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Immune-mediated β-cell destruction in vitro and in vivo—A pivotal role for galectin-3

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Abstract

Pro-apoptotic cytokines are toxic to the pancreatic β-cells and have been associated with the pathogenesis of Type 1 diabetes (T1D). Proteome analysis of IL-1β exposed isolated rat islets identified galectin-3 (gal-3) as the most up-regulated protein. Here analysis of human and rat islets and insulinoma cells confirmed IL-1β regulated gal-3 expression of several gal-3 isoforms and a complex in vivo expression profile during diabetes development in rats. Over-expression of gal-3 protected β-cells against IL-1β toxicity, with a complete blockage of JNK phosphorylation, essential for IL-1-mediated apoptosis. Mutation scanning of regulatory and coding regions of the gal-3 gene (LGALS3) identified six polymorphisms. A haplotype comprising three cSNPs showed significantly increased transmission to unaffected offspring in 257 T1D families and replicated in an independent set of 170 T1D families. In summary, combined proteome–transcriptome–genome and functional analyses identify gal-3 as a candidate gene/protein in T1D susceptibility that may prove valuable in future intervention/prevention strategies.

Keywords: BB-rats; Proteome analysis; Human islets; β-cell; Galectin; Cyto-protection; Cytokines; Diabetes; Transplantation; Autoimmunity; Linkage analysis

Type 1 diabetes mellitus (T1D)* [MIM 222100] is a complex autoimmune disorder associated with selective destruction of the insulin producing β-cells in the pancreatic islets of Langerhans. Pro-apoptotic cytokines, in particular IL-1β alone or in combination with TNFα and IFNγ released from infiltrating monocytes during inflammation of the islets, are exquisitely toxic to the β-cells [1–4] and accumulating evidence supports a role for cytokines released during insulitis in the pathogenesis of T1D. Several attempts have been done to clarify the molecular mechanisms and pathways behind the exquisite β-cell sensitivity to cytokines, including the use of proteome and transcriptome analyses of cytokine exposed islets and β-cells [5,6].

The combined analyses from such studies have yielded substantial new insight into the complex race between protective and deleterious pathways activated in response to

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cytokines [7,8]. Whereas this information may provide information about the pathogenesis of T1D, it may also identify novel targets for future intervention or prevention of the immune-mediated β-cell destruction in T1D in particular and/or in autoimmune diseases in general.

In order to obtain a global assessment of the cytokine-mediated β-cell damaging processes at the protein level, we previously performed proteome analyses of rat islet lysates [9–14]. The most up-regulated protein spot in our first 2D-gel electrophoresis analysis of IL-1β exposed rat islets [9,10] was identified by mass spectrometry to contain gal-3 [13]. Our recent proteome analysis of transplanted islets revealed a complex regulation of native and post-translationally modified gal-3 isoforms during the development of diabetes in diabetes-prone Bio-Breeding (BB-DP) rats [15].

Gal-3, also known as Mac-2, εBP, IgE binding protein, CBP-35, CBP30, L-34, and L-29, belongs to an evolutionary conserved family of β-galactoside-binding lectins with multifunctional properties expressed in a variety of tissues and cell types mainly in the cytoplasm but it may also be found in the nucleus, on the cell surface or in the extracellular environment [16–21]. Accumulating evidence supports a pivotal role of gal-3 in immuno-regulation and gal-3 has been shown to inhibit apoptosis induced by anti-Fas-antibodies, TNFα, and NO. This property may be due to homology to the BH1 domain of the anti-apoptotic Bcl-2 protein and similar translocation by synexin to the perinuclear membrane to prevent cytochrome *c* release [16–18]. Indeed, gal-3 has been implicated in several physiological and pathophysiological processes, including embryogenesis, cell adhesion, T-cell growth, pre-mRNA splicing, apoptosis, neoplastic transformation, inflammation, and immunomodulation [16–22]. Gal-3 has been shown to have both pro-and anti-apoptotic activities [16]. Thus, cytoplasmatic gal-3 expression protects T-cells against apoptosis [23], whereas membrane-bound or extracellular gal-3 induces T-cell apoptosis [24]. Gal-3 has been implicated in a number of autoimmune or inflammatory diseases, e.g., juvenile idiopathic arthritis [25], experimental allergic encephalomyelitis (EAE), and multiple sclerosis [26,27]. In patients with Crohn's disease titers of anti-gal-3 antibodies were high and correlated negatively with disease activity [28].

Hence, gal-3 may be a candidate gene/protein in T1D pathogenesis which we here have evaluated at different levels. First the gal-3 expression profiles in both rat and human pancreatic islets in vitro as well as in vivo during spontaneous development of diabetes were evaluated. The latter was accomplished by proteome analysis on a rat transplantation model where syngeneic islets from prediabetic BB-DP rats were transplanted under the kidney capsule of young BB-DP and diabetes-resistant (BB-DR) rats and the gal-3 expression profile followed over time. Second the functional significance of gal-3 was established following over-expression in rat β-cells and subsequent cytokine exposure. Finally polymorphisms of the gal-3 gene were identified and tested for linkage to T1D in large groups of Danish and Swedish T1D families.

The result demonstrated that gal-3 expression is highly influenced by IL-1β in both rat and human islets during the development of T1D. Over-expression of gal-3 protected β-cells against the cytotoxic effect of IL-1β and genetic analysis demonstrated linkage to T1D of identified polymorphisms in the gal-3 gene (*LGALS3*). Thus, gal-3 represents an interesting candidate gene/protein in T1D and potentially in other immune-mediated diseases.

Materials and methods

Islet isolation and cell culture. Neonatal islets from pancreata of 4- to 5-day-old BB-DP/Wor/Mol-BB and BB-DR/Wor/Mol-WB rats (M & B, Ll. Skensved, Denmark) were isolated [14,29].

Human islets were isolated from cadaver organ donors at the University of Miami, FL [30]. RIN cells (RIN-5AH-T2B) were cultured as previously described [31]. The cytokines added to the islets and RIN cells were recombinant human IL-1β (Novo Nordisk, Bagsværd, Denmark) and recombinant mouse IL-1β (BD Pharmingen, San Diego, CA) alone or in combination with recombinant mouse IFNγ and recombinant human TNFα (Genzyme, Cambridge, MA). The concentration of IL-1β used was in all experiments based on titrations to obtain approximately half-maximal effects in the different assays. Titration analysis comparing the two sources of IL-1β revealed a near identical biological activity in our assays (data not shown).

Transplantation procedure and graft retrieval. Thirty (range: 28–32)-day-old BB-DP and BB-DR rats were purchased (M & B) and housed under SPF-conditions until islet isolation.

Two hundred (range: 187–215) handpicked neonatal islets were transplanted under the kidney capsule of 30-day-old recipient rats under sterile conditions [14,15,29]. Blood glucose (BG) was measured every third day. Grafts ($n = 3$–6 at each time point) were retrieved 7, 12, 23, 37, 48, and 173 days after transplantation or at onset of diabetes (defined as BG > 14 mmol/L on two consecutive days) for 2D-gel analysis. Transplanted BB-DP and BB-DR islets were compared with regard to their protein expression profile over time and between strains.

Graft and islet protein labeling. The grafts were labeled with [^{35}S]methionine (Amersham Biosciences, Freiburg, Germany) immediately after retrieval. Cultured islets were labeled after 24 h of incubation with or without IL-1β [9,14].

2D-gel electrophoresis and mass spectrometry. Protein expression in islets or grafts was analyzed by 2D-gel electrophoresis and identified by MS [14]. The term percentage of the integrated optical density (% IOD) used in the manuscript (see Table 1) defines the level of protein in an individual spot relative to the total level in all the spots detected on a gel. It should be noted that the value is based on incorporation of [^{35}S]methionine into the protein. Note that native human gal-3 contains three methionine residues whereas rat gal-3 contains six.

RNA isolation and semi-quantitative RT-PCR analysis, cloning, and sequencing. Isolation of total RNA, cDNA synthesis, and semi-quantitative RT-PCR were carried out as previously reported [32]. As internal standards, TATA-binding protein (TBP), cyclophilin, and SP-1 were used based on their linear amplification in the same range as for the mRNAs of gal-3 and their unresponsiveness to cytokine treatment. For primers, see supplementary Table.

The full-length coding sequence of gal-3 was amplified from cytokine exposed rat islets by PFU-polymerase supported PCR using primers based on published rat and human gal-3 sequences with an added Kosac consensus sequence to the 5′-primer and an additional stop codon to the 3′-primer (for primers, see Supplementary Table). Amplicons were cloned into the pCRII vector (Invitrogen, Carlsbad, CA), and three clones with origin from different mRNA preparations were sequenced on an ABI PRISM 310 Genetic Analyzer using BigDye Chemistry (Applied Biosystems, Foster City, CA). No differences to previously published gal-3

Table 1
Nomenclature of gal-3 spots on 2D-gels

Species	Spot #	Total spots	% IOD			Previous spot #
			Control	IL-1β	Ratio	
WF-rat	*x*	2200	0.70	17.12	24.45	19 [9],298 [10,13]
BB-rat	*x*	1815	3.951	8.331	2.11	377 [11,14,15]
BB-rat	*y*	1815	0.494	1.941	3.93	381 [11,14,15]
BB-rat	*z*	1815	0.007	0.187	28.15	398 [11,14,15]
BB-rat	*w1*	1815	0.119	0.049	*ns*	Fig. 1
BB-rat	*w2*	1815	0.011	0.076	*ns*	Fig. 1
BB-rat	*w3*	1815	0.317	0.409	*ns*	Fig. 1
Human	24	4200	0.74	2.95	3.66	Fig. 2

The nomenclature of the different gal-3-containing spots identified and discussed in the present manuscript (column 2) with references to nomenclature used in previous publications (last column). The percentage of integrated optical density (% IOD) is given for each spot in the absence or presence of IL-1β and the ratio of up-regulation is provided. The % IOD is calculated based on the total IOD in all detectable spots on the gels (column 3). See 2D-gel section in Materials and methods for details.

sequences were observed. The full-length coding region was sub-cloned into the pcDNA3 expression vector in sense or reverse-orientation under the control of the CMV promoter (Invitrogen) for functional expression studies. The RIN-5AH-T2B cell line was transfected with the constructs using SuperFect (Qiagen, Valencia, CA) and stably transfected clones selected by hygromycin resistance.

Functional assays. Viability was measured using the MTT assay for mitochondrial activity (Promega Madison, WI) as well as a propidium iodide (PI) based assay [33].

Apoptosis was measured in RIN-cells (5×10^4 per well) cultured for 24 h in 96-well plates with or without cytokines using the Death Detection ELISA^{PLUS} (Roche, Basel, Switzerland). The assay detects inter-nucleosomal histone-associated genomic DNA fragments in the cytosolic fraction resulting from apoptotic degradation of genomic DNA. A phosphotransferase assay using GST c-jun (aa 1–79) (Calbiochem, San Diego, CA) as substrate was used to measure the influence of gal-3 on JNK-mediated MAPK activity in cytokine exposed β cells [34].

Family collections. Initial linkage analysis was performed on 408 T1D multiplex families comprising 464 affected sib-pairs [35]. Linkage disequilibrium mapping was carried out in a Danish T1D family collection (a total of 1143 individuals) comprising 154 sib pair families (a total of 725 individuals, 317 affected and 138 unaffected offspring) and 103 simplex families (a total of 418 individuals, 103 affected and 114 unaffected offspring). For replication studies 170 Swedish affected sib-pair families were used. All patients were diagnosed according to WHO criteria.

Genotyping of microsatellites. Microsatellites were analyzed using the biotinylated primer pairs listed in supplementary table. PCR amplifications were performed as follows: PCR mixture comprised 1.0 μM of each primer, 50 μM dNTPs, 0.5–2.0 mM $MgCl_2$, 0.5 U/sample *Taq* DNA polymerase (Promega) with 1× reaction buffer and 40 ng genomic DNA in a total reaction volume of 20 μL. The cycling conditions were 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 52–65 °C for 30 s, and 72 °C for 30 s, and finally 72 °C for 10 min. Gel electrophoresis and scoring of genotypes were performed as previously detailed [36].

Mutation detection. Eight hundred and two base pair of the promoter region, intron 2 (716 bp), the total coding region (752 bp), and 119 bp of the 3′UTR of *LGALS3* have been screened for mutations by single stranded conformation polymorphism (SSCP) and direct sequencing. Primers and conditions for PCR amplification are presented in Supplementary Table. SSCP screening was performed as previously reported [37]. Samples showing mobility shifts after SSCP analysis were sequenced to verify putative mutations.

Genotyping of identified polymorphisms. Analysis of the identified polymorphisms was performed by PCR amplification with the primers listed in the Supplementary Table under standard conditions. The PCR products were digested overnight with restriction enzymes *Mse*I (for the g—A715G polymorphism), *Eco0109I* (g—698-693del(TAAGGA); 689-684del(TGCCCC)), *Stu*I (g—C659T), *Sca*I (Gln201His), *Nco*I (His64Pro), and *Bsa*WI (Pro98Thr) and the samples were analyzed on a 1–2% agarose gel.

Statistical analysis. For evaluation of protein expression levels on 2-D gels Student's *t* test (paired) was applied and $P < 0.01$ was chosen as level of significance [10]. For the analysis of mRNA expression Student's *t* test was applied and $P < 0.05$ (two-tailed) was chosen as level of significance, and for the functional analysis a two-tailed Wilcoxon test with $p < 0.05$ chosen as level of significance was used. For the analysis of gal-3 expression in the transplants, a two-way ANOVA with $p < 0.05$ chosen as level of significance was applied. Trend analysis was performed by simple regression modeling. All data are given as means with standard deviations (SD).

Transmission of microsatellite marker alleles and single nucleotide polymorphism (SNP) alleles was assessed from heterozygous parents to both affected and unaffected offspring using the transmission disequilibrium test (TDT) [38,39]. Linkage was analyzed by the Genehunter software [40].

Ethics. All human studies were approved by the relevant Regional and National Ethics Committees and performed according to the principles expressed in the Helsinki Declaration. All animal experiments were carried out according to national and international law and ethical standards and approved by the Danish Council for Animal Welfare under the Ministry of Justice.

Results

Effect of cytokines on gal-3 expression in islets of Langerhans and β-cells

Our previous proteome analysis of Wistar–Furth (WF) rat islets, detecting approximately 1,500 different protein-spots [9], revealed a gal-3 containing spot to be the most up-regulated (24-fold) following IL-1β exposure based on % IOD [10,13] (Table 1). Subsequent analyses of IL-1β exposed islets from the BioBreeding diabetes prone (BB-DP) rat strain revealed a significant smaller IL-1β induced upregulation (2.1-fold; $p < 0.01$) of gal-3 expression (spot *x* in Fig. 1 and Table 1) [14], however the basal % IOD-level of gal-3 in this spot was also 5.6-fold higher than in WF islets. Furthermore, this study revealed two additional IL-1β up-regulated gal-3-containing spots (spots *y* and *z*, Fig. 1 and Table 1). We here identify three additional gal-3 containing spots (spots *w1–3*, Fig. 1) one of which (*w*3) is also significantly changed during diabetes

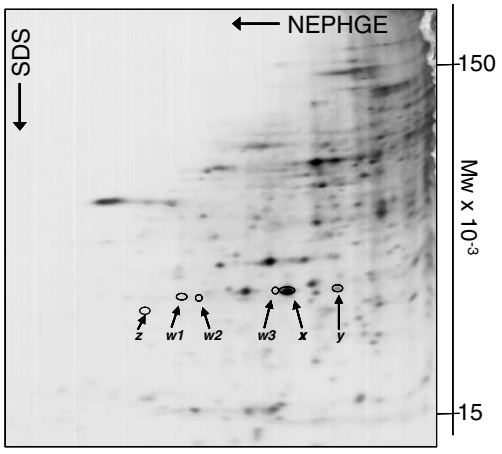

Fig. 1. 2D gel of basic proteins (pH 6.5–10.5) of BB-rat islet proteins. Spots marked *x*, *y*, and *z* (corresponding to the spot numbered 377, 381, and 398 in [14]) contains gal-3 isoforms previously demonstrated to be IL-1β up-regulated. Spot *w1–3* contains additional gal-3 isoforms, not influenced by IL-1β. Spot *x* is identical to the gal-3 spot originally demonstrated to be highly up-regulated by IL-1β in WF rat islets (previous nomenclature: spot 19 in [10] and spot 298 in [13]).

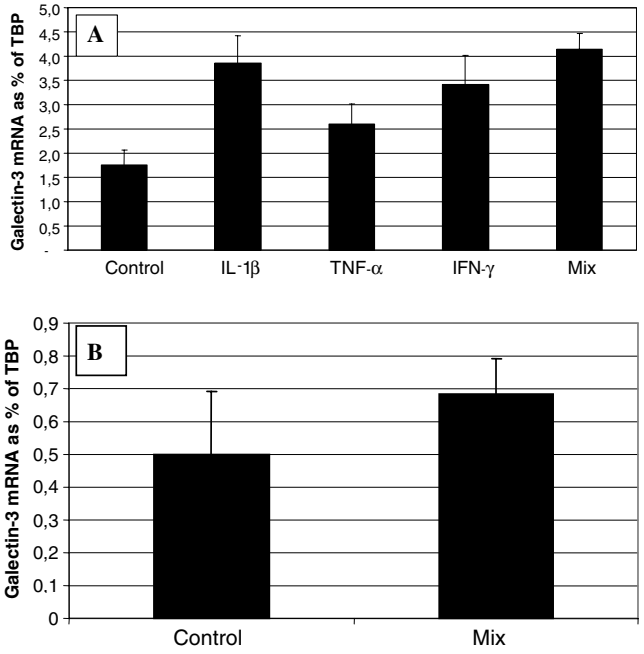

Fig. 3. Gal-3 mRNA expression in cytokine exposed RIN cells and human islets. (A) The quantitative data on steady-state gal-3 mRNA expression obtained from semi-quantitative RT-PCR analysis of RIN cells ($n = 6$) cultured in the absence or presence of 150 pg/ml IL-1β or 200 U/ml of either TNFα or IFNγ alone or mixed (mix). Data are relative to the housekeeping transcript TATA-binding protein. A significant ($p < 0.01$) increase in gal-3 mRNA expression was observed following all cytokine exposures. (B) Human islets ($n = 3$, analyzed in separate duplicate experiments) cultured for 24 h with or without 1500 pg/ml IL-1β in the presence of 200 U/ml of both TNFα and IFNγ (mix) are shown. Also here a significant ($p < 0.014$) up-regulation of gal-3 expression was detected. Data are means ± SD.

development of the BB-DP rat (see later). Thus, gal-3 exists in different most likely post-translationally modified forms in IL-1β exposed rat islets and during the development of T1D.

In search for gal-3 on 2D-gels prepared from human islets, we also identified gal-3 as a cytokine-up-regulated protein, although hitherto only in one spot. This spot (24 on Fig. 2) was up-regulated 3.7-fold ($p < 0.005$) and 4.0-fold ($p < 0.005$) in response to 1500 pg/ml IL-1β alone or in combination with IFNγ and TNFα, respectively (Fig. 2).

Semi-quantitative mRNA analysis using rat insulinoma (RIN) cells revealed a significant increase in gal-3 steady-state mRNA level following 24 h exposure to any of the cytokines as compared to the control culture in the absence of cytokines ($p < 0.01$, Fig. 3A). Similar data were obtained using SP-1 as the internal reference gene (data not shown). Analysis of gal-3 mRNA expression in isolated human islets, in which a mix of cytokines has been described as necessary for toxicity [41], confirmed the ability of cytokines to increase mRNA expression ($p = 0.014$; Fig. 3B). Similar data were obtained using cyclophilin as internal reference (data not shown).

Gal-3 expression profile during T1D development in vivo

To analyze the gal-3 expression profile in vivo in islets during T1D development, syngeneic rat islets were transplanted under the kidney capsule to young prediabetic diabetes-prone BB-DP or diabetes resistance BB-DR rats and the islet grafts excised for 2D-gel analyses at

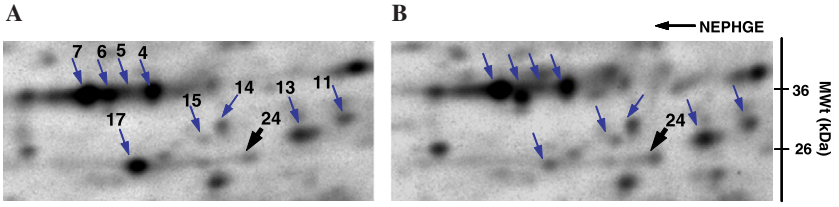

Fig. 2. 2D gel of basic proteins (pH 6.5–10.5) of human islets. The area on the gel covering the gal-3 expression profile is shown. (A) Control islets. (B) Islets exposed to 1500 pg/ml IL-1β for 24 h. Spot #24 contains gal-3 (IOD: 0.74 ± 0.52), which was significantly ($p < 0.005$) up-regulated in response to IL-1β alone (IOD: 2.71 ± 0.27; 3.7-fold) or in combination with IFNγ and TNFα (IOD: 2.95 ± 0.21; 4.0-fold). The identified proteins in some of the surrounding spots are; #4, 5, 6, and 7: glyceraldehyde-3-phosphate dehydrogenase (data base Accession No.; gi31645); # 11: VDAC2 protein (gi15277577); #13: Porin (gi238427); #14: carbonyl reductase 1 (gi4503599); #15: L-lactate dehydrogenase A chain (gi17369829); # 17: chain A of L3 hydroxyacyl-Coa dehydrogenase complexed with Acetoacetyl-Coa and NAD^+ (gi10120604). Data are means ± SD of three individual experiments.

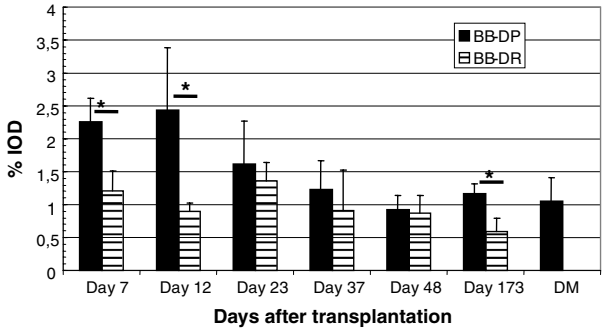

Fig. 4. Expression of gal-3 in syngeneic transplanted islets under the kidney capsule of BB-DP or -DR rats. Expression of the native gal-3 containing spot, spot *x*, was significantly different between the syngeneic transplants of BB-DP compared to BB-DR islets: between strains: $p < 0.0001$, time: $p < 0.0001$ and interaction $p < 0.038$; two-way ANOVA. Significant differences between BB-DP and BB-DR animals are indicated by asterisks. $*p < 0.05$. Data are means ± SD of $n = 4$–6 independent experiments. Day 173 includes the few BB-DP rats that escaped diabetes development. DM represents the BB-DP animals that developed diabetes (mean day 48).

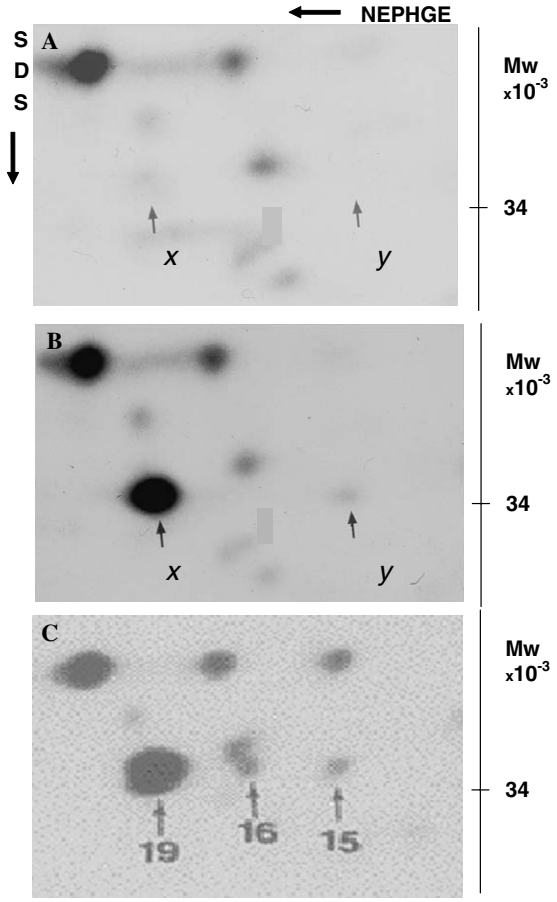

Fig. 5. Expression of gal-3 in RIN cells and WF rat islets. Section of 2D-gel showing gal-3 expression in RIN cells before (A) and after (B) stable transfection with gal-3 under a CMV-promoter. Arrows indicate gal-3 containing spots. By comparison with Fig. 1 it is evident that the native recombinant gal-3 (*x*) is identical to the most up regulated gal-3 containing spot (spot *x*) in BB-rat islets, corresponding to the gal-3 containing spot (spot 19) originally demonstrated in WF rat islets to be up regulated 24-fold in response to IL-1β (C) [9,10,13].

different time points. The gal-3 spot of highest expression, corresponding to spot *x* in Fig. 1, significantly changed expression level over time in the transplanted BB-DP rat islets but not in the BB-DR-islet transplants (Fig. 4). A significantly higher expression of spot *x* was observed in the transplanted BB-DP rat islets at the early time-points after transplantation. That the decreased expression of spot *x* over time after transplantation was not only the result of ongoing β-cell destruction was demonstrated by the simultaneous up-regulated expression levels over time of two of the less abundant post-translationally modified gal-3 spots, spot *y* ($p < 0.05$) and spot *w3* ($p < 0.001$). The 3 remaining gal-3 containing spots, *z*, *w*1, and *w*2, were not significantly changed during the diabetes development.

Functional studies

The ability of intracellular expressed gal-3 to influence cytokine induced β-cell toxicity was examined in two RIN-cell clones stably transfected with gal-3 expressed under a CMV promoter. Gal-3 expression under the constitutive CMV promoter resulted in significant up-regulation of a gal-3 containing spot (Figs. 5A and B) at a location similar to spot *x*, the most IL-1β upregulated spot in BB- [14]) and WF-rat islets (Fig. 5C spot 19; [9,10,13]). The presence and up-regulation of gal-3 in both clones used for the functional characterization was confirmed by Western blot analysis (data not shown). Note that the minor gal-3 containing spot (spot *y*) is only marginally affected in response to the over-expression of the non-modified form of gal-3 in the absence of cytokines.

Analysis of viability was performed using two independent assays, the MTT assay, measuring mitochondrial activity (Fig. 6A) and a PI assay measuring DNA binding (Fig. 6B). Combining data from two independent clones with stable gal-3 over-expression revealed that gal-3 fully protected the two clones against the toxic effects of 150 pg/ml IL-1β alone and partially against the combination of the three cytokines as compared to both the parental RIN cell line or two clones with gal-3 in reverse orientation. At higher IL-1β concentrations only partial protection of the gal-3 transfected cells was observed (data not shown). This result was not due to an increased proliferation rate, since the level of constitutively expressed gal-3 did not influence cell proliferation as compared to the control cells. Measurement of apoptosis based on the presence of histone-associated fragments demonstrated that gal-3 over-expression inhibited apoptosis following 24 h cytokine exposure by ~50% (Fig. 6C, $p < 0.04$). In addition, gal-3 over-expression reduced the basal apoptotic rate by ~30% ($p = 0.05$). The same trend was observed for exposure to IL-1β alone, although this did not reach statistical significance ($p = 0.06$).

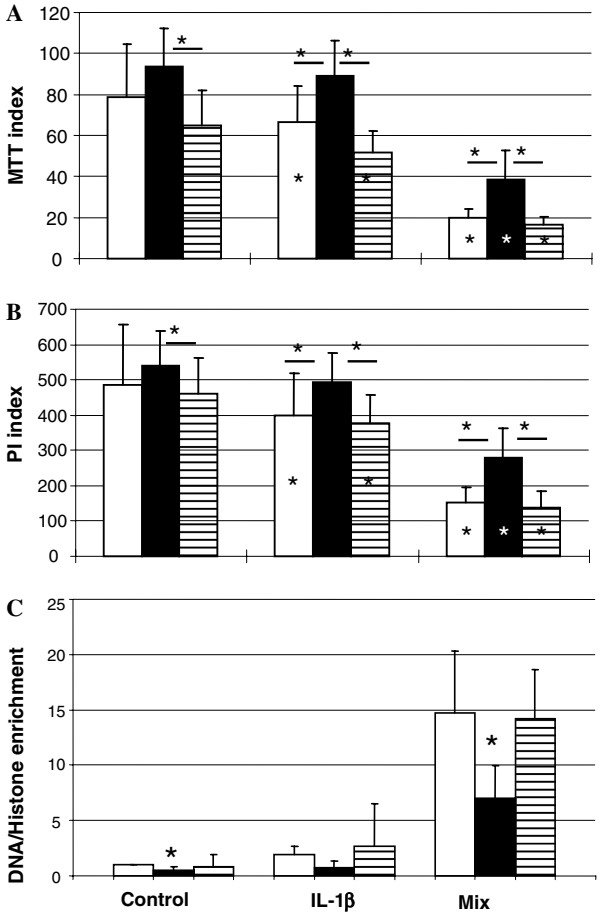

Fig. 6. Effect of gal-3 on cytokine mediated β-cell toxicity. Viability, based on mitochondrial MTT activity (A) or the PI assay (B), was determined in non-transfected RIN cells (white bars), stably transfected with gal-3 in sense- (black bars) or reverse- (striped bars) orientations, cultured in the absence or presence of cytokines (same concentrations as for Fig. 2) for 3 days. Significance relative to control is indicated by an asterisk within the bars, and differences among the three conditions indicated above the bars: $*p < 0.02$, Wilcoxon match-pairs. Pooled data from two separate stable clones of sense as well as reverse gal-3 expression are making basis for the analysis. Data are means ± SD of $n = 7$–9 independent experiments. (C) Measurement of induction of apoptosis was performed following exposure to IL-1β or cytokine mix (1 ng/ml IFNγ and 80 pg/ml IL-1β) for 24 h. Significance relative to non-transfected clone is indicated by an asterisk above the bars: $*p < 0.05$. Data are means ± SD of $n = 4$ independent experiments.

Phosphotransferase assay

Gal-3 expression has been shown to be regulated through JNK and NFκB activation in glioblastoma cells [42]. Since IL-1β activates mitogen-activated protein kinases (MAPK) and NFκB signal transduction pathways in which activation of JNK is essential for promoting β-cell apoptosis [43], we addressed the effect of gal-3 on this pathway in β-cells. Using c-jun as substrate for JNK, we demonstrated that gal-3 completely inhibited phosphorylation induced by 30 min exposure to two different IL-1β concentrations titrated to provide half-maximum and maximal activation of MAPK, respectively (Fig. 7).

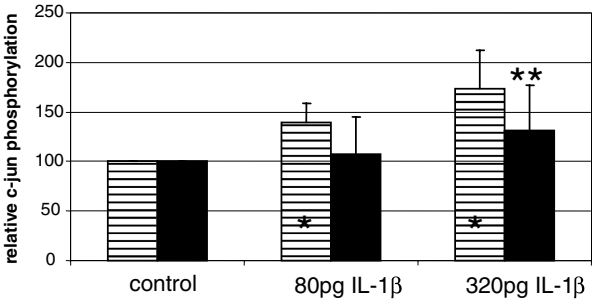

Fig. 7. Effect of gal-3 on IL-1β mediated MAPK activation. JNK mediated in vitro phosphorylation of c-Jun was analyzed following 30 min exposure to 80 and 320 pg/mL IL-1β in RIN cells with stable expression of gal-3 in sense- (black bars) or reverse- (striped bars) orientation. Significant changes relative to control conditions are indicated by asterisks within the bars, and differences between the two conditions indicated above the bars. $*p < 0.05$ and $**p < 0.01$. Data are means ± SD of $n = 4$ independent experiments.

Genetic analysis

The gal-3 gene (*LGALS3*) maps to human chromosome 14 to a region *(D14S70–D14S276)* where some evidence for linkage to T1D (MLS = 2.0) was observed in a UK whole genome screen [44]. Linkage was observed also in the genome scan of Scandinavian T1D families (*D14S276*, lod score = 2.69, $p = 0.006$) in the group with high-risk HLA alleles (DR3/4), but with a lod score less than 1.0 in the unconditional analysis [35]. We therefore addressed the possibility that polymorphisms in *LGALS3* might be responsible for this genetic linkage. By adding five new microsatellite markers around *D14S276* additional support for linkage was obtained by linkage disequilibrium mapping in 257 Danish T1D families. Of the new markers, *D14S980* showed significant association, $p = 0.04$, in the unconditioned data set supporting *LGALS3* as a candidate for the observed linkage.

The *LGALS3* was scanned for mutations by SSCP and heteroduplex analysis and by direct sequencing of the promoter and coding regions. Six polymorphisms were identified, three in the promoter region and three missense polymorphisms (Table 2). The promoter polymorphisms included a—715A>G, a—(698-693del(TAAGGA); 689-684del(TGCCCC)), and a—659C>T polymorphisms. The identified missense mutations were P64H, P98T, and Q201H. None of the promoter polymorphisms affected theoretical transcription factor binding sites (MatInspector version 2.2 [45]). Alignment of the human (P17931), rat (P08699), and mouse (P16110) gal-3 protein sequences showed 83% and 85% identity, respectively. In all three species, proline at position 64 and glutamine at position 201 were conserved, whereas proline at position 98 was conserved between human and rat, but substituted with serine in the mouse sequence, indicating that the three identified polymorphisms are located in well-conserved regions of the protein.

The six polymorphisms were genotyped in a Danish T1D family collection (Table 2). No significant T1D

Table 2
Transmission of alleles to T1D affected and unaffected offspring for the identified polymorphisms

		T1DM-offspring			Unaffected offspring		
		T (mc)	T (lc)	χ^2	T (mc)	T (lc)	χ^2
Promoter							
*Mse*I		154	140	0.67	105	98	0.24
	DK						
Eco0109I	DK	160	157	0.03	100	114	0.92
*Stu*I	DK	106	110	0.07	56	66 (54)	0.82
cDNA							
*Nco*I	DK	89	87	0.02	58	64	0.30
	S	94 (59)	66	4.90*	39 (68)	18	7.74**
	Total	183 (54)	153	2.68	97	82 (54)	1.26
BsaWI	DK	119	133	0.78	64	84 (57)	2.70
	S	72	84 (54)	0.92	18	39 (68)	7.74**
	Total	191	217	1.66	82	123 (60)	8.20**
*Sca*I	DK	50 (58)	37	2.68	22	25	0.19
	S	37	36	0.01	18 (69)	8	3.85*
	Total	87 (54)	73	1.23	40 (55)	33	0.44

T (mc), transmitted most common alleles; T (lc), transmitted least common alleles. Numbers in brackets are transmission frequencies of the allele given in percentage. Only frequencies $\geqslant 54\%$ are listed.
* $p < 0.05$.
** $p < 0.005$.

associations were observed, but increased transmission (58%) of the most common allele (allele 1) to affected offspring was observed for the *Sca*I polymorphism. Evaluating haplotypes, the 1-2-1 haplotype (i.e., allele 1 (*Nco*I −), allele 2 (*Bsa*WI +), and allele 1 (*Sca*I +)) showed significantly increased transmission to unaffected offspring, $p = 0.0015$.

A collection of Swedish T1D families ($n = 170$) was subsequently tested for the three cDNA polymorphisms. Significant associations were observed for the *Nco*I SNP in this population (Table 2). In the combined Danish and Swedish data set also a significant association of the 1-2-1 haplotype was observed ($p = 0.0039$).

Stratification by HLA high-risk (DR3/4 heterozygosity) and non-high risk (non-DR3/4 heterozygosity) genotypes did not change these observations (data not shown).

Discussion

Cytokines have been proposed as effector molecules in the destruction of pancreatic β-cells and to be important in the initiation of autoimmune diabetes [1,46]. By proteome-based characterization of the mechanisms involved in β-cell destruction [9–14] we identified gal-3 as the most up-regulated protein in WF-rat islets following cytokine exposure [13]. Subsequent analysis of islets transplanted to BB-DP rats further demonstrated a complex regulation of gal-3 during T1D development. Here, using a multi-disciplinary approach combining proteomics, transcriptomics with genetic and functional studies (reviewed in [5,6]), we present evidence strongly suggesting that gal-3 plays a role in both β-cell resistance to cytokines and genetic pre-disposition to T1D. Analysis of gal-3 expression in isolated human islets, previously described to require a combination of cytokines for to toxicity to occur [41], supported cytokine regulated gal-3 protein expression. In addition, mRNA expression analyses using cytokine exposed RIN cells demonstrated gal-3 to be regulated at the transcriptional level in this β-cell line. In addition, further up-regulation of recombinant gal-3 in this cell-line following stable transfection increased the resistance to cytokine induced toxicity.

In line with our findings, gal-3 (also known as IgE-binding protein) was identified as one of the most up-regulated transcripts (~10-fold) in cytokine exposed WF rat islets using high-density oligonucleotide arrays [47].

Over-expression of gal-3 in RIN β-cells demonstrated a protective role of gal-3 against the toxic effects of cytokines as demonstrated by several assays, including the measurement of reduced induction of apoptosis. It is previously described that phosphorylation of gal-3 significantly alters the interaction of gal-3 with its ligands and is required for its anti-apoptotic function [16–18,22]. Indeed, the ability to detect post-translational modifications often critical for protein activation (and hence potentially of pathogenetic relevance) is one of the hallmarks of proteome analysis. We identified gal-3 in six different protein spots in cytokine exposed and transplanted islets, five of which most likely represent post-translational modifications of the native protein. Based on molecular weight and isoelectric focusing positions, at least two of the modifications are probably phosphorylations. Nevertheless, here we have shown by forced over-expression of gal-3 in β-cells that increasing the intercellular pool of native gal-3 at the time of cytokine challenge is sufficient to increase the defense capacity of the β-cell. Future experiments should elucidate the nature, importance, and mechanism of the putative post-translational modifications.

In addition to gal-3, several other β-cell proteins are dependent on phosphorylation for their activation. Different MAPK phosphorylates and activates a range of transcription factors of importance for IL-1β-mediated signaling, including c-jun, activating transcription factor 2 (ATF-2), regulatory factor Elk-1, and activating transcription factor 1 (AP1) [48]. Three major MAPK are activated in β-cells in response to IL-1β, namely the JNK, p38, and ERK. Whereas JNK and p38 promote apoptosis, ERK activity inhibits apoptosis. An interesting target regulated by JNK-mediated phosphorylation is the Bcl-2 protein. Whereas declined Bcl-2 activity following phosphorylation has been demonstrated [49], phosphorylation at serine-6 seems to be needed for its anti-apoptotic activity in other studies [16–18,22]. The present data showed that gal-3 over-expression reduced IL-1β induced c-jun phosphorylation and therefore in part could exert its protective role through a negative feedback mechanism on JNK or NFκB signaling. In stress-activated glioblastoma cells with inhibited JNK and NFκB activation, decreased gal-3 expression has indeed been detected [42]. Furthermore, macrophages and mast cells from gal-3-deficient mice are more prone to apoptosis than cells from

wild-type animals [16–18,22]. It has previously been speculated that the anti-apoptotic role of gal-3 may be mediated through the NWGR amino acid sequence highly conserved within the BH1domain of the bcl-2 gene family [50] and indeed phosphorylation of gal-3 has been shown to be required for its anti-apoptotic function [51].

In addition to the protective role against cytokines demonstrated in our in vitro analyses, our transplantation model also supported a role of gal-3 in the development of T1D [15]. Further analysis using this model allowed us to demonstrate a significantly altered gal-3 expression profile during T1D development, suggesting a complex regulation of gal-3 expression in the islets during diabetes development associated with inflammation and cytokine release (insulitis) eventually resulting in diabetes.

It should, however, be kept in mind that gal-3 has also been identified in dendritic cell-derived exosomes, which have strong immuno-stimulatory properties [52]. In addition, extracellular gal-3 has been shown to be toxic to T-cells [24]. Thus, it cannot be excluded that increased gal-3 expression in vivo may both protect against or augment immune-mediated β-cell destruction, depending perhaps, on its intra- or extracellular expression profile. Indeed, gal-3 expression has been associated with different function of potential importance for development and complications associated with diabetes. In line with this gal-3 knock-out mice revealed an increased susceptibility to diabetes- and AGE-induced glomerulopathy [53,54]. Recently gal-3 ablation studies demonstrated a protective role of gal-3 expression against retinal angiogenesis in diabetes [55]. Thus, whereas the present study focuses on the protective effect of intracellular gal-3 expression, future studies should address the potential immuno-stimulatory and regulatory effect of membrane bound or secreted gal-3 in the pathogenesis of T1D as well as the influence of the here described post-translational modifications and polymorphisms within the gal-3 gene hereon. In line with this, our preliminary 2D-gel analyses of secreted BB-DP rat islet proteins suggest gal-3 to be released in a glucose-dependent manner (TS et al., unpublished data). Furthermore, array analysis of β-cells has revealed up-regulated expression of several chemokines and adhesion molecules in response to cytokines [8,56].

Evidence for linkage of chromosome 14q21, including the gal-3 gene, has been observed in whole genome scans [35,44]. The present analyses confirmed linkage and association to T1D of the chromosome 14q21 region in 257 Danish T1D families, supporting *LGALS3* as candidate for the observed linkage. We therefore screened the promoter region [57], exons 1–6, and intron 2 for mutations. Intron 2 was included as transcription can be initiated at multiple sites located in the second intron [58]. Six polymorphisms likely to have functional significance were identified—all with minor allele frequencies larger than 5%. No significant T1D association was found in Danish T1D families. In an independent replication dataset comprising 170 Swedish T1D families, transmission distortion was found for the *Nco*I SNP. However, in the combined dataset, the 1-2-1 haplotype, which included all three identified missense polymorphisms, showed significant distorted transmission to unaffected offspring ($p < 0.005$). The implication of this observation is not yet clear and future functional studies should address the significance of the identified polymorphisms.

In summary, the combined data strongly point to gal-3 as an important player in the intra-islet milieu and β-cell response to cytokines associated with T1D. Gal-3 expression may in part mediate its intrinsic anti-apoptotic effect on IL-1 signaling through inhibition of JNK phosphorylation and activation of the transcription factor c-jun and associated mitochondrial cytochrome *c* release in the β-cell. This is in line with data from a human breast epithelial cell line, where galectin-3 translocates to the perinuclear mitochondrial membranes and inhibits cytochrome *c* release following a variety of apoptotic stimuli [59]. Together with other activated transcription factors, c-jun forms the activating transcription factor 1 (AP-1) complex, shown to activate gal-3 transcription [60]. In β-cells IL-1β induced gal-3 expression may thus represent a negative regulatory feed-back loop on IL-1 signaling.

In conclusion, the present study demonstrates the value of combining proteome, transcriptome, and genome analyses to identify genes/proteins of relevance for the disease process leading to T1D [5]. Here we have identified gal-3 as a naturally but insufficiently up-regulated defense protein in β-cells exposed to cytokines and immunological mediators associated with T1D pathogenesis. Future studies may clarify the potential role of gal-3 in T1D and its value as a target for intervention/prevention in the disease. The approach as well as the observations made might also be useful in studies of other multi-factorial diseases with defined cellular targets.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.03.105.

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