Expression of an uncleavable N-terminal RasGAP fragment in insulin secreting cells increases their resistance towards apoptotic stimuli without affecting their glucose-induced insulin secretion.

Jiang-Yan Yang§, Joël Walicki§, Amar Abderrahmani§, Marion Cornu#, Gérard Waeber§¶, Bernard Thorens#, and Christian Widmann§*

§ Department of Cellular Biology and #Department of Physiology, Faculty of Biology and Medicine, Lausanne University, Switzerland. ¶ Department of Internal Medicine, Lausanne University Hospital (CHUV), Switzerland.

Keywords: Apoptosis, Beta cells, Caspases, Diabetes, RasGAP, Stress, Survival pathways.

*Address correspondence to: Christian Widmann, Department of Cellular Biology and Morphology, Biology and Medicine Faculty, University of Lausanne, Bugnon 9, 1005 Lausanne, Switzerland. Phone: +41 21 692 5123. FAX: +41 21 692 5255. E-mail: Christian.Widmann@unil.ch

Apoptosis of pancreatic β cells is implicated in the onset of type 1 and type 2 diabetes. Consequently, strategies aimed at increasing the resistance of β cells towards apoptosis could be beneficial in the treatment of diabetes. RasGAP, a regulator of Ras and Rho GTPases, is an atypical caspase substrate as it inhibits, rather than favors, apoptosis when it is partially cleaved by caspases-3 at position 455. The anti-apoptotic signal generated by the partial processing of RasGAP is mediated by the N-terminal fragment (fragment N) in a Ras-PI3K-Akt-dependent, but NF-κB-independent, manner. Further cleavage of fragment N at position 157 abrogates its anti-apoptotic properties. Here we demonstrate that an uncleavable form of fragment N activates Akt, represses NFκB activity and protects the conditionally immortalized pancreatic insulinoma βTC-1 cell line against various insults, including exposure to genotoxins, trophic support withdrawal, and incubation with inflammatory cytokines. Fragment N also induced Akt activity and protection against cytokine-induced apoptosis in primary pancreatic islet cells. Fragment N did not alter insulin cell content and insulin secretion in response to glucose.

These data indicate that fragment N protects β cells without affecting their function. The pathways regulated by fragment N are therefore promising targets for anti-diabetogenic therapy.

Apoptosis appears to be a critical determinant in the development of both type 1 and type 2 diabetes. Type 1 diabetes is a direct consequence of an auto-immune attack on pancreatic islet β-cells leading to their death (1). Analyses of pancreatic tissues from deceased human subjects has indicated that β cell mass is decreased in type 2 diabetes as well, and that the mechanism underlying this is an increase in β cell apoptosis (2). The higher apoptotic rate observed in β cells of diabetic patients could result from deregulated levels of various circulating fuel molecules (e.g. glucose and saturated fatty acids) and chronic activation of the innate immune system (3).

Apoptosis is induced when members of the caspase family of proteases are activated. These enzymes cleave a subset of cellular proteins (4,5), inducing the characteristic biochemical and morphological features of apoptosis. Cells can activate a series of regulatory mechanisms to maintain an adequate balance between pro- and anti-apoptotic signals. For
example, the ratio of pro-apoptotic vs. anti-apoptotic Bcl-2 family members can determine whether a cell survives or not (6). Many intracellular signaling pathways also regulate cell death (7). However, the activation of a given intracellular signaling pathway does not necessarily generate the same response in different cell types. For example, activation of NF-κB is protective in fibroblasts and T cells (8;9), but favors cells death in pancreatic β cells (10;11). The cellular context will likely modulate the way a given signaling pathway in a given cell type regulates apoptosis.

RasGAP, a regulator of Ras and Rho, is a caspase-3 substrate that functions as a sensor of caspase-3 activity in cells (12). RasGAP is cleaved in a stepwise manner as caspase activity increases. At low caspase-3 activity, RasGAP is cleaved only once at position 455, generating an N-terminal fragment, called fragment N, that induces a potent anti-apoptotic Ras-PI3K-Akt-dependent pathway (13;14). This protective pathway occurs independently of NF-κB activation as fragment N inhibits the ability of Akt to stimulate the NF-κB pathway (14). Generation of fragment N is crucial for cell survival in low stress conditions (15). At higher caspase activity, fragment N is further processed into two additional fragments, called fragments N1 and N2. Cleavage of fragment N abrogates its protective functions and hence the second cleavage of RasGAP promotes apoptosis (13).

Pancreatic β cells undergo apoptosis in response to a variety of stimuli, including nutrient deprivation and inflammatory cytokines. Counteracting the pro-apoptotic effects of caspases would therefore be advantageous to render β cells more resistant to a series of noxious stimuli. To assess whether fragment N has a beneficial function in insulin-secreting cells, we have assessed here whether an uncleavable form of this fragment renders the conditionally immortalized pancreatic insulinoma βTC-tet cell line more resistant to a series of adverse stimuli. We also compared the protection efficacy of fragment N with that of another anti-apoptotic protein, Bcl-2, which has been shown to induce survival signals in β cells (16) but which does not activate the Ras-PI3K-Akt pathway and therefore protects cells differently than fragment N (6;17). Finally, we have determined whether the combined expression of fragment N and Bcl-2 provides additive levels of protection in β cells.

Materials and Methods.

Plasmids.
HA-D157A.dn3 encodes RasGAP mutants that cannot be cleaved at positions 157. Two plasmids bearing the uncleavable form of fragment N have been used for the production of fragment N-encoding lentivirus. The first one, N-D157A.lti, bears the fragment N cDNA under the control of the phosphoglycerate kinase [PGK] promoter and has been described earlier (15). The second one, N-D157A.irs, has been generated by subcloning the BamHI-XhoI fragment of N-D157A.dn3 (14) in TRIP-PGK-IRESNEO-WHV opened with BamHI and SalI. This plasmid bears the neomycine resistance gene and the fragment N cDNA separated by an internal ribosomal entry site under the control of the PGK promoter. The plasmid used for the generation of Bcl-2-encoding lentivirus (SIN-PGK-hBcl-2-WHV) has been described previously (16). The plasmid encoding the dominant-negative kinase-dead mutant of Akt [HA-Akt1(K179M).cmv; previously called Akt-DN.cmv] has been described earlier (14).

Lentivirus.
Recombinant lentiviruses were produced as described (18). Briefly, 293T cells were co-transfected using the calcium phosphate DNA precipitation method (19) with 10 μg of the lentiviral vector containing the cDNA of interest (e.g. N-D157A.lti), 2.5 μg of the envelope protein-coding plasmid (pMD.G), and 7.5 μg of the packaging
construct (pCMVDR8.91). Two days after the transfection, the virus-containing medium was harvested. To determine how much of the virus preparations was needed to infect 100% of the cells, βTC-tet and βTC-tet/Bcl-2 seeded at a 50% confluency on coverslips placed in 6 wells plates were cultured overnight with various volumes of fragment N-encoding lentiviruses. After removal of the virus solution, the cells were maintained for 2 more days before fixation and immunocytochemical staining with antibodies directed at the N-terminal domain of RasGAP. The lowest volumes of the lentiviral preparations required to infect 100% of the cells were chosen for further experiments.

Cell lines.

βTC-tet and βTC-tet/Bcl-2 cells (16) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, catalog number D-5671) containing 15% decomplemented horse serum (HS) (Sigma, catalog n° H-1270), 2.5 % fetal calf serum (FCS) (Sigma, catalog number F-7524), 10 mM HEPES (Sigma, catalog n° H-3537), 2 mM L-Glutamine (Sigma, catalog n° G-7513), 1 mM sodium pyruvate (Sigma, catalog n° S-8636) at 37°C and 5% CO₂. Generation of fragment N-producing cell lines was obtained following infection with fragment N-encoding viruses in conditions leading to 100% infection efficiency (see the “Lentivirus” section). Generation of control cell lines were obtained using empty lentiviruses using the same volume loads as when cells were infected with fragment N-encoding viruses. When viruses constructed with plasmids derived from the TRIP-PGK-IRESNEO-WHV vector were used (e.g. N-D157A.irs), a selection step with 1 mg/ml neomycin for 14 days was additionally performed. Growth-arrest of the βTC-tet and their derivatives was achieved by incubating the cells for 4 days with 1 µg/ml tetracycline (Fluka, Buchs, Switzerland) (16). This represses the expression of the large T antigen and prevents the cells from proliferating (20).

Chemicals and antibodies.

The anti-phospho serine 473-Akt rabbit polyclonal IgG antibody was from Cell Signaling Technology (catalog n° 9271). The rabbit polyclonal IgG antibody recognizing Akt1/2 was from Santa-Cruz Biotechnology (catalog n° SC-8312). The anti-RasGAP antibody is directed at the SH domains of RasGAP and has been described before (21). Anti-Bcl-2 antibody was from Upstate (catalog n° 05 341). Cisplatin was from Sigma (catalog n° P4394). Cytokines (TNFα, IL-1β, INF-γ) were from ALEXIS (catalog n° ALX-520-002-C010, ALXALX-520-001-C010, PBL-11500-2, respectively). The antibody directed at the SV40 large T antigen was from Becton Dickinson (catalog n° 55414).

Apoptosis assay.

Percentage of apoptosis was determined by scoring cells displaying pycnotic nuclei (visualized with Hoechst 33342) (13). Apoptosis scoring of primary islets cells was performed after dissociating the agglomerated cells in 300 µl of cell dissociation PBS-based buffer (Gibco, cat. n° 13151-014) at 37°C for 10-15 minutes. The cells were then washed once in PBS before being resuspended in 100 µl of PBS containing 10 µg/ml Hoechst 33342. Scoring of apoptosis was performed on 20 µl of the stained cells deposited on slides and covered with coverslips.

NF-κB activity assay.

Cells were transfected with 0.5 µg of the NF-κB reporter plasmid prLUC (22) and 0.5 µg of pRL-TK encoding the Renilla luciferase (Promega) used as an internal control. One day following transfection, the cells were lysed in “passive lysis buffer” (Promega kit n° 1910). Measurement of the NF-κB activation was then performed with a Promega kit (catalog n° #E1910) using lysates (25 µg protein) according to the manufacturer’s protocol (http://www.promega.com/tbs/tm040/tm040).
The activity of the NF-κB reporter plasmid was normalized to the activity of the internal control.

**Western blot analysis.**

Cells were lysed in monoQ-c buffer (13) in which 1 mM Na₃VO₄ was freshly added. Western blotting was performed as described previously (23;24) using a homemade ECL reagent (13).

**Immunocytochemistry.**

The functional infectivity of the virus preparations was determined by immunocytochemistry. Sub-confluent β-Tc-tet or CDM3D cells seeded on coverslips in 6 wells plates were cultured overnight with various volumes of fragment N-encoding recombinant virus. After removal of the virus solution, the cells were maintained in culture for 2 more days. The next steps were performed at room temperature. The cells on coverslips were washed with 4 ml PBS, fixed with 3 ml PBS/3%formaldehyde/3% sucrose for 20 min, washed three times with PBS, incubated with 2 ml PBS/0.2% TX-100 for 10 min, washed once with PBS and incubated 15 minutes at room temperature with 3 ml of filtered serum-containing culture medium. The cells were then incubated 30 minutes in this medium in the presence of a 1/50 dilution of an anti-HA antibody prepared as described (13). After 3 washes with 4 ml PBS, the cells were incubated as above with an anti-mouse Cy3-labelled antibody (Jackson ImmunoResearch, catalog n° 715-165-151) at a 1:500 dilution. After 3 more washes over a period of 60 minutes, the coverslips were mounted in VECTASHIELD Mounting Medium (Vector Laboratories, Ltd., catalog n° H-1000) and visualized with a Zeiss Axioplan 2 imaging microscope equipped with a Plan-Neofluar 40x/0.17 oil ∞/0.17 lens and a Zeiss AxioCam HRC camera using the Zeiss AxioVision acquisition software.

**Giemsa staining.**

The cells were seeded in 6 well plates at a density of 200 cells/well. After two weeks, the colonies were stained as follows. The wells were washed with 5 ml PBS and air-dried. The wells were then incubated 15 minutes with ethanol and air-dried again, then incubated 15 minutes with 4 ml of a Giemsa stain solution (Giemsa Blood Stain solution; Baker Inc.; Phillipsburg, NJ) diluted 1:5 in methanol. The stain solution was then aspirated and the plates placed upside down on water for at least 20 min. The plates were finally extensively washed with tap water and air-dried. Colonies were counted with a Bio-Rad Fluor-S MultiImager using the Personal Molecular Imager FX colonies counting program.

**Cell counting.**

Cells in 6-well plates (200 cells/well) were trypsinized for 3 minutes in 500 µl of trypsin-EDTA solution 1x (Sigma, catalog n° T 3924), followed by the addition of 500 µl culture medium. The cells were then harvested, centrifuged at 700 x g for 5 minutes, and resuspended in 100 µl culture medium in the presence of 10 µl of Trypan Blue Solution (0.4%) (Sigma; catalog n° T8154). Living cells excluding the dye were scored using a Neubauer Improved counting chamber (Blue Brand, catalog n° MAR-0610710).

**Insulin quantitation.**

Cells were extracted with acid/ethanol (lysis in 400 µl 75% ethanol, 1.5% concentrated hydrochloric acid for 24 h at 4°C followed by a centrifugation at 700 g for 3 min). Insulin content of the cell extracts was assessed using a Linco ELISA kit according to the manufacturer’s protocol (Linco; ELISA kit, catalog n° EZRMI-13K and E6013).

**Insulin secretion assays.**

Growth-arrested cells were washed three times with a modified Krebs-Ringer/bicarbonate-HEPES buffer (KRH;
140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH₂PO₄, 0.5 mM MgSO₄, 1.5 mM CaCl₂, 2 mM NaHCO₃, 10 mM HEPES, 0.1% bovine serum albumin, pH 7.4) and pre-incubated with KRBH containing 2 mM of glucose for 1 hour at 37°C. Cells were then incubated for 35 minutes in KRBH, 2 mM glucose (basal response) or KRBH, 20 mM glucose, 10 µM forskolin (Sigma; catalog n° F-6886), 100 µM isobutylmethylxanthine (IBMX; Sigma; catalog n° I-7018) (stimulated response). The supernatant was harvested for the measurement of secreted insulin and the remaining cells were extracted with acid/ethanol for the measurement of the cellular insulin content, as described above.

Preparation of rat and mouse islets.
Wistar rats (200-350 g) were killed in a CO₂ atmosphere for 4 minutes. The abdominal cavity was then exposed and the proximal common bile duct was occluded with a Silkan thread (BBraun, Sempach, Switzerland, cat. n°01134027). A 18G butterfly needle was then inserted in the ampulla of Vater, tied with Silkan thread, and through which 10 ml of ice-cold collagenase (Roche; cat. n° 11 213 865 001) solution (1 mg/ml in Hanks' balanced salt solution [HBSS]-Hepes/glucose buffer [140 mM NaCl, 0.4 mM Na₂HPO₄, 4 mM NaHCO₃, 5 mM KCl, 0.4 mM KH₂PO₄, 0.8 mM MgSO₄•7H₂O, 1.3 mM CaCl₂•2H₂O, 2 mM glucose, 10 mM Hepes pH 7.2]) was injected. The pancreas was surgically removed and placed in 10 ml of ice-cold collagenase solution, then transferred to a 37°C water bath for 38 minutes. During this period, the sample was vortexed 4 times. Forty ml of ice-cold HBSS-Hepes/glucose buffer containing 4 mg/ml BSA (isolation buffer) were then added and the sample centrifuged at 65 g for 10 seconds. The next steps were all performed on ice. Part of the supernatant was aspirated so as to leave about 5 ml in the tube. The supernatant was discarded (about 20 ml), the remaining solution passed through the 5G needle twice, complemented with 20 ml of isolation buffer and centrifuged as described above. Twenty ml of the supernatant was again discarded and the remaining solution filtered through 2 layers of sterile gaze. The filter was washed twice with 10 ml of isolation buffer. The collected solution was centrifuged at 500 g for 10 seconds. The supernatant was discarded so as to leave about 5 ml in the tube. The sample was more tightly packed by a 10 second centrifugation at 400 g. The supernatant was decanted, the pellet was gently resuspended in 20 ml Histopaque 1077 solution (Sigma; cat. n°10771), layered carefully on 20 ml isolation buffer and spun in a Sigma 4K15 centrifuge at 1100 g for 15 minutes (with setting 0 for the acceleration and the deceleration). The lower islet-containing phase (but not the pellet) was then harvested with a Pasteur pipette and isolation buffer was added up to 40 ml. The sample was spun in a Sigma 4K15 centrifuge at 450 g for 1 minutes (with setting 3 for the acceleration and the deceleration). The supernatant was discarded so as to leave about 5 ml in the tube and isolation buffer was added up to 40 ml. The sample was then spun in a Sigma 4K15 centrifuge at 65 g for 30 seconds (with setting 0 for the acceleration and the deceleration). The islets were washed once with 40 ml RPMI (Gibco, cat. n°31870-025) supplemented with 10% FCS, 10 mM Hepes pH 7.4 (Sigma; cat. n°H3537), 1 mM sodium pyruvate (Sigma; cat. n°S8636), 2 mM glutamine (Sigma; cat. n°G7513), 50 µM β-mercaptoethanol (Gibco; cat. n°31350-010) and 1% penicilline/streptomycine solution (Sigma; cat. n°P0781) and centrifuged as above. The supernatant was discarded so as to leave about 5 ml in the tube. The islets were then transferred to a 3 cm-Petri dish, hand-picked (approximately 500 islets/rat) and placed in a new 3 cm-Petri dish. This procedure was repeated (5-10 times) until the majority of the exocrine cells was eliminated.
Dissociation of the islets was performed as follows. The islets were washed with 1 ml HBSS-Hepes containing 1 mM EGTA and 5 mM glucose, resuspended in 300 µl of the same buffer and incubated at 37°C for 3 minutes. The islets were then pipetted up and down until loosely dissociated. The reaction was stopped by the addition of 1 ml of supplemented RPMI. The islets were washed again with 1 ml supplemented RPMI, resuspended in the same medium at a concentration of about 20 islets/ml before being placed in 6 well-plates (2 ml/well).

Mice islets were prepared in a similar manner as described for the rat islets with the following differences. Two ml collagenase solution was injected through a 30G needle and the pancreas transferred to 2 ml of the collagenase solution. The reaction was stopped by the addition of 30 ml ice-cold HBSS-Hepes, centrifuged 2 minutes at 400 g at 4°C, washed 3 times with 40 ml of HBSS-Hepes and the pellet resuspended in 10 ml HBSS-Hepes. Following filtration through the gaze, the islets were not layered on Histopaque but directly resuspended in 10 ml supplemented RPMI and hand-picked and dissociated as described above.

Results.

Generation of fragment N-expressing lentiviruses.

Fragment N can be cleaved, and its anti-apoptotic properties abrogated, when caspase activity increases in cells (12). Fragment N cleavage can be prevented by introducing a point mutation in the caspase-3 recognition site at position 157 (13;14). In this study, the terminology “fragment N” refers to the uncleavable form.

Fragment N was introduced in βTC-tet cells, expressing or not Bcl-2 as a result of lentiviral infection (16), using 2 different types of fragment N-encoding lentiviruses. The first type expresses fragment N but no selection marker, while the second type bears a polycistron composed of fragment N followed by the neomycin resistance gene. Figure 1 shows that both types of viruses induced the production of fragment N in cells. The levels of fragment N expression were however lower in cells infected with the neomycine resistance encoding-viruses compared to the cells infected with the other virus (Figure 1). The two types of viruses provide therefore the opportunity to study the effect of high and low expression levels of fragment N on β cell survival.

Fragment N has been shown to induce Akt activity and repression of the NF-κB pathway in HeLa cells (14). Figure 2 shows that expression of fragment N in βTC-tet cells, whether or not they express Bcl-2, also led to Akt activation coupled with a reduction of basal NF-κB activity. There is therefore no difference in the way fragment N regulates Akt and NF-κB in the different cell types tested so far.

Fragment N increases the resistance of β cells towards various stresses.

We first determined the sensitivity of βTC-tet cells to cisplatin, a genotoxic compound inducing apoptosis in many cell types, in the presence or absence of fragment N. βTC-tet cells infected with empty viruses were as sensitive as control cells towards cisplatin-induced apoptosis (Figure 3A). In contrast, a 4 times higher cisplatin concentration was required to kill the cells infected with fragment N-encoding viruses compared to control cells or cells infected with empty viruses (Figure 3A). Cells infected with the fragment N-encoding virus bearing no selection marker were slightly more resistant towards cisplatin compared to cells infected with fragment N-encoding virus bearing the neomycin-resistant gene (Figure 3A). This is likely due to the lower expression of fragment N in the former cells compared to the latter (Figure 1).

We next assessed whether the protective effect of fragment N could synergize with those of Bcl-2, another anti-apoptotic molecule that has been shown to increase the resistance of βTC-tet towards
hypoxia- and cytokine-induced apoptosis (16). As expected, the presence of Bcl-2 rendered βTC-tet cells about 2.5 times less sensitive to cisplatin-mediated cell death (compare the EC50 of the red lines in Figure 3B). The Bcl-2-expressing cells could be rendered 4 times more resistant towards cisplatin in the presence of fragment N (Figure 3B, right panel). Therefore, the combined presence of Bcl-2 and fragment N rendered βTC-tet cells 10 times more resistant to cisplatin.

We next determined the capacity of βTC-tet cells to cope with cell crowding as a mean to assess their resistance towards a degradation of their trophic and growth conditions. Control βTC-tet cells grew more or less exponentially up to 7-8 days and their population collapsed thereafter within the following 3 days (Figure 4). The presence of Bcl-2 slightly delayed the collapse of the culture by 1-2 days. In contrast, cells expressing high or low levels of fragment N (i.e. cells derived after infections with non neomycin-encoding vs. neomycin-encoding viruses [see Figure 1]) appeared to grow faster and collapsed 2-3 days later and at cells densities that were 2-4 times higher compared to control cells. These results indicate that the presence of fragment N allows cells to grow and survive longer in degraded environments.

To assess the resistance of βTC-tet cells to an inflammatory environment, βTC-tet cells were incubated with a cocktail of inflammatory cytokines. Non-infected cells or cells infected with empty viruses encoding or not the neomycin resistance gene displayed a ~3 fold increase in the apoptotic rate in response to the cytokine cocktail (Figure 5A). In contrast, when the cells were infected with either type of viruses encoding fragment N, the cytokines only increased apoptosis by 1.5-1.6 fold. The presence of Bcl-2 did not protect cells from apoptosis in response to the concentrations of cytokines used here. Akt activity was required for the protection conferred by fragment N because a dominant-negative mutant of Akt prevented fragment N from inhibiting cytokine-induced apoptosis (Figure 5B).

It could be argued that fragment N may only confer a temporary protection (such as the one seen in Figure 5A) but no long-term survival advantage. To assess this point, βTC-tet and βTC-tet/Bcl-2 cells expressing or not fragment N were placed in Petri dishes and allowed to grow for about 2 weeks in the absence or in the continuous presence of inflammatory cytokines. As shown in Figure 6, the presence of inflammatory cytokines diminished the ability of βTC-tet to form colonies by a factor of about 10 regardless of whether the cells expressed Bcl-2 or not. In contrast, inflammatory cytokines diminished the capacity to form colonies by less than 2-fold in βTC-tet cells expressing fragment N. The co-expression of fragment N with Bcl-2 did not appear to further increase the capacity of βTC-tet cells to generate colonies (Figure 6). Altogether, these results demonstrate that fragment N confers long-term protection against the pro-apoptotic actions of inflammatory cytokines when other anti-apoptotic proteins (e.g. Bcl-2) cannot.

The βTC-tet cells express the large T antigen allowing them to proliferate. Even though it has been recently demonstrated that differentiated β cells have the capacity to proliferate to compensate for a loss in β cell mass (25), mature β cells have probably a low proliferation activity. βTC-tet cells can be growth arrested by repressing the expression of the large T antigen with tetracycline (Figure 7A) and in this state can secrete insulin with a normal glucose dose dependency (20). We therefore determined whether fragment N would also protect βTC-tet when growth arrested. As shown in Figure 7B, the presence of fragment N protected growth-arrested βTC-tet cells as efficiently as it did in proliferating βTC-tet cells. Importantly, the presence of fragment N did neither alter the insulin content of the cells (Figure 7C) nor their ability to secrete insulin in response to increased glucose concentrations (Figure 7D). Altogether, these results indicate that fragment N
potently protects β cells from a variety of stress and noxious stimuli without interfering with their insulin secretion capacity.

**Fragment N protects primary islet cells from cytokine-induced death.**

To determine whether fragment N also mediates protection in primary islet cells, mouse and rat islets of Langerhans were isolated, dissociated, infected with empty lentiviruses or virus encoding fragment N, and then incubated or not with inflammatory cytokines. Figure 8 shows that fragment N potently protected islet cells from both species from cytokine-induced apoptosis (panel A) and that this correlated with increased levels of phosphorylation of Akt at its activation sites (panel B). These results indicate that fragment N activates Akt and promotes survival in both transformed and primary β cells.

**Discussion.**

Elimination of pancreatic β cells by apoptosis is a culminating event leading to diabetes. The development of tools favoring β cell survival in patients is therefore of critical importance to delay or prevent the development of the disease (26). Moreover, compounds that increase β cell survival would be extremely useful in islet transplantation procedures, such as the Edmonton Protocol, to increase the yield of islet cells production from diseased donors and to ameliorate the rate of successful engraftment of the pancreatic islets in the host (26).

In the present study we describe a new strategy to protect insulin-secreting cells based on the expression of an anti-apoptotic N-terminal RasGAP fragment called fragment N. Efficient protection of the insulin-secreting βTC-tet cell line against genotoxins, degraded cellular environments, and inflammatory cytokines was achieved even with the lowest cellular expression of fragment N tested. It seems therefore not necessary to strongly express fragment N in order to protect insulin-secreting cells.

The potencies of fragment N and Bcl-2 to protect insulinoma cells were compared. Expression of both proteins in cells was achieved through infection with lentiviral vectors [this study and (16)]. The same methodology was therefore used to express these two proteins in the insulin-secreting cell line. In these conditions, it was observed that fragment N protected cells more efficiently against a variety of noxious stimuli than Bcl-2. The two proteins however clearly induced an additive protection signal when combined (e.g. against cisplatin-induced apoptosis or to counteract the negative effects of degraded environments). Strategies using two anti-apoptotic molecules are therefore not necessarily mutually exclusive and might in fact confer additional levels of β cell protection.

A very potent anti-apoptotic mediator is the Akt kinase (27). Akt is able to stimulate the NF-κB pathway (14;28;29) and in some cells types, Akt-induced NF-κB activation is required for cell survival (30). In β cells, however, NF-κB stimulation can be detrimental (3;11;31-33). Therefore in contrast to many cell types, expression of active Akt in β cells and a concomitant NF-κB activation could favor apoptosis. This has indeed been confirmed in vivo in mice expressing an Akt1 transgene under the control of the insulin promoter (34).

In HeLa cells, the N-terminal fragment of RasGAP generated following its partial cleavage by caspase-3 induces Akt but prevents Akt from activating the NF-κB pathway (14). We show here that fragment N does the same in insulin secreting cells. Expression of fragment N in pancreatic β cells would therefore allow Akt to fulfill its anti-apoptotic and proliferative functions, and at the same time, repress the potentially detrimental NF-κB-inducing activity of Akt (11;35). Our results indeed demonstrate that expression of fragment N in the βTC-tet insulinoma cell line confer long-term
protection from various adverse conditions and apoptotic stimuli, including inflammatory cytokines that are believed to be the patho-physiological mediators of β cell death leading to diabetes (3). Fragment N did not however affect the insulin secretion capacity of βTC-tet cells. This fragment represents therefore a potential therapeutical tool to protect β-cells in diabetogenic conditions without compromising their physiological properties.

Acknowledgements.
We thank Guy Niederhauser and Gilles Dubuis for technical assistance and Dr. Peter Clark for suggestions and comments. We also thank Dr. Isabelle Decosterd and Marie Pertin for their help with rat manipulations. This work was supported by the Swiss National Science Foundation (grant n° 3100-066797/1) and the Botnar Foundation (Lausanne, Switzerland).

Reference List

Figure Legends

Figure 1. Expression of fragment N in insulinoma βTC-tet cells following lentiviral infection.
A. βTC-tet cells were infected with the indicated quantities of HA-tagged fragment N-encoding lentiviruses that do (lower row) or do not (upper row) co-express the neomycin resistance gene. The expression levels of fragment N were assessed by immunocytochemistry analysis 72 hours after the infection using an anti-HA antibody and an anti-mouse IgG Cy3-labelled antibody. All pictures were taken with a 40x objective and with an exposure time of 4 s using a Zeiss Axiovision microscope.
B. βTC-tet cells and βTC-tet/Bcl-2 cells were infected as described in panel A. The cells were then lysed and expression of fragment N was assessed by Western blot using an anti-RasGAP antibody.

Figure 2. Fragment N induces Akt activation and NF-κB inhibition in insulin-secreting cells.
A. Half a million βTC-tet (-) or βTC-tet /Bcl-2 cells (+) stably infected with the indicated viruses were cultured in 6 well-plates for 24 hours and starved in DMEM for an additional 48 hour period to reduce basal Akt levels. The cells were then lysed in 150 µl monoQ-c lysis buffer. The levels of active Akt were then assessed by Western blot analysis using an anti-HA antibody.
antibody specific for the phosphorylated active form of Akt. The expression levels of Akt were also assessed by Western blot using an antibody recognizing all forms of Akt (total Akt).

B. βTC-tet and βTC-tet/Bcl-2 cells (0.5 x 10⁶) stably infected with the indicated viruses were placed in 6 wells plates. The following day, the cells were transfected with prLUC and pRL-TK plasmids and 24 hours later the cells were lysed and the NF-κB activity measured as described in the method section.

Figure 3. **Insulinoma cells expressing fragment N are more resistant to cisplatin induced apoptosis**

A. βTC-tet cells, βTC-tet infected with empty viruses bearing or not the neomycin (neo) resistance gene, and βTC-tet infected with viruses expressing fragment N virus (with or without the neomycin resistance gene) were incubated with increasing concentrations of cisplatin for 24 hours. The extent of apoptosis was then scored. Results correspond to the mean + SD of 4 independent experiments.

B. βTC-tet cells and βTC-tet cells stably expressing the Bcl-2 cDNA (βTC-tet /Bcl-2 cells) were either not infected, infected with an empty virus, or infected with a lentivirus encoding fragment N. The viruses used here did not encode the neomycin resistance gene. The cells were then incubated with increasing concentrations of cisplatin for 24 hours and the extent of apoptosis was scored. Results correspond to the mean + SD of 3 independent experiments.

Figure 4. **Expression of fragment N in insulinoma cells leads to increased expansion and survival.**

βTC-tet and βTC-tet /Bcl-2 cells infected with the indicated lentiviruses were seeded in 6 well (3.7 cm)-plates at a density of 50'000 cells per well. The cells in the wells were counted after the indicated periods of time in culture. The cells were left in the initial 2 ml culture medium until the end of the experiment.

Figure 5. **Expression of fragment N in insulinoma cells increases their resistance towards inflammatory cytokines.**

A. βTC-tet and βTC-tet/Bcl-2 cells stably infected with the indicated viruses were left untreated or incubated with 1000 U/ml of TNFα, IL-1β, and INF-γ. Apoptosis was scored one day later. Results correspond to the mean ± SD of 4 independent experiments.

B. βTC-tet cells stably infected with empty viruses (control cells) or lentiviruses encoding fragment N were infected with empty lentiviruses or lentiviruses encoding a dominant-negative mutant of Akt (DN-Akt). Forty-eight hours later, the cells were incubated with 1000 U/ml of TNFα, IL-1β, and INF-γ for 24 hours and the extent of apoptosis was scored. Results correspond to the mean ± SD of 2 independent experiments performed in duplicate. The significance of the differences between the two cell types infected with the dominant-negative Akt mutant and stimulated with cytokines was assessed by the Student t-test (NS, not significant; *, p < 0.05).

Figure 6. **Expression of fragment N in insulinoma cells confers long-term resistance towards inflammatory cytokines.**

Two hundred βTC-tet and βTC-tet /Bcl-2 cells stably infected with the indicated viruses were placed in p100 Petri dishes and left untreated or incubated with 1000 U/ml of TNFα, IL-1β, and INF-γ for 14 days. The colonies were then stained with Giemsa and counted. The upper panel depicts a representative experiment. The results presented in the lower panel are expressed as the
percentage of clones obtained in the absence of cytokines (average ± SD of 3 independent experiments).

Figure 7. Fragment N increases the resistance of growth-arrested βTC-tet cells towards inflammatory cytokines without affecting their glucose-induced insulin secretion. βTC-tet and βTC-tet/Bcl-2 cells stably infected with the indicated viruses were incubated with tetracycline (1 µg/ml) for 5 days to repress the expression of the large T antigen and to promote growth arrest (16). Expression of the large T antigen was assessed by Western blot (panel A). The cells were then treated as described in Figure 5 to assess their sensitivity towards cytokine-induced apoptosis (panel B). The insulin content of the growth-arrested cells was also determined (panel C). Results are expressed as the percentage of the insulin content in control cells and represent the mean ± SD of triplicate determinations. Alternatively, the growth-arrested cells were incubated in 2 mM glucose-containing medium for 1 hour and switched to a 2- or 20 mM glucose-containing medium for 35 minutes. The amount of insulin secreted was then measured by Elisa (panel D). Results are expressed as a percentage of cellular insulin content and correspond to the mean ± SD of triplicate determinations.

Figure 8. Fragment N protects primary islet cells from cytokine-induced apoptosis. Freshly dissociated rat and mouse islets were infected with empty lentiviruses or lentiviruses encoding fragment N. Twenty-four hours later, the cells were incubated or not with inflammatory cytokines (1'000 U/ml TNFα, 1’000 U/ml IL1β and 50 U/ml IFNγ) for an additional 24 hour period before scoring apoptosis (panel A). Results correspond to the mean ± SD of 3-4 independent determinations. The significance of the differences between cells types incubated or not with cytokines was assessed by the Student t-test (NS, not significant; **, p < 0.01). Alternatively, rat islet cells were lysed and the levels of active Akt and total Akt were assessed by Western blot analysis as described in Figure 2A (panel B).
### A.

<table>
<thead>
<tr>
<th>Virus load (ml)</th>
<th>0</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragment N-encoding viruses</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
</tr>
<tr>
<td>Fragment N-encoding viruses (neo)</td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
</tr>
</tbody>
</table>

### B.

<table>
<thead>
<tr>
<th></th>
<th>βTC-tet</th>
<th>βTC-tet/Bcl2</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-infected</td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>empty virus</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
</tr>
<tr>
<td>neo-encoding virus</td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
</tr>
<tr>
<td>fragment N-encoding virus</td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
</tr>
<tr>
<td>fragment N- and neo-encoding virus</td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
</tr>
<tr>
<td>non-infected</td>
<td><img src="image21.png" alt="Image" /></td>
<td><img src="image22.png" alt="Image" /></td>
</tr>
<tr>
<td>empty virus</td>
<td><img src="image23.png" alt="Image" /></td>
<td><img src="image24.png" alt="Image" /></td>
</tr>
<tr>
<td>neo-encoding virus</td>
<td><img src="image25.png" alt="Image" /></td>
<td><img src="image26.png" alt="Image" /></td>
</tr>
<tr>
<td>fragment N-encoding virus</td>
<td><img src="image27.png" alt="Image" /></td>
<td><img src="image28.png" alt="Image" /></td>
</tr>
<tr>
<td>fragment N- and neo-encoding virus</td>
<td><img src="image29.png" alt="Image" /></td>
<td><img src="image30.png" alt="Image" /></td>
</tr>
</tbody>
</table>

*Figure 1
Yang 2005*
Figure 2

Yang 2005
Figure 5
Yang 2005

A. Bar graph showing the percentage of apoptosis in cells infected with different viruses. The graph compares control and cytokines treatments in the context of βTC-tet and βTC-tet/Bcl-2 conditions.

B. Bar graph showing the percentage of apoptosis in control and DN-Akt expressing cells. The graph compares the response of control and cytokines treatments under normal and DN-Akt conditions.
Figure 6
Yang 2005

Number of colonies in the presence of 100 U/ml cytokine cocktail (% control)

βTC-tet
- cytokines
- cytokines

+ cytokines

βTC-tet/Bcl-2

- cytokines
+ cytokines

non-infected
empty virus
fragment N-encoding virus
neo-encoding virus
fragment N- and neo-encoding virus
non-infected
empty virus
fragment N-encoding virus
neo-encoding virus
fragment N- and neo-encoding virus
Figure 7 (A-B)

Yang 2005
A. A graph showing the percentage of apoptosis in rat and mouse under different conditions.

B. Western blot images showing Phospho-Akt and Total Akt levels under various conditions.

Figure 8
Yang 2005
Expression of an uncleavable N-terminal rasgap fragment in insulin secreting cells increases their resistance towards apoptotic stimuli without affecting their glucose-induced insulin secretion

Jiang-Yan Yang, Joël Walicki, Amar Abderrahmani, Marion Cornu, Gérard Waeber, Bernard Thorens and Christian Widmann

*J. Biol. Chem.* published online July 25, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M504058200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2005/07/25/jbc.M504058200.citation.full.html#ref-list-1