Proteins Linked to a Protein Transduction Domain Efficiently Transduce Pancreatic Islets

Jennifer Embury, Dagmar Klein, Antonello Pileggi, Melina Ribeiro, Sundararajan Jayaraman, R. Damaris Molano, Christopher Fraker, Norma Kenyon, Camillo Ricordi, Luca Inverardi, and Ricardo L. Pastori

The resounding success of a new immunosuppressive regimen known as the Edmonton protocol demonstrates that islet cell transplantation is becoming a therapeutic reality for diabetes. However, under the Edmonton protocol, a single donor does not provide enough islets to attain the insulin independence of a transplant recipient. This limitation is mainly caused by islet apoptosis triggered during isolation. In this study, we describe a highly efficient system of transiently transferring anti-apoptotic proteins into pancreatic islets, thus opening an exciting new therapeutic opportunity to improve the viability of transplantable islets. We fused β-galactosidase to the 11-amino acid residues that constitute the protease transduction domain (PTD) of the HIV/TAT protein and transduced pancreatic islets ex vivo with this fusion protein in a dose-dependent manner with >80% efficiency. We observed that transduction of the anti-apoptotic proteins Bcl-XL and PEA-15 fused to TAT/PTD prevented apoptosis induced by tumor necrosis factor-α in a pancreatic β-cell line, indicating that TAT/PTD anti-apoptotic proteins retain their biological activity. Finally, we demonstrated that TAT-fusion proteins did not affect the insulin secretion capability of islets, as determined by glucose static incubation and by reversion of hyperglycemia in diabetic immunodeficient mice. Diabetes 50:1706–1713, 2001

Transplantation of islets of Langerhans has long been considered a potential curative treatment for diabetes (1). Unfortunately, the results of several clinical trials showed that most transplant recipients failed to achieve complete insulin independence. Very recently, however, a new immunosuppressive regimen developed by Shapiro et al. (2) in Edmonton, Canada, has resulted in unprecedented success in achieving insulin independence with transplanted islets. The use of a glucocorticoid-free protocol that included the administration of low-dose tacrolimus, sirolimus, and antibody against the interleukin-2 receptor (daclizumab) was associated with sustained insulin independence in 100% of the patients receiving islet transplants.

Despite this success, some problems still persist and influence the outcome of islet transplantation. Patients achieved insulin independence only after receiving two or more islet transplants at 4–6 weeks after the first transplant. This observation suggests that despite the progress in islet isolation procedures, a single donor transplant may not provide enough functional islets to attain insulin independence. Moreover, islet primary nonfunction or early loss of islets has been reported in syngeneic islet transplant models (3) as well as in models of T-cell inactivation (4–5). Collectively, these observations indicate that in addition to the recurrence of autoimmunity and the toxicity of immunosuppressive agents, the intrinsic viability of islets plays a critical role in the outcome of islet transplantation.

There is substantial evidence linking early graft loss to apoptosis associated with isolation and purification procedures, which expose islets to osmotic, mechanical, and ischemic stresses. When pancreatic islets are harvested for transplantation, a variable fraction of the cells undergo apoptosis due to either enzymatic or mechanical stress during the cell separation process (6–7). In addition, detachment of the islets from their surrounding extracellular matrix (ECM) may be a significant factor in the loss of viability (8). The importance of cell-matrix interactions for sustaining β-cell function is well documented (9–10). The disruption of the integrin-mediated cell-matrix contact induces apoptosis in the cells that are detached, an event known as “anoikis” (11).

It has been shown that virus-mediated transfer of anti-apoptotic genes into islets in culture improved the viability of islets significantly (12–15). Proteins can be transferred directly to cells when they are linked to protein transduction domains (PTDs), small peptide domains that can freely cross cell membranes. Several PTDs have been identified that allow a fused protein to efficiently cross cell membranes in a process known as protein transduction (16–19). In particular, a PTD designated PTD-5, selected from an M13 phage peptide display library, was reported to successfully transduce eGFP into human islets (20).
Proteins fused to the 11-amino acid PTD of the HIV/TAT protein transduce several different tissues, crossing even the hemato-encephalic barrier (21) when administered to mice and rats (22). Transduction with PTD/TAT-fusion proteins is independent of receptors and transporters and is thought to take place across the lipid bi-layer of the cell membrane. This independence from membrane receptors is highly advantageous for ex vivo delivery of proteins to tissues, organs, or cells.

In this study, we show that TAT/PTD-fusion proteins have the capability to transduce pancreatic islets with great efficiency ex vivo, preserving the biological function of the transduced protein, without affecting the insulin secretion capability of the islets. These results raise the possibility of redirecting the islets from destructive apoptotic signals generated during islet isolation by transducing pancreatic tissue with proteins that are capable of blocking apoptosis.

**RESEARCH DESIGN AND METHODS**

**Cloning and related techniques.** The recombinant TAT/PTD anti-apoptotic protein constructs were generated by inserting the coding region of the Bcl-XL and PEA-15 DNAs in the EcoRI site of the pTAT expression vector in frame with the TAT leader peptide. The pTAT bacterial expression vector and the TAT/PTD β-galactosidase expression plasmid were generously provided by Steven Dowdy from Washington University School of Medicine, St Louis, Missouri. The human Bcl-XL DNA inserted into the pTAT vector was generated by polymerase chain reaction (PCR) amplification of the plasmid pBluescript/I-Bcl-XL (generously provided by Larry Boise, University of Miami, Miami, Florida). PCR was performed using the oligonucleotides GAATTCGAGTGCTCAGAGCAACCGG and GAATTCGATTCCGGCATGGAAG as forward and reverse primers, respectively.

The PEA/15 DNA plasmid was generated by PCR amplification of cDNA synthesized with an oligo (dT) primer from total RNA isolated from human islets and subcloned in the vector pCR2.1 (Invitrogen, Carlsbad, CA). The identity of the PEA/15 DNA was confirmed by DNA sequencing. PEA/15 PCR amplification was performed using the oligonucleotides GCACGATATCGCGTCTGGGAGGATACGGG and GAATTCTCATTTCCGA CTGAAGAG as forward and reverse primers, respectively. To generate the PEA/15 DNA construct for the recombinant TAT/PTD/PEA-15 construct, a PCR was performed using plasmid pBPEA as the template and oligonucleotides GAATTCGAGTGCTCAGAGCAACCGG and GAATTCCTGCTCCTCGG as forward and reverse primers, respectively.

The bacterial expression cassette of the TAT/PTD anti-apoptotic protein fusions was used to generate proteins with the structure Met–6(x)His-Gly–YGRKKRRQRRR-Gly-Bcl-XL or Met–6(x)His-Gly–YGRKKRRQRRR-Gly–β-galactosidase lacking the TAT/PTD domain, TAT/PTD–β-galactosidase labeled with Alexa-Fluor 488. To study the persistence of the fusion protein in the insulinoma cell line, βT3 cells were seeded in 25 cm² tissue culture flasks at a density of ~5 × 10⁶ cells per flasks with Delbacco’s modified Eagle’s medium (high glucose, 4.5 g/l), 15% horse serum, and 2.5% fetal bovine serum (FBS) at 37°C and 7.5% CO₂. Rat islet cells were dispersed into single-cell suspensions by incubation with 0.05% trypsin and 0.53 mmol/l EDTA for 4 min (Gibco-BRL, Gaithersburg, MD) and cultured in CMRL 1000 supplemented with 10% FBS.

**Transduction of TAT/PTD-fusion protein into pancreatic β-cells and islets.** βT3 cells (10⁶) were transduced for 30 min with an appropriate amount of TAT-PTD–β-galactosidase labeled with Alexa-Fluor 488. To study the persistence of the fusion protein in the insulinoma cell line, βT3 cells were seeded in 25 cm² tissue culture flasks at a density of ~5 × 10⁶ cells per flasks with Delbacco’s modified Eagle’s medium (DMEM) plus serum, and each plate was inoculated for 1 h. The medium was then replaced with 1% paraformaldehyde and then dissociated with 0.5% trypsin and 0.53 mmol/l EDTA in Hank’s salt solution for 4 min with continuous pipetting. The enzymatic reaction was stopped with 100% FBS, and the cells were washed in PBS. Altogether, 1 million cells of dissociated pancreatic islets were evaluated by flow cytometry.

Cells transduced with the TAT/PTD anti-apoptotic proteins were incubated for 3 h before tumor necrosis factor-α (TNF-α) cycloheximide (CHX) treatment to allow the internalized fusion protein to refold. For flow cytometry, cell samples were resuspended in PBS, washed twice in PBS, resuspended in 1% paraformaldehyde, and then analyzed for cell size (forward-angle light scatter), density (side-angle light scatter), and fluorescein isothiocyanate (FITC) intensity using an Epics Coulter (Coulter, Hialeah, FL) or FACSStar (Becton-Dickinson, Mountain View, CA) flow cytometer.

**Analysis of transduction assessed by β-galactosidase activity.** After the 4-h transduction, pancreatic islets were washed 1× with PBS, resuspended in 500 μl of PBS, and cytospined at 860 rpm for 5 min on Histogrip-treated slides. Then, they were dried for 15 min and fixed with freshly diluted fixative following instructions provided in the β-Gal staining kit (Stratagen, La Jolla, CA). Staining was developed for 2 h at 37°C or overnight at room temperature. For analysis of β-galactosidase activity using the βT3 cell line, transduced cells were washed 3× with PBS and fixed, and the staining was developed for 15 min at 37°C.

**Enzymatic detection of caspase-3 activity (DEVStar assay).** After the treatment, cells were washed twice with cold PBS, pelleted, and frozen at −80°C until assays were performed. The pellets were resuspended and brieﬂy sonicated in 10 mmol/l HEPES pH 7.4, 2 mmol/l EDTA, 0.1% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate, 5 mmol/l dithiothreitol, and the protease inhibitors PMSF, pepstatin A, aprotinin, and leupeptin. After centrifugation at 10,000 g for 10 min, the supernatant was removed to assay caspase-3 activity and protein content. Caspase-3 activity was measured in the presence or absence of the caspase-3 inhibitor Acetyl (Ac)-Asp-Glu-Val-Asp (DEVD)-fluoroketone (FMK). A 1000-fold dilution of the fluorogenic carbobenzoxy-DEVD–7-amino-4-trifluoromethyl coumarin (APC) peptide as substrate, according to the instructions contained in the FluorAce apopain assay kit (Bio-Rad, Hercules, CA). Caspase-3 activity values were adjusted according to the protein content.

**Assessment of apoptosis in pancreatic β-cells by binding of FITC-Val-DMV-FMK.** Detection of pancreatic β-cells with activated caspases was based on a modification of the previous protocol detailed by S.J. Anderson et al. (27). Cells were resuspended in 10 mmol/l of CaspACE FITC-Val-Ala-Asp (VDV)-FMK in situ marker (Promega, Madison, WI), incubated at 37°C for 20 min, centrifuged at 300g for 5 min, washed twice in PBS, and fixed in 1% paraformaldehyde. Cells were analyzed on a FACStar (Becton-Dickinson) flow cytometer.
Glucose levels were measured daily after transplantation using whole-blood specimens collected from the tail vein. Graft function was defined as nonfasting blood glucose <200 mg/dL. To rule out residual function of the native pancreas, survival nephrectomy was performed after 30 days from diabetes reversal in order to verify that a quick return to hyperglycemia was obtained.

**RESULTS**

**Transduction of TAT/PTD-β-galactosidase to pancreatic β-cells.** To test whether the TAT-fusion protein was capable of transducing pancreatic β-cells, insulinoma βTC-3 cells were incubated for 3 h with 0.8 μg of the fusion protein per 1,000 cells in order to allow for the proper refolding of the fusion protein (21), then they were stained for LacZ activity. As shown in Fig. 1A, the transduction of TAT/PTD-β-galactosidase was dependent on the presence of the PTD 11-amino acid domain. Practically 100% of the βTC-3 cells were transduced with TAT/PTD-β-galactosidase, whereas no activity was detected in cells transduced with β-galactosidase missing the TAT/PTD 11-amino acid domain.

To study the persistency of TAT/PTD-β-galactosidase transduction, cells were transduced with the fusion protein conjugated with the fluorescent marker Alexa-Flour 488 (similar to FITC). βTC-3 cells were transduced for 30 min with the labeled protein (0.8 μg per 1,000 cells). Cells were washed and cultured in medium free of TAT/PTD-fusion protein, then they were collected at different time points and analyzed by flow cytometry. The labeled protein was detectable for at least 48 h (Fig. 1B). Thus, it appears that TAT/PTD-mediated transduction is a viable alternative approach for the delivery of transgenes to target cells, particularly in the context of islet transplantation.
tein that inhibits apoptosis by binding the DEDs of both Bcl-XL and PEA/15. Both anti-apoptotic proteins exert their anti-apoptotic activities via distinct molecular mechanisms. Bcl-XL belongs to the Bcl-2 family, which plays an important role in regulating the response of different cell types (including pancreatic islets) to a wide variety of apoptotic stimuli signaling through the mitochondria (13,26). PEA/15 is a 15-kDa, death effector domain (DED)-containing protein that inhibits apoptosis by binding the DEDs of both the adapter molecule FADD (Fas-associated death domain) and the effector caspase-8 (27).

Both TAT/PTD-Bcl-XL and TAT/PTD-PEA/15 fusion proteins prevented activation of caspase-3, a key enzyme involved in TNF-α-induced apoptosis (28), in βTC-3 insulinoma cells. Inhibition of protein synthesis by CHX is necessary to trigger apoptosis by TNF-α in βTC-3 cells (D.K. and R.L.P., unpublished data), perhaps because it prevents the de novo synthesis of anti-apoptotic factors triggered by TNF-α, as previously described in other cell lines (29). Caspase-3 activity was assessed by measuring the release of AFC from the fluorogenic Ac-DEVD-AFC substrate as a consequence of the caspase-3-mediated cleavage. As shown in Fig. 3A, transduction of pancreatic β-cells with TAT/PTD-Bcl-XL or TAT/PTD-PEA/15 resulted in a 50 and 48% reduction in TNF-α-induced caspase-3 activity, respectively, whereas transduction with TAT/PTD-β-galactosidase did not prevent TNF-α-induced apoptosis. Similarly, we observed a general reduction in the activation of caspasas in cells treated with the TAT/PTD–Bcl-XL fusion protein, as assessed by the binding of the FITC conjugate of the pan-caspase inhibitor VAD-FMK to activated caspases (Fig. 3B-I). However, as expected, transduction with TAT/PTD–β-galactosidase did not have any preventive effect (Fig. 3B-II). Binding of VAD-FMK/FITC to activated caspases serves as an in situ marker of apoptosis. We did not study inhibition of caspase activation by TAT/PTD-PEA/15 using this method. Furthermore, pancreatic β-cells treated with TAT-Bcl-XL or TAT/PTD-PEA/15 fusion proteins had higher viability than control cells after incubation with TNF-α/CHX (Fig. 4). Using a viability test based on combined acridine orange and ethidium bromide staining (FluoRoQuench kit; One Lambda, Canoga Park, CA), we observed that the staining patterns of viable cells (green color) transduced with TAT/PTD–Bcl-XL or TAT/PTD-PEA/
15 fusion proteins were comparable to those of the control cells, whereas the morphology of cells treated with TNF-α/CHX in the absence of TAT-fusion protein corresponded to that of an advanced stage of apoptosis (yellow to orange). Similar results were obtained by assessing cell viability using a combination of calcein AM and ethidium homodimer-1 (Molecular Probes, Eugene, OR) (data not shown). The pictures shown in Fig. 4 are representative of three separate experiments. Cells transduced with Bcl-XL- and PEA/15-fusion proteins showed (means ± SD), respectively, 82 ± 8 and 59 ± 13% of the green (viable) cells compared with >93 ± 5% in control cells and only 15 ± 6% in TNF-α/CHX–treated cells. In addition, the green fluorescence of live cells from control and TAT/PTD-transduced cells was 45 ± 8% more intense than the fluorescence remaining in cells treated only with TNF-α/CHX, as assessed by the intensity of luminosity of areas of similar pixels using Photoshop 5.5 software. These results indicate that the two anti-apoptotic proteins investigated can be expressed as TAT/PTD-fusion proteins, retaining their biological function and protecting cells from apoptosis.

**TAT/PTD-fusion protein does not affect insulin secretion capabilities of islets.** A key requirement for any molecular manipulation of pancreatic islets is that insulin secretion capability not be affected. We therefore investigated whether the TAT-fusion protein transduction affected islet physiology and insulin secretion; we did this by testing the ability of transduced islets to reverse hyperglycemia in diabetic nude mice. As shown in Fig. 5A, islets transduced with TAT/PTD–Bcl-XL reversed hyperglycemia within the same time frame as control islets. Transduced and nontransduced islets were implanted under the kidney capsule of immunodeficient CB17- scid mice previously rendered diabetic through chemical induction. All animals returned to normoglycemia after implant of the islet graft in both groups, demonstrating that islet cell performance was not affected by exposure to the TAT/PTD-fusion protein in vitro. After removal on day 29 of the kidney bearing the graft, a prompt rise in blood glucose was observed in all animals, confirming that euglycemia was sustained by insulin produced by the grafted islets. Furthermore, analysis of hematoxylin-eosin–stained sections did not reveal any histological differences between control and TAT-transduced islets, nor was there any difference in the insulin staining between the two (data not shown).

To complement these studies, we performed static glucose challenge stimulation of rat islets transduced with TAT/PTD–Bcl-XL. Transduced islets maintained their ability to secrete insulin physiologically in a manner similar to control nontransduced islets (Fig. 5B). The same population of islets were subjected sequentially to low-glucose (2.2 mmol/l), then high-glucose (22 mmol/l), and again low-glucose (2.2 mmol/l) stimulation, thus allowing a more physiological evaluation of glucose-stimulated insulin secretion. Means of stimulation indexes (SIs) from five independent experiments using control and transduced islets (2.5 ± 0.42 [17%] and 2.8 ± 0.6 [22%], respectively) did not show a significant statistical difference (P = 0.19). Note that these values represent the SI, which is assessed without the supplementation of insulin secretagogues in the high-glucose medium (30). The Lewis rat islets used in these experiments were in good condition, lacking central core necrosis and maintaining intact islet capsule. The validity of the SI assessments is confirmed by their ability...
to return to basal insulin secretion levels when subjected to second low-glucose stimulation. These results indicate that the process of transduction of pancreatic islets with TAT/PTD–Bcl-XL fusion protein did not alter or impair insulin secretion in transduced islets.

DISCUSSION

In this study, we used TAT/PTD methodology to deliver proteins to pancreatic islets. Utilizing a TAT/PTD–β-galactosidase fusion protein labeled with Alexa 488, we found that TAT-fusion proteins are capable of transducing pancreatic β-cells (Fig. 1). The labeled protein was still detectable after 48 h of transduction when cultured in medium free of TAT/PTD-fusion protein (Fig. 1B). Interestingly, all of the cells were fluorescent, suggesting that the TAT/PTD-fusion protein was passed onto newly divided cells as well. Although we have not yet studied the turnover of any TAT/PTD-fusion proteins, we speculate that it depends on the intrinsic turnover of the expressed protein. Similar to that which was observed in transduction mediated by PTD-5 (20), the TAT/PTD domain mediated both cytoplasmic and nuclear localization of β-galactosidase in βTC-3 cells. Fluorescence microscopy showed that immediately after transduction, the fluorescence associated with the TAT/PTD–β-galactosidase fusion protein was present throughout the cell, although after 24 h it was found to be more localized in the nuclear compartment (J.E. and R.L.P., unpublished observations).

TAT/PTD–β-galactosidase also has the ability to transduce pancreatic islets by diffusion in culture with >80% efficiency (Fig. 2). These results indicate that transduction of islets in suspension using the TAT/PTD system is much more efficient than the viral transduction (31). Transduction of pancreatic cells or islets is totally dependent on the presence of the TAT/PTD domain in the protein (Figs. 1A and 2B).

Although the mechanism by which cells take up TAT/PTD fusion proteins is currently unknown, it has been suggested that an unfolding step is involved in its internalization (32) and that in vivo refolding of the protein is subsequently required (16). It was therefore important to investigate whether an anti-apoptotic protein, expressed as a TAT/PTD-fusion protein, retains biological activity after transduction into cells. The experiments shown in Fig. 3 and Fig. 4 indicate that Bcl-XL and PEA/15, two anti-apoptotic proteins that use different molecular mechanisms to block apoptosis, retain their anti-apoptotic properties once transduced to pancreatic cells.
Rat islets were incubated with 0.4 TAT/PTD–Bcl-XL. Lewis rat islets were transplanted under the kidney of chemically induced diabetic CB17-scid mice. Animals received islets cultured for 24 h either in the presence (triangles) or absence (circles) of TAT-fusion protein (day –1). TAT-treated islets restored normoglycemia (blood glucose <200 mg/dl) in the transplanted animals as well as in control islets 1 day after implantation (day 0). The arrow on day 29 indicates the removal of the kidney bearing the graft. TAT1 and TAT2 represent two independent experiments performed with the same fusion protein. B: Static glucose challenge of islets transduced with TAT/PTD-Bcl-XL fusion proteins. Rat islets were incubated with 0.4 µg TAT/PTD-Bcl-XL per islet and subsequently cultured overnight before being harvested for the static glucose challenge test. The experiment shown is representative of five independent experiments performed in duplicate with islets isolated from different pools of animals. Bars with pattern are control islets; empty bars represent transduced islets.

TAT/PTD-fusion proteins are delivered efficiently to islets and pancreatic tissues, without impairing islet function, as assessed by reversal of hyperglycemia in diabetic immunodeficient mice (Fig. 5A). We complemented these studies with a static glucose challenge performed on islets suspended in a Sepharose matrix (Fig. 5B). In this method, the same islets are subjected to low, then to high, and then again to low glucose. A better physiological islet profile can be obtained with this method than with the classical method. If the islets are not subjected again to low glucose, it cannot truly be said whether they are functioning in a physiological fashion because they may be experiencing a phenomenon known as “dying and dumping,” in which insulin is released from the cells over time and in an unregulated fashion at constantly higher levels. Rat islets transduced with TAT/PTD–Bcl-XL maintained their basal secretion value, responded to high glucose, and then returned to near basal levels when subjected again to low glucose (Fig. 5B). Unlike that which was reported in transgenic mice expressing Bcl-XL >10× the level of control mice (33), transduction with TAT/PTD–Bcl-XL did not impair insulin secretion, possibly reflecting the transient nature of TAT/PTD transduction or just a quantitative phenomenon.

It is clear that improving islet viability is of utmost importance for the successful outcome of islet transplantation. During islet isolation, islet viability could be improved by transduction of the pancreas with anti-apoptotic proteins, such as the TAT/PTD-fusion proteins that inhibit activation of the stress-activated protein kinases, either alone or in combination with TAT-fusion proteins that prevent mitochondria-mediated apoptosis. A distinct advantage of the TAT/PTD system is that the transduced proteins are present in the cells transiently. This is in contrast to classical gene therapy approaches, which would result in either permanent or long-term expression of the anti-apoptotic proteins. Long-term or permanent expression of anti-apoptotic proteins may be deleterious to islet physiology, and they may be potentially unsafe to the recipient. Recent reports by Zhou et al. (33) showed, for example, that constitutive overexpression of Bcl-XL prevented cell death but impaired mitochondrial signals necessary for insulin secretion.

Prevention of islet apoptosis due to isolation stress may yield a greater number of healthy functional islets per isolation, making it possible to perform islet transplantation in one or more recipients using the pancreas from a single donor. This would also significantly reduce transplantation waiting periods caused by shortage of organs.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Institutes of Health (DK-25802) and by the Diabetes Research Institute Foundation.

We thank Dr. Jan Klein for critical reading of the manuscript.

REFERENCES


