Original Article

Pro-Survival Role of Gelsolin in Mouse β-Cells

Barbara Yermen, Alejandra Tomas, and Philippe A. Halban

We have previously shown that the Ca^{2+}-dependent actin-severing protein gelsolin plays an important role in regulated insulin secretion. The aim of this study was to determine the role of gelsolin in β-cell survival as it has been shown to play a dual role in apoptosis in other cell types. MIN6 subclones B1 and C3, shown previously to express gelsolin at different levels (B1≫C3 cells), were used for this purpose. We demonstrate that B1 cells have lower levels of apoptosis and active caspase-3 when compared with C3 cells, in both standard (25 mmol/l glucose and 15% FCS) and deprived (5 mmol/l glucose and 1% FCS) conditions. Overexpression of gelsolin resulted in a decrease in the percentage of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling $(TUNEL)^+$ and active caspase-3^+ cells. Conversely, knockdown of gelsolin by RNA interference in B1 cells caused an increase in the number of $TUNEL^+$ and active caspase-3^+ cells. Finally, the anti-apoptotic role of gelsolin was confirmed in purified primary mouse β-cells where overexpression of gelsolin resulted in a decrease in the percentage of $TUNEL^+$ cells. In summary, our results show for the first time that gelsolin plays a pro-survival role in pancreatic β-cells. *Diabetes* 56:80–87, 2007

Decreased β-cell mass due to (at least in part) apoptosis is a hallmark of both major types of diabetes (1–4). Identifying key molecules regulating β-cell apoptosis will be critical for understanding why apoptosis is increased in diabetes and may allow for new ways to prevent it.

Gelsolin is a Ca^{2+}-dependent actin-severing protein that has been shown to be expressed in pancreatic β-cells (5,6). Its roles in different cell types include transduction of signals into dynamic rearrangement of the cytoskeletal architecture and regulation of cell motility, secretion, and apoptosis (7–10). After cleavage by caspase-3, the NH_2 terminus of gelsolin is constitutively active and pro-apoptotic, causing an increase in actin depolymerization and cell death (11–13). Overexpression of gelsolin has, however, been shown to play an anti-apoptotic role in some cell types by different pathways. Gelsolin has thus been shown to block a target upstream of caspase-3 (14), to inhibit activation of caspase-3 by forming a complex with phosphatidylinositol 4,5-bisphosphate (15), to block loss of mitochondrial membrane potential and inhibit the release of cytochrome c (16,17), and to bind to and block the mitochondrial voltage-dependent anion channel (VDAC) (18). Furthermore, the anti-apoptotic role of gelsolin has been demonstrated both in vitro (19,20) and in vivo (21) by using gelsolin-null mice models.

The aim of this study was to determine the role of gelsolin in pancreatic β-cell survival. To do this, we took advantage of two subclones, named B1 and C3, derived from the parental mouse pancreatic β-cell line MIN6 and shown previously to express gelsolin at different levels (B1≫C3 cells) (6,22). In addition, results obtained in B1 and C3 cells were further confirmed in primary purified mouse β-cells. We show for the first time that gelsolin plays a pro-survival role in pancreatic β-cells.

RESEARCH DESIGN AND METHODS

Reagents and antibodies. The following materials were used: In Situ Cell Death Detection kit for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, Cell Death Detection $ELISA^{PLUS}$ kit, complete Mini, EDTA-free Protease Inhibitor Cocktail Tablets, and mouse anti–hemagglutinin (HA)-tag from Roche (Basel, Switzerland); DC protein assay from Bio-Rad (Reinach, Switzerland); Lipofectamine 2000 from Invitrogen (Carlsbad, CA); Adeno-X Tet-ON expression system and Adeno-X Rapid Titer Kit from BD Biosciences (San Jose, CA); recombinant mouse interleukin-1β (IL-1β) from R&D Systems (Abingdon, U.K.); rabbit anti-cleaved caspase-3 (Asp 175), rabbit anti–pro-caspase-3 from Cell Signaling Technology–Bioconcept (Allschwill, Switzerland); sheep anti-mouse horseradish peroxidase (HRP) and donkey anti-rabbit HRP from Amersham Pharmacia Biotech (Dübendorf, Switzerland); goat anti-mouse Alexa Fluor 555, goat anti–guinea pig Alexa Fluor 488, goat anti-rabbit Alexa Fluor 633, and donkey anti-rabbit Alexa Fluor 488 from Molecular Probes (Eugene, OR); rabbit anti-gelsolin (a gift from C. Chapponier [University of Geneva Medical School, Geneva, Switzerland]); and guinea pig anti-insulin serum (made in the laboratory following the protocol of Wright et al. [23]).

MIN6 cell culture. Transformed mouse β-cell (MIN6) subclones B1 and C3, were cultured in Dulbecco's modified Eagle's medium supplemented with 15% FCS, 25 mmol/l glucose, 71 μmol/l 2-mercaptoethanol, 2 mmol/l glutamine, 100 units/ml penicillin, and 100 mg/l streptomycin at 37°C in an atmosphere of humidified air (95%) and CO_2 (5%) as previously described (22).

After 24 h of culture in standard conditions (25 mmol/l glucose and 15% FCS), apoptosis was induced by incubating cells in deprived conditions (5 mmol/l glucose and 1% FCS) or with 2 ng/ml IL-1β for 48 h.

Detection of apoptosis

TUNEL assay. MIN6 cells were seeded in 35-mm adherent plastic Petri dishes (1×10^6 cells/dish) 24 h before inducing apoptosis by lowering the concentration of glucose and FCS or incubating the cells with 2 ng/ml IL-1β. Cells were then washed with PBS and fixed with 4% paraformaldehyde (20 min at room temperature). After permeabilization with 0.5% Triton X-100 (4 min at room temperature), the TUNEL assay, which detects DNA strand breaks formed during apoptosis (24), was performed using the In Situ Cell Death Detection kit (Roche) according to the manufacturer's instructions. Hoechst 33342 (1 μg/ml; 10 min at room temperature) was used to stain nuclei. Results are expressed as percentage of $TUNEL^+$ cells per total number of cells.

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Control-RNAi, pSUPER-Control RNA interface; GFP, green fluorescent protein; Gsn-RNAi, pSUPER–gelsolin RNAi; HA, hemagglutinin; HA-Gsn, pcDNA3.1-HA-gelsolin; HRP, horseradish peroxidase; IL-1β, interleukin-1β; shRNA, small hairpin RNA; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; VDAC, voltage-dependent anion channel.

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Cell Detection ELISA^{PLUS} kit. Cells were seeded in 24-well plates (2×10^5cells/well) 24 h before inducing apoptosis with deprived conditions. Apoptosis was quantified using the Cell Detection ELISA^{PLUS} kit (Roche), which detects mono- and oligonucleosomes present in the cytoplasm of apoptotic cells, according to the manufacturer's instructions. In parallel, cells were trypsinized and counted. Results are expressed as absorbance (405–490 nm)/1×10^6 cells.

Caspase-3 activation. MIN6 cells were plated in 35-mm adherent Petri dishes (1×10^6 cells/dish) 24 h before inducing apoptosis by lowering the concentration of serum and glucose. Activation (cleavage) of caspase-3 was then determined by Western blot or by immunofluorescence using an antibody specific for the active cleaved form.

Western blot analysis. Cells were washed twice with PBS and then lysed with lysis buffer (20 mmol/l Tris, pH 7.5, 1 mmol/l EDTA, 1 mmol/l EGTA, 150 mmol/l NaCl, and 1% NP-40) complemented with complete Mini EDTA-free protease inhibitor cocktail. The amount of total protein in each sample was quantified using the detergent-compatible protein assay according to the manufacturer's instructions. Different protein amounts were added to sample buffer (62 mmol/l Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, and 1% β-mercaptoethanol) and boiled for 5 min before loading. To examine protein expression, samples were separated on a 12% polyacrylamide gel (active caspase-3 and pro-caspase-3) or a 10% polyacrylamide gel (gelsolin and HA-tag). Proteins were then transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). All antibodies were incubated in Tris-buffered saline (25 mmol/l Tris and 150 nmol/l NaCl, pH 7.4) with 0.1% (v/v) Tween-20 and 5% (w/v) dry milk. The enhanced chemiluminescence detection kit (Amersham Pharmacia Biosciences) and a Kodak image station were used to visualize the desired proteins. The membrane was stripped in 65 mmol/l Tris, pH 6.8, 2% SDS, and 0.7% β-mercaptoethanol (30 min at 50°C) before detection of pro-caspase-3 and HA-tag.

Immunofluorescence. For detection of active caspase-3, HA tag, gelsolin, and insulin, cells were washed twice with PBS, fixed with 4% paraformaldehyde (20 min at room temperature), and then permeabilized in 0.5% Triton (4 min at room temperature). Cells were blocked in 0.5% BSA-PBS (30 min at room temperature) and were then incubated with the primary antibody diluted in 0.5% BSA-PBS (2 h at room temperature). After rinsing the cells extensively with PBS, the cells were incubated with the secondary antibody in 0.5% BSA-PBS (1 h at room temperature), and the nuclei were then stained with Hoechst 33342 (1 μg/ml; 10 min at room temperature).

Transient overexpression of gelsolin. The plasmid pcDNA3.1-HA-gelsolin (HA-Gsn) was used for transient transfections to overexpress the fusion protein HA-tag–gelsolin (mouse) (6). Cells were seeded for 72 h at a density of 1×10^6 cells/dish in a 35-mm adherent Petri dish after which they were transfected using Lipofectamine 2000 according to the manufacturer's instructions (4 μg DNA/dish). One day after transfection, apoptosis was induced as described above.

Silencing of endogenous gelsolin expression with small interfering RNAs (siRNAs). The plasmid pSUPER–gelsolin RNAi (Gsn-RNAi) coding for a small hairpin RNA (shRNA) specific for mouse gelsolin was used as described previously by us to knock down gelsolin in MIN6 B1 cells (6). The pSUPER-Control RNAi (Control-RNAi) plasmid, coding for a nonspecific shRNA without mammalian homology (6), was used as a negative control. Cells were seeded for 72 h at a density of 1×10^6 cells/dish in a 35-mm adherent Petri dish after which they were co-transfected using Lipofectamine 2000 according to the manufacturer's instructions. Cells were co-transfected with a vector expressing phogrin-green fluorescent protein (GFP) and the pSUPER plasmid expressing either the gelsolin-specific or negative control shRNA sequence (1:3 DNA ratio). One day after transfection, apoptosis was induced as described above.

Recombinant adenovirus. The Adeno-X Tet-ON expression system from BD Biosciences was used to generate an inducible adenoviral system to overexpress gelsolin in the presence of doxycycline. The sequence encoding the HA-gelsolin–fused protein present in the HA-Gsn plasmid was cloned into the pTRE-Shuttle2 vector (BD Biosciences). The inducible cassette was then excised and cloned into the Adeno-X viral DNA to generate a recombinant adenovirus (Ad-HAGsn). Both Ad-HAGsn and the adenoviral construct harboring the reverse tetracycline controlled transactivator (Ad-TetON) were amplified in HEK 293 cells and titrated using the Adeno-X Rapid Titer Kit (BD Biosciences) according to the manufacturer's instructions.

Mouse islet isolation and β-cell purification. Adult male C57BL/6 mice (Janvier, Le Genest-St-Isle, France) were used to isolate islets of Langerhans from which primary β-cells were then purified as described by the method of Rouiller et al. (25). In brief, after islet isolation by collagenase digestion of the pancreases and purification by Ficoll, cells were dispersed by trypsinization. β-Cells were then purified using fluorescence-activated cell sorting (FACS) (FACStar-Plus; Becton Dickinson, Sunnyvale, CS) as described by Rouiller et al. (25), to yield a population of >90% β-cells.

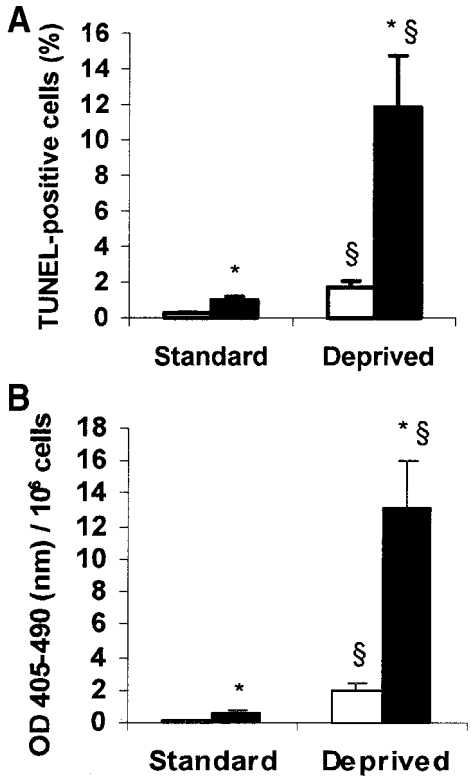

FIG. 1. Apoptosis is greater in MIN6 C3 cells than B1 cells. MIN6 B1 (□) and C3 (■) cells were incubated for 48 h in standard (25 mmol/l glucose and 15% FCS) or deprived (5 mmol/l glucose and 1% FCS) conditions. Apoptosis was measured by the TUNEL assay (*A*) or the Cell Death Detection ELISA^{PLUS} kit (*B*). $n = 4$, *$P < 0.02$ B1 vs. C3 cells in respective condition; §$P < 0.02$ deprived versus standard conditions.

Adenovirus infection of purified primary mouse β-cells. After cell sorting by FACS, primary mouse β-cells were left to recuperate in suspension for 2 h at 37°C in culture medium (RPMI supplemented with 20% FCS, 11.2 mmol/l glucose, 110 μg/ml sodium pyruvate, 50 μmol/l 2-mercaptoethanol, 2 mmol/l glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin) and were then co-infected with Ad-HAGsn (28 infectious units/cell) and Ad-TetON (56 infectious units/cell) in suspension for 90 min. After washing the cells twice with deprived RPMI (5 mmol/l glucose and 1% Tet System approved FBS), the cells were plated as 50-μl droplets, at a density of $0.5–1 \times 10^6$ cells/ml, on poly-L-lysine (0.1 mg/ml) precoated Petri dishes. After an overnight incubation at 37°C, the cells were washed twice and were further incubated for 48 h in deprived RPMI ± 0.5 μg/ml doxycycline. Cells were then fixed and analyzed by TUNEL and immunofluorescence.

Presentation of data and statistics. Data are presented as means ± SE for n independent experiments. Statistical significance for differences was evaluated by Student's two-tailed t test for unpaired groups with $P < 0.05$ considered significant.

RESULTS

Induction of apoptosis in MIN6 B1 and C3 cells. To determine whether apoptosis is induced in a similar manner in MIN6 B1 and C3 subclones, cells were cultured for 48 h in either standard (25 mmol/l glucose and 15% FCS) or deprived (5 mmol/l glucose and 1% FCS) conditions. Apoptosis was first quantified using the TUNEL assay, which detects DNA strand breaks formed during programmed cell death (24). As shown in Fig. 1*A*, the percentage of TUNEL$^+$ cells was greater in C3 compared with B1 cells when incubated in either standard (C3 1.7% vs. B1 0.3%) or deprived (C3 12% vs. B1 1%) conditions. This latter condition significantly increased apoptosis in both subclones when compared with standard conditions ($P <$ 0.02). These results were confirmed using the Cell Death

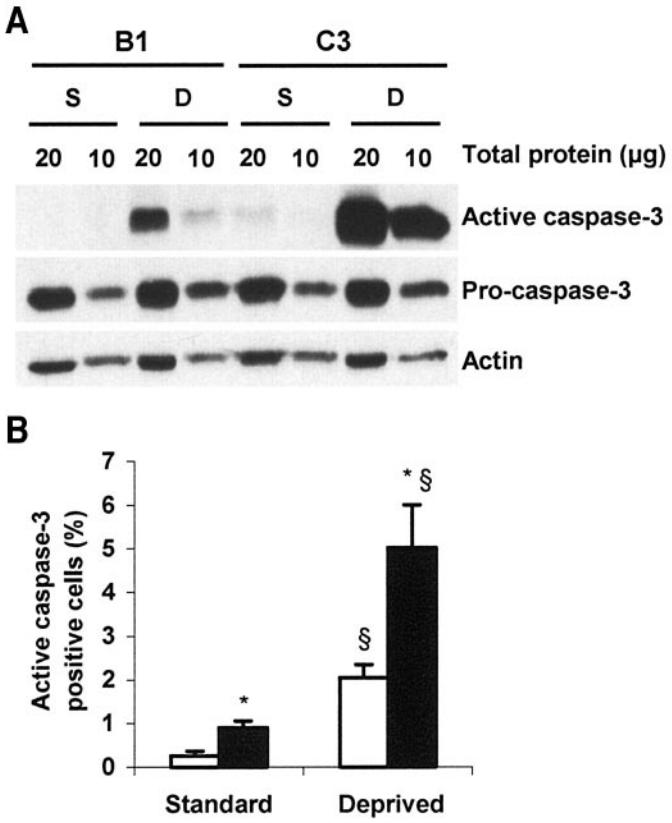

FIG. 2. Caspase-3 activation is greater in MIN6 C3 than B1 cells. MIN6 B1 (□) and C3 (■) cells were incubated for 48 h in standard conditions (S) or deprived (D) conditions. Active caspase-3 levels were examined by Western blot with pro-caspase-3 and actin as loading controls (*A*) and immunofluorescence (percentage of cells with active caspase-3) (*B*). $n = 4$, *$P < 0.02$ B1 vs. C3 cells in respective conditions; §$P < 0.01$ deprived versus standard.

Detection ELISA^{PLUS} kit, which detects mono- and oligonucleosomes present in the cytoplasm of apoptotic cells (Fig. 1*B*).

Because caspase-3 is known to be a main mediator of apoptosis, levels of its cleaved (active) form were examined. As expected, Western blot analysis showed that after induction of apoptosis, there was an increase of active caspase-3 levels in both C3 and B1 cells (Fig. 2*A*). C3 cells had higher levels of active caspase-3 when compared with B1 cells when incubated in either standard or deprived conditions, whereas levels of pro-caspase-3 (inactive form) and actin were equal in all conditions. These results were confirmed by immunofluorescence. The percentage of active caspase-3$^+$ cells increased after induction of apoptosis from 0.3 to 2% in B1 cells and from 1 to 5% C3 cells (Fig. 2*B*). These results show that deprived conditions cause an increase in apoptosis and active caspase-3 levels when compared with standard conditions in both B1 and C3 subclones. Furthermore, B1 cells are less prone to apoptosis when compared with C3 cells because they have a lower number of TUNEL$^+$ and active caspase-3$^+$ cells in either standard or deprived conditions.

Overexpression of gelsolin in MIN6 B1 and C3 cells decreases apoptosis. Previous studies from our group have shown that gelsolin is highly overexpressed in B1 cells compared with C3 cells at both the mRNA and protein levels (6,22). To determine whether gelsolin plays an anti-apoptotic role, which could explain the reduced apoptosis level in B1 versus C3 cells observed in this study

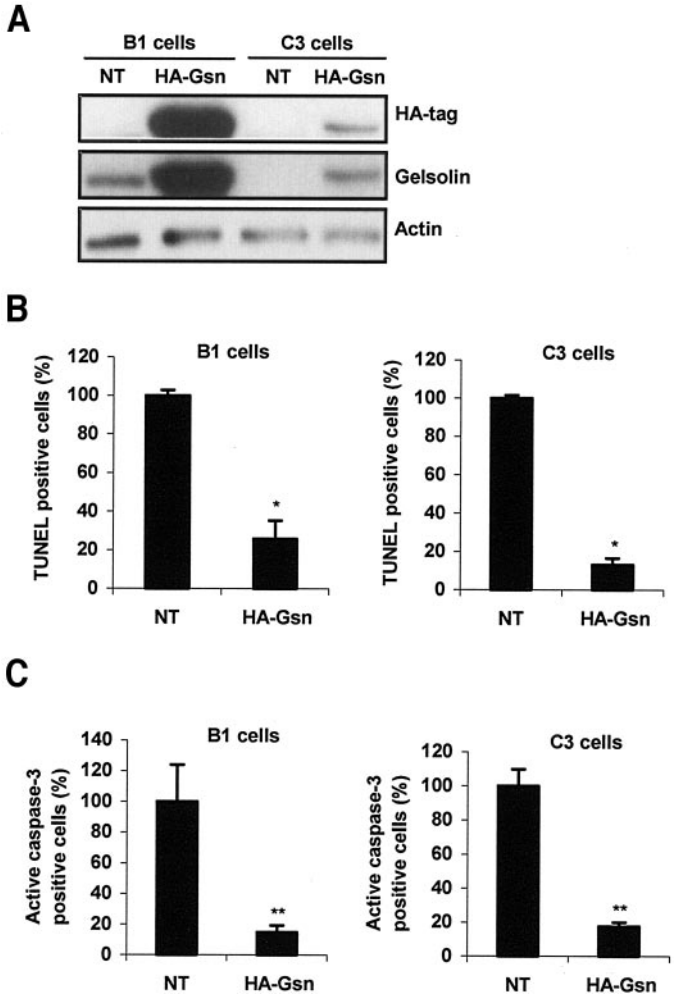

FIG. 3. Overexpression of gelsolin protects MIN6 cells from apoptosis and decreases the activation of caspase-3. MIN6 B1 and C3 cells were transfected with the HA-Gsn vector before apoptosis induction for 48 h by deprived conditions. *A*: Expression of HA-tag, gelsolin, and actin, the latter used as a loading control, was examined by Western blot in nontransfected (NT) and HA-Gsn–transfected B1 and C3 cells (10 μg protein/lane). *B*: Apoptosis was detected by TUNEL in HA$^+$ cells as well as NT cells from the same dish. *C*: Activation of caspase-3 was detected by immunofluorescence in both HA-Gsn and NT cells from the same dish. Results are normalized to NT cells incubated in deprived conditions. $n = 4$, *$P < 0.001$; **$P < 0.05$ for HA-Gsn vs. NT.

(refer to above), apoptosis was monitored specifically in cells overexpressing gelsolin after transfection. The HA-Gsn vector was used to overexpress the fused protein HA-tag gelsolin in B1 cells and C3 cells, which allowed transfected cells to be identified on the basis of the expression of the HA-tag (Fig. 3*A*). Note that C3 cells transfect less efficiently than B1 cells, hence the lower expression level of the HA-tag. After induction of apoptosis by deprived conditions, the number of TUNEL$^+$ cells decreased by >70% in gelsolin-overexpressing cells when compared with nontransfected cells, in either B1 or C3 cells (Fig. 3*B*, $P < 0.05$). There was no effect of gelsolin overexpression on TUNEL positivity when the cells were cultured in standard conditions (data not shown), where the percentage of apoptosis is low (<1%), suggesting that overexpression of gelsolin plays an anti-apoptotic role in B1 and C3 cells only after induction of apoptosis or that the assay system is not sufficiently sensitive to detect changes in this range.

In some cell types, it has been reported that gelsolin

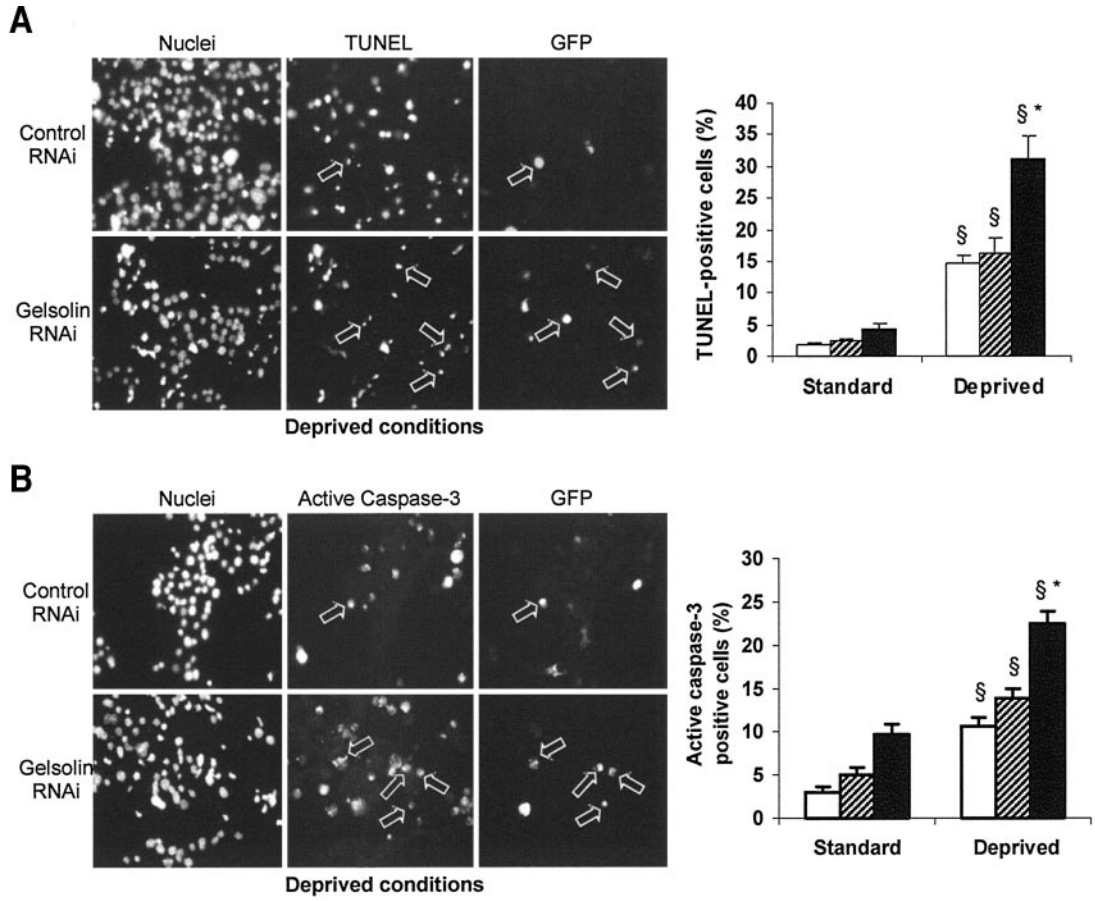

FIG. 4. Downregulation of gelsolin expression increases apoptosis in MIN6 B1 cells. MIN6 B1 cells were co-transfected with the phogrin-GFP plasmid and the Control-RNAi or the Gsn-RNAi plasmid and were then incubated for 48 h in either standard or deprived conditions. *A*: Apoptosis measured by TUNEL. *B*: Active caspase-3 measured by immunofluorescence. Nontransfected (□), Control-RNAi–transfected (▨), and Gsn-RNAi–transfected (■) cells were then analyzed in standard and deprived conditions. In the representative images shown beside each graph, arrows point to GFP and TUNEL or active caspase-3 double-positive cells. $n = 3–4$, §$P < 0.01$ deprived vs. standard conditions; *$P < 0.04$ vs. nontransfected and control-RNAi–transfected cells under the same conditions.

plays an anti-apoptotic role by inhibiting the activation of caspase-3, either directly or indirectly (14,15,17). To investigate this hypothesis in B1 and C3 cells, gelsolin was overexpressed, and the percentage of active caspase-3$^+$ cells was quantified in transfected cells. As shown in Fig. 3*C*, after induction of apoptosis by deprived conditions, overexpression of gelsolin in B1 and C3 cells decreased the number of active caspase-3$^+$ cells by 85 and 82%, respectively, when compared with nontransfected cells ($P < 0.001$). In agreement with the TUNEL results, there was no significant difference in the activation of caspase-3 between cells overexpressing gelsolin and nontransfected cells when cultured in standard conditions (data not shown).

Knockdown of gelsolin by siRNA in MIN6 B1 cells increases apoptosis. Because overexpression of gelsolin protects MIN6 cells from apoptosis, we predicted that cells with a downregulated expression of gelsolin would have a higher apoptotic rate. To confirm this, the RNA interference technique was used to knock down gelsolin in MIN6 B1 cells. The plasmid Gsn-RNAi, previously shown by our group to successfully knock down gelsolin in MIN6 B1 cells (6), was used along with the control plasmid Control-RNAi, encoding for a nonspecific shRNA of nonmammalian origin. Because the transfection efficiency with these plasmids is poor (5–10%) in MIN6 cells, transfected cells were tagged by co-transfection with a plasmid coding phogrin-GFP fusion protein, thereby targeting GFP to the secretory granules and preventing any nuclear localization (26). Preliminary experiments indicated that expression of GFP itself, rather than the fusion protein, interfered with the TUNEL assay because of the presence of GFP in the nucleus.

Apoptosis was once again compared under standard or deprived conditions (Fig. 4*A*). Control-RNAi–transfected cells did not differ in their percentage of TUNEL$^+$ cells when compared with nontransfected cells in either standard or deprived conditions, indicating that transfection with this plasmid has no effect on cell death. In standard culture conditions, the percentage of TUNEL$^+$ cells in Gsn-RNAi–transfected, Control-RNAi–transfected, or nontransfected cells was not significantly different. However, after incubation in deprived conditions, Gsn-RNAi–transfected cells had a twofold increase in the levels of apoptosis when compared with either Control-RNAi–transfected or nontransfected cells ($P < 0.04$). A similar trend was seen when evaluating active caspase-3 in Gsn-RNAi–transfected cells when compared with Control-RNAi–transfected and nontransfected cells (Fig. 4*B*). Taken together, these results show that knockdown of gelsolin causes an increase in apoptosis as well as active caspase-3 after incubation in deprived conditions, confirming the importance of gelsolin in the prevention of β-cell death.

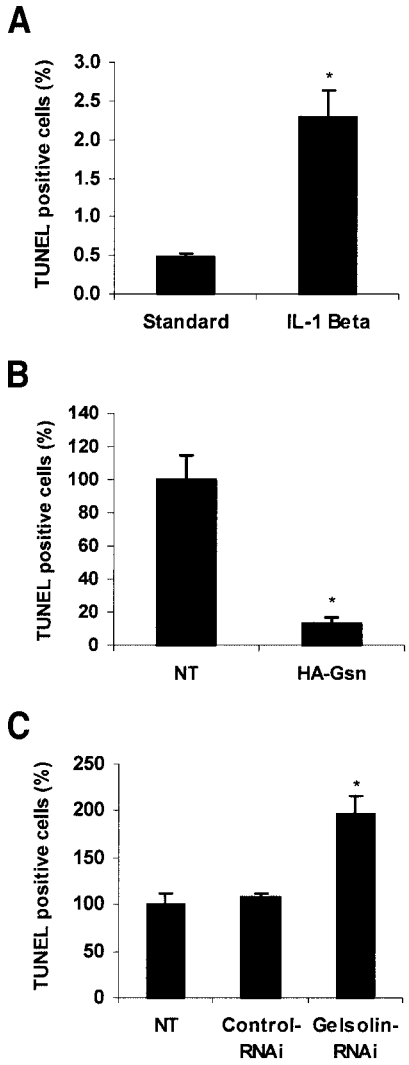

FIG. 5. Gelsolin plays an anti-apoptotic role in MIN6 B1 cells after IL-1β treatment. Apoptosis was measured by TUNEL after 48 h of treatment with 2 ng/ml IL-1β in MIN6 B1 cells. *A*: Control cells. *B*: Non-transfected (NT) and HA-Gsn–transfected cells. *C*: NT, control-RNAi–, and Gsn-RNAi–transfected cells. Results are normalized to NT cells incubated with 2 ng/ml Il-1β (*B* and *C*). $n = 3$, *$P < 0.03$.

Gelsolin plays a protective role in MIN6 B1 cells after induction of apoptosis by IL-1β. To further confirm the protective role of gelsolin in β-cells, apoptosis was induced by IL-1β treatment because it is a cytokine known to induce β-cell apoptosis in both type 1 and type 2 diabetes (3,27). As expected, 2 ng/ml IL-1β treatment of MIN6 B1 cells for 48 h resulted in a significant increase in apoptosis when compared with standard conditions (Fig. 5*A*).

To examine whether gelsolin protects cells from IL-1β–induced apoptosis, TUNEL positivity was specifically measured in HA-Gsn–transfected B1 cells. The percentage of $TUNEL^+$ cells decreased by 87% in gelsolin-overexpressing cells when compared with nontransfected cells (Fig. 5*B*, $P < 0.03$). To strengthen this result, the effect of knocking down gelsolin in B1 cells was assessed after IL-1β treatment. As shown in Fig. 5*C*, depletion of gelsolin causes an increase of >1.5-fold in apoptosis levels when compared with Control-RNAi–transfected cells, indicating again that gelsolin plays a protective role in β-cell survival.

Overexpression of gelsolin protects purified primary mouse β-cells from apoptosis. Even well-differentiated transformed cell lines such as MIN6 B1 cannot necessarily be considered a useful model of their primary cell counterpart. This can be true in particular when studying apoptosis. To address this, purified primary mouse β-cells were used to further investigate the role of gelsolin in β-cell survival. Because transient transfection efficiency is poor in primary β-cells, an inducible adenoviral Tet-ON system was used to overexpress gelsolin after addition of doxycycline. After co-infection of cells with the Ad-HAGsn and Ad-TetON viruses, a specific anti-gelsolin antibody previously characterized by Chaponnier et al. (28) was used along with a monoclonal anti-HA antibody to examine the expression of the HA-gelsolin–fused protein. In the presence of doxycycline, >45% of the cells overexpressed HA-gelsolin, whereas in the absence of the tetracycline analog, there was no detection of the HA-tag (Fig. 6). As expected for this population of cells sorted by FACS, most of the cells overexpressing gelsolin were β-cells because they also express insulin, as seen after staining with a previously characterized insulin antibody (29).

To examine the role of gelsolin in primary mouse β-cell survival, cells were cultured for 48 h in deprived medium (5 mmol/l glucose and 1% tetracycline free Tet System approved FBS) to increase apoptosis to a detectable level. Cells were infected with the inducible adenoviral Tet-ON system, and apoptosis was then measured by TUNEL (Fig. 7*A*). To exclude any toxic effect of doxycycline, cell death was measured in cells incubated with or without the tetracycline analog, and no change was observed in the percentage of apoptotic HA^- cells (data not shown). Overall, a 30% decrease in apoptosis was observed between doxycycline nontreated and treated cells when examining the total cell population (i.e., without identifying cells expressing HA-gelsolin), therefore not creating any discrimination between infected and noninfected cells. Furthermore, when cells overexpressing gelsolin were compared with noninfected cells from the same dish, apoptosis levels decreased by about 70% (Fig. 7*B*, $P < 0.01$). These results confirm the pro-survival role that gelsolin plays in primary mouse pancreatic β-cells.

DISCUSSION

Gelsolin has recently been shown by our group to play an important role in regulated insulin secretion of pancreatic β-cells (6). In the present study, we show for the first time that gelsolin also functions as a pro-survival protein in these cells, emphasizing its key role in maintaining β-cell function and viability.

The subclones derived from the parental mouse pancreatic β-cell line MIN6, named B1 and C3, have been previously shown by our group to differentially express gelsolin both at the mRNA (B1 >100 times higher than C3) and protein level (B1 cells express similar levels as primary β-cells with barely detectable amounts in C3 cells) and also to secrete insulin differently in response to glucose (B1 but not C3 cells respond to glucose in a concentration-dependent manner) (6,22). Here, we show that these subclones also differ in their levels of apoptosis when cultured in standard conditions (25 mmol/l glucose and 15% FCS), with B1 cells being less prone to apoptosis when compared with C3 cells. Deprived conditions (5 mmol/l glucose and 1% FCS), which are known to induce apoptosis in pancreatic β-cells (30–32), caused an in-

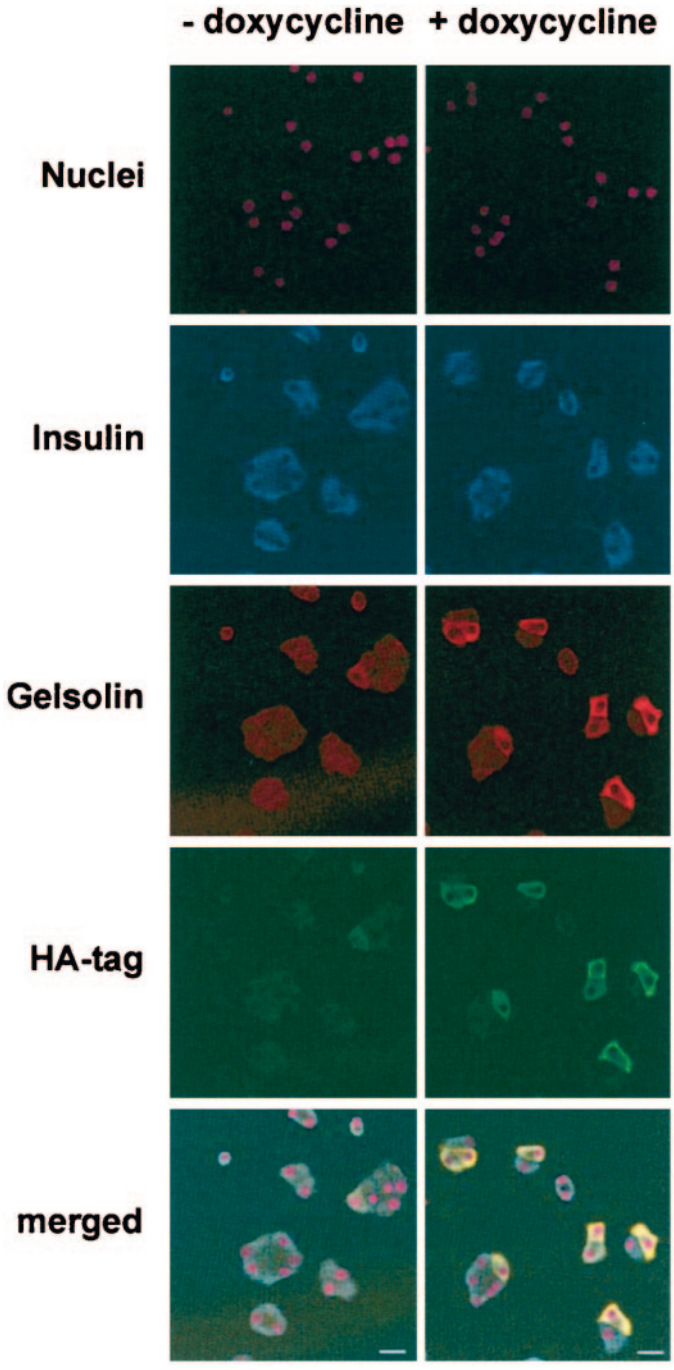

FIG. 6. Gelsolin overexpression in purified primary mouse β-cells. After cell sorting by FACS, purified primary mouse β-cells were co-infected with Ad-HAGsn and Ad-TetON viruses and incubated for 48 h in deprived conditions (5 mmol/l glucose and 1% Tet System approved FBS) with or without 0.5 μg/ml doxycycline. Overexpression of gelsolin was detected by immunofluorescence against the HA-tag and gelsolin. The identity of β-cells was confirmed by insulin staining. Bars = 20 μm.

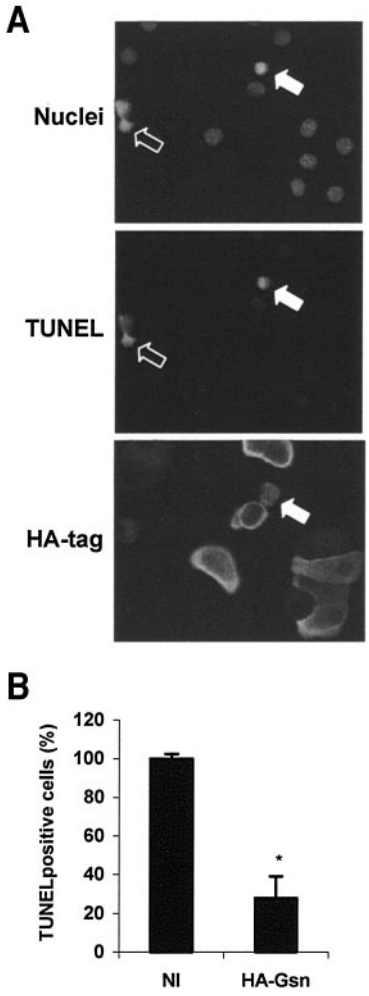

FIG. 7. Overexpression of gelsolin protects purified primary mouse β-cells from apoptosis. Purified primary mouse β-cells were co-infected with Ad-HAGsn and Ad-TetON viruses and incubated for 48 h in deprived conditions in the presence of 0.5 μg/ml doxycycline. *A*: Apoptosis was detected by TUNEL in gelsolin-overexpressing cells (HA-Gsn) and in noninfected cells (NI) from the same dish. Filled arrow is TUNEL$^+$ and HA$^+$; empty arrow is TUNEL$^+$ and HA$^-$. *B*: Results were quantified and normalized to NI cells incubated in deprived conditions. Absolute values, 6.0 ± 0.2% (NI) and 1.7 ± 0.7% (HA-Gsn). $n = 3$, *$P < 0.01$.

crease in the levels of apoptosis in both subclones, with C3 cells always being more susceptible to cell death when compared with B1 cells. As predicted, a similar tendency was observed when examining the expression of active caspase-3, a major mediator in the programmed cell death signaling pathway (33,34).

Clearly, the mere correlation of gelsolin levels and apoptosis does not prove any causal relationship. This was explored further by modulating levels of expression of gelsolin. Similar results were observed when apoptosis was induced by either deprived conditions or cytokine treatment (IL-1β), the latter being more relevant to diabetes. RNA interference was used to knock down gelsolin. Downregulation of gelsolin resulted in a significant increase in pancreatic β-cell apoptosis when compared with control cells after induction of apoptosis. These results are consistent with those obtained by Klampfer et al. in which Hke-3 cells (human colon carcinoma cell line–derived clone) are sensitized to butyrate–induced (35) or 5-FU–induced apoptosis (36) after gelsolin silencing. Experiments performed with gsn$^{-/-}$ mice also demonstrate that neurons lacking gelsolin are more susceptible to apoptosis, when compared with control, after glucose and oxygen deprivation (19). In our study, we also saw an increased activation of caspase-3 after knockdown of gelsolin by siRNA. This result is again consistent with a study showing that gsn$^{-/-}$ neurons are more susceptible to apoptosis after treatment with AF64A with an increase in the activation of caspase-3 (20).

As expected, overexpression of gelsolin resulted in the

opposite phenotype, with a decrease of about 70% in the percentage of TUNEL$^+$ cells and of about 80% in the levels of active caspase-3 in MIN6 cells. These observations are in agreement with those seen in gelsolin-overexpressing Jurkat cells in which gelsolin strongly inhibits apoptosis induced by several agents, such as anti-Fas antibody, thapsigargin, or VP-16 (14,17). In Jurkat cells overexpressing human gelsolin, it has been reported that gelsolin protects cells from apoptosis by inhibiting apoptotic mitochondrial changes via the closing of VDAC (18). However, the same group also showed that overexpression of mouse gelsolin in NIH3T3 cells has no effect on apoptosis. This discrepancy is probably due to the cell type and stimulus used to induce apoptosis, underlying the current controversy surrounding the role of gelsolin in apoptosis (13,14,37). In our case, further studies will be required to identify the pathway by which gelsolin protects pancreatic β-cells against apoptosis. This could possibly involve the remodeling of the actin cytoskeleton (20) or a direct inhibition of caspase-3 (15).

Although MIN6 B1 cells appear to be quite well differentiated, have low basal levels of apoptosis, and express levels of gelsolin similar to primary β-cells, to prove the physiological relevance of our studies on these transformed cells, we also evaluated the anti-apoptotic role of gelsolin in primary mouse β-cells. Obtaining purified primary mouse β-cells is quite challenging. We nevertheless managed to study the effect of gelsolin overexpression in purified primary mouse β-cell survival cultured in similar conditions to those shown to induce apoptosis in MIN6 cells. Because transient transfection is very poor in purified primary mouse β-cells, we used an adenovirus system to increase the number of cells overexpressing gelsolin, which allowed us to infect nearly 50% of the total number of cells. After measuring apoptosis, we obtained a 70% decrease in the percentage of TUNEL$^+$ cells compared with control cells. These results are comparable with those obtained in MIN6 B1 and C3 cells, confirming the anti-apoptotic role of gelsolin in primary pancreatic β-cells.

Type 2 diabetes occurs when the endocrine pancreas fails to secrete sufficient insulin to cope with the metabolic demand because of an acquired β-cell secretory dysfunction and/or decreased β-cell mass (38). Molecules and pathways that play a dual role in promoting both insulin secretion and β-cell survival are of particular interest in understanding such pathophysiology and would be logical targets for new therapeutic approaches for type 2 diabetes. An example of this is provided by outside-in signaling from the extracellular matrix, shown by us to enhance glucose-stimulated insulin secretion from β-cells (39) and to be anti-apoptotic (40). We now suggest such a dual role for gelsolin, having shown previously that this protein is implicated in regulated insulin secretion (6) and showing here that it also has an important role in β-cell survival and could be a major player in the development of type 2 diabetes.

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