The cancer/testis antigen CAGE induces MMP-2 through the activation of NF-κB and AP-1

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INTRODUCTION

The wide expression of cancer/testis antigens in various tumors, contrasted with their restricted expression in normal tissues, make them attractive targets for cancer therapy. The cancer-associated antigen CAGE is a novel cancer/testis gene that was identified by screening recombinant cDNA expression libraries with the sera of gastric cancer (1), endometrial cancer and melanoma patients (2). The expression of CAGE is typical of other cancer/testis antigens as it is restricted to normal tissues to the testis and widely observed in various tumor tissues and cancer cell lines (2). In addition, the expression of CAGE is subjected to epigenetic regulation. 5-aza-2-deoxycytidine restores the expression of CAGE in cancer cell lines not expressing it (2). The existence of methylated CAGE promoter sequences in premalignant lesions suggests that the expression status of CAGE could be a useful diagnostic marker for the early detection of cancer (2). The expression of CAGE in the sera of various cancer patients, including those with melanoma, has been confirmed by Western blot analysis (3). Although cancer/testis antigens are considered oncogenes, the functional roles for these antigens in cellular processes such as cell motility have not yet been investigated. The downstream targets of these cancer/testis antigens and their interacting proteins currently remain unknown. We have previously reported that CAGE increases the motility of hepatic cancer cells by activating focal adhesion kinase (FAK), ERK and p38 MAPK (4, 5). However, the mechanism of CAGE-promoted cell motility has not been investigated in detail. In this study, we have examined the molecular mechanisms associated with CAGE-promoted cancer cell motility.

RESULTS AND DISCUSSION

CAGE activates ERK and Akt to induce expression and secretion of MMP-2 and to enhance cellular invasion

Sperm-associated gene 1 is a highly expressed cancer/testis antigen gene in pancreatic tumor tissues and testis that is known to enhance cell motility (6). Therefore, to evaluate the potential role of cancer/testis antigens in cell motility, we investigated the molecular mechanisms of CAGE-promoted cancer cell invasion. Among various cancer cell lines, the SNU387 human hepatic cancer cell line showed negligible levels of CAGE expression (data not shown).

It has been observed that matrix metalloproteinases (MMPs) play a crucial role in cellular invasion (7, 8), and that CAGE enhances cancer cell invasion through the activation of MAPK signaling (5). In determining the effect of CAGE on MMPs, it was found that CAGE induced the expression and secretion of MMP-2 (Fig. 1A), but not of MMP-9 (data not shown). The induction of MMP-2 by CAGE occurred at the transcriptional level (Fig. 1A) through increased MMP-2 promoter activity (Supplementary Fig. 1) caused by ERK and Akt activation (Fig. 1B). CAGE-mediated cellular invasion was triggered by ERK and Akt, but was negatively affected by the MMP-2 inhibitor.
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Fig. 1. ERK and Akt are necessary for the induction of MMP-2 by CAGE. (A) SNU387 human hepatic cancer cells were pretreated with or without PD (10 μM) or LY (10 μM) for 1 h, followed by transfection with control vector (2 μg) or CAGE (2 μg). At 48 h after transfection, cell lysates were subjected to Western blot analysis. Supernatants were subjected to gelatin zymography. Total RNAs were subjected to RT-PCR. V denotes control vector transfection. (B) SNU387 cells were pretreated with or without GM6001 (10 μM), PD (20 μM) or LY (10 μM) for 1 h, followed by transfection with control vector (2 μg) or CAGE cDNA (2 μg). At 48 h after transfection, cells were subjected to chemoinvasion assays. The columns represent the mean of triplicate experiments; bars, standard deviation. *P < 0.05 versus control, **P < 0.005 versus control.

Fig. 2. NF-κB is responsible for the induction of MMP-2 by CAGE. (A) SNU387 cells were transiently transfected with MMP-2 promoter-luciferase constructs with or without CAGE. Cell lysates were subjected to luciferase activity assays. The columns represent the mean of triplicate experiments; bars, standard deviation; **P < 0.005 versus control. (B) SNU387 cells were transiently transfected with control vector or CAGE. Cell lysates from each fraction were subjected to Western blot analysis. (C) SNU387 cells were transiently transfected with CAGE with or without IκB super repressor cDNA tagged with HA. Cell lysates and supernatants were subjected to Western blot and gelatin zymography, respectively. Total RNAs were subjected to RT-PCR.

GM6001 (Fig. 1B). These results suggest that CAGE enhances cellular invasion by inducing MMP-2 in response to the activation of ERK and PI3 kinase/Akt.

NF-κB is necessary for induction of MMP-2 by CAGE
Based on our finding that CAGE increased phosphorylation of inhibitory kappa B (IκB) (data not shown), we examined the role of NF-κB in the induction of MMP-2. CAGE increased NF-κB promoter activity following the transient transfection of MMP-2 promoter luciferase construct into SNU387 cells (Fig. 2A). The p65 subunit of NF-κB was also shown to translocate into the nucleus after CAGE overexpression (Fig. 2B). Although the expression of CAGE is under epigenetic regulation, it does not entirely correspond to the methylation status of CAGE (2), suggesting that expression of CAGE is under autocrine and/or paracrine control.

EGF is known to increase the secretion of hyaluronic acid (HA). Based on our preliminary findings, overexpression of CAGE was found to correlate with the activation of EGFR while HA induced expression of CAGE in SNU387 cancer cells (data not shown). Based on these results, we confirmed that EGF induced the expression of CAGE (Supplementary Fig. 2). The p65 subunit of NF-κB was induced to translocate into the nucleus by EGF (Supplementary Fig. 3A). Inhibition of NF-κB by the IκB super repressor prevented CAGE from inducing MMP-2 expression, which increased phosphorylation of ERK and Jun N-terminal kinase (JNK) but not of Akt or p38MAPK (Fig. 2C). This suggests that NF-κB functions in a pathway downstream of Akt/p38MAPK and upstream of ERK and JNK. Both EGF and CAGE increased phosphorylation of IκBβ (Supplementary Fig. 3B), suggesting that phosphorylation of IκB results from the activation of IκBβ by CAGE. CAGE also induced an interaction between IκBβ and Akt (Supplementary Fig. 3C), suggesting that Akt interacts with IκBβ to activate NF-κB. These results indicate that NF-κB mediates CAGE-promoted cellular invasion through the induction of MMP-2.

CAGE induces Fra-1 by activating ERK and MMP-2 through the AP-1 subunit junB by activating Akt, p38 MAPK and JNK
JNK is necessary for the induction of MMP-2 (Supplementary Fig. 4A, B) and for enhanced cellular invasion mediated by CAGE (Supplementary Fig. 4C). We therefore examined the role of AP-1 in CAGE-promoted cellular invasion. AP-1 plays many roles during cellular invasion, including as a down-
CAGE induces Fra-1 through activation of ERK, and induces Jun B by activation of Akt and p38 MAPK. (A) SNU387 cells were transiently transfected with various MMP-2 promoter-luciferase constructs (2 μg) or a pGL3-Basic control vector (2 μg) together with a pSV-βgalactosidase vector (0.6 μg). Twenty-four hours after transfection, control vector or CAGE was transiently transfected into SNU387 cells. The columns represent the mean of triplicate experiments, bars, standard deviation, *P < 0.05 versus control. (B) SNU387 cells were pretreated without or with various signaling molecule inhibitors for 1 h, followed by transfection with a control vector or CAGE. Western blot analysis of cell lysates was performed. (C) SNU387 cells were transiently transfected with control siRNA (10 nM), Fra-1 siRNA (10 nM) or JunB siRNA (10 nM) with or without CAGE. Cell lysates were subjected to Western blot analysis.

Fig. 4. Increased DNA binding activity of AP-1 is associated with CAGE-promoted cellular invasion. (A) SNU387 cells were pretreated with or without various signaling molecule inhibitors for 1 h, followed by transfection with a AP-1-promoter luciferase construct with or without CAGE. SNU387 cells were also transfected with the AP-1-promoter luciferase construct in combination with various constructs as described. Luciferase activity assays were performed on cell lysates. The columns represent the mean of triplicate experiments, bars, standard deviation, *P < 0.05 versus control, **P < 0.005 versus control. (B) SNU387 cells were transiently transfected with a control vector or CAGE. The specific DNA binding activity of AP-1 transcription factor was determined by EMSA in the presence or absence of a 10-fold excess of cold competitors. Anti-JunB antibody (5 μg) was employed for supershift analysis. AP-1 denotes the DNA-protein complex. SS denotes Jun B-DNA complex.

In conclusion, the cancer/testis antigen gene CAGE activates NF-κB and AP-1 to induce MMP-2. The induction of MMP-2 is necessary for CAGE-promoted cellular invasion. In this study, we therefore found a novel mechanism associated with CAGE-promoted cellular invasion. Further dissection of the molecular mechanism leading to the activation of NF-κB and AP-1 is necessary for a better understanding of CAGE-promoted cancer cellular invasion.
MATERIALS AND METHODS

Cell lines and cell culture
Cancer cell lines used in this study were cultured in Dulbecco’s modified minimal essential medium (DMEM; Gibco, Gaithersburg, MD, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS, Gibco) and antibiotics at 37°C in a humidified incubator containing 95% air and 5% CO2.

Materials
Anti-Akt, anti-phospho Akt (Ser473), anti-ERK, anti-phospho ERK, anti-p38MAPK, anti-phospho p38MAPK, anti-JunB, and anti-Fra-1 antibodies were purchased from Cell Signaling Technology Company (Beverly, MA). All other antibodies and chemicals used in this study were purchased from Sigma (St. Louis, MO). Anti-mouse and anti-rabbit IgG-horse radish peroxidase-conjugate antibody were purchased from Pierce Company (Rockford, IL, USA). Enhanced chemiluminescence (ECL) kit was purchased from Amersham (Arlington Heights, IL, USA). Lipofectamin and Plus™ reagents were purchased from Invitrogen (Carlsbad, CA, USA). The transwell chamber system was purchased from Costar (Acton, MA, USA). Bioneer (Daejeon, Korea) synthesized all primers used in this study.

Western blot analysis
For polyacrylamide gel electrophoresis (PAGE) and western blotting, cell lysates were prepared in lysis buffer [62.5 mM Tris-HCl pH 6.8, 2% (v/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 50 mM Dithiothreitol (DTT), 0.01% (w/v) bromophenol blue, 10 mM sodium fluoride, 1% (v/v) protease inhibitor cocktail, 1 mM sodium orthovanadate]. The samples were boiled for 5 min, and equal amounts of protein (10 μg/well) were analyzed by 10% PAGE. Following electrophoresis, proteins were transferred onto a nitrocellulose membrane, blocked in 5% non-fat dry milk, and incubated with primary antibodies overnight. After extensive washing, blots were incubated with anti-mouse or anti-rabbit IgG-horseradish peroxidase-conjugate antibody at a 1:3,000 dilution for 1 h and developed using ECL kit (Amersham).

Chemoinvasion assay
The invasive potentials of various cancer cells were determined using a transwell chamber system with 8-μm pore polycarbonate filter inserts (Costar, Acton, MA, USA). The lower and upper sides of the filter were coated with gelatin and matrigel, respectively. Trypsinized cells [2×10^5] in serum-free RPMI containing 0.1% BSA were then added to each upper chamber of the transwell. RPMI supplemented with 10% FBS was placed in the lower chamber and incubated at 37°C for 16 h. The cells were fixed by methanol and invaded cells were stained and counted.

Preparation of small interfering RNA (siRNA) duplexes
siRNA duplexes were constructed using the following target sequences: Fra-1, sense (5’-AAG CAT CAA CAC CAT GAG TGG CCT GTG TC-3‘); antisense (5’-AAC GTA GTG GTG GTA CTC ACC CCT GTG TC-3‘); JunB, sense (5’-AAT GGA ACA GCC CCT CTA CCA CCT GTG TC-3‘); antisense (5’-AAA CCT TGT CGG GAA GAT GGT CCT GCT TC-3‘); control, sense (5’-AAACGTGACAGTGGAGAAGGACCTGTCTC-3‘). The construction of siRNAs was performed according to the instruction manual provided by the manufacturer (Ambion, Austin, TX). Transfection of the siRNA constructs was performed using lipofectamine.

Gelatin zymography
Conditioned medium from SNU387 cells or HeLa cells cultured in serum-free medium was mixed 3:1 with substrate gel sample buffer [40% (v/v) glycerol, 0.25 M Tris-HCl, pH 6.8, and 0.1% (w/v) bromophenol blue] and loaded onto a 7.5% SDS-PAGE containing type I gelatin (2 mg/ml). After electrophoresis, the gel was soaked in Triton X-100 with shaking for 30 min with one change of solution. The gel was rinsed and incubated for 24 h at 37°C in substrate buffer [50 mM Tris-HCl, pH 7.5, 5 mM CaCl2 and 0.02% NaN3]. After incubation, the gel was stained with 0.1% Coomassie brilliant blue G-250 and destained in a solution composed of 50% methanol, 10% acetic acid and 40% water.

RT-PCR
Total RNAs were isolated by Trizol reagent and converted into cDNA using Superscript reverse transcriptase. The following primer pairs were used for PCR: MMP-2, 5’-TGACAGCTGCACCTGAG-3’ and 5’-CTCCTGAATGCCCTTGATGT-3’; Gelatin zymography, 5’-AAACGTGACAGTGGAGAAGGACCTGTCTC-3‘. The construction of siRNAs was performed according to the instruction manual provided by the manufacturer (Ambion, Austin, TX). Transfection of the siRNA constructs was performed using lipofectamine.

Transient transfection and luciferase assay
pGL3 vector (2 μg; Promega, Madison, WI, USA) containing wild type or mutant MMP-2 promoter (2 μg) was transiently transfected into SNU387 cells, along with 0.6 μg of the pSV-β galactosidase control vector. Lipofectamine (Invitrogen) was used for transfections. After 24 h, cells were transfected with control vector (2 μg) or CAGE (2 μg), and a luciferase activity assay was performed according to the manufacturer’s instructions (Tropix, Applied Biosystems). A pGL3-basic control vector lacking an insert was used as a negative control and β-galactosidase was used to normalize luciferase activities. Transfections of other constructs, including AP-1 promoter constructs, for luciferase activity assays were performed as described above. To determine the effect of various signaling molecules, SNU387 cells were pretreated with PD (20 μM), LY (10 μM), SB (10 μM) or AG1478 (10 μM) for 1 h, followed by transfection with the AP-1 promoter-luciferase construct (2 μg).
To determine the effect of NF-κB on AP-1 promoter activity, SNU387 cells were transiently transfected with the AP-1 promoter construct along with IκB super repressor (2 μg). After 24 h, cells were transfected with control vector (2 μg) or CAGE (2 μg), and a luciferase activity assay was performed.

Electrophoretic mobility shift assay
 AP-1 binding assays were performed using nuclear extracts and biotin-labeled AP-1 oligonucleotides (Panomics, Fremont, CA, USA). EMSA was performed using a EMSA Gel-Shift Kit (Panomics). A 10 μl reaction mixture containing 10 μg of nuclear protein was incubated for 30 min at room temperature with a binding mixture containing 10 mM Tris, 50 mM KCl, 1 mM dithiothreitol, 5 mM MgCl₂, 2 μg poly (dI·dC) and 2 pmol oligonucleotide probe, with or without a non-labeled 10-fold excess of competitor oligonucleotides. Protein-DNA complexes were separated by electrophoresis on a 6% non-denaturing acrylamide gel containing 0.5 x TBE buffer (1 x TBE-90 mM Tris, 90 mM boric acid, 2.5 mM EDTA), transferred to Biotinyl nylon membranes (Pierce), and then cross-linked in a Stratagene cross-linker. Protein gel shifts were visualized using streptavidin-horseradish peroxidase followed by chemiluminescence detection. For antibody super shift assay, 5 μg of anti-junB antibody (Santa Cruz Biotechnology, Santa Cruz, CA USA) was added to the reactions, followed by incubation for 45 min at room temperature before gel loading. The nucleotide sequence of Biotin-labeled AP-1 was 5'-CGCTTGATGACGTCGCCCAG-3'. The AP-1 recognition sequence is underlined.

Chromatin immunoprecipitation (ChIP) assays
ChIP assays were performed according to manufacturer’s instructions (UBI, NY, USA). Briefly, one fourth of the chromatin solution was reserved for Total input. The remaining solution was precleared by protein A-agarose, subsequently incubated with the anti-JunB antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). This work was supported by a grant from the Regional Research Centers Program of the Ministry of Health and Welfare of Korea, a grant (FG06-2-23) from the 21C Frontier Functional Human Genome Project from the Ministry of Science & Technology in Korea. This work was also supported by the Regional Research Centers Program of the Ministry of Education, Science and Technology.

Cell Fractionation
Nuclear and cytosolic extracts were prepared using a nuclear/cytosol fractionation kit (Biovision, Mountain View, CA). Fourty-eight hours after transfection with CAGE, SNU387 human hepatic cancer cells were collected by centrifugation at 600 g for 5 min at 4℃. Cell pellets were washed twice in ice-cold PBS, followed by the addition of 0.2 ml of cytosol extraction buffer A and vigorous mixing for 5 s. Ice-cold cytosol extraction buffer B (11 μl) was then added to the solution. After mixing, nuclear and cytosolic fractions were separated by centrifugation at 16,000 g for 5 min (supernatants are the cytosolic fraction). Following this, nuclear extraction buffer was added to the nuclear fraction. After 40 min of vortexing, nuclei were centrifuged at 16,000 g for 10 min. Therefore, supernatants obtained at this point contained the nuclear fraction. The protein concentration of each fraction was determined using a DC Protein Assay Kit (Bio-Rad). Equal amounts of nuclear/cytosolic extracts were loaded for SDS-PAGE, followed by Western blot analysis. Purity of the cytosolic and nuclear fractions was confirmed by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and histone H1, respectively.

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