacrifice the extent of infiltration of immune cells into the pancreatic islets was scored according to an arbitrary scale on histological sections of the pancreases. We found no apparent difference in immune cell infiltration score between saline and Gleevec-treated mice. Next, to study if Gleevec could influence the increasing \(\beta\)-cell destruction leading to overt diabetes, we gavaged female NOD mice from 9 to 35 weeks of age with saline or Gleevec daily. At 35 weeks of age 4 out of 10 saline-treated mice had developed diabetes, whereas all 9 Gleevec-treated mice remained non-diabetic. We also scored the severity of insulitis and observed that Gleevec did not affect the extent of peri-insulitis or insulitis.

The possible immunomodulatory properties of Gleevec was further tested when we analyzed splenocyte production of IFN-\(\gamma\) and IL-10. Although there was a trend to increased production of IFN-\(\gamma\) and IL-10 in response to Gleevec, no statistically significant differences were observed. Also the \(\beta\)-cell area was similar in the 9 Gleevec-treated mice as compared to the 10 control mice. However, when comparing the 9 Gleevec mice with the 4 dia-
betic saline treated control mice, there was a significant larger β-cell area in the Gleevec treated animals. This suggests that the bulk of the β-cell destruction, which occurs as the NOD mice progress into overt diabetes, is counteracted by Gleevec. These data indicate that Gleevec may protect against diabetes in the NOD mouse affecting neither Th1/Th2-activities nor insulitis.

Using i.v administration of STZ and p.o treatment with Gleevec, a partial protection against the rise in blood glucose induced by STZ was found. As could be expected the β-cell area of mice treated with the high dose of STZ was markedly lowered as compared to control mice. Interestingly, this decrease was partially counteracted by Gleevec, which indicates that Gleevec protected against diabetes by preventing β-cell death.

We wanted to determine under which conditions Gleevec acts as a cell death inhibiting agent. For this purpose, we exposed human pancreatic islets to a NO-donor. The protective effect of Gleevec was statistically significant at 10 μM together with a 24 h pre-incubation period. Also, Gleevec did not affect islet viability by itself. Furthermore, when examining the effect of Gleevec in a dose-response experiment, we observed that Gleevec protects rat islets potently against a low (0.4 mM) concentration of STZ, but only weakly against the high (0.75 mM) concentration of STZ. This indicates that very high concentrations of STZ promote islet cell death by mechanisms not affected by Gleevec, possibly necrosis. Gleevec protected also against cell death induced by STZ in isolated human and mouse islets, probably dependent on c-Abl inhibition, since c-Abl d-siRNA treatment in dispersed islet cells also provided protection from STZ-induced toxicity. To further strengthen this hypothesis, we treated βTC-6 cells with either scrambled siRNA or siRNA specific for c-Abl and we observed a markedly decrease in c-Abl mRNA after 24 h. This RNAi approach also conferred protection from cytokine induced toxicity.

Our finding that a 24 h pre-incubation with Gleevec was necessary to promote protection against STZ, NO and cytokines, prompted us to investigate whether Gleevec induces a state similar to that of ischemic preconditioning. Indeed, Gleevec increased nitrite levels in the presence of cytokines. Our results show that the increased nitrite levels can be explained by an increased iNOS expression, and that Gleevec stimulates NF-κB signaling by enhancing the degradation of IκB. The importance of this NF-κB activation was shown when we blocked NF-κB activity using a synthetic NF-κB activation inhibitor (Tobe M 2003). Under these circumstances, Gleevec was unable to prevent cell death in response to NO-formation. Importantly, it was also demonstrated that Gleevec does not affect normal β-cell function, measured as the ability to release insulin in response to glucose stimulation.
COS cells transiently overexpressing Shb and c-Abl were immunoprecipitated for Shb. Western blot analysis revealed that c-Abl and Shb co-immunoprecipitate and that Shb becomes tyrosine phosphorylated when co-overexpressed with c-Abl, suggesting that Shb is a substrate for the c-Abl kinase. In the reciprocal experiment, the same lysates were immunoprecipitated using the c-Abl antibody. Indeed, Shb was found to co-immunoprecipitate with c-Abl under these conditions and in addition we observed that the tyrosine phosphorylation of c-Abl was increased by Shb overexpression. Transfection with wild type c-Abl (wt-Abl) resulted in decreased expression of c-Abl and Shb compared with transfection with kinase inactive c-Abl (kd-Abl). Shb tyrosine phosphorylation remained unchanged, however, after transfection with wild-type c-Abl and migrated with reduced mobility, indicating an increased relative Shb tyrosine phosphorylation.

Using Shb fusion proteins comprising the SH2 domain or PTB domain + proline rich region, we tried to pull down c-Abl. Shb GST-SH2 domain mediated pull-down of tyrosine phosphorylated c-Abl from pervanadate stimulated cells, and that this binding is phosphotyrosine specific, since it can be abolished by addition of free phosphotyrosine. In addition, we observe a constitutive and efficient association between the Shb GST-PTB domain + proline rich region and c-Abl. This c-Abl product is primarily unphosphorylated and its binding is not influenced by pervanadate or inhibited by free phosphotyrosine.

In order to try to pull down Shb, extracts of COS cells overexpressing Shb were incubated with GST-c-Abl-SH2+SH3. Indeed, the c-Abl-SH2+SH3 fusion protein specifically binds Shb. Using Shb mutants, in which one tyrosine residue had been mutated, we found that tyrosine 423 in Shb is the most important site for in vitro binding to the c-Abl SH2+SH3 domain since it showed the weakest association to the fusion protein. Also, it was observed that all tyrosine mutants displayed some degree of binding. This finding was strengthen in immunoprecipitation experiments, where all Shb mutants exhibit decreased binding to c-Abl compared with wild type control. Again, the Y423F-Shb mutant displayed the least efficient binding to c-Abl.

To assess whether Shb overexpression affects c-Abl activity, COS cells were transfected with c-Abl, c-Abl plus Shb and c-Abl plus Y423F-Shb. Transfection with c-Abl strongly increased c-Abl expression, but only caused a modest increase in pY245-Abl phosphorylation. Shb and c-Abl co-transfection reduced the amount of total c-Abl immunoreactivity. The pY245-Abl phosphorylation remained equally elevated, indicating that Shb increases the relative degree of c-Abl pY245-Abl phosphorylation. Co-transfection with the Y423F-Shb mutant that displays reduced c-Abl binding decreased pY245-Abl. In order to assess the functional importance of the c-
Abl/Shb complex, since both c-Abl and Shb independently have been shown to influence cell viability under various conditions, we evaluated cell viability in COS cells overexpressing Shb, c-Abl and Shb + c-Abl. As shown in Fig. 6, co-overexpression leads to substantially higher levels of spontaneous cell death. Treatment of these cells with hydrogen peroxide increased cell death further (Fig. 6). Again, c-Abl augmented the death response to Shb. Thus, it is conceivable that the c-Abl/Shb complex is part of a cellular stress response that increases cell death in response to increased ROS production. It then follows that disruption or inhibition of the c-Abl/Shb complex could protect the cells against cell death.

In order to silence Shb, we use a lentivirus-based expression system for short-hairpin RNA molecules directed against the Shb mRNA (Ventura A 2004). The lentiviral shRNA knockdown is Cre recombinase dependent, i.e. a Cre containing adenoviral vector (AdCre) induces the expression of the short hairpin RNA (shRNA), that is converted to short interfering RNA (siRNA) by the cellular machinery. Using GFP as a selection marker we were able to create populations of βTC-6 cells, which are insulin producing cells, transduced by lentiviruses coding for control (Sico) or Shb shRNA (Sico-Shb). The cells were then transduced with AdCre (10 MOI). Shb protein levels were reduced in the Sico-Shb cells upon AdCre virus addition. These cells were then treated with STI571 overnight, followed by exposure to cisplatin or tunicamycin. We observe that STI571 caused a slight reduction of cisplatin-induced death. Shb knockdown reduced cisplatin-induced cell death, and this rendered the cells unable to further respond to STI571. Shb knockdown reduced tunicamycin-induced cell death, an effect that was prevented by STI571 regardless of whether the Shb content had been depleted or not.
Discussion

Paper I, II, III

We here present the first report demonstrating efficient delivery and gene silencing of siRNA in primary islet cells. The high efficiency of liposome mediated siRNA delivery to dispersed islet cells may appear surprising considering that liposomes transfect islet cells with DNA-plasmids with low efficiency (Demeterco C 2001). However, DNA-plasmids need to be transported through the nuclear pore for expression, whereas siRNA needs only to reach the cytosol to silence gene expression. Indeed, liposomes are known to mediate efficient transfer over the plasma membrane, but not over the nuclear membrane (Zhdanov R 2002).

The presently observed decrease in c-Abl mRNA levels indicated a more or less complete knock-down of the messenger in the cells that had been transfected. Unfortunately, we could not determine whether the c-Abl protein levels were equally decreased, as c-Abl specific immunoreactivity could not be observed in islet cells using the immunoblot technique. Nevertheless, the efficient knockout of c-Abl using d-siRNA indicates that a pool of siRNA molecules mediates a more potent gene targeting effect than a single synthetic siRNA molecule. The d-siRNA technique will simplify the elucidation of gene function and promote understanding of β-cell stress-induced signaling components. One component which we believe might be crucial in this regard is the non-receptor tyrosine kinase c-Abl.

Inhibition of c-Abl using Gleevec is known to protect against genotoxic agent-, death receptor activation- and hydrogen peroxide-induced apoptosis in various cell types (Raina D 2005; Dan S 1999; Kumar S 2003). We observed that imatinib, at least partially, protected the islet cells from death induced by different toxins, which indicates that imatinib promotes survival by inhibiting a signaling event common for several pathways leading to β-cell death. Assuming that the activity of c-Abl is linked to death signaling in response to events such as ER stress, oxidative stress or a sustained elevation of the cytosolic Ca^{2+} concentration, it might be possible to block a multitude of death signals that participate in the pathogenesis of diabetes employing only one approach.

One death signal in Type 1 diabetes may be the pro-inflammatory cytokines IL-1β, IFN-γ and TNF-α (Eizirik and Mandrup-Poulsen, 2001). Inter-
Interestingly, we observed that imatinib potently prevented islet cell death induced by pro-inflammatory cytokines.

The activation of iNOS and ensuing NO formation in response to cytokine stimulation is known to be a major contributor of cytokine-induced β-cell death in rodents (Eizirik and Mandrup-Poulsen, 2001) and addition of either cytokines or NO-donors to islets cultured in vitro has been reported to activate the pro-apoptotic MAP kinases JNK and p38 (Welsh N 1996; Makeeva N 2006). In addition, in other cell types than β-cells activation of cytosolic c-Abl has been demonstrated to result in phosphorylation of the MAP and ERK Kinase-1 (MEKK-1), which in turn promotes MKK4 and JNK1/2 activation (Kharbanda S 2000).

We observed that imatinib significantly decreased JNK2 phosphorylation in response to DETA/NO. There was also a trend to a lowered DETA/NO-induced activation of p38 and ERK1/2 as a result of the imatinib treatment, but it did not reach statistical significance. The trend to a lowered p38 and ERK activation is line with the notion that imatinib acts at a site upstream of the MAP kinases, probably with c-Abl, rather than directly with JNK, ERK or p38. Thus, imatinib–mediated inhibition of JNK could be one mechanism by which imatinib prevents β-cell death. This is in line with a previous report stating that cell-permeable inhibitors of JNK protect against cytokine-induced cell death in insulin producing cells (Bonny et al., 2001).

In our in vivo studies, we found that Gleevec partially or completely counteracted the Type 1 diabetes that develops spontaneously in NOD mice or in response to a single STZ injection, and that this was paralleled by a partially preserved β-cell mass. Although it is possible that Gleevec affected signaling events pertinent to the control of peripheral insulin sensitivity and lipoprotein metabolism, it is generally agreed that the diabetes of the two Type 1 models presently investigated essentially results from β-cell destruction. Indeed, in the STZ-injected mouse the vast majority of the β-cells are rapidly killed in response to the specific toxin, and as Gleevec was given only one day before, the same day and one day after the STZ injection, effects on peripheral insulin sensitivity and lipoprotein metabolism were probably negligible during the subsequent 8 day Gleevec-free period. When it comes to the presently observed protection against diabetes of the NOD mouse, Gleevec did not seem to affect the immune system of these mice, as judged by the development of insulitis and the Th1/Th2 cytokine balance in resting or activated splenocytes. Thus, it is likely that Gleevec acted in both diabetes models by preventing β-cell death and dysfunction.

The model for the Gleevec mode of action presented above, although straightforward and conceptually attractive, is confounded by two findings. Firstly, we have in primary β-cells hitherto not been able to detect an increased c-Abl activity in response to DETA/NONOate, STZ or cytokines (results not shown). Although this could result from an especially low level of c-Abl expression in insulin producing cells and/or a low sensitivity of the
Tyr(245) c-Abl antibody, which we have used herein to detect c-Abl activity, we cannot exclude the possibility that c-Abl activity is unaffected by stress signals in primary β-cells. Secondly, an overnight pre-incubation in the presence of Gleevec was required for the anti-apoptotic effect. This speaks in favor for a more complex chain of events involving altered gene expression rather than only a direct Gleevec-induced inhibition of the stress-activated c-Abl kinase. The findings of this study support the notion that the alternative pathway, which might operate in parallel with the direct pathway, promotes a state resembling that of ischemic preconditioning originally observed in cardiomyocytes (Murphy E 2004). Ischemic preconditioning is defined as an increased tolerance to ischaemia and reperfusion induced by a previous sub-lethal ischemia (Riksen N 2004). On the molecular level, ischemic preconditioning is characterized by events such as NF-κB and PKC activation, opening of mitochondrial potassium channels, mitochondrial membrane depolarization and reduced ATP production, increased generation of ROS and release of cytochrome c from the mitochondria (Yellon D 2003; Baines C 1999). The late protective phase in response to a brief ischemic episode lasts for 24 to 72 hours (Baxter G 1997), and chemical preconditioning, induced by the pharmacological opening of mitochondrial potassium channels, is known to mimic ischemic preconditioning (Yellon D 2003). The findings of a previous report, demonstrating that K<sub>ATP</sub> channel openers protect islet cells from death (Kullin M 2003), indicate that a preconditioning pathway is operational also in β-cells. Moreover, an overnight exposure to Gleevec resulted in four characteristics for preconditioning, i.e. increased ROS production, decreased mitochondrial membrane potential, NF-κB activation and increased NO production. Among these, NF-κB activation appears to be a necessary event as the pharmacological inhibitor of NF-κB prevented the Gleevec-induced protection against NO. The Gleevec-induced NF-κB activation may have resulted from inhibition of c-Abl-mediated phosphorylation of IκB, an event that antagonizes NF-κB activation (Kawai H 2002).

In most cell types NF-κB is considered an anti-apoptotic transcription factor (Karin M 2002). In insulin producing β-cells, however, the prevailing view has been that NF-κB is pro-apoptotic (Eizirik D 2001). This opposite view might have evolved as a consequence to the important role of NF-κB in activation of the iNOS gene, an event that is highly cytotoxic to rodent β-cells (Darville M 1998). However, as NF-κB activation without a concomitant expression of the iNOS gene, which occurs for example in response to TNF-α receptor activation, does not result in increased β-cell death (Chang I 2003), and as the anti-apoptotic action of Gleevec required increased NF-κB activity, it may be that NF-κB should be considered an anti-apoptotic factor also in β-cells.

In summary, we report here that Gleevec treatment prevents β-cell apoptosis and thereby maintains the pancreas insulin secretion capacity in situations of β-cell stress. Gleevec may act by counteracting the pro-apoptotic
effects of c-Abl activation, but perhaps more importantly, Gleevec promotes NF-κB activation and an ischemic preconditioning-like state. Furthermore, in view of recent reports, it is also likely that Gleevec has an effect on the peripheral insulin action. Clinical findings, showing that chronic myeloid leukemia patients on Gleevec therapy improved both their Type 2 diabetes (Veneri D 2005; Breccia M 2004) and lipid status (Gottardi M 2005), support the notion that Gleevec might also act peripherally to enhance insulin sensitivity. Thus, further studies on Gleevec and Gleevec-related compounds may pave the way for a conceptually completely new diabetes drug that improves not only β-cell function, but also peripheral insulin signaling. Unfortunately, due to the side effects that have been observed in the clinic, it is not likely that imatinib will be used as a treatment for diabetes. Instead, the only curative treatment for Type 1 diabetes is today islet transplantation combined with immunosuppressive therapy (Shapiro A 2005). However, it has been reported that up to 60% of the transplanted islet mass is rapidly lost due to apoptosis, in part dependent on hypoxia-induced damage (Emmamaullee J 2005). The apoptosis rate in the graft reaches its peak 2-3 days after transplantation, then it declines until day 14 (Emmamaullee J 2005). Therefore, studies that could establish whether pre-treatment with imatinib, either ex vivo or in vivo, could enhance graft survival and function during the first two critical weeks post-transplantation are highly warranted. It has recently been observed that Gleevec attenuates PDGFR-induced phosphorylation of the low density lipoprotein receptor-related protein (LPR) (Loukinova E 2002), which in turn might lead to an improved lipoprotein metabolism (Gottardi M 2005), protection against atherosclerosis (Boucher P 2003) and regression of Type 2 diabetes (Veneri D 2005; Breccia M 2004).

Paper IV

Shb is a ubiquitously expressed adaptor protein, which forms signaling complexes, by recruiting other proteins through binding to its domains (SH2, PTB and proline rich domains). In addition, Shb has four putative tyrosine phosphorylation sites and binding of the SH2 proteins CrkII, Ras-GAP and SLP-76 to phosphorylated Shb has been previously demonstrated (Lu L 2000; Lindholm C 2002; Anneren C 2003). Besides the traditional Src-family kinases, FRK (Anneren C 2000), and currently c-Abl have been shown to phosphorylate Shb. Shb regulates apoptosis in fibroblasts (Karls- son T 1996), endothelial cells (Claesson-Welsh 1998) and in β-cells (Welsh M 1999). A transgenic mouse overexpressing Shb in β-cells under control of the rat insulin promoter exhibited increased susceptibility to cytotoxic cytokines (Welsh M 1999) and the β-cell toxin streptozotocin (Welsh M 1999; Anneren C 2002). So far, the mechanisms underlying the regulatory role of
Shb in apoptosis have remained obscure. The present investigation may shed light on this.

We currently demonstrate interactions between Shb and c-Abl, that c-Abl causes tyrosine phosphorylation of Shb and that Shb regulates c-Abl phosphorylation, and thus presumably its kinase activity. Curiously, Shb has four potential tyrosine phosphorylation sites consisting of the c-Abl preferred target motif Y-X-X-P (Lindholm C 2000). The mode of interaction between c-Abl and Shb appears complex and involves binding of the c-Abl SH3 domain to the Shb proline-rich motifs, the c-Abl SH2 domain primarily to phosphorylated Y-423 and the Shb SH2 domain binding phosphorylated c-Abl. In concert, these interactions appear to exert a regulatory role on c-Abl activity. The biological significance of the c-Abl/Shb interaction was presently tested in both overexpression and Shb knockdown experiments and was found to regulate cell death particularly under conditions of oxidative, genotoxic or ER-stress. Accordingly, we propose that upon stress, Shb and c-Abl associate via multiple interactions. This modulates c-Abl kinase activity and death signaling via downstream pathways. Shb knockdown will alter these interactions in such a manner that the cells will become insensitive to STI571 upon exposure to the genotoxic agent cisplatin. However, in the ER-stress setting, ie tunicamycin exposure, Shb depletion reduces cell death, an effect that is further accentuated by STI571 treatment. Thus it appears as if Shb and c-Abl participate in two parallel pathways causing cell death in response to ER stress.

Several lines of investigation have suggested that ER-stress is an important mediator of β-cell dysfunction. The insulin producing β-cell has a high level of protein synthesis and proteins associated with ER-stress are highly expressed in the cell (Oyadomari S 2001). Evidence that ER-stress contributes to the development of diabetes, stems from recent findings where pro-inflammatory cytokines (IL-1β and IFN-γ), known mediators of β-cell dysfunction, induce ER-stress via an NO-dependent mechanism, that could be prevented using an inducible NO synthase blocker (Cardozo A 2005). There are also reports that exposure of INS-1E cells to free fatty acids induce ER-stress in an NO-independent manner (Kahrroubi I 2004) which could possibly link β-cell dysfunction in type 2 diabetes to ER-stress.
Conclusions

- The d-siRNA technique can successfully be used for the silencing of genes in primary β-cells.
- Gleevec treatment can prevent β-cell death in response to various stress-inducing agents.
- Inhibition of JNK can in part explain the protective action of Gleevec. Another protective effect could be mediated via ischemic preconditioning.
- Gleevec can prevent diabetes development in the NOD mouse model of type 1 diabetes.
- The adaptor protein Shb and c-Abl can form a complex, that is implicated in β-cell stress responses.
Acknowledgements

This work was carried out at the Department of Medical Cell Biology, Uppsala University. I wish to express my sincere gratitude to:

Professor Nils Welsh, my supervisor, for his (almost)endless patience and vast knowledge in both theoretical and practical aspects of research. You have developed my creativity, and I will always be grateful for that.

Professor Michael Welsh, my co-supervisor, whose great enthusiasm and driving force sets an example for us all. You have shown me that hard work pays off.

Professor Stellan Sandler, my co-supervisor, who always takes his time and shares his ideas. You have shown me that it is important to keep your feet on the ground when doing research.

I would also like to thank professor Arne Andersson for running the department smoothly and creating a pleasant atmosphere. Leif Jansson for generous advice and interesting discussions. Astrid Nordin and Birgitta Bodin for teaching me how to work with animals and pleasant company. Eva Törnelius for helping me with histology. Carina Carlsson who is always encouraging me. Johnny Sternesjö and Malin Flodström, you got me started doing research and provided valuable discussions and generous support in the beginning. Natalia Makeeva, Dariush Mokhtari and Anders Tengholm, my co-authors for continuos support and new ideas. Past and present members of Nils Welsh lab, especially Dariush Mokhtari, Johan Olerud, Natalia Makeeva and Rickard Fred for valuable friendship, and for making the lab a happy place. I would also like to thank Aileen King, Martin Blixt, Andreas Börjesson and Björn Åkerblom for pleasant company at network meetings. Nina Ågren and Richard Olsson, for sharing my interest in running. Agneta Bäfve for excellent handling of the administration and for always smiling. Former and present PhD students at the department, with special thanks to Annika Andersson, Cecilia Anneren, Andreea Barbu, Mattias Gareskog, Kristina Holmqvist, Magnus Johansson, Ella Karlsson, Vitek Kriz, Cecilia Lindholm, Lingge Lu, Eva Ludvigsen, Göran Mattson, Hanna Nyblom, Henrik Ortsäter, Tobias Rydgren, Johan Saldeen, Monica Sandberg, E-ri Sol, Tea Sundsten, Sophia Thore, Lina Thorvaldson, Linda Tillmar and Björn Tyrberg.

At last, my family, including my loving wife Annika and our daughters Anna-Klara and Vera, you matter the most!
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