The Regulatory Mechanism of the LY6K Gene Expression in Human Breast Cancer Cells*

Hyun Kyung Kong, Sukjoon Yoon, and Jong Hoon Park

From the Department of Biological Science, Sookmyung Women’s University, Chungpa-dong, Yongsan-gu, Seoul 140-742, Korea

Background: LY6K is a candidate cancer biomarker that promotes invasion and metastasis. Results: AP-1 activation is required for the LY6K expression and interfered by SNP242 or methylation. Conclusion: AP-1 promotes LY6K expression that regulates cell mobility, whereas SNP242 or methylation reduces the metastasis. Significance: Understanding the regulatory mechanisms of the LY6K is important for investigating breast cancer risk.

LY6K is a cancer biomarker and a therapeutic target that induces invasion and metastasis. However, the molecular mechanisms that determine human LY6K transcription are completely unknown. To elucidate the mechanisms involved in human LY6K gene regulation and expression, multiple cis-elements were predicted using TRANSFAC software, and the LY6K regulatory region was identified using the luciferase assay in the human LY6K gene promoter. We performed ChIP, EMSA, and supershift assays to investigate the transcription factor activity on the LY6K promoter, and the effect of a SNP and CpG site methylation on AP-1 transcription factor binding affinity. AP-1 and the CREB transcription factor bound to LY6K promoter within −550/−1, which was essential for LY6K expression, but only the AP-1 heterodimer, JunD, and Fra-1, modulates LY6K gene transcriptional level. A decrease in LY6K was associated with the SNP242 C allele, a polymorphic G/C-SNP at the 242 nucleotide in the LY6K promoter region (rs2585175), or methylation of the CpG site, which was closely located with the AP-1 site by interfering with binding of the AP-1 transcription factor to the LY6K promoter. Our findings reveal an important role for AP-1 activation in promoting LY6K gene expression that regulates cell mobility of breast cancer cells, whereas the SNP242 C allele or methylation of the CpG site may reduce the risk of invasion or metastasis by interfering AP-1 activation.

The human LY6K (lymphocyte antigen 6 complex locus K) belongs to the Ly-6/urokinase-type plasminogen activator receptor (uPAR)* superfamily, which can be divided into two subfamilies based on the glycosylphosphatidylinositol-anchor-

---

* This work was supported by the National Research Foundation of Korea grant funded by the Korean government (Ministry of Education, Science and Technology) (Science Research Center program, 2011-0001382); the Bio & Medical Technology Development Program (Korea Genetically Engineered Mouse Center Program), and the National Research Foundation of Korea 2011-0028816 (National Leading Research Lab program). This article contains supplemental Table 1 and Figs. 1–3.

1 To whom correspondence should be addressed: Dept. of Biological Science, Sookmyung Women’s University, Seoul 140-742, Korea. Tel.: 82-2-710-9414; Fax: 82-2-2077-7322; E-mail: parkjh@sookmyung.ac.kr.

2 The abbreviations used are: uPAR, urokinase-type plasminogen activator receptor; CREB, cAMP-responsive element-binding protein; AP-1, activating protein-1; luc, luciferase; NE, nuclear extract; ADR, Adriamycin resistance; mt, mutant.

---

© 2012 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
LY6K Expression Regulated by AP-1 Activation

tool for analyzing the human genome and identifying specific genes and genomic regions linked to cancer phenotypes (21). Functional SNPs located on the promoter modulate gene expression leading to hormone or drug sensitivity and disease susceptibility (22, 23).

DNA methylation occurs when a methyl group is added to a CpG site and is an epigenetic event that plays an important role in gene regulation. In particular, methylation of the gene promoter CpG islands is tightly linked to histone modifications and nucleosome remodeling mechanisms that cause gene silencing (24, 25). Aberrant DNA methylation in human cancers is one of the most consistent epigenetic mechanisms that activates or silences gene expression (25).

In the present study, we identified the regulatory region essential for LY6K promoter activity. We also provide the first evidence that LY6K gene expression is regulated by SNP or DNA methylation in the proximal promoter region. Our findings suggest that the AP-1 transcription factor affects invasion and metastasis by regulating LY6K expression in breast cancer cells and that SNP242 or DNA methylation of a specific CpG site within the LY6K promoter down-regulates LY6K by effectively interfering with AP-1 binding, suggesting a reduced risk of breast cancer.

EXPERIMENTAL PROCEDURES

Cell Culture—The human breast carcinoma cells used in this study included the MCF7 and MCF7-ADR, sublines, which are adriamycin-resistant (obtained from Roswell Park Cancer Institute, Buffalo, NY). Cells were grown in Dulbecco’s modified Eagle’s medium (WelGENE, Inc., Daejeon, Korea) containing 10% fetal bovine serum (WelGENE) and maintained at 37 °C in a humidified atmosphere with 5% CO2 and 95% air.

LY6K Promoter Constructs—Promoter constructs encompassing the region from −2.0 kb to 0.2 kb relative to the transcription start site of the human LY6K gene were amplified from human genomic DNA using specifically designed forward and reverse primers containing the XhoI and HindIII restriction enzyme sites, respectively. Each fragment was digested with XhoI and HindIII purified using the HiYield Gel/PCR extraction kit (RBC Bioscience, Taipei, Taiwan) according to the manufacturer’s instructions and cloned into the pGL3-LY6K-0.5 vector. The PCR cycling parameters were as follows: 1 cycle of 95 °C for 30 s, 12 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 1 min for point mutation. The insert sequence was verified by DNA sequence analysis.

Chromatin Immunoprecipitation Assay—The imprint chromatin immunoprecipitation kit (CHP1, Sigma-Aldrich) was used for the ChIP assay. Briefly, cells were fixed with 1% formaldehyde and quenched with 1.25 mM glycine, and the DNA was sheared by sonication on ice (total time, 15 min; on time, 30 s; off time, 2 min). The sheared DNA was incubated with IgG as a negative control, and c-Jun (sc-1694), JunB (sc-8051), JunD (sc-74), c-Fos (sc-52), FosB (sc-28213), Fra-1 (sc-183), Fra-2 (sc-604), and PAX3 (sc-81351) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) in a strip well overnight at 4 °C. The strip well was washed, the cross-link was reversed, and the DNA was purified with the GenElute Binding Column G (C6863, Sigma-Aldrich). Prepared samples were amplified using specifically designed ChIP primers for the region of interest (supplemental Table 1). PCR conditions for the ChIP analysis were as follows: 1 cycle of 95 °C for 2 min, 50 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, 72 °C for 5 min, and storage at 4 °C.

Nuclear Extracts and Electrophoretic Mobility Shift Analysis—Nuclear extracts were prepared using a nuclear extraction kit (Panomics, Inc., Santa Clara, CA), according to the manufacturer’s instructions. The sequences of the predicted transcription binding site probe within the 550-bp human LY6K promoter region are presented in Table 1. Double-stranded oligonucleotides containing the AP-1 and CREB consensus sites were purchased from Promega. The sequences of the wild type and mutant probes containing the transcription binding site are presented in supplemental Table 1. For binding assays, 10 µg of nuclear extract samples were incubated with purified γ-32P-labeled probe using G-50 Sephadex columns (Roche Applied Science) at room temperature for 30 min in a buffer containing 100 mM KCl, 30 mM HEPES, 1.5 mM MgCl2, 0.3 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 1 µM DTT, proteinase inhibitors (1 mM PMSF, 1 µg/ml aprotinin, and 1 µg/ml leupeptin) and 0.2 µg poly(dI-dC) in a 20-μl reaction volume. DNA-protein complexes were analyzed on 4% non-denaturing polyacrylamide gels that had been prerun at 200 V for 30 min in 0.5× Tris borate-EDTA buffer. The gels ran at 150 V for 2 h, dried under vacuum at 80 °C for 1 h, and then exposed to a FLA-7000 cassette. For competition assays, 10 µg of nuclear extract was incubated with unlabeled probe for 15 min before adding the purified γ-32P-labeled probe.

Supershift Assay—Antibodies against the AP-1 factor (Santa Cruz Biotechnology), CREB (9104, Cell Signaling Technology, Inc., Danvers, MA), CBP (sc-369, Santa Cruz Biotechnology) or PAX3 (sc-81351, Santa Cruz Biotechnology), and 10 µg of nuclear extracts were incubated for 90 min and then added to the purified γ-32P-labeled probe for 15 min at room tempera-
tured. The gels were run at 180 V for 3 h, dried under vacuum at 80 °C for 1 h, and then exposed to a FLA-7000 cassette.

RNA Interference—MCF7-ADR cells were transfected with several small interfering RNA (siRNAs) at a concentration of 30 nM for 48 h using Lipofectamine RNAi Max (Invitrogen), according to the manufacturer’s protocol. siRNAs specific to LY6K (sc-77440), JunD (sc-35728), c-Fos (sc-29221), Fra-1 (sc-35405), and Fra-2 (sc-35407) were used to silence target genes. A control siRNA (sc-37007), which had no considerable homology to any known human sequence, was transfected as the negative control. All siRNAs were purchased from Santa Cruz Biotechnology.

Real-time RT-PCR—Total cellular RNAs were extracted with a NucleoSpin RNA/Protein kit (Macherey-Nagel, Düren, Germany), according to the manufacturer’s instructions. A total of 5 μg of RNA was used for reverse transcription with the M-MLV Reverse Transcription kit (Promega). A 100–300-ng portion of template cDNA was mixed with 1 μl of primer mix of forward and reverse preimers (supplemental Table 1) and 5 μl of SYBR Green Mastermix (Applied Biosystems, Foster City, CA).

Co-immunoprecipitation—Reciprocal IP was performed using 100-mm dishes of MCF7-ADR cells. The cells were washed with 1× cold PBS and harvested in 1 ml of 1× cold PBS following centrifugation at 12,000 rpm for 1 min at 4°C. The pellets were lysed by vortexing with 100 μl of IP150 buffer/Nonidet P-40 (150 mM NaCl, 25 mM Tris-HCl (pH 8.0), 10% glycerol, 1% Nonidet P-40, and 1 mM EDTA) and incubated for 15 min in ice. Lysates were diluted with 400 μl of IP150 buffer without Nonidet P-40 (37.5 mM NaCl, 6.25 mM Tris-HCl (pH 8.0), 2.5% glycerol, and 0.25 mM EDTA) and centrifuged at 12,000 rpm for 5 min at 4°C to remove debris. The supernatants were incubated at 4°C with anti-JunD rabbit polyclonal antibody (sc-74), anti-Fra-1 rabbit polyclonal antibody (sc-183), and anti-IgG rabbit polyclonal antibody as a negative control for 90 min. Immunoglobulin G (IgG)-Sepharose beads were used to immunoprecipitate the proteins. Following the incubation, the beads were washed three times with IP wash buffer (225 mM NaCl, 5 mM Tris-HCl (pH 8.0), 0.125% Nonidet P-40 and 0.25 mM EDTA). The washed beads containing the bound proteins were suspended in 20 μl of 6× SDS gel loading buffer and heated at 100°C for 5 min. The released polypeptides in 15 μl of heated sample were resolved on 10–12% polyacrylamide gels.

Western Blot Analysis—Proteins from breast cancer cells were extracted using a NucleoSpin RNA/protein kit, (Macherey-Nagel), according to the manufacturer’s instructions. Equal amounts of extracted protein were loaded on polyacrylamide gels and transferred to polyvinyl difluoride membranes. The membranes were blocked with 5% skim milk and incubated with specific primary antibodies. The antibody bound membranes were washed and then incubated with secondary antibodies and an anti-biotin marker. The membranes with bound antibodies were analyzed using the Amersham Biosciences ECL Plus Western blotting detection system (GE Healthcare). The primary antibodies were LY6K (sc-87282), JunD (sc-74), c-Fos (sc-52), Fra-1 (sc-183), and Fra-2 (sc-604) from Santa Cruz Biotechnology and anti-biotin (7075) from Cell Signaling Technology.

LY6K Expression Regulated by AP-1 Activation

RESULTS

Identification of the Putative LY6K Promoter Region—We have previously reported that LY6K is a potential breast cancer biomarker that affects invasive ability and metastasis (8). To investigate the regulatory mechanism of the LY6K gene, a series of LY6K-luc promoter constructs −2050/+182 were transfected into MCF7-ADR cells, and the levels of promoter activity were measured. The pGL3-control vector and the pGL3-basic vector were transfected as positive and negative controls, respectively. Transcriptional activity of LY6K-luc was normalized with the pGL3-basic vector, and we confirmed that the activity significantly increased −550/−200 and −1550/−1050 bps (Fig. 1A). Then, truncated constructs were specifically designed with deletions from −500 to −1 and from −1550 to −1050 to identify a more important site for controlling gene expression. The deletion from −550 to −1 sharply and significantly reduced promoter activity. However, deletion of −1550/−1050 did not alter promoter activity (Fig. 1B). These findings suggest the presence of a putative transcription binding site within the −550/−1 that regulates basal LY6K gene expression.

The AP-1 Transcription Factor Regulates LY6K Gene Expression—Given that promoter activity was reduced in −550/−1 deleted constructs, we investigated the DNA binding motif using TRANSFAC gene tool software, which is schematically listed in Table 1 and presented in Fig. 1C. We examined which transcription factor bound the LY6K promoter using EMSA (supplemental Fig. S1). We observed the formation of a prominent DNA-protein complex for the double-stranded oligonucleotide probes containing AP-1, cAMP-responsive element (CRE), and TATA sequences of the LY6K gene had the rs2585175 SNP located at nucleotide 242 within the LY6K promoter (called SNP242). We designed mutant constructs for the region encompassing nucleotides 312 to 133 of the LY6K promoter. Various sized LY6K promoter constructs ligated to the luciferase reporter gene were transiently transfected into MCF7-ADR cells (Fig. 1C). The shorter pGL3−73 construct, which encompassed nucleotides −73 to +182, contained a minimal TATA box. This construct represented basal LY6K promoter activity. The pGL3−133 construct showed basal-like activity in the MCF7-ADR cell line. The pGL3−312 construct directed a 5-fold increase above basal transcription activity, suggesting the presence of important cis-regulatory elements, AP-1 and NF-kB, between nucleotides −133 and −312 of the LY6K promoter (Fig. 1C). Based on these results, we focused our study on the region encompassing nucleotides −312 to −133 of the LY6K promoter. The LY6K gene had the rs2585175 SNP located at nucleotide 242 within −312 to −133 of the LY6K promoter (called SNP242). We designed mutant constructs for AP-1 and SNP242 using site-directed mutagenesis to deter-
mine the functional role of AP-1 and SNP242 in regulating the ly6k promoter. The pGL3-basic vector was transfected as the negative control, and transcriptional activity was measured. As shown in Fig. 1D, the normalized promoter activity of the AP-1 mutated ly6k-luc vector significantly decreased ~8-fold, suggesting that the transcription factor bound to the AP-1 conser-

FIGURE 1. Transcriptional activity of the ly6k-luc reporters in MCF7-ADR cells. A, series constructs of the human ly6k gene promoter from −2050 to +182 cloned into the pGL3-luc vector. Transcriptional activity of the ly6k promoter was determined by transient transfection into the MCF7-ADR cell line and by quantifying the ly6k promoter linked to firefly luciferase activity. Promoter activity of ly6k-luc in MCF7-ADR cells was normalized with the pGL3-basic vector, which was transfected as a negative control. B, pGL3-Δ500 and Δ1000 are truncated constructs of the ly6k promoter which were missing bp −500 to −1 and −1550 to −1050, respectively. C, deletion analysis of the human ly6k promoter. Schematic presentation of the promoter elements in the ly6k promoter, based on the predicted ly6k promoter sequence. D, site-directed mutated ly6k-luc constructs. Substitution of two base pairs of the AP-1 binding site GA for CT, or a single base pair of SNP242 G for C. Site-directed mutated ly6k-luc reporter was co-transfected with phRL-CMV into MCF7-ADR cells. Cell lysates were measured using the luciferase assay.
TABLE 1
Consensus sequences found in the 550-bp human LY6K promoter region (−550/−1)

| Binding sites | Sequence | Location (bp) |
|---------------|----------|--------------|
| TATA box      | 5′-TATAA-3′ | −30 to −25  |
| AML1a         | 5′-TGTGGT-3′ | −63 to −58  |
| p300          | 5′-GTACCTCA-3′ | −123 to −116 |
| CREB          | 5′-GGGAGACCC-3′ | −230 to −221 |
| NF-κB         | 5′-AGATAG-3′ | −519 to −514 |
| AP-1          | 5′-TGAGTCA-3′ | −252 to −246, −502 to −492 |
| GATA3         | 5′-AGATAG-3′ | −519 to −514 |

LY6K Expression Regulated by AP-1 Activation

Interestingly, the single base pair mutated LY6K-luc reporter SNP242 decreased transcriptional activity 2-fold in MCF7-ADR cells. Taken together, we conclude that a transcription factor bound to the AP-1 binding site within the LY6K promoter and regulated expression of the LY6K gene and that the SNP242 mutation (G→C) reduced levels of the LY6K gene transcript.
Identified AP-1 Binding Site within 22 bp—The EMSA was conducted with the wt and mt AP-1 probe to confirm that the AP-1 binding site was within the LY6K promoter. First, it was determined whether the nuclear extract (NE) from MCF7-ADR cells had the AP-1 factor that bound to the consensus AP-1 probe in the presence or absence of unlabeled probe (Fig. 2A). Then, binding of NE from MCF7-ADR cells to the wt 22-bp probe within the LY6K promoter was investigated (Fig. 2B). A shifted band was observed in lane 3, which was a mixture of NE from MCF7-ADR cells with the wt 22-bp oligonucleotide probe (Fig. 2B). The shifted band disappeared in lanes 4 and 5 in the presence of the unlabeled or AP-1 mutated probe. Therefore, it was concluded that the shifted band was a complex of the transcription factor and radiolabeled oligonucleotide. The EMSA was performed with the wild type and mutant CREB probes to confirm which transcription factors bound the CRE element of the LY6K promoter. The CRE binding factors present within the NE of MCF7-ADR cells demonstrated significant binding to a consensus CRE probe (supplemental Fig. 2A). We observed the formation of one prominent DNA-protein complex for the wild type CRE probe analyzed (supplemental Fig. 2B). In a supershift experiment, we confirmed that adding anti-CREB antibody produced a supershift complex but that the anti-CREB binding protein CBP antibody had no effect (supplemental Fig. 2, C and D). These results suggest that the AP-1 and CREB transcription factors bind to the LY6K promoter within –253/–246 and –130/–111 but that only AP-1 modulates LY6K gene transcription levels.

AP-1 Factor Binds to the AP-1 Binding Site within the LY6K Promoter—ChIP and supershift assays were used to demonstrate in vitro interactions between the AP-1 transcription factor and the AP-1 binding site within the LY6K promoter. First, a ChIP analysis using c-Jun, JunB, JunD, c-Fos, FosB, Fra-1, and Fra-2 antibodies was carried out and confirmed by PCR. Input and IgG were used as positive and negative controls, respectively. The results revealed that JunD directly bound to the DNA fragment containing the AP-1 binding site of LY6K (Fig. 2C). c-Fos and Fra-1 also bound to the LY6K promoter, but the signal was weak (Fig. 2C). Next, we examined which AP-1 transcription factors bound to the wt 22-bp probe contained at the AP-1 binding site. As shown in Fig. 2D, mixtures with JunD, Fra-1, and Fra-2 antibodies were supershifted. In the presence of the mutant AP-1 probe, supershifted bands were completely abolished compared with that of the wt AP-1 probe (Fig. 2E). Taken together, AP-1 factor binding to the proximal AP-1 binding site of the LY6K promoter suggested that these factors might be important in regulating LY6K promoter activity.

Functional Role of JunD and Fra-1 in LY6K Expression—To determine which AP-1 transcription factor forms a dimer with JunD, we used siRNAs specifically directed against LY6K, JunD, c-Fos, Fra-1, and Fra-2 to silence their expression in MCF7-ADR cells. Transfection of several siRNAs abrogated LY6K, JunD, c-Fos, Fra-1, and Fra-2 mRNA and protein expres-
sion, respectively, in MCF7-ADR cells assayed by real-time PCR and Western blotting, respectively (Fig. 3, A and B). Interestingly, we found that blocking JunD or Fra-1 expression induced a decrease in LY6K mRNA and protein level in MCF7-ADR cells, whereas transfection of the control siRNA did not alter this expression (Fig. 3, A and B). Endogenous proteins were immunoprecipitated to confirm the endogenous interaction between JunD and Fra-1. Western blot analysis of endogenous protein immunoprecipitates obtained with either anti-JunD or anti-Fra-1 antibodies revealed that JunD strongly co-immunoprecipitated with Fra-1, suggesting that these proteins may co-function in LY6K expression (Fig. 3C). The JunD and Fra-1 expression vectors were transfected in MCF7 cells to investigate whether increased levels of JunD and Fra-1 are able to modulate LY6K. We confirmed that LY6K expression level was up-regulated by elevated JunD or Fra-1 RNA and protein (Fig. 3, D and E). Therefore, it was concluded that the heterodimer of the AP-1 factors, JunD and Fra-1, regulates LY6K gene expression by directly binding to the LY6K promoter.

The Effect of SNP242 on AP-1 Binding Activity—Transcriptional activity of a single base pair of mutated constructs, pGL3-luc-mtSNP242, significantly decreased (Fig. 1D). SNP242 was closely located at a distance of three nucleotides from the AP-1 binding site; thus, we hypothesized that substituting G for C in SNP242 might interfere with AP-1 binding in the LY6K promoter. The newly designed EMSA probes, wt SNP242 and mt SNP242 containing the AP-1 binding site, were used to investigate whether they affected AP-1 binding activity. The density of the shifted band in lane 4 was weaker than that in lane 3, indicating that the AP-1 factor bound less to the probes (Fig. 4A). Then, a supershift assay was performed with the AP-1wtSNP242wt probe using the AP-1 family factor (Fig. 4B).
and the AP-1 wt SNP242 mt probes using c-Jun, JunD, Fra-1, and Fra-2 antibodies, which bind to the LY6K gene promoter (Fig. 4C). We observed that the mixture of antibodies in lanes 6, 8, and 10 produced weaker bands than those in lanes 5, 7, and 9 (Fig. 4C). These results suggest that AP-1 bound to the LY6K promoter and activated transcription and that the presence of SNP242 (G→C) repressed AP-1 activity by reducing its DNA binding affinity to the LY6K promoter.

**Functional Role of JunD and Fra-1 on the LY6K Promoter**—To further examine the functional role of AP-1 factor, JunD and Fra-1, and the identified AP-1 binding site within the LY6K promoter, we compared the activity of the pGL3-0.5, the mutated AP-1 or the mutated SNP242 constructs in the absence or presence of the JunD or Fra-1 expression vector (Fig. 5). Overexpressed JunD or Fra-1 significantly up-regulates the transcriptional activity of the pGL3-0.5 vector, respectively, with slightly effects on the activity of the mutated AP-1 and mutated SNP242 LY6K-luc constructs (Fig. 5). Similarly, in other breast cancer cell lines, MCF7 and MDA-MB-231, overexpression of JunD or Fra-1 up-regulated the luciferase activity of the pGL3-0.5, without any significant effect on the activity of the mutated AP-1 and mutated SNP242 LY6K-luc constructs (supplemental Fig. 3). Therefore, these results support the conclusion that AP-1 factor, JunD and Fra-1, directly bind to identified AP-1 binding site within the LY6K promoter, and its binding affinity was affected by SNP242 in MCF7-ADR cells.

**SNP242 Is Associated with LY6K Down-regulation**—The EMSA was performed using SNP242 wt and SNP242 mt probes to confirm the hypothesis that substitution of G for C might generate transcription factor binding sites. In the presence of the SNP242-mutated probe, the shifted band appeared in lane 4 (Fig. 6A). NE protein from MCF7-ADR cells bound to the SNP242 mt probe, and the band shifted disappeared in the presence of competitor and the SNP242 wt probe (Fig. 6A). Therefore, it was concluded that the shifted band was a complex of transcription factors and radiolabeled oligonucleotides. We investigated the DNA binding motif using TRANSFAC software to predict the transcription factor that binds to *de novo* transcription binding sites. PAX3, a member of the paired-box transcription factor family, binding sites are created by SNP242 mutation. The PAX3 protein was used in the supershift assay to confirm PAX3 protein binding to the *de novo* PAX3 site (Fig. 6B). PAX3, which bound to the SNP242 mt probe, slightly supershifted in the presence of anti-PAX3 antibody (Fig. 6B). The LY6K miRNA level was compared in four cell lines that were wt for SNP242 (G/G) to the levels found in five cell lines for SNP242 (C/C) and measured by real-time PCR. The presence of SNP242 correlated with low LY6K transcript expression when compared with the levels seen in cells that were wt for SNP242 (G/G) (Fig. 6C). A ChIP analysis using PAX3 antibodies was carried out and confirmed by PCR using the MCF7-ADR SNP242 G/G) and MCF7 SNP242 C/C) cell lines. Input and IgG were used as positive and negative controls, respectively. The results revealed that PAX3 directly bound to the SNP242 (C/C) DNA fragment of LY6K (Fig. 6D). Taken together, PAX3 transcription factor bound to the *de novo* PAX3 binding site generated by mutated SNP242 (C/C), which decreased AP-1 activity on the LY6K promoter by interfering with AP-1 factor binding.

**The Effect of CpG Site Methylation on AP-1 Binding Activity**—The pGL3-LY6K promoter plasmids were in *vitro*-methylated by SssI methylase to further investigate the role of epigenetic modification in the regulation of gene expression. After MCF7-ADR cells were transfected with the methylated plasmids, the luciferase activities in these transfectants significantly decreased compared with those found in the cells transfected with unmethylated pGL3-312 and pGL3-550 (Fig. 7A). The probe containing the AP-1 binding site was methylated at two CpG sites by SssI methylase to investigate the effect of methylation on AP-1 binding activity. The two CpG sites were located 2 and 10 bp downstream of the AP-1 binding site, respectively. The methylated probe was incubated with the nuclear proteins from MCF7-ADR cells (Fig. 7B). Binding of the nuclear proteins to the methylated oligonucleotide probe significantly reduced compared with binding to the unmethylated probe (Fig. 7B). This result suggests that methylation at the CpG sites close to the AP-1 binding site interfered with AP-1 transcription factor binding to the AP-1 site.

**DISCUSSION**

LY6K has been reported as a testicular cancer antigen that is not only a target antigen for head-and-neck squamous cell carcinoma but also a diagnostic marker for breast cancer and a serologic biomarker and therapeutic target for lung and esophageal carcinomas (7–9). Activating LY6K results in an increase in cell invasive motility and metastasis, whereas a low level of LY6K reduces cell mobility in breast cancer cells (12).

The interaction of vascular endothelial growth factor (VEGF) with its type-2 receptor activates the MAPK pathway to stimulate uPAR expression, which induces angiogenesis (26). uPAR, a member of the Ly-6 superfamily, has AP-1 and NF-kB putative binding sites at the 5′-flanking region within the GC-rich
sequence and fewer TATA and CAAT boxes (27, 28). Similarly, a computer analysis of transcription factor binding sites screening the \textit{LY6K} promoter showed fewer CAAT boxes and TATA boxes and putative AP-1 binding sites within the GC-rich region. Therefore, \textit{LY6K} expression related to angiogenesis may be regulated by the VEGF signaling pathway similar to uPAR synthesis.

Promoter activity assays using a series of reporter gene constructs containing progressive 5' deletions or specific mutations allowed us to determine regions in the \textit{LY6K} promoter necessary for efficient activity. EMSA assays were used to detect one prominent complex corresponding to a specific protein-DNA interaction with the AP-1 and CRE sites (supplemental Fig. S1). Our results suggest that the AP-1 transcriptional activator directly binds the AP-1 binding site within the cis-element of the \textit{LY6K} promoter. In contrast, CREB-1 did not affect \textit{LY6K} expression but bound to the CRE motif. Therefore, the activity of the AP-1 transcription factor, an upstream transducer of \textit{LY6K} and a member of the Ly-6/uPAR family, is critical for activating \textit{LY6K}.

The AP-1 binding site identified in this study appears in the regulatory regions of many genes involved in proliferation, cell growth, cellular mobility, and the stress response. More recent studies have been carried out to identify the relationship between AP-1 transcription factors and breast cancer cells. Studies reported to date suggest that AP-1 plays a critical role in breast cancer, including breast cell transformation and breast cancer cell growth, proliferation, survival, invasion, and metastasis (29, 30). Based on our results, we suggest that the AP-1 family members JunD and Fra-1 play an important role in cell mobility of breast cancer cells by promoting \textit{LY6K} gene expression.
The Jun/Fos combination of the AP-1 complex induces strong proliferation, malignant transformation, and a powerful aggressiveness phenotype (31, 32). This is because the Jun/Fos heterodimer is more stable with a longer half-life and stronger DNA binding affinity within the promoter than those of the Jun/Jun homodimer (33). The Jun and Fos protein dimer binding affinity to the asymmetric heptanucleotide (TGA(C/G)TCA) is higher than that to the symmetric octanucleotide (TGACGTCA) (34). We conclude that the JunD/Fra-1 heterodimer complex is an AP-1 transcription factor that binds with the highest affinity within the \textit{LY6K} promoter, indicating that \textit{LY6K} gene expression is unregulated by the combination of JunD/Fra-1, which could promote the aggressiveness phenotype.

A functional SNP is a useful polymorphic marker for diagnosis and personalized cancer treatment. In particular, a localized promoter of specific genes contributes to the cancer phenotype and regulates gene expression by disrupting or creating transcription factor sites or interfering with transcription factor binding (21). Our results demonstrate that \textit{LY6K} expression is dependent not on the AP-1 transcription factor but on SNP242 by creating a \textit{de novo} PAX-3 binding site that leads to a decrease in AP-1 binding affinity. This observation suggests that the \textit{LY6K} SNP242 C allele may reduce the carcinogenesis risk of breast cancer and metastasis (Fig. 8).

DNA methylation, which leads to gene silencing, is one of the epigenetic modifications, and at least three hypotheses explain this silencing (25). First, the suppression is mediated by binding between methylated DNA and a family of methyl-CpG binding proteins, leading to a change in chromatin structure. Second, gene silencing is caused by inhibiting the binding of sequence-specific transcription factors to their binding sites containing CpG (35). Third, CpG site modifications affect transcription factor binding affinity but are not part of the binding site (36). We also demonstrated that methylation at a CpG site closely located to the AP-1 binding site may reduce the risk
of breast cancer by effectively interfering with AP-1 binding (Fig. 8).

In summary, we investigated the molecular mechanisms that govern LY6K gene transcription by demonstrating that the binding of the JunD and Fra-1 transcription factors to the LY6K promoter regulated gene activation in vitro. Activated JunD and Fra-1 promoted LY6K expression at the RNA and protein levels. CREB also bound to the CRE within the LY6K promoter but did not affect LY6K expression. We also demonstrated that the SNP242 C allele and methylation of a CpG site close to the promoter regulated gene activation in vitro.

3. Horie, M., Okutomi, K., Taniguchi, Y., Ohbuchi, Y., Suzuki, M., and Taka-hashi, E. (1998) Isolation and characterization of a new member of the human Ly6 gene family (LY6H).

4. Adermann, K., Watterl, F., Watterl, S., Heine, G., Meyer, M., Forssmann, W. G., and Nehls, M. (1999) Structural and phylogenetic characterization of human SLURP-1, the first secreted mammalian member of the Ly-6/GPRL family.

5. Ploug, M., and Ellis, V. (1994) Structure-function relationships in the human Ly6 gene family (LY6H).

6. Sawai, H., Okada, Y., Funahashi, H., Matsuo, Y., Takahashi, H., Takeyama, H., and Manabe, T. (2006) Interleukin-1α enhances the aggressive behavior of pancreatic cancer cells by regulating the activation of the NF-κB-integrin and urokinase plasminogen activator receptor expression.

7. de Nooij-van Dalen, A. G., van Dongen, G. A., Smeets, S. J., Nieuwenhuis, E. J., Stigt-van Walsum, M., Snow, G. B., and Brakenhoff, R. H. (2003) Characterization of the human Ly-6 antigens, the newly annotated member Ly-6K included, as molecular markers for head-and-neck squamous cell carcinoma. Int. J. Cancer 103, 768–774

8. Lee, J. W., Lee, Y. S., Yoo, K. H., Lee, K. H., Park, K., Ahn, T., Ko, C., and Park, J. H. (2006) LY-6K gene: a novel molecular marker for human breast cancer. Oncol. Rep. 16, 1211–1214.

9. Ishikawa, N., Takano, A., Yasui, W., Inai, K., Nishimura, H., Ito, H., Miyagi, Y., Nakayama, H., Fujita, M., Hosokawa, M., Tsuchiya, E., Kohno, N., Nakamura, Y., and Daigo, Y. (2007) Cancer-testis antigen lymphocyte antigen 6 complex locus K is a serologic biomarker and a therapeutic target for lung and esophageal carcinomas. Cancer Res. 67, 11601–11611.

10. Matsuda, R., Enokida, H., Chiyomaru, T., Kikkawa, N., Sugimoto, T., Kawakami, K., Tatara, S., Yoshino, H., Toki, U., Uchida, Y., Kawahara, K., Nishiyama, K., Seki, N., and Nakagawa, M. (2011) LY6K is a novel molecular target in bladder cancer on basis of integrate genome-wide profiling. Br. J. Cancer 104, 376–386

11. Zhang, B., Zhang, Z., Zhang, X., Gao, X., Fernandine, K. H., and Zhong, L. (2012) Serological antibodies against LY6K as a diagnostic biomarker in esophageal squamous cell carcinoma. Biomarkers 17, 372–378

12. Choi, S. H., Hong, H. K., Park, S. Y., and Park, J. H. (2009) Metastatic effect of LY-6K gene in breast cancer cells. Int. J. Oncol. 35, 601–607

13. Karin, M. (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. J. Biol. Chem. 270, 16483–16486

14. Shaulian, E., and Karin, M. (2001) AP-1 in cell proliferation and survival. Oncogene 20, 2390–2400

15. Chinenov, Y., and Kerppola, T. K. (2001) Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. Oncogene 20, 2438–2452

16. Vogt, P. K. (2002) Fortuitous convergences: the beginnings of JUN. Nat. Rev. Cancer 2, 465–469

17. Lin, F., Xiao, D., Kolluri, S. K., and Zhang, X. (2000) Unique anti-activator protein-1 activity of retinoic acid receptor β. Cancer Res. 60, 31–38

18. Smith, L. M., Wise, S. C., Hendricks, D. T., Sabichi, A. L., Bos, T., Reddy, P., Brown, P. H., and Birrer, M. J. (1999) c-Jun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive, and hormone-resistant phenotype. Oncogene 18, 6036–6070

19. Liu, Y., Ludes-Meyers, J., Zhang, Y., Munoz-Medellin, D., Kim, H. T., Lu, C., Ge, G., Schiff, R., Hilsenbeck, S. G., Osborne, C. K., and Brown, P. H. (2002) Inhibition of AP-1 transcription factor causes blockade of multiple signal transduction pathways and inhibits breast cancer growth. Oncogene 21, 7680–7689

20. Cargill, M., Altshuler, D., Ireland, J., Sklar, P., Ardlie, K., Patil, N., Shaw, L. H., Lin, J. R., Lee, E. P., Kalyanaraman, N., Nemesh, J., Ziaugra, L., Friedland, L., Wolf, A., Lipshutz, R., Daley, G. Q., and Lander, E. S. (1999) Characterization of single-nucleotide polymorphisms in coding regions of human genes. Nat. Genet. 22, 231–238

21. Engle, L. J., Simpson, C. L., and Landers, J. E. (2006) Using high-throughput SNP technologies to study cancer. Oncogene 25, 1594–1601

22. Bontant, M., Ramos, O. H., Lecoeur, C., Vaillant, E., Philippe, J., Zhang, P., Perilhou, A., Valcarcel, B., Sebert, S., Jarvelin, M. R., Baulk, B., Scott, D., Froguet, P., Vaxillaire, M., and Vasseur-Cognet, M. (2012) Glucose-dependent regulation of NRP23 promoter and influence of SNP-rs3743462 on whole body insulin sensitivity. PLoS One 7, e35810

23. Nayak, M. S., Yang, J. M., and Haid, W. N. (2007) Effect of a single nucleotide polymorphism in the murine double minute 2 promoter (SNP309) on the sensitivity to topoisomerase II-targeting drugs. Cancer Res. 67, 5831–5839

24. Bird, A. (1992) The essentials of DNA methylation. Cell 70, 5–8

25. Jones, P. A., and Baylin, S. B. (2007) The epigenomics of cancer. Cell 128, 683–692

26. Del Rosso, M. (2011) uPAR in angiogenesis regulation. Blood 117, 3941–3943

27. Lengyel, E., Wang, H., Stepp, E., Juarez, J., Wang, Y., Doe, W., Pfarr, C. M., and Boyd, D. (1996) Requirement of an upstream AP-1 motif for the constitutive and phorbol ester-inducible expression of the uroki-nase-type plasminogen activator receptor gene. J. Biol. Chem. 271, 23176–23184

28. Dang, J., Boyd, D., Wang, H., Allgayer, H., Doe, W. F., and Wang, Y. (1999) A region between –141 and –61 bp containing a proximal AP-1 is essential for constitutive expression of urokinase-type plasminogen activator receptor. Eur. J. Biochem. 264, 92–99

29. Ludes-Meyers, J. H., Liu, Y., Muñoz-Medellín, D., Hilsenbeck, S. G., and Brown, P. H. (2001) AP-1 blockade inhibits the growth of normal and malignant breast cells. Oncogene 20, 2771–2780

30. Shen, Q., Uray, I. P., Li, Y., Krisko, T. I., Strecker, T. E., Kim, H. T., and Brown, P. H. (2008) The AP-1 transcription factor regulates breast cancer cell growth via cyclins and E2F factors. Oncogene 27, 366–377

31. Mechat, F., Lallemend, D., Pfarr, C. M., and Yaniv, M. (1997) Transcription by fos modifies AP-1 binding and AP-1 activity. Oncogene 14, 837–847

32. Pospelova, T. V., Medvedev, A. V., Kukushkin, A. N., Svetlikova, S. B., van der Eb, A. J., Dorsman, J. C., and Pospelov, V. A. (1999) E1A + CHa-ras
transformed rat embryo fibroblast cells are characterized by high and constitutive DNA binding activities of AP-1 dimers with significantly altered composition. *Gene. Expr.* 8, 19–32
33. Ryseck, R. P., and Bravo, R. (1991) c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. *Oncogene* 6, 533–542
34. Nakabeppu, Y., Ryder, K., and Nathans, D. (1988) DNA binding activities of three murine Jun proteins: stimulation by Fos. *Cell* 55, 907–915
35. Tate, P. H., and Bird, A. P. (1993) Effects of DNA methylation on DNA-binding proteins and gene expression. *Curr. Opin. Genet. Dev.* 3, 226–231
36. Deng, G., Chen, A., Pong, E., and Kim, Y. S. (2001) Methylation in hMLH1 promoter interferes with its binding to transcription factor CBF and inhibits gene expression. *Oncogene* 20, 7120–7127