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Clast5/Stra13 Is a Negative Regulator of B Lymphocyte Activation

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CD40 is a member of the tumor necrosis factor receptor family and mediates a variety of functions of B cells, including B cell survival, proliferation, immunoglobulin gene class switching, memory B cell formation, and regulation of Fas-mediated apoptosis. To begin to elucidate the molecular mechanism governing such diverse functions of CD40, we have isolated a gene from mouse splenic B cells, termed Clast5, whose expression is strongly repressed during B cell activation. Clast5 is identical with Stra13, a recently identified member of the basic helix-loop-helix family of transcription factors. Clast5/Stra13 is highly expressed in unstimulated, resting B cells and is rapidly downregulated by a variety of stimuli that activate B cells, including CD40 ligand, anti-IgM antibodies, lipopolysaccharides and interleukin-4. Forced expression of Clast5/Stra13 in B cells delayed the cell cycle progression into S phase and strongly suppressed Fas-mediated apoptosis. Moreover, Clast5/Stra13 inhibited the colony formation in fibroblasts. Our results suggest that Clast5/Stra13 functions as a negative regulator of B cell activation by inhibiting cell cycle progression and cell growth.

Key Words: **CD40; Fas; proliferation; cell cycle; apoptosis; helix-loop-helix; transcription factor.**

CD40 is a 50 kDa type I membrane protein belonging to the tumor necrosis factor receptor (TNF-R) family (1). It is mainly expressed by B cells, dendritic cells, follicular dendritic cells, monocytes and macrophages. The ligand for CD40 (CD40L) is a 35 kDa type II membrane protein that belongs to the TNF family, and is expressed by activated T cells (1). Studies in the past few years have revealed crucial roles for CD40/CD40L interaction in B cell survival, proliferation, immunoglobulin (Ig) gene class switching and memory B cell formation (2, 3). The significance of CD40/CD40L interaction is further illustrated by the finding that the X-linked hyper-IgM syndrome (HIGM) is caused by mutations in CD40L gene (4–7). HIGM is an immunodeficient disorder characterized by a severe impairment of T cell dependent antibody responses, lack of B cell memory, deficiency in immunoglobulin gene somatic mutations, and little or no circulating IgG, IgA, or IgE antibodies. Mice deficient in either CD40 (8) or CD40L (9) exhibit profound immunodeficiency similar to human HIGM, indicating that CD40 function is conserved between human and mice.

CD40 not only mediates B cell activation, but also plays a crucial role in the regulation of B cell apoptosis. CD40 ligation on B cells rapidly induces Fas expression and enhances Fas susceptibility (11–13). The Fas-mediated apoptosis is critical for the activation-induced cell death of B and T cells, a process that eliminates antigen-nonspecific B cells (14, 15) and terminates immune responses (16–18). CD40 thus positively and negatively regulates B cell function. Consistent with its dual functions, patients with HIGM frequently suffer from autoimmune disorders.

Despite the biological importance of CD40, relatively little is known about how CD40 mediates such diverse and opposite functions. To identify the cellular effector molecules that act downstream of CD40 signals, we have isolated a gene, termed Clast5 (GenBank Accession No. AF364051), whose expression is downregulated in normal splenic B cells by CD40L stimulation. Clast5 turned out to be identical with the recently described Stra13 gene, a member of the basic helix-

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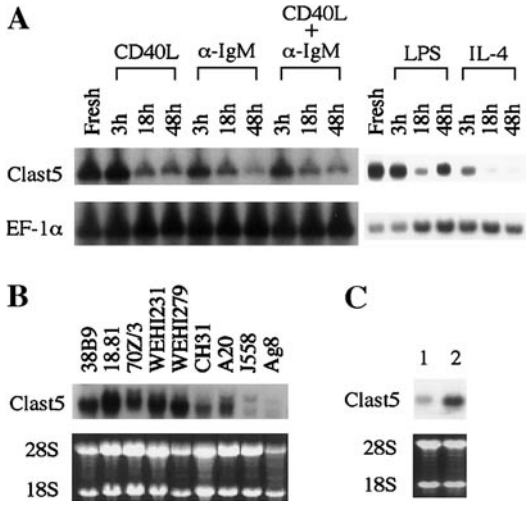

FIG. 1. (A) Clast5/Stra13 expression is downregulated upon B cell activation. Fresh splenic B cells or B cells cultured in the presence of soluble CD40L, anti-IgM antibodies (5 μg/ml), CD40L plus anti-IgM antibodies, LPS (10 μg/ml) or IL-4 (100 units/ml) for the indicated time. (B) Clast5/Stra13 expression in a panel of B lineage cell lines. Northern blot analysis was performed as described under Materials and Methods. (C) Clast5/Stra13 expression in unsynchronized (lane 1) and G1 arrested (lane 2) WEHI231 B cells. The G1 arrest was induced by serum deprivation for 48 h.

loop-helix family of transcription factors that is inducible by all-*trans* retinoic acid in P19 embryonal carcinoma cells (19). We show here that Clast5/Stra13 is a negative regulator of B cell activation.

MATERIALS AND METHODS

Reagents. Goat F(ab′)2 anti-mouse IgM antibodies were purchased from Southern Biotechnology Associates. (SBA, Birmingham, AL) and were extensively dialyzed in PBS. Culture supernatants from a myeloma cell line producing soluble CD40 ligand-CD8 fusion protein (CD40L) were used at a three fold dilution as described previously (20). Recombinant interleukin-4 (IL-4) was purchased from R&D Systems (Minneapolis, MN). The anti-Flag antibodies (M5 and M2) were obtained from Sigma (St. Louis, MO). The anti-mouse CD40 antibody (IC10) and anti-Fas antibody (Jo-2) were purchased from PharMingen (San Diego, CA). The horseradish peroxidase (HRP)-labeled antibodies to mouse IgG1 were obtained from SBA.

Purification of splenic B cells and cell culture. Splenic B cells were isolated from 8- to 12-week-old C57BL/6 mice after T cell depletion with anti-Thy-1 antibodies and complement treatment, followed by Ficoll–Hypaque density centrifugation. The cells were cultured at 37°C with 5% CO_2 in a humidified atmosphere, in RPMI 1640 medium containing 5×10^{-5} M 2-mercaptoethanol, 50 units of penicillin–streptomycin, supplemented with 10% fetal calf serum (FCS) (Life Technologies, Gaithersburg, MD).

Isolation of Clast5/Stra13 gene. Total RNA was extracted using TRIzol reagent (Life Technologies) from fresh splenic B cells and B cells cultured in the presence of CD40L for 2 days. Poly(A)$^+$ mRNA was then purified using a poly(dT) column. cDNA syntheses and subtraction procedures were performed using the PCR-Select cDNA subtraction kit (Clontech, Palo Alto, CA) according to the company's protocol. Candidate gene fragments were resolved in 1% agarose gels, transferred simultaneously to two nylon membranes. Each membrane was subjected to a reverse Northern blot with ^{32}P-labeled total cDNA from either fresh splenic B cells or B cells after CD40L treatment as a probe. Gene fragments that exhibited differential hybridization signals were used as probes in Northern blot to confirm their differential expression before and after CD40L treatment, and to isolate the full-length cDNA.

Northern blot analysis. Twenty μg of total RNA was resolved in a formaldehyde-agarose gel, transferred onto a Hybond N$^+$ nylon membrane (Amersham, Buckinghamshire, UK), and hybridized with a ^{32}P-labeled Clast5/Stra13 probe.

Western blot analysis. Cells were lysed in a buffer containing 50 mM Tris–HCl (pH 6.8), 2% SDS, 1 M 2-mercaptoethanol, 10% glycerol and a cocktail of protease inhibitors (Sigma). The lysate proteins were sonicated for 2 min, heated at 100°C for 5 min and separated on an 8% SDS polyacrylamide gel. The resolved proteins were transferred to a cellulose nitrate membrane with a semi-dry blotter, blocked with 5% nonfat milk, reacted with the anti-Flag antibody (M5) and then with HRP-labeled antibodies. The membrane was developed with the ECL system (Amersham) and exposed to autoradiography.

Construction of expression vectors and colony assays Full-length mouse Clast5/Stra13 cDNA, a cDNA with unknown function (irrelevant cDNA), or BAX cDNA was cloned into the pCXN2 expression vector. NIH3T3 cells were transfected with 5 μg of DNA using Lipofectin Reagent (Life Technologies) and 2 days later selected with 500 μg/ml of Geneticin. Two weeks after the selection, the dishes were washed with phosphate buffered saline (PBS), fixed with 100% Methanol, stained with Giemsa's solution, and the colony number was counted.

Establishment of Clast5/Stra13 stable transfectants and apoptosis assays. WEHI231 cells were transfected with 5 μg of the pCXN2-Flag-Clast5 plasmid DNA using LipofectAmine Reagent (Life Technologies), and 2 days later selected with 800 μg/ml of Geneticin. Two weeks later, isolated colonies were picked up using cloning cylinders and subjected to Western blot analysis. For anti-IgM-mediated apoptosis, cells (1.5×10^6/ml, 0.5ml) were seeded in 24 well plates and cultured in the presence of anti-IgM antibodies (5 μg/ml) or the anti-CD40 antibody (5 μg/ml). After 48 h, cells were collected and incubated in PBS containing 0.1% RNase A and 0.1% Triton at room temperature for 10 min, and then in PBS containing 100 μg/ml of propidium iodide (PI) on ice for 30 min. DNA content was analyzed with a FACScan flow cytometer (Becton–Dickinson, Mountain View, CA). For serum deprivation assay, cells were washed once with serum free RPMI medium and cultured in the same medium at a density of 5×10^5/ml in 24-well plates. Cells were collected after 24, 48, and 72 h and stained with 1 μg/ml of PI and PI positive (dead) cells were analyzed with a FACScan flow cytometer. For Fas-mediated apoptosis, cells (1×10^5, 0.5 ml) were seeded in 24-well

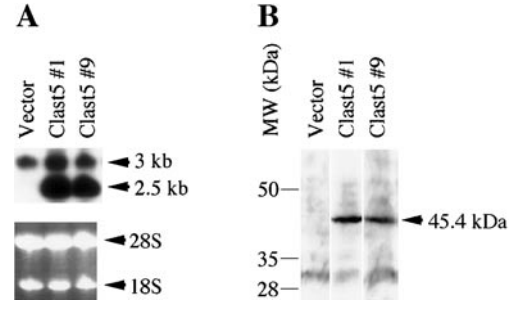

FIG. 2. Establishment of Clast5/Stra13 stable transfectants in WEHI231 B lymphoma cells. Northern (A) and Western (B) blot analyses of Clast5/Stra13 (#1 and #9) and vector control transfectants (vector).

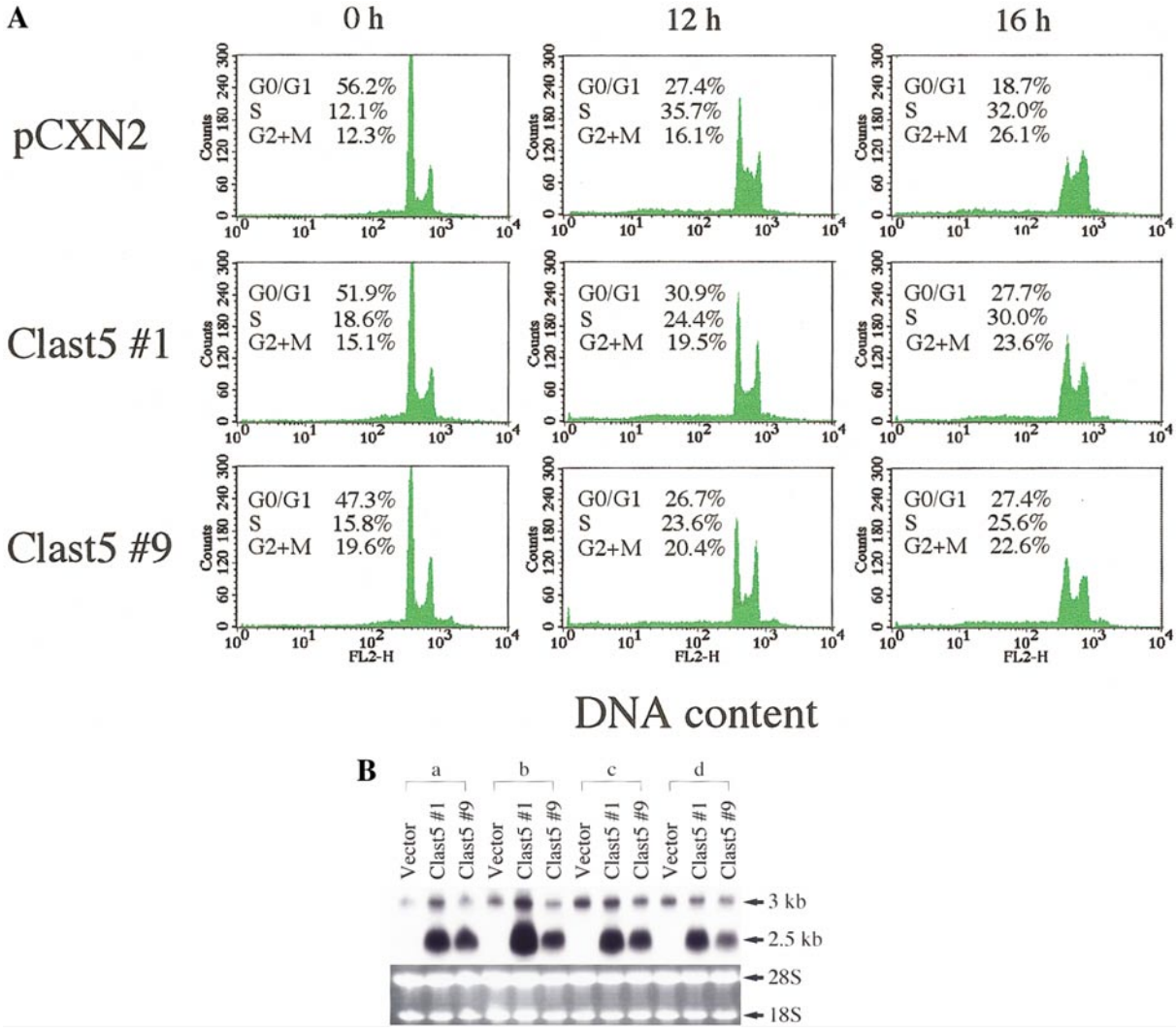

FIG. 3. Clast5/Stra13 inhibits cell cycle progression in B cells. (A) Clast5/Stra13 and control transfectants were deprived of serum for 48 h to induce cell cycle arrest (0 h). Serum was then added and cell cycle progression was monitored at 12 and 16 h. (B) The expression of endogenous (3-kb) and the exogenous (2.5-kb) Clast5/Stra13 RNA in unsynchronized cells (a), 48 h after serum deprivation (b), and 3 h (c) and 8 h (d) after serum addition.

plates and cultured in the presence of 5 μg/ml of the anti-CD40 antibody. After 48 h, anti-Fas antibody (Jo-2) was added at a final concentration of 1 μg/ml. After over night culture, cells were stained with PI and analyzed with a FACScan flow cytometer.

Analysis of cell cycle progression. WEHI231 transfectants were washed once with serum free RPMI medium, cultured for 48 h in RPMI medium containing 0.1% FCS to induce G0/G1 arrest, and then subjected to Ficoll–Hypaque density centrifugation to remove dead cells as previously described (20). The resulting live cells were resuspended in RPMI supplemented with 10% FCS and seeded in a 24-well plate at a density of 7.5×10^4 cell/well. DNA content was analyzed as described in the preceding section.

RESULTS

Clast5/Stra13 Expression Is Rapidly Downregulated upon B Cell Activation

Clast5 was highly expressed in fresh, unstimulated splenic B cells, and was downregulated by CD40L treatment (Fig. 1A). In addition, Clast5 expression was rapidly repressed by anti-IgM antibodies, lipopolysaccharides (LPS) and IL-4 (Fig. 1A). Each of these stimuli induces B cell survival and proliferation, and in combination, they promote B cell terminal differentiation such as Ig gene class switching and memory B cell and plasma cell formation. Therefore, downregulation of Clast5 expression is associated with B cell activation and differentiation. In agreement with this observation, Clast5/Stra13 expression was barely detectable in terminally differentiated plasma cells, J558 and Ag8 (Fig. 1B), while it was expressed in all the earlier stages of B cell development, including progenitor (38B9), precursor (18.81, 70Z/3), immature (WEHI231) and mature B cells (WEHI279, CH31 and A20). In addition, Clast5/Stra13 expression was upregulated upon G1 arrest in WEHI231 B cells (Fig. 1C), suggest-

ing a link between growth arrest and the upregulation of Clast5/Stra13.

Forced Expression of Clast5/Stra13 Inhibits Cell Cycle Progression in B Lymphoma Cells

Since Clast5/Stra13 expression was strongly repressed in normal splenic B cells undergoing proliferation (Fig. 1A) and is upregulated upon growth arrest (Fig. 1C), we investigated whether Clast5/Stra13 could inhibit the cell cycle progression. We established Clast5/Stra13 transfectants in WEHI231 B lymphoma cells. Two independent stable clones, #1 and #9, as well as a pCXN2 vector control transfectants, were examined. Northern blot analysis revealed much more abundant expression of the 2.5-kb Clast5/Stra13 transcript derived from the transfected gene, compared with that of the endogenous 3-kb Clast5/Stra13 RNA (Fig. 2A). Western blot analysis detected the Flag-tagged 45.4 kDa Clast5/Stra13 protein in Clast5/Stra13 transfectants but not in vector control (Fig. 2B). The two Clast5 transfectants grew slightly slower than did the vector control, but the difference did not reach statistical significance (data not shown).

Clast5/Stra13 as well as control transfectants were deprived of serum for 48 h to induce cell cycle arrest predominantly at the G0/G1 phase (Fig. 3A, 0 h). Serum was then added and the cell cycle progression was followed as a function of time. At 12 h after serum addition, there was a striking increase of the S phase cells in control transfectants (from 12.1 to 35.7%), which was accompanied by a decrease of the cells at the G0/G1 phase. In contrast, only a slight increase of the S-phase cells was observed in the two Clast5/Stra13 transfectants (from 18.6 to 24.4% and 15.8 to 23.6%). At 16 h after serum addition, there was a further decrease of G0/G1 cells and an increase of G2-M phase cells in control transfectants, suggesting that cells at the G0/G1 phase progressed through S phase and entered the G2-M phase. At this time, an increase of the S phase cells became evident in Clast5/Stra13 transfectants, indicating that there was a delay in the entering of S phase in these cells compared with the control transfectants of WEHI231 cells. Similar inhibitory effects were observed in Clast5/Stra13 transfectants of NIH3T3 fibroblasts (data not shown). The transfected Clast5/Stra13 gene was expressed at much higher levels than was the endogenous gene (Fig. 3B), in unsynchronized cells (a), after serum deprivation (b), or 3 h (c) and 8 h (d) after serum addition. Forced expression of Clast5/Stra13 thus resulted in delayed cell cycle progression into S phase.

Overexpression of Clast5/Stra13 Does Not Affect the Apoptosis Triggered by Anti-IgM Antibodies or Serum Deprivation, but Suppresses Fas-Mediated Apoptosis

Since Clast5/Stra13 expression was repressed by anti-IgM antibodies, we next examined whether B cell apoptosis triggered by antigen receptor (BCR) crosslinking was modulated by forced expression of Clast5/Stra13. Using WEHI231 cells which are known to undergo programmed cell death by BCR crosslinking with the anti-IgM antibodies, we found no significant differences in the percentages of apoptotic cells (shown as a sub G1 fraction) between vector control (77.6%) and the two Clast5/Stra13 stable transfectants (79.3 and 72.3%). In addition, Clast5/Stra13 overexpression did not affect the apoptosis induced by serum deprivation (data not shown).

CD40 ligation induces B cell survival and proliferation, and simultaneously upregulates Fas expression and Fas susceptibility in B cells. The downregulation of Clast5/Stra13 by CD40L treatment suggests that Clast5 could be inhibitory to CD40-mediated enhancement of Fas sensitivity. We have previously shown that WEHI231 cells became more susceptible to Fas-mediated apoptosis after CD40 crosslinking (21). Treatment with the anti-CD40 antibody decreased the expression of the endogenous 3-kb Clast5/Stra13 transcript but the 2.5-kb RNA species derived from the transfected gene remained highly expressed (Fig. 4A). These cells were then treated with the anti-Fas antibody, which revealed a significantly reduced sensitivity to Fas-mediated apoptosis in Clast5/Stra13 transfectants compared with parental cells or vector control transfectants (Fig. 4B). Clast5/Stra13 thus inhibited CD40-triggered Fas sensitivity in B lymphoma cells.

Clast5/Stra13 Inhibits Colony Formation in NIH3T3 Cells

Inhibition of cell cycle progression by Clast5/Stra13 suggests that Clast5/Stra13 may suppress cell growth. We therefore performed the colony formation assays. Expression of Bax, a pro-apoptotic protein, decreased the number of colonies to approximately 30% of that of vector control (Fig. 5). Expression of Clast5/Stra13 resulted in a less dramatic, but statistically significant decrease in the number of colonies (Fig. 5). This inhibitory effect was not observed when the Clast5/Stra13 gene was cloned in an anti-sense direction (Clast5 as), or when an irrelevant DNA fragment was expressed. We concluded that forced expression of Clast5/Stra13 inhibited cell growth.

Clast5/Stra13 Localizes into Nuclear Foci

To verify the cellular localization of Clast5/Stra13, we expressed a Flag-tagged Clast5/Stra13 protein in COS-7 cells. Western blot analysis confirmed the expression of the 45.4 kDa Clast5/Stra13 protein (Fig. 6A). Immunostaining with the anti-Flag antibody (M2) and confocal analysis revealed that Clast5/Stra13 was localized in nuclei, forming nuclear foci rather than diffusely distributed (Fig. 6B). This staining pattern is

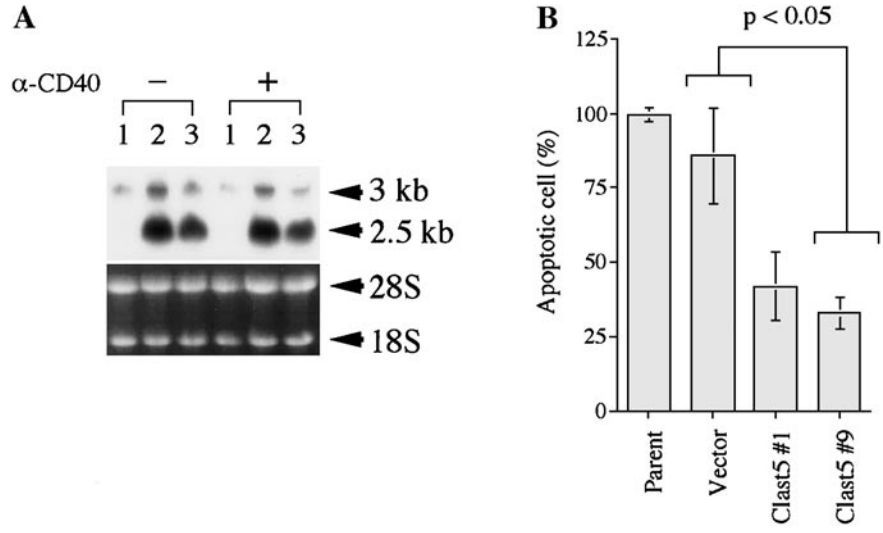

FIG. 4. Effects of forced expression of Clast5/Stra13 on Fas-mediated apoptosis in WEHI231 B lymphoma cells. (A) Endogenous (3-kb) and transfected (2.5-kb) Clast5/Stra13 gene expression before and after treatment with the anti-CD40 antibody. (B) Induction of apoptosis by the anti-Fas antibody.

consistent with the recent finding that Clast5/Stra13 is a transcription factor (22).

DISCUSSION

In the present study, we have identified Clast5/Stra13 as a unique gene whose expression is rapidly downregulated by a variety of stimuli that activate B cells, including CD40L, anti-IgM antibodies, IL-4 and LPS. Consistent with its downregulation upon B cell activation, Clast5/Stra13 overexpression inhibited cell cycle progression into S phase in B cells and suppressed cell growth in fibroblasts. In addition, Clast5/Stra13 expression is sharply downregulated in plasma cells, suggesting a role for Clast5/Stra13 in the inhibition of B cell terminal differentiation. Clast5/Stra13 thus appears to be a negative regulator for B cell proliferation and differentiation.

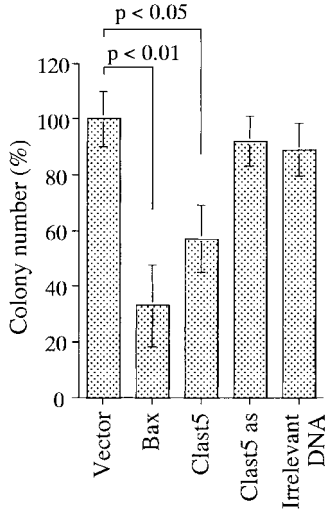

FIG. 5. Clast5/Stra13 suppresses cell growth. Colony formation assays were performed as described under Materials and Methods.

The biological significance of CD40 in the immune system has attracted many groups to isolate novel genes that act downstream of CD40. Several CD40-inducible genes have been identified, including NF-κB and A20 (23, 24), which are transcription factors, and bcl-xL and A1/Bf1-1 (25–28), which regulate apoptosis. However, few genes have so far been reported that are downregulated by CD40L stimulation. Clast5/Stra13 thus represents a unique gene whose expression shows an inverse correlation with the activation and terminal differentiation of normal B lymphocytes.

Stra13 was originally identified as a novel retinoic acid-inducible gene of the basic helix-loop-helix (bHLH) family. The amino acid sequence of Stra13 shows the highest homology in the bHLH domain with *Drosophila Hairy, Enhancer* of *Split*, and mouse Hes1 proteins (19). Overexpression of Stra13 in P19 embryonal carcinoma cells enhanced the neuronal differentiation, accompanied by altered expression of mesodermal and neuronal markers (19). This gene is also known as a new member of target genes of the Von Hippel–Lindau tumor suppressor gene (VHL) (29). More recently, Stra13 was shown to be a transcriptional repressor through histone deacetylase-dependent and independent mechanisms, and its overexpression repressed cell growth in NIH3T3 cells (22). The results obtained in the present study, using both B lymphoma cells and fibroblasts, confirm and expand previous finding, further demonstrating that Clast5/Stra13 expression inhibits cell cycle progression into S phase. However,

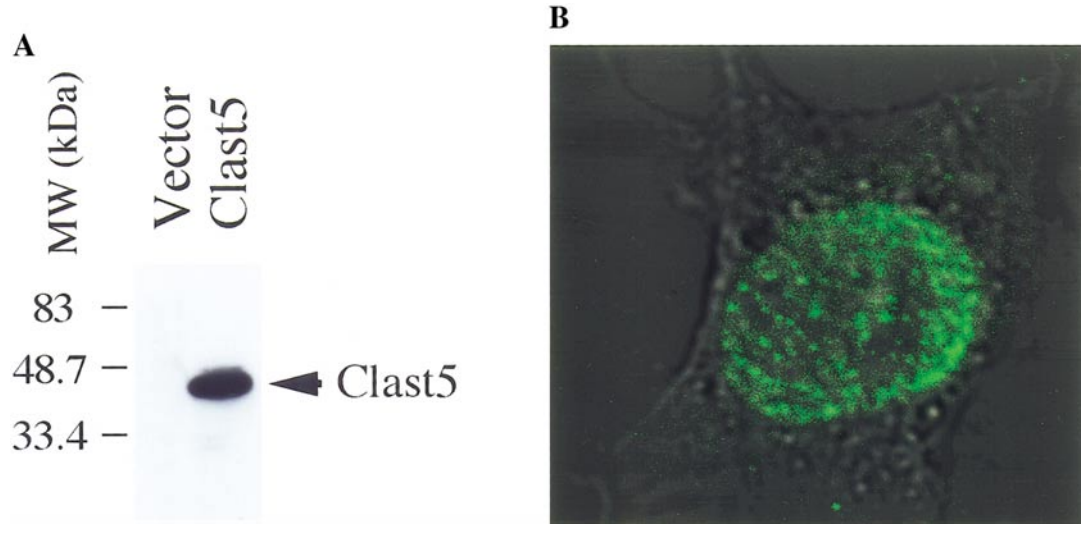

FIG. 6. Clast5/Stra13 localizes into nuclear foci. (A) Western blot analysis of Clast5/Stra13 protein expressed in COS-7 cells. (B) Immunostaining and confocal microscopy of COS-7 cells transfected with Clast5/Stra13.

since the growth rate between Clast5/Stra13 transfectants and the vector control was not significantly different, the observed delay in S phase entry may not simply be attributable to an inhibition of G1 to S transition, but may be due to an inhibition of G0 to cell cycle entry. Further studies are required to clarify this point.

CD40 ligation of B cells induces elevated Fas expression and enhances sensitivity to Fas-mediated apoptosis. In the present study, we found that CD40 ligation downregulated Clast5/Stra13 expression, suggesting a link between elevated Fas susceptibility and decreased Clast5/Stra13 expression. Indeed, forced expression of Clast5/Stra13 in WEHI231 B lymphoma cells significantly reduced CD40-triggered sensitivity to Fas-mediated apoptosis. As a transcriptional repressor, Clast5/Stra13 may inhibit the upregulation of Fas expression induced by CD40L. Alternatively, Clast5/Stra13 may modulate the expression of genes that are directly or indirectly involved in the Fas susceptibility. We have generated Clast5/Stra13 transgenic mice and it would be interesting to examine whether transgenic B cells have altered Fas expression and Fas susceptibility.

It is intriguing that Clast5/Stra13 expression is differentially regulated in neural cells and B lymphocytes. Stra13 expression is upregulated by retinoic acid (RA), which induces neuronal differentiation. The RA-inducible Clast5/Stra13 appears to facilitate neural cell differentiation by regulating the expression of other genes (19). In contrast to neural cells, Clast5/Stra13 expression is strongly downregulated by a variety of stimuli that induce B cell activation and terminal differentiation. Clast5/Stra13 thus appears to have opposite functions in the neural cells and B lymphocytes. Since Clast5/Stra13 is also expressed in T cells, macrophages and fibroblasts (data not shown), it may have a broad function in the regulation of growth and differentiation of many other cell types.

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