Expression of the N-terminal fragment of RasGAP in pancreatic beta cells increases their resistance to stresses and protects mice from diabetes

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Objective - Our laboratory has previously established *in vitro* that a caspase-generated RasGAP N-terminal moiety, called fragment N, potently protects cells, including insulinomas, from apoptotic stress. We aimed to determine whether fragment N can increase the resistance of pancreatic beta cells in a physiological setting.

Research design and methods - A mouse line, called RIP-N, was generated that bears a transgene containing the rat insulin promoter followed by the cDNA encoding fragment N. The histology, functionality, and resistance to stress of RIP-N islets were then assessed.

Results - Pancreatic beta cells of RIP-N mice express fragment N, activate Akt and block NFκB activity without affecting islet cell proliferation or the morphology and cellular composition of islets. Intraperitoneal glucose tolerance tests revealed that RIP-N mice control their glycemia similarly as wild-type mice throughout their lifespan. Moreover, islets isolated from RIP-N mice showed normal glucose-induced insulin secretory capacities. They however displayed increased resistance to apoptosis induced by a series of stresses including inflammatory cytokines, fatty acids, and hyperglycemia. RIP-N mice were also protected from multiple low-dose streptozotocin injection-induced diabetes and this was associated with reduced *in vivo* beta cell apoptosis.

Conclusions - Fragment N efficiently increases the overall resistance of beta cells to noxious stimuli without interfering with the physiological functions of the cells. Fragment N and the pathway it regulates represent therefore a potential target for the development of anti-diabetic tools.
Elimination of pancreatic β cells by apoptosis is a culminating event leading to type 1 diabetes (1) and possibly type 2 diabetes (2;3). The development of tools favoring β cell survival in patients is therefore of critical importance to delay or prevent the development of the disease.

Apoptosis is induced when a family of proteases called the caspases is activated (4;5). These enzymes cleave a subset of cellular proteins, inducing the characteristic biochemical and morphological features of apoptosis. Pancreatic islet cells undergo apoptosis in response to many stimuli (6), including anoxia (7), nutrient deprivation (8), hyperglycemia (9) and inflammatory cytokines (10). Counteracting the pro-apoptotic effects of caspases would therefore be advantageous to render islet cells more resistant to a series of noxious stimuli.

Many pro-apoptotic signaling pathways have been characterized in beta cells. These include the Fas death receptor pathway, the ER stress response, and the activation of the NFκB transcription factor (6;11). The detrimental effect of sustained NFκB activity observed in beta cells contrasts with the pro-survival effect of NFκB activation in many other cell types (7;8). An elegant in vivo support for the notion that NFκB can be deleterious in beta cells comes from the demonstration that transgenic mice expressing specifically in beta cells a degradation-resistant NFκB inhibitor are protected from diabetogenic agents (12).

On the other hand, anti-apoptotic pathways can be induced in beta cells to allow for survival in stress conditions. Akt is a kinase that inhibits apoptosis in many cell types by regulating a vast variety of pro- and anti-apoptotic molecules (13;14). Expression of a constitutively active form of Akt in beta cells in mice protected them from experimentally induced diabetes (15;16). In one of the models at least, this was accompanied by disturbed beta cell and islet morphology, islet hyperplasia and, paradoxically, by a very significant increase in the basal beta cell apoptotic rate (15). The increased rate of proliferation was therefore compensating for the loss of cells through apoptosis. These data indicate that expression of an active form of Akt1 in β cells generates two opposing forces: an increase in basal apoptosis and a stimulation of proliferation/growth. The latter effect eventually promotes the development of insulinomas (17). The potential beneficial effects of Akt activity in β cells are therefore mitigated by a predisposition towards malignancy and by an increased susceptibility to cell death that is most likely mediated by the concomitant activation of NFκB (6).

RasGAP, a regulator of Ras and Rho, is a caspase-3 substrate bearing two cleavage sites. RasGAP is cleaved in a stepwise manner as caspase activity increases in cells. At low caspase-3 activity, RasGAP is cleaved only once, generating an N-terminal fragment, called fragment N, that induces a potent anti-apoptotic response (18;19). At higher caspase activity, fragment N is further processed into two additional fragments, called fragments N1 and N2, that no longer protect cells (18;20). It is however possible to prevent cleavage of fragment N by replacing, in the second caspase cleavage site, the aspartate residue at position 157 with an alanine (18). Fragment N induces cell survival by activating the Ras-PI3K-Akt pathway (19).

Importantly, not only does fragment N not require NFκB activity for its anti-apoptotic
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properties, it inhibits the ability of Akt to activate NFκB (19). This indicates that different ways of activating Akt (i.e. via expression of an active mutant of Akt or via expression of fragment N) does not lead to the same cellular responses. We have recently demonstrated that expression of fragment N in β cells in vitro leads to the stimulation of Akt-dependent protective signals while blocking the ability of Akt to activate the pro-apoptotic NFκB pathway (21). To determine whether fragment N would display its protective functions in an in vivo setting, a transgenic mouse was generated that expresses an uncleavable form of fragment N under the control of the rat insulin promoter to restrict its expression in pancreatic β cells. This mouse model displayed an increased resistance to experimentally induced diabetes and its β cells were less susceptible to apoptosis induced by a variety of death stimuli.

RESEARCH DESIGN AND METHODS

Cell Culture, Chemicals and antibodies, Transgene detection by PCR, Quantitative PCR, Mouse islet isolation and dissociation, Preparation of tissue sections and immunochemistry, Insulin quantitation, Western Blot Analysis, Southern Blot, Nuclear protein extract preparation and electromobility shift assay (EMSA). See supplemental methods in the online appendix which is available at http://diabetes.diabetesjournals.org.

Apoptosis Assay. Apoptosis ex vivo was assessed by scoring the number of cells with pyknotic nuclei after Hoechst 33342 staining (20). Apoptosis in vivo was assessed by TUNEL assay (DeadEnd Fluorometric TUNEL system, Promega Switzerland, cat. n° G3250) on islet paraffin sections as per the manufacturer’s protocol.

Animal experimentation. All procedures on mice were performed according to the Swiss legislation for animal experimentation. Unless noted otherwise, the animals were used at an age of 8-12 weeks.

Transgenic lines. The transgenic construct [RIP-HA-N(D157A).xf3] bears fragment N of RasGAP under the control of the rat insulin promoter (RIP). It was obtained by ligation of a blunt-ended BamHI/SalI 1.4 kb fragment from plasmid HA-N(D157A).bs (22) with a blunt-ended XbaI/HindIII 4 kb fragment from RIP-vMos.xf3 plasmid. The correctness and functionality of the plasmid were controlled by sequencing and transfection into insulinoma cell lines. Finally a BamHI 2.8 kb fragment from RIP-N.xf3 was microinjected into FVB/N oocytes at the Transgenic Animal Facility of the Lausanne University. Four independent RIP-N expressing founders were obtained. Founders #1 and #2 were used in the experiments described here.

Blood glucose level measurements and intraperitoneal glucose tolerance test (IPGTT). Blood glucose content of mice under feeding or fasting (16 hours) conditions was determined with an Accu-Check Compact Plus glucometer (Roche Diagnostics). For the IPGTTs, fasted (16 hours) animals were injected intraperitoneally with 2 mg/kg glucose. Blood glucose levels were determined from a blood drop taken after a short incision of the tail tip at increasing time intervals (-30, 0, 15, 30, 60, 90, 120, 150 minutes) following glucose injection.

Streptozotocin-induced diabetes. Type 1-like diabetes was induced by multiple low dose streptozotocin injections. Briefly, 4 hour-fasted female RIP-N mice were injected intraperitoneally with 50 mg streptozotocin per kg of mice. This procedure was repeated every day for a total period of 5 days. Streptozotocin was prepared and diluted in citrate buffer pH 4.5 (sodium citrate 25 mM, citric acid 23 mM) just before injection. Control mice were injected with the citrate
buffer alone. Blood glucose levels were assessed biweekly. 

In vitro insulin secretion measurement. Islets were isolated from mice pancreas as described above. The islets (200 per 100 mm dish in 10 ml culture medium) were incubated over night at 37°C, 5% CO2. The next day, the islets were hand picked and cultured in KRBH-BSA (120 mM NaCl, 4 mM KH2PO4, 20 mM HEPES, 1 mM MgCl2, 1 mM CaCl2, 5 mM NAHCO3, pH: 7.4 with 0.5 % BSA) at 37°C and 5% CO2. The following day, well preserved and good quality islets were again hand picked and placed into 12 well-plates (10 islets/well) in 1 ml KRBH-BSA containing 2.8 mM glucose for 1 hour. The islets were then transferred to new wells containing 2.8 mM or 20 mM glucose with or without 10 nM exendin-4 (Bachem, cat. n°H-8370) in 1 ml KRBH-BSA and incubated for 2 additional hours. The supernatant and islets were collected into separate tubes and placed on ice. The islets were lysed in 500 µl acid/ethanol (75% ethanol/1.5% concentrated HCl) and sonicated 15" (using a W-375 cell disruptor from Kontron equipped with a 3 mm tip). Insulin in the supernatant and extracted islets was measured using an RIA kit LINCO (cat. n° RI-13K).

Statistical Analysis. Unless stated otherwise, the statistical analyses were done with Microsoft Office Excel 2003 SP1 using the two-tailed unpaired Student’s t test. Significance is indicated by an asterisk when p < 0.05/n, where p is the probability derived from the t test analysis and n is the number of comparisons done (Bonferroni correction). All the other statistical analyses were performed with the SAS/STAT software v9.1.3, SAS Institute, Inc. (Cary, NC).

RESULTS

Generation of a transgenic mouse expressing fragment N in pancreatic β cells. A transgenic vector was constructed (see Materials and Methods) so as to encode an HA-tagged form of fragment N bearing the D157A mutation (preventing it from being cleaved by caspases) under the control of the rat insulin promoter (RIP) and regulatory sequences of the simian virus 40 (SV40) gene (Figure 1A). The construct was injected into FVB/N oocytes and transgene-positive mice where identified by Southern blotting (Figure 1B). In total 4 founder mice were obtained. The results presented here all include data from founder #1 (labelled mouse 5 in Figure 1B). When indicated some experiments were also performed with mice derived from founder #2 (labelled mouse 28 in Supplemental Figure 1). By comparison with the endogenous insulin promoters, it was estimated that founder #1 and #2 bore 12-15 and 1 copies of the transgene in their genome, respectively (Figure 1B and Supplemental Figure 1 in the online appendix which is available at http://diabetes.diabetesjournals.org).

To determine the expression pattern of fragment N in the transgenic line, lysates from pancreatic islets, liver, brain, and spleen were analyzed by Western blotting using antibodies specific for the HA tag or for the N-terminal part of RasGAP. Figure 1C, shows that fragment N was, as expected, only expressed in islet cells. Immuno-fluorescence analysis of both founders revealed that fragment N was restricted to the endocrine part of the pancreas (Figure 1D and Supplemental Figure 2 in the online appendix) and that the vast majority of fragment N-expressing cells corresponded to β cells (i.e. insulin-containing cells) (Figure 1E).

Regulation of Akt and NFκB by fragment N in RIP-N β cells. In various cell types, fragment N, when ectopically expressed or when generated in response to mild-stress, activates Akt (19;21;23). As adaptive mechanisms can take place in vivo, it was important to determine whether fragment
N could induce a chronic Akt activity in islet cells in mice. Islets isolated from control and RIP-N mice were therefore analyzed for the presence of activated Akt. As shown in Figure 2A, there was a significant ~3 fold increase in Akt activity in islet cells from RIP-N compared to control islets. This indicates that fragment N can stimulate Akt on a long-term basis when expressed in vivo.

A potential important property of fragment N in the context of β cell protection is its ability to block NF-κB activation. This property however had so far only been evidenced in cultured immortalized cell lines (19;21). As shown in Figure 2B, binding of nuclear factors to NF-κB binding elements was markedly diminished in nuclear extracts from RIP-N mouse-isolated islet cells stimulated with cytokines compared to similarly treated islets isolated from control mice. Moreover, cytokine-induced expression of the transcript encoding iNOS, which participates in β cells apoptosis (24) and whose gene is a NFκB target (25), also appeared to be impaired in islets cells isolated from RIP mice compared to wild-type islets (Figure 2C).

These results indicate that fragment N regulates Akt and NFκB in β cells in vivo in a manner similar to what has been described using cultured cell lines. As cytokine can induce apoptosis of β cells via NF-κB-mediated nitric oxide production (24), these results also suggest that the ability of fragment N to protect β cells might rely, at least in part, on its capacity to target the NF-κB-iNOS axis.

No detection of fragment N in the brain of RIP-N mice. It was reported in transgenic models done using RIP-Cre mice that the RIP promoter can also be active in the brain (more specifically in the hypothalamus) (26;27). Immuno-histochemical analysis however did not reveal the presence of fragment N in hypothalamic sections from adult RIP-N mice (Supplemental Figure 3). This indicates that the RIP-N transgene is not expressed in adult mouse brain or, if it is expressed, at levels that are much lower than those detected in the endocrine pancreas and that are under the sensitivity limit of our assay.

**Fragment N expression does not affect islet morphology and cellularity.** Expression of fragment N in insulinomas and islet cells leads to Akt activation [Figure 1F and (21)]. Since Akt signalling has the potential to stimulate cell survival and proliferation (28) and since transgenic mice expressing a constitutively active form of Akt (myr-Akt) show an increase in both β-cell size and total islet mass (15), the presence of fragment N in islets might affect the morphology and cellularity of the endocrine pancreas. However, neither the proportion of α and β cells (Figure 3A), nor the insulin content of the pancreas (Figure 3B) were affected by the presence of fragment N. Moreover, the size of the islets did not appear to be different in RIP-N transgenic mice compared to control mice (Figure 3C). Finally, the percentage of cells positive for the nuclear protein Ki67 that is preferentially expressed in dividing cells was similar in both types of mice (Figure 3D). These results indicate that fragment N does not favour β cell proliferation in an in vivo setting and that it does not affect the normal development of the endocrine pancreas. Consistent with this notion is the observation that RIP-N mice did not develop insulinomas over a 18 month-period (as assessed by a drop in glucose blood level and increased mortality; see Figure 8).

Islets from RIP-N transgenic mice display increased resistance to basal- and stress-induced apoptosis. In wild-type mice, the basal apoptotic rate in islets is very low (less than 0.5%; Figure 6B) or undetectable (15). In contrast, islets from transgenic mice expressing a constitutively active form of Akt show a marked increase in β cell apoptosis (15). Despite the ability of fragment N to
activate Akt (Figure 2A), there was no associated increase in the basal apoptotic rate in islets from RIP-N mice compared to the wild-type controls, either in vitro or in vivo (Figure 4A and first two bars of Figure 6B). Moreover, islets isolated from RIP-N mice were more resistant than those isolated from control mice when subjected to a variety of stress stimuli, including inflammatory cytokines, the free fatty acid palmitate, and high glucose concentrations (Figure 4). These results demonstrate that fragment N efficiently protects pancreatic β cells against various conditions and stimuli, including some that are associated with the development of type 1 and type 2 diabetes (e.g. inflammatory cytokines and free fatty acids).

**Fragment N does not adversely affect β cell functions in vivo.** Transgenic mice expressing a non-degradable form of IκBα under the control of Pdx1 promoter, which drives its expression in the β cells of the pancreas, display impaired glucose-induced insulin secretion (29). Fragment-N by blocking NFκB activity (19;21) could potentially similarly affect insulin secretion. However, fragment N expression in β cells did not modify glycemia under non-fasted (Figure 5A) or fasted (Figure 5B, first points in the graphs) conditions. Moreover, the ability of the transgenic mice to metabolize glucose, assessed by intraperitoneal glucose tolerance tests, was not negatively affected by the presence of the transgene in β cells (Figure 5B). Finally, islets isolated from control and RIP-N transgenic mice had a similar ability to secrete insulin in response to glucose and the gluco-incretin exendin-4 (Figure 5C). These results indicate that fragment N does not compromise the ability of β cells to secrete insulin in response to augmented glucose levels.

**RIP-N transgenic mice are protected against streptozotocin-induced diabetes.** Multiple low dose-streptozotocin injections in mice induce islet inflammation, ultimately leading to cell loss and diabetes (30;31). This model is thought to mimic the development of type 1 diabetes in humans (32). Using this protocol, it was found that RIP-N mice were resistant to diabetes induction compared to control mice (Figure 6A). Assessment of apoptosis by the TUNEL method showed that the percentage of β cell apoptosis induced by streptozotocin in vivo was significantly reduced in the RIP-N mice compared to the wild-type controls (Figure 6B).

To further characterize the reduced sensitivity to streptozotocin-induced diabetes in RIP-N mice, pancreas sections from control and RIP-N mice treated or not with streptozotocin were prepared. The mice were sacrificed 3 days after the last streptozotocin injection at a time were increased apoptosis can be detected in wild-type mice (see Figure 6B) but before the apparition of an overt diabetes (see Figure 6A). At this time, leucocytic infiltration can be visualized in the islets of wild-type streptozotocin-treated mice. This was accompanied by a loosening of the islet structure. This was not seen in RIP-N mice (Figure 7A). Additionally, insulin-staining was reduced in the islets of wild-type streptozotocin-treated mice (Figure 7B). Moreover, there was almost no sign of insulinitis in the transgenic mice after the streptozotocin injections, while in similarly treated control mice a strong insulinitis developed (Figure 7C). Finally, there was a significant reduction in CD3-positive leukocyte infiltration in RIP-N mice compared to the control mice after the streptozotocin treatment (Figure 7D). Taken together, these results indicate that streptozotocin induces less damage in RIP-N islets, which results in a weaker inflammatory response, compared to wild-type islets.

**The RIP-N transgene does not alter glucose homeostasis or the life-span of mice.** In relatively young mice (8-12 weeks),
glucose homeostasis and insulin secretion is unaffected by the presence of fragment N in beta cells (see Figure 5). To determine if the transgene could nevertheless negatively affect the function of pancreatic beta cells on a longer term basis, the glycemia of a cohort of female and male wild-type and RIP-N mice was followed for up to 130 weeks (Figure 8A). Males displayed higher glycemia values than females. There was also a significant decrease in glycemia as the mice aged. However, the glycemia between wild-type and RIP-N mice for a given sex was not statistically different. This indicates that fragment N does not negatively affect the function of the islets of Langerhans. Consistent with this is the observation that IPGTT tests performed on very old animals did not reveal differences between wild-type and RIP-N mice (Figure 8B).

Constitutive expression of Akt in pancreatic beta cells increases the likelihood of insulinoma development leading to a reduction of the life time expectancy of the mice (17). As fragment N activates Akt, it was relevant to check if fragment N would have a negative impact on the survival of the mice. Figure 8C shows that this is not the case. While females lived significantly longer than males, the presence of the transgene did not affect the percent survival rate of the mice. Finally, there was no histological difference that could be evidenced on islets from very old wild-type and RIP-N mice (Figure 8D). Altogether these results indicate that fragment N expressed in pancreatic beta cells displays no negative effect throughout the lifespan of mice.

**DISCUSSION**

Apoptosis, which is the cause of β cells death in patients with type 1 diabetes (33), might also participate in the loss of β cell mass observed in type 2 diabetes (34-37). The notion, however, that there is a decrease in β cell mass in type 2 diabetes has been controversial for a number of years. Nevertheless, if one considers those studies using well-preserved pancreases obtained from autopsies, it appears that there is a 3-10 fold increase in the rate of β cell apoptosis in type 2 diabetic patients compared to control subjects (38). These results indicate that failure to compensate for insulin resistance could result from decreased β cell mass mediated by apoptosis.

Understanding the pathways leading to β cell death and β cell protection might therefore be of crucial importance to find new approaches to treat diabetic patients. Procedures to block β cell death could not only potentially inhibit the development of diabetes but might also be useful in the context of islet transplantation where apoptosis has been shown to adversely affect the number of islets that can be implanted in patients. Here we present an *in vivo* model - the RIP-N transgenic mice - where the N-terminal fragment of RasGAP (called fragment N) effectively protects pancreatic β cell against apoptosis without affecting their ability to appropriately secrete insulin in physiological conditions and without favouring excessive proliferation.

Hyperlipidaemia, a risk factor for the development of diabetes (39), can cause β cell apoptosis (40;41). Islets isolated from RIP-N mice underwent less apoptosis in conditions mimicking hyperlipidemia (i.e. high concentrations of palmitate) compared to control islets. Hyperglicemia, which can lead to β cells dysfunction and death (6), also induced less apoptosis in fragment N-expressing islets. Finally, RIP-N transgenic islets were more resistant to IL1β, TNFα, and IFNγ-induced death. Interestingly, these inflammatory cytokines, known to be involved in the development of type 1 diabetes, have also been shown to be produced at high concentrations in diabetic-prone obese patients (42;43). These observations suggest that the protective
signals elicited by fragment N can counteract pro-diabetic conditions (e.g. hyperglycemia, hyperlipidemia, presence of inflammatory cytokines) that are deleterious for β cells.

Much work on the cellular mechanisms controlling cell death and survival has been performed within the last few years. This knowledge has been used to manipulate β cells in order to increase their survival capacities. One strategy was based on Akt because it is a potent anti-apoptotic kinase in many cell types (13). Transgenic mice expressing an active form of Akt (myr-Akt) in β cells in mice have larger β cells and bigger islets (15) and this ultimately favors the development of insulinomas (17). These transgenic mice are resistant to experimentally induced diabetes but, paradoxically, their β cells have a much increased basal apoptotic rate (15). Conceivably, this higher apoptotic rate is compensated by increased β cell renewal to maintain an adequate β cell mass. As NFκB activation can induce β cell death (6) and because Akt stimulates NFκB (44), the increased apoptosis response observed in myr-Akt-expressing β cells likely results from the stimulation of the NFκB pathway. Indeed, prevention of NFκB activation using a dominant-negative IκB mutant allows mice to resist streptozotocin-induced diabetes (12;45). NFκB inhibition might however not always be protective against diabetes as indicated by the increased susceptibility of NOD mice to develop diabetes when their beta cells express the dominant-negative IκB mutant (45). Additionally, it has been shown that expression of a dominant-negative IκB mutant in beta cells in mice can inhibit glucose-stimulated insulin secretion (29).

The RIP-N mice appear to lack some of the defects associated with the models described above. The activation of Akt by fragment N in the beta cells of RIP-N mice is not accompanied by an increased basal apoptotic rate, most likely because fragment N blocks Akt from stimulating NFκB. Moreover, in contrast to mice expressing an active form of Akt in beta cells (17), RIP-N mice do not display islet and beta cell hyperplasia, they do not develop insulinomas, and have normal lifespan. Finally, RIP-N mice have no defect in glucose-induced insulin-secretion and display normal glucose homeostasis even in very old animals. Transgenic mice over-expressing proteins of the IAP (inhibitor of apoptosis) family specifically in the β cells have been generated. Similarly to RIP-N mice, their beta cells are less susceptible to apoptosis and this is not accompanied by alterations in islet morphology and function (46). It will therefore be important to define if there is a link between fragment N and IAPs that could explain the protective function of fragment N in beta cells. A detailed characterization at the molecular level of the pathways regulated by fragment N might ultimately lead to the identification of new strategies to preserve beta cell mass.

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Figure Legends

**Figure 1: Expression and function of fragment N in RIP-N mice.**

A. Schematic representation of the RIP-N transgene together with the strategy for its detection by Southern blot. A HA-tagged form of fragment N (amino acids 1 to 455 of RasGAP) followed by an SV40 derived poly-A sequence was placed under the control of the rat insulin promoter (RIP). Band A corresponds to the transgene-specific EcoRI Southern blot fragment. B1 and B2 are examples of EcoRI Southern blot fragments derived from random insertions of the transgene into the host’s genome.

B. Identification of RIP-N transgenic mice. The progeny of the injected pseudo-pregnant mice were genotyped by Southern blot (see Material and Methods for details). Band A (2.8 kb) is specific for the transgene. Founder #1 (mouse #5) was able to transmit the transgene to the F1 generation.

C. Tissue expression of fragment N. Lysates from the indicated tissues were analysed for the presence of fragment-N by Western blot using anti-HA and anti-RasGAP antibodies.

D. Expression of fragment-N in the pancreas. The presence of fragment N was assessed by immunofluorescence analysis of paraformaldehyde-fixed cryosections using an antibody recognizing the HA tag born by fragment N.

E. Colocalization of insulin and fragment N. The specific location of fragment-N in pancreatic β-cells was determined by immunofluorescence of paraformaldehyde-fixed cryo-sections from RIP-N mice using anti-insulin and anti-HA antibodies.

**Figure 2: Fragment N activates Akt and inhibits NF-κB in islet β cells.**

A. Lysates from islets isolated from the indicated mice were analysed by Western blot for the presence of fragment N using an HA-specific antibody and for the activation of Akt using a phospho-specific anti-Akt antibody (p-Akt). An Akt-specific antibody was used to assess evenness in loading (total Akt). The numbers under the blots correspond to the quantitation (arbitrary units) of the detected bands (mean ± SD of three independent determinations). The asterisk indicates a statistically significant difference as assessed by a paired t-test analysis.

B. Islets isolated from wild-type (+/+) and RIP-N mice (+/RIP-N) were stimulated or not for 30 minutes with inflammatory cytokines (1,000 units/ml TNFα, 1,000 units/ml interleukin-1β and 50 units/ml interferon-γ). The ability of nuclear proteins to interact with a NF-κB binding element-bearing radioactive probe was then monitored by EMSA as described in the methods. The locations of p65-p50 and p50-p50 complexes are indicated. The asterisk denotes a non-specific band. This experiment was repeated once with similar results.

C. Islets isolated from wild-type (+/+) and RIP-N mice (+/RIP-N) were stimulated or not for the indicated periods of time with 1,000 units/ml of interleukin-1β. The expression of iNOS mRNA was then measured by quantitative real-time PCR, normalized as described in the methods and expressed as percent of the 6 hour-values. The results correspond to the mean ± SEM of 3 independent experiments performed in triplicate. The asterisk indicates a significant difference as determined by a non-parametrical Wilcoxon signed-rank test.

**Figure 3: Fragment N expression does not affect islet morphology and cellularity.**
A. The identification of α- and β-cells was determined by immuno-histochemistry of paraffin-embedded pancreas sections with antibodies directed against glucagon (dark brown staining) and insulin (purple-red staining). The graph depicts the proportion of α- and β-cells in islets derived from the analysis of an average of 25 islets per 9-12 weeks old wild-type and +/RIP-N mice. Data from individual mice are shown (the numbers in the sex symbols indicate which founder the animals are derived from) as well as the mean ± SD values (indicated by the µ symbol in the grey area).

B. Freshly isolated pancreases where homogenized and extracted with acid ethanol. Insulin concentration in the supernatant was determined by enzyme-linked immuno-sorbent assay. The results correspond to the mean ± SD of 9 (wild-type) and 6 (RIP-N) pancreases.

C. The graph depicts the number of cells per islet section counted on HE stained paraffin embedded pancreas sections. The results are presented as in panel A and were derived from the analysis of 9-12 weeks old wild-type and +/RIP-N animals (12 mice per genotype; an average of 90 islets per mouse were analyzed).

D. The percentage of proliferating cells was determined by scoring Ki67-positive cells on paraffin-embedded pancreas sections (the arrow points to a Ki67-positive cell). The bar graph depicts the percentage of proliferating cells in islets (mean ± SD) derived from the analysis of at least 20 histological slices obtained from 9 mice per genotype.

Figure 4: RIP-N islet cells are more resistant to stress-induced apoptosis.
A. Freshly isolated islets were loosely dissociated (see the methods) and incubated or not with inflammatory cytokines (1,000 units/ml TNFα, 1,000 units/ml interleukin-1β and 50 units/ml interferon-γ) for an additional 24 hour-period. The islets were then stained with Hoechst 33342 and apoptosis scored. The results correspond to the mean ± SD of 3 independent experiments (statistic analyses were performed for the control and stimulated conditions between wild-type and RIP-N mice [2 comparisons]).

B. Freshly isolated islet were loosely dissociated and treated with vehicle (C; ethanol 1%) or incubated during 72 hours with 33 mM glucose (G) or 1 mM palmitate (P). Apoptosis was then assessed as above. The results correspond to the mean ± SD of 3 independent experiments (statistic analysis was performed for each condition between wild-type and RIP-N mice [3 comparisons]).

Figure 5. Glycemia and glucose tolerance of RIP-N mice.
A. Non-fasting glycemia of wild-type and RIP-N males and females was determined as described in the methods.

B. Mice were subjected to an intra-peritoneal glucose tolerance test (IPGTT) to analyse their response to hyperglycaemic conditions. Results correspond to the mean ± SD of 6 independent experiments. Statistic analysis was performed for each time point between wild-type and RIP-N mice (8 comparisons). No significant differences were recorded.

C. Islets from wild-type and RIP-N female mice were stimulated with low or high glucose concentration in the presence or in the absence of exendin-4 (see Research Design and Methods). Insulin secretion was then determined. Results are expressed as the amount of insulin secreted normalized to the initial cellular insulin content (mean ± SD of quadruplicate determinations). The # signs indicate no statistical differences between
insulin secretion of wild-type and RIP-N islets for a given stimulation regimen [4 comparisons].

**Figure 6. Resistance of RIP-N mice to streptozotocin-induced diabetes.**

Wild-type (+/+) and RIP-N (+/RIP-N) females (nine each) were subjected to multiple low-dose injections of streptozotocin (see materials and methods). Glucose blood levels were then determined at the indicated times. The results are expressed as the mean ± SD (statistic analysis was performed for each time point between wild-type and RIP-N mice [8 comparisons]). This experiment has been repeated two more times with similar results (panel A). Alternatively, the mice were sacrificed 8 days after the first streptozotocin-injection and apoptosis on islet sections was determined by the TUNEL assay (panel B). The results shown in the graph correspond to the mean ± SD of the quantitation performed on 3 and 4 mice for the control and streptozotocin treatment, respectively (an average of 24 islets per mouse were analyzed). Statistic analysis was performed on the indicated groups. As reported by others (47), we note that the percentage of apoptotic islet cells in vivo is more than ten fold lower than what is detected in in vitro cultured islets (compare this figure with Figure 4).

**Figure 7. Reduced insulinitis in streptozotocin-treated RIP-N mice.**

Control and RIP-N mice were subjected or not to multiple low-dose injections of streptozotocin (see materials and methods) and sacrificed 8 days after the first streptozotocin-injection. Sections were then prepared for histology and immuno-histochemistry analyses. Representative images are shown. Panel A shows haematoxylin-eosin stained paraffin-embedded pancreas sections. The red arrows point to infiltrating leukocytes. Note also the loosened structure of islets from wild-type streptozotocin-injected mice. Panel B depicts insulin immunofluorescence staining of paraformaldehyde-fixed cryosections. Panel C shows aldehyde fuchsin stained sections. These were used to score insulinitis in the streptozotocin-treated animals as described in reference (48) (right part of the panel; the higher the grade, the stronger the insulinitis; more than 100 islets from 2-3 mice per genotype were analyzed). Panel D depicts CD3 stained sections of paraffin-embedded pancreas sections (there was a high background staining in the exocrine pancreas but this was not seen in the islets). Representative images are shown (arrows indicate CD3-positive cells; the inset shows an enlargement of the indicate region). The number of CD3-positive cells within and at the immediate periphery of the islets was counted (graph on the right). The results correspond to mean ± SD of four mice (at least 25 islets per mouse were scored).

**Figure 8. The RIP-N transgene does not affect glucose homeostasis in old mice.**

In the experiments presented in this figure, a cohort of 8 and 12 wild-type (+/+) and transgenic (+/RIP-N) female mice, respectively and 11 and 10 wild-type and transgenic male mice, respectively, was used. In panel A, their non-fasting glycemia was measured at the indicated times. The average values for the indicated groups are shown. Males had significantly higher glycemia values than females (p<0.0001). There was also a significant decrease in glycemia as the mice aged (p<0.0001). However, the glycemia between wild-type and RIP-N mice for a given sex was not statistically different (the p values are indicated on the figure). The statistical test used was ANOVA (repeated measures with a 1st-order autoregressive co-variance structure). In panel B, four and three 106-109 week-old female wild-type and transgenic mice, respectively were subjected to an intra-peritoneal glucose tolerance test (IPGTT). Statistic analysis (t-tests) was performed for each time point between wild-type and RIP-N mice (8 comparisons).
significant differences were recorded. This experiment was repeated once on 111-114 week-old females with similar results. In panel C, the survival rate of the mice is presented. The statistical test used was a test of equality over strata (lifetest procedure of the SAS/STAT software, including a Log-Rank test and a Wilcoxon test). Males survived significantly less than females (p=0.0007 for the Log-Rank test and p=0.0013 for the Wilcoxon test). However, the transgene did not affect survival within a given sex (p=0.21 and p=0.24 for the Log-Rank test and p=0.20 and 0.33 for the Wilcoxon test, for females and males, respectively; NS: not significant). In panel D, haematoxylin-eosin stained paraffin-embedded pancreas sections from 130 week old female control and RIP-N mice were produced. Representative images are shown. No histological differences were detected between wild-type and RIP-N islets.

Figure 1
Fragment N protects beta cells

Figure 2

A

|       | HA      | p-Akt  |
|-------|---------|--------|
| +/-   | x/++    | x/++   |
| +/-   | x/RIP-N | x/RIP-N|

- 130 kDa
- 100 kDa
- 95 kDa
- 72 kDa
- 55 kDa
- 40 kDa
- 33 kDa
- 24 kDa

25±1  67±5

B

|       | +/-        | +/-        |
|-------|------------|------------|
| +/-   | cytokines  | +/-        |

- p50-p65
- p50-p50

Free probe

C

iNOS mRNA levels
(normalized to 36B levels)

% value at 6 hour

Time (hours)

Figure 2
Yang 2009
Figure 3

A

Islets

B

Insulin content (ng insulin per mg pancreas)

C

% total islet cells

D

Ki67

Fragment N protects beta cells

Yang 2009
Fragment N protects beta cells

Figure 4

A

% apoptotic cells

+/

+/RIP-N

Control

Cytokines

B

% apoptotic cells

C

P

G

+/+

+/RIP-N

Figure 4
Yang 2009
Fragment N protects beta cells

**Figure 5**

|         | +/+     | +/RIP-N |
|---------|---------|---------|
| **♀**   | 7.3 ± 0.8 | 7.4 ± 0.9 |
| n=18    | 7.3 ± 0.8 | 7.4 ± 0.9 |
| **♂**   | 8.4 ± 1.1 | 8.1 ± 0.3 |
| n=3     | 8.4 ± 1.1 | 8.1 ± 0.3 |

**Figure 5**

**A**

![Graph showing glucose levels over time for different genotypes](image)

**B**

![Graph showing glucose levels over time for different genotypes](image)

**C**

![Bar graph showing insulin secretion](image)

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Yang 2009
Figure 6

A

![Graph showing glucose levels](image)

B

![Images showing Hoechst 33342 and TUNEL staining](image)

Fragment N protects beta cells

Yang 2009
Figure 7

Fragment N protects beta cells

A

Non-injected  Streptozocin
+/+
+/RIP-N

Hematoxilin-eosin staining

B

Non-injected  Streptozocin
+/+
+/RIP-N

Insulin staining

C

Non-injected  Streptozocin
+/+
+/RIP-N

Aldehyde fuchsin staining

D

Hoechst / CD3

+/+
+/RIP-N

Number of CD3+ cells within and around islet

Figure 7
Yang 2009
Fragment N protects beta cells

Figure 8

A

Females

Glycemia (mM)

Weeks

+/+

+/RIP-N

p=0.34

Males

Glycemia (mM)

Weeks

+/+

+/RIP-N

p=0.98

B

Glycemia (mM)

Minutes post-injection

+/+

+/RIP-N

106-109 week-old females

C

% survival rate

Weeks

Females +/+

Females +/RIP-N

Males +/+

Males +/RIP-N

NS

D

HE

+/+

+/RIP-N

Yang 2008