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Studies on autoimmunity for initiation of beta-cell destruction X. Delayed expression of a membrane-bound islet cell-specific 38 kDa autoantigen that precedes insulitis and diabetes in the diabetes-prone BB rat

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Summary The diabetic syndrome in the DP-BB rat results from progressive beta-cell destruction by autoimmune responses. However, the initial events causing the autoimmune destruction of beta cells remain largely unknown. Our recent experimental results suggest that the delayed expression of a beta-cell-specific autoantigen may result in the initiation of beta-cell-specific autoimmunity. The present investigation was initiated to identify such an autoantigen. Islets were isolated from DP-BB rats of several different ages, and protein extracts from the membrane fraction of the islet preparations were immunoprecipitated with sera from diabetic DP-BB rats. We have found that a membrane-bound islet cell-specific 38 kDa autoantigen is not expressed early in the life of DP-BB rats, but is delayed-expressed by approximately 30 days of age, the time at which immunological effectors begin to recognize beta cells. In contrast, a 64 kDa islet cell protein is expressed from birth in DP-BB rats. On the basis of these observations, we suggest that delayed expression of a gene encoding for the membrane-bound islet cell-specific 38 kDa autoantigen may result in a breakdown of self-tolerance, leading to beta-cell-specific autoimmune IDDM in the BB rat. [Diabetologia (1994) 37: 460–465]

Key words Islet cell-specific 38 kDa autoantigen, immunological effectors, BB rat, autoimmune IDDM.

Cumulative evidence suggests that IDDM results from progressive autoimmune destruction of insulin-producing pancreatic beta cells [1–4]. Animal models for IDDM, such as the DP-BB rat and the non-obese diabetic mouse, support the autoimmune hypothesis. Although it is well known that both humoral and cell-mediated immune responses are involved in the destruction of beta cells, the initial cause of autoimmune destruction of beta cells remains unknown. Our recent experimental results [5] showed that islet grafts from neonatal DP-BB rats remained intact and insulitis-free when transplanted into the renal subcapsular space of acutely diabetic DP-BB rats. In contrast, islet grafts from adult DP-BB rats, which had received silica to prevent insulitis, developed severe insulitis and were rapidly destroyed after transplantation [5]. These results suggest that the delayed expression of a beta-cell-specific autoantigen may result in the initiation of beta-cell-specific autoimmunity. We therefore attempted to identify such an autoantigen, and we now report the existence of a membrane-bound islet cell-specific 38 kDa autoantigen, which is not expressed early in the life of DP-BB rats, but which is expressed at approximately 30 days of age, the time at which immunological effectors begin to recognize beta cells.

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Abbreviations: IDDM, Insulin-dependent diabetes mellitus; BB, BioBreeding; WF, Wistar Furth; DP-BB, diabetes-prone BioBreeding; DR-BB, diabetes-resistant BioBreeding; GAD, glutamic acid decarboxylase; APC, antigen-presenting cell.

Materials and methods

Animals

Our DP-BB and DR-BB rat colonies were produced from breeding stock purchased from the University of Massachusetts (Worcester, Mass., USA). These animals were maintained on regular rat chow and tap water ad libitum, at the animal care facilities of the University of Calgary. The overall incidence of diabetes in our DP-BB colony is approximately 80%, and the mean age at detection of diabetes is 95 days (range: 70–110 days). WF rats were purchased from the Harlan-Sprague-Dawley Laboratory (Indianapolis, Ind., USA). For the preparation of islets free from lymphocytic infiltration, DP-BB rats were injected with silica (100 mg/kg body weight, i.p.) (Steinkohle-Bergbau-Verein, Essen, Germany) starting at 30 days of age. The rats continued to receive silica once per week until they were killed at 60 or 80–100 days of age [6]. We had previously confirmed that this treatment completely prevents autoimmune beta-cell destruction in DP-BB rats [6, 7].

Preparation of islets

Islets from neonatal (3–5-day-old) or 10-day-old DP-BB, DR-BB, and WF rats were isolated by a modification of a method previously described [5, 8]. Briefly, pancreata from these rats were finely minced in Hank's balanced salt solution and incubated for 5–7 days in RPMI 1640 medium supplemented with 5% fetal bovine serum, 20 mmol/l HEPES, 2 mmol/l glutamine, and 50 μg/ml gentamicin. The islets were handpicked twice to obtain pure islets from surrounding fibroblasts and exocrine acinar cells. Islets from young and adult (20-, 30-, 60-, or 80–100-day-old) DP-BB, DR-BB, and WF rats were isolated by a modification of the collagenase Ficoll gradient method described elsewhere [5, 9].

Identification of 38 kDa autoantigen

Subcellular fractionation of islets was performed following a procedure described elsewhere [10] with modification. Islets ($n = 2000$) were isolated from neonatal (3–5-day-old), or 80–100-day-old DP-BB rats as described above. The islets were labelled with ^{35}S-methionine (2.5 mCi/2000 islets) in 5 ml of RPMI 1640 labelling medium without methionine, supplemented with 5% dialysed fetal bovine serum, 20 mmol/l HEPES, 2 mmol/l glutamine, and 50 μg/ml gentamicin for 10 h at 37°C. The islets were washed with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline, containing 2 mmol/l cold methionine, kept 30 min on ice, and homogenized in 400 μl of homogenization buffer [20 mmol/l Tris.HCl (pH 7.4), 5 mmol/l EDTA, 1000 kallikrein inhibitory units/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 2 μg/ml trypsin-chymotrypsin inhibitor] using a Dounce homogenizer (200 strokes, tight pestle). Unbroken cells were removed by centrifugation for 5 min at 100 × *g*. The supernatant was centrifuged at 36,000 × *g* for 30 min (30,000 rev/min, TLA 100.1 rotor, TL-100 Beckman Ultracentrifuge; Beckman, Fullerton, Calif., USA). The resultant supernatant was used as the cytosolic component of the islet cells. For preparation of the membrane fraction, the pellet was dissolved in 200 μl of homogenization buffer containing 1% Nonidet P-40, solubilized for 2 h at 4°C, and ultracentrifuged at 100,000 × *g* for 30 min (55,000 rev/min, TLA 100.1 rotor). The supernatant was used as the detergent-solubilized membrane fraction. After preclearing with pooled WF rat sera ($n = 7$; 60–100-day-old; anti-38 kDa antibody negative), the cytosolic fraction and the islet cell membrane fraction were incubated with pooled DP-BB rat sera ($n = 12$; 70–110-day-old) (10: 1) containing anti-38 kDa islet cell antibody, precipitated with protein A-sepharose, and separated by SDS-PAGE as previously described [11]. After SDS-PAGE, the gel was soaked in 1 mol/l sodium salicylate for 30 min, dried, and fluorographed.

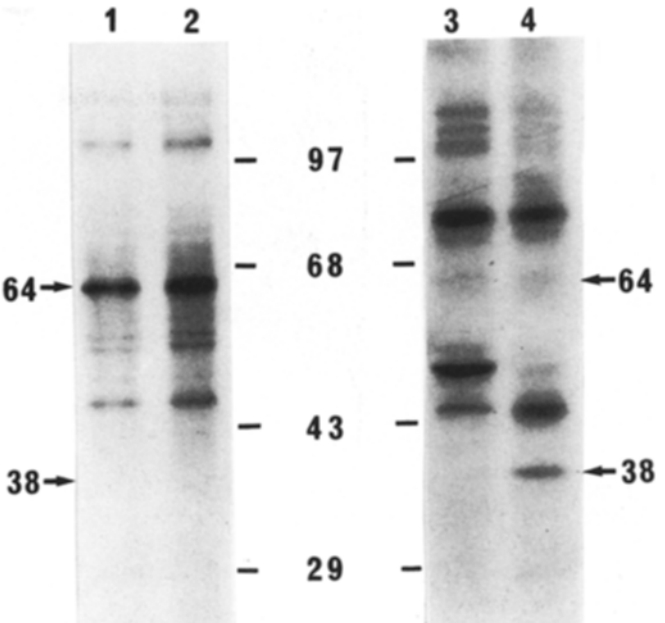

Fig. 1. Fluorograph of immunoprecipitation of neonatal and adult islets from DP-BB rats. A protein of molecular size 64 kDa was detected primarily in the cytosolic fraction of neonatal (lane 1) and adult (lane 2) DP-BB rat islets. A faint band of 64 kDa was also detected in the membrane fraction of both neonatal and adult islets (lanes 3 and 4). In contrast, a 38 kDa protein was not detected in the cytosolic fraction from neonatal (lane 1) or adult (lane 2) DP-BB rat islets. A 38 kDa band was not detected in the membrane fraction from neonatal DP-BB rat islets (lane 3), but a 38 kDa band was detected in the membrane fraction of adult DP-BB rat islets (lane 4). Relative molecular mass markers are indicated in thousands (kDa)

Examination of the tissue specificity of 38 kDa autoantigen expression

Islets ($n = 2000$), rat splenocytes, or kidney primary cells from neonatal, 10-, 20-, 30- or 60-day-old WF, DR-BB and DP-BB rats were labelled with ^{35}S-methionine in RPMI 1640 medium, as described above. The membrane fractions of the cell lysates were prepared and precleared with pooled WF rat sera as described above, followed by immunoprecipitation with pooled anti-38 kDa antibody-positive DP-BB rat sera as previously described [11]. The immunoprecipitated complexes were analysed by SDS-PAGE and fluorographed.

Transplantation of pancreatic islets

Three groups, each comprised of seven diabetic DP-BB rats, received islets from 10-day-old, 30-day-old, or 60-day-old DP-BB rats. Islets from donor rats in these groups were isolated as de-

Table 1. Tissue specific expression of 38 kDa autoantigen at different ages of BB and WF rats[a]

Rat strain	Tissues	Age (days) Neonate	10	20	30	60
DP-BB	Islet	–[b]	–	–	+[b]	+
	Kidney	–	–	–	–	–
	Spleen	–	–	–	–	–
WF	Islet	+	+	+	+	+
	Kidney	–	–	–	–	–
	Spleen	–	–	–	–	–
	Lymphocytes	–	–	–	–	–
DR-BB	Islet	+	+	+	+	+

[a] Each tissue was biosynthetically labelled with ^{35}S-methionine and lysed as described in Materials and methods. The metabolically labelled cell lysates were then precleared with pooled control WF rat sera, incubated with DP-BB rat sera containing anti-38 kDa islet cell autoantibody, precipitated with protein A-sepharose, and separated by SDS-PAGE. After SDS-PAGE, the gel was soaked in 1 mol/l sodium salicylate, dried, and fluorographed.

[b] "–" represents absence of 38 kDa autoantigen and " + " represents presence of 38 kDa autoantigen

scribed above and transferred to a pipette tip. With the acutely diabetic recipient DP-BB rat under light ether anaesthesia, 500 islets were placed under the kidney capsule by injecting the islets settled in the pipette tip through a hole made in the capsule. The recipients were monitored and treated with insulin as necessary. Three weeks following transplantation, the islet grafts were removed and examined histologically, following fixation in Bouin's solution and staining with haematoxylin and eosin to demonstrate general morphology and the presence of infiltrating mononuclear cells. A graded scheme was used to assess the degree of mononuclear cell infiltration and beta-cell survival.

Results

Our previous results showed that beta cells from neonatal DP-BB rats are different from beta cells from adult DP-BB rats with regard to their recognition by diabetogenic effectors [5]. In order to identify any differences in islet cell proteins between neonatal (3–5-day-old) and adult (80-day-old) DP-BB rats, the protein extract from the membrane or cytosolic components of neonatal or adult DP-BB rat islet preparations was immunoprecipitated with diabetic DP-BB rat sera containing anti-38 kDa islet cell antibody. When the cytosolic protein extract from either age was immunoprecipitated, a 38 kDa band was not detected (Fig. 1, lanes 1 and 2). In contrast, a 38 kDa protein was consistently immunoprecipitated from the membrane fraction of adult islets (Fig. 1, lane 4), but not neonatal preparations (Fig. 1, lane 3). A protein band of 64 kDa was detected primarily in the cytosolic fractions from all rats (Fig. 1, lanes 1 and 2), and to a lesser extent, in the membrane fraction of both neonatal and adult DP-BB islets (Fig. 1, lanes 3 and 4). These results show that the 38 kDa protein is localized in the plasma membrane,

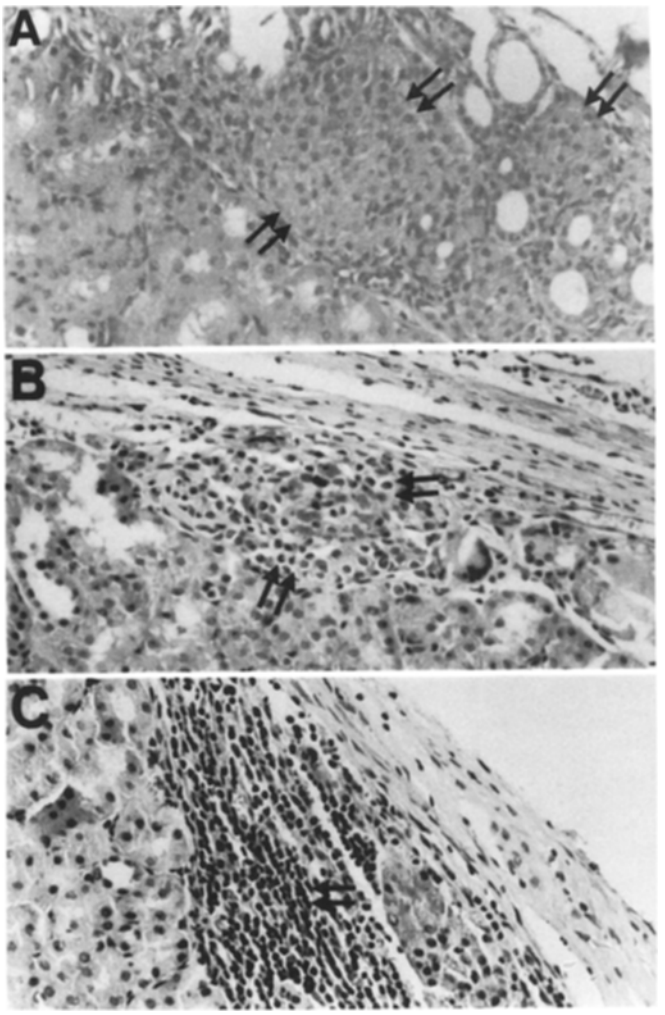

Fig. 2 A–C. Photomicrograph of islet grafts 3 weeks after transplantation into syngeneic diabetic DP-BB rats (haematoxylin-eosin staining; × 400). **A** Islet grafts from 10-day-old DP-BB rats; healthy islet cells with no mononuclear infiltration (arrows) are shown; **B** Islet grafts from 30-day-old DP-BB rats; islet cells at periphery were destroyed and show moderate mononuclear cell infiltration (arrows); **C** Islet grafts from 60-day-old silica-treated DP-BB rats; islets were extensively destroyed and show severe mononuclear cell infiltration (arrows)

rather than in the cytoplasm, of adult DP-BB rat islet cells, and that it is delayed-expressed.

In addition, the density of several protein bands differed between the plasma membrane fractions prepared from neonatal DP-BB rat islets (Fig. 1, lane 3) and adult DP-BB rat islets (Fig. 1, lane 4). A 77 kDa band appeared to be slightly denser in lane 3 than lane 4. However this effect was not consistently seen and appears to be an artifact. A 55 kDa band and high molecular weight bands (> 97 kDa) appeared to have decreased in the adult islet membrane preparations. This was most likely a result of the expression of the genes encoding these proteins diminishing with age for some unknown reason. It is unlikely that these proteins play a pathogenic role in the process of beta-cell-specific

Table 2. Correlation between expression of islet cell-specific 38 kDa autoantigen and recognition of immune effectors

Donor		Recipient	
Age (days)	Presence of 38 kDa antigen[a]	Animal	Histological score of islet-graft[b]
10	–	1	0
		2	0
		3	1
		4	0
		5	0
		6	1
		7	0
30	+	1	1
		2	2
		3	2
		4	1
		5	2
		6	1
		7	3
60	+	1	3
		2	4
		3	4
		4	1
		5	4
		6	3
		7	4

[a]Approximately 2000 islets were isolated from 8–30 DP-BB rats at 10, 30, or 60 days of age. The 38 kDa antigen was identified in the membrane fraction of the islet preparation by the immunoprecipitation method at each age. "–" represents absence of 38 kDa autoantigen and "+" represents presence of 38 kDa autoantigen.

[b]A graded scheme was used to assess the degree of mononuclear cell infiltration and beta-cell destruction as follows: 0 – intact islets with no mononuclear cell infiltration and no beta-cell destruction; 1 – intact islets with minor focal mononuclear cell infiltration at peripheral areas of islets; 2 – intact islets with moderate mononuclear cell infiltration; 3 – damaged islets with extensive mononuclear cell infiltration; 4 – end-stage islets with few beta cells and some mononuclear cells

autoimmunity, as antibodies against these proteins are present in both DP-BB and control WF rats, regardless of the development of diabetes. The difference in density of the 45 kDa protein band in the two lanes is interesting. This protein does consistently increase with age, but it is still present in neonates, so the expression of the gene encoding this 45 kDa protein could be enhanced with age. However, this protein is unlikely to play a critical role in the initiation of beta cell-specific autoimmunity, since it is believed that once a protein is expressed, tolerance to it is usually induced.

To determine when the 38 kDa autoantigen is expressed in DP-BB rats, protein extracts from the membrane fraction of islet preparations from rats aged 3–5 (neonate), 10, 20, 30, or 60 days were immunoprecipitated with anti-38 kDa islet cell antibody positive DP-BB rat sera. A 38 kDa protein was present in islet preparations from 30- and 60-day-old rats, but not from 10- or 20-day-old rats (Table 1), indicating that the antigen is expressed between 20 and 30 days of age. In contrast, a 38 kDa protein was expressed equally in islet preparations from both neonatal and adult DR-BB rats and all ages of WF rats (Table 1). These results indicate that the 38 kDa autoantigen is only delayed-expressed in pancreatic islets from DP-BB rats, not in those from DR-BB or WF rats. To see whether anti-38 kDa islet cell-specific antibody reacts with protein from other tissues, the membrane fractions of cell lysates from kidney and spleen of DP-BB and WF rats at different ages were immunoprecipitated with sera containing anti-38 kDa islet cell antibody. None of these tissues expressed detectable levels of a 38 kDa protein at any age in either DP-BB or WF rats, indicating that the protein is likely to be islet cell-specific (Table 1).

As the direct interaction of the delayed-expressed 38 kDa autoantigen with immune effector cells cannot be tested because the 38 kDa protein cannot be collected in sufficient quantity, an alternative approach was devised to determine whether diabetogenic effector cells recognize islets from 30- and 60-day-old DP-BB rats (which express the 38 kDa autoantigen), but not islets from 10-day-old DP-BB rats (which do not express the 38 kDa autoantigen). Histological examination of the grafts obtained from 10-day-old rats revealed that five of seven had healthy islet cells with no mononuclear cell infiltration (Fig. 2A; Table 2). The remaining two grafts from 10-day-old rats had minor focal mononuclear cell infiltration around thick layers of islet cells (Table 2). In contrast, three of seven grafts obtained from 30-day-old rats showed moderate mononuclear cell infiltration (Fig. 2B; Table 2), and one of seven revealed extensive mononuclear cell infiltration (Table 2). The remaining three showed minor mononuclear cell infiltration (Table 2). Examination of grafts obtained from silica-treated 60-day-old donors showed extensive mononuclear cell infiltration or severe beta-cell destruction in six of seven grafts (Fig. 2C; Table 2), while the remaining one showed minor mononuclear cell infiltration (Table 2).

Discussion

In this study, we show for the first time that a membrane-bound islet cell-specific 38 kDa autoantigen in DP-BB rats is delayed-expressed at about the time when immunological effectors begin to recognize beta cells. Adams et al.[12] showed that delayed expression of a transgene in beta cells resulted in a failure to establish self-tolerance and consequently produced autoimmune lesions in the pancreatic islets of several lines of mice. In contrast, mice that expressed this transgene early in life were tolerant. In non-diabetic WF and DR-BB rats, the 38 kDa islet cell-specific protein is expressed in both neonatal and adult islets. This early expression of the 38 kDa protein is comparable to early expression of the transgene seen in the transgenic mice studied by

Adams et al. [12]. Buschard et al. [13] reported that diabetes in DP-BB rats could be prevented by neonatal stimulation of beta cells. This procedure is thought to induce early antigen expression on beta cells and subsequent tolerance. On the basis of this information and our own observations, we suggest that in DP-BB rats, an antigen is absent, or insufficiently expressed early in life, and the later expression and accumulation of the antigen, as beta cells mature functionally, may trigger beta-cell-specific autoimmune diabetes.

Our previous study [11] showed that autoantibodies to an islet cell protein of approximately 38 kDa molecular weight were detectable by immunoprecipitation at about 30 days of age in the sera of BB rats, but were not detectable in rats aged 5–20 days, nor during the advanced stages of the disease when beta cells have been completely destroyed [11]. These results indicated that the presence of anti-38 kDa islet cell autoantibodies is beta-cell dependent. Moreover, about 90% of the rats in which autoantibodies were detected eventually became diabetic [11]. However, we did not know the expression time of the 38 kDa antigen to which the antibody is directed. The present study correlates well with our previous work, in that the 38 kDa antigen is expressed at about 30 days of age, which is the same time circulating antibodies are first detected. Similar autoantibodies have been reported in humans. In a previous study [14], autoantibodies in the sera from all four tested newly onset IDDM patients immunoprecipitated a 38 kDa islet cell protein prepared from HLA-DR3-positive donor islets.

To date, we have found that the 38 kDa protein is the only delayed-expressed islet cell autoantigen whose antibody is consistently found in acutely diabetic DP-BB rats. WF rats which do express the 38 kDa autoantigen from birth do not develop diabetes. Thus, we propose that the presentation of 38 kDa autoantigens, released during spontaneous turnover of beta cells, through APC such as macrophages and dendritic cells to CD4+ T helper cells, in conjunction with MHC class II molecules, might be the initial step in the development of autoimmune IDDM in DP-BB rats. Our current hypothesis on this initial step in the pathogenesis of IDDM in the DP-BB rat is supported by earlier reports from ourselves and others that the inactivation of macrophages by silica treatment stops further immune processes leading to the prevention of insulitis and diabetes [6, 15]. The activated CD4+ helper T cells can secrete cytokines, such as interleukin-2. While this process is taking place, 38 kDa autoantigen-specific CD8+ cytotoxic T cells may be generated and amplified by the interleukin-2. The 38 kDa autoantigen-specific CD8+ cytotoxic T cells, as final effectors, may recognize the 38 kDa antigens expressed on the unaffected beta cells, in conjunction with MHC class I molecules. Free radicals and cytokines, such as interleukin-1, produced by macrophages and monocytes may work synergistically with the action of CD8+ effector T cells, leading to the destruction of pancreatic beta cells. However, we cannot exclude the possibility that other autoantigens are involved in the generation of final effectors responsible for the destruction of pancreatic beta cells. Whether the 38 kDa autoantigen alone, or another autoantigen(s), or both, are involved in the generation of these final effectors remains to be determined.

Regarding a 64 kDa protein, which has been the subject of much research, we found that it is present in neonatal as well as adult DP-BB islets. This 64 kDa protein has been identified as glutamic acid decarboxylase (GAD), the biosynthesizing enzyme to the inhibitory neurotransmitter gamma-aminobutyric acid [10]. GAD is not beta-cell-specific, but is expressed in the testes, ovaries, thymus and stomach [16, 17], as well as in human pancreatic alpha, delta and polypeptide producing cells [18] and in *Escherichia coli* strains present in the gut [19]. The precise role of GAD in the pathogenesis of autoimmune IDDM in the DP-BB rat is not known, although two recent studies using the non-obese diabetic mouse have suggested that GAD is the key target antigen in the induction of murine IDDM [20, 21].

Roep et al. [22] identified a 38 kDa antigen that is recognized by a T-cell clone established from a newly diagnosed IDDM patient. Subcellular fractionation studies using rat insulinoma cells indicated that the antigenic determinant recognized by this T-cell clone is an integral membrane component of the insulin secretory granule [22]. Granular membrane proteins are transiently exposed to the cell surface during exocytosis, and their accessibility to components of the immune system is thought to be a function of the secretory activity of beta cells. Whether there are any molecular similarities between this 38 kDa protein isolated from insulinoma cells and our membrane-bound, delayed-expressed 38 kDa islet cell autoantigen remains to be determined.

In conclusion, we suggest that the delayed expression of a gene encoding for a beta-cell-specific 38 kDa autoantigen may result in a breakdown in self-tolerance, leading to beta-cell-specific autoimmune IDDM in the DP-BB rat.

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