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A Graves' Disease-Associated Kozak Sequence Single-Nucleotide Polymorphism Enhances the Efficiency of *CD40* Gene Translation: A Case for Translational Pathophysiology

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We analyzed the mechanism by which a Graves' disease-associated C/T polymorphism in the Kozak sequence of *CD40* affects CD40 expression. CD40 expression levels on B cells in individuals with CT and TT genotypes were decreased by 13.3 and 39.4%, respectively, compared with the levels in CC genotypes ($P = 0.012$). Similarly, Rat-2 fibroblasts transfected with T-allele cDNA expressed 32.2% less CD40 compared with their C-allele-transfected counterparts ($P = 0.004$). Additionally, an *in vitro* transcription/translation system showed that the T-allele makes 15.5% less CD40 than the C-allele ($P < 0.001$), demonstrating that the effect of the single-nucleotide polymorphism (SNP) on CD40 expression is at the level of translation. However, the SNP did not affect transcription, because the mRNA levels of *CD40*, as measured by quantitative RT-PCR, were independent of genotype. Therefore, our results may suggest that the C allele of the *CD40* Kozak SNP, which is associated with Graves' disease, could predispose to disease by increasing the efficiency of translation of *CD40* mRNA. (*Endocrinology* 146: 2684–2691, 2005)

OF THE CONSTELLATION of surface receptor molecules involved in B cell regulation, CD40, perhaps, has been dealt the most versatile and central role. A member of the TNF-R receptor family of molecules (1), CD40 is expressed on all nonterminally differentiated B cells. CD40 plays a fundamental role in B cell activation because its ligation provides the necessary costimulatory signal for B cell proliferation, Ig class switching, antibody secretion, the prevention of apoptosis of germinal-center B cells, affinity maturation, and the generation of long-lived memory cells (reviewed in Ref. 2). As underscored by the immune system defects seen both in HIGM3 (hyper IgM) patients harboring mutations in *CD40* and in *CD40* gene-targeting experiments in mice (reviewed in Ref. 3), CD40 is absolutely required for the development of a humoral immune response.

Activation of B cells requires the engagement of their surface CD40 molecules by its ligand CD154, expressed on activated $CD4^+$ T helper cells (4). Upon activation of the CD40 pathway, B cells enter lymphoid germinal centers and undergo differentiation into plasma cells that secrete large titers of high-affinity antibodies (5). A stimulated B cell, receiving no secondary CD40 signal, will have a limited lifespan and is restricted to making modest amounts of low-affinity antibodies. Indeed, blocking CD40-CD154 engagement prevented the development of both primary and secondary antibody responses (6).

The pivotal role of CD40 in the regulation of B cells has been shown to be relevant in autoimmune diseases with a strong humoral component. For example, blocking CD154 ameliorated experimental autoimmune myasthenia gravis (7). Another antibody-mediated autoimmune disease in which CD40 has been postulated to play a role is Graves' disease (GD) (8, 9), an antibody-mediated autoimmune disease characterized by hyperthyroidism, diffuse goiter, lymphocytic infiltration of the thyroid, and hallmark TSH receptor-stimulating antibodies, mimicking the action of TSH (10). Indeed, CD40 has been shown to be up-regulated in the thyroids of patients with GD. Recently, we (11) and others (12, 13) have shown that the *CD40* gene locus was linked and associated with GD. Sequencing the entire *CD40* gene led to the identification of a C/T polymorphism in the Kozak sequence of the 5′ untranslated region (UTR). Subsequently, case-control association studies have demonstrated an association of the C allele with GD (11, 12). Because the Kozak sequence is known to be of importance for the initiation of translation of a nascent mRNA molecule (14), we hypothesized that the strategic location of the polymorphism may affect *CD40* translational efficiency. Our data suggest that the CD40 polymorphism acts at the level of translation rather than transcription and could exert its influence on disease etiology by subtly affecting the amount of CD40 protein present.

Materials and Methods

Sequencing of the mouse CD40 5′ UTR region

To detect whether Kozak polymorphisms in CD40 exist in other species, we analyzed the mouse CD40 5′ UTR region sequence in 19

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Abbreviations: EAT, Experimental autoimmune thyroiditis; FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GD, Graves' disease; GFP, green fluorescent protein; IRES, internal ribosome entry site; PE, R-phycoerythrin; PMBC, peripheral mononuclear blood cell; SLE, systemic lupus erythematosus; SNP, single-nucleotide polymorphism; TCA, trichloroacetic acid; UTR, untranslated region.

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distinct strains of mice (see *Results*). Genomic mouse DNA was purchased from The Jackson Laboratory (Bar Harbor, ME). A total of 168 bp of 5′ UTR, encompassing the Kozak sequence, was sequenced from each strain. Mouse genomic DNA was amplified as described above for the CD40 Kozak single-nucleotide polymorphism (SNP) analysis using the following primers: forward primer, CTCCTAGCAGGGACTTTGGA; reverse primer, AGCCCTAACTCCCTTGGAGG. The PCR product was purified using the QIAquick gel extraction kit (QIAGEN, Valencia, CA). It was then sequenced using the PerkinElmer DNA sequencing kit (PE Applied Biosystems, Foster City, CA) and separated on an ABI-310 automated sequencer.

CD40 constructs and subcloning

All primers used were synthesized by PROLIGO Primers and Probes (Boulder, CO). The human *CD40* cDNA construct (pCD40.neo) was generously provided by Gail Bishop (Department of Microbiology, University of Iowa, Iowa City, IA). Fifty nanograms of *Sal*I-linearized pCD40.neo were used as a template with 250 nM forward primer (5′-TCC**CCGCGG**GCGCCCAGTGGTCCTGCCGCC-3′, *Sac*II site in *bold*) and 250 nM reverse primer (5′-ATT**CTCGAG**CTGTCTCTCCTGCACT-GAGATGCG-3′, *Xho*I site in *bold*); Deep Vent DNA polymerase (2 U; catalog no. M0258, New England Biolabs, Beverly, MA) was added per reaction. PCR cycling was as follows: 95 C for 5 min, followed by 36 cycles with 95 C for 30 sec, 55 C for 30 sec, 72 C for 1 min; after cycling, a 72 C incubation was performed for 10 min. The amplicon was subcloned into the *Sac*II/*Xho*I sites of pIRES-hrGFP-1a (catalog no. 240031; Stratagene, La Jolla, CA). The construct, termed CD40pIRES.C, includes the 39 bases of 5′ UTR and has the C Kozak polymorphism. The T Kozak polymorphism was introduced into CD40.pIRES.C, making CD40.pIRES.T, using the QuikChangeII XL Site-Directed Mutagenesis Kit (Stratagene catalog no. 200521), as per the manufacturer's instructions, and primers 5′-CTGGTCTCACCTCGC**T**ATGGTTCGTCTGCCT-3′ and 5′-AGGCAGAC-GAACCAT**A**GCGAGGTGAGACCAG-3′, where the base incorporating the C>T change is *bold*. Subsequently, CD40.pIRES.C and CD40.pIRES.T were used as templates to transfer the CD40 into *Xba*I/*Eco*RV sites pcDNA3.1(−) (catalog no. V795-20; Invitrogen, Carlsbad, CA), using forward primer 5′-TCC**TCTAGA**GCGCCCAGTGGTCCTGCCGCC-3′ and reverse primer 5′-AAAGGG**GATATC**TCACTGTCTCTCCTGCA-C-3′, where the respective *Xba*I and *Eco*RV sites are *bold* and the stop codon is *underlined*. The CD40 constructs in pCDNA 3.1(−) were termed CD40.pcDNA.C and CD40.pcDNA.T, respectively. All constructs used in this study were sequenced, so as to verify the integrity of their *CD40* inserts. The following primers were employed during sequencing: hCD40rev876 (5′-CTGTCTCTCCTGCACTGAGATGCG-3′), CD40fw600 (5′-TGTGGTCCCCAGGATCGGCTG-3′), CD40rev600 (5′-CAGCCGATC-GTGGGGACCACA-3′), and hCD40rev100 (5′-TGCAGTGGGTGGTT-CTGGATG-3′).

In vitro transcription/translation assays

The efficiency of CD40 protein synthesis was assessed using a reticulocyte lysate system, by comparing CD40.pcDNA.C and CD40.pcDNA.T as the templates. Reactions were performed, according to the manufacturer's instructions, with 1 μg of plasmid template and the inclusion of 50 μCi of ^{35}S-labeled cysteine (catalog no. SJ232; Amersham, Piscataway, NJ) to the reaction mixture (50 μl total volume), supplemented with a cysteine-free amino acid mix. Negative controls consisted of adding an equivalent volume of H_2O in lieu of the plasmid. Reactions were allowed to proceed for 60 min. Protein synthesis was quantified by two separate means. First, 2 μl of translation reaction were trichloroacetic acid (TCA)-precipitated and transferred to a 24-mm GF/c glass fiber filter (catalog no. 1822024; Whatman, Clifton, NJ). Acid-precipitable counts, reflecting total protein synthesis, were measured using a liquid scintillation counter (Tri-Carb 4000; Packard, Meriden, CT). Second, 15 μl of translation mixture were run on a 12% polyacrylamide gel under reducing conditions. After electrophoresis, the contents of the gel were transferred to a 0.45-μm nitrocellulose filter (catalog no. 162-0214; Bio-Rad, Hercules, CA). Subsequently, the filter was exposed to x-ray film (CL-Xposure, catalog no. 34092; Pierce, Rockford, IL). X-ray film analysis revealed the presence of a discrete band, migrating at approximately 40 kDa, that was nonexistent in negative control samples. The intensity of the 40-kDa band was quantified using the program Unscanit, where the intensity of the band was measured and the intensity of a clear region of film was subtracted away, so as to account for background contributions. The 40-kDa product is smaller than the reported 48-kDa CD40 size, as the protein, *in vivo*, exists in glycosylated form and the rabbit reticulocyte system lacks necessary the machinery for full glycosylation.

Antibodies

The antibody used to detect surface CD40 expression was clone G28-5 (catalog no. HB-9110; American Type Culture Collection, Manassas, VA), originally raised against human erythrocyte-rosette-negative tonsillar lymphocytes and later demonstrated to be specific for the Bp50 (CD40) antigen on human B cells (15). G28-5 hybridomas were grown in RPMI 1640 (catalog no. 31800-022; Invitrogen). RPMI 1640 was adjusted to 1.5 g $NaHCO_3$ per liter, 10 mM HEPES acid, 4.5 g/liter glucose, 1 mM sodium pyruvate, 1% penicillin/streptomycin, and 10% heat-inactivated FBS (catalog no. APA20428; Hyclone, Logan, UT). Supernatant from G28-5 was used at 1:2 dilution. Secondary antibodies used were R-phycoerythrin-conjugated $F(ab')_2$ goat antimouse IgG (catalog no. 115-116-071; Jackson ImmunoResearch Laboratories, West Grove, PA) and R-phycoerythrin-conjugated whole-molecule goat antimouse IgG (Jackson ImmunoResearch catalog no. 115-115-164). CD19 was detected with AlexaFluor-conjugated mouse antihuman CD19 (catalog no. 557697; BD Biosciences, San Diego, CA). Isotype controls consisted of Alexa-fluor conjugated IgG1 (catalog no. MG120; Caltag, Burlingame, CA) and an unconjugated, nonspecific mouse IgG1 (Takao Ando, Mount Sinai School of Medicine; personal gift).

Cell transfections and analysis

The cell line used in this study was the rat embryonic fibroblast cell line, Rat2 (ATCC catalog no. CRL-1764). Rat2 cells are a highly transfectable cell line that have been previously demonstrated to be capable of high cell surface levels of human proteins (16). Cells were grown in DMEM (Invitrogen catalog no. 11965092) with high glucose containing L-glutamine and pyridoxine-HCL. Cells were grown at 37 C with 5% CO_2 and 90% humidity. Cells were transfected with lipofectamine 2000 (Invitrogen catalog no. 11668-027), according to the manufacturer's protocol. In brief, cells were plated into 24-well tissue-culture-treated plates (catalog no. 353047; Falcon, Franklin Lakes, NJ) and were transfected with either 0.5 or 1 μg of CD40 plasmid DNA per well. Cells were analyzed 48 h after transfection. Fluorescence-activated cell sorting (FACS) (FACScan; Becton Dickinson Immunocytometry Systems, Mountain View, CA) was used to assess the level of CD40 surface expression. Staining was performed directly on the tissue culture plates. Initially, medium was aspirated off, and the cell monolayer was washed with tissue-culture grade PBS (catalog no. 21-040-CV; Mediatech, Herndon, VA). Cells were blocked through the addition of 150 μl per well of FACS buffer, PBS, containing 0.1% BSA (catalog no. A-7888; Sigma, St. Louis, MO) and 0.01% sodium azide, for 15 min at room temperature. After the block, 150 μl of G28-5 supernatant was added to each well and allowed to incubate overnight at 4 C. After the incubation with primary antibody, cells were washed twice with PBS. Subsequently, R-phycoerythrin (PE)-conjugated whole-molecule goat antimouse IgG was diluted 1:200 in FACS buffer and 200 μl was added per well and allowed to incubate for 30 min at room temperature. After two washes with PBS, cells were detached through the addition of 200 μl of 2 mM EGTA/EDTA in PBS. Cells were centrifuged for 5 min at 1000 $\times$ *g* and were resuspended in 500 μl of PBS before FACS analysis. Negative controls consisted of cells transfected with empty plasmid DNA and incubation with primary and secondary antibodies.

For transfections done with CD40.pIRES.C/T constructs, the methodology was identical to that described above. However, because the signal was typically lower than seen with the CD40.pCDNA constructs (signal was close to two orders of magnitude greater than background), background corrections were employed in the analyses. The RNA transcribed from the internal ribosome entry site (IRES) vectors is bicistronic, due to the presence of an IRES preceding a hrGFP cDNA. Both CD40 and green fluorescent protein (GFP) will be translated from a single mRNA; normalizing to GFP fluorescence should account for any differences that may exist between mRNA levels and, moreover will elucidate whether the polymorphism is acting at the level of translation. Cells transfected with empty pcDNA vector and stained with both primary and second-

ary antibodies were used to obtain background levels for green and PE fluorescence.

B cell isolation, stimulations, and analysis

Twenty milliliters of blood were drawn from healthy volunteers and were mixed in heparinized tubes at room temperature. The anticoagulated whole blood was diluted 1:2 with PBS and was layered on an equal volume of Ficoll-Paque (Amersham catalog no. 171440-02), and peripheral mononuclear blood cells (PMBCs) were isolated according to the manufacturer's instructions. Purified PMBCs were resuspended in 2 vol of PBS and filtered through a 70-μm nylon cell strainer (BD Falcon catalog no. 352350), and centrifugation at 100 $\times$ *g* for 10 min at 20 C followed. The pellet was then washed and resuspended in 2 vol of IMag buffer (PBS supplemented with 0.5% BSA and 2 mM EDTA) (BD Biosciences) and centrifuged as described previously. IMag buffer was aspirated off and the cell pellet was resuspended in 50 μl of antihuman CD19-conjugated magnetic nanoparticles (BD Biosciences catalog no. 551520), in fresh IMag buffer, and incubated at room temperature for 30–45 min. B cells expressing CD19 were selectively removed from the PMBC mixture by exposing the cells to a magnetic field (BD Biosciences catalog no. 552311), according to the manufacturer's instructions. After three rounds of binding and washing, cells were resuspended in 1 ml of RPMI 1640 medium, supplemented with 10% FBS and 1% peniciliin/streptomycin. FACS analysis confirmed that more than 95% of the cells were positive for CD19 expression. Typical B cell yields ranged from 1×10^5 to 1×10^6 cells per 20 ml of blood. Purified B cells were grown in cell culture for 16 h after harvesting in ultra-low-attachment sterile plates (catalog no. 3471; Corning, Corning, NY). The B cells were split so that 0.5 ml were added to 3.5 ml of RPMI 1640, and the other 0.5 ml were added to 3.5 ml of RPMI 1640 containing 200 U/ml of recombinant human interferon-γ (catalog no. 300-02; Peprotech, Rocky Hill, NJ). Hence, B cells, with respect to CD40 expression, were examined both in resting and in stimulating conditions.

CD40 surface expression, on B cells, was assessed by employing FACS (Becton Dickinson FACScan). Cells were stained for CD19 and CD40, and only those that were doubly positive were analyzed. Antibodies used are described above and all steps were carried out at room temperature. The method used for detecting CD19 and CD40 is a modification of Stewart's protocol for labeling cells (Stewart CC, Stewart SJ. Methods Cell Biol 1994; 41: 39–60), where one antibody is unconjugated and the other is directly conjugated. After the 16-h incubation with or without interferon-γ, cells were pelleted and then resuspended in 200 μl of PBS to which 12 μl of purified goat IgG (Jackson ImmunoResearch catalog no. 005-000-003) had been added. After a 10- to 15-min incubation, 200 μl of G28-5 supernatant was added, whereas control cells received 200 μl of RPMI 1640 medium with 20 μl of IgG1-purified isotype control (0.1 μg/ml). Incubation proceeded for 30 min. Cells were centrifuged twice, as described previously, and washed with PBS. Cells were resuspended in 100 μl of RPMI 1640 with 10% FBS, containing a 1:200 dilution of PE-conjugated $F(ab')_2$ goat antimouse IgG. Incubation proceeded for 15 min. Cells were pelleted and washed twice and resuspended in 100 μl of 10% FBS/RPMI 1640, with the addition of 6 μl of purified mouse IgG (11.0 mg/ml; Jackson ImmunoResearch catalog no. 015-000-003). After 10 min, 5 μl of AlexaFluor-conjugated antihuman CD19 was added, whereas control cells received AlexaFluor-conjugated IgG1 isotype control. Cells were centrifuged and washed twice with PBS and were resuspended in 600 μl of PBS before FACS analysis. Cells singly stained for CD40 showed no crossover into the fluorescein channel, and cells stained singly against CD19 showed no absorbance on the rhodamine channel.

Quantitative RT-PCR

mRNA was isolated from purified B cells using the Absolutely RNA RT-PCR miniprep kit (Stratagene catalog no. 400800). After isolation, mRNA was quantified on a Beckman (Fullerton, CA) DU 640 spectrophotometer by measuring absorbance at 260 nm. mRNA was used as a template for single-stranded cDNA synthesis using the Sensiscript Reverse Transcriptase kit (QIAGEN catalog no. 205211). All of the cDNA reactions were done concurrently and on the same heat block.

For quantitative RT-PCR, an Applied Biosystems Prism 7900 HT sequence detection system was used, using the default thermocycling protocol, for 31 cycles. The Applied Biosystems software program Primer express 2.0 was used to design a probe and primers that would amplify across the boundaries of exons 1 and 2 of the *CD40* gene. The probe used was synthesized by Applied Biosystems and had the following sequence: 5′-*6FAM*-CGACCCCAACCTAGGGCTTCGG-*TAMRA*-3′, where the FAM was the reporter dye and TAMRA was the quencher. The forward primer was 5′-CACTGCCACCAGCACAAATACT-3′ and the reverse primer used was 5′-CTGTTTCTGAGGTGCCCTTCT-3′. Reactions were performed using TaqMan universal PCR mix (Applied Biosystems catalog no. 4304437), with 100 nM probe concentration and primer concentrations of 50 nM. Amounts of CD40 cDNA present were calculated against a standard curve, generated by linear regression analysis, that was constructed by using known amounts (10^3, 10^4, 10^5, 10^6, and 10^7 molecules) of plasmid CD40pIRES.C as data points. Copies of CD40 transcripts were normalized against glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) levels. *GAPDH* was detected using a human GAPDH predesigned assay kit (Applied Biosystems catalog no. 4310884E). Quantification was performed against a standard curve constructed from know amounts of Raji cell mRNA (Stratagene catalog no. 735403) converted to cDNA. All standard curves used in the quantitative RT-PCR analysis had R factors greater than 0.995.

Statistical analyses of CD40 expression

The comparison of CD40 expression levels, as a function of genotype, were performed using either the two-tailed paired or the unpaired Student's *t* test, as specified. $P < 0.05$ was considered significant.

Results

Sequence analysis of the CD40 5′ UTR in mice

To examine whether *CD40* 5′ UTR variants could affect the development of experimental GD and experimental autoimmune thyroiditis (EAT) in mice, we sequenced the *CD40* 5′ UTR in 19 different strains of mice known to be susceptible or resistant to experimental GD and EAT. Of the 19 strains sequenced, two are susceptible to experimental GD (BALB/c and C57BL/6) and two are resistant to experimental GD (CBA/J and DBA/1J) (17); 10 strains are susceptible to EAT (CBA/J, C3H/HeJ, C3HeB/FeJ, AKR/J, B10.BR/SgSnJ, CE/J, MA/MyJ, RF/J, ST/bJ, B6C3F1/J), and nine are resistant to EAT (C57BL/6J, 129/J, C57L/J, LP/J, BALB/cJ, DBA/2J, C57BLKS/J, PL/J, DBA/1J) (18). Sequence analysis demonstrated no evidence of any SNPs in the 5′ UTR of the murine *CD40* gene, at least in the 19 strains we examined. Hence, the presence of the Kozak sequence polymorphism is not conserved between mouse (**CCTGCGATGG**) and human (**CTCGCC$_T$ATGG**).

Analysis of the effect of the CD40 Kozak SNP on CD40 translation by an in vitro transcription/translation system

Initially, to test the effect of the C and T alleles of the *CD40* Kozak SNP on *CD40* translation, we made use of a rabbit reticulocyte lysate system, in which we supplied *CD40*, on a plasmid (CD40.pcDNA.C/T) whose mRNA expression was driven by the addition of T7 polymerase, whereas the ribosomal machinery of the lysate system drove the translation of the nascent mRNA. Because the hCD40 protein only has one methionine (at the start of translation), we elected to label translated protein with ^{35}S cysteine in an effort to obtain a more robust signal. Total protein synthesis was measured by TCA-precipitating protein on glass filter papers and measuring the counts per minute on a liquid scintillation counter. Our analyses, summarized in Fig. 1A, consistently have shown significantly less CD40 protein synthesis with the T

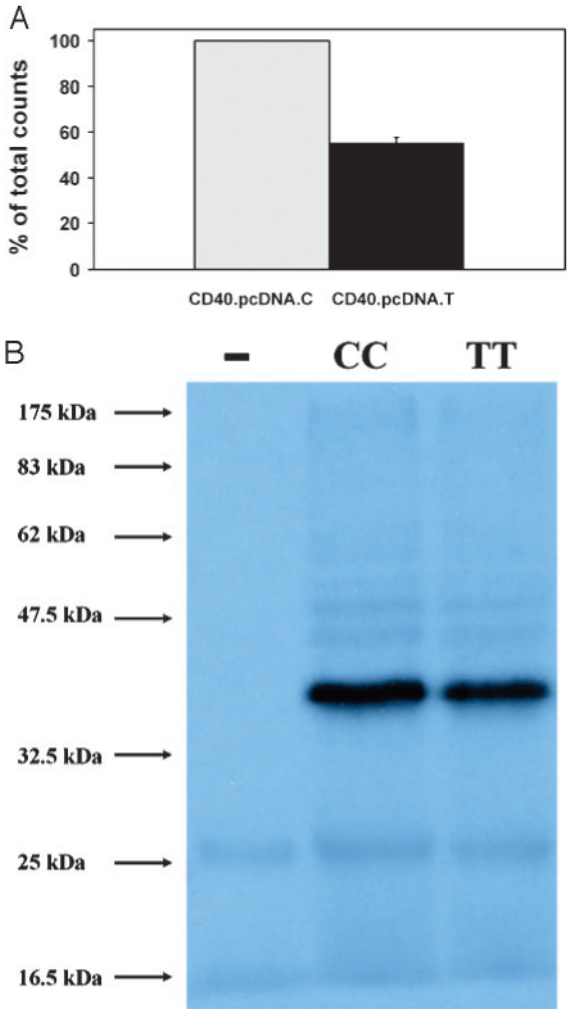

FIG. 1. A, Effect of the Kozak SNP on total protein synthesis. One microgram of either CD40.pcDNA.C (*gray bar*) or CD40.pcDNA.T (*black bar*) was used to drive an *in vitro* transcription/translation reaction. After a 1-h incubation, aliquots were taken, TCA was precipitated on filters, and total protein synthesis was quantified by scintillation counting of ^{35}S. Data represent the results obtained from two independent experiments. The C allele was taken to be 100% and the T allele was normalized with respect to the C allele. On average, the reaction containing the T allele was found to synthesize protein 45.5% less efficiently than the reaction containing the C allele (shown are means + SE; $P = 0.01$, based on a nonpaired two-tailed *t* test). B, PAGE analysis of *in vitro*-translated CD40. Typical autoradiogram of the ^{35}S-labeled products of the translation reaction. Lane CC had 1 μg of CD40.pcDNA.C added to the mixture, whereas lane TT had 1 μg of CD40.pcDNA.T. The control lane had no added plasmid. Relative size standards are indicated to the *left* of the autoradiogram. The gel shows a single discrete protein product, migrating at approximately 40 kDa. In total, three separate reactions were performed, with two gels run for each reaction. Bands were quantified by densitometry, as described in *Materials and Methods*. On average, the T allele made protein 15.5% less efficiently than the C allele ($P < 0.001$, based on a nonpaired two-tailed *t* test).

allele (45.5% less, $P = 0.01$, based on a nonpaired two-tailed *t* test), compared with the levels seen with the C allele.

To measure the level of specific protein product synthesized, we ran 15 μl of the translation mixture on a polyacrylamide gel under reducing conditions. The contents of the gel were transferred to a nitrocellulose membrane, which was subsequently exposed to x-ray film. Translation reactions showed the presence of a discrete band (Fig. 1B) migrating at 40 kDa that was absent from the negative control reactions, lacking plasmid in the translation mixture. The *hCD40* gene product itself is a rather modestly sized polypeptide consisting of some 277 amino acids, with an expected molecular mass of 30,600 Da (19); however, as the nascent protein undergoes extensive glycosylation, the fully processed species attains a final molecular mass of 48 kDa. The fact that the main protein product migrated at a lower molecular mass than the reported CD40 size is, ostensibly, due the fact that CD40 cannot be fully glycosylated in the rabbit reticulocyte lysate system. Densitometric analysis showed, over the course of multiple experiments, that significantly less CD40 protein was produced using CD40.pcDNA.T *vs.* CD40.pcDNA.C (15.5% less with the T allele, $P < 0.001$, based on a nonpaired two-tailed *t* test).

Effect of the CD40 Kozak SNP on CD40 surface expression in transfected fibroblasts

Next, we measured the effect of the Kozak polymorphism on surface CD40 expression using the rat embryonic kidney fibroblast cell line, Rat2, transfected with CD40.pcDNA.C or CD40.pCDNA.T. CD40 surface expression was measured by mean fluorescent intensities, obtained by FACS analysis. Multiple experiments, using 1 or 0.5 μg of plasmid repeatedly, demonstrated that the C allele gave more surface CD40 expression than the T allele; on average the T allele produced 32.3% less CD40 than the C allele ($P = 0.004$, based on a nonpaired two-tailed *t* test, Fig. 2).

In addition to the transfections done with vectors CD40.pcDNA.C/T, a single transfection experiment was performed, using 0.5 μg of vector CD40.pIRES.C or CD40.pIRES.T, to account for any differences in mRNA levels. CD40 protein levels, normalized to GFP fluorescence, again showed a decreased efficiency of translation of the T allele with respect to the C allele (35.0% less translation with

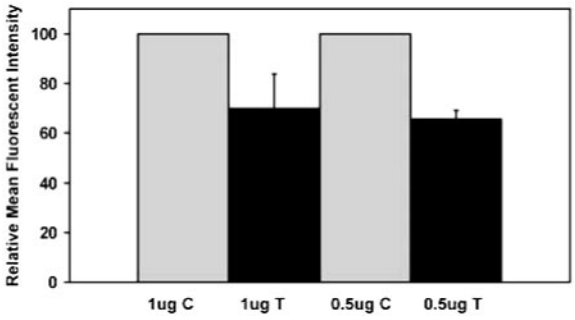

FIG. 2. Summary of transfection data. Analysis of three independent transfections (one analysis per reaction, pooling six wells of transfected cells) demonstrated that the T allele synthesized protein 30.1 and 34.4% less efficiently than the C allele, when transfected with 1 or 0.5 μg, respectively. Averaging the results of the three transfections showed that the T allele transfectants had 32.3% less CD40 surface expression compared with the C allele (shown are means + SE; $P = 0.004$, based on a nonpaired two-tailed *t* test). The *gray bar* represents normalized values for CD40.pcDNA.C, and the *black bar* represents the relative values obtained with CD40.pcDNA.T. The *two bars on the left* represent results obtained with 1 μg of cDNA and the *two rightmost bars* represent results from 0.5 μg of cDNA.

the T allele compared with the C allele), consistent with our earlier results and our hypothesis that the polymorphism affects translational efficiency.

Effect of the CD40 Kozak SNP on CD40 surface expression on B cells

Finally, the effect of the Kozak SNP on CD40 expression on human B cells was examined. We analyzed the surface expression of CD40 on B cells obtained from 23 healthy individuals harboring CC, CT, and TT genotypes. B cells were purified from PMBCs and were dually labeled for CD19 and CD40; those staining doubly positive had their CD40 expression levels assessed through FACS analysis. Cells were cultured for 16 h in a resting state (just RPMI 1640 medium) and in an activated state, where RPMI 1640 was supplemented with 200 U/ml of interferon-γ. This enabled us to examine whether the Kozak SNP genotype influences B cell CD40 expression and, if so, whether any putative difference is enhanced or decreased during periods of rapid CD40 synthesis (*i.e.* after interferon-γ stimulation). The levels of CD40 expression were significantly higher on B cells, both under resting and stimulating conditions, from individuals with the CC phenotype when compared with individuals with the CT or TT phenotype. Under resting conditions, the CT and TT genotypes had CD40 expression levels that were lower by 13.3 and 39.4%, respectively, compared with the CC genotype (using the two-tailed paired *t* test, CC *vs.* CT $P = 0.021$ and CC *vs.* CT + TT $P = 0.012$, Fig. 3); whereas under stimulatory conditions, the respective CT and TT genotype CD40 levels were lower by 12.8 and 27.0% compared with the CC genotype (using the two-tailed paired *t* test, CC *vs.* CT $P = 0.046$ and CC *vs.* CT + TT $P = 0.042$, Fig. 3**;** note that because the range of the absolute values of the B cell expression data varied between experiments, we used the two-tailed paired *t* test, in which each pair represented the averaged raw data from one experiment). Due to the small number of individuals with the TT genotype, we could not compare them separately to individuals with the CC or CT genotypes.

Effect of the CD40 Kozak SNP on CD40 mRNA levels in B cells

Purified B cells were grown in culture, with or without interferon-γ, as previously described. mRNA was isolated from the B cells after 16 h in culture. Because the CD40 Kozak SNP is transcribed in the nascent mRNA transcript, we studied whether the SNP is associated with a change in the level of mRNA transcripts in the cell. Such a change could conceivably arise due to an effect on mRNA stability or, alternatively, by a feedback mechanism, where less available protein causes a compensatory up-regulation of transcription. Quantitative RT-PCR was used to determine the number of transcripts present per nanogram of total cellular mRNA. Transcript copy number was normalized with respect to GAPDH. In total, B cells from 11 individuals (comprised of genotypes four CC, five CT, and two TTs) were tested. Our data showed no correlation between *CD40* mRNA levels and *CD40* Kozak genotype (two-tailed paired *t* tests used to compare CC *vs.* CT + TT gave *P* values of 0.14 and 0.39 for unstimulated and stimulated B cells, respectively, Fig. 4), thus providing evidence that the polymorphism acts solely at the level of translation.

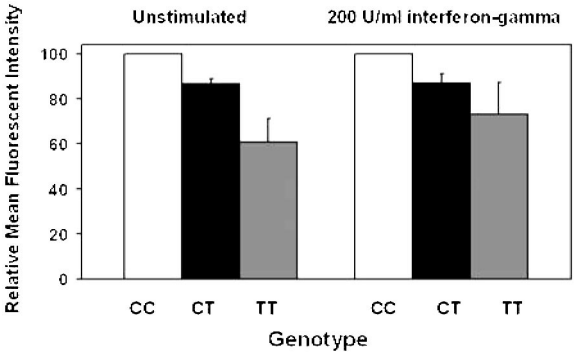

FIG. 3. Summary of CD40 expression on B cells from 23 individuals. B cells were isolated from 23 healthy individuals (11 CCs, 11 CTs, and two TTs) and grown in culture with either plain medium, or in medium supplemented with 200 U/ml of interferon-γ. Isolations were done in groups of five to seven individuals, over four independent experiments. Because each experiment showed a CD40 expression level pattern that was, on average, CC > CT > TT, the average expression level of the CC genotype was set to 100% and the averaged CT and TT genotypes were normalized with respect to the averaged CC value. The averaged individual genotype levels from the four separate experiments were then averaged together. Under conditions with no stimulation, CD40 expression from the respective CT and TT genotypes was found to be 13.3 and 39.4% lower than the levels seen with the CC genotype (shown are means + SE; using the two-tailed paired *t* test, CC *vs.* CT $P = 0.021$ and CC *vs.* CT + TT $P = 0.012$). An identical trend was observed under conditions of stimulation, with CT and TT genotypes expressing 12.7 and 27.0%, respectively, less CD40 when compared with the CC genotype (shown are means + SE; using the two-tailed paired *t* test, CC *vs.* CT $P = 0.046$ and CC *vs.* CT + TT $P = 0.042$).

Discussion

The immune system is forced to walk a razor's edge. On one hand, it must allow for the expansion of the B and T cells

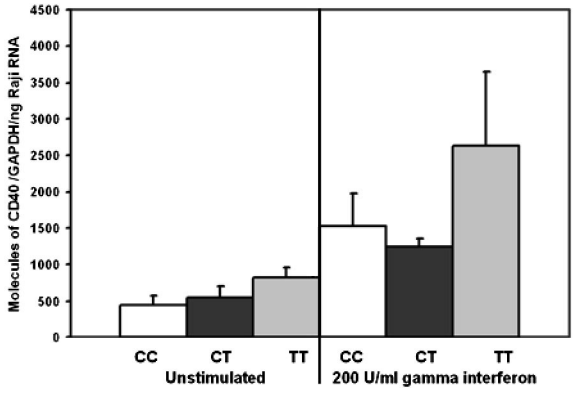

FIG. 4. mRNA levels of CD40 expression in B cells. mRNA transcripts of CD40 were normalized with respect to GAPDH. In total, B cells from 11 individuals (comprised of genotypes four CC, five CT, and two TTs) were tested. All individuals were compared with one another in a single analysis for CD40 mRNA levels, and in a single analysis for GAPDH mRNA levels. Our data showed no dependence of CD40 mRNA levels on CD40 Kozak genotype (shown are means + SE; using two-tailed paired *t* tests to compare CC *vs.* CT + TT, gave *P* values of 0.14 and 0.39 for unstimulated and stimulated B cells, respectively).

needed to mount a robust immune response to eliminate invading pathogens and cancerous cells; yet, at the same time, it must severely curtail the activity of any autoreactive lymphocytes in the periphery. Hence, a delicate balance between tolerance and immunity must be maintained; if that balance is perturbed, then a breakdown in tolerance, *i.e.* autoimmunity, could arise as a consequence. Perhaps not surprisingly, given the exquisite levels of cellular coordination and control needed, autoimmune diseases are a fairly common occurrence, affecting at least 5% of the population (20). In this report, we have focused on the immunogenetic basis of GD, which is among the most common of autoimmune diseases (present in ~1% of the population in the United States) (10, 21).

Genetic factors have been shown to play a major role in the etiology of GD (21), and recently it was reported that a SNP residing in the 5′ UTR of the *CD40* gene is associated with GD. Very recently, the authors of two additional published studies, both from the United Kingdom, claimed to find no association between the *CD40* SNP and GD (22, 23). However, reanalysis of the data described in the study by Heward *et al.* (22) clearly showed a replication of the association between the CC genotype of the *CD40* SNP and GD (24). Moreover, pooling of the data of all published studies on the *CD40* SNP in GD (a total of 1537 GD patients and 1513 controls) showed a highly significant increase in the frequency of the CC genotype in GD patients when compared with controls (58.4 *vs.* 52.7%, $\chi^2 = 9.75$, $P = 0.0018$, odds ratio = 1.26) (24). These studies, which were performed in unselected GD patients, showed a low relative risk (1.3). What could be the reason for this weak association? We believe that this reflects the fact that CD40 contributes to the genetic risk for GD only in a subset of patients, and when this subgroup is diluted by testing all GD patients the association is weakened. Indeed, when we reanalyzed the CD40 Kozak SNP in a subset our GD patients who were positive for thyroid peroxidase or thyroglobulin antibodies after treatment for GD, the CC genotype was associated with disease with a higher relative risk of 2.5 ($P = 0.0002$). Also, GD patients carrying the C allele were reported to have higher TSH receptor antibody levels compared with patients carrying the T allele (12). Hence, this polymorphism might be implicated in autoantibody production in GD patients.

The 5′ UTR SNP of *CD40*, associated with GD, is located at the −1 position within the Kozak sequence. Of fundamental importance to the initiation of translation, the Kozak sequence represents a consensus sequence (GCCACCATGG), compiled from the study of some 699 vertebrate genes, that flanks the starting methionine (AUG) codon (14). Even subtle variations in this sequence can have significant effects on translation (25). Therefore, we examined the effect of the two alleles of the *CD40* Kozak polymorphism on *CD40* translation. Using a battery of methods, including *in vitro* transcription-translation assays, surface expression analyses of cells transfected with the two alleles, and analyses of B cells from individuals with different SNP genotypes, we have consistently seen a significantly decreased *CD40* translational efficiency with the T allele, compared with the C allele. In contrast, analysis of *CD40* mRNA levels has shown no effect of the SNP on gene transcription. Thus, our data demonstrated that the presence of the T allele of CD40 is associated with significantly reduced gene product, mediated by a reduction in the efficiency of *CD40* translation. Mechanistically, as illustrated in Fig. 5, the Kozak SNP would be expected to interfere with the ability of the ribosome to initiate translation, although not affecting the ability of RNA polymerase to transcribe mRNA.

Based on recent findings, increased CD40 expression levels could, conceivably, overstimulate B cells and play a role in the initiation of GD. Indeed, a direct link between the overstimulation of B cells by CD40 activation and autoimmune disease is emerging. Previous reports have shown that overstimulation of murine B cells, by ectopically expressing a *CD154* transgene, led to experimental systemic lupus erythematosus (SLE) (26). Additionally, a polymorphism in the 3′ UTR of *hCD154*, which is associated with SLE, causes more prolonged hCD154 expression in activated lymphocytes of patients *vs.* those in controls (27). Finally, a soluble form of CD154 is functional and exists at elevated levels in the sera of SLE patients (28). Hence, the *CD40* signaling pathway could represent a molecular checkpoint or stopgap in the balance between normal B cell function and the initiation of autoimmunity. Indeed, there exist a number of B cell surface molecules whose expression levels have been shown to affect tolerance (29, 30).

Although we have focused on B lymphocytes, because GD is characterized by high levels of autoantibodies we cannot neglect the contribution that other CD40-expressing cell types could make toward the etiology of the disease. CD40 has been documented to be expressed on thyroid follicular cells (31, 32) and thyroid fibroblasts (32, 33), and CD40 was shown to be up-regulated in thyroid tissues from GD patients (8). Whether this thyroidal CD40 expression is simply a byproduct of the course of disease or, alternatively, is important

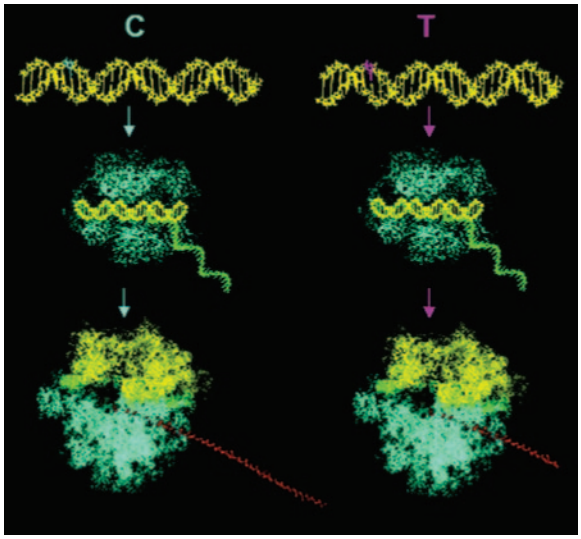

FIG. 5. Mechanism of the hCD40 Kozak SNP action. The hCD40 Kozak SNP exerts its effects at the level of translation. The C (turquoise) and the T polymorphisms (*magenta*) are transcribed equivalently by RNA polymerase. The mRNAs produced are shown in *green*. In contrast, however, differences exist at the level of translation. The ribosome is able to initiate translation more efficiently in the presence of the C polymorphism and results in the formation of greater amounts of CD40 protein, represented by the *red* peptide.

TABLE 1. Effects Kozak polymorphisms on translation and relevance to disease

Gene	Polymorphism	Result	Relevance to disease	Ref.
Annexin V	GTCGCC$_T$ATGG	40% Increase in cell-free transcription/ translation system; 62% increase in CT *vs.* CC genotypes	Risk of myocardial infarction at early age	43
Factor XII	AC$_T$GCCATGA	31% & 66% Decrease in CT and TT genotypes with respect to CC	Factor XII deficiency; increased risk of thrombosis?	44
BRCA1	AAAG$_T$AAATGG	70% Decrease in cell-free assay; polysome recruitment defect	Sporadic breast cancer	45
Androgen receptor	TCAAGGATGG$_A$	20–50% Reduction in Cos cells	Partial androgen insensitivity syndrome	46
CD40	CTCGCC$_T$ATGG	15% Decrease in cell-free system; 32% decrease in rat2 cells; 23% and 39% decrease in CT and TT; genotypes with respect to CC	Potentiation of GD	

in its potentiation, is not known. However, in view of the up-regulation of CD40 expression on thyrocytes, one can hypothesize that, in the context of the C allele of the Kozak SNP, CD40 could initiate a heightened inflammatory response during thyroidal injury (*e.g.* due to infection). This inflammatory process could then function to attract peripheral lymphocytes and up-regulate the expression of CD40 itself (34, 35), along with major histocompatibility complex class II molecules, on thyrocytes (36, 37). Infiltrating T cells could then be activated by thyrocytes, which by virtue of their CD40 and major histocompatibility complex II expression, could function as antigen-presenting cells, presenting thyroidal proteins as antigen. Finally, infiltrating B cells expressing higher levels of CD40 (*i.e.* with the C Kozak polymorphism) could have a lower threshold for activation. Indeed, it has been shown that infiltrating lymphocytes colocalized with human leukocyte antigen-DR-expressing thyrocytes (38) or thyrocytes expressing both human leukocyte antigen-DR and CD40 without undergoing apoptosis (8).

Because the observed relative risk conferred by the C allele of the Kozak SNP is 1.3, a large percentage of individuals with the C allele do not develop GD. Hence, it is clear that CD40 may act only as a potentiation factor, in concert with polymorphisms of other genes, as well as other factors such as infections (39) and dietary iodine (40). Future studies that dissect the contribution of the SNP in syngeneic mouse strains would help clarify the role of CD40 in the development of GD.

On a conclusory note, our data suggest that differences in CD40 expression levels, by virtue of a SNP in the Kozak sequence, may play a role in the development of GD. Such a mechanism has been shown to be important in several diseases (Table 1). In light of our findings, we suggest that the pathogenesis of GD might be influenced by translational pathophysiology (41, 42), where even a modest change in the efficiency of translation of an mRNA transcript may manifest itself in the development of, or predisposition to, disease.

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