Ezrin, encoded by VIL2, is a membrane-cytoskeletal linker protein that has been suggested to be involved in tumorigenesis. Ezrin expression in esophageal squamous cell carcinoma (ESCC) was described recently, but its clinical significance and the molecular mechanism underlying its regulated expression remain unclear. Thus, we retrospectively evaluated ezrin expression by immunohistochemistry in a tissue microarray representing 193 ESCCs. Ezrin overexpression in 90 of 193 tumors (46.6%) was associated with poor survival (p = 0.048). We then explored the mechanism by which ezrin expression is controlled in ESCC by assessing the transcriptional regulatory regions of human VIL2 by fusing deletions or site-directed mutants of the 5′-flanking region of the gene to a luciferase reporter. We found that the region -87/-32 containing consensus Sp1 (-75/-69) and AP-1 (-64/-58) binding sites is crucial for VIL2 promoter activity in esophageal carcinoma cells (EC109) derived from ESCC. AP-1 is comprised of c-Jun and c-Fos. Electrophoretic mobility shift and chromatin immunoprecipitation experiments demonstrated that Sp1 and c-Jun bound specifically to their respective binding sites within the VIL2 promoter. In addition, transient expression of Sp1, c-Jun, or c-Fos increased ezrin expression and VIL2 promoter activity. Use of selective inhibitors revealed that VIL2 transactivation required the MEK1/2 signal transduction pathway but not JNK or p38 MAPK. Taken together, we propose a possible signal transduction pathway whereby MEK1/2 phosphorylates ERK1/2, which phosphorylates Sp1 and AP-1 that in turn bind to their respective binding sites to regulate the expression of human VIL2 in ESCC cells.

Ezrin, encoded by VIL2, is a membrane-cytoskeletal linker protein belonging to the ezrin-radixin-moesin family (1). By linking the cytoplasmic face of the plasma membrane to the actin cytoskeleton, ezrin acts as both a structural scaffold and a platform for the transmission of signals in response to extracellular cues (2). Ezrin is involved in a wide variety of cellular processes such as adhesion (3), survival (4), motility (5), and signal transduction (6–8). Furthermore, recent biochemical and functional data have identified a novel role for ezrin in the control of cyclin A gene transcription and endothelial cell proliferation (9).

Ezrin is often aberrantly expressed in human cancers. There is a relationship between high expression of ezrin and metastatic potential of some carcinomas, including hepatocellular carcinoma (10), lung cancer (11), breast carcinoma (12), pancreatic adenocarcinoma (13), and endometrial cancer (14). We also have demonstrated that ezrin is overexpressed in a malignant transformed esophageal epithelial cell line compared with an immortalized cell line (15). Our more recent study on esophageal squamous cell carcinoma (ESCC)3 samples showed that ezrin tends to translocate from the plasma membrane to the cytoplasm in the progression from normal epithelium to invasive carcinoma of the esophagus (16). Moreover, both in vivo and in vitro experiments suggest that ezrin may affect tumor formation and tumor invasiveness directly (17). These findings of ezrin up-regulation associated with epithelial tumor metastasis and invasion make ezrin a potentially new prognostic marker and/or therapeutic target for some carcinomas (12, 18, 19).

Although much is known about how ezrin functions, there have been few reports about how ezrin expression is regulated. It has been reported that human ezrin expression can be regulated by cytokines, interleukin 2 (IL-2), IL-8, IL-10, and insulin-like growth factor 1 inhibit ezrin expression in human colon cancer cells, whereas epidermal growth factor and IL-11 increase cellular ezrin levels (20). Moreover, tumor necrosis factor-α treatment of human endothelial cells elevates ezrin expression.

3 The abbreviations used are: ESCC, esophageal squamous cell carcinoma; AP-1, activating protein-1; Sp1, specific protein-1; EMSA, electrophoretic mobility shift assay; MAPK, mitogen-activated kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IL, interleukin; DMSO, dimethyl sulfoxide; RT, reverse transcription; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; rh, recombinant human.
expression (9). In disseminated osteosarcoma, ezrin is strongly stained by immunohistochemistry and has been proposed as a crucial factor for osteosarcoma metastasis (21). Ogino et al. (22) demonstrated a high level of ezrin mRNA expression in an osteosarcoma biopsy sample with lung metastasis, which was compatible with previous reports analyzing ezrin protein levels (21). These data suggest that ezrin levels are controlled at the transcriptional level. Stable transformation of Rat-1 fibroblasts by Fos results in increased expression of ezrin (23, 24). Mouse ezrin expression correlates with Six1 expression in rhabdomyosarcoma (25). Six1, a homeodomain-containing transcription factor required for skeletal muscle development, can bind to the mouse Vil2 promoter between −1106 and −870, a region containing the MEF3-like motif TTCAGGA, and regulate ezrin expression (26). Sequence alignment showed that the 5′-flanking regions of human Vil2 and mouse Vil2 are highly diverged (supplemental Fig. S1). Also the MEF3-like motif TTCAGGA present in the mouse sequence does not exist in the human Vil2 promoter. These sequence differences imply that the transcriptional regulation mechanism probably differs between human Vil2 and mouse Vil2. However, no study has addressed the transcriptional regulation of human Vil2, the key transcriptional regulatory regions of the gene, or the regulatory mechanisms governing its expression.

In the present study, we evaluated the clinical significance of ezrin overexpression in ESCCs and explored the importance of DNA sequence elements, transcription factors, and the mitogen-activated protein kinase (MAPK) signal transduction pathway in regulating ezrin expression in human esophageal carcinoma cells (EC109 cells), which are derived from ESCC (27). We found that ezrin overexpression in ESCCs was associated with decreased survival and that consensus transcription factor Sp1 (−75/−69) and AP-1 (−64/−58) binding sites are essential for human Vil2 promoter activity. We further demonstrated that the cooperativity of Sp1 and AP-1 (c-Jun/c-Fos heterodimer) regulate Vil2 promoter activity and ezrin expression and that mitogen-activated protein kinase kinase (MEK1/2) and extracellular signal-regulated kinase (ERK1/2) are upstream kinases that control human Vil2 transcriptional activation in ESCC cells.

EXPERIMENTAL PROCEDURES

Materials—Expression plasmid CMV-Sp1 was kindly provided by Dr. Guntram Suske (Philips University, Marburg, Germany). Plasmid pcDNA3 was purchased from Invitrogen. Plasmids pGL3-Basic and pRL-TK, recombinant human (rh) proteins rhSp1 and rhAP-1 (c-Jun), and kinase inhibitors U0126, PD98059, and SB203580 were purchased from Promega (Madison, WI). c-Jun N-terminal kinase (JNK) inhibitor II SP600125 was purchased from Calbiochem (La Jolla, CA). Antibodies against Sp1, c-Jun, c-Fos, phospho-ERK1/2, ERK1/2, and ezrin, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-Actin was purchased from Sigma. All other reagents were of analytical grade.

Tissue Specimens and Immunohistochemical Staining—Surgically removed tumors embedded in paraffin wax blocks from 193 ESCC cases were retrieved from the archives of the Department of Pathology of the Central Hospital of Shantou City, China. The cases were received between 1987 and 1997. The cases were selected to build tissue microarrays as described (28) and were included in this study only if a follow-up was obtained and clinical data were available. Mean age at surgery was 53 years (range 35–70), and 127 patients were male and 66 were female. The SuperPicTure Polymer Detection kit and Liquid Substrate kit (Invitrogen) were used to conduct immunohistochemistry according to the manufacturer’s instructions. Staining was scored using the following scale: 0, no staining; 1+, minimal staining; 2+, moderate staining in at least 20% of cells; 3+, strong staining in at least 50% of cells. Cases scored as 0, 1+, or 2+ were classified as “non-overexpression,” and cases with 3+ staining were classified as “overexpression.” The study was approved by the ethical committee of the Central Hospital of Shantou City and the Medical College of Shantou University, and written informed consent was obtained from all surgical patients to use resected samples for research.

Expression Vectors and Reporter Gene Constructs—The human Vil2 5′-flanking region plus 134 bp of transcribed human Vil2 sequence was generated by PCR using the following primers: Fezr, 5′-CGGGGTACCAAGCTTACCGCCACGATGATGTTCTC-3′; Rezr, 5′-CGGGATCCCTTCCCTGCCCCCTC-3′, and the resulting plasmid was named pGLB-hE(−1759/+134). The luciferase reporter plasmids, pGLB-hE(−324/+134), pGLB-hE(−890/+134), pGLB-hE(−696/+134), pGLB-hE(−213/+134), pGLB-hE(−146/+134), pGLB-hE(−97/+134), pGLB-hE(−87/+134), and pGLB-hE(−32/+134) were generated from pGLB-hE(−1759/+134) using the Erase-a-Base® System (Promega). Site-directed mutagenesis to obtain sequences (−87/+134)Sm, (−87/+134)Am, and (−87/+134)SAm was performed by PCR using primer “Rezr” along with the following primers: Fezr-Sm, 5′-GCAATGCTAATAATTTCGTC-ACTCACCAGGCCCCG-3′, Fezr-Am, 5′-GCAATGCTG-GCGGGCGCGCGTGGATCCCGGCGCCCCGATTGCGG-GTTTCTC-3′; and Fezr-SAm, 5′-GCAATGCTAATATTTCGTCGGTCCAGATCCGCCCCCCGCGGTCCC-3′ (position −83 is indicated; mutated bases are underlined). The amplified fragments obtained using Pfu DNA polymerase (Promega) were digested with HindIII and inserted into the Smal/HindIII sites of pGL3-basic, and the resulting plasmid was named pGLB-hE(−1759/+134). The luciferase reporter plasmids, pGLB-hE(−324/+134), pGLB-hE(−890/+134), pGLB-hE(−696/+134), pGLB-hE(−213/+134), pGLB-hE(−146/+134), pGLB-hE(−97/+134), pGLB-hE(−87/+134), and pGLB-hE(−32/+134) were generated from pGLB-hE(−1759/+134) using the Erase-a-Base® System (Promega). Site-directed mutagenesis to obtain sequences (−87/+134)Sm, (−87/+134)Am, and (−87/+134)SAm was performed by PCR using primer “Rezr” along with the following primers: Fezr-Sm, 5′-GCAATGCTAATAATTTCGTC-ACTCACCAGGCCCCG-3′, Fezr-Am, 5′-GCAATGCTG-GCGGGCGCGCGTGGATCCCGGCGCCCCGATTGCGG-GTTTCTC-3′; and Fezr-SAm, 5′-GCAATGCTAATATTTCGTCGGTCCAGATCCGCCCCCCGCGGTCCC-3′ (position −83 is indicated; mutated bases are underlined). The amplified fragments obtained using Pfu DNA polymerase (Promega) were digested with HindIII and inserted into the Smal/HindIII sites of pGL3-basic, and the resulting plasmid was named pGLB-hE(−87/+134)Sm, pGLB-hE(−87/+134)Am, and pGLB-hE(−87/+134)SAm; in the constructs the sequence upstream of the sense primer was GCCC, which is the same sequence as −87/−84 of the human Vil2 5′-flanking region. The c-Fos and c-Jun expression vectors were constructed by cloning full-length c-Fos or c-Jun cDNA in the pcDNA3 plasmid. Primers for c-Fos were 5′-CCAAAGCTTACCGCAGGATGTGTTCTC-3′ (HindIII site underlined) and 5′-CCGGATCTTCTTCCGTCCCCCTCTACA-3′ (BamHI site underlined). Primers for c-Jun were 5′-CCAAAGCTTACCGCAGGATGTGTTCTC-3′ (HindIII site underlined) and 5′-CCGGATCTTCTTCCGTCCCCCTCTACA-3′ (BamHI site underlined).
Cell Culture and Transfection—EC109 cells were maintained in 199 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C in a 5% CO₂ environment. For transfection, cells were seeded in 96-well plates at 1.5 × 10⁵ cells/ml, grown to 50–80% confluency and transfected with the plasmids described above using Superfect Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Following transfection, cells were incubated for another 48 h before being harvested for the luciferase assay or gene expression assay. Alternatively, after transfection for 24 h, cells were treated with a kinase inhibitor for another 24 h before being harvested.

Luciferase Assay—Transfected cells were harvested in Passive Lysis Buffer (Promega) and the cell lysates analyzed for luciferase activity with the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s recommendations.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts from human esophageal cancer cells were produced according to standard methods (29). Extracts were aliquoted and stored at −70 °C. Equimolar amounts of complementary, single-stranded oligonucleotides were annealed and labeled with digoxigenin (DIG)-ddUTP by terminal transferase using a DIG Gel Shift kit (Roche). The oligonucleotide probes used in EMSA were as follows: probe W, 5’-GCCCGCGACTGCTGGCAGTGCTGACTCACCCGGGCCGCGGG-3’ (mutated Sp1 binding site underlined); probe Sm, 5’-GCCGCAGTGCTGGGCGGGGCGCTGACTCACCCGGGCCGCGGG-3’ (mutated AP-1 binding site underlined); and probe SAm, 5’-GCCCGCATGCTAAATTTTGCGCTACCTACCCGGGCGCGGG-3’ (mutated Sp1 binding site underlined). These probes corresponded to the human VIL2 5’-flanking sequence from −87 to −46. Each binding mixture (20 μl) for EMSA contained 5–10 μg of nuclear extract or 0.2–0.3 μg of recombinant protein, 20 mM HEPES (pH 7.9), 1 mM EDTA (pH 8.0), 1 mM dithiothreitol, 10 mM (NH₄)₂SO₄, 0.2% (w/v) Tween 20, 30 mM KCl, 1 μg poly[d(I-C)], 0.1 μg of poly-l-lysine, and 0.05 pmol of labeled double-stranded oligonucleotide probe. Samples were incubated at room temperature for 30 min, and complexes were analyzed by electrophoresis on 6% non-denaturing polyacrylamide gels (acrylamide/bis-acrylamide ratio of 29:1) in 0.5 × TBE at 80 V for 180 min at 70 °C. Equimolar amounts of complementary, single-stranded oligonucleotides were annealed and labeled with digoxigenin (DIG)-ddUTP by terminal transferase using a DIG Gel Shift kit (Roche). The oligonucleotide probes used in EMSA were as follows: probe W, 5’-GCCCGCGACTGCTGGCAGTGCTGACTCACCCGGGCCGCGGG-3’ (mutated Sp1 binding site underlined); probe Sm, 5’-GCCGCAGTGCTGGGCGGGGCGCTGACTCACCCGGGCCGCGGG-3’ (mutated AP-1 binding site underlined); and probe SAm, 5’-GCCCGCATGCTAAATTTTGCGCTACCTACCCGGGCGCGGG-3’ (mutated Sp1 binding site underlined). These probes corresponded to the human VIL2 5’-flanking sequence from −87 to −46. Each binding mixture (20 μl) for EMSA contained 5–10 μg of nuclear extract or 0.2–0.3 μg of recombinant protein, 20 mM HEPES (pH 7.9), 1 mM EDTA (pH 8.0), 1 mM dithiothreitol, 10 mM (NH₄)₂SO₄, 0.2% (w/v) Tween 20, 30 mM KCl, 1 μg poly[d(I-C)], 0.1 μg of poly-l-lysine, and 0.05 pmol of labeled double-stranded oligonucleotide probe. Samples were incubated at room temperature for 30 min, and complexes were analyzed by electrophoresis on 6% non-denaturing polyacrylamide gels (acrylamide/bis-acrylamide ratio of 29:1) in 0.5 × TBE at 80 V for 180 min at 70 °C. Equimolar amounts of complementary, single-stranded oligonucleotides were annealed and labeled with digoxigenin (DIG)-ddUTP by terminal transferase using a DIG Gel Shift kit (Roche). The oligonucleotide probes used in EMSA were as follows: probe W, 5’-GCCCGCGACTGCTGGCAGTGCTGACTCACCCGGGCCGCGGG-3’ (mutated Sp1 binding site underlined); probe Sm, 5’-GCCGCAGTGCTGGGCGGGGCGCTGACTCACCCGGGCCGCGGG-3’ (mutated AP-1 binding site underlined); and probe SAm, 5’-GCCCGCATGCTAAATTTTGCGCTACCTACCCGGGCGCGGG-3’ (mutated Sp1 binding site underlined). These probes corresponded to the human VIL2 5’-flanking sequence from −87 to −46. Each binding mixture (20 μl) for EMSA contained 5–10 μg of nuclear extract or 0.2–0.3 μg of recombinant protein, 20 mM HEPES (pH 7.9), 1 mM EDTA (pH 8.0), 1 mM dithiothreitol, 10 mM (NH₄)₂SO₄, 0.2% (w/v) Tween 20, 30 mM KCl, 1 μg poly[d(I-C)], 0.1 μg of poly-l-lysine, and 0.05 pmol of labeled double-stranded oligonucleotide probe. Samples were incubated at room temperature for 30 min, and complexes were analyzed by electrophoresis on 6% non-denaturing polyacrylamide gels (acrylamide/bis-acrylamide ratio of 29:1) in 0.5 × TBE at 80 V for 180 min at 4 °C. The gels were then transferred to a positively charged polyvinylidene difluoride membrane (Immobilon, pore size 0.45 μm, Millipore, Bedford, MA) using a constant voltage of 60 V for 120 min. The membranes were then blocked in 5% nonfat milk in phosphate-buffered saline containing 0.1% Tween 20 for 1 h at room temperature followed by the addition of the primary antibody for 1 h at room temperature. The membranes were then washed and incubated with a secondary antibody coupled to horseradish peroxidase for 1 h at room temperature. Antigen-antibody complexes were detected by Western blot luminol reagent (Santa Cruz Biotechnology).

Western Blot Analysis—Whole cell protein extracts were boiled for 5 min with Laemmli buffer and subjected to 12% SDS-polycrylamide gel electrophoresis using standard methodology. Proteins were then transferred electrophoretically onto a polyvinylidene difluoride membrane (Immobilon, pore size 0.45 μm, Millipore, Bedford, MA) using a constant voltage of 60 V for 120 min. The membranes were then blocked in 5% nonfat milk in phosphate-buffered saline containing 0.1% Tween 20 for 1 h at room temperature followed by the addition of the primary antibody for 1 h at room temperature. The membranes were then washed and incubated with a secondary antibody coupled to horseradish peroxidase for 1 h at room temperature. Antigen-antibody complexes were detected by Western blot luminol reagent (Santa Cruz Biotechnology).

Statistical Analysis—Data analysis was performed using SPSS 13.0 (SPSS, Inc., Chicago, IL). A two-tailed independent-sample t test was used to determine the significance of differences between groups. Differences were considered statistically significant at p < 0.05. Data are plotted as mean ± S.D. Survival was assessed by Kaplan-Meier analysis, and the log-rank score was used to determine statistical significance.

Regulation of VIL2 by Sp1 and AP-1

Briefly, the enzymatically sheared chromatin was preclreated with protein G beads and an aliquot saved as a positive control (input DNA). Aliquots of the preclreated sheared chromatin were then immunoprecipitated using 2 μg of antibodies against IgG, Sp1, c-Jun, or c-Fos. The resulting DNA was used for PCR analysis, and the amplified DNA fragments were visualized on an agarose gel. PCR of immunoprecipitated DNA were carried out using human VIL2 promoter-specific primers and negative control primers flanking a region of genomic DNA between GAPDH and the chromosome condensation-related SMC-associated protein gene. The human VIL2 promoter-specific primers were 5’-CTCCCCATGCGCCGACGTGCT-3’ (VIL2 −95/−76 sequence) and 5’-GGTGAGTATCTCGATCCCC-GAATA-3’ (VIL2 +123/+99 sequence). The negative control primers were 5’-ATGGTTGCGACTGGGATCT-3’ and 5’-TGCCAAAGGCTAGGGGAGA-3’.

Real-time Reverse Transcription (RT)-Polymerase Chain Reaction—Total cellular RNA was extracted from EC109 cells with TRIzol reagent (Invitrogen) and reverse transcribed to cDNA using the PrimerScript™ RT-PCR kit (TaKaRa, Dalian, China). The real-time RT-PCR assay was carried out with the Rotor-Gene 6000 system (Corbett Life Science, Sydney, Australia) using SYBR® Premix Ex Taq™ (TaKaRa) according to the manufacturer’s instructions. All PCRs were done in triplicate. The sequences of VIL2 primers were designed based on the human VIL2 mRNA sequence (GenBank accession number NM_003379) as follows: VIL2 forward, 5’-AGCCGATCATGAGCCGAG-3’ and reverse, 5’-GCCCAGCTTTGTCTGG-3’. The sequences of GAPDH primers were designed based on the human GAPDH mRNA sequence (GenBank accession number NM_000246) as follows: GAPDH forward, 5’-GGACCCCTCAAGGGTGAACAC-3’ and reverse, 5’-TGGAAGACGGCAGGGAA-3’. The absolute levels of mRNAs of VIL2 were normalized to that of GAPDH mRNA. The relative value from the vehicle-treated control group was considered equal to one arbitrary unit.

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Overexpression of ezrin in ESCCs was associated with poor survival. Kaplan-Meier survival analysis (Fig. 2) demonstrated that ezrin overexpression was associated with decreased survival \( (p = 0.048) \).

Consensus Sp1 \((-75/-69\) and AP-1 \((-64/-58\) Binding Sites Are Essential for Human VIL2 Promoter Activity—To better understand the transcriptional regulation of ezrin in ESCCs, 1759 bp of the 5′-flanking region of human VIL2 and 134 bp of the transcribed sequence were cloned from EC109 cells (derived from ESCC) and sequenced (GenBank accession number EF184645). NCBI BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) showed that the cloned fragment had 99% identity with the corresponding segment of human VIL2 AL589931. The CpGPlot Program (www.ebi.ac.uk/emboss/cpgplot) revealed this fragment to be highly GC rich, with CpG islands located at \(-1564/-581, -558/-303\), and \(-203/+77\) (supplemental Fig. S2). Potential transcription factor binding sites within human VIL2 were identified through gene-regulation (www.gene-regulation.com/pub/programs/alibaba2). The analysis revealed multiple potential transcription factor binding sites in this fragment, many of them overlapping, and one site that is a putative target of several factors (supplemental Fig. S3). Moreover, the human VIL2 promoter region lacks a typical TATA box but contains numerous potential Sp1 binding sites, as is common with many GC-rich promoters (30).

Transient transfection of EC109 cells showed that the 5′-flanking region of human VIL2 \((−1.8\, \text{kb})\) could drive transcription of a luciferase reporter. To localize the regulatory region of promoter activity, a series of 5′-deletion mutants was constructed and analyzed. In EC109 cells, the \(-1324/+134\) region of VIL2 directed maximum luciferase activity (Fig. 3A). The region \(-87/+134\) had considerable reporter activity. Sequence deletion from \(-1324\) to \(-890\) caused an ~50% reduction in luciferase activity, whereas 5′-deletion from \(-87\) to \(-32\) nearly abolished the activity. When compared with region \(-890/+134\), further deletions, i.e., \(-696/+134\), \(-213/+134\), \(-146/+134\), \(-97/+134\), and \(-87/+134\), did not markedly change the reporter activity. These data suggest that the region \(-1324/−890\) positively regulates transcription and that the region \(-87/−32\) regulates the promoter activity of human VIL2 in EC109 cells.

To further investigate the role of specific 5′-flanking regions in the promoter activity of human VIL2, two candidate sites were chosen: a consensus Sp1 binding site \((-75/−69\), GGGCGGGG) and a consensus AP-1 binding site \((-64/−58\), TGACTCA) (Fig. 3B). Constructs containing 87 bp of VIL2 5′-flanking region with site-directed mutations in the Sp1 site and/or the AP-1 were used for transfection, and expression of the constructs was detected by luciferase assays in EC109 cells. The Sp1 and AP-1 sites were essential for efficient luciferase activity; when either site was mutated, luciferase activity was reduced by 30–60% (Fig. 3C), and when both sites were simultaneously mutated the luciferase activity was greatly reduced. Similar data were obtained using cervical carcinoma HeLa cells (supplemental Fig. S4). These results imply that the Sp1 \((-75/−69\) and AP-1 sites \((-64/−58\) are essential for human VIL2 promoter activity in EC109 and HeLa cells.

Interaction between Sp1 and the Sp1 Site \((-75/−69\) and between AP-1 and the AP-1 Site \((-64/−58\) of VIL2—Because the region between \(-87\) and \(-32\) seemed to be critical for VIL2

### RESULTS

**Ezrin Overexpression in ESCCs Is Associated with Poor Survival**—Our recent study on ESCC samples showed that ezrin tends to translocate from the plasma membrane to the cytoplasm in the progression from normal epithelium to invasive carcinoma of the esophagus (16). Here, we confirmed this finding and further evaluated clinical significance. Ezrin immunoreactivity in normal esophageal epithelial tissue was weak to moderate in the membrane and cytoplasm (data not shown). However, ESCC tumors displayed one of four distinct immunostaining phenotypes (Fig. 1): intense diffuse membranous and/or cytoplasmic staining (Fig. 1A), moderate cytoplasmic staining (Fig. 1B), weak cytoplasmic staining (Fig. 1C), or no staining (Fig. 1D). Ezrin overexpression was defined as intense diffuse cytoplasmic and/or plasma membrane staining in >50% of tumor cells. In all, 90 of 193 tumors were designated as ezrin overexpressors. Kaplan-Meier survival analysis (Fig. 2) demonstrated that ezrin overexpression was associated with decreased survival \( (p = 0.048) \).

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Interaction between Sp1 and the Sp1 Site \((-75/−69\) and between AP-1 and the AP-1 Site \((-64/−58\) of VIL2—Because the region between \(-87\) and \(-32\) seemed to be critical for VIL2

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**FIGURE 1. Immunohistochemical staining of ezrin in ESCC.** A, a case with 3+ staining, B, a case with 2+ staining, C, a case with 1+ staining, D, a negative-staining case. Bar = 50 μm.

**FIGURE 2. Kaplan-Meier estimates of the survival by ezrin status.** The survival rate for patients overexpressing ezrin was significantly lower than that for patients not overexpressing ezrin.
Regulation of VIL2 by Sp1 and AP-1

To assess the binding of Sp1 and AP-1 to their respective sites, recombinant proteins rhSp1 and rhAP-1 (c-Jun) were individually incubated with probe W, probe Sm, probe Am, or probe SAm, which is mutated in the AP-1 site (−64/−58) and which sequence (Fig. 4A).

We next analyzed complex formation in the presence of mithramycin A, a Sp1-specific chemical inhibitor that binds to the promoter GC box and blocks Sp1 or other Sp family proteins from binding (31). In EMSAs, mithramycin A inhibited the formation of complex I in a dose-dependent manner but did not visibly change c-Jun expression. Interestingly, ezrin expression was increased in all transfectants and cotransfectants. These data indicate that Sp1, c-Jun, and c-Fos up-regulate ezrin expression in EC109 cells. However, compared with transfections involving a single plasmid encoding Sp1, c-Jun, or c-Fos, ezrin expression by Western blotting was increased in all transfectants and cotransfectants. These data indicate that Sp1, c-Jun, and c-Fos expression did not increase substantially upon co-transfection with c-Jun/c-Fos or Sp1/c-Jun/c-Fos plasmids.

Sp1 and AP-1 Regulate Human VIL2 Promoter Activity Through Their Respective Sp1 and AP-1 Sites—To define the respective roles of Sp1, c-Jun, and c-Fos in VIL2 transactivation, and to examine whether transactivation occurs through the respective Sp1 and AP-1 sites, EC109 cells were cotransfected with pGLB-hE(−87/+134), which was set to 100%. Each value represents the mean ± S.D. The data are representative of at least two independent experiments. Transfections were carried out in triplicate for each experiment. ***, p < 0.001.

Similarly, two distinct complexes formed in the presence of rhAP-1 (c-Jun) with probe W, but no complex formed with probes Am or SAm in which the AP-1 site (−64/−58) is mutated. These results suggest that Sp1 and AP-1 bind specifically to their respective sites.

To investigate whether Sp1 and AP-1 bind the VIL2 promoter region in EC109 cells, we performed a ChIP assay. Immunoprecipitated chromosomal DNA was subjected to PCR using primers designed to amplify the VIL2 promoter region harboring the Sp1 and AP-1 binding sites or negative control primers flanking a region of genomic DNA between GAPDH and the gene encoding chromosome condensation-related SMC-associated protein. Sp1 and c-Jun indeed bound to the VIL2 promoter region containing the Sp1 and AP-1 sites (Fig. 4D), whereas c-Fos did not.

Transient Expression of Sp1, c-Jun, or c-Fos Up-regulates Ezrin Expression—To explore the effect of transcription factors Sp1, c-Jun, and c-Fos on ezrin expression, EC109 cells were cotransfected with expression vectors CMV-Sp1, pcDNA3-c-Jun, or pcDNA3-c-Fos. Backbone vector pcDNA3 was used as a negative control. Total protein was extracted for analysis of Sp1, c-Jun, c-Fos, and ezrin expression by Western blotting in EC109 cell lysates.
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FIGURE 4. Binding activity of the human VIL2 promoter region. A, binding of nuclear extract proteins from EC109 cells to the −87/−66 sequence of human VIL2. Digoxigenin-labeled probe W was incubated without (lane 1) or with (lanes 2 and 3) nuclear extract prepared from EC109 cells and then analyzed by EMSA. Unlabeled probe W, as a specific competitor, was used at 100-fold molar excess (lane 3). B, effect of mithramycin A on binding of nuclear extract from EC109 cells to probe W. Digoxigenin-labeled probe W was preincubated with increasing concentrations of mithramycin A (0.1, 1, or 10 μM) for 1 h at 4 °C before addition to the EMSA reaction. Specific competitor was added at 100-fold molar excess (lane 1). C, binding specificity of rhSp1 and rhAP-1 (c-Jun) for the Sp1 and AP-1 sites. Recombinant protein rhSp1 or rhAP-1 (c-Jun) (0.2–0.3 μg) was incubated with probe W, probe Sm, probe Am, or probe SAm, and then analyzed by EMSA. D, association of Sp1 and AP-1 with the human VIL2 promoter in cells using the ChIP assay. Cross-linked chromatin isolated from EC109 cells was immunoprecipitated with anti-Sp1 (lane 4), anti-c-Jun (lane 5), or anti-c-Fos (lane 6). The associated chromosomal DNA fragments were amplified with a human VIL2 promoter-specific primer pair resulting in a 218-bp fragment or a negative control primer pair resulting in a 174-bp fragment. The control reaction (no DNA, lane 1), chromosomal DNA input (lane 2, described under “Experimental Procedures”), and ChIP DNA with nonspecific IgG (lane 3) were subjected to the same PCR amplification. PCR products were separated on a 3% agarose gel containing ethidium bromide and detected via ultraviolet illumination.

FIGURE 5. Transient expression of Sp1, c-Jun, or c-Fos up-regulates ezrin expression in EC109 cells. EC109 cells were transfected without (−) or with 600 ng (+) of transcription factor expression vectors pcDNA3, CMV-Sp1, pcDNA3-c-Jun, or pcDNA3-c-Fos. Total protein from EC109 cells was collected and analyzed by Western blotting using 20 μg of protein/lane. β-Actin is shown as a loading control. Fold-change in ezrin indicates the ratio of band intensity of ezrin to β-actin. Experiments were repeated two to three times with similar results.
MEK/ERK1/2 Signaling Pathway Regulates VIL2 Promoter Activity and Ezrin Expression—ERK1/2, JNK, and p38 belong to a superfamily of serine-threonine kinases known as the MAPKs. These kinases participate in signal transduction pathways that control intracellular events, including acute responses to hormones and major developmental changes in organisms (32). To explore which MAPK activates the VIL2 promoter leading to VIL2 transcription via phosphorylation of Sp1 and AP-1, EC109 cells were cotransfected with constructs pGL3-Sp1 and pRL-TK. After 24 h of transfection, the cells were cultured for another 24 h in the absence or presence of the MAPK inhibitors: U0126 (MEK1/2 inhibitor), PD98059 (p38 MAPK inhibitor), SB203580 (p38 MAPK inhibitor), or SP600125 (JNK inhibitor). The inhibitor concentrations (10 or 40 μM) used in these experiments did not affect cell viability (data not shown). U0126 (10 μM or 40 μM) or PD98059 (40 μM) significantly inhibited human VIL2 promoter activity (p < 0.001), whereas SB203580 or SP600125 did not (Fig. 7A). U0126 and PD98059 specifically block MEK1/2 activation and therefore inhibit the subsequent phosphorylation and activation of ERK1/2 (33, 34). These preliminary findings indicated that transactivation of the VIL2 promoter requires ERK1/2 and that p38 MAPK and JNK have no role in the activation.

To further determine whether U0126 and PD98059 inhibit the phosphorylation of ERK1/2 to impact ezrin expression, EC109 cells were cultured for another 24 h in the absence or presence of the inhibitors (40 μM) for 24 h before being harvested. Total cellular RNA and proteins were extracted for real-time RT-PCR and Western blot analysis, respectively. The VIL2 mRNA level was significantly lower in MEK1/2 inhibitor-treated cells relative to DMSO-treated cells (Fig. 7B, p < 0.05). Moreover, U0126 and PD98059 not only inhibited the phosphorylation of ERK1/2 but also down-regulated the expression of ezrin protein (Fig. 7C). These results suggest that reduced phosphorylation of ERK1/2 decreases ezrin expression and VIL2 promoter activity and that ERK1/2 may be the upstream kinase that phosphorylates Sp1 and AP-1, which in turn activate human VIL2 transcription in EC109 cells.

DISCUSSION

Ezrin is often aberrantly expressed in human cancers and thus may be a potentially new prognostic marker and/or therapeutic target for some carcinomas (12, 18, 19). In this study, we also found that ezrin overexpression in ESCCs was associated with poor survival. Although it is well established that ezrin is implicated in many aspects of cancer cell biology (3–8), the transcription factors and signal transduction pathways that regulate VIL2 transcription remain poorly understood. Here, we showed that transcription factors Sp1 and AP-1 (c-Jun/c-Fos) can transactivate the VIL2 promoter through the Sp1 site and the adjacent AP-1 site, respectively, and that the MEK/ERK1/2 signaling pathway is implicated in this process.

Truncation experiments showed that the cloned human VIL2 5′-flanking sequence contains a positive regulatory region at −1324/−890 and a promoter regulatory region at −87/−32 (Fig. 3A). The human VIL2 promoter is a GC-rich TATA-less promoter. Similar promoters are found in many mammalian genes such as the human cystathionine β-synthase gene (35) and the human survivin gene (36). We studied the regulatory region for VIL2 promoter activity and showed that a consensus Sp1 binding site at −75/−69 and a consensus AP-1 binding site at −64/−58 contribute to VIL2 promoter activity in EC109 cells (Fig. 3C). Using Sp1 expression plasmid CMV-Sp1 and constructs containing the human VIL2 5′-flanking region with a wild-type or mutated Sp1 site, we found that the Sp1-mediated increase in VIL2 promoter activity depended on the Sp1 binding site (Fig. 6, A–C). Sp1 and Sp3 recognize consensus Sp1

FIGURE 6. Effect of transient expression of Sp1, c-Jun, and c-Fos on human VIL2 promoter activity in EC109 cells. Constructs pGL3-Sp1, pGL3-c-Jun, and pGL3-c-Fos were cotransfected into EC109 cells with pRL-TK and with the indicated amounts of pcDNA3, pcDNA3-Sp1, pcDNA3-c-Jun, and/or pcDNA3-c-Fos. Luciferase activity was normalized to Renilla luciferase activity and then shown relative to that of cells cotransfected with pcDNA3 and pGL3-hE−87+134)Am (C), which was set at 100%. Each value represents the mean ± S.D. The data are representative of at least two independent experiments. Transfections were carried out in triplicate for each experiment. **, p < 0.01; ***, p < 0.001.
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FIGURE 7. ERK1/2 inhibitors down-regulate VIL2 expression in EC109 cells. A, effect of MAPK inhibitors on human VIL2 promoter activity. Construct pGLB-hE(−87/+134) was transfected with pRL-TK into EC109 cells for 24 h before ERK1/2 inhibitors U0126 and PD98059, p38 inhibitor SB203580, JNK inhibitor SP600125, and/or DMSO were added to the cultures for another 24 h before harvesting the cells. Luciferase activity of pGLB-hE(−87/+134) was normalized to Renilla luciferase activity and then shown relative to that of cells transfected with the construct without inhibitors, which was set to 100%. Each value represents the mean ± S.D. The data are representative of at least two independent experiments. Transfections were carried out in triplicate for each experiment. B, real-time RT-PCR for VIL2 mRNA expression. EC109 cells were incubated with ERK1/2 inhibitors U0126, PD98059, and/or DMSO for 24 h before being harvested. VIL2 mRNA expression was analyzed by real-time RT-PCR, and expression was recorded as the fold-change normalized to mRNA from vehicle (DMSO)-treated cells. C, Western blot for phospho-ERK1/2 and ezrin expression. Total protein from EC109 cells treated as in B was analyzed by Western blotting using 25 μg of protein per lane. β-Actin is shown as a loading control. Fold-change in ezrin indicates the ratio of band intensity of ezrin to β-actin. All inhibitors used in the experiments were dissolved in DMSO, and the final concentration of DMSO in EC109 cell cultures was 0.45%. Experiments were done two to three times with similar results. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

binding sites with identical affinity (37). Sp1 is a general activator of transcription, whereas Sp3 can act as an activator or as a repressor of Sp1-mediated activation, depending on the cellular context (38). To determine whether Sp3 also has a role in VIL2 promoter activity, EC109 cells were transfected with plasmid CMV-Sp3; CMV-Sp3 slightly increased VIL2 promoter activity even in the presence of the mutated Sp1 site (data not shown), suggesting that Sp3 does not compete with Sp1 for binding to the Sp1 site at −75/−69 within the VIL2 promoter. Thus, only Sp1 mediates VIL2 transactivation through the Sp1 site.

Regulation of gene expression through the use of combinations of different transcription factors has been widely observed. Indeed, Sp1 can interact and functionally cooperate with other transcription factors, including nuclear factor-κB (39), Ets-1 (40), STAT1 (41), G-C homopolymer binding factors (42), CCAAT/enhancer binding protein β (43), and Egr-1 (44). Furthermore, the cooperativity between Sp1 and AP-1 in the transcriptional activation of human genes encoding leukocyte integrin CD11c (45), involucrin (46), and loricrin (47) has been reported. AP-1 is a sequence-specific transcription factor consisting of a heterodimer of proteins from the Jun family (c-Jun, JunD, and JunB) (48, 49) and the Fos family (c-Fos, FosB, Fra-1, and Fra-2) (50, 51). Moreover, members of the Jun family form homo- or heterodimers that recognize the AP-1 site (consensus DNA sequence TGAGTCA) or related sequences. In this study, luciferase expression experiments also showed that c-Jun with c-Fos, and Sp1 with AP-1 cooperated to activate the human VIL2 promoter (Fig. 6D). We did not examine whether the c-Jun/c-Fos or Sp1/AP-1 complex exists in EC109 cells, or whether the binding of one transcription factor to a VIL2 DNA element facilitated binding of other factors to their corresponding DNA elements; as such, the synergistic cooperation between c-Jun and c-Fos or between Sp1 and AP-1 needs further investigation. Previous studies have shown that the cooperation seems not to depend on the spacing between functional Sp1 and AP-1 binding sites (45, 46); one proposed concept to explain the interaction is that Sp1 induces a conformational change in the DNA that contributes to the activation, perhaps by enhancing AP-1 binding.

Sp1 and AP-1 are often final targets of signal-transducing kinase cascades, and upon phosphorylation they become activated and bind to their respective target promoters and trigger expression of the corresponding genes (52, 53). It has been reported that Sp1 can be phosphorylated by ERK1/2, JNK, and/or p38 MAPK (54, 55), depending on the target gene in different cells. AP-1 activity can be regulated by altering the expression of specific AP-1 components as well as by protein phosphorylation as a result of the activation of intracellular signaling cascades (56–58). For example, phosphorylation of c-Jun by UV-activated JNK permits c-Jun to bind to AP-1 sequences present in the c-Jun promoter (56). ERK1/2 can also phosphorylate c-Jun, but not as effectively as JNK (59). c-Fos was recently shown to be a target of ERK2 activity upon mitogenic stimulus (57). In addition, p38 MAPK also mediates stress-induced c-Fos phosphorylation that then activates transcription of specific genes (60).
To understand the events upstream of the phosphorylation of Sp1 and AP-1 leading to VIL2 transcriptional activation in EC109 cells, we investigated the role of ERK1/2, JNK, and p38 MAPK using specific inhibitors. Analysis of human VIL2 promoter-luciferase constructs revealed that VIL2 transcription activation requires the MEK/ERK1/2 signal transduction pathway but not JNK or p38 MAPK (Fig. 7A). Previous studies have shown that ERK1/2 can phosphorylate Sp1 (55), c-Jun (59), and c-Fos (57) in some cell types. Additionally, activation of MEK/ERK1/2 has been shown to involve the induction of various signaling pathways, depending on the stimulus and cell type. Several extracellular stimuli such as cytokines tumor necrosis factor-α, epidermal growth factor, insulin-like growth factor 1, and IL-1 modulate human ezrin expression (9, 20). Our present results showed that a MEK1/2 inhibitor not only inhibited the phosphorylation of ERK1/2 but also down-regulated the expression of VIL2 mRNA and ezrin protein (Fig. 7, B and C). Based on these results, we propose a possible signal transduction pathway for transactivation of VIL2 in EC109 cells whereby extracellular stimuli activate MEK1/2 and then MEK1/2 phosphorylates ERK1/2. Subsequently, phosphorylated ERK1/2 phosphorylates Sp1 and AP-1, which bind to their respective sites in the VIL2 promoter, resulting in VIL2 transcriptional activation (Fig. 8). Perhaps, this pathway is just one route by which ezrin expression is regulated in EC109 cells, as evidenced by the fact that CpG islands and enhancer sequences exist in the VIL2 5′-flanking region and that additive cooperation between Sp1 and AP-1 for VIL2 transactivation does not coincide with ezrin expression.

REFERENCES
Regulation of VIL2 by Sp1 and AP-1

Sp1 and AP-1 Regulate Expression of the Human Gene VIL2 in Esophageal Carcinoma Cells
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doi: 10.1074/jbc.M809734200 originally published online January 21, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M809734200

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