Citrullinated glucose-regulated protein 78 is an autoantigen in type 1 diabetes.

Running title: GRP78 is an autoantigen in type 1 diabetes

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Main text: 4000 words

Abstract: 146 words

Number of figures and tables: 8
Abstract

Post-translational modifications of self-proteins play a substantial role in the initiation or propagation of the autoimmune attack in several autoimmune diseases, but their contribution to type 1 diabetes is only recently emerging. In the present study we demonstrate that inflammatory stress, induced by the cytokines IL-1β and IFNγ, leads to citrullination of GRP78 in beta-cells. This is coupled with translocation of this endoplasmic reticulum chaperone to the beta-cell plasma membrane and subsequent secretion. Importantly, expression and activity of peptidylarginine deiminase 2, one of the 5 enzymes responsible for citrullination and a candidate gene for type 1 diabetes in mice, is increased in islets from diabetes-prone NOD mice. Finally, (pre-)diabetic NOD mice have autoantibodies and effector T-cells that react against citrullinated GRP78, indicating that inflammation-induced citrullination of GRP78 in beta-cells generates a novel autoantigen in type 1 diabetes, opening new avenues for biomarker development and therapeutic intervention.
Type 1 diabetes is an autoimmune endocrine disease in which loss of central and peripheral tolerance towards beta-cell antigens is proposed as the underlying mechanism. However, beta-cells themselves also contribute to trigger and/or propagate the autoimmune attack, leading to a dialogue with immune infiltrating cells that may amplify local inflammation (insulitis) in genetically predisposed individuals (1). Insulin (or pro-insulin) is probably the primary autoantigen in type 1 diabetes (2), but antigen spreading occurs as the autoimmune assault progresses, with autoantibodies appearing against several non-beta-cell specific autoantigens, such as GAD65 (3), islet antigen 2 (IA2) (4), heat shock protein 60 (HSP60) (5) and chromogranin A (ChgA) (6).

During insulitis, local production of inflammatory mediators, such as the cytokines IL-1β and IFNγ, triggers beta-cell oxidative and endoplasmatic reticulum (ER) stress. These, and other signals, may lead to alternative splicing and misfolding of beta-cell proteins as well as post-translational modifications (PTM) (7-9). In other autoimmune diseases, like rheumatoid arthritis (RA), multiple sclerosis (MS) and celiac disease, such post-translationally modified proteins behave as autoantigens (10; 11), but their relevance in type 1 diabetes is only starting to be explored (12-15).

Building on our previous observation that the ER chaperone 78 kDa glucose-regulated protein (GRP78; also named binding immunoglobulin protein (BiP)) is post-translationally modified in cytokine-exposed insulin-producing INS-1E cells (9), we now identify this modification as citrullination and show that inflammation-induced citrullinated GRP78 is an autoantigen in type 1 diabetes. These findings suggest a novel role for GRP78, beyond its well-known function in the ER, leading to the loss of tolerance to beta-cells in type 1 diabetes.
Research Design and Methods

Western blotting, mass spectrometry, GRP78 cloning, expression, purification and \textit{in vitro} citrullination are available as supplementary data.

Reagents and Antibodies

Primary antibodies were: mouse anti-Poly(ADP-ribose) monoclonal antibody (mAb) (Enzo Life Sciences, Antwerp, Belgium), rabbit anti-GRP78 and anti-CHOP polyclonal antibody (pAb) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-eIF2\(\alpha\) pAb, anti-p-eIF2\(\alpha\)(Ser51) pAb, anti-PERK mAb and anti-p-PERK(Thr980) mAb (Cell Signaling, Beverly, MA, USA) and mouse anti-actin mAb (Sigma-Aldrich, Diegem, Belgium) for Western blotting; rabbit anti-\(\beta\)-catenin mAb (Cell Signaling Technology) and mouse anti-GRP78 pAb (Abcam, Cambridge, UK) for immunocytochemistry. Anti-Cit(510)-GRP78 was raised in rabbits against the following peptides, C-aminohexanoic acid-IDVNGIL[Citrulline]VTAEDKG-amide and Acetyl-IDVNGIL[Citrulline]VTAEDKG-aminohexanoic acid-C-amide, through 5 subsequent injections (21st Century Biochemicals). Specificity of the antibody was confirmed by dot blot against the citrullinated peptide and its native counterpart. Secondary antibodies were: donkey anti-rabbit horseradish peroxidase, donkey anti-mouse Alexa Fluor 488 and donkey anti-rabbit Alexa Fluor 555 (Invitrogen, Merelbeke, Belgium). Ovalbumin and rabbit PAD enzyme were from Sigma.

Cell Lines and Culture Conditions

Rat INS-1E cells, a kind gift from Prof. Wollheim (CMU, Geneva, Switzerland), were cultured as described (9). INS-1E cells were exposed to recombinant rat IFN\(\gamma\) (R&D Systems, 500 U/ml), recombinant human IL-1\(\beta\) (R&D Systems, 10 U/ml), thapsigargin (Tg) (Sigma, 15 and 50 nmol/l), tunicamycin (Tn) (Sigma, 2 and 5 \(\mu\)g/ml), high glucose (HG) (Sigma, 25 mmol/l), palmitate (Pa) (Sigma, 0.5 mmol/l). Mouse MIN6 cells, a kind gift from Dr.
Miyazaki (Osaka University, Japan), were cultured in DMEM (Invitrogen) containing 15% (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 70 µmol/l β-mercaptoethanol. MIN6 cells were exposed to recombinant mouse IFNγ (R&D Systems, 500 U/ml) and human recombinant IL-1β (10 U/ml).

**Apoptosis measurements**

The percentage of living and apoptotic cells was assessed as described (9).

**Mice**

C57Bl/6 mice were obtained from Harlan Laboratories (Horst, The Netherlands) and non-obese-resistant (NOR) mice from Jackson Laboratory (Maine, USA). Non-obese diabetic (NOD) mice are inbred in our animal facility since 1989 and are kept under semi-barrier conditions. For all experiments a mix of male and female mice were used. All animal manipulations were in compliance with the principles of laboratory care and approved by the Institutional Animal Ethics Committee of KU Leuven.

**Islet Isolation and Culture**

Pancreatic islets were isolated from 3- or 10-week old C57Bl/6, NOD and NOR mice. Islet isolation and culture was performed as described (16). C57Bl/6 islets were exposed to recombinant mouse IFNγ (1000 U/ml) and recombinant human IL-1β (50 U/ml).

**Immunofluorescence**

Immunofluorescence on isolated islets was performed as described (17). Fixed INS-1E cells or sectioned islets were incubated with primary antibody in 1% BSA for 1 h, followed by four washes in PBS before incubation with the secondary antibody in 1% BSA for another hour. Nuclei were detected with DNA-binding dye DRAQ5TM (Biostatus Ltd., UK). Specificity
was confirmed by including negative controls with secondary antibodies alone. INS-1E samples were observed under a Zeiss LSM 510 microscope using a Plan-Neofluar 40x/1.3 Oil DIC lens. Images were acquired and processed using Lsm510 software (Carl Zeiss AG, Germany). Mouse islet sections were observed under a Nikon Eclipse Ti microscope using a Plan-Fluor 40x/0.75 DIC lens and images were acquired and processed using Nis-Elements Viewer 4.20 software (Nikon Instruments Inc.).

**Cell Surface Biotinylation**

INS-1E or MIN6 cells were incubated with the indicated stressors for 12-15 h and then treated as described (18).

**GRP78 ELISA**

To determine GRP78 concentration in conditioned media from INS-1E cells, the GRP78 ELISA kit (Enzo Life Sciences, Antwerp, Belgium) was used, following the manufacturer’s protocol.

**2-Dimensional gel electrophoresis analysis**

2-Dimensional gel electrophoresis analysis was performed as described (9).

**Quantitative RT-PCR**

Quantitative RT-PCR was performed as described (19).

**Measurement of peptidylarginine deiminase (PAD) activity**

To determine PAD activity levels in islets and pancreata from C57Bl/6, NOR and NOD mice, the Antibody Based Assay for PAD activity (ABAP) (ModiQuest Research, Oss, The Netherlands) was used, following the manufacturer’s protocol.

**Autoantibody ELISA against native or citrullinated GRP78**

Serum autoantibodies against two native and citrullinated GRP78 peptides (amino acids 500-519 TFEIDVNGILRVTAEDKGTG and amino acids 295-314
AKRALSSQHQARIEIESFYE) were determined by ELISA as described (20). For the citrullinated forms, arg510 or arg306 were replaced by citrulline (synthesized by PolyPeptide Laboratories, France).

**Interferon-γ measurement**

Splenocytes from 10-week-old and new-onset diabetic NOD mice and age-matched C57Bl/6 and NOR mice were cultured in a flat bottom 96-well plates (1 x 10^6 cells/well) in the absence or presence of the indicated stimuli. IFNγ levels were measured in cell culture supernatant after 48 h of culture, using Meso Scale Discovery technology (Rockville, MD, USA), following the manufacturer’s protocol.

**Statistics**

Statistical analyses of data were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Data are expressed as means ± s.e.m. and were analyzed by a Kruskal-Wallis test followed by a Dunn’s multiple comparisons test, unless stated otherwise in the figure legend. P-values < 0.05 were considered significant.
Results

*GRP78 is translocated to the beta-cell surface upon cytokine exposure*

We investigated cytokine-mediated regulation of GRP78 at both transcriptional and translational levels after 12-15 h exposure and at cytokine concentrations that induced only minor apoptosis (Fig. 1A). No significant increase in GRP78 mRNA (Fig. 1B) and total protein expression (Fig. 1C central panel and 1D) were observed as compared to control INS-1E cells. However, a detailed analysis of the plasma membrane fraction revealed a limited presence of GRP78 at the cell surface under basal conditions, which was increased upon IL-1β+IFNγ exposure, but not upon single cytokine exposure (Fig. 1C lower panel and Fig. 1E). This was confirmed in MIN6 cells after 12 h treatment (Fig. 1F), a pre-apoptotic time point (Fig. 1G). In parallel with the increased plasma membrane translocation of GRP78, we also observed an increase in GRP78 secretion upon cytokine exposure of INS-1E cells (Fig. 1H).

These observations were confirmed by immunocytochemistry showing increased GRP78 staining on the plasma membrane of non-permeabilized cytokine-exposed INS-1E cells (Fig. 2A), and a clear increase in co-localization between GRP78 and the plasma membrane marker beta-catenin in cytokine-exposed INS-1E cells (Fig. 2B). Importantly, a similar membrane translocation was observed in cytokine-exposed C57Bl/6 mouse islets (Fig. 2C), again at an early and pre-apoptotic time point (Fig 1I). Thus, surface expression of GRP78 upon cytokine exposure is not solely taking place in clonal beta-cell models but also in primary beta-cells, and is not a consequence of non-specific changes associated with cell death, increasing the relevance of these findings.

*GRP78 is translocated to the plasma membrane upon chemical ER stress, but not upon metabolic stress.*
Cytokine exposure of INS-1E cells and primary rat, mouse and human beta-cells is known to induce ER stress-dependent apoptosis (21-25). We further evaluated the contribution of ER stress to the observed cytokine-induced GRP78 membrane translocation, by investigating the effect of the chemical ER stressors thapsigargin (Tg) and tunicamycin (Tn). Cytokines (12-15 h) induced a clear activation of the PERK-eIF2α-CHOP pathway, at the dose tested (Suppl. Fig. 1). For Tg there was a dose-response effect both in terms of apoptosis (Fig 3A) and ER stress induction (Fig 3B and Suppl. Fig 1), with minor ER stress at 15 nmol/l but a clear induction of the PERK-eIF2α-CHOP branch at 50 nmol/l Tg, towards levels similar to those observed upon cytokine exposure. Of note, Xbp1 splicing was induced by Tg at 50 nmol/l, which was not the case upon cytokine exposure (Suppl Fig. 1B). INS-1E cells were more resistant to Tn, with less apoptosis (Fig 3A) and an intermediate activation of PERK-eIF2α-CHOP and Xbp1 splicing, both at 2 and 5 µg/ml (Suppl. Fig. 1), as compared to the higher doses of Tg and cytokines. As illustrated in Fig. 3C-E, 15 nmol/l Tg and 2 µg/ml Tn did not induce membrane translocation of GRP78. Importantly, the higher dose of 50 nmol/l Tg led to a clear membrane translocation, which was paralleled by more marked expression of ER stress markers (see above). A similar effect was observed with 5 µg/ml Tn, although less pronounced.

Finally, upon metabolic stress (palmitate (Pa, 0.5 mmol/l) or the combination of high glucose (HG, 25 mmol/l) + palmitate), most of the ER stress markers were increased, although the increase in CHOP mRNA and protein was less marked than that observed with chemical ER stressors or cytokines (Suppl. Fig. 1). On the other hand, the total and membrane-associated GRP78 protein levels remained unaltered (Fig. 3F-H). Taken together, these findings indicate that GRP78 membrane translocation occurs upon inflammation- and severe chemical-induced ER stress, but not upon metabolic stress.
**GRP78 is post-translationally modified in cytokine-exposed beta-cells.**

Besides the above described membrane translocation of GRP78, we observed extensive post-translational modification (PTM) of GRP78 upon cytokine exposure of INS-1E cells (Fig. 4A and previously described (9)) and C57Bl/6 mouse islets (Fig. 4B). This was also the case, although to a lesser degree, for IL-1β or IFNγ exposure alone (Fig. 4A). Of particular interest, 2D-GE analysis of the plasma membrane fraction of control and cytokine-exposed INS-1E (Fig. 4C) and MIN6 cells (Fig. 4D) not only demonstrated the presence of the three different cytokine-responsive GRP78 isoforms, but also showed a cytokine-mediated upregulation of numerous acidic GRP78 isoforms as compared to control cells. On the other hand, metabolic- and chemical (both at low and high concentrations)-induced ER stress did not induce detectable PTMs of GRP78 (Fig. 4E), confirming the specific effects of pro-inflammatory cytokines.

**GRP78 is citrullinated in cytokine-exposed INS-1E cells**

In order to identify the nature and site of cytokine-induced PTMs in GRP78, we subjected the three different GRP78 isoforms observed in INS-1E cells to mass spectrometry (MS) analysis. Sequence coverage ranged from 62.54% to 74.46% (n=2). When comparing the resulting peptide profiles of isoform 1 (I1) vs. isoform 2 (I2) and I1 vs. isoform 3 (I3), one specific peptide, VTAEDKG(T)GNK (AA511-521), was identified exclusively in I1. This was confirmed by a quantitative differential analysis using trypsin digestion in combination with differential N-butrylation and endoproteinase Lys-C digestion combined with differential N-propionylation (Fig. 5A and 5B). As the peptide upstream of VTAEDKG(T)GNK could not be identified using both methods, we hypothesized the presence of a PTM in this region, possibly on Arg510, which would prevent tryptic digestion in I2 and I3, thereby rendering the resulting peptide (AA493-521) too long for retrieval and detection by MS. Of note, also a second
peptide of interest was found, which was identified almost exclusively in I2 and I3, while hardly in I1 (AA307-324, Fig. 5A and 5B). Since this would point to the presence of a PTM in the basic but not acidic forms, we did not further analyze the relevance of this peptide.

Based on these findings, we then investigated the nature of PTM present in the identified region. We neither succeeded in retrieving by MS the longer, Arg510-containing peptide (AA493-521), nor a spiked tryptic peptide with heavy label (AA493-521) used as internal control. This is probably caused by insolubility and inability to analyze on a C18 column, and forced us to use instead specific enzymatic and antibody-based assays. We focused on three potential PTMs consistent with an acidic shift in isoelectric point without change in molecular weight and described to occur in GRP78 in other cell types, namely ADP-ribosylation, phosphorylation and citrullination (26-29). Both ADP-ribosylation and citrullination occur on Arg residues, while phosphorylation may occur on Thr 518.

We initially investigated ADP-ribosylation and phosphorylation of GRP78, but the absence of a positive signal in 2D-Western blots of cytokine-exposed INS-1E cells with anti-poly(ADP-Ribose) (Fig. 6A) and the absence of GRP78 staining by Pro-Q Diamond in control- and cytokine-exposed INS-1E cells (Fig. 6B), as well as persistence of the modified isoforms upon treatment with calf intestinal alkaline phosphatase (CIAP) (Fig. 6C) or λ-phosphatase (Data not shown), argued against both modifications.

To verify the implication of citrullination, an antibody that specifically recognizes citrullinated GRP78 at Arg510 (selection based on the obtained MS data, see Fig. 5) was raised in rabbits. 2D-Western blots from control and cytokine-exposed INS-1E cells with anti-cit510-GRP78 clearly indicated that the cytokine-induced acidic isoforms of GRP78 correspond to citrullinated GRP78 at residue Arg510, whereas no reactivity was observed against the most basic, non-modified GRP78 isoform 1 (Fig. 6D).
Next, we investigated the potential role of citrullination in the diabetes-prone NOD mouse. Elevated Padi2 mRNA expression was observed in islets of 3- and 10-week old pre-diabetic NOD mice as compared to islets from age-matched C57Bl/6 and NOR control mice (Fig. 7A and B). No differences between the strains were observed for Padi1, Padi3, Padi4 and Padi6 expression, which were either low or undetectable. Further analysis in other tissues revealed an overall very low expression of Padi2 in the immune related tissues thymus, lymph nodes and spleen. Except for kidney, no elevated levels of Padi2 were observed in the other tissues analyzed in NOD as compared to C57Bl/6 mice. In addition, NOR mice showed even lower/undetectable Padi2 expression in most of the tissues analyzed (Fig. 7C). Furthermore, elevated Padi2 mRNA expression in NOD islets corresponded to higher PAD activity in total pancreases (Fig. 7D and E) and islets (Fig. 7F and G) of 3- and 10-week old NOD mice, compared to both C57Bl/6 and NOR mice, indicating a marked increase of PAD activity in islets of NOD mice immediately before and during insulitis. Of note, 3-week old NOD mice did not show any sign of inflammation in the islets, as measured by IL-1β (Fig. 7H) and IFNγ (Fig. 7I) mRNA expression. In 10-week-old NOD mice, on the other hand, clear signs of immune infiltration were observed, as evidenced by high expression of IFNγ and IL-1β mRNA, confirming previous findings from our group (30).

NOD mice have circulating autoantibodies and autoreactive T-cells against citrullinated GRP78

To evaluate whether citrullinated GRP78 contributes to the autoimmune response in NOD mice, serum samples from pre-diabetic and new-onset diabetic NOD mice and age-matched C57Bl/6 and NOR mice were analyzed for the presence of autoantibodies against the native and citrullinated peptide containing the epitope of interest (p500-519). Serum levels of anti-
GRP78 antibodies recognizing this citrullinated epitope were significantly higher in new-onset diabetic NOD mice as compared to age-matched C57Bl/6 or NOR mice (Fig. 8A, right panel). Furthermore, in diabetic NOD mice significant higher serum antibody levels to the citrullinated peptide were found compared with those of the native peptide. No such differences were observed in case of another irrelevant (citrullinated) GRP78 peptide (p295-314) tested (Suppl. Fig. 2). These findings provide evidence that this specific citrullinated epitope is important for autoantibody generation during type 1 diabetes development in NOD mice.

Next, to determine if NOD mice have autoreactive T-cells against native or citrullinated GRP78, freshly isolated splenocytes from pre-diabetic and new-onset diabetic NOD mice and age-matched C57Bl/6 and NOR mice were stimulated with various concentrations of native and \textit{in vitro} citrullinated recombinant mouse GRP78 protein. Secretion of IFN\(\gamma\) was used as a measure of effector T-cell activation. While little to no effector T-cell activation was observed in the three different strains when culturing splenocytes with different concentrations of native GRP78 (Fig. 8B, red line and upper right graph), a clear, dose-responsive increase in IFN\(\gamma\) secretion was observed when splenocytes from pre-diabetic and diabetic NOD mice were cultured in the presence of citrullinated GRP78 (Fig. 8B, blue line and lower right graph). C57Bl/6 splenocytes were unresponsive to citrullinated GRP78, while a minor IFN\(\gamma\) response was detected in NOR splenocytes. Absence of a IFN\(\gamma\) response against both the PAD-enzyme alone and the control protein ovalbumin, either native or \textit{in vitro} citrullinated (at 0.1; 1 and 5 \(\mu\)g/ml) (data not shown), suggests that the observed autoreactive T-cell response is specifically generated against citrullinated GRP78.
**Discussion**

The role of post-translationally modified proteins is well established in several human autoimmune diseases and evidence for similar phenomena in the development of type 1 diabetes is accumulating (12-14). Most importantly for this study, McGinty and colleagues (15) recently demonstrated the relevance of citrullination in patients with type 1 diabetes, by showing an increased response to citrullinated GAD65 peptides. We show that the ER chaperone GRP78 is citrullinated specifically upon exposure of beta-cells to inflammatory stress. This is paralleled by translocation of GRP78 to the beta-cell plasma membrane and eventually its secretion. Under these circumstances, a specific cross-talk between the beta-cell and the immune system is initiated, resulting in the generation of autoantibodies and induction of T-cell autoreactivity against citrullinated GRP78 (Fig. 8C). Importantly, we also observed a marked up-regulation of Padi2 in islets of NOD mice, providing a strong argument for PADI2 being the diabetes susceptibility gene in the recently identified Idd25 locus on mouse chromosome 4 (31) and adding to a potential role for citrullination in type 1 diabetes (15).

In previous studies we have shown that GRP78 is post-translationally modified in INS-1E cells exposed to cytokines (9), a PTM which we now identified as citrullination. Cytokines contribute to beta-cell dysfunction and death at least in part through inducing ER stress (23-25). However, citrullination of GRP78 is neither induced by chemical ER stressors (Tg or Tn) nor by metabolic stress via exposure to high glucose and/or palmitate, suggesting that cytokine-induced GRP78 citrullination occurs through a mechanism independent of its ER stress-inducing capacities. Preliminary data suggest that this citrullination is not mediated by direct up-regulation of the PADI2 enzyme by cytokines (data not shown), but needs to be the consequence of increased PAD activity in beta-cells exposed to cytokines. Since PAD activity is highly Ca$^{2+}$ dependent, changes in Ca$^{2+}$ fluxes induced in cytokine-exposed beta-cells might play a role. The present findings, together with the recent report on post-translationally
modified GAD65 (15), add type 1 diabetes to the list of autoimmune diseases involving citrullination, i.e. rheumatoid arthritis (RA), multiple sclerosis (MS) and systemic erythematous (SLE) (32). This suggests that citrullination is not a specific disease-related event, but rather an inflammation-dependent process occurring preferentially in autoimmune target tissues. The role of citrullination in the induction of autoantigenicity has been best described in RA, with several citrullinated autoantigens - including GRP78 - already identified (20; 33). These citrullinated peptide epitopes are better accommodated in the HLA-pocket of HLA-DR4 type individuals, determining the strength of the immune response to citrullinated peptides and providing a molecular basis for the genetic predisposition of HLA-DR4 individuals to RA (34). This mechanism has recently also been described in type 1 diabetes (15), where HLA-DR4 is an important risk allele for the disease (35).

In addition to citrullination, cytokine-induced translocation of GRP78 to the beta-cell plasma membrane may be a crucial step for GRP78 to become an autoantigen. This translocation is shown to be an early event in response to inflammation, suggesting that it is an active process at least in part independent from “protein leakage” by apoptotic beta-cells. GRP78 membrane translocation, but not citrullination, was found to be ER stress-dependent and paralleled to the increased CHOP expression. Membrane-associated GRP78 has been described in different tumor cell types (36-38) as well as in exocrine pancreatic cells (39) and proliferating endothelial and monocytic cells (40; 41). In these models membrane-associated GRP78 acts, among other functions, as a cell surface signaling receptor for different ligands such as activated α2-macroglobulin (42; 43) and coxsackie A9 virus (44; 45) and is found associated with the major histocompatibility complex class I (MHC-I) (44). Of interest, changes in the topography of membrane-associated GRP78, caused by PTMs, may convert GRP78 into a receptor with autoantigenic properties. This has been observed in cancer cells where GRP78 is modified by O-linked glycosylation (38), leading to the generation of GRP78
autoantibodies. The present observations that inflammation induces extensive GRP78 citrullination, translocation to the plasma membrane and secretion, underscore its putative function as an autoantigen in type 1 diabetes. Furthermore, based on the proposed transmembrane model for GRP78 (46), the reactive p500-519 epitope, against which GRP78-autoantibodies are generated in NOD mice, is located in the extracellular domain, thus being exposed to infiltrating immune cells.

The possible role for citrullinated GRP78 as an autoantigen in type 1 diabetes is supported by the present observations showing the generation of autoantibodies as well as CD4+ T-cell autoreactivity against citrullinated GRP78 in NOD mice. No T-cell reactivity is observed in C57Bl/6 mice, whereas NOR mice show a minor T-cell response. This supports the idea that inflammation is necessary to initiate this process, as low levels of IFNγ and IL-1β are detected in islets of 10-week old NOR mice, a phenomenon referred to as protracted, non-invasive insulitis (47). A marked up-regulation of PAD2, a key enzyme for protein citrullination, is observed exclusively in islets from NOD mice, as compared to NOR and C57Bl/6 islets. PAD-activity is further increased in NOD islets with increasing age and insulitis suggesting that inflammation plays a role for this phenomenon, perhaps by increasing cytosolic Ca^{2+} concentrations due to cytokine-mediated calcium depletion from the ER (24). Interestingly, expression of Padi2 was very low in NOD thymus and not different from C57Bl/6 and NOR thymus. This may explain the escape of citrullinated GRP78 from thymic tolerization in developing thymocytes, thereby clarifying why citrullinated GRP78 can be recognized as a beta-cell specific autoantigen while native GRP78 is ubiquitously expressed. A similar mechanism has been proposed for chromogranin A, where exposure of a naturally occurring cleavage product (peptide WE14) to transglutaminase, expressed in beta-cells, forms high- and low-molecular weight aggregates, thus rendering the peptide highly antigenic in NOD mice (13).
Whether these observed processes are also applicable to other ER chaperones or heat shock proteins, such as HSP60, requires further investigation. It will be of utmost importance to determine whether similar processes are involved in human type 1 diabetes. The knowledge that inflammation-mediated beta-cell stress is taking place in human type 1 diabetes (48) and the high overlap between autoantigens identified to date in NOD mice and type 1 diabetes patients (49), are in support of this hypothesis.

In conclusion, local inflammation in the islets induces citrullination of GRP78 in the stressed beta-cells, turning citrullinated GRP78 into an autoantigen. This modified GRP78 is recognized by both B- and T-cells, thus propagating and amplifying the ongoing autoimmune attack against the beta-cells. Our findings support and provide mechanistic evidence for the concept that inflammation-induced beta-cell stress initiates a specific communication between the beta-cell and the immune system, which will aggravate and accelerate the development of type 1 diabetes. A genetic predisposition for increased citrullination in the islets, as shown here for NOD mice, is expected to further exacerbate this process. This proposed mechanism (Fig. 8C), implicating tissue specific and inflammation-induced protein modification, cell surface translocation and secretion, would also explain why different tissue specific autoimmune diseases can have similar autoantigens.
**Author contributions.** D.R. and I.C. designed and performed research, analyzed data and wrote the paper. W.D.H., G.B.F., A.S. and A.D.G. performed research, D.L.E. analyzed data, wrote and edited the paper, P.A. and K.G. edited the paper. L.O. and C.M. designed research and wrote and edited the paper. L.O. and C.M. are guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

**Acknowledgements.** The technical assistance of Frea Coun, Martine Gilis, Jos Laureys, Willem Van Den Berghe, Wim Werckx and Farah-Deborah Lok (Laboratory of Clinical and Experimental Endocrinology, KU Leuven) is greatly appreciated. We thank Katleen Lemaire (Gene Expression Group, KU Leuven) and Monique Beullens (Laboratory for Biosignaling & Therapeutics, KU Leuven) for advice on GRP78 cloning in pET vector. We thank the Cell Imaging Core (KU Leuven) for providing technical assistance with confocal microscopy.

**Funding.** This work was supported by the Juvenile Diabetes Research Foundation International (17-2012-129 and 17-2013-515), the European Community’s 7th Framework Programme NAIMIT under grant agreement n° 241447, the KU Leuven (Geconcerteerde Onderzoeksactie GOA 12/24 and a F+ fellowship for D.R.), and the Flemish Research Foundation (G.0619.12, a post-doctoral fellowship for G.B.F. and W.D.H. and a clinical research fellowship for C.M.).

**Duality of interest.** No potential conflicts of interest relevant to this article are to be reported.
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Figure Legends

Figure 1 Cytokine exposure induces membrane translocation of GRP78 in insulin-secreting INS-1E and MIN6 cells. (A) Apoptosis levels in INS-1E cells exposed for 12-15 h (white bars) or 24 h (black bars) to IL-1β (10 U/ml) and/or IFN-γ (500 U/ml) (n=3-8 independent experiments; each biological replicate is the mean of two technical duplicates). (B) GRP78 mRNA expression in INS-1E cells treated for 12-15 h as described above (n=7, each biological replicate is the mean of 2 technical duplicates). (C) Total and plasma membrane-associated (PM) GRP78 protein expression in INS-1E cells exposed to the indicated stressors. A representative Western blot from 4 independent experiments is shown. (D)-(E) The relative intensities of the different protein bands were quantified by densitometry and expressed as a ratio (n=4). (F) Total and plasma membrane-associated (PM) GRP78 protein levels in control and cytokine-exposed MIN6 cells. A representative Western blot from 2 independent experiments is shown. (G) Apoptosis levels in MIN6 cells exposed for 12 h (white bars) and 24 h (black bars) to IL-1β and IFN-γ (n=5-8 independent experiments, each biological replicate is the mean of two technical duplicates). (H) GRP78 protein concentration in the culture medium of control and cytokine-exposed INS-1E cells. Data are expressed as means ± s.e.m. and were analyzed by a two-tailed paired t test (n=10 independent experiments). (I) Apoptosis levels in C57BL/6 mouse islets exposed for 24 h (white bars) and 72 h (black bars) to IL-1β (50 U/ml) and IFN-γ (1000 U/ml) (n=3-5 independent experiments, each biological replicate is the mean of two technical duplicates). *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 vs. respective Ctrl.

Figure 2 Microscopic imaging of cytokine-induced GRP78 membrane translocation in INS-1E cells and mouse islets of Langerhans. (A) Unpermeabilized control and cytokine-exposed INS-1E cells (15 h) were stained for GRP78 (green) while the nuclei (blue) were stained with Hoechst 33342. Images shown are representative for two independent experiments. Bar = 10 µm (B) Control and cytokine-exposed INS-1E cells (15 h) and (B) intact mouse islets (24 h) were stained for GRP78 (green) and β-catenin (red). Nuclei (blue) were stained with Hoechst 33342. Images shown are representative for three independent experiments. Bar = 20 µm

Figure 3 Chemical ER stress, but not metabolic stress, induces membrane translocation of GRP78 in insulin-secreting INS-1E cells. (A) Apoptosis levels in INS-1E cells exposed for 12-15 h (white bars) or 24 h (black bars) to thapsigargin (Tg; 15 or 50 nmol/l), tunicamycin (Tn; 2 or 5 µg/ml), high glucose-(HG; 25 mmol/l), palmitate- (Pa; 0,5 mmol/l) or the combination (HG+Pa) (n=5-9 independent experiments; each biological replicate is the mean of two technical duplicates). (B) GRP78 mRNA expression in INS-1E cells treated for 12-15 h as described above (n=4-10, each biological replicate is the mean of 2 technical duplicates). (C and F) Total and plasma membrane-associated (PM) GRP78 protein expression in INS-1E cells exposed to the indicated stressors. A representative Western blot from 4 independent experiments is shown. (D)-(E) and (G)-(H) The relative intensities of the different protein
bands were quantified by densitometry and expressed as a ratio ($n=4\text{-}7$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ vs. respective Ctrl.

**Figure 4** Cytokine exposure induces post-translational modification of GRP78 in INS-1E cells and in intact mouse islets. Representative images from 2D-DIGE analysis (selected region of 24 cm pH 4-7, 12.5% SDS-PAGE) of GRP78 in control and cytokine-exposed (A) INS-1E cells and (B) intact mouse islets with corresponding three-dimensional view and Graph view of the DeCyder analysis. For each of the three isoforms (I1, I2 and I3), the fold-increase or decrease is shown and statistical analysis was performed using a two-tailed unpaired $t$ test ($n=4$ independent experiments; *$P < 0.05$ and **$P < 0.01$ vs. Control). (C-D) Representative images of 2D-GE analysis (selected region of 24 cm pH 4-7, 12.5% SDS-PAGE) of intracellular and membrane-associated GRP78 in control and cytokine-exposed INS-1E cells (C, one representative experiment out of 5 independent experiments is shown) and MIN6 cells (D, one representative experiment out of 2 independent experiments is shown). (E) Representative 2D-GE analysis (selected region of 24 cm pH 4-7, 12.5% SDS-PAGE) with corresponding three-dimensional view of GRP78 in total lysates from control and differentially exposed (as indicated) INS-1E cells. One representative experiment out of 3 independent experiments is shown.

**Figure 5** Quantitative mass spectrometric analysis of the three GRP78 isoforms I1, I2 and I3 using trypsin digestion combined with differential N-butyrylation and endoproteinase Lys-C digestion combined with differential N-propionylation. For each dataset the ratios (Light (I1)/Heavy (I2 or I3)) were converted to their log 2 value, in order to render a normal distribution. In the volcano plots the size of the fold change is compared to the statistical significance level. (A) Comparison between the most basic isoform of GRP78 (I1) and the first more acidic isoform (I2) and (B) between I1 and the second more acidic GRP78 isoform (I3) with a sequence coverage of 77.67% and 71.70%, respectively (494 and 456 out of 654 amino acids of the GRP78_RAT sequence, respectively, with omission of the 18 amino acid signal peptide). The identified tryptic peptides are marked in red, the identified Endo-LysC peptides in bold and the signal peptide in blue. The two peptides with the highest z-score and fold change are depicted on the volcano plot and underlined in the sequence.

**Figure 6** GRP78 is citrullinated upon cytokine exposure of INS-1E cells. (A) Representative 2D-Western blot (11 cm pH 4-7, 4-12.5% SDS-PAGE) of cytokine-exposed INS-1E lysate detected with anti-Poly(ADP-Ribose) antibody (upper panel) followed by anti-GRP78 (bottom panel) (one representative experiment out of 3 independent experiments is shown). (B) 2D-GE gel of cytokine-exposed INS-1E cells (24 cm pH 4-7; 12.5% SDS-PAGE) stained using Sypro Ruby to visualize all proteins (upper panel) and Pro-Q Diamond dye to detect phosphoproteins (bottom panel) (one representative experiment out of 2 independent experiments is shown). (C) 2D-GE analysis of GRP78 in control and cytokine-exposed INS-1E cells (selected region of pH 4-7; 12.5% SDS-PAGE is shown), treated or not with Calf Intestinal Alkaline Phosphatase (CIAP) (one representative experiment out of 2 independent
experiments is shown). (D) 2D-GE analysis of citrullinated GRP78 in control and cytokine-exposed INS-1E cells with anti-Cit510-GRP78 (upper panels) and total GRP78 Ab (bottom panels) (one representative experiment out of 7 independent experiments is shown).

**Figure 7** NOD islets have high Padi2 mRNA expression and PAD activity. (A) and (B) Padi1, 2, 3, 4 and 6 mRNA expression in islets of Langerhans of respectively 3- and 10-week old C57BI/6 (black bars), NOR (grey bars) and NOD (white bars) mice (n=5-8). (C) Padi2 mRNA levels in different tissues of 3-week old C57BI/6 (black bars), NOR (grey bars) and NOD (white bars) mice (n=5-10, each sample consists of tissue isolated from a single mouse). (D) and (E) Pancreatic PAD activity in respectively 3- and 10-week old C57BI/6 (black bars), NOR (grey bars) and NOD (white bars) mice. Data are expressed as means ± s.e.m. and were analyzed by a one-way ANOVA followed by a Bonferroni multiple comparisons test (n=4-9). (F) and (G) Islet PAD activity in respectively 3- and 10-week old C57BI/6 (black bars), NOR (grey bars) and NOD (white bars) mice (n=4). All replicates refer to biological replicates with samples (islets or pancreas) obtained from individual mice. The PAD activity experiments were performed at least 3 times. ND=not detectable. (H) IL-1β and (I) IFNγ mRNA levels in 3- and 10-week old C57BI/6 (black bars), NOR (grey bars) and NOD (white bars) mice (n=5-8, each sample consists of islets isolated from a single mouse). *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001

**Figure 8** NOD mice have circulating autoantibodies and autoreactive T-cells against citrullinated GRP78 (A) Comparison between the serum levels of anti-native and anti-citrullinated peptide (AA500-519) antibodies in pre-diabetic and diabetic NOD mice and age-matched C57BI/6 and NOR mice grouped according to age. Each dot indicates the value of a single mouse. Four independent experiments were performed, each containing samples of all experimental groups. (B) IFNγ response of splenocytes from pre-diabetic (n=9, light grey bars) and diabetic NOD (n=11, white bars) mice and age-matched C57BI/6 (n=8, black bars) and NOR (n=11, dark grey bars) mice stimulated with various concentrations of native (red) and citrullinated (blue) recombinant mouse GRP78. Four independent experiments were performed, with 2-3 animals per group per experiment. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. (C) Proposed model for the role of beta-cell citrullinated GRP78 in autoantibody generation and T-cell activation. Exposure of beta-cells to cytokines induces citrullination, membrane translocation and secretion of GRP78. Citrullinated membrane-associated or secreted GRP78 is taken up and processed by B-cells and antigen presenting cells (APCs) resulting in the generation of specific anti-citrullinated GRP78 autoantibodies and release of IFNγ by activated effector T-cells, respectively.

**Suppl. Fig. 1** Analysis of different ER stress markers upon exposure of INS-1E cells to diverse ER stressors. (A) CHOP and (B) Xbp1 spliced mRNA expression in control (Ctrl), cytokine (IL-1β+IFNγ, IL-1β (10 U/ml) and IFNγ (500 U/ml)), chemical ER stress (thapsigargin (Tg; 15 and 50 nmol/l) and tunicamycin (Tn; 2 and 5 µg/ml)), or metabolic stress (high glucose (HG; 25 mmol/l), palmitate (Pa; 0.5 mmol/l) or both) exposed INS-1E
cells (12-15 h) \( n=4-10 \) independent experiments; each biological replicate is the mean of two technical duplicates). (C) Western blot analysis of CHOP and the phosphorylation status of PERK and eIF2\( \alpha \) in INS-1E cells exposed as described above. A representative Western blot from 3 independent experiments is shown. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) and ****\( P < 0.0001 \)

**Suppl. Fig. 2** Comparison between the serum levels of anti-native and anti-citrullinated peptide (AA295-314) antibodies in 12-15-week-old C57Bl/6, NOR and diabetic NOD mice. Each dot indicates the value of a single mouse.
Figure 1
Figure 2
Figure 3
Figure 4

A

B

C

D

E

Plasma membrane GRP78
Intracellular GRP78
Merged

IL-1β+PYY Control

Plasma membrane GRP78
Intracellular GRP78
Merged

IL-1β+PYY Control

Control
Tg (15 nmol/l)
Tg (60 nmol/l)
Tn (2 µg/ml)
Tn (5 µg/ml)
HG
Pa
HG + Pa

227x280mm (300 x 300 DPI)
Figure 5

Volcano plot: z vs log2 (ratio 11/12)

Volcano plot: z vs log2 (ratio 11/13)
Figure 6

129x94mm (300 x 300 DPI)
Figure 7

103x57mm (600 x 600 DPI)
**SDS-PAGE and Western blotting**

Protein samples were separated on 4-12% Bis-Tris gels (Invitrogen), blotted onto a PVDF membrane (Hybond-ECL; GE Healthcare), and probed with the indicated antibodies. Western blots were incubated with the Western lightning™ Plus-ECL detection system (PerkinElmer, Zaventem, Belgium) and analyzed with the ImageQuant LAS 500 system (GE Healthcare, Diegem, Belgium).

**Analysis of post-translational modifications of GRP78 by mass spectrometry**

INS-1E cells were lysed, 300 µg protein lysates were run as described above and stained with G250 Coomassie Brilliant Blue. The three isoforms of GRP78 were excised manually and analyzed by mass spectrometry. Spots were numbered from the basic to acidic side, with isoform 1 being the most basic and isoform 3 being the most acidic isoform. The proteins were cleaved using trypsin (Promega, Madison, WI, USA) and the resulting peptides were analyzed using LC-MS/MS (LTQ-Orbitrap Velos). MS/MS data were analyzed with Mascot against the SwissProt database (version 2011_12) with taxonomy set on *Rattus* (7687 sequences). Variable modifications were set on formation of pyroglutamate (N-terminal glutamine) and methionine oxidation to methionine-sulfoxide. No fixed modifications were taken into account. Peptide mass tolerance was set on 10 ppm and fragment mass tolerance on 0.5 Da with a maximum of 1 missed cleavage. Mascot was used at 99% confidence settings.

In parallel, trypsin digestion in combination with N-butyrylation labeling and endoproteinase Lys-C digestion in combination with N-propionylation (1; 2) was performed on the three spots. LC-MS/MS test analyses were done in order to adjust the GRP78 peptides observed when comparing two differentially labeled samples to equal amounts prior to the final LC-MS/MS analysis. The following comparisons were done for each digestion enzyme: isoform 1 *versus* isoform 2 and isoform 1 *versus* isoform 3. Labeling was such that peptides from the most basic isoform (I1) contained $^{12}$C$_4$ butyrate groups on their alpha-amines (peptide N-
terminus) and on their epsilon-amine (side-chain of lysine). Peptides generated from the more acidic isoforms (I2 and I3) contained $^{13}$C$_4$ butyrate groups, thereby creating a difference of 4 Da (Arg-ending peptides) or 8 Da (Lys-ending peptides) for most of the peptides generated following trypsin digestion. In case of endoproteinase Lys-C digestion, a difference of 3 Da was created per primary amino group, rendering a difference of 6 Da per peptide.

For each dataset the calculated peptide ratios (light (I1)/heavy (I2 or I3)) were converted to their log$_2$ value, which yields a normal distribution of peptide ratios. The robust statistic parameters, median and the Huber scale, were calculated and once determined, lower and upper limits were calculated to identify significantly ($P \leq 0.01$) regulated peptides.

**GRP78 cloning, expression, purification and *in vitro* citrullination**

To express recombinant mouse GRP78-His$_6$, full length mouse GRP78 cDNA was amplified by PCR using the forward and reverse infusion primers TATCGAAGGTCGTCATATGATGAAGTTCACTGTGGTGGC and GTTAGCAG CCGGATCC TTACAAACTC ATCTTTTTCTGATGTATCC and Platinum® Pfx DNA polymerase (Invitrogen). After confirmation of the cDNA sequence, the full length GRP78 PCR-fragment was cloned into NdeI and BamHI sites in pET16 (Novagen) using In-fusion® (Clontech), following the manufacturer’s protocol. Endotoxins were removed using a Detoxi-Gel Endotoxin removing column (Thermo Scientific) and purity of recombinant GRP78 was verified by SDS-PAGE and Coomassie Blue staining. For *in vitro* citrullination of recombinant mouse GRP78, 2 U of rabbit PADI2 (Sigma-Aldrich) were added to 1 mg of protein and incubated for 2 h at 50 °C in citrullination buffer (0.1 M Tris-HCl, pH 7.4; 10 mM CaCl$_2$ and 5 mM DTT).
References