14-3-3 proteins are essential signalling hubs for beta cell survival

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Abstract

Aims/hypothesis Diabetes is characterised by pancreatic beta cell death and dysfunction, resulting from unbalanced pro-survival and pro-death signalling. The 14-3-3 proteins are molecular adaptors that integrate numerous signalling pathways, including the v-raf-leukaemia viral oncogene 1 (RAF1)/B cell leukaemia/lymphoma 2 (BCL-2)-associated agonist of cell death (BAD) pathway, which we have previously implicated in insulin-dependent beta cell survival. Nevertheless, the roles of 14-3-3 proteins in beta cell fate and function have not been investigated.

Methods We examined the abundance, localisation, modulation and roles of 14-3-3 proteins using quantitative RT-PCR, immunoblot or imaging. MIN6 cells or mouse islets cells were manipulated with inhibitors, short interfering RNA (siRNA) or plasmids overexpressing 14-3-3.

Results We first characterised the abundance and subcellular location of all seven 14-3-3 isoforms in mouse and human beta cells. Most isoforms were cytoplasmic, except 14-3-3 σ , which appeared to be nuclear. Analysis of 14-3-3 abundance under stress conditions revealed distinct modulation in mouse islets and MIN6 cells. Generalised 14-3-3 inhibition promoted apoptosis and dysfunction, and siRNA-mediated knockdown revealed isoform-specific roles in

caspase-3-dependent beta cell apoptosis, with a clear role for 14-3-3 ζ . Overabundance of 14-3-3 ζ sequestered BAD–BCL2-associated X protein (BAX) from mitochondria, attenuated *Dp5* (also known as *Hrk*) and *Puma* (also known as *Bbc3*) induction, and increased survival in response to pro-inflammatory cytokines or thapsigargin. Anti-apoptotic insulin treatment increased the sequestration of BAD/BAX by 14-3-3 ζ . BAD mutants that were unable to bind 14-3-3 ζ localised to mitochondria and induced apoptosis.

Conclusions/interpretation This first study of the 14-3-3 family in beta cells revealed specific regulation, localisation and anti-apoptotic roles among the isoforms. Focus on 14-3-3 ζ revealed its importance in preventing BAD–BAX mitochondrial localisation and protecting beta cells from multiple stresses. Thus, some 14-3-3 proteins are pro-survival signalling hubs.

Keywords 14-3-3 ζ · BAD · BAX · Insulin signalling · Mitochondria · Molecular adaptors

Abbreviations

ASK1	Apoptosis signal-regulated kinase-1
BAD	BCL2-associated agonist of cell death
BAX	BCL2-associated X protein
BCL-2	B cell leukaemia/lymphoma 2
Bcl _x _L	B-cell lymphoma-extra large
BH3	Bcl-2 homology domain 3
CHOP	CCAAT/-enhancer-binding protein homologous protein
COX-IV	Cytochrome c oxidase subunit IV
dsRed	Red fluorescent protein from <i>Discosoma</i> coral
ER	Endoplasmic reticulum
ERK	Extracellular signal-related kinase
GFP	Green fluorescent protein
HA	Haemagglutinin
JNK	c-Jun N-terminal kinase
mRFP	Monomeric red fluorescent protein

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NFκB	Nuclear factor κB
RAF1	v-Raf-leukaemia viral oncogene 1
SAPK	Stress-activated protein kinase
siRNA	Short interfering RNA
YFP	Yellow fluorescent protein

Introduction

The causes of diabetes are complex, with multiple factors contributing to the death and dysfunction of pancreatic beta cells [1, 2]. Despite recent advances in elucidating the mechanisms underlying beta cell death, attempts to prevent the onset of diabetes have been unsuccessful. Numerous hormones promote cell survival, but it is not understood how their downstream signalling pathways are coordinated. Molecular adaptor proteins are key factors that facilitate and integrate multiple signalling cues by recognising specific proteins based on their post-translational modifications. These adaptors coordinate the cellular localisation of specific proteins, leading to the accurate transduction of multiple parallel signals [3]. The roles of such adaptor proteins remain largely unexplored in beta cells.

The seven members of the mammalian 14-3-3 protein family are molecular adaptors that recognise proteins bearing phospho-serine or phospho-threonine motifs [4]. 14-3-3 dimers simultaneously bind multiple proteins and coordinate their orientation and activity [5]. All 14-3-3 proteins have a similar structure, but the extent to which they have evolved distinct roles remains unclear [6]. Evidence from lower organisms and mammalian cell lines shows specific and overlapping roles of each isoform [7, 8]. However, a thorough side-by-side comparison of each endogenous isoform has yet to be performed in any endocrine cell type. Studies have demonstrated the involvement of 14-3-3 proteins in many cellular functions, including positive and negative roles in programmed cell death through their effects on protein stability and protein localisation [4, 9–11]. To mediate cell survival, 14-3-3 isoforms bind to and inhibit the pro-apoptotic activity of B cell leukaemia/lymphoma 2 (BCL2)-associated agonist of cell death (BAD), following its phosphorylation by v-raf-leukaemia viral oncogene 1 (RAF1) or Akt [10, 12]. Of the seven isoforms, 14-3-3ζ is known to modulate the activity of RAF1 [13], which can regulate the production and activity of BAD in beta cells [14–17]. It remains to be determined whether 14-3-3ζ is critical in the regulation of cell survival. The above evidence suggests that 14-3-3 proteins serve as unique integration points to coordinate kinase activity and phospho-dependent survival cues [18]. Therefore, we tested the hypothesis that 14-3-3 proteins are essential for coordinating beta cell survival as the core of a signalling node that integrates multiple survival signals.

We report the first side-by-side comparison of all 14-3-3 isoforms in any endocrine cell type and identified a critical anti-apoptotic role for 14-3-3ζ. These findings suggest that increasing 14-3-3ζ levels or activity could be a novel approach to the prevention of beta cell death that occurs in diabetes.

Methods

Cell culture, transient transfection and reagents MIN6 cells were cultured as described [14]. Experiments were repeated across multiple passages (28 to 45). Mouse islets were isolated from C57/BL6 mice and purified by filtration [15]. Islets were maintained in 10 mmol/l glucose RPMI with 10% FBS (vol./vol.) and penicillin/streptomycin. Human islets (60–80% pure) were collected by G. Warnock (Vancouver General Hospital, Vancouver, BC, Canada) with informed consent from donors, who were 40 to 60 years of age and had no history of diabetes. All mouse islet protocols were performed in accordance with guidelines of the University of British Columbia Animal Care committee. Studies with human islets had institutional review board approval and followed principles from the Declaration of Helsinki of 2000.

MIN6 cells were transfected with commercially available short interfering RNA (siRNA) against each 14-3-3 isoform using Lipofectamine 2000 (Life Technologies, Burlington, ON, Canada). A scrambled, non-targeting siRNA served as the negative control. All experiments were performed 48 h after transfection. MIN6 cells and mouse islets were electroporated (Neon System; Life Technologies) to deliver plasmids encoding: (1) difopein–yellow fluorescent protein (YFP) (gift from H. Fu, Emory University); (2) Haemagglutinin (HA)-14-3-3ζ (gift from A. Muslin, Washington University in St Louis); (3) green fluorescent protein (GFP)-14-3-3ε (Genecopeia, Rockville, MD, USA); (4) BAD–GFP mutants (gift from A. Tolkovsky, University of Cambridge); (5) mitochondria-targeted dsRed (from H. McBride, University of Ottawa); or (6) their respective control plasmids.

In studies examining the response to insulin (Sigma-Aldrich, St Louis, MO, USA), MIN6 cells were starved overnight with 5 mmol/l glucose DMEM with 0.5% FBS (vol./vol.), followed by 2 h incubation in 0 mmol/l glucose KRB supplemented with 0.2% BSA (wt/vol.). The 14-3-3 antagonist, I 2–5, was from EMD/Millipore (Billerica, MA, USA). Cytokines (25 ng/ml TNFα, 10 ng/ml IL-1β, 10 ng/ml IFN-γ) were from R&D Systems (Minneapolis, MN, USA). Thapsigargin (1 μmol/l) and anisomycin (1 μmol/l) were from Sigma (St Louis, MO, USA).

Quantitative real-time PCR and gene analysis RNA was isolated from MIN6 cells or islets using a kit (RNeasy;

Qiagen, Mississauga, ON, Canada). cDNA was generated with a kit (qScript cDNA Synthesis; Quanta Biosciences, Gaithersburg, MD, USA) and transcript levels were measured with SYBR Green fluorescence (StepOnePlus Real-Time PCR System; Applied Biosystems, Carlsbad, CA, USA). All quantitative PCR data were normalised to *Hprt* via the $2^{-\Delta C_t}$ method; expression of *Hprt* was not changed under any experimental conditions. Mature mouse or human beta cells were identified after infection with a *Pdx1*-mRFP-*Ins1*-eGFP dual reporter lentivirus and fluorescence-activated cell sorting using a Cytospea Influx device (BD Biosciences, San Jose, CA, USA) [19, 20]. The relative levels of each 14-3-3 isoform were collected from gene array data (Illumina MouseWG-6 version 2.0 Expression BeadChip, Illumina, San Diego, CA, USA). Other observations from that gene array experiment have been published [20].

Insulin secretion analysis MIN6 cells or dispersed mouse islets were pre-incubated for 2 h in 0 mmol/l glucose KRB with 0.2% BSA (wt/vol.). Cells were subsequently washed with PBS and incubated for 2 h in 3 or 16 mmol/l glucose made with KRB-BSA. The medium was collected and centrifuged at 900g (3,000 rpm). Secreted insulin in supernatant fractions and insulin content in cell lysates were measured by radioimmunoassay (Millipore). Secretion data were normalised to total islet protein.

Western blot and immunoprecipitation MIN6 cells were washed with PBS prior to lysis with RIPA buffer, supplemented with protease inhibitors (Roche Applied Sciences, Laval, QC, Canada). Immunoprecipitation experiments were performed as previously described [15]. Pull-down of HA-14-3-3 ζ was performed with anti-HA-conjugated agarose beads (Sigma-Aldrich). Cytoplasmic and mitochondrial fractions were separated from crude cell lysates with a kit (Mitochondria/Cytosol Fractionation Kit; Biovision, Mountain View, CA, USA). Proteins were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes and probed with antibodies against: cleaved caspase-3, pSer112 BAD, pSer136 BAD, BAD, BCL2-associated X protein (BAX), cytochrome c oxidase subunit IV (COX-IV), pAkt (Ser473), Akt, p-extracellular signal-related kinase (ERK)1/2 (Thr202/Tyr 204), p-c-Jun N-terminal kinase (JNK)1/2, JNK1/2, 14-3-3 β , 14-3-3 γ , 14-3-3 ϵ , 14-3-3 ζ , 14-3-3 η and 14-3-3 θ (all from Cell Signaling Technology, Danvers, MA, USA); and 14-3-3 σ (Millipore); β -actin (Novus Biologicals, Littleton, CO, USA); HA (Santa Cruz Biotechnology, Santa Cruz, CA, USA); CHOP (Millipore); and β -tubulin (Sigma-Aldrich).

Fluorescent imaging Mouse pancreatic sections from C57/BL6 mice were de-paraffinised, rehydrated, subjected to sodium citrate antigen retrieval, incubated in serum-free

protein block (DAKO, Burlington, ON, Canada) and incubated overnight with antibodies against 14-3-3 isoforms (Cell Signaling Technology and Millipore) and insulin (Sigma-Aldrich), followed by Alexafluor-conjugated secondary antibodies (Life Technologies). Dispersed islets or MIN6 cells were seeded on glass coverslips or in 96-well plates. All images were taken with identical exposure times using an inverted microscope (200M; Zeiss, Toronto, ON, Canada) and deconvolved with SlideBook software (Intelligent Imaging Innovations, Denver, CO, USA). Some images were obtained with an inverted confocal microscope (FluoView FV1000 Laser Scanning, Olympus, Markham, ON, Canada). Live-cell analysis of cell death was measured in MIN6 cells or dispersed mouse islets incubated in 25 mmol/l glucose DMEM or 10 mmol/l glucose RPMI, respectively, which contained propidium iodide and Hoechst 33342. Dying cells (positive for propidium iodide and Hoechst 33342) were determined from the total number of cells counted from 30 fields in a well from a 96-well plate, using a live-cell imager (ImageXpress^{MICRO}; Molecular Devices, Sunnyvale, CA, USA); quantification was done with a software package (MetaXpress; Molecular Devices). A $\times 10$ objective was used to capture a large number of cells. However, a disadvantage of this high-throughput approach is that the type of cell death could not be discerned by nuclear morphology (Hoechst 33342).

Statistical analysis Data are expressed as mean $\pm$ SEM and were analysed by Student's *t* test or ANOVA, followed by post-hoc analysis where appropriate. Results were considered statistically significant at $p < 0.05$.

Results

14-3-3 isoforms in pancreatic beta cells To date, no published study has compared the roles of all 14-3-3 isoforms. As a first step, using quantitative PCR, we found that all 14-3-3 isoforms are present in isolated mouse islets and in MIN6 cells (Fig. 1a, b), with similar results found in gene array analysis of FACS-purified mouse and human beta cells [20] (Fig. 1c, d). The localisation of each 14-3-3 protein was characterised in beta cells (Fig. 1e). The 14-3-3 σ isoform was predominantly localised to the nucleus, whereas the γ and ζ isoforms were distributed between the cytosol and nucleus; the remaining isoforms were cytoplasmic. The 14-3-3 group of proteins exists as homo- or heterodimers, which influences their repertoire of binding partners [5]. As expected, co-immunoprecipitation of 14-3-3 ζ from whole MIN6 cell lysates revealed the presence of heterodimers with other isoforms (Fig. 1f). Previous studies have suggested that 14-3-3 protein levels can be modulated during cell death [21, 22]. Thus, we assessed whether the

Fig. 1 14-3-3 proteins are present in pancreatic beta cells. **(a)** Isolated RNA from mouse islets and **(b)** MIN6 cells was subjected to quantitative PCR for all 14-3-3 isoforms. All data were normalised to *Hprt* as the endogenous control ($n=4$ per group). **(c)** FACS-purified mature human ($n=2$) and **(d)** mouse ($n=3$) beta cells were subjected to Illumina gene arrays. [AU, arbitrary units; Rel. exp., relative expression] **(e)** Immunostaining for insulin (red) and each 14-3-3 isoform as indicated (green) was performed on wild-type C57/BL6 mouse pancreatic sections. DAPI was used to visualise nuclei. Scale bars 10 μ m. Insets, $\times 50$ magnification of a single cell. **(f)** Immunoprecipitation (IP) of endogenous 14-3-3 ζ was performed on MIN6 cell lysates and probed for the remaining isoforms (blot representative of $n=3$ per isoform). IB, immunoblot. **(g)** MIN6 cells were treated for 24 h with

cytokines (25 ng/ml TNF- α , 10 ng/ml IFN- γ or 10 ng/ml IL-1 β), 1 μ mol/l thapsigargin or 1 μ mol/l anisomycin, and markers of apoptosis (cleaved caspase-3 [Cl. Casp-3]) and ER stress (CHOP) were measured by immunoblotting; $n=4$ per group; $*p<0.05$ compared with control cells. **(h–k)** Protein levels for 14-3-3 isoform transcripts **(h, i)** (14-3-3 σ [also known as *Sfn*]; 14-3-3 ζ [also known as *Ywhaz*]; 14-3-3 β [also known as *Ywhab*]; 14-3-3 γ [also known as *Ywhag*]; 14-3-3 η [also known as *Ywhah*]; 14-3-3 ϵ [also known as *Ywhae*]; 14-3-3 θ [also known as *Ywhaq*]), and protein levels **(j, k)** in MIN6 cells **(h, j)** and mouse islets **(i, k)** were measured by quantitative PCR or immunoblotting, following exposure to cytokines (black bars), thapsigargin (light grey bars) or anisomycin (dark grey bars); $n=4$ per group; $*p<0.05$ compared with control (white bars) cells

abundance of 14-3-3 isoforms can be affected by three cellular stresses: pro-inflammatory cytokines to mimic type 1 diabetes initiation; thapsigargin to induce endoplasmic reticulum (ER) stress; and anisomycin to model JNK1/2-dependent apoptosis. All stressors induced apoptosis, as detected by increases in cleaved caspase-3. However, the induction of ER stress, as determined by CHOP levels, was only attributed to thapsigargin treatment at the time tested (Fig. 1g). The induction of CHOP by cytokines may depend on culture conditions or species differences [23, 24]. In MIN6 cells and mouse islets, changes in 14-3-3 isoform mRNA were detectable (Fig. 1h, i). Analysis of 14-3-3 isoforms at the protein level revealed distinct changes, including stress-induced reductions in 14-3-3 ζ in primary islets (Fig. 1j, k). Collectively, these data demonstrate that all 14-3-3 proteins are present and their levels are regulated independently by distinct stresses in transformed and primary beta cells. Since we only analysed a single time point in our experiments, it should be noted that any apparent differences between species could be accounted for by differences in the kinetics of these responses.

Inhibition of 14-3-3 proteins decreases beta cell survival To ascertain whether 14-3-3 proteins are required in beta cell fate and function, we transfected cells with difopein, a pan-14-3-3 inhibitor [11]. This induced apoptotic cell death, demonstrated by significant propidium iodide incorporation, caspase-3 activation and BAD translocation to mitochondria (Fig. 2a–d). This is consistent with other studies suggesting that 14-3-3 proteins promote survival by sequestering pro-apoptotic BCL-2 proteins, such as BAD and BAX, in the cytoplasm [4, 25, 26]. Similar results were obtained after incubating MIN6 cells with a cell-permeable 14-3-3 antagonist (Fig. 2e, f). The observation that 14-3-3 inhibition causes apoptosis does not preclude roles in other forms of cell death.

We also assessed whether inhibition of 14-3-3 proteins affects insulin secretion, given their known roles in catecholamine and neurotransmitter exocytosis [27]. Analysis of glucose- and KCl-stimulated insulin release from difopein-producing MIN6 cells or mouse islets showed significant impairments in glucose-induced insulin release due to inhibition of 14-3-3 proteins (Fig. 2g, i). No effects on insulin content were observed (Fig. 2h, j).

To specifically examine the role of each 14-3-3 isoform, we performed a side-by-side comparison of cells with isoform-specific siRNA-mediated knockdown, which in each case significantly reduced the mRNA and protein levels of the respective targets by at least 50% and 30%, respectively (Fig. 3a, b). Knockdown of each isoform did not cause any non-specific decreases in the expression of the remaining isoforms at the mRNA level (Fig. 3a), but in some cases we observed a compensatory increase in another

14-3-3 isoform, consistent with previous studies involving overexpression in other cell types [7]. Knockdown of 14-3-3 γ , 14-3-3 η , 14-3-3 ϵ and 14-3-3 ζ was associated with significant induction of apoptosis (Fig. 3c). The degree of knockdown for each isoform was not identical, thus precluding an absolute ranking of their importance in beta cell survival (i.e. comparing 14-3-3 γ with 14-3-3 ζ). Reductions in the θ , σ and β isoforms did not induce significant apoptosis. Taken together, these findings demonstrate that certain 14-3-3 proteins regulate beta cell survival.

Overexpression of 14-3-3 ζ promotes beta cell survival The high levels of the 14-3-3 ζ isoform in purified human and mouse beta cells (Fig. 1a–d), and the robust effects of 14-3-3 ζ RNA interference (Fig. 3) prompted additional efforts to define the mechanisms by which 14-3-3 ζ is involved in beta cell survival. 14-3-3 ζ is also known to regulate the kinase activity of RAF1 [13], which we have shown to be essential for beta cell survival under serum-free in vitro conditions, where the only source of growth factors is the islets themselves (e.g. autocrine insulin signalling) [14–16, 28]. Specifically, RAF1 can prevent apoptosis by phosphorylating BAD on key serine residues that promote binding to 14-3-3 ζ [14–17]. Thus, we sought to test whether 14-3-3 ζ would be sufficient to protect beta cells from death under severe stress conditions. Indeed, at the time points examined, MIN6 cells overproducing 14-3-3 ζ displayed significantly reduced cell death following exposure to cytokines or thapsigargin (Fig. 4a, b), as well as decreased levels of activated caspase-3 (Fig. 4c). To examine whether this protective effect was specific to 14-3-3 ζ , we performed similar experiments with another isoform, 14-3-3 ϵ . However, MIN6 cells overproducing GFP–14-3-3 ϵ were not protected from cytokine- or thapsigargin-mediated cell death (Fig. 4d, e). Thus, 14-3-3 ζ , but not all other 14-3-3 isoforms can protect beta cells.

Pro-inflammatory cytokines activate intrinsic and extrinsic apoptotic pathways to initiate cell death [29, 30]. Previous studies on beta cells have shown that cytokines induce the dephosphorylation of BAD at Ser112 and Ser136, followed by cytoplasm-to-mitochondrial translocation of BAD to initiate apoptosis [31]. Here we confirmed that cytokine exposure stimulated the translocation of BAD to mitochondria in MIN6 cells (Fig. 5a, b). The sequestration of BAD in the cytoplasm by 14-3-3 ζ is influenced by the phosphorylation of Ser112 and Ser136, which are RAF1- and Akt-targeted residues, respectively [10, 32]. Treatment of MIN6 cells with low and high doses of insulin, which we have previously demonstrated to promote the differential activation of canonical insulin signalling pathways [15, 33], induced the phosphorylation of BAD at Ser112 and Ser136 (Fig. 5c). Interestingly, overexpression of HA-14-3-3 ζ in MIN6 cells was associated with increased RAF1 activity as implied by the elevated basal ERK1/2

Fig. 2 14-3-3 proteins are required for beta cell survival and function. **(a)** MIN6 and **(c)** dispersed mouse islet cells were transfected with difopein-YFP, a pan-14-3-3 inhibitor, or with the empty YFP vector alone, and incubated with propidium iodide (PI) and Hoechst 33342. Dying cells were scored as being positive for PI, YFP and Hoechst 33342. **(b)** Cell lysates from difopein-producing MIN6 cells were resolved by SDS-PAGE and probed for levels of cleaved caspase-3 (Cl. Casp-3). **(a–c)** $n=6$ per group, $*p<0.05$. **(d)** Mitochondrial (Mito.) and cytoplasmic (Cyto.) fractions from control and difopein (Difo)-producing cells were resolved by SDS-PAGE and probed for BAD and BAX. COX-IV and β -tubulin were used as mitochondrial and cytoplasmic loading controls, respectively. Graphs show quantifications of the mitochondrial fraction. Results are representative of $n=4$ experiments; $*p<0.05$. **(e)** MIN6 cells were treated for 24 h with a cell-

permeable 14-3-3 antagonist (14-3-3i) or thapsigargin (Thap.; 1 $\mu\text{mol/l}$), followed by immunoblotting detection of activated caspase-3 from cell lysates; $n=4$ per group; $*p<0.05$. **(f)** MIN6 cells co-producing BAD-GFP or GFP with mitochondria-targeted (Mito)-dsRED to visualise mitochondria were treated for 24 h with a 14-3-3 antagonist to detect BAD translocation to mitochondria. DAPI was used to visualise nuclei. Scale bars 10 μm . **(g, h)** MIN6 cells and **(i, j)** dispersed mouse islets were transfected with plasmids encoding YFP or difopein-YFP and treated with 16 mmol/l glucose or 25 mmol/l KCl for 2 h. Secreted insulin in medium **(g, i)** or insulin content in cell lysates **(h, j)** were measured by radioimmunoassay and normalised to total protein; $n=3–6$ per group; $*p<0.05$ compared with the untreated control, $^{\dagger}p<0.05$ compared with control vector-treated cells

phosphorylation and Ser112 phosphorylation on BAD (Fig. 5c). This observation is in line with the known ability of 14-3-3 ζ to modulate the kinase activity of RAF1 [13]. Moreover, insulin treatment of MIN6 cells promoted the association of pro-apoptotic BAD and BAX with endogenous 14-3-3 ζ (Fig. 5e, f). We next tested whether the increased cell survival conferred by 14-3-3 ζ overabundance was associated with altered BAD and BAX localisation. Mitochondrial fractions from cytokine-treated cells were purified from whole-cell lysates,

and when compared with control vector-transfected cells, overabundance of 14-3-3 ζ prevented cytokine-induced translocation of BAD and BAX to mitochondria and led to an increase in their cytoplasmic distribution (Fig. 5d). Co-immunoprecipitation showed that insulin promoted the association of HA-14-3-3 ζ with BAD and BAX (Fig. 5g). Together, these data suggest that overabundance of 14-3-3 ζ can inhibit cytokine-mediated cell death through sequestration of BAD and BAX in the cytoplasm.

Fig. 3 Knockdown of each 14-3-3 isoform demonstrates differential roles in cell survival.

(a) Knockdown efficiency (50% or greater) and expression of remaining isoform transcripts as indicated following transfection with isoform-specific siRNAs (100 nmol/l) were measured by quantitative PCR; $n=4$ per group; $*p<0.05$ compared with scrambled (siCon)-transfected cells. (b) Lysates from siRNA-transfected cells were resolved by SDS-PAGE and probed for the corresponding 14-3-3 isoform. Actin was used as a loading control and the fold change in abundance of each isoform was normalised to scrambled (siCon) siRNA-transfected cells; $n=4$ per group. (c) Apoptosis was measured in siRNA-transfected cells by immunoblotting for cleaved caspase-3 (Cl. casp-3). Data were normalised to actin as a loading control; $n=4$ per group; $*p<0.05$

To further examine whether the serine phosphorylation of BAD is critical for its localisation and apoptotic activity, BAD-GFP mutants containing single serine-to-alanine mutations at S112A or S136A or a double S112A/S136A mutation, were overproduced in MIN6 cells. A triple mutant (S112A/S136A/S155A) lacking a PKA-phosphorylation site served as a control, as it lacks all inhibitory phosphorylation sites [34]. Wild-type BAD-GFP was predominantly retained in the cytoplasm; however, all other mutants showed increased localisation to mitochondria (Fig. 6a, b). Overabundance of wild-type BAD or Ser112A BAD resulted in modest increases in programmed cell death, as measured by propidium iodide incorporation or activated cleaved caspase-3 levels (Fig. 6c, d), but cells producing the S136A, double mutation or triple mutation BAD-GFP proteins demonstrated much larger increases ($p<0.05$) in dying cells and cleaved caspase-3 levels. These data suggest that in the beta cell, the interaction of BAD with 14-3-3 ζ is a fundamental signalling event that regulates survival.

Regulation of BCL-2 protein levels by 14-3-3 ζ Apart from their ability to bind pro-apoptotic BCL-2 proteins, 14-3-3 proteins can also regulate the nuclear factor κ B (NF κ B) and JNK1/2 signalling pathways [35, 36], both of which mediate cytokine-induced expression of apoptosis-regulating genes, including members of the BCL-2 family [37–39]. Both pathways were clearly activated by cytokines in MIN6 cells (Fig. 7a–c), as evidenced by increased phosphorylation of the p65/RelA transcription factor and by increased phosphorylation of JNK1/2. These events were attenuated in cells overproducing 14-3-3 ζ (Fig. 7b, c). Overabundance of 14-3-3 ζ did not affect the cytokine-induced transcription of a large number of canonical apoptosis-regulating genes (Fig. 7d–i). However, we did find that 14-3-3 ζ overabundance did abrogate the cytokine-induced increase in mRNA levels of *Puma* (also known as *Bbc3*) and the BH3-only sensitiser, *Dp5* (also known as *Hrk*) (Fig. 7n, o) [38–40]. Taken together, these findings suggest that

Fig. 4 Overabundance of 14-3-3ζ promotes beta cell survival. **(a)** MIN6 cells were transfected with plasmids encoding HA-14-3-3ζ or the control vector, and cell death, as assessed by propidium iodide (PI) incorporation, was measured in cells exposed to cytokines (25 ng/ml TNF-α, 10 ng/ml IL-1β, 10 ng/ml IFN-γ) or thapsigargin (1 μmol/l) for 24 h or **(b)** for 48 h; $n=4-6$ per group; $*p<0.05$ compared with the empty vector or untreated control; $^{\dagger}p<0.05$ compared with the empty vector treatment. **(c)** MIN6 cells were transfected with plasmids encoding HA-14-3-3ζ or the control vector (pcl) and treated with cytokines at various time points. Cell lysates were resolved by SDS-PAGE and

immunoblotted for cleaved caspase-3 (Cl. casp-3). Actin was used as a loading control. Results are representative of $n=4$ experiments; $*p<0.05$ compared with control vector-treated cells. **(d)** MIN6 cells were transfected with plasmids encoding GFP or GFP-14-3-3ε, and subsequently treated with cytokines or thapsigargin for 24 h. Cell death was measured by determining PI incorporation in GFP-positive cells or **(e)** by detecting levels of activated caspase-3 in cell lysates by SDS-PAGE; $n=4$ per group; $*p<0.05$ compared with untreated, GFP-transfected cells

14-3-3ζ may promote cell survival through the suppression of specific pro-apoptotic BCL-2 genes.

Discussion

In the present study, we sought to survey all 14-3-3 protein isoforms and we chose a single isoform for detailed mechanistic studies. Our data demonstrate that all seven members are present in pancreatic beta cells, with distinct distribution patterns and differential regulation and roles in cell survival. We identified 14-3-3ζ as a critical signalling hub, which prevents beta cell death through multiple mechanisms. Our findings suggest that modulating the activity or levels of 14-3-3ζ could be a way to prevent beta cell apoptosis and diabetes.

The 14-3-3 family of proteins, first characterised in brain [4], consists of molecular adaptors that recognise phosphorylated proteins (e.g. kinases, transcription factors and receptors). They are able to coordinate almost limitless combinations of protein complexes, which accounts for their functional diversity [3–5, 26]. A previous report established that one isoform is present in beta cells [41], but without an in-depth investigation of its physiological function. In the

present study, inhibition of 14-3-3 function with difopein or a cell-permeable inhibitor promoted cell death, but the differential regulation of isoform levels by cellular stressors

Fig. 5 14-3-3ζ facilitates cell survival through its interactions with BAD. **(a)** MIN6 cells producing GFP or **(b)** wild-type BAD-GFP, and **(a, b)** dsRed targeted to mitochondria (Mito-dsRed) were treated with cytokines for 24 h and imaged. DAPI was used to visualise nuclei. Scale bars 10 μm. **(c)** MIN6 cells transfected with the control vector or plasmids encoding HA-14-3-3ζ were treated with insulin for 20 min and subsequently lysed. Lysates were resolved by SDS-PAGE and immunoblotted for activation markers of the canonical insulin signalling pathways as indicated; $n=4$ per group; $*p<0.05$ compared with untreated, control cells; $^{\dagger}p<0.05$ compared with insulin-treated control cells. **(d)** Mitochondrial and cytoplasmic fractions were purified from whole-cell lysates from control and cytokine-treated MIN6 cells transfected with the vector control or HA-14-3-3ζ plasmids, and immunoblotting was used to measure the abundance of BAD and BAX in each fraction. COX-IV and β-tubulin were used as mitochondrial and cytoplasmic loading controls, respectively; $n=4$ per group; $*p<0.05$. **(e, f)** Immunoprecipitation (IP) of 14-3-3ζ, **(e)** or BAD or BAX from MIN6 lysates, followed by immunoblot analysis, was performed to determine the association of BAD and BAX with 14-3-3ζ after treatment with insulin or glucagon-like peptide 1 (GLP-1). Blots are representative of $n=4$ experiments; $*p<0.05$ when compared to untreated cells. **(g)** HA pull-down assays were performed on MIN6 cells that were overabundant in HA-14-3-3ζ and were treated with insulin, with immunoblotting used to measure BAD and BAX association. Blots are representative of $n=4$ experiments; $*p<0.05$ when compared to untreated cells

suggested isoform-specific roles in apoptosis. In primary islets, 14-3-3 ζ levels were significantly reduced by cytokines and thapsigargin, suggesting a possible role for this isoform in survival. While differences between MIN6 cells and mouse islets were observed in response to the cellular stressors at the time point tested, our data do not rule out similar responses at different time points.

The roles of each endogenous 14-3-3 protein in cell survival were tested by isoform-specific RNA interference that was validated for off-target effects against remaining family members. Different degrees of cell death were observed following knockdown of each isoform, an unexpected finding, given a previous report that in Cos7 cells each isoform inhibits BAD-induced apoptosis to equal degrees

Fig. 6 Impaired interactions of BAD with 14-3-3 ζ promotes cell death. **(a)** The localisation of wild-type (WT) BAD–GFP and Ser-Ala point mutants was visualised in MIN6 cells co-transfected with mitochondria-targeted (Mito)-dsRED. DAPI was used to visualise nuclei. Scale bars 10 μ m. DM, double mutation; TM, triple mutation. **(b)** MIN6 cells overproducing wild-type BAD–GFP or Ser-Ala mutants were lysed and the distribution of GFP-conjugated proteins was examined in mitochondrial and cytoplasmic fractions. COX-IV and β -tubulin were used as loading controls for mitochondrial and cytoplasmic fractions, respectively, to quantify the relative levels of each BAD mutant in mitochondrial fractions; $n=4$ per group; $*p<0.05$ compared with wild-type BAD–GFP. **(c)** Apoptosis induced by overabundance of wild-type BAD–GFP or the Ser-Ala mutants was measured in MIN6 cells by propidium iodide (PI) incorporation or **(d)** by immunoblotting for cleaved caspase-3 (Cl. casp-3); $n=4$ per group; $*p<0.05$

[7]. The differences observed in cell death can be attributed to each isoform having unique interactomes, as structural analysis of the 14-3-3 family revealed a highly variable region between α -helices 8 and 9, which determines binding specificity [42]. This concept has been validated in screens of human 14-3-3 binding partners, which revealed common and unique protein $\times$ protein interactions among the tested isoforms [43]. Further work is required to examine the interactome of each 14-3-3 isoform in beta cells.

One of the defining characteristics of type 1 diabetes is the profound loss of beta cell mass induced by pro-inflammatory cytokines and linked, by some investigators, to ER stress [1, 2, 29]. The survival of pancreatic beta cells under normal and diabetic conditions is influenced by the balance of pro-survival growth factors, including insulin itself [1, 2, 14–17]. We have previously identified the key insulin signalling component, RAF1, as a context-dependent regulator of pancreatic beta cell survival and function [14–17]. Our decision to focus on 14-3-3 ζ was in

part due to its ability to regulate the kinase activity of RAF1, which phosphorylates pro-apoptotic BAD on Ser112, a 14-3-3 binding site [13, 44]. The 14-3-3 ζ isoform was also the most highly abundant isoform in FACS-purified human beta cells. Although knockdown of 14-3-3 γ led to the highest degree of caspase-3 activity, its abundance in mouse and human beta cells was one of the lowest among 14-3-3 isoforms, thus reinforcing our decision to focus on the ζ isoform. Our observations that 14-3-3 ζ knockdown or overabundance promoted apoptosis or cell survival was consistent with evidence from other cell types [9, 45], suggesting an essential role for 14-3-3 ζ in beta cell survival.

Life and death decisions in pancreatic beta cells are modulated by proteins of the BCL-2 family [29, 30, 46]. Following phosphorylation by pro-survival signals, BAD dissociates from BCLXL and is sequestered by 14-3-3 proteins in the cytosol [10, 25, 44]. In the present study, phosphorylation of BAD following insulin exposure induced binding with 14-3-3 ζ , thus demonstrating a direct

Fig. 7 Effects of HA-14-3-3 ζ overexpression on BCL family gene expression. (**a–c**) MIN6 cells transfected with plasmids encoding HA-14-3-3 ζ (black bars) or the control vector (white bars) were treated with cytokines. Cell lysates were resolved by SDS-PAGE and probed for markers of activation of the NF κ B (**a**, **b**) and SAPK–JNK1/2 (**c**) signalling pathways; $n=4–6$ per group; $*p<0.05$ compared with empty vector-treated cells. IkB α , nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; t, total. (**d–i**) Transcript levels of canonical pro-apoptotic and survival pathways, and (**j–s**) of members of the BCL-2 protein family as indicated by labelling were

measured by quantitative PCR using isolated RNA from MIN6 cells transfected with the control vector (white bars) or with plasmids encoding HA-14-3-3 ζ (black bars) and subsequently treated with cytokines for 24 h. All data (arbitrary units [AU]) were normalised to *Hprt*; $n=4$ per group; $*p<0.05$ compared with untreated control cells; $^{\dagger}p<0.05$ compared with control treated cells. *Mcp-1*, also known as *Ccl2*; *iNos*, also known as *Nos2*; *A20*, also known as *Tnfrsf25*; *clap2*, also known as *Birc3*; *Bclxl*, also known as *Bcl2l1*; *Bim*, also known as *Bcl2l11*; *Bak*, also known as *Bak1*

relationship between these proteins. Binding with 14-3-3 ζ does not require Ser112 and Ser136 to be doubly phosphorylated [44], and it is unclear which serine residues play a predominant role in 14-3-3 binding [10, 12]. Our data suggest that, in beta cells, Ser136 is critical for survival, as overabundance of the S136A mutant was associated with the greatest degree of cell death. It should be noted that the pro-survival actions of 14-3-3 ζ are not solely limited to its effect on BAD, as overabundance of 14-3-3 ζ retained BAX in the cytoplasm following cytokine exposure. Furthermore, overabundance of 14-3-3 ζ also suppressed cytokine-mediated increases in *Dp5* and *Puma* mRNA, which are

dependent on activation of the ASK1–JNK and NF κ B signalling pathways, respectively [38–40].

Interestingly, transgenic mice overexpressing the triple Ser-Ala (112/136/155) BAD mutant were glucose-intolerant due to decreased glucokinase activity in islets [47]. This observed decrease in glucokinase activity was due to the phosphorylation status of BAD, which we found to be critical for its cellular localisation in beta cells (Fig. 6a, b). Similarly, difopein overproduction in beta cells blunted glucose-stimulated insulin release and promoted BAD translocation to mitochondria. Taken together, these findings suggest that 14-3-3 ζ may act as a unique scaffold of BAD

that determines cellular localisation and, ultimately, 14-3-3 ζ 's dual roles in metabolism and cell survival.

Collectively, our data demonstrate an important role of the 14-3-3 proteins in pancreatic beta cell survival and function. To date, the regulatory mechanisms and pathways that control the abundance of each isoform are not fully known, nor are the prevalence and mechanisms of isoform-specific functions well understood [6]. We determined multiple mechanisms by which 14-3-3 ζ promotes survival (electronic supplementary material [ESM] Fig. 1). Further work is required to dissect the roles of the remaining 14-3-3 isoforms. Our observations raise the possibility that therapeutic approaches, designed to increase the levels or activity of 14-3-3 proteins, could protect beta cells from death during diabetes [2]. Interestingly, the inhibition of 14-3-3 proteins has been proposed as a potential novel approach for the treatment of some cancers [48], an approach that, based on our results, could have deleterious effects on beta cell survival and potentially on glucose homeostasis. Further work is therefore required to evaluate the role of 14-3-3 proteins in other tissues important for glucose homeostasis.

In conclusion, we report for the first time that certain 14-3-3 proteins are required for the determination of beta cell fate and function. Despite structural similarities, 14-3-3 family members appear to have distinct roles in beta cell survival. Overabundance of 14-3-3 ζ increased survival via actions on BCL-2 proteins. Modulation of the levels or function of this isoform, and perhaps others, could be a novel approach to prevent beta cell death.

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