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Role of CAGE, a Novel Cancer/Testis Antigen, in Various Cellular Processes, Including Tumorigenesis, Cytolytic T Lymphocyte Induction, and Cell Motility

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A cancer-associated antigen gene (*CAGE*) was identified by serological analysis of a recombinant cDNA expression library (SEREX). The gene was identified by screening cDNA expression libraries of human testis and gastric cancer cell lines with sera from patients with gastric cancer. *CAGE* was found to contain a D-E-A-D box domain and encodes a putative protein of 630 amino acids with possible helicase activity. The *CAGE* gene is widely expressed in various cancer tissues and cancer cell lines. Demethylation plays a role in the activation of *CAGE* in certain cancer cell lines where the gene is not expressed. The functional roles of *CAGE* in tumorigenesis, the molecular mechanisms of *CAGE* expression, and cell motility are also discussed.

Keywords: Cancer-associated antigen gene, cell cycle, cell motility, cytolytic T lymphocyte, signaling network, tumorigenesis

There is a great deal of evidence that the immune system interacts with tumor cells during the course of a disease, and the presence of tumor-infiltrating lymphocytes leads to a better prognosis and the prolonged survival of individual cancer patients. Several experimental strategies have already been employed to identify antigens recognized by T cells. For example, T-cell epitope cloning methods have successfully identified tumor antigens recognized by cytolytic T lymphocytes (CTLs) [5], where MAGE-1 was the first antigen identified by such a method [5], followed by BAGE and GAGE [4, 77]. These antigens all share a common characteristic, as their expression in normal tissue is restricted to the testes, ovaries, or placenta, whereas they are widely expressed in various tumor tissues. Therefore, this group of antigens is referred to as cancer/testis antigens. Furthermore, the identification of tumor antigens that elicit spontaneous T-cell responses in cancer patients and the discovery of the mechanisms of antigen expression, processing, and MHC class I-restricted presentation of antigen-derived peptides have made it possible to design peptide-based immunotherapies.

Thus, patients with antigen-positive tumors can be vaccinated with the relevant peptides that are presented in the context of MHC I molecules [31]. Moreover, it has been shown that immunization with the relevant peptides derived from cancer/testis antigens can induce a cellular immune response [53, 55, 58]. Meanwhile, the development of a new cloning technology called SEREX (serological analysis of recombinant cDNA expression library) has made it possible to identify a multitude of tumor antigens that induce a humoral immune response in patients. These antigens can be recognized by CTLs ($CD8^+$ T cells), and more than 2,000 SEREX-defined antigens have already been identified (http://www.licr.org/SEREX.html).

Characteristics of Cancer/Testis Antigens

Certain cancer/testis antigen (CT) gene families contain multiple members (*e.g.*, *MAGEA*, *GAGE1*), as well as splice variants (*e.g.*, *XAGE1a*, *XAGE1b*), and 89 distinct transcripts are currently known to be encoded by the CT genes. The CT antigens share the following characteristics: (i) predominant mRNA expression in the testes, and not in other normal somatic tissues, (ii) mRNA expression in a wide range of human tumor types, (iii) existence of multigene families, and (iv) with rare exceptions, localization of the coding genes in the X chromosome. Table 1 shows a partial list of the CT antigens that have already been identified using various methods, including SEREX [12, 17, 27, 44, 52, 64]. Many testis-specific transcripts and proteins are under such tight regulatory control that they are almost never expressed in cancers other than in germ cell tumors. However, the frequent expression of CT antigens in various types of tumors is an exception to this general rule, suggesting that CT antigens, most with unknown functions at present, are a distinct group of proteins in terms of their regulation and possibly their biological function.

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Table 1. Partial list of genes identified by SEREX.

CT antigen	Number of genes	Chromosome	Immunity in cancer patients
MAGE-A [58]	15	Xq28	Cellular and humoral
MAGE-B [58]	17	Xp21	Cellular
SSX-2 [58]	5	Xp11.2	Cellular and humoral
SCP-1 [29]	3	1p12-13	Humoral
NY-ESO-1 [58]	3	Xq28	Cellular and humoral
CT10 [41]	1	Xq27	Humoral
SAGE [49]	1	Xq28	Unknown
cTAGE-1 [17]	1	18p11.2	Humoral
GAGE-A [58]	8	Xp11.4	Cellular
CT16 [41]	2	Xp11.2	Unknown
CT17 [41]	1	21q11	Unknown
HOM-TES-85 [27]	1	Xq24	Humoral
CT7/MAGE-C1 [58]	7	Xp26-27	Humoral
CAGE [12]	1	Xp22	Humoral

These genes were detected by CTLs or antibodies.
Numbers in parentheses denote references.

Identification of *CAGE* by SEREX

Evidence related to the immune regulation of different cancers continues to grow. The presence of cancer-specific and immune-stimulating genes was first reported in melanomas [75], and more than 2,000 tumor antigens have since been identified. The identification of tumor antigens is critical for diagnosis and therapy. Since tumor antigens are recognized by antibodies [8, 15, 31, 39, 48] and CTL [5, 9], SEREX has been used to identify tumor antigens recognized by autologous antibodies in cancer patients [36, 43, 63]. The SEREX approach offers the following features: (i) the use of fresh tumor specimens restricts the analysis to genes that are expressed by the tumor cells *in vivo*, (ii) the use of patient serum allows the identification of multiple antigens, and (iii) the screening is restricted to antigens against which patients have raised high-titer

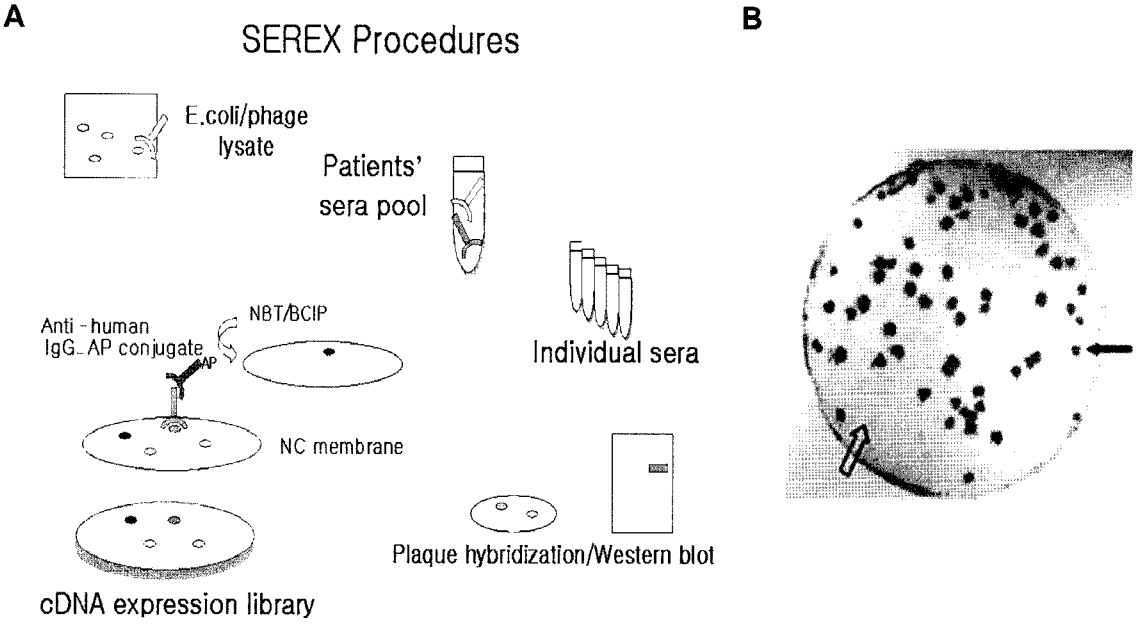

Fig. 1. Identification of SEREX-defined antigens.
A. cDNA expression libraries were constructed from gastric cancer cell lines or human testis tissue. Poly(A)$^+$ RNA (5 μg) was converted into cDNA by reverse transcriptase. The resulting cDNA library was cloned into a λ ZAP expression vector. Each library usually consisted of 2×10^6 primary recombinants on average, and 5×10^5 of the recombinants were used for immunoscreening. Each recombinant cDNA library was transformed into *E. coli* to yield a recombinant cDNA expression library. **B.** Seroreactivity of gastric cancer patients for *CAGE*. Phages without an insert were mixed with test clones and served as negative controls. Assays were only scored positive when the test clones were clearly distinguishable from the control phages. The bold arrow indicates a test clone, whereas the blank arrow indicates a control clone.

antibody responses. SEREX has been applied to a range of tumor types, including melanomas [6], esophageal cancer [10], lung cancer [24, 59], and gastric cancer [56]. SEREX provides broad targets for a large-scale analysis of the humoral response in cancer patients and healthy individuals, plus it employs a bacteriophage recombinant cDNA expression library prepared from tumor tissues, tumor cell lines, and testis tissues (Fig. 1). The use of tumor cell lines for a SEREX analysis also has benefits, including the absence of contaminating normal-cell types, invariably present in tumor specimens, and the elimination of B cells that give rise to false-positive IgG-expressing clones in the expression library. The cDNA expression library is used to transduce *E. coli*. A recombinant protein library is then induced and transferred to nitrocellulose membranes, followed by incubation with diluted (1:100–1:1,000) extensively pre-adsorbed pooled serum from the autologous patient. The clones reactive with high-titer antibodies are identified using an enzyme (an alkaline phosphatase-conjugated secondary antibody) specific for human IgG, and the positive clones are then subjected to DNA sequencing. The sequence information of a DNA insert can be used to determine the expression profile of the transcript and to evaluate the incidence of antibody responses to the respective antigens.

Tumor antigens recognized by antibodies and CTLs are classified into differentiation antigens, mutational antigens, overexpressed antigens, and cancer/testis antigens. The first CT antigens were identified in melanomas, where CT antigens, such as MAGE and BAGE, show a higher expression in metastatic melanomas than in primary melanomas. Since these antigens are also likely to be oncogenic, as spermatogenic cells do not express HLA molecules, they are excellent targets for the development of cancer therapeutics. Gastric cancers are one of the most common cancers in Asian countries, and are typically resistant to chemotherapy and radiation therapy. Therefore, the identification of tumor antigens specific for gastric cancers is critical for the development of diagnostics and therapeutics. As only a few antigens are currently known to be associated with gastric cancers, the identification of multiple tumor antigens is critical for the development of diagnostics and therapeutics for gastric cancers. Moreover, only a few genetic alterations have so far been reported related to gastric cancers. Accordingly, in an effort to identify such antigens, a SEREX analysis (Fig. 1A) was performed. Cancer-restricted recognition suggests common origins for immunogenicity, such as gene mutation or aberrant expression, indicating that serological methods of gene discovery can be used to identify molecules of etiologic relevance to cancer. Yet, the current authors were interested in identifying cancer/testis antigens.

To identify such antigens, cDNA libraries constructed from gastric cancer cell lines and human testis tissues were screened with pooled sera from patients with gastric cancer. Thirty-nine clones were identified that reacted with the pooled sera from five gastric cancer patients. The positive clones were then excised *in vivo* in a plasmid form. In this screening, the most frequently isolated genes were genes encoding ADP ribosyl transferase, RBP JK/H-2k binding factor 2, and the poly(A)-binding protein, comprising 14%, 9%, and 33% of the clones, respectively. An RT-PCR and EST database search revealed that most of the clones identified showed ubiquitous expression patterns.

Table 2. SEREX-defined genes identified by screening sera from patients with gastric cancer, using cDNA expression libraries constructed from human gastric cancer cell lines or human testis tissue.

Designation	Accession No.	Gene	SEREX DB	No. clones
St-1	XM010732	ADPRT	Yes	15
St-2	BC000672	G prot.	No	5
St-4	NM006940	SOX5	No	1
St-8	XM003095	ZNF288	No	7
St-9	AF309034	SOX6	No	1
St-15	XM007263	KSN2	No	3
St-17	XM008359	HDAC5	Yes	1
St-19	XM008972	DDXL	No	2
St-21	AY039237	Novel	No	1
St-28	NM002752	JNK2	No	8
St-30	XM018280	Poly(A) BP	No	35
St-31	NM015874	RBPJK	Yes	11

Table 2 shows a partial list of the genes identified by the SEREX of the sera from the gastric cancer patients. The cDNA libraries were constructed from human gastric

Table 3. Expression pattern of *CAGE* in various normal tissues, cancer cell lines, and tumor tissues.

Normal tissues	Freq.	Cancer cell lines	Freq.	Cancer tissues	Freq.
Liver	0/2	Stomach	9/10	Stomach	17/19
Kidney	0/2	Lung	3/4	Lung	4/4
Large intestine	0/2	Hepatic	9/10	Cervical	20/20
Small intestine	0/2	Cervical	6/7		
Lung	0/2	Melanoma	0/5		
Ovary	0/2	Breast	0/4		
Spleen	0/3	Kidney	2/5		
Trachea	0/2	Colon	2/4		
Muscle	0/2	Leukemia	0/12		
Stomach	0/3	Myeloma	0/5		
Temporal lobe	0/2	Prostate	0/2		
Parietal lobe	0/2	Pancreatic	0/6		
Testis	2/2	Sarcoma	1/1		
Thymus	0/1				
Skin	0/1				
Spinal cord	0/1				

An RT-PCR analysis was performed to determine the expression of *CAGE*.

cancer cell lines and human testis tissues. None of the clones reacted with sera from healthy individuals or lung cancer patients. Among the clones, a novel gene (*st-21*, later named *CAGE*) attracted particular interest based on its reactivity with the sera from the gastric cancer patients (Fig. 1B).

An RT-PCR was performed to determine the expression profile of *CAGE*, which was found to be widely expressed among cancer cell lines and cancer tissues, yet restricted to testis tissue among normal tissues (Table 3). Next, RH-panel mapping was performed to determine the localization of *CAGE* in human chromosomes, and shown to be located in the X chromosome [12], which is typical of cancer/testis antigen genes. Meanwhile, a DNA sequencing analysis showed that *CAGE* contained a helicase motif, suggesting a role in RNA processing [12]. After the DNA sequencing, it was also found that CAGE encoded a protein containing a D-E-A-D box domain. DEAD box-containing proteins are known to play important roles in various cellular processes, including RNA processing, embryogenesis, and cell growth [47]. In addition, proteins with a DEAD box domain usually display helicase activity [26]. The CAGE protein was also found to exhibit ATPase activity (unpublished observation). A Northern blot analysis revealed the presence of a 2.4 kb transcript. Similar to *HAGE*, *CAGE* did not contain any intron sequences [12]. Furthermore, Southern blot hybridization showed that *CAGE* existed as a single copy in the genome.

Iwata *et al.* [29] subsequently cloned *CAGE* by screening cDNA expression libraries made from testis or endometrial cancer cell lines using sera from patients with endometrial cancer or melanomas. As a result, anti-CAGE IgG antibodies were detected in the sera from 5 of 45 endometrial cancer patients, 2 of 24 melanoma patients, and 2 of 33 colon cancer patients, yet not in the sera from healthy individuals. Meanwhile, based on an ELISA analysis, anti-CAGE antibodies were detected in the sera from 12 of 45 endometrial cancer patients, 2 of 20 melanoma patients, and 4 of 33 colon cancer patients. Thus, when taken together, these results suggest that CAGE may be a valuable marker for the diagnosis and prognosis of cancer patients.

Expression Regulation of *CAGE*

DNA methylation is well recognized as a possible mechanism of tumorigenesis [1, 2, 14, 18–20, 39, 41], and is involved in a variety of cellular activities, including genomic imprinting [40, 46], mutagenesis [3], aging, and the regulation of tissue-specific gene expression [16]. The expression of cancer/testis antigens, such as MAGE, has already been reported to be under epigenetic regulation [23, 49, 67, 78]. The methylation of DNA at the CpG dinucleotide is a post replication event, catalyzed by the DNA (cytosine-5)-methyltransferase [68]. There is also a close association between DNA methylation and gene silencing [34, 70]. Genes that are not expressed display methylation predominantly at the CpG dinucleotides, and the associated transcription repression manifests in stable and heritable changes in the local chromatin structure [79]. Transcription repression caused by DNA methylation appears to be mediated by the inhibition of the binding of a transcription factor to the methylated CpG-containing DNA motifs, or by the recruitment of CpG-binding proteins, which subsequently recruit histone deacetylase containing corepressor complexes to the methylated DNA [21, 32, 37, 50, 56, 71, 72]. Thus, since *CAGE* was only expressed in testis tissue among the normal tissues, the expression of CAGE may be under epigenetic regulation, just like MAGE. Furthermore, treatment with 5-aza-2-deoxycytidone increased the expression of *CAGE* in certain cancer cell lines that do not normally express *CAGE* [11], also indicating that the expression of *CAGE* is under epigenetic regulation. A methylation-specific PCR (MSP) showed that the expression of *CAGE* was closely related with the methylation pattern (Fig. 2A), because when examining the relationship between the extent of expression and the methylation, the methylation status generally reflected the expression of *CAGE*. SNU886 cells, which do not express *CAGE*, displayed heavy methylation of CpG sites, where C33a cells, which express *CAGE*, displayed a low level of methylation of CpG sites. However, the methylation status of *CAGE* was not completely associated with expression, suggesting the presence of other factors regulating the expression of *CAGE*. The methylation status of paired gastric tissues was also determined, where gastric cancer tissues showed a higher hypomethylation frequency than normal mucosa tissues (Fig. 2C, lower panel). An RT-PCR also showed that the expression of *CAGE* was higher in gastric cancer tissues than in normal tissue (Fig. 2C, upper panel), whereas an MSP analysis using various tissues revealed that hypomethylation of *CAGE* was seen in premalignant tissues [11], suggesting that the expression of *CAGE* is associated with the progression of cancer. Methylation of the promoter by SSs1 methylase greatly decreased the expression of *CAGE*, as determined in luciferase activity assays [11], which indicates that methylation of CpG sites prevents access by transcription factors. The *CAGE* promoter sequences contain consensus sequences for the binding of transcription factors, including GATA, ELK-1, and Ets. Thus, the methylation status of the CpG sites of the *CAGE* promoter was closely associated with the lack of *CAGE* expression (Figs. 2A and 2C). Furthermore, an electrophoretic mobility shift assay (EMSA) analysis revealed that the methylation of the *CAGE* promoter prevented any binding of nuclear factors [11].

From an MSP analysis of archival samples, high frequencies of hypomethylation were found [11] for breast cancer (20/24, 83%), lung cancer (18/25, 72%), and hepatic carcinomas (19/31, 61%), whereas low frequencies of

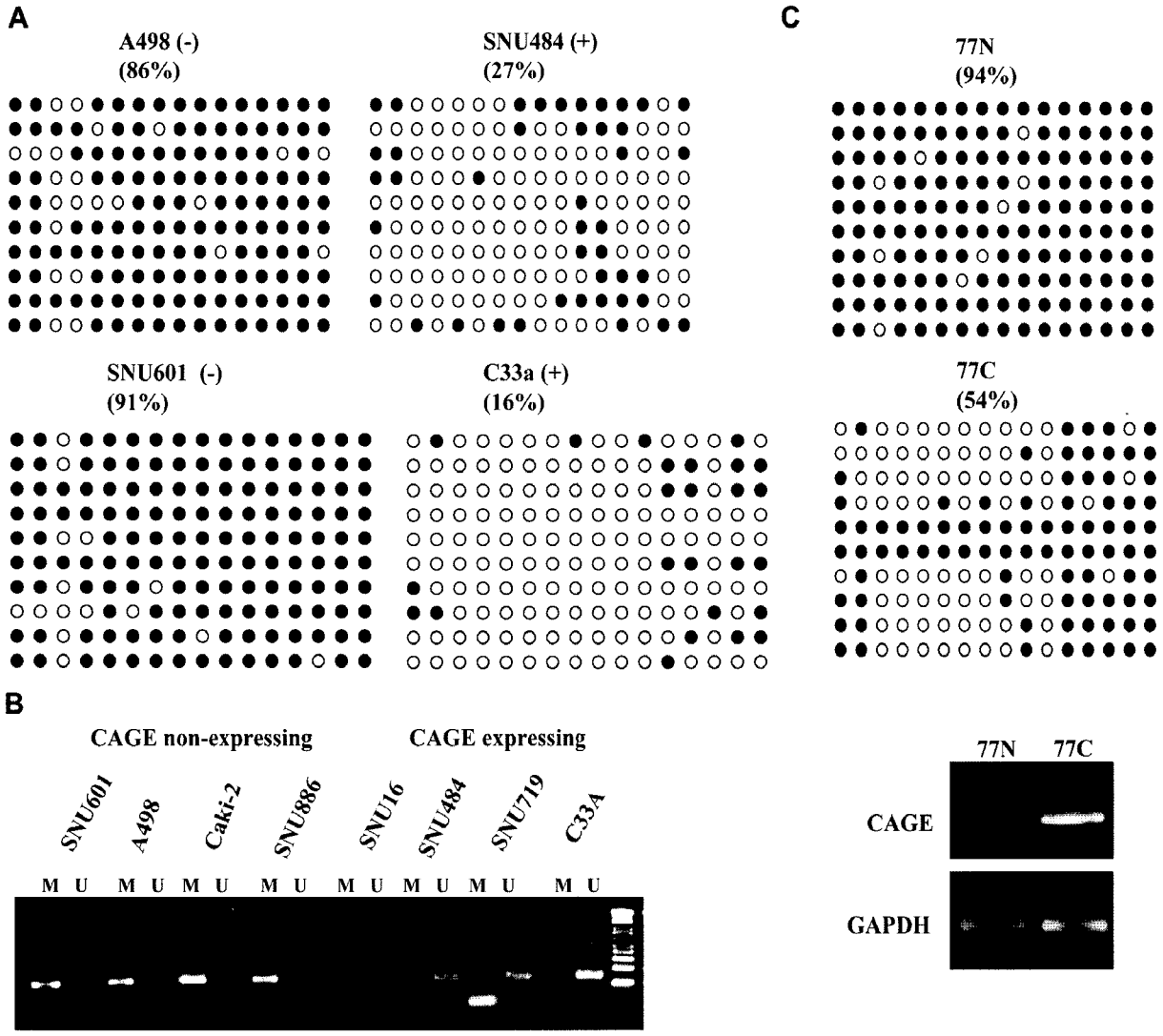

Fig. 2. Correlation of methylation status and expression of *CAGE*.
A. Comparison of methylation status of 16 CpG sites of *CAGE*-expressing and *CAGE*-nonexpressing cancer cell lines. Each row of circles represents a single plasmid cloned and sequenced from the PCR products of the amplified DNA following sodium bisulfite treatment. An open circle denotes unmethylated cytosine, whereas a closed circle denotes methylated cytosine. To determine the DNA methylation status, PCR-amplified products from each cell line were transformed into *E. coli*. The resulting plasmids were then subjected to DNA sequencing. The numbers in parentheses denote the frequency of methylation. **B.** Methylation-specific PCR of various cancer cell lines. The PCR yielded a 150 bp product. **C.** Methylation-specific PCRs of gastric cancer tissue and corresponding mucosa tissue. The lower panel shows an RT-PCR of gastric cancer tissue and normal tissue.

hypomethylation were found for uterine cervix cancer (2/22, 9%), larynx cancer (4/19, 21%), colorectal cancer (4/16, 25%), and prostate cancer (8/23, 35%). A normal prostate (0/14), chronic hepatitis (0/11), and normal colon (0/14) showed no hypomethylation. Therefore, the absence of any hypomethylation of *CAGE* in the normal prostate and normal colon suggests that the hypomethylation of *CAGE* may be associated with the development of prostate and colon cancers. From an MSP analysis using fresh-frozen tissues, higher frequencies of hypomethylation were found for hepatic carcinomas (5/6, 83%), gastric cancer (50/64, 78%), and colorectal cancer (8/9, 89%). The hypomethylation of *CAGE* was also found in blood samples from cancer patients [11], suggesting that the methylation of *CAGE* may be a valuable marker for the diagnosis of cancers.

CTL-inducing Activity of *CAGE*

The immune system has been shown to interact with tumor cells during the course of disease. The following are general properties of cancer antigens: (i) cancer antigens contain epitopes that bind to various HLA alleles and (ii) tumor-infiltrating T lymphocytes contain various cancer antigens. A large number of tumor-infiltrating lymphocytes (TILs) from tumors greatly increase T-cell populations capable of recognizing cancer antigens [28, 53].

CTLs recognize antigens presented as peptides (8–10 mer) on HLA class I molecules. The peptide-HLA complex

is then recognized by the T cell receptor (TCR) on the surface of a CTL. The nonamer peptides of GAGE, BAGE, and MAGE have already been shown to be recognized by CTLs [4, 74, 77]. Moreover, MAGE-A4- and SAGE-derived peptides have been shown to induce CTL activity towards human tumor cell lines [53], RAGE-1- and MAGE-9-derived peptides were shown to induce CTL activity towards renal tumor cell lines [58], and MAGE1-derived peptides were shown to be recognized by CTLs on human HLA-A2 tumor cells [60]. The identification of peptides derived from tumor-associated antigens (TAAs) expressed by different human tumors is valuable for active immunotherapy, and immunization studies using these tumor-associated antigen (TAA) peptides have already been relatively successful in generating high levels of T cells against cancer antigens [33, 62].

In the case of gastric cancer, peptides from SEREX-defined antigens are able to induce CTL responses, indicating their potential uses as cancer vaccines in certain patients with gastric cancer [38]. Since many cancer/testis antigens elicit CTL responses, it was expected that CAGE would be able to elicit CTL responses to kill tumor cells that express *CAGE*. Therefore, various CAGE antigen-derived peptides were designed and synthesized. The peptides were designed to bind to HLA-A2 molecules, and the CTL-inducing activity of these peptides was then determined. Fig. 3A shows the experimental schemes used for the study. The CAGE-derived peptides (A2-1, A2-2) were found to induce CTL responses (Fig. 3B). When using cancer cell lines expressing the *CAGE* gene at a different level, the peptides exerted cytotoxic effects against those cell lines with a high expression of *CAGE* [64], thereby suggesting that CAGE-derived peptides may be valuable as cancer therapeutics.

Molecular Functions Associated with CAGE

Cell synchronization studies have found that *CAGE* shows cell-cycle-specific expression, with an expression peak in the late G1 phase [12]. Moreover, expression analyses have shown that *CAGE* expression is higher in gastric cancer tissue than in normal mucosa tissue (Fig. 2C), whereas sera from patients with endometrial cancer contained high titers of antibodies against the CAGE antigen (Fig. 1B). Therefore, all these findings would seem to indicate that *CAGE* is an oncogene. Thus, to determine the oncogenic potential of CAGE, mouse L929 cells stably expressing

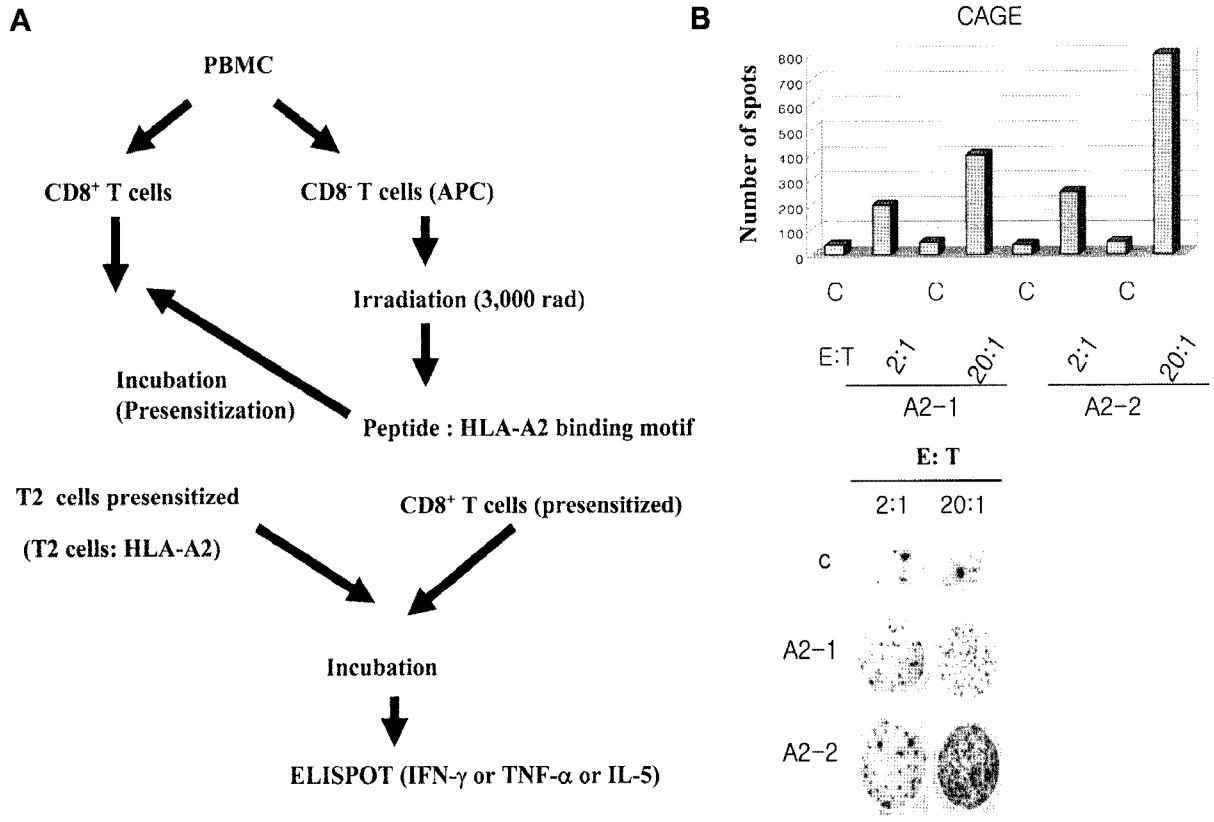

Fig. 3. CTL-inducing activity of CAGE-derived peptides.
A. $CD8^+$ T cells were isolated from peripheral blood lymphocytes (PBLs) using magnetic beads and seeded onto 48-well plates. The PBLs depleted of $CD8^+$ T cells were then irradiated and incubated with β2-microglobulin (2.5 μg/ml) and peptide (10 μg/ml) for 1 h and added to plates for $CD8^-$ T cell presensitization. **B.** $CD8^-$ T cells isolated from healthy individuals were sensitized with HLA-A2-restricted peptides derived from CAGE and tested against T2 cells sensitized with the same peptides. C denotes the control without peptide stimulation. Each bar represents the average of spots in triplicate wells.

CAGE were generated. The cells stably expressing CAGE showed enhanced growth rates, anchorage independence, and migration [65]. In addition, CAGE further enhanced the tumorigenicity of various cancer cells in nude mice assays (unpublished observation). Since *CAGE* also exhibits cell-cycle-specific expression [12], *CAGE* expression may be associated with cyclins. Hence, when using a Tet-on induction system, CAGE overexpression was found to increase the expression of G1 cyclins, including cyclin D and cyclin E (unpublished observation). Moreover, cyclin D/E induced by CAGE caused the phosphorylation of Rb through E2F-1 (unpublished observation). Consequently, all these findings suggest an oncogenic potential for CAGE. Fig. 5 shows a hypothetical model of the effect of CAGE on the cell cycle. In detail, CAGE activates cyclin D-CDK4 and cyclin E-CDk2. The activated cyclin-CDK phosphorylates serines on the Rb protein. This frees E2F, a transcription factor, from inhibitory Rb, to induce the expression of various genes involved in cellular growth. Since the oncogenic potential is closely related with enhanced cell motility, the effect of CAGE on cell motility was examined, and the overexpression of CAGE using a Tet-on system was shown to enhance mouse L929 cell motility [66]. The overexpression of CAGE was also found to enhance the motility of cancer cells, such as HeLa (unpublished observation). Next, the molecules that mediated the effect of CAGE on cell motility were determined. Focal adhesion kinase (FAK) is well known to be associated with cell motility [51], and the overexpression of CAGE increased the phosphorylation of FAK (Fig. 4B), whereas the overexpression of FRNK, an endogenous inhibitor of FAK, decreased the motility of cancer cells transfected with *CAGE* (Fig. 4A, lower panel). The inhibition of ERK and p38 MAK showed the same effect on cell motility (Fig. 4B, lower panel), whereas the overexpression of CAGE increased the phosphorylation of ERK and p38 MAPK (Fig. 4B, upper panel). However, further identification of the targets of CAGE is required for a better understanding of the mechanisms associated with CAGE-promoted cell motility.

Current Progress and Future Work

CAGE exhibits oncogenic potential [65] and enhanced cell motility *via* a pathway involving FAK, ERK, and p38 MAPK [66]. The oncogenic potential of CAGE is further supported by the findings of a tissue array analysis, where

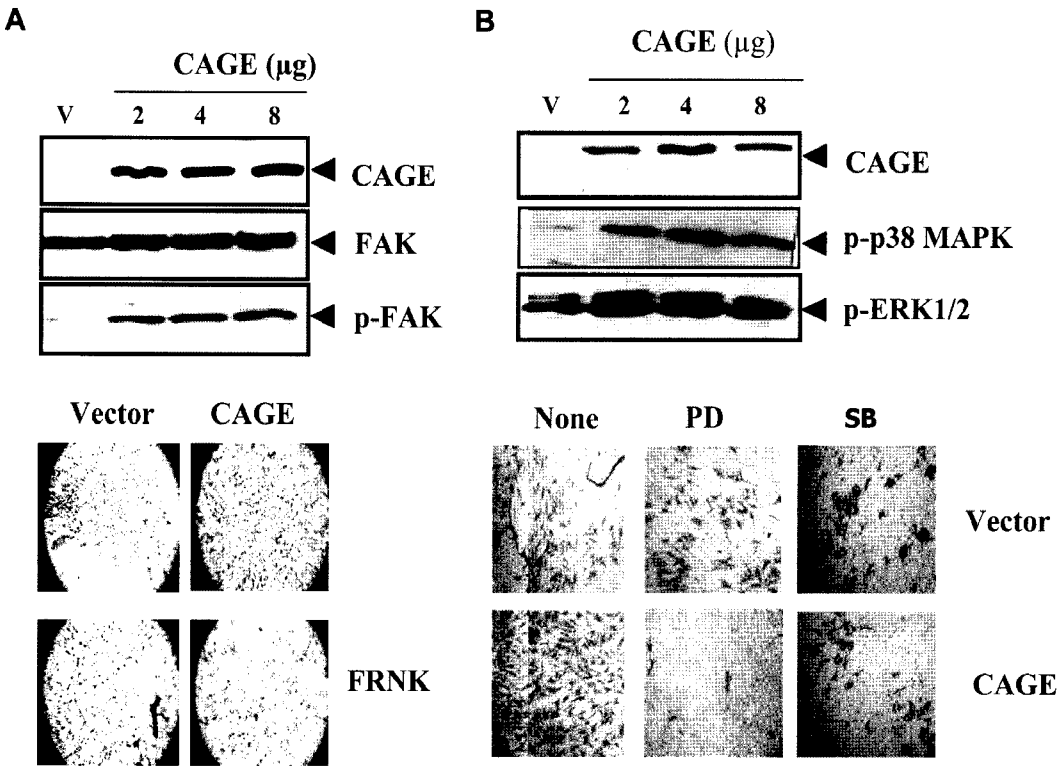

Fig. 4. CAGE promotes cell motility *via* a pathway involving FAK, ERK, and p38 MAPK.
A. SNU387 human hepatic cancer cells were transiently transfected with a control vector or *CAGE* vector. After transfection (48 h), cell lysates were prepared and subjected to a Western blot analysis (upper panel). The same cells were transiently transfected with a control vector or FRNK, an endogenous inhibitor of FAK. After transfection (24 h), the cells were transfected with a vector containing the *CAGE* gene. The next day, the cells were subjected to chemoinvasion assays (lower panel). **B.** Western blot analysis of SNU387 cells transiently transfected with a control vector or CAGE (upper panel). The SNU387 cells were pretreated with PD98059 (10 μM) or SB203580 (20 μM) for 1 h, and then transfected with a control vector or vector containing the *CAGE* gene. After transfection (48 h), chemoinvasion assays were performed.

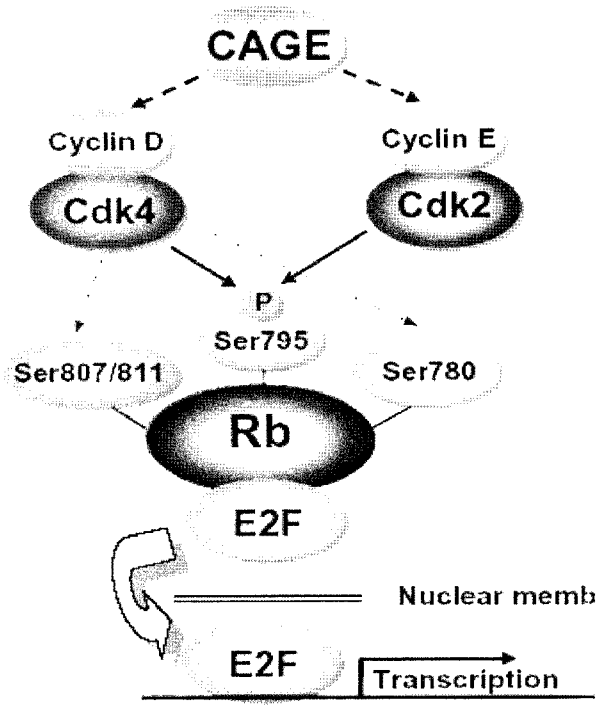

Fig. 5. Hypothetical role of CAGE in the cell cycle.

the expression of CAGE was highest in metastatic melanoma tissue, followed by malignant melanoma tissue and finally normal tissue (unpublished observation). Meanwhile, the overexpression of CAGE in HeLa cells enhanced the tumorigenic potential of HeLa cells in nude mice assays (unpublished observation). However, the molecular mechanism associated with CAGE-promoted cell motility and tumorigenesis still needs to be studied in further detail, along with the factor(s) regulating the expression of CAGE, and the pathway(s) in which CAGE may play an important role.

Hyaluronic acid (HA) is a glycosaminoglycan found in the extracellular matrix of the human brain. HA is already known to promote the proliferation of certain tumor cell lines [54, 75] and epithelial cells [22], and inhibits osteoclast cell differentiation [7]. Tumor-specific accumulation of HA has also been widely observed in human tumors, including colon cancer [35] and breast cancer [13], making studies with HA an excellent model for the mechanisms associated with cell motility. Thus, since CAGE was shown to promote cell motility *via* a pathway involving FAK, ERK, and p38 MAPK [65], it was interesting to determine the relationship between HA and CAGE in promoting cell motility. In a preliminary study, HA was found to induce the expression of *CAGE* (unpublished observation). HA increased Rac1 activity and the level of reactive oxygen species (ROS) (unpublished observation). PKCα and -δ were also shown to regulate Rac1 and ROS in melanoma cells (unpublished observation). Furthermore, the overexpression of Rac1 increased the expression of CAGE, whereas the inhibition of Rac1 by its dominant negative construct prevented HA from increasing the expression of CAGE (unpublished observation), suggesting that Rac1 is responsible for the increased expression of CAGE by HA. Rac1 is already known to be responsible for the generation of ROS in many cell lines [42]. Meanwhile, exposure to hydrogen peroxide (H_2O_2) increased the expression of *CAGE* (unpublished observation). However, although the expression of the *CAGE* gene was shown to be under epigenetic regulation, the expression of *CAGE* was not completely associated with the methylation status of its promoter, indicating the existence of other factors regulating the expression of *CAGE*. Thus, ROS regulation of the expression of *CAGE* will lead to the identification of additional factors regulating the expression of *CAGE*.

Epithelial-mesenchymal transition (EMT) occurs during tumorigenesis, which is closely related with the expression of EMT-related proteins [74], and HA has been found to affect the expression of proteins involved in EMT (unpublished observation). Thus, it would be interesting to examine whether CAGE affects the expression of these proteins.

The downregulation of CAGE also exerted a negative effect on HA-promoted cell motility (unpublished observation). Therefore, all these findings suggest that CAGE is an important constituent in the signaling network involving HA. Transglutaminase II (TGase II) is involved in diverse cellular functions, including wound healing [25, 69] and tissue inflammation [61]. HA increases the expression of TGase II (unpublished observation), and the induction of TGase II by HA occurs later than that of CAGE (unpublished observation). In addition, the overexpression of CAGE was found to increase the expression of TGase II, whereas the downregulation of CAGE suppressed the induction of TGase II by HA (unpublished observation). Therefore, further identification of the downstream targets of CAGE is critical for a better understanding of the function of CAGE. Furthermore, the identification of the molecules interacting with CAGE is essential for understanding the functional role of CAGE. In a preliminary study, CAGE exhibited interaction with Rac1 (unpublished observation), and Rac1 has been shown to affect the expression of EMT-related proteins, suggesting a potential role of CAGE in EMT (unpublished observations). CAGE-derived peptides exert cytotoxic effects on tumor cells that express the CAGE protein [65], making it necessary to design more CAGE-derived peptides with various HLA specificities to develop effective therapeutic vaccines. A combination of these peptides may also prove to be a functional therapeutic vaccine against various solid tumors, including gastric cancers.

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