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Glucose-Dependent Insulin Release from Genetically Engineered K Cells

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Genetic engineering of non-β cells to release insulin upon feeding could be a therapeutic modality for patients with diabetes. A tumor-derived K-cell line was induced to produce human insulin by providing the cells with the human insulin gene linked to the 5′-regulatory region of the gene encoding glucose-dependent insulinotropic polypeptide (GIP). Mice expressing this transgene produced human insulin specifically in gut K cells. This insulin protected the mice from developing diabetes and maintained glucose tolerance after destruction of the native insulin-producing β cells.

Diabetes mellitus (DM) is a debilitating metabolic disease caused by absent (type 1) or insufficient (type 2) insulin production from pancreatic β cells. In these patients, glucose control depends on careful coordination of insulin doses, food intake, and physical activity and close monitoring of blood glucose concentrations. Ideal glucose levels are rarely attainable in patients requiring insulin injections (*1*). As a result, diabetic patients are presently still at risk for the development of serious long-term complications, such as cardiovascular disorders, kidney disease, and blindness.

A number of studies have addressed the feasibility of in vivo gene therapy for the delivery of insulin to diabetic patients. Engineering of ectopic insulin production and secretion in autologous non-β cells is expected to create cells that evade immune destruction and to provide a steady supply of insulin. Target tissues tested include liver, muscle, pituitary, hematopoietic stem cells, fibroblasts, and exocrine glands of the gastrointestinal tract (*2–7*). However, achieving glucose-dependent insulin release continues to limit the clinical application of these approaches. Some researchers have attempted to derive glucose-regulated insulin production by driving insulin gene expression with various glucose-sensitive promoter elements (*8*). However, the slow time course of transcriptional control by glucose makes synchronizing insulin production with the periodic fluctuations in blood glucose levels an extremely difficult task. The timing of insulin delivery is crucial for optimal regulation of glucose homeostasis; late delivery of insulin can lead to impaired glucose tolerance and potentially lethal episodes of hypoglycemic shock. Therefore, what is needed for insulin gene therapy is a target endocrine cell that is capable of processing and storing insulin and of releasing it in such a way that normal glucose homeostasis is maintained.

Other than β cells, there are very few glucose-responsive native endocrine cells in the body. K cells located primarily in the stomach, duodenum, and jejunum secrete the hormone GIP (*9*, *10*), which normally functions to potentiate insulin release after a meal (*11*). Notably, the secretion kinetics of GIP in humans closely parallels that of insulin, rising within a few minutes after glucose ingestion and returning to basal levels within 2 hours (*12*). GIP expression (*13*) and release (*14*) have also been shown to be glucose-dependent in vitro. However, the mechanism that governs such glucose-responsiveness is unclear. We made an interesting observation of glucokinase (GK) expression in gut K cells (Fig. 1A). GK, a rate-limiting enzyme of glucose metabolism in β cells, is recognized as the pancreatic "glucose-sensor" (*15*). This observation raises the possibility that GK may also confer glucose-responsiveness to these gut endocrine cells. Given the similarities between K cells and pancreatic β cells, we proposed to use K cells in the gut as target cells for insulin gene therapy.

A GIP-expressing cell line was established to investigate whether the GIP promoter is effective in targeting insulin gene expression to K cells. This cell line was cloned from the murine intestinal cell line STC-1, a mixed population of gut endocrine cells (*16*). K cells in this population were visually identified by transfection of an expression plasmid containing ~2.5 kb of the rat GIP promoter fused to the gene encoding the enhanced green fluorescent protein (EGFP) (*17*). After clonal expansion of the transiently fluorescent cells, clones were analyzed for the expression of GIP mRNA by Northern blotting (*18*). The amount of GIP mRNA in one clone (GIP tumor cells; GTC-1) was ~8 times that in the parental heterogeneous STC-1 cells (Fig. 1B). Transfection of GTC-1 cells with the human genomic preproinsulin gene linked to the 3′ end of ~2.5 kb of the rat GIP promoter (Fig. 1C, GIP/Ins) resulted in a correctly processed human preproinsulin mRNA transcript (*19*) (Fig. 1D). When the same GIP/Ins construct was transfected into a β-cell line (INS-1), a liver cell line (HepG2), and a rat fibroblast (3T3-L1) cell line, little human preproinsulin mRNA was detectable (*20*). These observations suggest that the GIP promoter used is cell-specific and is likely to be effective in targeting transgene expression specifically to K cells in vivo. Western blot analysis revealed that the proprotein convertases required for correct processing of proinsulin to mature insulin (PC1/3 and PC2) (*21*) were expressed in GTC-1 cells (Fig. 1E) (*22*). Consistent with this observation, a similar molar ratio of human insulin and C pep-

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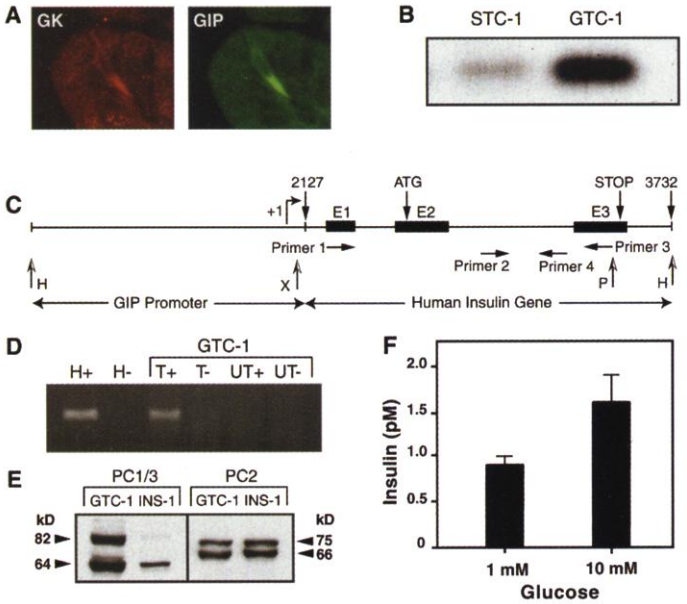

Fig. 1. Expression of human insulin in tumor-derived K cells. (**A**) Immunofluorescence staining for glucokinase (GK, red) and GIP (green) in mouse duodenal sections. (**B**) Northern blot analysis of GIP mRNA in STC-1 and GTC-1 cells. K-cell enrichment was determined by comparing the amount of GIP mRNA in the parental cell line (STC-1) with that of the newly subcloned K-cell lines. (**C**) Schematic diagram of the plasmid (GIP/Ins) used for targeting human insulin expression to K cells. The rat GIP promoter (~2.5 kb) was fused to the genomic human preproinsulin gene, which comprises 1.6 kb of the genomic sequence extending from nucleotides 2127 to 3732 including the native polyadenylation site. The three exons are denoted by filled boxes (E1, E2, and E3). The positions of primers used for RT-PCR detection of proinsulin mRNA are indicated. Hind III (H), Xho I (X), and Pvu II (P) sites are shown. Positions of start (ATG) and stop codons are indicated. (**D**) RT-PCR analysis of cDNA from human islets (H) and GTC-1 cells either transfected (T) or untransfected (UT) with the GIP/Ins construct. Samples were prepared either in the presence (+) or absence (−) of reverse transcriptase. (**E**) Western blot analysis of proprotein convertases PC1/3 and PC2 expression in a β-cell line (INS-1) and GTC-1 cells. Arrowheads indicate products at the predicted size for PC1/3 isoforms (64 and 82 kD) and PC2 isoforms (66 and 75 kD). (**F**) Effects of glucose on insulin secretion from GTC-1 cells stably transfected with the GIP/Ins construct. Triplicate wells of cells were incubated in media containing either 1 or 10 mM glucose (*22*). Medium was collected after 2 hours in each condition and assayed for human insulin. Values are means ± SEM; $P < 0.03$.

tide was observed in culture medium from cells transfected with the GIP/Ins construct. Furthermore, release of insulin from these cells was glucose-dependent (Fig. 1F) (*23*).

To determine whether the GIP/Ins transgene can specifically target expression of human insulin to gut K cells in vivo, we generated transgenic mice by injecting the linearized GIP/Ins fragment into pronuclei of fertilized mouse embryos (*24*). In the resulting transgenic mice, human insulin was expressed in duodenum and stomach, but not in other tissues examined (Fig. 2A). The insulin mRNA detected in the duodenum sample from the transgenic mice was confirmed by reverse transcription–polymerase chain reaction (RT-PCR) to be a product of the transgene and not contamination from adjacent mouse pancreas (*25*) (Fig. 2B). This tissue distribution of insulin gene expression in transgenic animals corresponds to the known tissue expression pattern of GIP (*9*, *10*). The cellular localization of human insulin protein was determined in tissue samples from transgenic mice by using antisera to human insulin. Insulin immunoreactivity was detected in distinct endocrine cells in sections from stomach (Fig. 2C, left) and duodenum (Fig. 2C, middle) of transgenic animals. Furthermore, these cells were identified as K cells by the coexpression of immunoreactive GIP (*26*) (Fig. 2C, right), confirming that human insulin production was specifically targeted to gut K cells. Plasma levels of human insulin in pooled samples collected after an oral glucose challenge were 39.0 ± 9.8 pM (n = 10, mean ± SEM) in transgenic and undetectable in controls (n = 5). It is interesting that amounts of mouse C peptide after an oral glucose load in transgenics were ~30% lower than those of controls (227.1 ± 31.5 pM versus 361.5 ± 31.2 pM, n = 3 in each group, mean ± SEM) (*27*). This observation suggests that human insulin produced from the gut may have led to compensatory down-regulation of endogenous insulin production.

Whether human insulin production from gut K cells was capable of protecting transgenic mice from diabetes was investigated. Streptozotocin (STZ), a β-cell toxin, was administered to transgenic mice and age-matched controls. In control animals, STZ treatment resulted in fasting hyperglycemia (26.2 ± 1.52 mM, n = 3, mean ± SEM) and the presence of glucose in the urine within 3 to 4 days, indicating the development of diabetes. When left untreated, these animals deteriorated rapidly and died within 7 to 10 days. In contrast, neither glucosuria nor fasting hyperglycemia (9.52 ± 0.67 mM, n = 5, mean ± SEM) was detected in transgenic mice for up to 3 months after STZ treatment, and they continued to gain weight normally. To determine whether insulin production from K cells was able to maintain oral glucose tolerance in these mice, despite the severe β-cell damage by STZ, mice were challenged with an oral glucose load (*28*). Control mice given STZ were severely hyperglycemic both before and after the glucose ingestion (Fig. 3A). In contrast, STZ-treated transgenic mice had normal blood glucose levels and rapidly disposed of the oral glucose load as did normal age-matched control mice (Fig. 3A). To ensure that the STZ treatment effectively destroyed the β cells in these experimental animals, pancreatic sections from controls and STZ-treated transgenic animals were immunostained for mouse insulin (*26*). The number of cell clusters positively stained for mouse insulin was substantially lower in STZ-treated animals when compared with sham-treated controls (Fig. 3B). Total insulin in the pancreas (*29*) in STZ-treated transgenic mice was only 0.5% that of the sham-treated controls (0.18 versus 34.0 μg insulin per pancreas, n = 2). These STZ-treated transgenic mice disposed of oral glucose in the same way that normal mice do, despite having virtually no pancreatic β cells, which indicates that human insulin produced from the gut was sufficient to maintain normal glucose tolerance. Previous attempts to replace insulin by gene therapy prevented glucosuria and lethal consequences of diabetes, such as ketoacidosis, but were unable to restore normal glucose tolerance (*2*). Our findings suggest that insulin production from gut K cells may correct diabetes to the extent of restoring normal glucose tolerance.

The identification of a glucose-responsive endocrine cell target for endogenous insulin production represents an important step toward a potential gene therapy for DM. However, an effective means of therapeutic gene delivery to gastrointestinal cells needs to be developed. There are many features of the upper gastrointestinal tract that make it an attractive target tissue for gene therapy. This region of the gut is readily accessible by noninvasive techniques, such as oral formulations or endoscopic procedures, for therapeutic gene transfer. The gut epithelium is also one of the most rapidly renewing tissues in the body and has a large number of proliferative cells, thus allowing the deployment of retroviral vectors that are approved for human investigation. Indeed, the gut—the largest endocrine organ—may have the highest concentration of stem cells

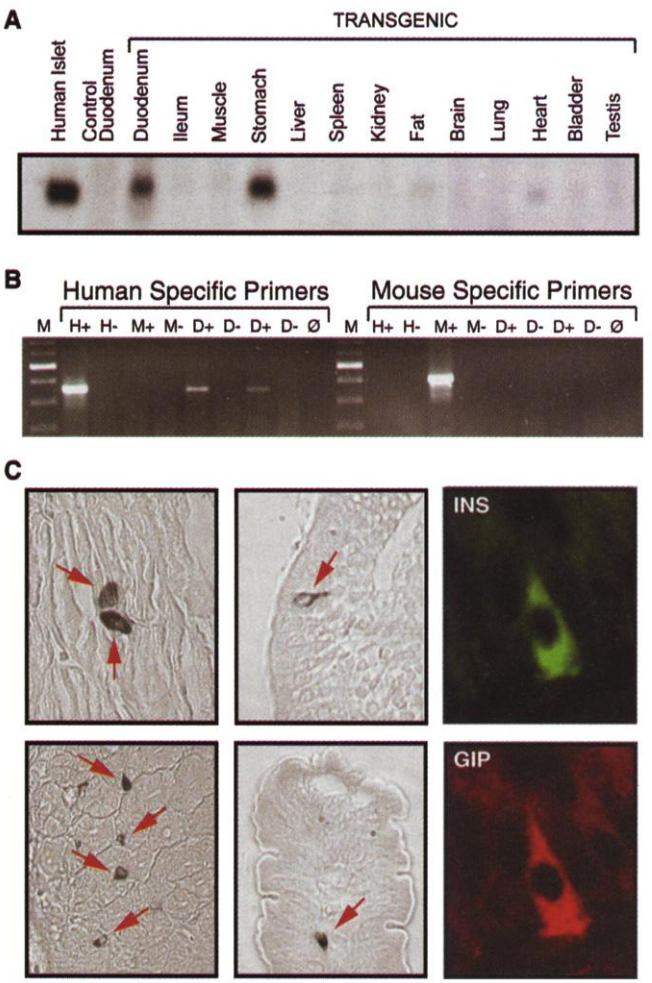

Fig. 2. Targeted expression of human insulin to K cells in transgenic mice harboring the GIP/Ins transgene. (**A**) Northern blot analysis for human insulin gene expression in human islet, control mouse duodenum, and transgenic mouse tissues. The blot was probed with a 333-base pair cDNA fragment encompassing exons 1 and 2 and part of exon 3 of the human preproinsulin gene. (**B**) RT-PCR analysis of cDNA from human islets (H), mouse islets (M), and duodenum samples (D) from two transgenic mice, with primers specific for human or mouse proinsulin. Samples were prepared either in the presence (+) or absence (−) of reverse transcriptase. ø, no DNA; M, markers. (**C**) Immunohistochemical staining for human insulin in sections of stomach (left column) and duodenum (middle column) from a transgenic mouse. Arrows indicate human insulin immunoreactive cells. Duodenal sections from the same animal were also examined by immunofluorescence microscopy (right column). Tissue sections were costained with antisera specific for insulin (INS, green) and GIP (red).

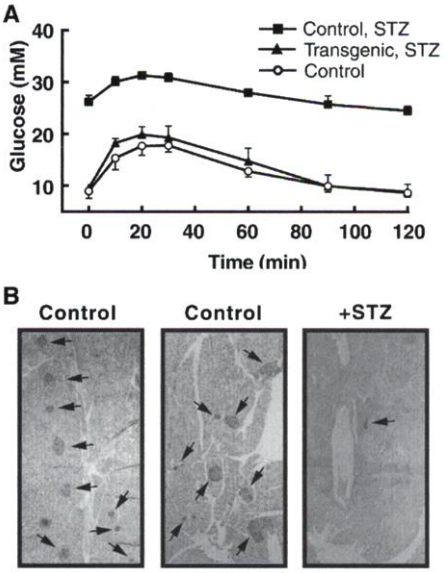

Fig. 3. Production of human insulin from K cells protects transgenic mice from diabetes induced by destruction of pancreatic β cells. (**A**) Oral glucose tolerance tests. Mice were given intraperitoneal injection of streptozotocin (STZ, 200 mg/kg) or an equal volume of saline. On the fifth day after treatment, after overnight food deprivation, glucose (1.5 g/kg body weight) was administered orally by feeding tube at 0 min. Results are means (±SEM) from at least three animals in each group. (**B**) Immunohistochemical staining for mouse insulin in pancreatic sections from control mice and an STZ-treated transgenic mouse. Arrows indicate mouse islets.

found anywhere in the body (*30*). These cells, which give rise to the various cells lining the gut epithelium, including billions of K cells (*31*), are situated in the crypts of Lieberkühn (*30*). Successful transduction of these stem cells should allow long-term expression of the transgene, as occurs in our transgenic mice. Viral vectors have already been developed that deliver genes to cells of the intestinal tract, including the stem cells (*32*, *33*). Given the massive number of K cells, appropriately regulated insulin secretion from a fraction of these cells may be sufficient for adequate insulin replacement for patients with diabetes. This gene therapy approach is also amenable to the expression of alternate insulin analogs, which could have more potent glucose-lowering activity and/or longer duration of action, as required. Therefore, genetic engineering of gut K cells to secrete insulin may represent a viable mode of therapy for diabetes, freeing patients from repeated insulin injections and reducing or even eliminating the associated debilitating complications.

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17. The rat GIP promoter was obtained from a rat genomic λ DASH library (Stratagene, La Jolla, CA) by plaque hybridization with the rat GIP cDNA clone as described previously [M. O. Boylan *et al.*, *J. Biol. Chem.* **273**, 17438 (1997)]. The GIP promoter was subcloned into the promoterless pEGFP-1 plasmid (Clontech, Palo Alto, CA). The resulting reporter vector was transfected into STC-1 cells (gift from D. Drucker, University of Toronto) using LipofectAMINE reagent (GIBCO BRL/Life Technologies, Rockville, MD). Cells were dispersed with trypsin/EDTA, and fluorescent cells expressing EGFP were doubly hand-picked and placed into individual dishes for clonal expansion.
18. Total RNA from GTC-1 and STC-1 cells was isolated with Trizol (GIBCO) according to manufacturer's instructions. Total cell RNA (20 μg from each sample) was electrophoretically separated and transferred to nylon membrane. Hybridization was performed with the radiolabeled 660-bp Eco RI fragment of the rat GIP cDNA that was random-primed with [α-^{32}P]deoxycytidine 5′-triphosphate (dCTP). After hybridization, membranes were washed and exposed to x-ray film.
19. Reverse transcription–PCR analysis was used to determine whether the preproinsulin gene is appropriately transcribed and processed in transfected cells. Total RNA was isolated with Trizol. Total RNA (5 μg) isolated from transfected and nontransfected cells and human islets was reverse-transcribed with oligo(dT) primer by using superscript II reverse transcriptase (GIBCO). The cDNA product (2 μl) was then amplified with human preproinsulin gene-specific primers (primers 1 and 3, Fig. 1C).
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22. Cells were lysed in ice-cold radioimmunoprecipitation assay buffer and supernatants were assayed for total protein content by using the Bradford method [M. Bradford, *Anal. Biochem.* **72**, 248 (1976)]. Cell lysate protein (50 μg) was fractionated on 10%

SDS–polyacrylamide gel electrophoresis. After gel separation, proteins were electroblotted onto nitrocellulose membranes and incubated with polyclonal antibodies that recognize PC1/3 and PC2 (provided by I. Lindberg, Louisiana State Medical Center). Membranes were washed and then incubated with goat antiserum to rabbit coupled to horseradish peroxidase (Amersham Pharmacia Biotech, Uppsala, Sweden). The blots were then developed with a chemiluminescence Western blotting detection kit.

23. GTC-1 cells grown to 70 to 80% confluence in 12-well plates were given restricted nutrients for 2 hours in Dulbecco's minimum essential medium (DMEM) with 1.0 mM glucose and 1% fetal calf serum (FCS). Cells were washed and then incubated in 0.5 ml of release media (DMEM plus 1% FCS with either 1.0 or 10.0 mM of glucose) for 2 hours. Insulin levels in media were measured using the human-specific insulin ELISA kit [American Laboratory Products Company (ALPCO), Windham, NH] according to supplier's instructions.
24. The GIP/Ins fragment (4.2 kb) was excised with Hind III and gel-purified. Transgenic mice were generated by pronuclear microinjection of the purified transgene into fertilized embryos that were then implanted into pseudopregnant females. Transgenic mice were identified by Southern blot analysis. Ear sections were digested, and the purified DNA was cut with Xho I and Pvu II (Fig. 1C), electrophoretically separated, and transferred to nylon membrane. For the detection of the transgene, a 416-bp human insulin gene fragment encompassing intron 2 was amplified by using primers 2 and 4 (Fig. 1C). The PCR product was prepared as a probe by radiolabeling with [α-^{32}P]dCTP, and bands were detected by autoradiography. Southern analysis results were further confirmed by PCR amplification of the genomic DNA using primers 2 and 4. Positive founders were outbred with wild-type FVB/N mice to establish transgenic lines.
25. Primers used were human proinsulin–specific, forward 5′-CCAGCCGCAGCCTTTGTGA-3′ and reverse 5′-GGTACAGCATTGTTCCACAATG-3′; mouse proinsulin–specific, forward 5′-ACCACCAGCCCTAAGTGAT-3′ and reverse 5′-CTAGTTGCAGTAGTTCTCCAGC-3′. PCR conditions were as follows: denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min for 45 cycles. PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining. The human- and mouse-specific primer sets yield 350-bp and 396-bp products, respectively.
26. Tissues were fixed in Bouin's solution overnight and embedded in paraffin. Tissue sections 5 μm thick were mounted on glass slides. For immunohistochemistry, the avidin-biotin complex method was used with peroxidase and diaminobenzidine as the chromogen. Sections were incubated with guinea pig antibody to insulin (1:500, Linco Research, St. Charles, MO) or mouse antibody to GIP (1:200, a gift from R. Pederson, University of British Columbia) for 30 min and appropriate secondary antibodies for 20 min at room temperature. Biotinylated secondary antibodies were used for immunohistochemistry, and fluorescein- or Cy3-conjugated secondary antibodies were used for immunofluorescence.
27. Plasma insulin levels were measured using the ultrasensitive human-specific insulin ELISA kit (ALPCO) according to supplier's instructions. This assay has <0.01% cross-reactivity with human proinsulin and C peptide and does not detect mouse insulin. Plasma C-peptide measurements were made with a rat/mouse C-peptide radioimmunoassay kit (Linco Research). The assay displays no cross-reactivity with human C peptide.
28. Streptozotocin was administered to 8-week-old transgenic and age-matched control mice via an intraperitoneal injection at a dose of 200 mg/kg body weight in citrate buffer. At this high dose of streptozotocin, mice typically display glucosuria within 3 days after injection. For oral glucose tolerance tests, glucose was administered orally by feeding tube (1.5 g/kg body weight) as a 50% solution (w/v) to mice that had been without food for 14 hours. Blood samples (40 μl) were collected from the tail vein of conscious mice at 0, 10, 20, 30, 60, 90, and 120 min after the glucose load. Plasma glucose levels were determined by enzymatic, colorimetric assay (Sigma), and plasma insulin levels were measured using the ultrasensitive human-specific insulin ELISA kit (*27*).
29. Pancreata were homogenized and then sonicated at 4°C in 2 mM acetic acid containing 0.25% bovine serum albumin. After incubation for 2 hours on ice, tissue homogenates were resonicated and centrifuged (8000*g*, 20 min), and supernatants were assayed for insulin by radioimmunoassay.

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Response to RAG-Mediated V(D)J Cleavage by NBS1 and γ-H2AX

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Genetic disorders affecting cellular responses to DNA damage are characterized by high rates of translocations involving antigen receptor loci and increased susceptibility to lymphoid malignancies. We report that the Nijmegen breakage syndrome protein (NBS1) and histone γ-H2AX, which associate with irradiation-induced DNA double-strand breaks (DSBs), are also found at sites of V(D)J (variable, diversity, joining) recombination–induced DSBs. In developing thymocytes, NBS1 and γ-H2AX form nuclear foci that colocalize with the T cell receptor α locus in response to recombination activating gene (RAG) protein–mediated V(D)J cleavage. Our results suggest that surveillance of T cell receptor recombination intermediates by NBS1 and γ-H2AX may be important for preventing oncogenic translocations.

V(D)J recombination is initiated by lymphoid-specific recombination activating gene 1 (RAG1) and RAG2 proteins, which introduce DSBs precisely between immunoglobulin and T cell receptor (TCR) coding gene segments and flanking recombination signal sequences. RAG-mediated cleavage generates four broken-end intermediates: two blunt signal ends and two covalently closed coding hairpin ends (*1*). The subsequent resolution of V(D)J ends into coding and signal joints requires ubiquitously expressed factors that function in general DSB repair (*2*, *3*). Although V(D)J recombination generates DNA damage, it has been presumed that broken DNA intermediates, which associate with RAG proteins within a postcleavage synaptic complex (*4*, *5*), are sequestered from the DNA damage surveillance machinery. Primary DNA damage sensors include histone H2AX, which becomes rapidly phosphorylated (γ-H2AX) in response to external damage (*6*, *7*), and the MRE11/RAD50/NBS1 complex, which forms ionizing irradiation-induced foci at DSBs (*8*, *9*). Although γ-H2AX and MRE11/RAD50/NBS1 appear to play an important role in monitoring chromosome integrity, the physiological conditions that activate these DNA damage surveillance/signaling factors have not been described.

To determine whether NBS1 and γ-H2AX are present at antigen receptor gene–specific breaks introduced during V(D)J recombination, we examined wild-type thymocytes by immunofluorescence analysis (*10*). We found that approximately 20% of freshly isolated thymocytes showed intense NBS1 and γ-H2AX foci (Fig. 1, A and B). Dual immunostaining revealed that H2AX was generally phosphorylated in the same nuclear domains where NBS1 foci were found (Fig. 1, I through K). The majority of thymocytes with intense NBS1/γ-H2AX staining contained one distinct spot, although cells were occasionally found to contain two, and less frequently, three or more foci. In contrast, multiple foci were distributed throughout

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