NIM) Triton X-100 was added to 900 μl of sperm suspension in NIM [see (*12*)] and mixed by trituration for 30 s on ice. Cells were pelleted by centrifugation for 1 min at 20,000*g*, 2°C and thoroughly resuspended in 2 ml of ice-cold NIM before repelleting for 2 min at 20,000*g*, 2°C. We resuspended the final pellet in 400 μl of CZB or NIM. Before microinjection, we mixed 1 μl of the DNA fragment with 9 μl of sperm suspension (containing 2 to 5 × 10^5 spermatozoa in CZB or NIM) by pipetting, to give a final DNA fragment concentration of 7 ng/μl. We incubated the DNA-sperm mixture at room temperature (about 25°C) or on ice for 1 min and then mixed it with a polyvinylpyrrolidone (PVP; average M_r 360,000) solution to give a final concentration of about 10% (w/v) PVP; it was then placed on the microscope stage for microinjection. All injections were done in CZB-H at room temperature within 1 hour of sperm-DNA mixing or within 1 hour of sperm-Triton X-100 mixing.

15. Purified *lac*Z-harboring fragments of pxCANLacZ, linearized by digestion either with Sal GI or Xho I and Sal GI, were mixed with spermatozoa at concentrations of 4.5 and 9 ng/μl, respectively, and microinjected as described in (*14*); both fragments gave similar results; the pxCANLacZ Xho I–Sal GI fragment lacks a replication origin. The β-galactosidase encoded by pxCANLacZ contains a nuclear localization signal. We assessed pxCANLacZ β-galactosidase expression in day 3 embryos [T. Tsukui, Y. Kanegae, I. Saito, Y. Toyoda, *Nature Biotechnol.* **14**, 982 (1996)] after a 5-min fixation at room temperature in phosphate-buffered saline (PBS) (pH 7.6) containing 1% (v/v) formaldehyde, 0.2% (v/v) glutaraldehyde, and bovine serum albumin (BSA) (5 mg/ml). Fixed embryos were washed thoroughly in PBS containing BSA (5 mg/ml) and stained by incubation for 5 hours at 37°C in PBS containing BSA (5 mg/ml), 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (1 mg/ml). We examined and scored embryos by light microscopy.
16. We used single-shot double transgenesis to generate embryos coexpressing two tg's after a single microinjection as described in (*14*). Sperm heads were coinjected with a DNA solution containing pCX-EGFP Sal GI–Bam HI fragment (2.5 ng/μl) and pCX-LacZ Sal GI–Pst I fragment (2.5 ng/μl). pCX-LacZ is a derivative of pCX-EGFP in which the *EGFP* gene is replaced by one that encodes β-galactosidase [M. Okabe, unpublished data]. After culture in vitro, we first scored embryos for GFP expression and then for β-galactosidase expression as described in (*12, 15*). For photography, we mounted embryos between a microscope slide and a coverslip and collected images to show development and GFP expression before fixation and staining to show LacZ expression.
17. M. Lavitrano, D. French, M. Zani, L. Frati, C. Spadafora, *Mol. Reprod. Dev.* **31**, 161 (1992).
18. We divided the sperm suspension in each washing experiment into two 5-μl aliquots immediately after mixing and incubating with pCX-EGFP DNA for 1 min. One aliquot (washed sperm) was diluted and washed by mixing well with 50 μl of ice-cold, fresh CZB or NIM. We then pelleted both aliquots for 2 min at 20,000*g*, 2°C. The supernatant from the washed sperm aliquot was carefully removed and replaced with 5 μl of fresh CZB or NIM; we used the supernatant from the second aliquot to resuspend its own pellet (therefore, this sample was not washed).
19. We mixed a fresh dilution of the Sal GI–Bam HI fragment of plasmid pCX-EGFP (7 ng/μl in NIM) with an equal volume of PVP 20% (w/v) and injected about 1 pl per oocyte. After a recovery time of 5 to 10 min at room temperature, we transferred oocytes to Ca^{2+}-free CZB containing 10 mM $SrCl_2$ and the cytokinesis-blocking agent cytochalasin B at 5 μg/ml and incubated them for 6 hours at 37°C [A. Bos-Mikich, D. G. Whittingham, K. T. Jones, *Dev. Biol.* **182**, 172 (1997)]. We then transferred them to CZB and incubation continued under standard embryo culture conditions. We scored embryos for GFP expression after 3.5 days as described (*12*).
20. F. J. Longo, G. Krohne, W. W. Franke, *J. Cell Biol.* **105**, 1105 (1987).
21. T. Wakayama and A. C. F. Perry, unpublished data; B. Maione, C. Pittoggi, L. Achene, R. Lorenzini, C. Spadafora, *DNA Cell Biol.* **16**, 1087 (1997).
22. S. Ladha, P. S. James, D. Clark, E. A. Howes, R. Jones, *J. Cell Sci.* **110**, 1041 (1997).
23. R. Y. Tsien, *Annu. Rev. Biochem.* **67**, 509 (1998).
24. Ectopic GFP expression in skin was clearly discernible as a green color when pups were examined 1 to 4 days after delivery by incidental illumination from a UV light source (480 nm).
25. M. Okabe, unpublished data.
26. Tail-tip biopsies from 3- to 6-week-old, randomly selected green pups and their nongreen littermates were used for extraction of total, genomic DNA. Photography of tails was under a fluorescent stereomicroscope equipped with a 480/440-nm filter. In Southern blot analysis, 10 μg of genomic DNA per sample was digested with Eco RI and probed with the 733-base-pair Eco RI fragment of pCX-EGFP. We used forward (TTGAATTCGCCACCATGGTGAGC) and reverse (TTGAATTCTTACTTGTACAGCTCGTCC) oligonucleotide primers detection of the *GFP* gene by PCR of 1 μg of genomic DNA per reaction. Reaction parameters were 95°C for 9 min (1 cycle) and 94°C for 45 s, 60°C for 30 s, 72°C for 45 s (40 cycles). Electrophoretically separated products were visualized after staining with ethidium bromide.
27. K. L. Kroll and E. Amaya, *Development* **122**, 3173 (1996).
28. R. Yanagimachi in *The Physiology of Reproduction*, E. Knobil and J. D. Neill, Eds. (Raven Press, New York, ed. 2, 1994), pp. 189–317.
29. K. Goto, A. Kinoshita, Y. Takuma, K. Ogawa, *Vet. Rec.* **127**, 517 (1990); N.-H. Kim, J. W. Lee, S. H. Jun, H. T. Lee, K. S. Chung, *Mol. Reprod. Dev.* **51**, 436 (1998).
30. Y. Kimura and R. Yanagimachi, *Biol. Reprod.* **52**, 709 (1995).
31. S. Kuretake, Y. Kimura, K. Hoshi, R. Yanagimachi, *ibid.* **55**, 789 (1996).
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Control of Autoimmune Diabetes in NOD Mice by GAD Expression or Suppression in β Cells

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Glutamic acid decarboxylase (GAD) is a pancreatic β cell autoantigen in humans and nonobese diabetic (NOD) mice. β Cell–specific suppression of GAD expression in two lines of antisense GAD transgenic NOD mice prevented autoimmune diabetes, whereas persistent GAD expression in the β cells in the other four lines of antisense GAD transgenic NOD mice resulted in diabetes, similar to that seen in transgene-negative NOD mice. Complete suppression of β cell GAD expression blocked the generation of diabetogenic T cells and protected islet grafts from autoimmune injury. Thus, β cell–specific GAD expression is required for the development of autoimmune diabetes in NOD mice, and modulation of GAD might, therefore, have therapeutic value in type 1 diabetes.

Type 1 diabetes, or insulin-dependent diabetes mellitus, is the consequence of progressive T cell–mediated autoimmune destruction of pancreatic β cells (*1*, *2*). However, the initial events that trigger the destruction of β cells are incompletely understood. Several β cell autoantigens have been implicated in the triggering of β cell–specific autoimmunity (*1*, *3*). GAD is the strongest candidate in both humans and the NOD mouse, which is considered the best animal model of the human disease (*3*–*5*). In NOD mice, GAD, as compared with other β cell autoantigens examined, provokes the earliest T cell proliferative response (*4*, *5*). However, no unequivocal evidence exists to indicate that the β cell expression of GAD is required for the initiation of diabetes in NOD mice. To address this issue, we examined the effect of selectively suppressing GAD expression in the β cells of diabetes-prone NOD mice.

The suppression of β cell GAD expression was achieved by producing transgenic

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NOD mice with an antisense GAD transgene (*6*) for both isoforms of rat GAD cDNA (rGAD65 and rGAD67) under the control of the rat insulin promoter (RIP) (*7*) (Fig. 1A). Six lines of antisense GAD65.67 transgenic NOD mice were established, defined by relative amount of transgene expression (Fig. 1, B and C). The first three lines of transgenic mice with high, medium, and low levels of expression of antisense GAD65.67 in the β cells were designated H-AS-GAD-NOD, M-AS-GAD-NOD, and L-AS-GAD-NOD, respectively (Fig. 1, C and D), and the second three lines of antisense GAD65.67 transgenic mice were designated H_k-AS-GAD-NOD, M_k-AS-GAD-NOD, and L_k-AS-GAD-NOD, respectively (Fig. 1, B and C). Protein immunoblot analysis (*8*) revealed the complete suppression of β cell GAD expression in islets of H-AS-GAD-NOD mice, whereas moderate and low suppression was found in M- and L-AS-GAD-NOD transgenic mice, respectively (Fig. 1E). In contrast, GAD expression was detected equally in the brain tissue of transgene-negative NOD mice and the three lines of AS-GAD-NOD mice (Fig. 1E). The β cell–specific suppression of GAD expression was confirmed in H-AS-GAD-NOD mice, whereas different amounts of GAD expression were seen in transgene-negative, L-AS-GAD-NOD, and M-AS-GAD-NOD mice by immunohistochemical staining with antibodies to GAD and insulin (Fig. 1F) (*9*). The three lines of AS-GAD-NOD mice were indistinguishable from the transgene-negative littermates in pancreatic insulin content [H, 446 ± 46; M, 461 ± 51; L, 458 ± 47; control, 451 ± 42 (SD) (micrograms of insulin per gram of pancreas)] and plasma insulin concentrations [H, 4.1 ± 0.21; M, 4.3 ± 0.19; L, 4.6 ± 0.22; control, 4.5 ± 0.18 (SD) (nanograms of insulin per milliliter of plasma)]. Similar results regarding the suppression of GAD expression in β cells and insulin content in pancreas and plasma were obtained in the second three lines of antisense GAD65.67 transgenic NOD mice.

To determine whether GAD expression in the β cells was required for the development of autoimmune diabetes in NOD mice, we monitored disease development in the three lines of AS-GAD-NOD mice and in transgene-negative littermates. None (0 of 15) of the H-AS-GAD-NOD mice developed diabetes by 40 weeks of age. In contrast, 67% (12 of 18) of the M-AS-GAD-NOD mice, 75% (12 of 16) of the L-AS-GAD-NOD mice, and 81% (17 of 21) of the transgene-negative littermates developed diabetes by the same age (Fig. 2A). We examined the islet histology of the above groups at 20 weeks of age. Over 80% of the examined H-AS-GAD-NOD islets were intact (Fig. 2, B and E), and less than 20% of the islets showed periinsulitis (Fig. 2B). In contrast, most of the M-AS-GAD-NOD and L-AS-GAD-NOD islets examined showed moderate to severe insulitis, as did transgene-negative littermates (Fig. 2, B and F). In the second three lines of AS-GAD-NOD mice, diabetes appeared in 2.8% (1 of 36) of the H_k-AS-GAD-NOD mice, 83.3% (15 of 18) of the M_k-AS-GAD-NOD mice, and 80.8% (21 of 26) of the L_k-AS-GAD-NOD mice by 40 weeks of age, whereas 85.7% (18 of 21) of the transgene-negative littermates developed diabetes (Fig. 2C). One animal in the H_k-AS-GAD-NOD group did develop diabetes; whether this small difference between the H-AS-GAD-NOD and H_k-AS-GAD-NOD groups is due to leakiness in the H_k-AS-GAD-NOD group or differences in the susceptibility genes is uncertain. We also examined the islet histology of the H_k-, M_k-, and L_k-AS-GAD-NOD mice at 19 to 20 weeks of age (Fig. 2D) and found no significant difference in the extent of insulitis from that in the H-, M-, and L-AS-GAD-NOD groups (Fig. 2B).

Our findings indicate that β cell GAD expression is a requirement for the development of diabetes in NOD mice. The complete prevention of diabetes in H-AS-GAD-NOD mice and the near complete prevention of diabetes in H_k-AS-GAD-NOD mice are not likely to be due to a nonspecific effect of the antisense transgene incorporated into the chromosomal DNA, because low or moderate suppression of GAD expression in M-, M_k-, L-, and L_k-AS-GAD-NOD mice carrying the same antisense transgene did not result in the prevention of diabetes (Fig. 2, A and C). To examine this issue further, we developed another control for the antisense GAD transgenic NOD mice, namely, antisense transgenic NOD mice carrying the antisense endogenous murine leukemia proviral *env* region DNA under the control of the RIP.

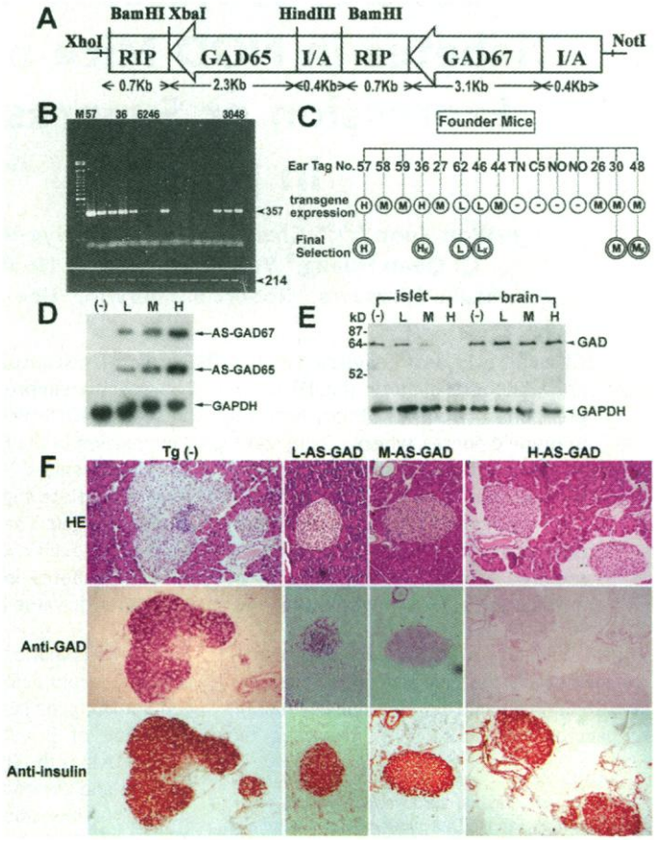

Fig. 1. Structure of RIP-antisense GAD65.67 transgene, pedigree of antisense GAD transgenic mouse lines, the expression of antisense GAD mRNA, and the suppression of GAD expression in β cells of AS-GAD transgenic NOD mice. (**A**) Diagram of RIP-antisense GAD65.67 transgene structure: RIP, SV40 small *t* intron, and a polyadenylation site (I/A). (**B**) Expression of antisense GAD transcript by reverse transcriptase PCR. Two micrograms of total RNA was converted to cDNA with sense rat GAD67 primer (5′-ATGACGTCTCCTACGATACA-3′), and the cDNA was amplified with sense and antisense GAD67 primers (5′-CCCCTTGAGGCTGGTAACCA-3′). As an internal standard, hypoxanthine-guanine phosphoribosyl-transferase mRNA was amplified with the following primers: sense, 5′-GTAATGATCAGTCAACGGGGGAC-3′; and antisense, 5′-CCAGCAAGCTTGCAACCTTAACCA-3′. Lane M, 100-bp ladder. Each lane corresponds to the ear tag number shown in (C). Numbers at right are the size of amplified product in base pairs. (**C**) Pedigree of AS-GAD transgenic mice. Eleven positive founder mice were obtained; six mice were selected on the basis of the expression of the antisense transcript and backcrossed with NOD mice. Ear tag number: TN, transgene-negative littermates; C5, C57BL/6 mice; and NO, nontransgenic NOD mice. Transgene expression: H, high; M, intermediate; L, low; and −, no expression of transgene. (**D**) Northern (RNA) blot analysis (*17*) of antisense GAD transcripts in the first three different lines of transgenic NOD mice (H-, M-, and L-AS-GAD-NOD) and transgene-negative littermates (−). (**E**) Protein immunoblot analysis (*8*) of the suppression of GAD expression in pancreatic islets and brain tissue in the first three different lines of transgenic NOD mice (H-, M-, and L-AS-GAD-NOD) and transgene-negative littermates (−). (**F**) Immunohistochemical staining (*9*) of pancreatic islets from 10-week-old H-, M-, and L-AS-GAD-NOD mice and transgene-negative [Tg (−)] littermates. Serial islet sections were stained with either hematoxylin and eosin (HE), antibody to GAD, or antibody to insulin. Magnification, ×200.

Endogenous retroviral *env* protein, a putative β cell autoantigen, is expressed in the β cells of NOD mice (*10*). These antisense transgenic NOD mice, unlike their GAD-suppressed counterparts, developed diabetes (79%, 15 of 19), as did the transgene-negative littermates (82%, 9 of 11) (*11*), even though the antisense transgene was highly expressed and effectively blocked the endogenous synthesis of viral protein. These results support the view that the prevention of diabetes in antisense GAD transgenic NOD mice is not due to the nonspecific effect of an antisense transgene incorporated into chromosomal DNA.

To determine whether β cell–specific suppression of GAD expression specifically affects β cell–specific autoimmunity, we examined the salivary gland, which also shows lymphocytic infiltration in diabetes-prone NOD mice. In contrast to the β cell, lymphocytic infiltration in the salivary gland of H-AS-GAD-NOD mice was not prevented (Fig. 2G), and sialitis was similar to that of transgene-negative littermates (Fig. 2H), indicating that autoimmunity was not affected in other tissues.

We next examined whether the suppression of GAD expression in the β cells inhibits disease development by blocking the generation of β cell–specific diabetogenic T cells. Splenocytes from 20-week-old nondiabetic female H-AS-GAD-NOD mice and age-matched nondiabetic transgene-negative littermates were transfused into 6- to 8-week-old NOD–severe combined deficiency disease (NOD.*scid*) mice (*12*). None of the NOD.*scid* recipients (0 of 8) of splenocytes from H-AS-GAD-NOD mice developed diabetes by 10 weeks after the transfer of splenocytes, whereas 90% (9 of 10) of the NOD.*scid* recipients of splenocytes from transgene-negative NOD mice developed diabetes within 9 weeks after transfer (Fig. 3A), as did NOD.*scid* recipients of splenocytes from acutely diabetic NOD mice. Similar results were obtained when we used splenocytes from H_k-AS-GAD-NOD mice. Thus, the generation of T cells capable of adoptively transferring diabetes is blocked in the absence of GAD expression in the β cells. In addition, we determined which T cell subsets ($CD4^+$ and $CD8^+$) are affected in H-AS-GAD-NOD mice. We found that the generation of both diabetogenic $CD4^+$ and $CD8^+$ T cells was blocked in the absence of GAD expression in the β cells (*11*).

Intravenous or intrathymus immunization of NOD mice with GAD suppresses T cell responses to GAD, heat shock protein (HSP) 60, carboxypeptidase H, and peripherin (*4*, *5*). To determine whether other β cell autoantigen-specific T cells developed in the absence of GAD in the β cells, we examined the proliferative response of splenocytes from 8- (Fig. 3B), 12- (Fig. 3C), and 15- (Fig. 3D) week-old H-AS-GAD-NOD mice, transgene-negative littermates, and control NOD mice to GAD and other β cell autoantigens (HSP60 and insulin) (*13*). In contrast to the transgene-negative control group, no proliferative response to GAD was detected in H-AS-GAD-NOD mice at any age tested. T cells only from the latter transgenic mice at 15 weeks of age showed a small but insignificant proliferative response to HSP60 or insulin (Fig. 3, C and D). Similar results were obtained when we used splenocytes from H_k-AS-GAD-NOD mice. Thus, β cell–specific suppression of GAD gene expression diminishes the T cell immune response to other β cell autoantigens as well as GAD.

The susceptibility of GAD-suppressed β cells to attack by diabetogenic T cells derived from acutely diabetic NOD mice was evaluated by transplanting GAD-suppressed islets from H-AS-GAD-NOD mice or GAD-expressing islets from young, transgene-negative male NOD mice into the renal subcapsular region of acutely diabetic NOD mice (*14*). All recipients (6 of 6) of GAD-expressing islets showed a recurrence of diabetes (Fig. 4A); most of the grafted islets showed massive infiltration by mononuclear cells within 1 week and were destroyed within 2 weeks (Fig. 4, B and C, bottom). In contrast, none of the recipients (0 of 7) of GAD-suppressed islets showed a recurrence of diabetes up to 40 days after transplantation, at the termination of the experiment (Fig. 4A). Furthermore, over 80% of the grafted GAD-suppressed islets remained intact, and about 20% showed periinsulitis (Fig. 4, B and C, top). Most of these islets were positively stained by antibody to insulin. When we transplanted GAD-suppressed islets from H_k-AS-GAD-NOD mice into the renal subcapsular region

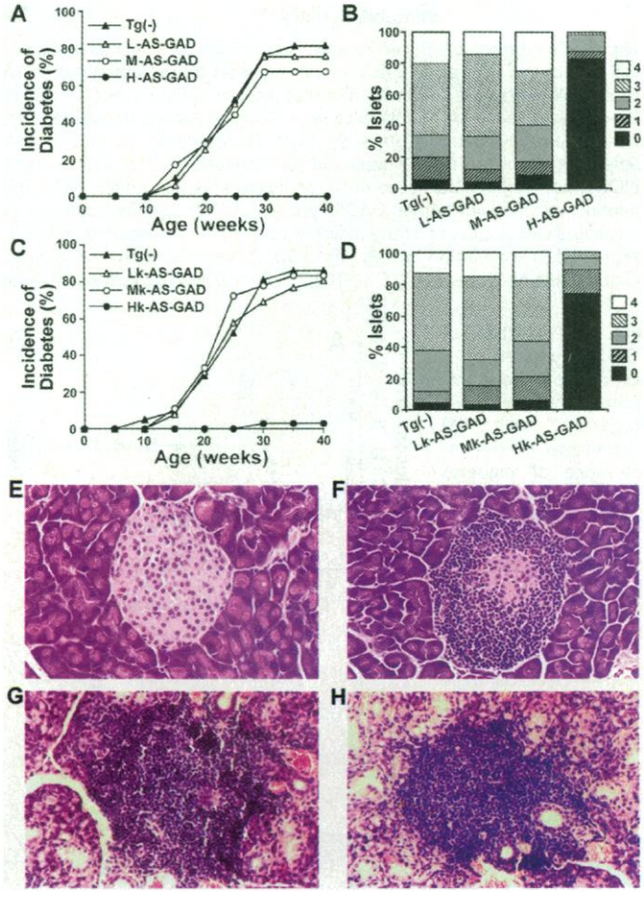

Fig. 2. The effect of β cell–specific suppression of GAD expression on the development of diabetes and insulitis. (**A**) The incidence of diabetes in the first three different lines of AS-GAD-NOD mice, at the seventh generation with a NOD background. Cumulative incidence of diabetes was determined by positive glycosuria and confirmed by hyperglycemia (nonfasting blood glucose > 16.7 mM) on 2 consecutive days up to 40 weeks of age. (**B**) Histological examination of insulitis in H-, M- and L-AS-GAD-NOD mice and transgene-negative littermates. Histological examination of pancreatic islets at 20 weeks of age; shown are results from five randomly selected nondiabetic mice at 20 weeks of age (at least 20 islets per mouse examined). Grade: 0, normal islets; 1, mononuclear infiltration, largely in the periphery, in less than 25% of the islet; 2, 25 to 50% of islet showing mononuclear infiltration; 3, over 50% of islet showing mononuclear infiltration; and 4, small, retracted islet with few mononuclear cells. (**C**) Incidence of diabetes in the second three different lines of AS-GAD-NOD mice at the seventh generation with a NOD background. (**D**) Histological examination of insulitis in H_k-, M_k-, and L_k-AS-GAD-NOD mice and transgene-negative littermates. (**E** to **H**) Photomicrographs of representative pancreatic islet (E and F) and salivary gland (G and H) sections from H-AS-GAD-NOD mice and transgene-negative NOD littermates. Paraffin sections of pancreas or salivary gland were stained by HE. (E) H-AS-GAD-NOD pancreatic section (intact islets). (F) Transgene-negative NOD littermate pancreatic section (severe lymphocytic infiltration). (G) Salivary gland sections of H-AS-GAD-NOD mice (severe lymphocytic infiltration). (H) Salivary gland sections of transgene-negative NOD littermate (severe lymphocytic infiltration). Magnification, ×400.

of acutely diabetic NOD mice, similar results were observed. In contrast, the transplantation of *env*-suppressed islets from antisense *env* transgenic NOD mice resulted in their destruction within 2 weeks (*11*). Thus, the *env*-suppressed islets were not resistant to the cytotoxic effect of diabetogenic T cells, suggesting that the resistance of GAD transgenic NOD islets is a specific rather than a nonspecific effect. In keeping with these results, when splenocytes from acutely diabetic NOD mice were transfused into 6-week-old, irradiated, male H-AS-GAD-NOD mice and age- and sex-matched transgene-negative littermates, none of the H-AS-GAD-NOD mice (0 of 9) developed diabetes, whereas 71% (5 of 7) of the transgene-negative control recipients developed diabetes within 4 weeks after transfer (Fig. 4D), again demonstrating that GAD expression is required for autoimmune destruction of β cells.

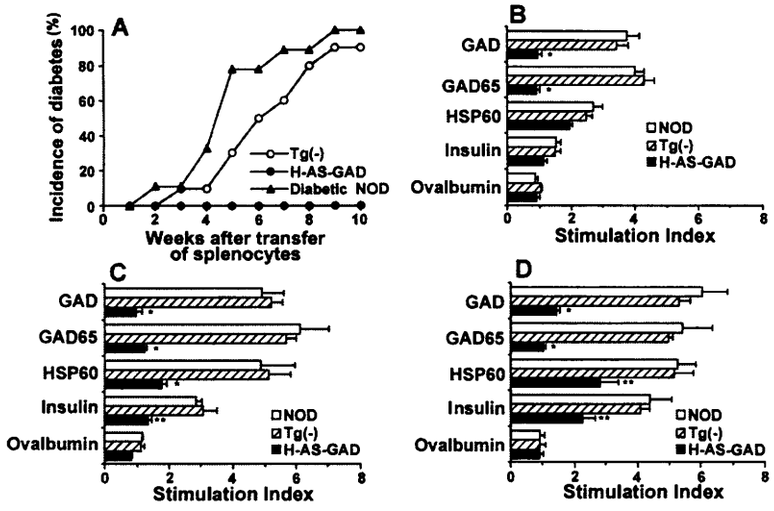

Fig. 3. The effect of β cell–specific suppression of GAD expression on the development of β cell–cytotoxic T cells and T cell immune responses to islet autoantigens. (**A**) Incidence of diabetes in 6- to 8-week-old female NOD.*scid* mice that received splenocytes (1×10^7 cells per mouse) isolated from 20-week-old H-AS-GAD-NOD mice ($n = 8$), age-matched transgene-negative littermates ($n = 10$), or newly diabetic NOD mice ($n = 9$). (**B** to **D**) Splenic T cell proliferative response to islet antigens. Splenocytes isolated from 8-week-old (B), 12-week-old (C), and 15-week-old (D) female H-AS-GAD-NOD mice, female transgene-negative littermates, or female NOD mice were reacted with GAD peptide, recombinant human GAD65 protein, HSP60, porcine insulin, or ovalbumin, and the cells were incubated with 1 μCi of [^{3}H]thymidine. Proliferation was determined by [^{3}H]thymidine uptake. Data are expressed as stimulation indices (SI) ± SD of the mean from five individual mice, tested in triplicate. Cutoff value of SI was 2.0. *, $P < 0.01$; **, $P < 0.05$ as compared with transgene-negative littermates.

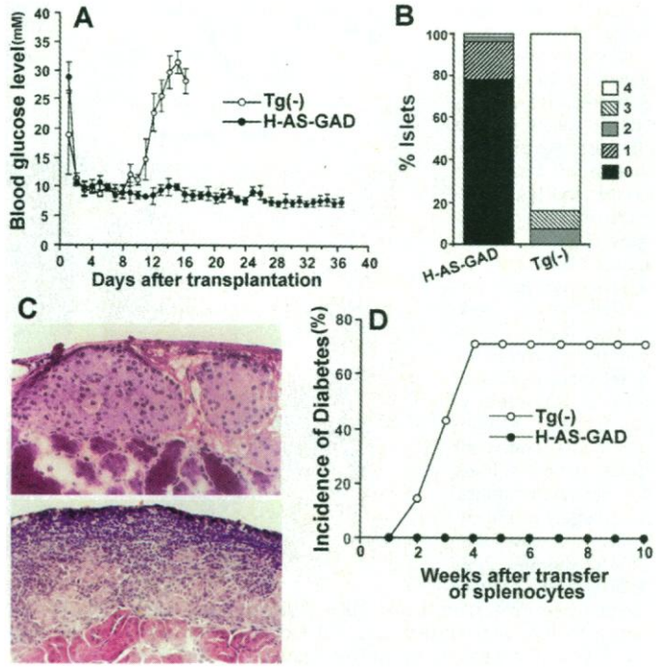

Fig. 4. Protection of GAD-suppressed β cells from autoimmune attack by diabetogenic T cells. (**A**) Prevention of the recurrence of diabetes by the transplantation of GAD-suppressed islets into the subrenal capsule of acutely diabetic NOD mice. Acutely diabetic NOD mice received islets (400 islets per mouse) from 4-week-old male H-AS-GAD-NOD ($n = 7$) or age- and sex-matched transgene-negative littermates ($n = 6$). Blood glucose was measured every other day after islet transplantation. (**B**) Insulitis grade in islet grafts from H-AS-GAD-NOD mice and transgene-negative littermates. The insulitis grades are described in Fig. 2B. (**C**) Photomicrographs of representative islet grafts from H-AS-GAD-NOD mice (top) (intact islets in the kidney capsule) and transgene-negative littermates (bottom) (massive infiltration of islets by mononuclear cells in the kidney capsule). Magnification, ×400. (**D**) Adoptive transfer of diabetes to H-AS-GAD-NOD mice or transgene-negative littermates by acutely diabetic splenocytes. Irradiated, 6-week-old male H-AS-GAD-NOD mice ($n = 9$) or transgene-negative littermates ($n = 7$) received splenocytes (1×10^7 cells per mouse) from acutely diabetic NOD mice.

Previous studies involving GAD immunization (*4*, *5*, *15*) and GAD-reactive T cells (*16*) support a role for GAD in the induction of autoimmune diabetes in NOD mice. Our data show that β cell–specific suppression of GAD expression is sufficient to nearly completely prevent autoimmune diabetes in NOD mice. This occurs in association with the suppression of GAD-reactive T cells. Thus, GAD expression is essential for the induction of diabetogenic T cells, and diabetogenic T cells cannot provoke diabetes in NOD mice in the absence of GAD from β cells.

References and Notes

1. R. Tisch and H. McDevitt, *Cell* **85**, 291 (1996); A. A. Rossini, D. L. Greiner, H. P. Friedman, J. P. Mordes, *Diabetes Rev.* **1**, 43 (1993); F. S. Wong and C. A. Janeway Jr., *Res. Immunol.* **148**, 327 (1997); J. W. Yoon, H. S. Jun, P. S. Santamaria, *Autoimmunity* **27**, 109 (1998).
2. M. Nagata and J. W. Yoon, *Diabetes* **41**, 998 (1992).
3. S. Baekkeskov *et al.*, *Nature* **347**, 151 (1990); S. Baekkeskov *et al.*, *J. Clin. Invest.* **79**, 926 (1987); M. A. Atkinson, N. K. Maclaren, D. Scharp, P. E. Lacy, W. J. Riley, *Lancet* **335**, 1357 (1990); M. A. Atkinson *et al.*, *ibid.* **339**, 458 (1992); J. Endl *et al.*, *J. Clin. Invest.* **99**, 2405 (1997).
4. D. L. Kaufman *et al.*, *Nature* **366**, 69 (1993).
5. R. Tisch *et al.*, *ibid.*, p. 72.
6. The 7.6-kb RIP-AS-GAD65.67 full-length transgene was microinjected into fertilized eggs of (C57BL/6 × SJL)F2 mice [J. W. Gordon, *Methods Enzymol.* **225**, 747 (1993)]). The founder mice were screened for the incorporation of the transgene into the genomic DNA by Southern blotting and polymerase chain reaction (PCR). Six selected transgene-positive founder mice were then backcrossed with NOD mice for seven generations to establish AS-GAD65/67 transgenic NOD mice (AS-GAD-NOD). The mice were screened for the transmission of the transgene with PCR, with rGAD67-specific primers. Female mice were used to determine the incidence of diabetes unless specifically mentioned otherwise. Immunoreactive insulin content in the pancreas and plasma insulin concentration (seven mice per group) were determined, as previously described [J. W. Yoon, M. A. Lesniak, R. Fussganger, A. L. Notkins, *Nature* **264**, 178 (1976)].
7. A recombinant RIP-DIPA/pXF3 plasmid vector [D. Hanahan, *Nature* **315**, 115 (1985)], which carries the RIP sequence [695 base pairs (bp)], 770 bp of diphtheria toxin gene (DIPA), the simian virus 40 (SV40) early gene terminator (400 bp) with a small *t* intron, and a polyadenylation site (I/A), was partially digested with Bam HI. The RIP-DIPA-I/A fragment (1.9 kb) isolated from the RIP-DIPA/pXF3 vector was cloned into the Xba I–Hind III site of the pBluescriptIISK vector (Stratagene). After removing the DIPA gene, we inserted rGAD65 cDNA (2.3 kb) (RIP-AS-GAD65) or rGAD67 cDNA (3.1 kb) (RIP-AS-GAD67) into the vector in antisense orientation. To construct the RIP-

antisense GAD65.67 transgene, we ligated antisense GAD65 complete transcription unit, which had been isolated from RIP-AS-GAD65 by digestion with Sal I and Not I, at the Sal I site of RIP-AS-GAD67 to produce RIP-AS-GAD65.67. The 7.6-kb RIP-AS-GAD65.67 full-length transgene (Fig. 1A) was separated from pBluescriptIISK by digestion with Xho I and Not I.

8. The total protein extracts from the islets or brain tissue of H-, M-, and L-AS-GAD-NOD mice as well as H_k-, M_k- and L_k-AS-GAD-NOD mice and their respective transgene-negative littermates were prepared, and 20 μg of protein was separated by 10% SDS–polyacrylamide gel electrophoresis. After transfer to a nitrocellulose membrane (Amersham), the membrane was reacted with a 1:3000 dilution of polyclonal rabbit antibody to GAD67 (Chemicon), and the GAD protein was detected by the biotin-streptavidin-peroxidase method, with a chemoluminescence system. As an internal control, the same membrane was probed with antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon).
9. Paraffin blocks were prepared and sectioned [P. Gilon, M. Tappaz, C. Remacle, *Histochemistry* **96**, 355 (1991)], the sections were reacted with GAD1 monoclonal antibody (ATCC), and GAD expression was detected by the avidin-biotin-peroxidase complex method with a Vectastain Elite ABC kit (Vector Laboratories). Serial sections of the pancreas were also stained with guinea pig polyclonal antibody to insulin (Vector).
10. Y. Kang, K. S. Kim, K. H. Kim, J. W. Yoon, unpublished data.
11. The data are available at www.sciencemag.org/feature/data/986073.shl
12. To measure the generation of diabetogenic T cells in GAD-suppressed transgenic NOD mice, we did adoptive transfer [T. Kawamura, M. Nagata, T. Utsugi, J. W. Yoon, *J. Immunol.* **151**, 4362 (1993); M. Nagata, P. Santamaria, T. Utsugi, J. W. Yoon, *ibid.* **152**, 2042 (1994)]. Splenocytes isolated from 20-week-old nondiabetic female H-AS-GAD-NOD mice, H_k-AS-GAD-NOD mice, and their respective age-matched, transgene-negative littermates were transfused intravenously (1×10^7 cells per mouse) into 6- to 8-week-old NOD.*scid* mice. The animals were monitored three times per week for glycosuria (>+2) and hyperglycemia (>16.7 mM) [J. W. Yoon, M. M. Rodrigues, C. Currier, A. Notkins, *Nature* **296**, 566 (1982)]. To determine whether the GAD-suppressed β cells are protected from autoimmune attack by diabetogenic T cells, we transfused splenocytes (1×10^7 cells per mouse) from acutely diabetic NOD mice intravenously into 6-week-old, irradiated, male H-AS-GAD-NOD mice and age-matched transgene-negative control male NOD mice. The animals were monitored as described above.
13. Splenocytes were isolated from individual 8-, 12-, and 15-week-old H-AS-GAD-NOD mice or H_k-AS-GAD-NOD mice, their respective transgene-negative littermates, and control NOD mice, and a proliferation assay was performed as described previously (*2*, *4*, *5*). The splenocytes (1×10^6 cells per well) were plated in 200 μl of culture medium in triplicate and reacted with GAD peptide (mixed 17, 34, and 35 peptides; 7 μM) (*4*), recombinant human GAD65 protein (Syntax), HSP60 (StressGen), porcine insulin (Sigma), or ovalbumin (Sigma) at 20 μg/ml for 72 hours and pulsed with 1 μCi of [^{3}H]thymidine. The incorporation of [^{3}H]thymidine was measured.
14. Islets were isolated from 4-week-old male H-AS-GAD-NOD or H_k-AS-GAD-NOD mice or age- and sex-matched transgene-negative littermates and transplanted (400 islets per mouse) into the renal subcapsular region of acutely diabetic NOD mice [T. Utsugi, M. Nagata, T. Kawamura, J. W. Yoon, *Transplantation* **57**, 1799 (1994)]. The animals were monitored for glycosuria and hyperglycemia as described above. Sections of the kidney capsules containing the transplanted islets were stained with haematoxylin and eosin and examined for lymphocytic infiltration.
15. J. F. Elliott *et al.*, *Diabetes* **43**, 1494 (1994).
16. D. Zekzer *et al.*, *J. Clin. Invest.* **101**, 68 (1998).
17. The total RNA was isolated from the pancreas of H-, M-, and L-AS-GAD-NOD mice and transgene-negative littermates with Trizol (Gibco BRL). The total RNA (20 μg) was separated by agarose-formaldehyde gel electrophoresis, transferred to a nylon membrane, and probed with in vitro transcribed sense rGAD65 or rGAD67 RNA. As an internal control, the same membrane was probed with antisense GAPDH RNA.
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Inactivation of Misselected CD8 T Cells by CD8 Gene Methylation and Cell Death

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Misselected CD8 cells that express T cell receptors (TCRs) that do not recognize class I major histocompatibility complex (MHC) protein can emerge from thymic selection. A postthymic quality control mechanism that purges these cells from the repertoire is defined here. The failure of mature CD8 cells to simultaneously engage their TCR and CD8 coreceptor triggers an activation process that begins with inhibition of CD8 gene expression through remethylation and concludes with up-regulation of surface Fas and Fas ligand and cellular apoptosis. Thus, inhibition of a death signal through continued TCR-CD8 coengagement of MHC molecules is a key checkpoint for the continued survival of correctly selected T cells. Molecular defects that prevent delivery of the death signal to mistakenly selected T cells underlie the expansion of double-negative T cells, which is the cellular signature of a subset of systemic autoimmune diseases.

The selection process in the thymus that regulates the repertoire of T cell subsets is effective but not foolproof. Thymocytes that coexpress CD8 normally recognize MHC class I, but class II–reactive CD8 cells can mistakenly arise through interactions either with strong peptide agonists or with self-peptides that antagonize CD4 but not CD8 cell activation (*1*), perhaps reflecting reduced $p56^{lck}$ signaling (*2*) or up-regulation of Notch 1 (*3*). T cells bearing antigen receptors that do not functionally engage self-MHC products can also emerge from the thymus (*4*). Although CD8 cells that bear mismatched TCRs may be functionally active in vitro (*1*, *5*), they do not contribute substantially to normal immune responses (*6*). Thus, CD8 cells that express TCRs that fail to engage class I may normally be inactivated by a postthymic mechanism.

We first examined the fate of CD8 cells that express a TCR that does not interact with class I MHC. CD8 cells bearing the class II–restricted DO11.10 TCR transgene survive thymic selection and are exported in substantial numbers to peripheral tissues (*2*, *7*). However, our observations indicate that they do not express a stable CD8 phenotype. Within 2 to 3 days after the adoptive transfer of purified $DO11^+$ CD8 cells into syngeneic Balb/C (H-2^d, $\beta_2M^{+/+}$) or MHC-deficient (C57BL/6J $\beta_2M^{-/-} \times$ I-$A_\beta^{b-/-}$) hosts, these cells down-regulate CD8 coreceptor expression (Fig. 1A). The kinetics of CD8 down-regulation in syngeneic hosts and in hosts deficient in both class I and class II MHC are equivalent despite the availability in normal hosts of class I MHC for CD8 engagement and class II MHC for TCR engagement. In contrast, CD8 cells that express a TCR transgene specific for a class I–restricted antigen [histocompatibility-Y antigen (H-Y)] (*8*) continue to express the CD8 coreceptor after a similar period in adoptive syngeneic (female) hosts (Fig. 1). Thus, CD8 engagement seems necessary for stable CD8 expression.

To further test the hypothesis that continued expression of the CD8 receptor may require coengagement of CD8 and TCR by MHC class I peptide complexes in peripheral tissues, we tested the effects of class I deficiency on continued expression of CD8 by peripheral T cells. CD8 cells from $\beta_2M^{+/+}$ ($Thy1.1^+$) mice were infused into syngeneic $\beta_2M^{-/-}$ or $\beta_2M^{+/+}$ ($Thy\ 1.2^+$) hosts. Within 5 to 10 days, virtually all donor cells recov-

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