Expression of Claudin7 Is Tightly Associated with Epithelial Structures in Synovial Sarcomas and Regulated by an Ets Family Transcription Factor, ELF3*

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Synovial sarcoma, a soft tissue sarcoma that develops in adults, is pathologically subclassified into monophasic spindle synovial sarcoma and biphasic synovial sarcoma with epithelial components. The molecular mechanism building the epithelial components in biphasic synovial sarcoma is totally unknown. Here we investigated claudins, critical molecules in the tight junction, in biphasic synovial sarcoma. Expression profiles of 21 claudins in 17 synovial sarcoma tumor samples, including 9 biphasic tumors, identified claudin4, claudin7, and claudin10 as biphasic tumor-related claudins, and immunohistochemical analyses demonstrated the localization of these claudins in the epithelial component in biphasic tumors, with claudin7 the most closely associated with the epithelial component. The mRNA expression and protein localization of claudin7 coincided with those of the ELF3, an epithelia-specific member of the Ets family of transcription factors. Luciferase reporter assays demonstrated that the presence of the Ets-binding site at −150 in the promoter region of the claudin7 gene was critical for the transcriptional activity, and gel shift and chromatin immunoprecipitation assays confirmed the binding of ELF3 to the Ets site at −150. Inhibition of ELF3 expression by small interfering RNA simultaneously down-regulated the mRNA expression of the claudin7 gene, and the introduction of ELF3 expression in claudin7-negative cell lines induced mRNA expression of the claudin7 gene. Therefore, the induction of claudin7 expression by ELF3 appears critical to the formation of the epithelial structures in biphasic synovial sarcoma.

In some rare types of malignant tumors, carcinomatous and sarcomatous elements are found simultaneously at an early stage. These tumors are classified as carcinosarcomas (1). A prototype among soft tissue sarcomas is synovial sarcoma (SS) (2, 3). SS is a malignant spindle cell sarcoma predominantly affecting the lower extremities of adolescents and young adults and accounts for 5–10% of soft tissue sarcomas (3). Although the nomenclature for this tumor has stemmed from the morphological similarity to synovial tissues (4), ultrastructural and immunohistochemical analyses have clearly excluded a synovial origin for this type of tumor (5, 6), and currently SS is classified as a miscellaneous tumor of unknown histological origin (3). Gene expression profiling of soft tissue sarcomas revealed that SS was clustered with malignant peripheral nerve sheath tumor (7), the cellular origin of which is Schwann cells, one of the descendants of neural crest cells (8). Indeed, most of the genes whose expression is up-regulated in SS are related to the neural crest (7), suggesting that the precursor cells of SS might be neural crest-derived cells.

Based on the presence or absence of an epithelial component, SS is classified into two major subtypes as follows: biphasic SS composed of distinct epithelial and spindle tumor cells, and monophasic spindle SS solely composed of spindle tumor cells (3). Epithelial cells of biphasic SS are cuboidal to tall and columnar, and are disposed in solid cords, nests, or granular structures that contain granular or homogeneous eosinophilic secretions (3). Epithelial cells are sometimes flattened bearing a resemblance to normal synovium, which may be the reason for the misnomer (4). Although its biological significance is unknown, this unique feature has been used to distinguish SS from other soft tissue sarcomas, and the expression of epithelial structure-related molecules such as cytokeratin, epithelial membrane antigen, and E-cadherin has been analyzed (9, 10).

Despite the pathological heterogeneity, almost all SSs share an oncogenic fusion gene, the SYT-SSX gene, which is created by the reciprocal chromosomal translocation t(X;18)(p11.2; q11.2), resulting in the fusion of the SYT gene on chromosome.
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18 (18q11) with either the SSX1, SSX2, or SSX4 gene on chromosome X (Xp11) (11, 12). Because of the high sensitivity and specificity, the SYT-SSX fusion gene was used as a reliable diagnostic marker for SS (13), although the precise function of SYT-SSX is still to be investigated. Data from clinical samples suggested a link between the function of the SYT-SSX protein and epithelial components; the SYT-SSX1 transcript was found in both monophasic and biphasic tumors, whereas almost all SS tumors with SYT-SSX2 were monophasic (14). The molecular mechanism of this association, however, is not known.

The formation of epithelial structures requires tight cell-to-cell adhesion, which is produced by three components, the tight junction (TJ), adherens junction, and desmosome (15). Adherens junctions mechanically link adjacent cells, in which cadherins and nectins play crucial roles (16, 17). The TJ is formed at the most apical side, where a set of continuous, anastomosing intramembranous particle strands exists (TJ strands) (18). Each TJ strand within a plasma membrane associates laterally with another TJ strand in the opposing membrane of an adjacent cell to form a paired TJ strand, making a tight seal separating luminal and basal sides of epithelial tissues (18). Three integral membrane proteins have been found as components of TJs as follows: occludin (19), claudin (CLDN) (20), and junctional adhesion molecule (21), of which CLDN plays a central role. CLDN has four transmembrane domains and short amino- and carboxyl-terminal cytoplasmic domains (22, 23). CLDN4, -7, and -10 as biphasic SS-related CLDNs, among which CLDN7 seemed to be indispensable. In addition, we found that the expression of CLDN7 was associated with the expression of a transcription factor, ELF3. Therefore, the induction of CLDN7 expression by ELF3 is an indispensable step in the formation of epithelial components in SS.

EXPERIMENTAL PROCEDURES

Tissue Samples and Cell Lines—Primary tumor tissues from 17 SS were obtained at either biopsy or resection. Informed consent was obtained from each patient, and tumor samples were approved for analysis by the Ethics Committee of the Faculty of Medicine, Kyoto University. SS tumors were subclassified into either monophasic spindle or biphasic tumors based on the presence of epithelial structures by one pathologist (Y. Nakashima). Tumor specimens were frozen quickly and kept at −80 °C until nucleic acid was extracted. Six human SS cell lines were used in this study. YaFuSS was established in our laboratory from monophasic SS (25), and HS-SY-II was provided from H. Sonobe (Kochi University, Japan) (26), SYO-1 from A. Kawai (National Cancer Center, Japan) (27), Fuji from S. Tanaka (Hokkaido University, Japan) (28), 1273/99 from O. Larsson (Karolinska Institute, Sweden), and OUSS from N. Hashimoto (Osaka University, Japan). Among the control cell lines, NMS-2 (a malignant peripheral nerve sheath tumor) was provided by A. Ogose (Niigata University, Japan) (29), and Saos2 (osteosarcoma), HT1080 (fibrosarcoma), COLO205 (colon cancer), SW480 (colon cancer), HCT15 (colon cancer), and NT2/D1 (embryonal carcinoma) cells were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained either in RPMI 1640 medium (Sigma) or Dulbecco’s modified Eagle’s medium (Sigma) with 10% fetal bovine serum (HyClone, Logan, UT).

Reverse Transcription (RT)-PCR—RNA was isolated using Sepazol reagent (Nacalai Tesque, Kyoto, Japan) from frozen tumor tissues and cell lines. After the treatment with DNase I (Nippon Gene, Osaka, Japan), 1 μg of total RNA was reverse-transcribed for single-stranded cDNA using an oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen). PCR was done using 1 μl of the RT product in a final volume of 25 μl containing 20 pmol each of the sense and antisense primers, 2.5 mmol/liter of MgCl₂, 0.2 mmol/liter of each deoxynucleotide triphosphate, and 1 unit of Taq polymerase (Toyobo, Osaka, Japan). All PCRs were done using GeneAmp PCR System 9700 (PerkinElmer Life Sciences). Information on the primers is available upon request.

Immunohistochemistry—Slides of paraffin-embedded SS surgical specimens were processed for antigen retrieval with the boiling method (boiling for 10 min followed by cooling at room temperature for 20 min) and incubated with antibodies against CLDN4 (Abcam, Cambridge, UK) at a concentration of 1:200, CLDN7 (Zymed Laboratories Inc.) at 1:500, CLDN10 (Abcam) at 1:100, or ELF3 (Novus Biologicals, Littleton, CO) at 1:750. Subsequently, mouse EnVision™ polymer reagent (Dako, Glostrup, Denmark) for CLDN7 or rabbit EnVision™ polymer reagent (Dako) for CLDN4, CLDN10, and ELF3 was added and visualized with peroxidase substrate (3,3′-diaminobenzidine tetrahydrochloride). Slides were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted in Entellan® (Merck).

Western Blotting—Whole cell lysates were prepared from each cell line, separated by SDS-PAGE using a 10% SDS-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 5% skim milk, the membrane was probed with anti-CLDN7 antibody at 1:500 or with anti-ESE1 antibody at 1:500 overnight at 4 °C and then probed with peroxidase-conjugated anti-mouse (for CLDN7) or anti-rabbit (for ELF3) immunoglobulin at 1:2000, respectively, for 1 h. Immunoreactive bands were visualized with Enhanced Chemiluminescence Plus (Amersham Biosciences).

Luciferase Assay—Information on the 5′ putative regulatory region of the CLDN7 gene was obtained from the GenBank™ database (accession number NT010718), and a search for putative binding sites of transcription factors was made with TFSEARCH. DNA fragments of different size were amplified by PCR using sense primers with an XhoI site, antisense primers with a HindIII site, and KOD plus DNA polymerase (TOYOBO, Osaka, Japan), digested by XhoI and HindIII (CLDN7), and cloned into a luciferase reporter plasmid vector,
PGV-B (Toyo Ink, Tokyo, Japan). CLDN7(−1136 mutated) having a mutation at the ETS site (−150) (TTCCG→TTAAG) was generated by a site-directed mutagenesis. Briefly, using CLDN7(−1136)-PGV-B as template DNA, PCR was performed with a sense and antisense primer containing the mutation (−150 site, TTCCG→TTAAG) using KOD plus DNA polymerase (TOYOBO). Amplified DNA was digested by DpnI (TOYOBO) and transformed to competent high DH5α (TOYOBO). Amplified DNA was digested by DpnI and incubating for 1 h at room temperature. Then the nuclear extract (5 μg) was generated by a site-directed mutagenesis. Briefly, using double-stranded oligonucleotides (OND) corresponding to the wild-type sequence from −159 to −138 (ETS-WT) were synthesized by annealing two single ONDs (5′-GGCCACGATTCGGGTTCAGGT-3′ and 5′-ACCTG-AACCGGAGTCTGGCC-3′). A fragment mutant with a mutated Ets site at −150 (ETS-MUT) was created by annealing two single ONDs (5′-GGCCACGATTCGGGTTCAGGT-3′ and 5′-ACCTGAAACATGGTCCAGG-3′). Each single OND was treated with the biotin 3′ end labeling kit (Pierce) and annealed with the corresponding OND by mixing together and incubating for 1 h at room temperature. Then the nuclear extract (5 μg) of SW480 was incubated with OND for 20 min at room temperature. The mixtures were electrophoresed in 5% polyacrylamide with 2.5% gelatin in 0.5% Tris borate EDTA at 85 V for 1.5 h and transferred to a nylon membrane. The biotin-labeled OND was detected with a LightShift chemiluminescent system (Promega, Madison, WI). Firefly luciferase activity and SeaPansy luciferase activity were measured as relative light units with a luminometer (STRATEC Biomedical Systems, Birkenfeld, Germany). The firefly luciferase activity was normalized for transfection efficiency based on the SeaPansy luciferase activity. Each experiment was performed in duplicate.

Electrophoresis Mobility Shift Assay—Nuclear extracts of SW480 were prepared with the NE-PER nuclear extraction reagent (Pierce). Double-stranded oligonucleotides (OND) corresponding to the wild-type sequence from −159 to −138 (ETS-WT) were synthesized by annealing two single ONDs (5′-GGCCACGATTCGGGTTCAGGT-3′ and 5′-ACCTG-GACCTGAGTATATGTA-3′). A fragment mutant with a mutated Ets site at −150 (ETS-MUT) was created by annealing two single ONDs (5′-GGCCACGATTCGGGTTCAGGT-3′ and 5′-ACCTGAAACATGGTCCAGG-3′). Each single OND was biotinylated by the biotin 3′ end labeling kit (Pierce) and annealed with the corresponding OND by mixing together and incubating for 1 h at room temperature. Then the nuclear extract (5 μg) of SW480 was incubated with OND for 20 min at room temperature. The mixtures were electrophoresed in 5% polyacrylamide with 2.5% glycerol in 0.5% Tris borate EDTA at 85 V for 1.5 h and transferred to a nylon membrane. The biotin-labeled OND was detected with a LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce). For the competition assay, the OND-protein complex was produced in the same way in the presence of given amounts of unlabeled OND. In the supershift assay, nuclear extracts were incubated with 3 μg of anti-ELF3 antibody and labeled OND overnight at 4 °C.

Chromatin Immunoprecipitation (ChIP) Assay—Cells were harvested and mixed with formaldehyde at a final concentration of 1.0% for 15 min at 37 °C to cross-link protein to DNA. Cells were suspended in 0.2 ml of SDS lysis buffer and settled on ice for 10 min. DNA cross-linked with protein was sonicated into fragments of 200–1000 bp. One-tenth of the sample was set aside as an input control, and the rest was pre-cleaned with salmon sperm DNA protein A-Sepharose beads (Upstate Biotechnology, Inc., Lake Placid, NY) for 30 min with agitation. The soluble DNA fraction was mixed with anti-ELF3 antibody at 4 °C overnight with rotation. Immune complexes were then collected with salmon sperm DNA protein A-Sepharose beads, and washed with the manufacturer’s low salt, high salt, LiCl buffers and then twice with TE buffer (10 mM Tris-Cl and 1 mM EDTA). The chromatin-antibody complexes were eluted with elution buffer (1% SDS and 0.1 M NaHCO3). Protein DNA cross-links were reversed with 5 M NaCl at 65 °C for 4 h. After proteinase K treatment and phenol/chloroform extraction, DNA was precipitated in ethanol and used as template for PCR amplification using primers specific for the CLDN7 promoter (sense, 5′-GTCAAAAACCGGCAAGCGAA-3′, −237 to −218; antisense, 5′-GGCACCTGAGTATATGTA-GGG-3′, −40 to −20). The reaction was performed with an initial denaturation of 5 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 56 °C, and 1 min at 72 °C with a final extension at 72 °C for 7 min.

RNA Interference and Quantitative RT-PCR—Small interfering RNAs (siRNAs) for the ELF3 gene were designed and produced by HP Guaranteed siRNA system (Qiagen). Transient transfections of SW480 cells (1 × 105) with siRNAs (100 pmol) were performed using Lipofectamine 2000 (Invitrogen). The knockdown effect of siRNA on the expression of ELF3 and CLDN7 was assessed with the 7300 real time PCR system (Applied Biosystems, Foster City, CA). PCR was done using 2 μl of RT product diluted 10-fold in a final volume of 25 μl containing 12.5 μl of 2× Power SYBR Green PCR Master Mix (Applied Biosystems). Primers were the same as those used in the standard RT-PCR, and the β-actin gene was used as the internal control. All of the reactions were run in triplicate. The ratio of ELF3 or CLDN7/β-actin in each sample was calculated, and the expression level was demonstrated as a relative value using the ELF3 or CLDN7/β-actin ratio in samples without treatment as a standard (1.0).

RESULTS

Expression of CLDN Genes in SS Tumors—The mRNA expression of 21 CLDN genes in SS tumor tissue samples was analyzed by conventional RT-PCR (Fig. 1A). The expression profiles of CLDN genes in SS differed considerably among tumors. Some tumors such as SS188 and SS259 expressed only one of the 21 genes, whereas the clear expression of nine CLDNs, among which the expression of CLDN7 was most specific in biphasic SS. All nine biphasic tumors. Some tumors such as SS188 and SS259 expressed only one of the 21 genes, whereas the clear expression of nine CLDNs, among which the expression of CLDN7 was most specific in biphasic SS. All nine biphasic profiles of CLDN7 were found with high frequency, among which the expression of CLDN12 was detected also in monophasic tumors (8:8). Therefore, we considered the remaining three CLDNs, CLDN4, -7, and -10, to be biphasic phenotype-related CLDNs, among which CLDN7 was most specific in biphasic SS. All nine biphasic tumors were positive, but only a faint signal was detected in one of eight monophasic tumors. These results indicated the strong association of CLDN7 with the epithelial structures in SS.

Expression of CLDN Genes in Mesenchymal and Epithelial Tumor Cell Lines—The expression of the 21 CLDN genes was analyzed in cell lines of various histological origin, including six
SS cell lines (Fig. 1B). Carcinoma cell lines (COLO205, SW480, SKGIIIa, and HeLa) expressed multiple CLDN genes, including the CLDN4 and CLDN7 genes. Fibrosarcoma (HT1080) and osteosarcoma (Saos2) cell lines expressed only the CLDN12 gene. A cell line derived from malignant peripheral nerve sheath tumor (NMS-2) expressed many CLDN genes as well as a cell line of embryonal carcinoma (NT2/D1). Among SS cell lines, only 1273/99 expressed multiple CLDN genes, including the CLDN7 and -10 genes, whereas other SS cell lines expressed only the CLDN11 and -12 genes. It is interesting that the expression of the CLDN11 gene was detected only in two of 17 SS tumor tissues, whereas all six cell lines expressed the CLDN11 gene in vitro.

Localization of CLDN4, -7, and -10 in SS Tumor Tissues—To analyze the localization of CLDN4, -7, and -10 proteins, immunohistochemical staining using the antibody for each CLDN was performed in typical biphasic and monophasic SS tumors (Fig. 2).

Biphasic SS Tumors—SS334 showed a single columnar epithelial structure surrounded by spindle cells (Fig. 2, a–c). The expression of CLDN4 was found mainly in epithelial cells but also in some spindle cells (Fig. 2a). In contrast, the expression of CLDN7 was intense in epithelial cells but barely detectable in spindle cells (Fig. 2b). The signal of CLDN10 was also strongly detected in epithelial cells, although some spindle cells also expressed CLDN10 (Fig. 2c). KS822 showed a single flattened epithelial structure (Fig. 2, d–f). As in the case of KS334, localization of CLDN4, -7, and -10 in SS Tumor Tissues—To analyze the localization of CLDN4, -7, and -10 proteins, immunohistochemical staining using the antibody for each CLDN was performed in typical biphasic and monophasic SS tumors (Fig. 2).

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CLDN4 was faintly detected in some epithelial cells (Fig. 2d), and the signal of CLDN7 (Fig. 2e) and -10 (Fig. 2f) was strongly and consistently detected in epithelial cells.

Monophasic SS Tumors—No signal from CLDN4, -7, or -10 was detected in KS334 (monophasic SS with SYT-SSX1) (Fig. 2, g–i) and KS646 (monophasic SS with SYT-SSX2) (Fig. 2, j–l). These results indicated that CLDN4, -7, and -10 were localized to epithelial cells in biphasic SS tumors, with the expression of CLDN7 highly specific to these cells.

Association of CLND7 with ELF3—ELF3 (E74-like factor 3) is a member of the Ets family of transcription factors, whose expression is restricted to epithelial cells, and known to be involved in the regulation of epithelium-specific gene products such as keratins (30, 31). Gene expression profiles of SS identified the ELF3 gene as one of the biphasic SS-specific genes (32), suggesting the involvement of ELF3 in the regulation of epithelial structure-related genes such as CLDN genes. First, the mRNA expression of the ELF3 gene along with other members of the ELF family (ELF1, -2, -4, and -5 genes) in SS tumor tissues and cell lines was investigated (Fig. 3). Consistent with previous reports, the mRNA expression of the ELF3 gene was observed in all biphasic tumors (Fig. 3A) and associated with the expression of the CLDN7 gene. Interestingly, this association was also observed in a monophasic tumor expressing the CLDN7 gene (SS720). The expression of the ELF1 genes was detected in most tumors, and some SS tumors expressed the ELF2, ELF4, or ELF5 genes, but there was no clear relationship between the expression of any of these ELF3 genes and histological classification or the expression of the CLDN7 gene. Identical association was also observed in the analyses of cell lines (Fig. 3B). In contrast with the data in tumor tissues, the expression of the ELF4 gene was observed constantly in all cell lines. As in the case of tumor tissues, a complete association was observed between the expression of the ELF3 gene and that of the CLND7 gene but not the CLDN4 or CDN10 gene.

To further investigate the association of ELF3 with CLDN7, the localization of ELF3 was analyzed by immunohistochemistry. In biphasic SS (SS334 and SS822), the staining of ELF3 protein was observed in the nucleus of epithelial cells as well as some apparently nonepithelial cells, although purely spindle cells were negative for the staining (Fig. 3C, a and c). Compared with the staining of CLDN7 (Fig. 3C, b and d), there seemed to be three types of cells as follows: epithelial cells expressing both ELF3 and CLDN7 (indicated by a closed arrowhead), spindle cells negative for both proteins (indicated by an open arrowhead), and cells with an intermediate morphology expressing ELF3 but not CLDN7 (indicated by an arrow). These results suggested that ELF3 was necessary, but itself not enough, for the expression of the CLDN7 gene. No signal from ELF3 was detected in CLDN7-negative monophasic tumors (data not shown).

Identification of the Transcriptional Regulatory Element of the CLND7 Gene—To investigate the possible role of ELF3 in the transcriptional regulation of the CLND7 gene, the transcriptional regulatory element of the CLND7 gene was investigated using the luciferase reporter assay. Computer analyses in the 5′ putative regulatory region of the CLND7 locus revealed that the region from -1136 to +428 (ATG site) was CpG-rich and contained several putative binding sites of known transcription factors, including an Ets site at -150 (Fig. 4A). From this region, seven DNA fragments of different lengths were cloned into the PGV vector, and the transcriptional activity of each fragment was analyzed using SW480 expressing the endogenous CLDN7 gene (Fig. 4B). The longest fragment showed the strongest activity, and the shorter fragments showed less activity, but the decrease was not remarkable until...
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The fragment lost the region between −201 and −144, suggesting that the loss of the Ets site at −150 influenced the activity (Fig. 4B).

To investigate the role of the Ets site at −150, mutant fragments with modified sequences at −150 were created, and the transcriptional activity was analyzed by conducting a co-transfection assay using the ELF3 expression vector, pCMV-ELF3 (Fig. 5). When pCMV-ELF3 was co-transfected into SW480 with the luciferase reporter vector containing the longest wild-type fragment with wild-type Ets site at −150 (Fig. 5A, b), the transcriptional activity was enhanced remarkably compared with that observed on co-transfection with empty vector (pCMV) (Fig. 5A, a). In contrast, the basal level of activity of the mutant fragment (Fig. 5A, c) was lower than that of wild-type fragment (Fig. 5A, a), and no enhancement by pCMV-ELF3 was observed (Fig. 5A, d). Similar results were obtained when the shorter fragment containing the Ets site at −150 was used. The basal transcriptional activity of the wild-type fragment (Fig. 5A, e) was almost identical with that of the longest wild-type fragment (Fig. 5A, a), and pCMV-ELF3 enhanced the activity (Fig. 5A, f), whereas the mutant fragment showed weak activity (Fig. 5A, g) equal to that of the shorter fragment (−144) lacking the −150 site (Fig. 5A, i), and no enhancement by pCMV-ELF3 was observed (Fig. 5A, h). Although the level of basal activity was much lower, a similar result was obtained when HT1080 was used instead of SW480, which lacked the expression of endogenous ELF3 and CLDN7 genes (Fig. 5B). These findings strongly suggested that the ELF3 was a positive transcription factor promoting the expression of the CLDN7 gene by binding to an Ets site at −150.

Binding of ELF3 to the Ets Site at −150—The binding of ELF3 protein to the Ets site at −150 was directly analyzed by electrophoresis mobility shift assay (Fig. 6). A 22-bp OND corresponding to −150 to −138 (ETS-WT) was synthesized, end-labeled, and mixed with nuclear extracts prepared from SW480. Electrophoresis mobility shift assay showed that ETS-WT bound to nuclear protein (Fig. 6A, lane 2), and the binding was inhibited by competition with unlabeled ETS-WT in a dose-dependent manner (Fig. 6A, lanes 4–7). On the other hand, OND with a mutated Ets site at −150 (ETS-MUT) failed to form a DNA-protein complex (Fig. 6A, lane 3), and the binding of ETS-WT with nuclear protein was not inhibited by competition with unlabeled ETS-MUT complex even at 200 times the amount (Fig. 6A, lane 8), suggesting the importance of the Ets site at −150 for the binding. When the antibody for ELF3 was added to the ETS-WT/protein mixture, the intensity of the original bands (arrowhead) decreased and a supershifted band (arrow) appeared (Fig. 6B, lane 3), which was not observed when nonimmune IgG was used instead of anti-ELF3 antibody (Fig. 6B, lane 4). These results suggested that ELF3 bound to the Ets site at −150.

To confirm the binding of ELF3 to the promoter region containing the Ets site at −150 in vivo, ChIP assay was performed (Fig. 6C). In SW480 expressing the CLDN7 gene, a DNA fragment of an expected size (217 bp) was amplified using anti-ELF3 antibody, whereas no PCR fragment was observed when the CLDN7-negative cell line, HT1080, was used instead of SW480. These results further confirmed the binding of ELF3 to the Ets site at −150.

Regulation of Endogenous Expression of the CLDN7 Gene by ELF3—To investigate the regulatory role of ELF3 in the expression of the endogenous CLDN7 gene, the expression of ELF3 was inhibited by siRNAs. Four different double-stranded siRNAs for the ELF3 gene were created and transfected into SW480 one by one. The level of down-regulation of the ELF3 gene differed

FIGURE 4. Transcriptional activity of the 5′-flanking region of the CLDN7 gene. A, a genetic information on the putative regulatory region of the CLDN7 locus. Upper scheme indicates the position of CpG dinucleotides (black bar) in the region from −3000 to +428 of the CLDN7 gene. Lower scheme shows the location of putative binding sites of transcriptional regulators, including the Ets site at −1136 and +428. The location of the E-box (at −350, −142, −27, and −16) is indicated by a black box. B, transcriptional activity of fragments cloned from the 5′-flanking region of the CLDN7 gene. Numbers in parentheses indicate the position of the 5′ end of the fragment relative to the transcription start site, with the 3′ end of the fragment being +428 in all cases. Results were demonstrated as the fold increase by setting the level of luciferase (Luc) activity in the experiment using PGV-8 and each construct as 1.0.
Finally, we analyzed whether the forced expression of the ELF3 gene into CLDN7-negative cells can induce the expression of the endogenous CLDN7 gene. pCMV-ELF3 was transfected into HT1080 and SYO-1, and RNA was extracted 48 h after transfection. In both cell lines, a weak but clear induction of CLDN7 gene expression was observed (Fig. 8A). No induction of the CLDN4 or -10 gene was detected, suggesting the specific induction of the CLDN7 gene by ELF3 (Fig. 8A). At the protein level, however, no induction of CLDN7 was observed in either cell line (Fig. 8B).

**DISCUSSION**

We have identified CLDN4, -7, and -10 as major constituents of the epithelial structures in biphasic SS. CLDN4 was initially cloned as a receptor for Clostridium perfringens enterotoxin (CPETR1) (33) and later identified as a member of the CLDN family by a data base search using homology with the CLDN1 and -2 genes (34). CLDN4 is selectively removed with treatment of the COOH-terminal half of C. perfringens enterotoxin (35), which destroys the permeability of body fluid and therefore causes diarrhea. CLDN7 was also identified by a data base search as a member of the CLDN family (34). Forced expression of the CLDN7 gene up-regulated the expression of the prostate-specific antigen gene in a prostate-specific antigen-positive prostate cell line, suggesting a role for CLDN7 in the regulation of the gene (36). CLDN10 was formally deposited in GenBankTM as oligodendrocyte-specific protein (OSP)-like protein (OSP-L) homologous to OSP, which was isolated as the product of a gene with high and specific expression in myelinating oligodendrocytes (37) and later found to be a member of the CLDN family (34), although only limited information was available. Phylogenetic analysis showed no similarity among these CLDNs (24), but the three were expressed in several of the same tissues such as pancreas, prostate, kidney, and lung, with a high level of expression in the kidney common to all three.

In kidney, these three CLDNs were expressed in renal tubules (38) as follows: CLDN4 in the ascending limb of Henle and collecting duct; CLDN7 in the distal tubules, collecting duct, and thick ascending limb of Henle; and CLDN10 in the proximal tubules and thick ascending limb of Henle. During develop-
Development, renal tubules are formed by mesenchymal cells of the intermediate mesoderm (39). Some of these cells become epithelial cells via a process known as mesenchymal-epithelial transition (39), but the rest remain mesenchymal creating stromal tissues to support the epithelial structure, which in histological features may resemble biphasic SS. It is worth analyzing “epithelial inducers” such as Wnt-4 or PAX3 during formation of the renal tubules in biphasic SS (40, 41).

Among the three CLDNs commonly expressed in biphasic SS, CLDN7 associated most tightly with the formation of epithelial structures and therefore may be an indispensable CLDN. We have demonstrated that ELF3 was a main inducer of the CLDN7 gene. ELF3, also called ESE-1 (epithelium-specific ets-1) (42), ESX (epithelium-restricted with serine box) (43), Jen (44), or ERT (ets-related transcription factor) (45), is a member of the Ets family of transcription factors, which has nearly 40 members, and binds to the consensus Ets-binding sequence, \((\text{C/A})\text{GGA (A/T)}\) (46), which lies in the −150 region of the CLDN7 promoter. ELF3, unlike most other members of the Ets family, is not expressed in hematopoietic cells and organs and is specifically expressed in cell lines of epithelial origin and in organs such as the lung, stomach, intestine, and kidney that have specialized epithelial cells (31). ELF3 regulates the transcription of several epithelium-specific genes such as the keratin gene (30). About 30% of Elf3-deficient mice died at around embryonic day 11.5. The other 70% were born with severe alterations of tissue architecture in the small intestine, manifested by the poor formation of villi and abnormal morphogenesis and mucus-secreting goblet cells (47).

ELF3 was identified as a biphasic SS-specific gene by a microarray analysis (32), and the result was confirmed by a quantitative RT-PCR analysis (48). In this study, we also confirmed the association of ELF3 with biphasic SS in mRNA analyses by RT-PCR. mRNA expression patterns of other members of the ELF family (ELF1, ELF2, ELF4, and ELF5) were not associated with the subtype of SS (Fig. 3, A and B), further confirming the specific association of ELF3 with biphasic SS, and our data indicated that CLDN7 was a factor connecting ELF3 with biphasic SS. The immunohistochemical staining of ELF3 and CLDN7, however, showed that the association between the two was not simple. As expected, cells expressing both ELF3 and CLDN7 were mostly in the epithelial component, and purely spindle cells were negative for the two proteins. In between these two types of cells, however, there were some nonepithelial cells expressing ELF3 but not CLDN7, indicating that the expression of ELF3 is not enough to induce the expression of the CLDN7 gene. This may be related to our failure to induce the CLDN7 protein expression by exog-

![FIGURE 6. Binding of ELF3 to the core promoter region of the CLDN7 gene in vitro and in vivo.](image)

A, specificity of the binding in vitro. A 22-bp biotin-labeled double-stranded DNA fragment corresponding to the region from −159 to −138 (ETS-WT) or mutant fragment from the same region with a mutated Ets site at −150 (ETS-MUT) was incubated with nuclear extract of SW480, and the DNA-protein complex was detected by chemiluminescence. For the competition assay, unlabeled ETS-WT with the indicated amount (up to 200-fold) (lanes 4–7) or 200-fold the amount of unlabeled ETS-MUT was added to the mixture. B, identification of ELF3 as the protein binding to ETS-WT. The DNA-protein complex was preincubated with anti-ELF3 antibody (lane 3) or nonimmune IgG (lane 4). C, ELF3 binds to the core promoter region of the CLDN7 gene in vivo. The DNA-protein complex was formed through the treatment of SW480 (endogenous CLDN7-positive) or HT1080 (endogenous CLDN7-negative) with formaldehyde and subjected to a ChIP assay as described under “Experimental Procedures.” PCR for the core promoter region of the CLDN7 gene was performed using DNA extracted from the DNA-protein complex immunoprecipitated using no antibody (No Ab), nonimmune IgG, or anti-ELF3 antibody.
interfere with the binding of ELF3 to the Ets site at therefore the expression of the gene. We are currently investigating this issue in detail using siRNA for the Snail gene. It is also important to investigate the pathophysiological effects of CLDN7 expression induced by ELF3 in SS cells. The histopathological findings of tumors produced by SS cell lines expressing the exogenous ELF3 gene may provide the information related to this issue. Finally, our findings may provide a clue as to the role of the SYT-SSX fusion protein in the formation of epithelial structures. Because SYT-SSX2 was negatively associated with biphasic SS (14) and ELF3 was a main inducer of the expression of the CLDN7 gene, it is worth investigating whether SYT-SSX2 regulates the expression and/or function of ELF3. The reporter system used in this study will be suitable material to analyze whether SYT-SSX protein can inhibit the function of ELF3 in SS cells.

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