Targeted Disruption of the IA-2β Gene Causes Glucose Intolerance and Impairs Insulin Secretion but Does Not Prevent the Development of Diabetes in NOD Mice

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Insulinoma-associated protein (IA)-2β, also known as phogrin, is an enzymatically inactive member of the transmembrane protein tyrosine phosphatase family and is located in dense-core secretory vesicles. In patients with type 1 diabetes, autoantibodies to IA-2β appear years before the development of clinical disease. The genomic structure and function of IA-2β, however, is not known. In the present study, we determined the genomic structure of IA-2β and found that both human and mouse IA-2β consist of 23 exons and span ~1,000 and 800 kb, respectively. With this information, we prepared a targeting construct and inactivated the mouse IA-2β gene as demonstrated by lack of IA-2β mRNA and protein expression. The IA-2β$^{-/-}$ mice, in contrast to wild-type controls, showed mild glucose intolerance and impaired glucose-stimulated insulin secretion. Knockout of the IA-2β gene in NOD mice, the most widely studied animal model for human type 1 diabetes, failed to prevent the development of cyclophosphamide-induced diabetes. We conclude that IA-2β is involved in insulin secretion, but despite its importance as a major autoantigen in human type 1 diabetes, it is not required for the development of diabetes in NOD mice. *Diabetes* 53:1684–1691, 2004

Insulinoma-associated protein (IA)-2 is a major autoantigen in type 1 diabetes (1,2). It is 979 amino acids in length, consists of an intracellular transmembrane and extracellular domain, and is found in dense-core secretory vesicles of β-cells and other neuroendocrine cells (3). It is located on chromosome 1 in mice and 2q35 in humans (4,5). Based on sequence data, IA-2 is a member of the transmembrane protein tyrosine phosphatase (PTP) family, but is enzymatically inactive because of two critical amino acid substitutions in the conserved PTP domain. Approximately 70% of newly diagnosed patients with type 1 diabetes have autoantibodies to IA-2. These autoantibodies appear years before the development of clinical disease and, in combination with autoantibodies to GAD, have become important diagnostic and predictive markers. Based on population screening, it is estimated that individuals with autoantibodies to both IA-2 and GAD are at about a 50% risk of developing type 1 diabetes within 5 years (6). Recent knockout studies (7) in mice showed that IA-2 is involved in glucose-stimulated insulin secretion.

IA-2β (also known as phogrin, PTP-NP, ICAAR, and IAR) is closely related to IA-2 (8–12). Its intracellular and extracellular domains are 74 and 26% identical, respectively, to IA-2. It too is enzymatically inactive and is found in dense-core secretory vesicles of β-cells and other neuroendocrine cells (13). IA-2β is located on chromosome 12F in mice and 7q36 in humans (14,15). About 50% of newly diagnosed patients with type 1 diabetes have autoantibodies to IA-2β, and these antibodies appear years before the development of clinical disease (16). However, the genomic structure and function of IA-2β has not been elucidated.

In the present study we determined the genomic structure of human and mouse IA-2β and succeeded in knocking out the mouse gene. We showed that IA-2β is involved in insulin secretion, but is not required for the development of diabetes in NOD mice.

RESEARCH DESIGN AND METHODS

Determination of genomic structure. Various restriction fragments isolated from mouse IA-2β cDNA clones (8,14) were used to screen a mouse 129– genomic cosmid library in vector pTCF (a gift of Dr. F. Grosveld) using conditions described previously (5). P1 genomic clones were identified in a gridded mouse 129 genomic library (Leiden Genome Technology Center, Wassenaarseweg, the Netherlands) by PCR. Positive clones were isolated, and appropriate fragments were subcloned and used for automated dideoxy sequence determination. In addition, the basic local alignment search tool (BLAST) program (17,18) was used to identify exon-containing genomic segments in public and commercial databases. Mouse strain C57BL/6 genomic sequences were determined through PCR amplification and sequencing of the relevant fragments. Resulting sequences were analyzed using Vector NTI software (Informax, Oxford, U.K.). Exon-intron junctions were determined by comparison with cDNA sequences, complying maximally to the "cagGT" rule for 3′ splice acceptor sites. Intron sizes were estimated from the sequence data, assuming that ambiguous stretches (n repeats) in the genomic entries were deposited proportionally to their length. Genomic sequences were compared lastly to version 14.30.1 of the mouse genome database (released 6 May 2003; http://www.ensembl.org), and the sequence contig annotated for mouse C57BL/6 *Ptprn2* (resulting from the current work) was submitted to the database (accession no. AJ583055).

Animals. The IA-2β targeting construct was prepared by ligating genomic IA-2β fragments (2.4 and 6.7 kb) to the 5′ and 3′ ends of the vector,

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IA, insulinoma-associated protein; PTP, protein tyrosine phosphatase.

TABLE 1
Exon and Intron sizes of mouse and human IA-2β–encoding gene

Mouse IA-2β				Human IA-2β				Mouse IA-2	
Exon	Size (bp)	Intron	Size (bp)	Exon	Size (bp)	Intron	Size (bp)	Exon	Size (bp)
1	≥254			1	≥216			1	~184
2	51	1	94,652	2	51	1	97,772	2	51
3	114	2	146,748	3	114	2	172,802	3	114
4	103	3	99,548	4	103	3	111,545	4	97
5	163	4	16,823	5	169	4	12,675	5	259
6	349	5	18,110	6	361	5	25,035	6	352
7	201	6	12,761	7	222	6	28,415	7	132
8	41	7	1,514	8	41	7	1,598	8	38
9	383	8	2,300	9	383	8	2,595	9	275
10	87	9	12,466	10	87	9	22,761	10	87
11	80	10	12,312	11	80	10	29,450	11	80
12	65	11	120,908	12	65	11	168,560	12	65
13	213	12	135,998	13	213	12	215,423	13	219
14	195	13	22,870	14	195	13	26,173	14	201
15	148	14	27,089	15	148	14	34,847	15	148
16	74	15	15,551	16	74	15	17,286	16	74
17	78	16	6,063	17	78	16	8,686	17	78
18	120	17	14,471	18	120	17	17,097	18	120
19	167	18	751	19	167	18	1,241	19	167
20	54	19	4,902	20	54	19	5,119	20	54
21	65	20	1,889	21	65	20	2,473	21	65
22	74	21	13,898	22	74	21	19,880	22	74
23	>246	22	6,908	23	~1,730	22	8,160	23	578

respectively. Homologous recombination resulted from the replacement of a portion of the 5′ upstream region, exon 1, and part of intron 1 of the IA-2β gene with a neomycin-resistant gene cassette into the *Eco*RI-*Kpn*I site of the targeted locus. Jl ES cells were transfected with the linearized targeting vector by electroporation and grown under double-selection conditions (350 μg/ml G418, 2 μmol/l gancyclovir). G418/gancyclovir-resistant ES cell clones were screened by Southern blot analysis. ES cells carrying the disrupted IA-2β allele were then injected into blastocysts to prepare chimeric mice. Chimeric mice were bred to C57BL/6Nci mice, and offspring that carried the disrupted IA-2β allele were backcrossed to C57BL/6Nci for five generations. Offspring also were backcrossed to NOD/LtJ mice (The Jackson Laboratories, Bar Harbor, ME) for four generations, and littermates were used. All protocols were approved by our institutional animal care and use committee.

Northern and Western blot analysis. Total RNA and proteins from brains were prepared and analyzed using specific probes or antibodies. Antibodies against mouse IA-2β (pol.IA-2β) were raised in rabbits by immunization with the recombinant intracellular domain of mouse IA-2β and absorbed with brain lysate from IA-2β$^{-/-}$ mice.

Analysis of blood glucose and hormones. Blood glucose was measured with a One Touch II Glucometer (Lifescan, Milpitas, CA). Serum insulin levels were determined with an enzyme-linked immunosorbent assay kit using a mouse insulin standard (Crystal Chem, Downers Grove, IL). For glucagon, blood samples were collected in tubes containing aprotinin, and the serum was stored at −80°C until radioimmunoassay (Linco Research, St. Charles, MO).

Histological and immunohistochemical analysis. All of the major organs and tissues were collected in 10% neutral-buffer formalin and processed for paraffin embedding. Sections were stained with hematoxylin and eosin. Pancreatic sections were incubated with antibodies to insulin, glucagon, somatostatin, and pancreatic polypeptide (Dako, Carpinteria, CA), followed by biotin-conjugated second antibody and streptavidin horseradish peroxidase.

Intraperitoneal glucose tolerance test and insulin release. Mice (3 months and 6–10 months of age) were fasted overnight, followed by intraperitoneal glucose injection (2 g/kg body wt). Blood was drawn at 0, 15, 30, 60, and 120 min after the injection, and blood glucose was measured using the One Touch II Glucometer. Insulin secretion was measured by injecting glucose (3 g/kg body wt i.p.) into male and female mice at 3 months and 6–10 months of age. Venous blood was collected at 0, 2, 5, and 20 min in heparinized tubes. After centrifugation, plasma was stored at −20°C. Insulin levels were measured with an enzyme immunoassay kit using a mouse insulin standard (Alpco Diagnostics, Windham, NH).

Glucose-stimulated insulin secretion from isolated pancreatic islets. Pancreatic islets from IA-2β$^{+/+}$ or IA-2β$^{-/-}$ mice were isolated using percoll density gradient separation and hand picked under a stereomicroscope (7). Insulin secretion was measured by placing 10 islets in each well (4–10 wells/group). Islets then were incubated in 1.0 ml of Krebs-Ringer bicarbonate buffer supplemented with 3.3 mmol/l glucose for 1 h. Subsequently, the islets were transferred to media containing 27.7 mmol/l glucose and incubated for 1 h. Aliquots were removed and stored at −20°C for quantitation of insulin by enzyme-linked immunosorbent assay.

Statistical analysis. Statistical analysis was performed using the Student's *t* test for unpaired comparisons. Values are presented as means ± SE. $P < 0.05$ was considered significant.

Cyclophosphamide-accelerated diabetes. To accelerate the development of diabetes, 8-week-old IA-2β$^{+/+}$ and IA-2β$^{-/-}$ (male and female) mice received one intraperitoneal injection of cyclophosphamide (250 mg/kg) (Sigma, St. Louis, MO). Two weeks later, mice remaining nondiabetic were given a second injection of cyclophosphamide and followed for an additional 3 weeks. Blood glucose levels were measured with the Elite XL Blood Glucose Meter (Bayer, West Haven, CT). Diabetes was defined as glucose levels of >250 mg/dl on two consecutive readings.

RESULTS

To determine the structure of the mouse IA-2β gene, which has been physically mapped to chromosome region 12F (14,15), we assembled information from the mouse genomic database and used mouse IA-2β cDNA sequences to screen genomic DNA libraries. The IA-2β gene (*Ptprn2*), similar to the closely related mouse IA-2 gene (*Ptprn*), consists of 23 exons (Fig. 1*A* and Table 1) (19), but the distance between the exons of the two genes differs enormously. Mouse *Ptprn2* spans ~800 kb, whereas *Ptprn* covers only 20 kb. The *Ptprn2* introns vary in size from 751 bp (intron 18) to 146.7 kb (intron 2). We also determined the structure of the human IA-2β gene (*PTPRN2*), located on chromosome 7q36, and found that it too consists of 23 exons distributed over some 1,000 kb (Table 1).

The first mouse IA-2β exon harbors the AUG translation start codon, followed by 106 additional protein-coding nucleotides and a 5′ untranslated region of at least 145 nucleotides. We chose this region of the mouse IA-2β gene

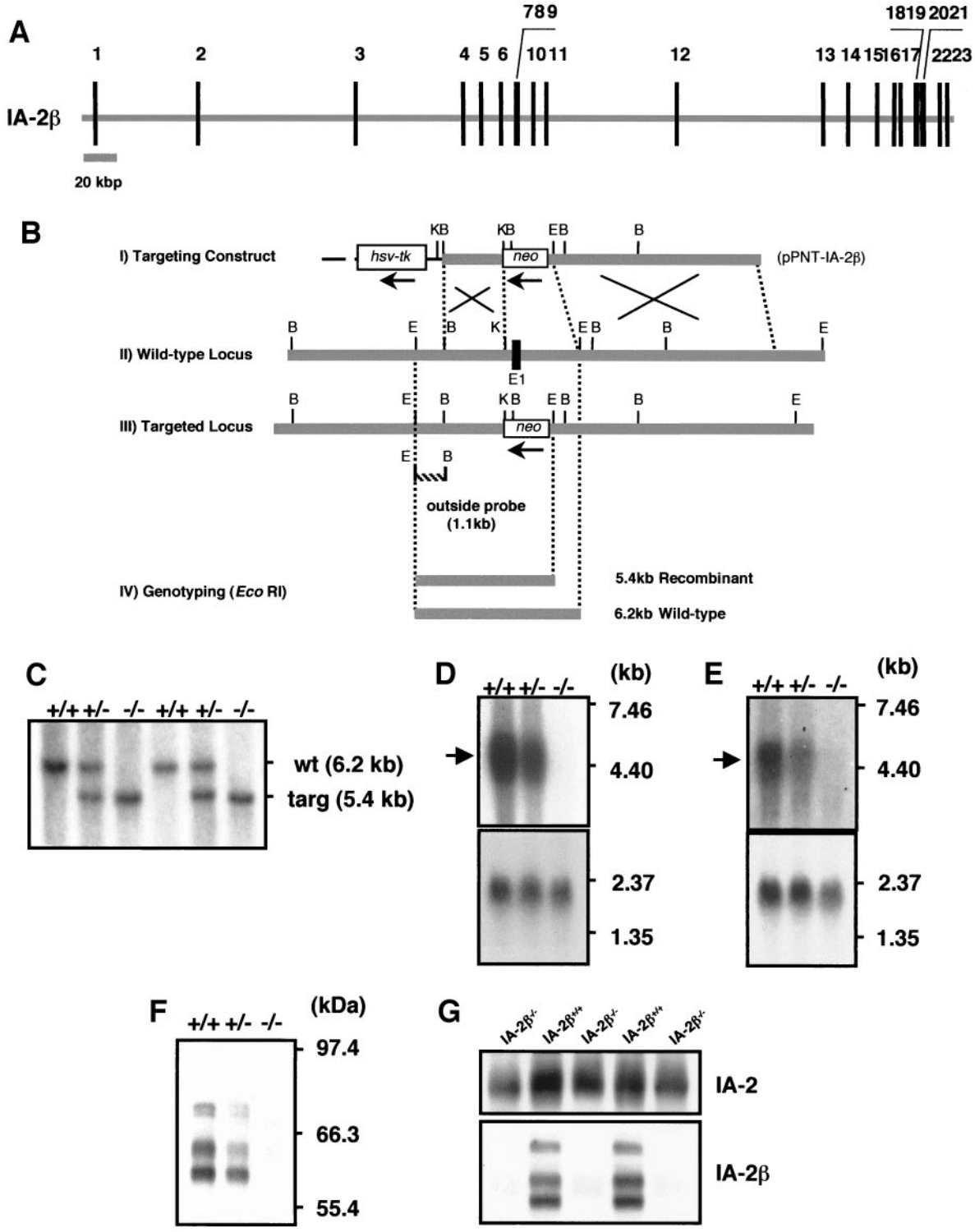

FIG. 1. Genomic structure and targeted disruption of the IA-2β gene. *A*: Mouse IA-2β genomic structure. The horizontal gray line depicts the 800-kb genomic segment on chromosome 12 band F, in which the gene resides. Vertical bars represent exonic sequences, which are numbered. Underneath exon 1 is a dark horizontal line indicating the 20-kbp DNA segment that was used in the targeting strategy and is shown enlarged as the wild-type locus (*B*, II) below. The paralogous IA-2 gene, *Ptprn*, has an identical exon-intron distribution but spans only 20 kb (not shown). *B*: *I*: Targeting construct shows that a portion of the 5′ upstream promoter, exon 1, and part of intron 1 are replaced with a neomycin cassette. *II*: Wild-type locus. Dashed lines with a large "X" show areas where homologous recombination takes place. E1 indicates exon 1. *III*: The targeted locus shows the integration of the targeting construct, containing the neomycin cassette, into the wild-type locus. *IV*: Size of restriction fragments when wild-type locus and targeted locus are cleaved with *Eco*RI and hybridized with the 1.1-kb outside probe. Restriction enzyme sites are indicated by letters: K, *Kpn*I; E, *Eco*RI; B, *Bam*HI. *C*: Southern blot analysis. DNA from the IA-2β$^{+/+}$, IA-2β$^{+/-}$, and IA-2β$^{-/-}$ mice were digested with *Eco*RI and hybridized with the 1.1-kb outside probe. IA-2β$^{+/+}$ mice yielded the expected 6.2-kb band, whereas DNA from the IA-2β$^{-/-}$ mice gave a 5.4-kb band. IA-2β$^{+/-}$ mice showed both bands. *D* and *E*: Northern blot analysis. Probes generated from either the extracellular (*D*) or3′ noncoding region (*E*) of IA-2β (*top panel*) showed a strong 5.5-kb band with IA-2β$^{+/+}$, a weaker band with IA-2β$^{+/-}$ mice, and no band with the IA-2β$^{-/-}$ mice. Actin mRNA (*bottom panel*) served as an internal control. *F*: Western blot analysis. Antibody to IA-2β recognizes three bands of the IA-2β protein in brain tissue from the IA-2β$^{+/+}$ and IA-2β$^{+/-}$ mice, but not from the IA-2β$^{-/-}$ mice. *G*: IA-2 protein in IA-2β$^{-/-}$ mice. Brain from IA-2β$^{+/+}$ and IA-2β$^{-/-}$ mice were analyzed by immunoblotting with anti–IA-2 (*top panel*) and anti–IA-2β (*bottom panel*) antibodies.

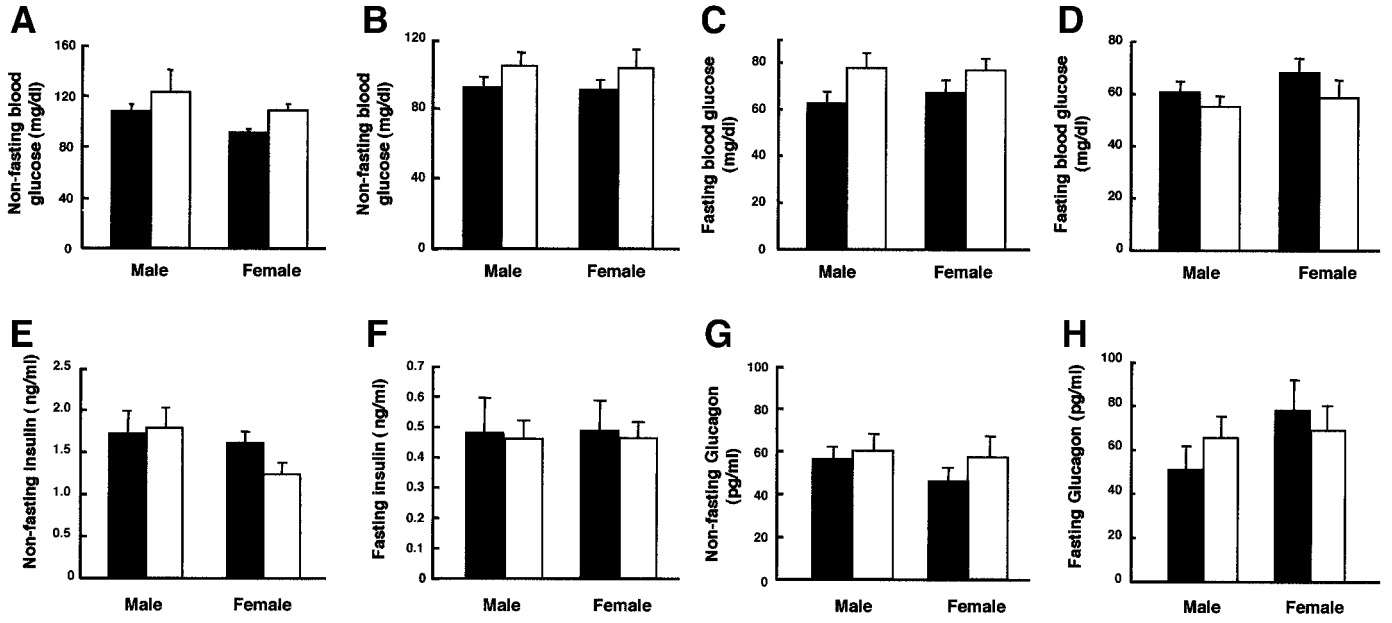

FIG. 2. Glucose, insulin, and glucagon levels in IA-2β$^{+/+}$ (■) and IA-2β$^{-/-}$ (□) mice (n = 4–10 per group). *A*: Nonfasting blood glucose at 3 months. *B*: Nonfasting blood glucose at 10 months of age. *C*: Fasting blood glucose at 3 months. *D*: Fasting blood glucose at 10 months of age. *E*: Nonfasting blood insulin at 3 months of age. *F*: Fasting blood insulin at 3 months of age. *G*: Nonfasting blood glucagon at 3 months of age. *H*: Fasting blood glucagon at 3 months of age.

as the target for disruption by homologous recombination. In brief, a targeting construct was prepared by replacing a portion of the 5′ upstream promoter region, exon 1, and a portion of intron 1 with a neomycin cassette (Fig. 1*B*). ES cells then were transfected with the targeting construct, and successful homologous recombination was determined by Southern blot analysis (data not shown). IA-2β$^{+/-}$ ES cells were then injected into blastocysts, resulting in chimeric mice that transmitted the disrupted IA-2β allele to their offspring. Wild-type (IA-2β$^{+/+}$), heterozygous (IA-2β$^{+/-}$), and homozygous (IA-2β$^{-/-}$) mice were identified by tail DNA PCR with appropriate primers (data not shown) and confirmed by Southern blot analysis using the 1.1-kb *Eco*RI-*Bam*HI fragment (outside probe) (Fig. 1*C*). Further evidence that the homologous recombination was successful and that the IA-2β gene had been disrupted was obtained by Northern and Western blot analysis. Hybridization of total brain RNA with probes corresponding to the extracellular region (nucleotides 141-1540) and 3′ noncoding region (nucleotides 3040–3143) of IA-2β resulted in a strong 5.5-kb band with IA-2β$^{+/+}$ RNA, a markedly reduced signal with IA-2β$^{+/-}$ RNA, and no signal with IA-2β$^{-/-}$ RNA (Fig. 1*D* and *E*). Western blot analysis, using rabbit anti-mouse IA-2β antibody, recognized three strong bands (55–75 kDa) in brain lysates of IA-2β$^{+/+}$ mice that are weaker in IA-2β$^{+/-}$ lysates and absent in IA-2β$^{-/-}$ lysates (Fig. 1*F*). To see if the deletion of IA-2β had any effect on the level of IA-2, we analyzed brain homogenates from IA-2β$^{-/-}$ and IA-2β$^{+/+}$ mice by Northern and Western blotting. Little if any change in IA-2 mRNA (data not shown) or protein was found in IA-2β$^{-/-}$ mice (Fig. 1*G*).

Physical examination of the IA-2β$^{-/-}$ mice revealed no gross abnormalities. Body weight of the IA-2β$^{-/-}$ mice did not differ from wild-type controls at 8 weeks of age (IA-2β$^{+/+}$ males, 24.0 ± 0.7 g; IA-2β$^{-/-}$ males, 24.1 ± 0.3 g; IA-2β$^{+/+}$ females, 17.5 ± 0.3 g; and IA-2β$^{-/-}$ females, 18.5 ± 0.8 g). Intercrossing the IA-2β$^{-/-}$ mice yielded the expected litter size (average 8.6 pups, 14 pairs). Nonfasting blood glucose levels of the IA-2β$^{-/-}$ male and female mice, measured at 3 and 10 months of age, were slightly elevated as compared with IA-2β$^{+/+}$ mice, but did not fall within the diabetic range (Fig. 2*A* and *B*). Fasting blood glucose levels also fell within the normal range (Fig. 2*C* and *D*). Similarly, nonfasting and fasting insulin (Fig. 2*E* and *F*) and nonfasting and fasting glucagon (Fig. 2*G* and *H*) levels of IA-2β$^{-/-}$ male and female mice did not differ significantly from those of IA-2β$^{+/+}$ male and female mice.

Histological studies failed to reveal any abnormality in the neuronal and neuroendocrine cells of the IA-2β$^{-/-}$ mice, including hippocampus (Fig. 3*A*), anterior pituitary (Fig. 3*B*), adrenal gland (Fig. 3*C*), and pancreatic islets (Fig. 3*D*). Immunohistochemical studies on the IA-2β$^{-/-}$ mice (Fig. 3*E–H*) also revealed normal-appearing pancreatic islets, with no difference in the morphology or staining pattern for insulin, glucagon, somatostatin, or pancreatic polypeptide as compared with IA-2β$^{+/+}$ mice (data not shown).

In contrast to the fasting and nonfasting blood glucose and insulin levels, an intraperitoneal glucose tolerance test revealed elevated glucose levels and depressed release of insulin in IA-2β$^{-/-}$ mice. Blood glucose levels were significantly elevated in IA-2β$^{-/-}$ female mice at both 3 months (Fig. 4*B*) and 6–10 months (Fig. 4*D*) of age, but not in male IA-2β$^{-/-}$ mice (Fig. 4*A* and *C*). Insulin levels were mildly depressed (Fig. 4*E–H*), but only reached statistical significance at 20 min in the female mice (Fig. 4*F* and *H*). Statistically significant differences, however, were found when cultured pancreatic islets isolated from IA-2β$^{+/+}$ and IA-2β$^{-/-}$ mice were stimulated with glucose. Male and female IA-2β$^{-/-}$ mice released 33 and 38% less insulin,

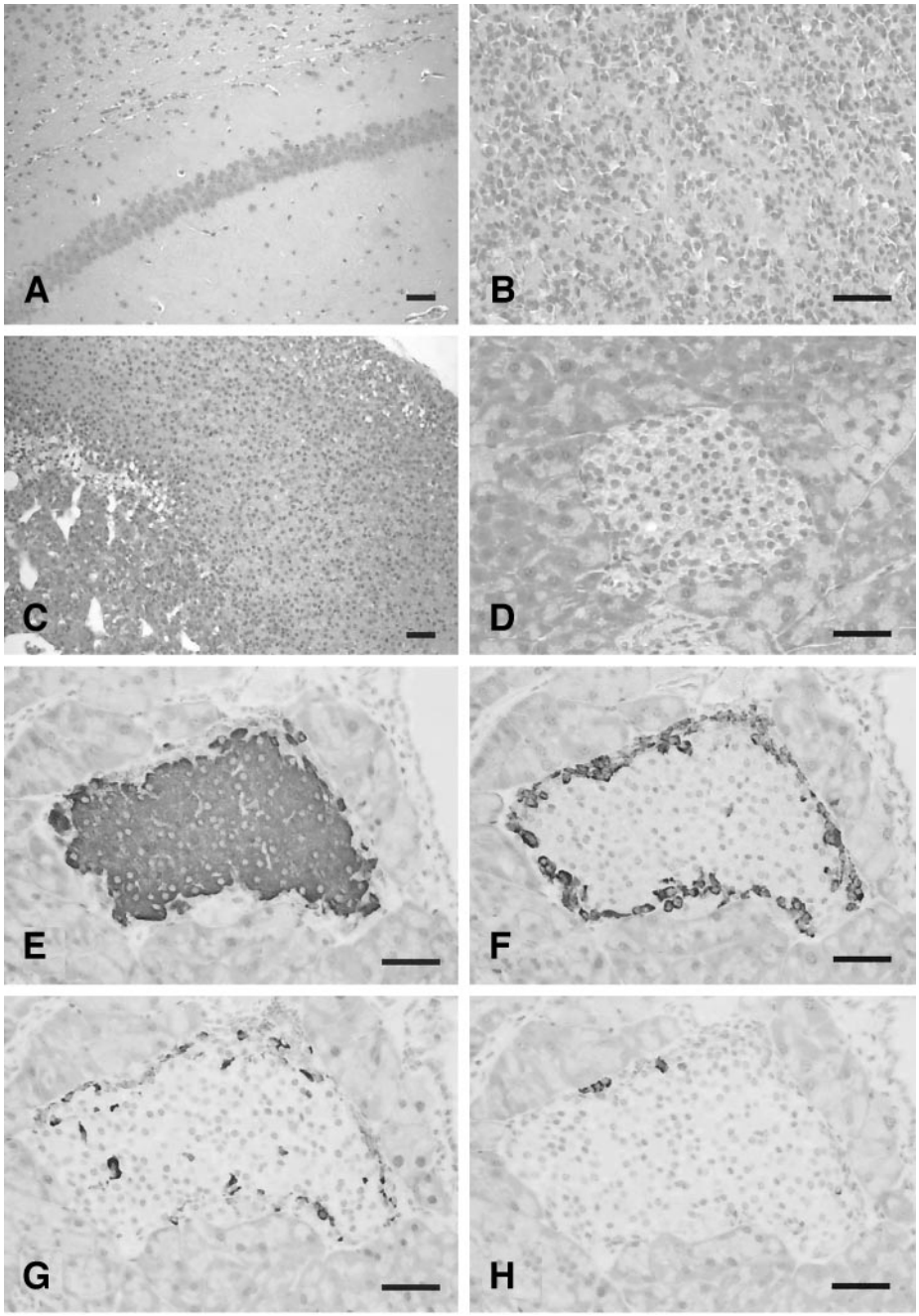

FIG. 3. Histology and immunostaining of tissues from IA-2β$^{-/-}$ mice. *A*: Hippocampus. *B*: Anterior pituitary gland. *C*: Adrenal gland. *D*: Pancreas stained with hematoxylin and eosin. Sections of pancreas were incubated with antibody to insulin (*E*), glucagon (*F*), somatostatin (*G*), and pancreatic polypeptide (*H*), followed by biotin-conjugated second antibody and streptavidin horseradish peroxidase. Scale bar, 100 μm.

respectively, than IA-2β$^{+/+}$ mice when switched from basal glucose (3.3 mmol/l) to high glucose (27.7 mmol/l) (Fig. 5).

Chimeric mice were bred to C57BL/6Nci mice, and the offspring that carried the disrupted IA-2β allele were backcrossed to NOD/LtJ mice for four generations. IA-2β$^{+/-}$ NOD mice then were intercrossed to obtain IA-2β$^{-/-}$ NOD mice as well as IA-2β$^{+/+}$ NOD mice. Heterozygous crosses tend to level any confounding genetic background effect that might still be present from 129-derived sequences, even though the most likely effect of the residual 129 genes would be protection from diabetes. In addition to genetic typing (data not shown), the lack of IA-2β protein expression was confirmed by Western blot analysis (Fig. 6*A*). Mice then were treated with 250 mg/kg i.p. of cyclophosphamide, a known accelerator of diabetes (20). Approximately 57% of IA-2β$^{+/+}$ male, 62% of IA-2β$^{-/-}$ male, 58% of IA-2β$^{+/+}$ female, and 53% of IA-2β$^{-/-}$ female NOD mice developed diabetes (Fig. 6*B*). Although the colony is still small, five IA-2β$^{-/-}$ NOD mice that were not given cyclophosphamide spontaneously developed diabetes between 16 and 36 weeks of age. Although not yet tested, since the knockout of IA-2β results in impaired secretion of insulin, the IA-2β$^{-/-}$ NOD mice might develop even more severe or accelerated diabetes than the IA-2β$^{+/+}$ NOD mice because of the superimposition of impaired insulin secretion on the NOD diabetes background.

DISCUSSION

Recently, we showed (7) that targeted disruption of IA-2 resulted in elevated glucose tolerance tests and impaired

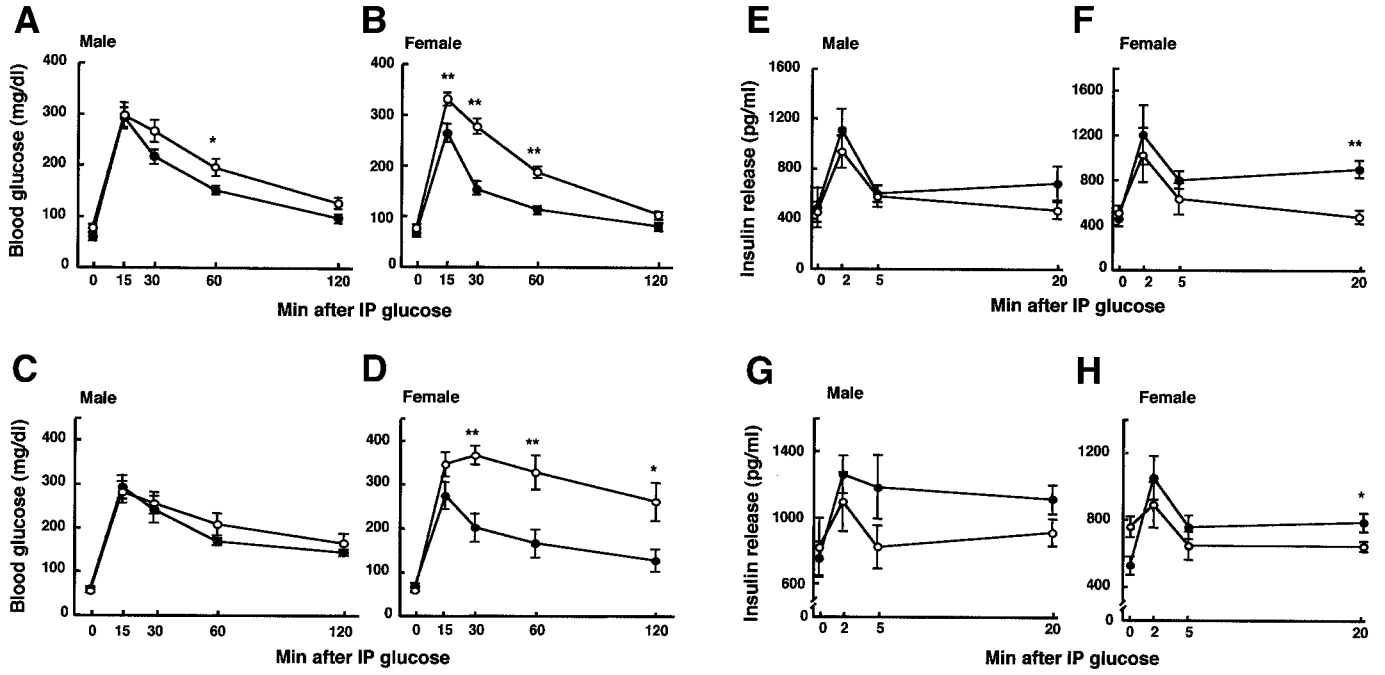

FIG. 4. Glucose tolerance and insulin secretion tests. After overnight fasting, male and female IA-2β$^{+/+}$ (●) and IA-2β$^{-/-}$ (○) mice at 3 months (*A* and *B*) and 6–10 months (*C* and *D*) of age were injected intraperitoneally with D-glucose (2 g/kg body wt), and blood glucose levels were measured at different times thereafter (n = 7–11 per group). Insulin secretion in response to intraperitoneal D-glucose (3 g/kg body wt) in IA-2β$^{+/+}$ (●) and IA-2β$^{-/-}$ (○) mice at 3 months (*E* and *F*) and 6–10 months (*G* and *H*) of age were measured at different times thereafter (n = 7–9 mice per group). Values are means ± SE. *P < 0.05; **P < 0.01.

secretion of insulin. The targeted disruption of IA-2β, reported here, also showed mild impairment of glucose tolerance tests and insulin secretion, resembling our findings in the IA-2 knockout mice and arguing that the two proteins have similar functions but act independently. This, in part, is supported by the fact that the knockout of IA-2 did not alter the expression of IA-2β mRNA (7), and, as shown here, the absence of IA-2β had little effect on the expression of IA-2. The possibility that IA-2β and IA-2 act in a complementary fashion is now being explored with double-knockout mice, and preliminary results show that glucose tolerance test abnormalities are considerably more pronounced in the double-knockout than in the single-knockout mice.

Precisely how IA-2 and IA-2β exert their effect on insulin secretion is not known. Recent protein interaction experiments suggest two alternative models. In the first, IA-2, as an intrinsic membrane protein of secretory vesicles, may be linked to cytoskeletal proteins and thereby aid in regulating secretory vesicle mobilization and recruitment in exocytosis (21). In the second, IA-2 and IA-2β may heterodimerize with receptor-type PTPs and consequently influence the signaling pathways involved in regulating neuropeptide or peptide hormone exocytosis (22).

The partial impairment of the insulin secretory process in both IA-2β– and IA-2–deficient animals may reflect functional redundancy. Indeed, IA-2β and IA-2 are almost 75% identical in their intracellular PTP-like segment, even though the underlying gene duplication event preceded vertebrate radiation (23). Although the amino acid sequence identity in the NH_2-terminal luminal segment is only 26%, the overall exon-intron segmentation of the two genes is well conserved. In sharp contrast, the gene sizes differ by no less than 40-fold in mice and by as much as

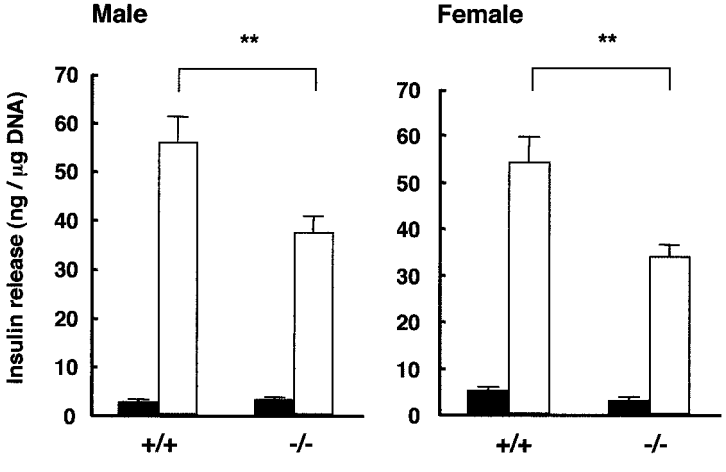

FIG. 5. Glucose-stimulated insulin release in vitro. Pancreatic islets isolated from IA-2β$^{+/+}$ and IA-2β$^{-/-}$ mice were incubated in Krebs-Ringer bicarbonate buffer with 3.3 mmol/l glucose (■). At the end of 1 h, insulin levels in the supernatants were measured. The islets were then transferred to Krebs-Ringer bicarbonate buffer containing 27.7 mmol/l glucose (□), and 1 h later, insulin levels were again measured. The results represent the average of three independent experiments. Values are the mean ± SE. **P < 0.01.

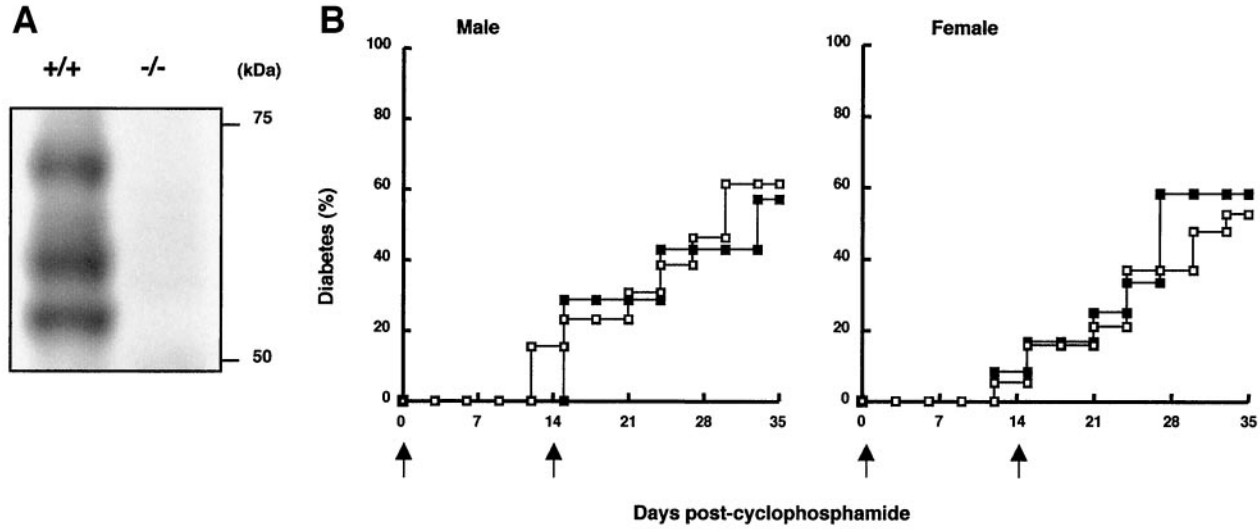

FIG. 6. *A*: **Western blot analysis demonstrates normal expression of IA-2β protein in the brain of IA-2β$^{+/+}$ NOD mice, but absence of expression in the brain of IA-2β$^{-/-}$ NOD mice.** *B*: **Diabetes incidence in cyclophosphamide-treated, 8-week-old IA-2β$^{+/+}$ and IA-2β$^{-/-}$ NOD mice. Cyclophosphamide (250 mg/kg) was administered twice (arrows). Males: IA-2β$^{+/+}$ (n = 7, ■) and IA-2β$^{-/-}$ (n = 13, □). Females: IA-2β$^{+/+}$ (n = 12, ■) and IA-2β$^{-/-}$ (n = 19, □).**

50-fold in humans. This is primarily due to the presence of five introns in IA-2β that equal or exceed 100 kb. At least six potential coding regions for still-unknown genes are annotated within the 1,000 kb of the human IA-2β gene. To date, reports (24,25) on intron size divergence mostly relate to interspecies comparisons between orthologous genes, but the mammalian talin and phospholipase D genes represent comparable cases of unusual size differences (of 6- and 20-fold, respectively) between paralogous genes. The underlying reason or mechanism remains unknown, although the impact of repeat sequences on intron length is well documented (26). Furthermore, analysis of the human genome has shown that the average intron size is inversely related to the G-C content of the genomic area (27), and indeed the 40-fold expansion of the IA-2β–encoding gene as compared with that of IA-2 is paralleled by a drop in G-C content from 51 to 45%. Whether the remarkable size difference merely reflects an epiphenomenon, a coevolution of the IA-2β and IA-2 genes (with their distinct genomic surroundings), or the acquisition of unique discriminatory differences at the regulatory level is not known. It is of interest in this context that differences in the gene size of the IA-2 and IA-2β orthologs are also apparent in the Japanese pufferfish (Fugu rubripes) (7 and 63 kb, respectively), which has a genome one-eighth the size of the human genome and diverged from the human lineage about 450 million years ago (28).

Since IA-2β is a major autoantigen in type 1 diabetes and the NOD mouse is the most widely used animal model for human type 1 diabetes (29), the development of IA-2β knockout mice gave us the opportunity to determine whether the absence of IA-2β would influence the development of diabetes in NOD mice. Although none of the known NOD susceptibility loci (i.e., *Idd1–Idd21*) are found on chromosome 12 (i.e., the location of IA-2β), our knockout experiments clearly show that IA-2β is not required for the development of cyclophosphamide-induced diabetes in these mice. Recent knockout experiments (30) showed that IA-2 is also not required for the development of diabetes in NOD mice. Moreover, by targeted gene disruption, GAD65, another major autoantigen in human type 1 diabetes, has been shown (31) not to be required for diabetes in NOD mice. In humans, the autoantibodies to IA-2, IA-2β, and GAD65 are important diagnostic and predictive markers for type 1 diabetes (32), but not all individuals with type 1 diabetes have these autoantibodies. At least in the case of the NOD mouse, it now appears that neither these proteins nor the humoral immune responses to them (33) are required for the development of diabetes. Taken together, these findings suggest that at the human level the autoimmune response to IA-2, IA-2β, and GAD65 may be a consequence of and/or a contributor to type 1 diabetes rather than being required for the development of the disease.

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