Targeted Disruption of the Protein Tyrosine Phosphatase-Like Molecule IA-2 Results in Alterations in Glucose Tolerance Tests and Insulin Secretion

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IA-2 is a major autoantigen in type 1 diabetes. Autoantibodies to IA-2 appear years before the development of clinical disease and are being widely used as predictive markers to identify individuals at risk for developing type 1 diabetes. IA-2 is an enzymatically inactive member of the transmembrane protein tyrosine phosphatase family and is an integral component of secretory granules in neuroendocrine cells. To study its function, we generated IA-2−/− deficient mice. Northern and Western blot analysis showed that neither IA-2 mRNA nor protein was expressed. Physical examination of the IA-2−/− animals and histological examination of tissues failed to reveal any abnormalities. Nonfasting blood glucose levels, measured over 6 months, were slightly elevated in male IA-2−/− as compared to IA-2+/− littermates, but remained within the nondiabetic range. Glucose tolerance tests, however, revealed statistically significant elevation of glucose in both male and female IA-2−/− mice and depressed insulin release. In vitro glucose stimulation of isolated islets showed that male and female mice carrying the disrupted gene released 48% (P < 0.001) and 42% (P < 0.01) less insulin, respectively, than mice carrying the wild-type gene. We concluded that IA-2 is involved in glucose-stimulated insulin secretion. Diabetes 51:1842–1850, 2002

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ELISA, enzyme-linked immunosorbent assay; ES, embryonic stem; KRBB, Krebs-Ringer bicarbonate buffer; PTP, protein tyrosine phosphatase.

recombinant IA-2 and GAD is being widely used to predict risk for type 1 diabetes (8,9).

IA-2 is a 979–amino acid transmembrane protein. It has an extracellular, transmembrane, and intracellular domain consisting of 576, 24, and 379 amino acids, respectively (1). Autoantibodies to IA-2 are directed exclusively to the intracellular domain, primarily to the COOH-terminus and, to a somewhat lesser extent, to the juxtamembrane region (10–12). The gene encoding IA-2 is located on human chromosome 2q35. The coding region extends over ~20 kb and consists of 23 exons (13). Exons 1–12 encode the extracellular domain; exon 13, the transmembrane domain; and exons 14 through 23, the intracellular domain. A region extending ~200 bp 5′-upstream from the translation start site and a region encompassing exon 1 and intron 1 have been shown to have strong promoter activity (R. Alam and A.L.N., unpublished data; 13).

The function of IA-2 is not known. Based on sequence analysis, IA-2 belongs to a subgroup of the transmembrane protein tyrosine phosphatase (PTP) family. Homologs have been found in cows, rats, mice, macaca, zebrafish, Drosophila, and Caenorhabditis elegans and show 99, 98, 97, 73, 82, 58, and 46% identity, respectively, to human IA-2 (14). IA-2 differs from other PTPs in that it is enzymatically inactive because of substitution of amino acids (Ala 911→Asp and Asp 877→Ala) at conserved sites known to be critical for enzymatic activity (15). Electron microscopic studies and immunohistochemical studies have localized the IA-2 protein (also known as ICA512) to the secretory vesicles of neuroendocrine cells (16).

The present study was initiated to determine the function of IA-2 by targeted disruption of the mouse IA-2 gene. Mouse IA-2 is very similar to human IA-2. It is 981 amino acids in length, consists of 23 exons, and is located on mouse chromosome 1 (17). A targeting construct, in which the 5′-upstream promoter region, exons 1–3 and introns 1, 2, and most of 3, were replaced with a neomycin cassette, was used to transfect embryonic stem (ES) cells by homologous recombination. ES cells in which the IA-2 construct had become integrated were injected into blastocysts, and the resulting chimeric animals and their offspring were bred and tested for evidence of homologous recombination and germ-line transmission. Here we describe the successful targeted disruption of the IA-2 gene in mice and provide a description of the resulting phenotype.
RESEARCH DESIGN AND METHODS

Generation of IA-2−/− deficient mice. A 129SvJ mouse genomic library (Stratagene, La Jolla, CA) was screened with a mouse IA-2 cDNA probe. A 17-kb fragment of mouse IA-2 containing the coding sequence of exons 1–13 was used to construct a targeting vector. A 3.5-kb SalI-EcoRI fragment upstream of the first exon was subcloned into pBluescript SK+ (Stratagene). From this plasmid, the 5′-segment was subcloned as a 3.5-kb KpnI-BamHI fragment into pPNT vector (18), resulting in pPNT-IA-2A. Subsequently, a 3.3-kb XhoI-XhoI fragment containing exons 4–6 was subcloned into pPNT-5′IA-2 vector, resulting in pPNT-IA-2. Homologous recombination resulted in the replacement of IA-2 exons 1–3 and exons 1, 2, and most of 3 with the neo-resistant vector from the pPNT-IA-2 vector into the EcoRI-XhoI site of the targeted locus. The targeting plasmid was linearized using the unique NotI site before electroporation. The HSV-tk cassette was used for negative selection.

J1 ES cells (19) were transfected with the linearized targeting vector (50 μg/1.5 × 1012 cells) using a BioRad Gene Pulser and grown under double-selection conditions (350 μg/ml G418, 2 μmol/l ganciclovir). After 7–10 days, G418-ganciclovir-resistant ES cell clones were picked and expanded (20).

Genomic DNA from individual ES cell clones was extracted and analyzed by Southern blot hybridization. A 1.1-kb XhoI-HindIII fragment (outside probe) containing exons 7 and 8 just downstream of the XhoI-XhoI segment was used for screening. Homologous recombinant clones selected by the outside probe were also analyzed with a 1.9-kb neo cassette probe to check for multiple integrations. Five ES cell clones were injected into C57BL/6 mice blastocyst-stage chimeras. Germline transmission was proved by tail DNA PCR and Southern blot analysis. As shown in Fig. 2A, a 6.9-kb band with IA-2+/− DNA, a 10.7-kb band with IA-2−/− DNA, and both 6.9- and 10.7-kb bands with IA-2+/− DNA were seen in Fig. 2A.

FURTHER evidence that the homologous recombination was successful and disrupted the IA-2 allele to their offspring. Wild-type (IA-2+/+) and IA-2−/− mice were identified by tail DNA PCR with appropriate primers. As seen in Fig. 2A, wild-type IA-2+/+ mice were identified by a 1.3-kb PCR product; homozygous IA-2−/− mice, by a 3.5-kb PCR product; and heterozygous IA-2−/+ mice, by both 1.3- and 3.5-kb PCR products. The identification of these mice was confirmed by Southern blot analysis. As seen in Fig. 2B, tail DNA digested with KpnI and hybridized with the 1.1-kb XhoI-HindIII fragment (outside probe) and a 1.9-kb neo probe (data not shown). Three IA-2−/− ES cells clones were injected into blastocysts to make chimeric mice that transmitted the modified IA-2 allele to their offspring. Wild-type (IA-2+/+), heterozygous (IA-2−/+), and homozygous (IA-2−−) mice were identified by tail DNA PCR with appropriate primers.
IA-2 knockout and was expressed equally in IA-2+/+ and IA-2−/− mice (Fig. 2E). Western blot analysis, using rabbit anti-IA-2 sera to measure protein expression in mouse brain extract, showed no expression of IA-2 protein in IA-2−/− mice as compared to IA-2+/+ mice (Fig. 2F).

Physical examination of the IA-2−/− mice revealed no gross abnormalities. Litter size (data not shown) and body weight did not differ from the IA-2−/− knockout and was expressed equally in IA-2+/+ and IA-2−/− mice (Fig. 2E). Western blot analysis, using rabbit anti-IA-2 sera to measure protein expression in mouse brain extract, showed no expression of IA-2 protein in IA-2−/− mice as compared to IA-2+/+ mice (Fig. 2F).

Nonfasting blood glucose levels of the IA-2−/− male mice, measured over a 22-week period, were slightly elevated as compared to IA-2+/+ mice, but they did not fall within the diabetic range (Fig. 3B). Glucose tolerance tests, however, showed that glucose was significantly elevated at 15 and 30 min in both male and female IA-2−/− mice (Fig. 5A). Moreover, after glucose injection, acute insulin release was depressed in both male and female IA-2−/− mice, with statistically significant differences in the female mice and near significance (P = 0.055 at 15 min) in the male mice (Fig. 5B). In glucose-stimulated insulin secretion assays, pancreatic islets isolated from IA-2−/− mice also responded less vigorously to glucose stimulation. As shown in Fig. 6, male and female IA-2−/− mice released 48 and 42% less insulin, respectively, than IA-2+/+ mice when switched from basal (3.3 mmol/l) to high (27.7 mmol/l) glucose.

DISCUSSION
Insulin is located in the dense-core secretory granules of pancreatic β-cells. Dense-core granules are complex structures, but a number of their constituents are now known (25). IA-2, a transmembrane glycoprotein, is an integral component of dense-core granules (16). The intracellular domain of IA-2 is thought to protrude into the cytoplasm of the cell, whereas at least a portion of the extracellular (luminal) domain resides within dense-core granules. There are two potential dibasic (KK) cleavage sites located at amino acid positions 386 and 448-449 of the extracellular domain. Transfection and pulse chase experiments have shown that IA-2 is expressed as a 120-kDa glycosylated protein that is then processed into a predominant 64-kDa fragment and several smaller fragments (26). Whether these fragments from the extracellular domain remain within the cytoplasm or are retained within the secretory granules is still not clear, nor is their function known. Similar posttranslational modifications have been observed in bovine pituitary cells (27). Except for these studies and a reported correlation between secretagogue stimulation of β-cells and upregulation of IA-2 mRNA (28,29), very little is known about the cell biology of IA-2.

In the present study, we succeeded in deleting the IA-2 gene by targeted gene disruption. Northern and Western
A blot analysis showed that neither IA-2 mRNA nor protein was expressed. Although nonfasting blood glucose levels remained in the normal range, glucose tolerance tests revealed statistically significant elevated blood glucose levels in both male and female IA-2⁻/⁻ mice. Glucose-stimulated insulin secretion showed statistically depressed insulin release in female IA-2⁻/⁻ mice, with a similar trend, just missing statistical significance, in male
IA-2\(^{-/-}\) mice. Islets from both male and female IA-2\(^{-/-}\) mice also showed significantly lower insulin release than islets from IA-2\(^{+/+}\) mice when the cultures were switched from basal (3.3 mmol/l) to high (27.7 mmol/l) glucose levels. These findings, taken together with the known location of IA-2 in dense-core secretory granules, argue that IA-2 plays a role in insulin secretion. Although the alterations in glucose tolerance tests and insulin release are statistically significant, they are mild, and this may explain why IA-2\(^{-/-}\) mice do not develop elevated nonfasting blood glucose levels and overt diabetes.

The findings with IA-2\(^{-/-}\) mice raise questions about the contribution of IA-2\(\beta\), also known as phogrin, to the secretory process. IA-2\(\beta\) is structurally similar to IA-2, showing 74% identity within the intracellular domain. This protein also is an integral component of dense-core granules and is phosphorylated in a Ca\(^{2+}\)-sensitive manner in response to secretagogue stimulation of \(\beta\)-cells (30). It is therefore possible that IA-2 and IA-2\(\beta\) work together or that one serves in a compensatory capacity for the other. However, at least at the mRNA level, IA-2 knockout does not result in a compensatory increase in IA-2\(\beta\) mRNA (Fig. 2E). Recently we succeeded in deleting the IA-2\(\beta\) gene (A.K. and A.L.N., unpublished data), and experiments are now underway to determine whether this protein also is involved in insulin secretion.

In terms of the pathogenesis of type 1 diabetes, it is of interest to ask whether there is any relationship between the autoimmune response to IA-2 and the functional role of IA-2 in secretion. It is known that autoantibodies to IA-2 are directed exclusively to the intracellular domain of the molecule that protrudes into the cytoplasm. Because there is no evidence that these autoantibodies are internalized and act within the cytoplasm, it seems unlikely that the autoimmune response to IA-2 would have any effect on the secretory function of the IA-2 molecule.Depressed insulin secretion, however, is one of the features of type 2 diabetes. Although

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**FIG. 3.** Body weight and nonfasting blood glucose levels. Body weight (A) and nonfasting blood glucose levels (B) in male \((n = 6)\) and female \((n = 6)\) IA-2\(^{-/-}\) (■) and male \((n = 6)\) and female \((n = 6)\) IA-2\(^{+/+}\) (□) mice measured over 22 weeks. Data are means ± SE.
FIG. 4. Histological appearance and immunostaining of pancreases and brain from IA-2−/− mice. Sections of pancreases were fixed and stained with hematoxylin and eosin (A) or incubated with anti-insulin antibody (B), anti-glucagon antibody (C), or anti-somatostatin antibody (D) followed by biotin-conjugated second antibody and streptavidin horseradish peroxidase. Sections of brain (hippocampus [E] and cerebellum [F]) were fixed and stained with hematoxylin and eosin (magnification ×200 [A–E] and ×300 [F]).
no association between IA-2 and type 2 diabetes has been recognized thus far, any gene that is involved in insulin secretion becomes a possible candidate gene for type 2 diabetes.

The process of secretion is complex and encompasses a number of different signals and pathways (25). Secretory vesicles are transported from the Golgi to the plasma membrane, where docking, priming, and fusion take place. Insulin is then secreted by exocytosis, a process that involves Ca\(^{2+}\) influx through voltage-dependent channels and regulation of exocytosis by a variety of phosphorylation events (31). Where in this multistep process IA-2 plays a role is not known. Recently, by use of the yeast two-hybrid system and co-immunoprecipitation, several proteins have been identified that bind to IA-2. This includes \(\beta\)IV spectrin and the PDZ domains of \(\beta\)2-syntrophin and neuronal nitric oxide synthase. Solimena and colleagues (32,33) have postulated that IA-2 may link the secretory granules with the actin cystoskeleton through its association with \(\beta\)IV spectrin or \(\beta\)2-syntrophin, and that this might affect granule traffic and exocytosis. Alternatively, because nitric oxide regulates the release of certain hormones, it is possible that the association of IA-2 with nitric oxide synthase may modulate insulin secretion.

The demonstration in the present study that deletion of IA-2 affects insulin secretion and the identification in other studies of IA-2 binding proteins (32,33) begin to provide insight into the function and possible mechanism of action of IA-2. The fact that IA-2 is present in the secretory granules of many different neuroendocrine cells raises the possibility that IA-2 may be involved not only in the secretion of insulin from \(\beta\)-cells, but also in the secretion of hormones from a broad range of neuroendocrine cells.

FIG. 5. Glucose tolerance and insulin secretion tests. A: Intraperitoneal (IP) glucose tolerance tests in male \((n = 20)\) and female \((n = 22)\) IA-2\(^{+/+}\) (C) and male \((n = 15)\) and female \((n = 19)\) IA-2\(^{+/-}\) (●) mice. After overnight fasting, 2 g/kg body wt of glucose was injected intraperitoneally, and blood glucose levels were measured at different time points. B: Acute insulin secretion in response to intraperitoneal glucose in male \((n = 17)\) and female \((n = 17)\) IA-2\(^{+/+}\) (○) and male \((n = 17)\) and female \((n = 14)\) IA-2\(^{+/-}\) (●) mice. Blood samples were drawn from the tail vein using heparinized capillary tubes before and after glucose injection (3 g/kg body wt). The results represent the average of three independent experiments. Data are means ± SE. *\(P < 0.01\); **\(P < 0.001\).
FIG. 6. Glucose-stimulated insulin release in vitro. Pancreatic islets isolated from IA-2+/+ and IA-2−/− mice were placed in five wells, each containing 10 islets, in KRBB containing 3.3 mmol/l glucose (□), and at the end of 1 h insulin levels were again measured. The results represent the average of three independent experiments. Data are means ± SE. *P < 0.01; **P < 0.001.

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