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Transcriptional activation of the human *claudin-18* gene promoter through two AP-1 motifs in PMA-stimulated MKN45 gastric cancer cells

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Yano K, Imaeda T, Niimi T. Transcriptional activation of the human *claudin-18* gene promoter through two AP-1 motifs in PMA-stimulated MKN45 gastric cancer cells. *Am J Physiol Gastrointest Liver Physiol* 294: G336–G343, 2008. First published November 21, 2007; doi:10.1152/ajpgi.00328.2007.—Claudin-18 (*CLDN18*), a member of the claudin family of proteins that are structural components of tight junctions, has two alternatively spliced variants, claudin-18a1 and claudin-18a2, which are highly expressed in lung and stomach, respectively. Downregulation of claudin-18a2 is associated with gastric cancers of an intestinal phenotype; however, the mechanisms regulating its expression have not been defined. Here, we found that phorbol 12-myristate 13-acetate (PMA) treatment of MKN45 human gastric cancer cell line increased claudin-18a2 expression. In addition, this study aimed to characterize the human *CLDN18a2* promoter. Using reporter gene assays and deletion analysis, we mapped the critical promoter region of the PMA-stimulated claudin-18a2 expression to the −923/−286 region. Electrophoretic mobility shift assays and mutational analyses revealed that two activator protein (AP)-1 binding sites played an important role in the expression of claudin-18a2 in PMA-stimulated MKN45 cells. Protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) inhibitors suppressed the upregulation of claudin-18a2. These results indicate that the PKC/MAPK/AP-1 dependent pathway regulates claudin-18a2 expression in gastric cells.

activator protein-1; phorbol 12-myristate 13-acetate; stomach; tight junction

TIGHT JUNCTIONS (TJs), which are specialized membrane domains at the most apical region of polarized epithelial and endothelial cells, create a primary barrier to paracellular transport of solutes and ions (19, 30). Claudins are major integral membrane proteins of TJ strands (37, 39). These proteins can form homodimers or heterodimers to produce paired strands between adjacent cells, thereby determining the characteristic permeability properties of different epithelial tissues (9). Mutations in the *claudin* gene can lead to various genetic disorders. For example, claudin-1-deficient mice die within 1 day of birth, because of defects in the epidermal barrier (8). Mutations in the human *CLDN14* gene lead to nonsyndromic recessive deafness, and the mutated *CLDN16* gene can also cause hereditary renal hypomagnesemia with hypercalciuria (32, 42). To date, at least 23 members of the claudin gene family have been identified in mammals. However, many of these claudins have not yet been examined in detail and the functional differences are largely unknown.

We previously identified *CLDN18* (encodes claudin-18) as a downstream target gene for the NKX2.1 homeodomain transcription factor in the lung (25). We then reported that *CLDN18* has two tissue-specific isoforms, claudin-18a1 (lung isoform) and claudin-18a2 (stomach isoform). The expression of claudin-18a1 and -18a2 depends on alternative promoter usage of the unique exon 1 (1a and 1b, respectively). Both isoforms are highly expressed and may be the most prominent claudin species in their respective tissues, which suggests that they play an important role in the structure and function of TJs in the lung or stomach (3, 14). Although several tissues and cell types, including the inner ear, hair follicles, and radial glia, have been shown to express claudin-18 protein by immunohistochemical analyses, it is still unknown exactly which isoform is expressed in these tissues (12, 15, 36). Claudin-10 has also been reported to undergo a similar splicing pattern, and the resulting isoforms have different tissue distributions and physiological functions (40).

Several claudin genes have been shown to exhibit abnormal expression in human cancers (14, 23, 33). For example, claudin-1 is downregulated in various cancers but elevated in colon carcinoma and melanoma (4, 17). Claudin-3 and -4 have been described to be frequently upregulated in ovarian, breast, and pancreatic tumors (16, 21, 28). Although the exact role of claudins in cancer is still under investigation, it is believed that they could be used as targets for cancer detection, diagnosis, and therapy (14, 23, 33). Recently, claudin-18a2 was shown to be frequently downregulated in gastric cancer of an intestinal phenotype, suggesting that claudin-18a2 would be a good marker for gastric cancer (29). The mechanisms, however, that result in claudin-18a2 downregulation in gastric cancer, and even basal transcriptional regulation, remain to be shown.

In the present study, we isolated the 5′-flanking region located upstream of exon 1b of the human *CLDN18* gene (designated as the *CLDN18a2* promoter) and investigated the transcriptional regulation of the *CLDN18a2* promoter in MKN45 human gastric cancer cells. We demonstrated that phorbol 12-myristate 13-acetate (PMA) treatment increased mRNA and protein levels of claudin-18a2 in MKN45 cells. Reporter gene assays and deletion analyses indicated that two activator protein (AP)-1 sites in the 5′-flanking region are critical for *CLDN18a2* promoter activity. In addition, results show that PMA-stimulated *CLDN18a2* upregulation was mediated by protein kinase C (PKC) and mitogen-activated protein kinase (MAPK)-dependent signal transduction pathways.

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MATERIALS AND METHODS

Reagents. PMA was obtained from Sigma (St. Louis, MO). Bisindolylmaleimide I, PD-98059, ERK inhibitor II, SB-203580, and JNK inhibitor II (SP-600125) were purchased from Calbiochem (San Diego, CA). Antibodies specific to c-*jun* (H-79), c-*fos* (4), and ATF-1 (H-60) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-claudin-18 (Mid), anti-claudin-1, and anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated antibodies were purchased from Zymed Laboratories (South San Francisco, CA).

Cell culture. The human gastric cancer cell lines MKN45 (passage 24), MKN74 (passage 21), NUGC-3 (passage 37), and AZ-521 (passage 14) were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and, with the exception of AZ-521, were maintained in RPMI-1640 medium (Sigma), supplemented with 10% fetal calf serum. AZ-521 was maintained in Eagle's minimum essential medium (Sigma), supplemented with nonessential amino acids and 10% fetal calf serum. The AGS cell line was obtained from American Type Culture Collection (Rockville, MD) and cultured in Nutrient Mixture F-12K (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum.

RT-PCR. Reverse transcription of mRNA was carried out with ReverTra Ace (Toyobo, Osaka, Japan) and random primers. Single-stranded cDNA was amplified by PCR using *Taq* DNA polymerase (New England Biolabs, Ipswich, MA) under the following conditions: denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min; these steps were repeated for 30 cycles. The oligonucleotide primers were as follows: claudin-18a2, 5′-TGTGCGCCACCATGGCCGTG-3′ (forward) and 5′-GTGCTGAGAGGTCTTAGAGC-3′ (reverse); claudin-1, 5′-TTCTCGCCTTCCTGGGATGG-3′ (forward) and 5′-AGCAAAGTAGGGCACCTCCC-3′ (reverse); claudin-3, 5′-TCGGCAGCAACATCATCACG-3′ (forward) and 5′-TGGTGGCCGTGTACTTCTTC-3′ (reverse); GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-GACCACAGTCCATGCCATCAC-3′ (forward) and 5′-GTCCACCACCCTGTTGCTGTA-3′ (reverse); vimentin, 5′-TCGCCAACTACATCGACAAGGTGC-3′ (forward) and 5′-AGCCGTGAGGTCAGGCTTGGAAAC-3′ (reverse); E-cadherin, 5′-CCAGGAACCTCTGTGATGGAGGTC-3′ (forward) and 5′-GGCCTCAAAATCCAAGCCCTTTGC-3′ (reverse).

Western blot analysis. Total cell lysates were prepared in Laemmli sample buffer, separated on 12% SDS-polyacrylamide gels, and electrophoretically transferred to nitrocellulose membrane (GE Healthcare, Piscataway, NJ). The filter was blocked in PBS containing 5% skim milk and subsequently incubated for 1 h with claudin-18 and claudin-1 polyclonal antibodies (1:250) (Zymed Laboratories). The filter was washed in PBS containing 0.1% Tween 20, incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000) (GE Healthcare), and subsequently washed with the same buffer. Protein bands were detected by using an ECL Western Blotting Detection Reagent (GE Healthcare).

Immunofluorescence staining. MKN45 cells were plated on 35-mm dishes with coverslips and were treated with or without 100 nM PMA. After 24 h, cells that had adhered to the coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were then washed with 0.5% bovine serum albumin (BSA)/PBS and incubated with anti-claudin-18 antibody for 1 h at room temperature. After being washed with 0.5% BSA/PBS three times, cells were incubated with anti-rabbit IgG FITC-conjugated antibody for 1 h. Cells were washed three times with 0.5% BSA/PBS and then mounted on glass slides with Vectashield (Vector Laboratory, Burlingame, CA). Specimens were observed by use of the Axiovert 200M photomicroscope (Carl Zeiss, Oberkochen, Germany).

Plasmids. The human *CLDN18a2* promoter fragment was generated by PCR amplification, using the 461M2 human RPCI-11 bacterial artificial chromosome clone as a template. The following primers were employed: 5′-AAAACGCGTTGCTGTGCAGAAGCAAATG-3′ (forward) and 5′-AAACTCGAGCGCACAGTGTCGAGACAGAGCC-3′ (reverse). Genomic DNA fragments were then subcloned into the *Mlu* I-*Xho* I site of the pGL3-Basic vector (Promega, Madison, WI) to generate −5126/+49 plasmids. Three 5′-deletion constructs were generated in the −5126/+49 plasmid by using the endogenous restriction sites and the appropriate restriction sites in the polylinker. All DNA sequencing was carried out by use of an ABI prism dye terminator cycle sequencing kit and a model 3100 DNA sequencer (Applied Biosystems, Foster City, CA).

The nucleotide sequence for the human *CLDN18a2* promoter reported in this paper appears in the GenBank nucleotide sequence database with the accession number AB332393.

Site-directed mutagenesis of potential AP-1 binding sites was carried out in the −923/+49 plasmid using the GeneTailor Site-Directed Mutagenesis System (Invitrogen). The sequences of the mutagenic primers are as follows: distal AP-1, 5′-TACTTCCCCTATGGGCAATGCAGTATTCAC-3′ (forward) and 5′-CATTGCCCATAGGGGAAGTACAGGGTCAGG-3′ (reverse); proximal AP-1, 5′-TATAGAACGTAACTACCATGAACTAGAATC-3′ (forward) and 5′-CATGGTAGTTACGTTCTATAAAGTCACAAA-3′ (reverse). All mutations were verified by sequencing.

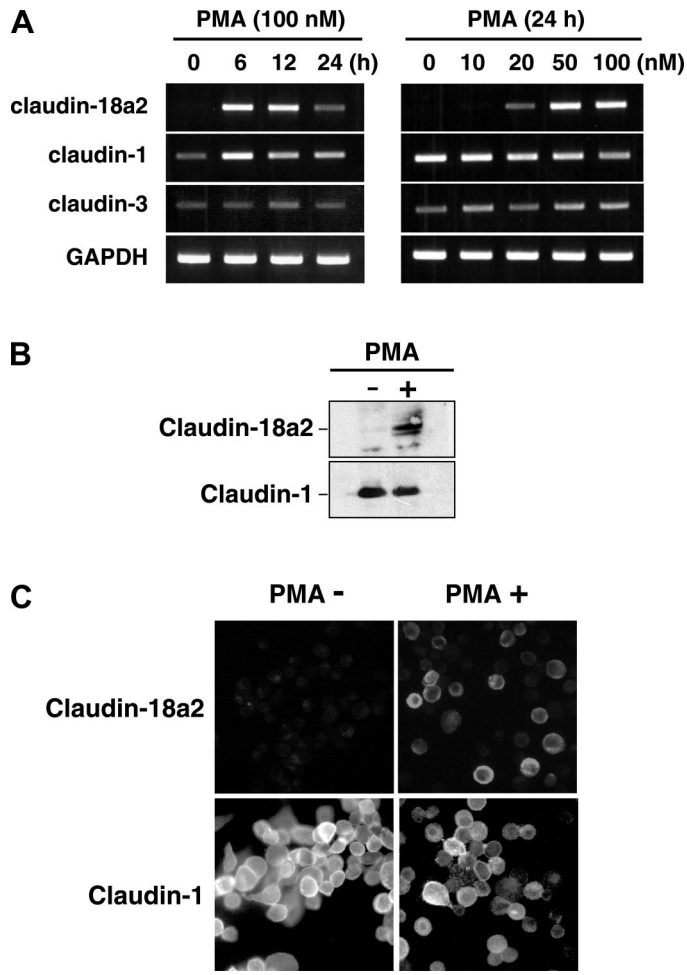

Fig. 1. Phorbol 12-myristate 13-acetate (PMA) induces claudin-18a2 mRNA and protein expression in MKN45 cells. *A*: MKN45 cells were treated with PMA for different time periods and with various concentrations, as indicated. Claudin-18a2, -1, and -3 mRNA levels were evaluated by RT-PCR analyses. GAPDH was used as internal control. *B*: Western blot analysis of claudin-18 and claudin-1 expression in MKN45 cells treated with or without PMA (100 nM for 24 h). *C*: immunofluorescent stainings with claudin-18- and claudin-1-specific antibodies in MKN45 cells treated with or without PMA (100 nM for 24 h).

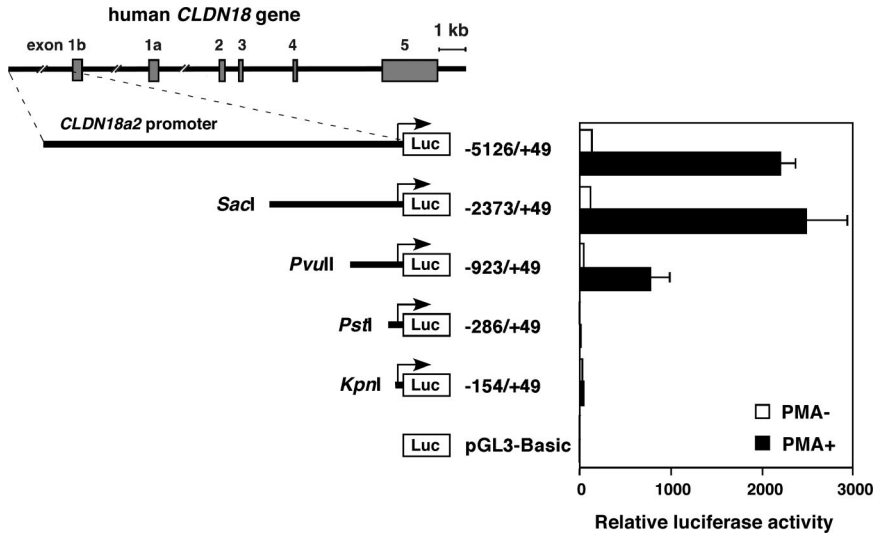

Fig. 2. Identification of a transcriptional regulatory region of the human *CLDN18a2* promoter. A series of human *CLDN18a2* promoter-reporter genes were constructed and transfected into MKN45 cells. One day after transfection, the cells were stimulated with 100 nM of PMA for 24 h and assessed for luciferase activity. The relative luciferase activities are shown as the means ± SE of 3 separate experiments (duplicate samples).

To construct an expression vector for c-*jun*, full-length mouse c-*jun* coding sequences were amplified by RT-PCR and cloned into the pcDNA3.1 (+) mammalian expression vector (Invitrogen).

RACE analysis. To determine the transcriptional initiation site for human claudin-18a2, 5′-rapid amplification of cDNA ends (5′-RACE) was performed using total RNA from the human stomach (Ambion, Austin, TX). The GeneRacer kit (Invitrogen) was used according to the manufacturer's protocol.

Transfection and reporter gene assays. Cells were grown in 24-well plates, which are 50–70% confluent, and were transfected via the FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN) with 100 ng of reporter plasmid and 5 ng of the *Renilla* luciferase expression vector phRL-null (Promega) as an internal control. In the *trans*-activation assay, 100 ng of reporter plasmid, 100 ng of expression plasmid, and 5 ng of the *Renilla* luciferase expression vector phRL-null were cotransfected. Forty-eight hours later, the cells were harvested in Passive Lysis Buffer (Promega), and the lysates were assayed for luciferase activity via the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity from various human *CLDN18a2* promoter constructs was normalized to that of *Renilla* luciferase and expressed as 1, based on activity of the pGL3-Basic plasmid. Data are expressed as the mean values of at least three experiments (duplicate samples) ± standard errors.

EMSA. Nuclear extracts of MKN45 cells were prepared as previously described (5). Single-stranded oligonucleotides were annealed at a concentration of 10 μM in annealing buffer [1 mM Tris·HCl (pH 7.5), 1 mM $MgCl_2$, and 5 mM NaCl] at 85°C for 5 min and then cooled to room temperature. Double-stranded DNA was end labeled with [α-^{32}P]-dCTP and the DNA polymerase Klenow fragment (New England Biolabs). Labeled DNA was separated from free dCTP by filtration through a ProbeQuant G-50 Micro Column (GE Healthcare).

Nuclear extracts (2 μg) and, when indicated, unlabeled oligonucleotide competitors were preincubated in 23 μl of gel mobility shift assay buffer [10 mM HEPES-KOH (pH 7.9), 50 mM KCl, 0.6 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 5 mM DTT, and 80 ng/μl poly(dI-dC) (GE Healthcare)] for 10 min on ice. An oligonucleotide probe (1×10^5 cpm) was added to the mixture and incubated for an additional 30 min at room temperature. For the antibody supershift analysis, 1 μl of antibody was added and the incubation was continued for an additional hour. DNA-protein complexes were separated from the free probe by 5% nondenaturing polyacrylamide gel electrophoresis. After electrophoresis, the gel was blotted onto Whatman No. 3MM paper, dried, and analyzed via a BAS2000 Image Analyzer (Fuji Film, Tokyo, Japan).

RESULTS

PMA induction of claudin-18a2 expression in MKN45 cells. To understand the transcriptional regulation of human claudin-18a2, expression of claudin-18a2 in various gastric cancer cell lines was first investigated, including AGS, AZ-521, MKN45, MKN74, and NUGC-3. All cell lines expressed low levels of claudin-18a2, according to RT-PCR analyses (data not shown). Therefore, claudin-18a2 mRNA levels were increased through the addition of various growth factors and reagents, such as epidermal growth factor, fibroblast growth factor-2, tumor necrosis factor-α, H_2O_2, and cAMP, all of which have been shown to be potent mitogens and activators of gastric gene expression. Subsequently, we determined that PMA treatment

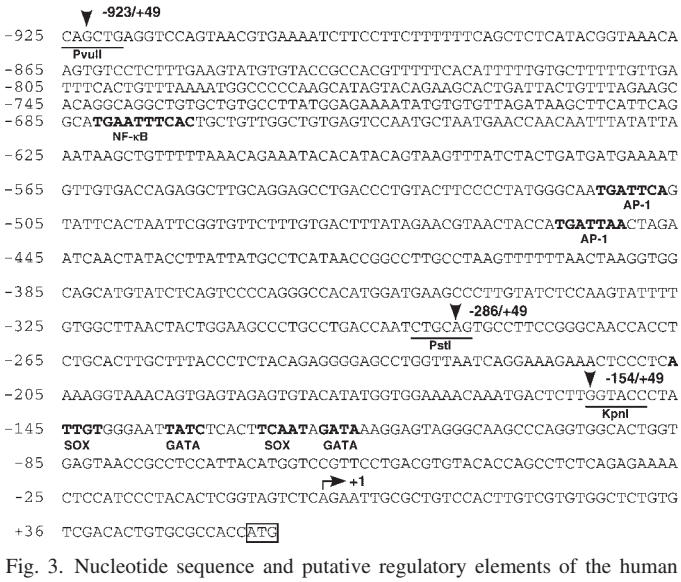

Fig. 3. Nucleotide sequence and putative regulatory elements of the human *CLDN18a2* promoter. Arrowheads indicate positions of the −923/+49, −286/+49, and −154/+49 deletion constructs. The restriction sites depicted in Fig. 2 are underlined. The bent arrow with +1 indicates the major transcription start site. The putative activator protein (AP)-1, NF-κB, SOX, and GATA transcription factor binding sites are shown in bold. The ATG initiation codon is boxed.

increased claudin-18a2 expression only in MKN45 cells; therefore, further experiments were designed to study the mechanisms regulating PMA-stimulated expression of claudin-18a2.

To determine the effects of PMA on claudin-18a2 levels in MKN45 cells, we performed RT-PCR analysis in cells that were incubated for different time periods and with varying concentrations of PMA (Fig. 1*A*). Claudin-18a2 was barely detectable in cells cultured in the absence of PMA. As shown in Fig. 1*A*, *left*, 100 nM of PMA induced a robust expression of claudin-18a2 mRNA within 6 h. When MKN45 cells were treated with different concentrations of PMA for 24 h, a slight increase in claudin-18a2 mRNA levels was detected at 20 nM, which appeared to plateau with 50 nM (Fig. 1*A*, *right*). In contrast, there was no significant induction of claudin-1 and -3 expression levels compared with claudin-18a2. These results suggest that PMA upregulated claudin-18a2 mRNA levels in a time- and dose-dependent manner.

To examine whether PMA treatment induces claudin-18a2 protein expression, Western blot analysis of cell lysates from MKN45 cells was performed. As expected, claudin-18a2 protein was barely detected in MKN45 cells; however, PMA significantly enhanced claudin-18a2 protein levels (Fig. 1*B*). The level of claudin-1 protein expression was not significantly altered. Immunofluorescent staining of MKN45 cells revealed that claudin-18a2 was expressed in almost all cell membranes from PMA-stimulated MKN45 cells: however, some expression was cytoplasmic as well (Fig. 1*C*). In addition, all cells did not uniformly expressed claudin-18a2 following PMA induction. It is unclear why all cells do not express claudin-18a2: however, this might be a result of reactivity of the anti-claudin-18 antibody or heterogeneity among the MKN45 cell population.

Identification of cis-elements that control expression of claudin-18a2 in MKN45 cells. The promoter region that controls human claudin-18a2 expression was obtained by PCR amplification, through the use of primers specific to the 5′-flanking region located upstream of exon 1b of the human *CLDN18* gene (available from public domain, locus ID: 51208, see Fig. 2). To precisely determine the transcriptional initiation site of the human claudin-18a2 transcript, 5′ -RACE analysis was performed using mRNA from human stomach tissue. Sequencing of the RACE products revealed that the majority of the transcripts were initiated from a site that was 53 bp upstream of the translation initiation codon. We have designated this site as +1.

To identify the *cis*-regulatory elements involved in the observed PMA-induction of claudin-18a2 expression, a series of reporter plasmids driven by the human *CLDN18a2* promoter of different lengths were constructed (−5126/+49, −2373/+49, −923/+49, −286/+49, and −154/+49), and transfected into MKN45 cells. The cells were subsequently stimulated with or without PMA and tested for luciferase reporter activity. When the MKN45 cells were treated with PMA, the transcriptional activity was dramatically elevated in the constructs −5126/+49 and −2373/+49, although such potentiation of transcriptional activity was not observed in the absence of PMA (Fig. 2). Deletion of up to −923 bp retained considerable transcriptional activity; however, further deletion up to −286 bp resulted in no activity. These results indicate that the 637-bp region encompassing −923 to −286 bp of the

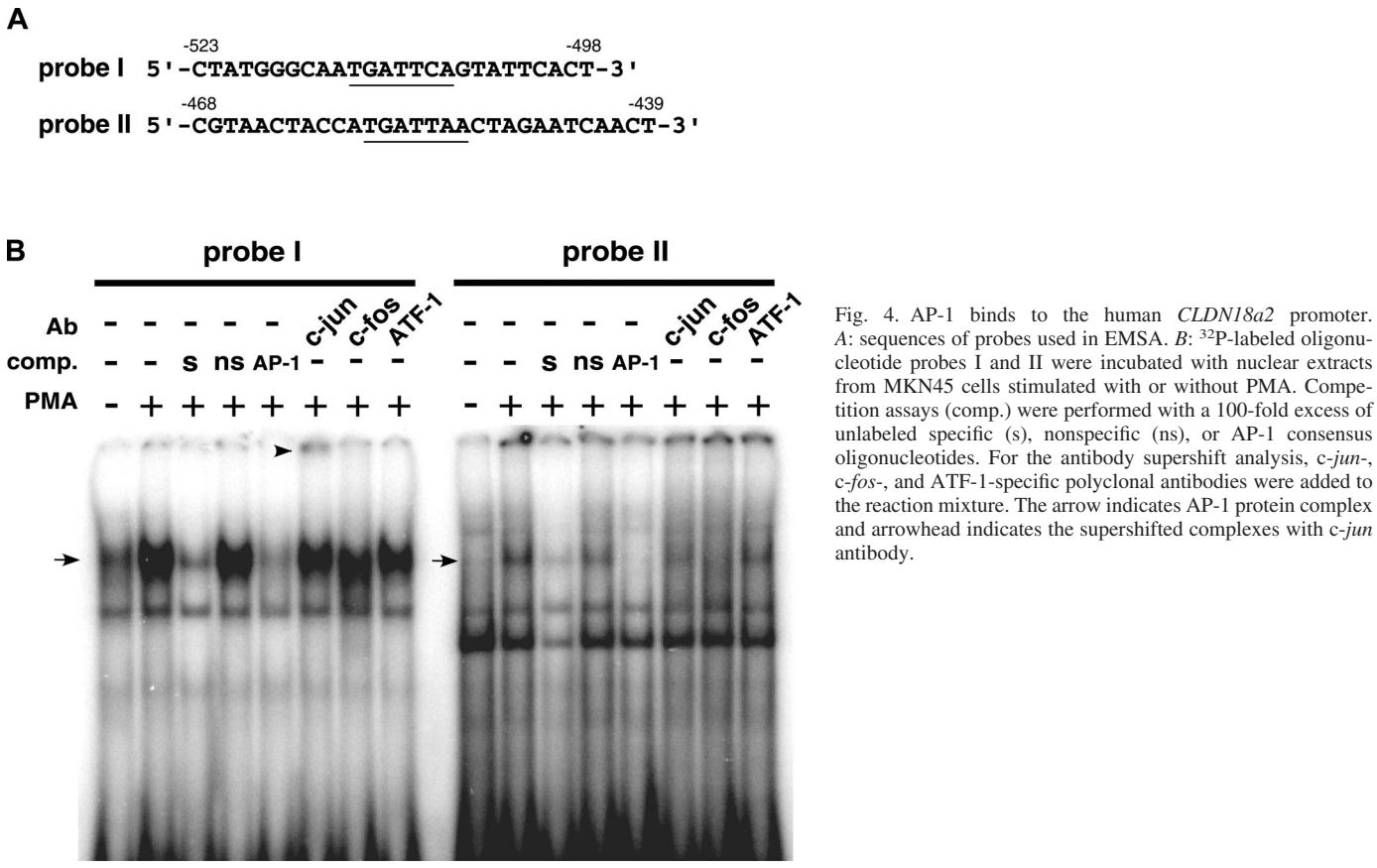

Fig. 4. AP-1 binds to the human *CLDN18a2* promoter. *A*: sequences of probes used in EMSA. *B*: ^{32}P-labeled oligonucleotide probes I and II were incubated with nuclear extracts from MKN45 cells stimulated with or without PMA. Competition assays (comp.) were performed with a 100-fold excess of unlabeled specific (s), nonspecific (ns), or AP-1 consensus oligonucleotides. For the antibody supershift analysis, c-*jun*-, c-*fos*-, and ATF-1-specific polyclonal antibodies were added to the reaction mixture. The arrow indicates AP-1 protein complex and arrowhead indicates the supershifted complexes with c-*jun* antibody.

human *CLDN18a2* promoter contained *cis*-regulatory elements that were involved in PMA responsiveness of MKN45 cells.

Characterization of AP-1 transcription factor binding to the CLDN18a2 promoter region in vitro. Nucleotide sequence analyses of the human *CLDN18a2* promoter revealed several potential binding sites for factors that are involved in the inflammatory process and immune response, including one NF-κB site and two AP-1 sites (Fig. 3). To test whether these transcription factors bind to this region, EMSA was performed with nuclear extracts, which were prepared from MKN45 cells stimulated with or without PMA. Although the probe containing the putative NF-κB binding site failed to form a protein-DNA complex (data not shown), the probes containing one of each AP-1 binding sites formed specific bands corresponding to AP-1-DNA complexes (Fig. 4). The proximal AP-1 binding site (probe II) showed relatively weak interaction, compared with the distal AP-1 binding site (probe I). Competitor oligonucleotides, which contained an authentic AP-1 binding motif (TGACTCA), abolished protein binding to both probes. Furthermore, antibodies to c-*jun* produced supershifted complexes, whereas antibodies to c-*fos* and ATF-1 did not. Therefore, it is most likely that c-*jun* forms homodimers or heterodimers with another Fos gene family protein and binds to these consensus sequences for AP-1 binding motifs.

Functional characterization of AP-1 for the CLDN18a2 promoter activity. To confirm the functional relationship between these AP-1 binding sites and the *CLDN18a2* promoter activity, mutations were introduced into the AP-1 binding sites of the −923/+49 reporter constructs (Fig. 5*A*). Disruption of the distal AP-1 binding site (mut 1) by site-directed mutagenesis eliminated most of the PMA-induced transcriptional activity. A mutation of the proximal AP-1 binding site (mut 2) showed an incomplete, yet significant, reduction of transcriptional activity. Mutations of both AP-1 binding sites (mut 3) resulted in an effect similar to mut 1. These data are consistent with the EMSA results, which demonstrated that probe I was more effective in causing a band shift than probe II.

We next examined whether exogenous expression of c-*jun* drives the *CLDN18a2* promoter activity in MKN45 cells. When the c-*jun* expression plasmid was cotransfected with the −923/+49 reporter constructs, c-*jun* increased transcriptional activity of the *CLDN18a2* promoter (Fig. 5*B*). This *trans*-activation was not observed with the −923/+49 reporter constructs containing mutations of both AP-1 binding sites (mut 3). Together, these results indicate that the synergy of the two AP-1 binding sites was critical for promoter activity of the human claudin-18a2 in PMA-stimulated MKN45 cells.

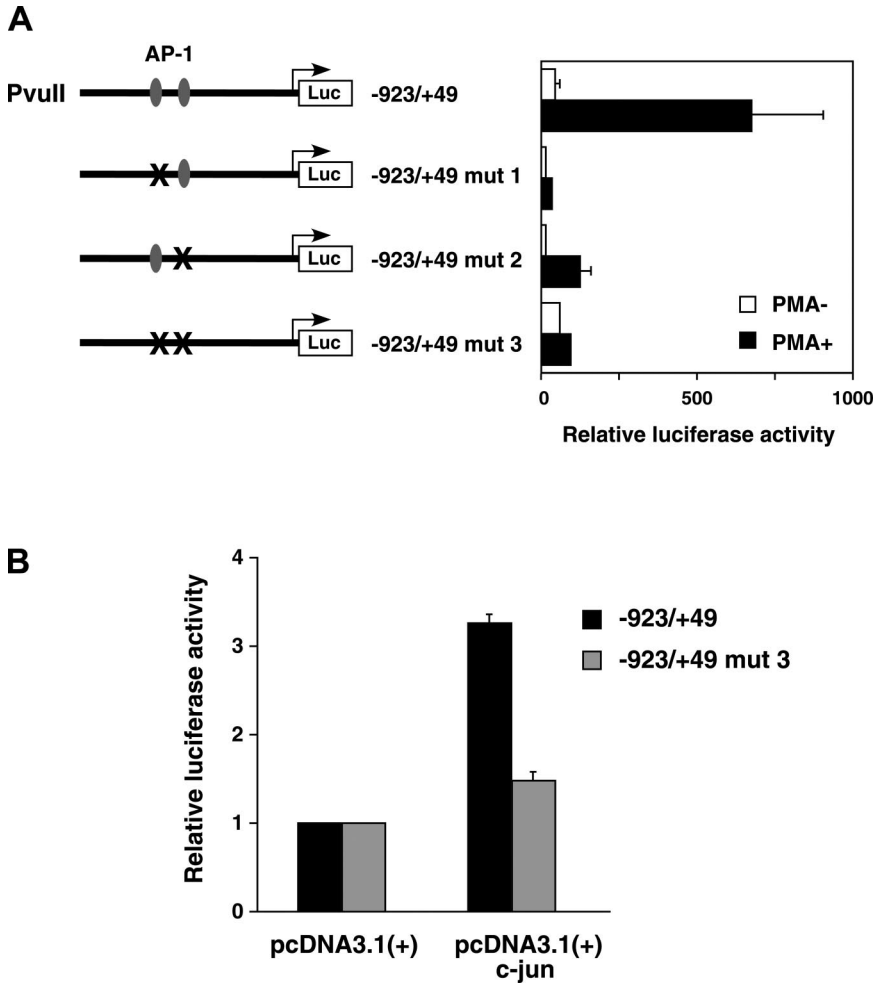

Fig. 5. Functional analysis of AP-1 binding sites on human *CLDN18a2* promoter activity. *A*: pGL3-Basic plasmids containing the wild-type *CLDN18a2* promoter (−923/+49) and the mutated promoters (mut 1 to mut 3) were transfected into MKN45 cells. One day after transfection, the cells were stimulated with 100 nM of PMA for 24 h and assayed for luciferase activity. Regions altered by site-specific mutagenesis are indicated by X. *B*: effect of c-*jun* on human *CLDN18a2* promoter activity. pGL3-Basic plasmids containing the wild-type *CLDN18a2* promoter (−923/+49) or the mutated promoters (−923/+49 mut 3), in conjunction with the c-*jun* expression plasmid, were cotransfected into MKN45 cells and assayed for luciferase activity. Relative luciferase activities are depicted as the means ± SE of 3 separate experiments (duplicate samples).

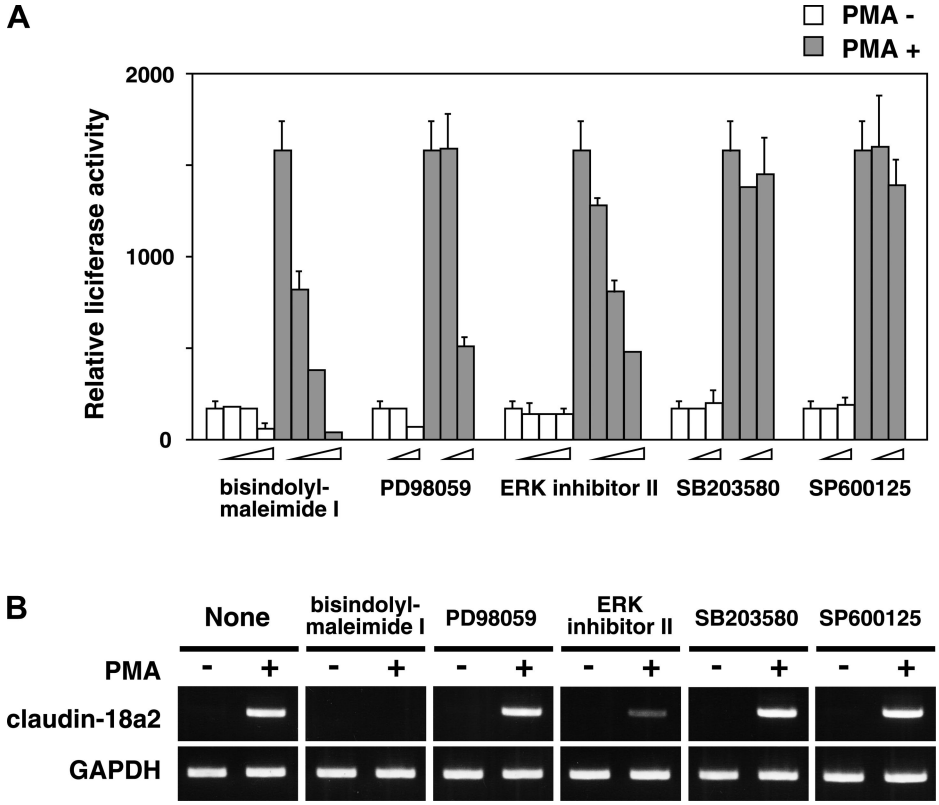

Fig. 6. PMA-induced *CLDN18a2* promoter activity is reduced by PKC and MAPK signaling pathway inhibitors. *A*: pGL3-Basic plasmids containing the *CLDN18a2* promoter (−923/+49) were transfected into MKN45 cells. One day after transfection, the cells were pretreated for 1 h with increasing concentrations of inhibitors and then treated with PMA (100 nM) for 24 h. Cells were assayed for luciferase activity. Inhibitor concentrations were as follows: bisindolylmaleimide I (0, 0.1, 1, and 10 μM); PD-98059 (0, 10, and 100 μM); ERK inhibitor II (0, 0.2, 2, and 20 μM); SB-203580 (0, 1, and 10 μM); and SP-600125 (0, 1, and 10 μM). The relative luciferase activities are depicted as the means ± SE from 3 separate experiments (duplicate samples). *B*: RT-PCR analysis of RNA from MKN45 cells pretreated with inhibitors, as above, prior to isolation of RNA. Inhibitor concentrations were as follows: bisindolylmaleimide I (10 μM); PD-98059 (100 μM); ERK inhibitor II (20 μM); SB-203580 (10 μM); and SP-600125 (10 μM).

PKC and MAPK signaling pathway mediates the PMA-stimulated activation of the CLDN18a2 promoter. PMA has been shown to activate PKC, which in turn mediates transmission of signals to MAPKs. Distinct groups of MAPKs, including extracellular signal-regulated kinase (ERK), p38, and c-Jun NH_2-terminal kinase (JNK), activate the downstream AP-1 transcription factor via phosphorylation. To understand whether these signaling pathways are involved in PMA-mediated claudin-18a2 upregulation, the effects of various chemical inhibitors on PMA-stimulated claudin-18a2 transcription were examined (Fig. 6). Treatment with the PKC inhibitor bisindolylmaleimide I (10 nM) completely abolished the PMA-stimulated *CLDN18a2* promoter activity, whereas the MAPK/ERK (MEK) inhibitor PD-98059 and ERK inhibitor II partially inhibited the activity (Fig. 6*A*). In contrast, neither the p38 kinase inhibitor (SB-203580) nor the JNK inhibitor (SP-600125) resulted in an alteration of the PMA-stimulated *CLDN18a2* promoter activity. RT-PCR analysis revealed that bisindolylmaleimide I and ERK inhibitor II caused a clear reduction in endogenous claudin-18a2 transcript levels (Fig. 6*B*). These results suggest that PMA-stimulated *CLDN18a2* promoter activity was regulated through the PKC and ERK-MAPK pathway.

PMA costimulates the mesenchymal marker vimentin and claudin-18a2 mRNA expression in MKN45 cells. A correlation between PKC activity and tumor metastasis has been previously reported in several tumor cell lines (6, 11). To determine the physiological significance of a PMA-dependent increase in claudin-18a2 expression, RT-PCR analysis was performed with primer pairs for the mesenchymal marker, vimentin, and the epithelial marker, E-cadherin. Interestingly, increased expression of vimentin, along with claudin-18a2, was observed in PMA-stimulated MKN45 cells, but not in MKN74 and NUGC-3 cells (Fig. 7). E-cadherin expression was not significantly altered in any cell lines. These results suggest that claudin-18a2 might contribute to invasion and metastasis of tumor cells in a cell-dependent manner.

DISCUSSION

It is well established that PMA activates several PKC isoenzymes, which in turn initiate a signaling cascade that regulates the expression of genes involved in cell growth and differentiation. Many studies have shown that the PKC pathway plays a key role in localization and transcription of TJ components.

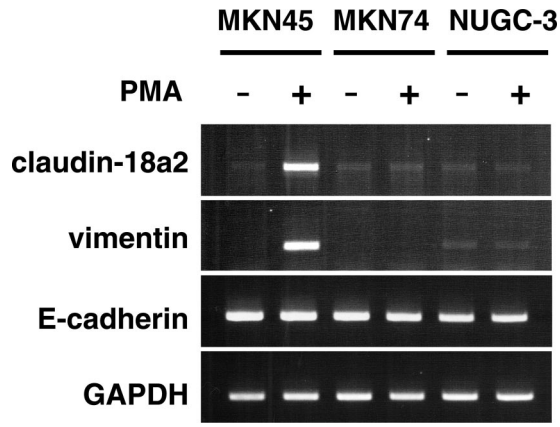

Fig. 7. PMA induces vimentin mRNA expression in MKN45 cells. MKN45, MKN74, and NUGC-3 cells were treated with 100 nM PMA for 24 h. Claudin-18a2, vimentin, and E-cadherin mRNA levels were evaluated by RT-PCR analyses. GAPDH was used as internal control.

The activation of PKC with PMA treatment led to an upregulation of claudin-1, in contrast to a downregulation of claudin-2 expression, in epithelial cells from rat choroid plexus (2). In addition, PKC activation by PMA resulted in an increase in claudin-1 transcription in human melanoma cells (17). In the present study, it was demonstrated in the MKN45 gastric cancer cell line that PMA could regulate claudin-18a2 expression through the PKC and ERK-MAPK pathway. PMA-induced claudin-18a2 transcription was directly controlled by two AP-1 sites located at −923/−286 in the *CLDN18a2* promoter. It has been previously reported that *Helicobacter pylori* infection induces AP-1 activity. This could represent a crucial step in the development of gastric neoplasias (20, 22, 24). It has also been shown that PMA injections in the rat stomach could cause gastric ulcers (34). Although the biological function of claudin-18 has not yet been determined, the PMA-stimulated upregulation of claudin-18a2 in gastric cells might cause a disturbance in TJ integrity and function, which might contribute to the development of gastric disease.

We demonstrated that AP-1 plays a crucial role in the activation of the *CLDN18a2* promoter. The AP-1 transcription factor is a dimeric complex, which is comprised of members of the Jun, Fos, ATF, and MAF protein families. Recent studies indicated that AP-1 could function as an oncogene by providing signals for cell survival and could also function as an anti-oncogene by inducing apoptosis, both of which depend on AP-1 dimer composition, as well as the cellular and genetic context (7, 13, 31). Thus AP-1-mediated upregulation of claudin-18a2 might be related to tumor suppressor activity. EMSA analysis demonstrated that c-*jun*, but not c-*fos*, can bind to one of the AP-1 binding sites in the *CLDN18a2* promoter. Further characterization of the AP-1 complex that binds to these sites will uncover the mechanisms of claudin-18a2 downregulation in gastric cancer, thereby providing clues for understanding the functions of claudin-18a2 under normal conditions and during tumor development.

The present study has characterized the human *CLDN18a2* promoter for the first time and demonstrated that the minimal promoter region, which is critical for expression in PMA-stimulated MKN45 cells, was mapped to the −923/+49 region. However, the regulation mechanisms of stomach-specific expression of claudin-18a2 remain unclear. Several studies have shown that AP-1 sites are required for cell-specific gene expression. Virolle et al. (41) demonstrated that DNA conformation, through the cooperative effect of AP-1 binding sites, triggers keratinocyte-specific expression of the human *LAMA3* gene. Zutter et al. (43) reported that two tandem AP-1 sites with dyad symmetry are critical for megakaryocyte/platelet-specific enhancer activity. These results led us to investigate whether the AP-1 sites in the *CLDN18a2* promoter participate in the stomach-specific enhancer. These sites did not comprise a cell-specific enhancer, however, since AP-1-mediated upregulation of promoter activity was observed in a nonstomach cell line, such as lung-derived NCI-H441 cells (data not shown). Sequence analysis revealed that several other consensus sequences, such as those encoding GATA and SOX transcription factors, were located in the proximal region of the human *CLDN18a2* promoter (see Fig. 3). GATA-4, -5, and -6 have been implicated in the transcription of genes in the gastrointestinal tract (10, 18). Recently, SOX2 has been shown to regulate stomach-specific *pepsinogen A* gene expression (35). We are currently investigating whether these factors are involved in the regulation of stomach-specific expression of claudin-18a2.

Changes in permeability properties and loss of cell polarity are hallmarks of epithelial cell tumorigenesis. TJs are believed to be critical for these functions in epithelial cells; however, recent reports indicate that TJs might not be important for epithelial polarization (38). Therefore, one could speculate that claudins might possess other functions in tumorigenesis. In fact, several claudins have been shown to increase cell motility through the activation of matrix metalloproteinase (MMP)-2 and/or membrane-type MMP-1 in cancer (1, 17, 27). MKN45, a round-shaped tumorigenic epithelial cell line, was derived from poorly differentiated adenocarcinoma; it is unable to form TJs, most likely because of low cell-cell adhesion activity caused by a mutation in the *E-cadherin* gene (26). Therefore, we examined the relationship between claudin-18a2 expression and vimentin. Results indicate that PMA-induced expression of claudin-18a2 was associated with mesenchymal gene expression. It has been previously shown that increased claudin-1 expression in colon cancer cell lines is concomitant with the upregulation of vimentin, as well as the regulation of metastatic behavior (4). It is important to note that PMA-induced expression of claudin-18a2 and vimentin was observed only in the MKN45 cell line. The mechanism of this cell-dependent response is unclear but could be the result of endogenous unknown factor(s) in MKN45 cells. Further studies are required to determine the mechanism of increased claudin-18a2 expression in metastasis and to identify the factor(s) in MKN45 cells. Although this cell system is unable to analyze the permeability function of claudin-18a2, it could serve as a useful resource for functional studies of gastric tumorigenesis.

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