Claudin-1 Acts through c-Abl-Protein Kinase Cδ (PKCδ) Signaling and Has a Causal Role in the Acquisition of Invasive Capacity in Human Liver Cells*§

Claudins are identified as members of the tetraspanin family of proteins, which are integral to the structure and function of tight junction. Recent studies showed an increase in expression of claudins during tumorigenesis, which is associated with loss of cell-cell contact, dedifferentiation, and invasiveness. However, the molecular basis for the causal relationship between claudin expression and cancer progression is not fully understood yet. In this study, we show that claudin-1 plays a causal role in the acquisition of invasive capacity in human liver cells and that c-Abl-protein kinase Cδ (PKCδ) signaling is critical for the malignant progression induced by claudin-1. Overexpression of claudin-1 clearly induced expression of matrix metalloproteinase-2 (MMP-2) and cell invasion and migration in normal liver cells as well as in non-invasive human hepatocellular carcinoma (HCC) cells. Conversely, small interfering RNA targeting of claudin-1 in invasive HCC cells completely inhibited cell invasion. Both c-Abl and PKCδ are found to be activated in normal liver cell line clones that stably overexpress claudin-1. Inhibition of either c-Abl or PKCδ alone clearly attenuated MMP-2 activation and impeded cell invasion and migration in both human HCC and normal liver cells expressing claudin-1. These results indicate that claudin-1 is both necessary and sufficient to induce invasive behavior in human liver cells and that activation of c-Abl-PKCδ signaling pathway is critically required for the claudin-1-induced acquisition of the malignant phenotype. The present observations raise the possibility of exploiting claudin-1 as a potential biomarker for the spread of liver cancer and might provide pivotal points for therapeutic intervention in HCC.

Metastasis is the spread of cancer from its primary site to other places in the body, a process that is common in the late stages of cancer (1–4). It is a multistep process that involves migration of cancer cells from the primary tumor site, penetration into the vascular or lymphatic system, dispersal through the circulation, and extravasation and growth of malignant cells in the target organ (2, 3, 5). However, little is known about how malignant cells leave the primary site and begin to grow at distant sites; moreover, how the process of metastatic progression develops in cells is unknown. Thus, understanding the molecular basis for the spread of cancer, especially the development of invasive properties, is one of the most important issues in cancer research.

Claudins (CLDs)² are a family of integral membrane proteins central to the formation of tight junctions, structures that are critical for the maintenance of cellular polarity, and are involved in paracellular transport and cellular growth and differentiation (6–8). Recent studies have provided evidence that claudins are aberrantly expressed in diverse types of human cancers, including hepatocellular carcinomas (HCCs) (9–11), and are associated with the development and progression of cancer. In this context, it has been shown that decreased or abnormal expression of claudin-4 (CLD4) or claudin-7 (CLD7) is correlated with liver metastases (12–14). Moreover, downregulation of claudin-2 (CLD2) has been implicated in the development and progression of breast carcinomas (4). Other reports, however, have suggested that increased CLD4 expression is associated with poor prognosis and high tumor grade in human breast cancer (15). In addition, overexpression of claudin-1 (CLD1) is associated with advanced stage disease in oral squamous cell carcinomas (16, 17) and with angiolymphatic and perineural invasion, consistent with an aggressive tumor phenotype (16–19). Moreover, CLD2, in combination with the epidermal growth factor receptor, has been shown to participate in tumor colonization in non-small cell lung cancer (20). These results indicate that the expression and functional significance of claudins may be highly specific for tumor cell type and depend on tumor grade.

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2 The abbreviations used are: CLD, claudin; HCC, hepatocellular carcinoma; PKC, protein kinase C; MMP, matrix-metalloproteinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; siRNA, small interfering RNA; MBP, myelin basic protein.
Proteins of the protein kinase C (PKC) family of serine/threonine kinases are involved in a variety of cellular processes related to tumor promotion and progression, including cell adhesion (21, 22), cell survival (23), invasion (24), and metastasis (25). PKC activation mediates the phorbol ester-induced increase in D54 human glioblastoma cell invasion in vitro by regulating the matrix metalloproteinase/tissue inhibitors of the metalloproteinase system (26). Moreover, phosphatidylinositol 3-kinase-dependent activation of the PKCε subtype is essential for breast carcinoma cell migration and invasion (27, 28), and PKCδ has a critical role in prostate cancer cell migration and invasion (29). It has also recently been demonstrated that PKC is associated with CLD1 expression in melanoma (30) and thereby contributes to cell motility. PKC up-regulates CLD1 expression in melanoma, which correlates with increased matrix metalloproteinase 2 (MMP-2) secretion and activation, which leads to melanoma cell invasion and migration (30–32). However, the molecular circuitry that links PKC with claudin expression and the subsequent regulation of cancer development and progression have not yet been fully determined.

In this study, we investigated the relationship between CLD1 expression and cell invasion in human liver cells. We demonstrate that CLD1 has a causal role in the acquisition of invasive capacity in human liver cells and that activation of c-Abl-PKCε signaling is critically involved in CLD1-dependent malignant progression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—Human hepatocellular carcinoma cell lines, SNU-354, SNU-398, SNU-423, SNU-449, SNU-475, and Chang, were established from the primary tumors of Korean patients. HCCs and normal Chang cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. After incubation for 24 h, cells were removed by washing with phosphate-buffered saline, and fresh complete medium was added. Cells were incubated at 37 °C for 24 h. Cells were imaged with phase-contrast microscopy (Leica Microsystems).

**Wound-healing Assay**—Cells were plated in a 60-mm culture dish and grown to 80% confluence in complete medium. A “wound” was made by scraping with a P200/yellow pipette tip in the middle of the cell monolayer. Floating cells were removed by washing with phosphate-buffered saline, and fresh complete medium was added. Cells were incubated at 37 °C for 24 h. Cells were imaged with phase-contrast microscopy (Leica Microsystems).

**Gelatin Zymography**—Production of MMPs in CLD1-overexpressing clones was analyzed by gelatin zymography. Subconfluent cells (about 70% confluence) were washed, replenished with serum-free Dulbecco’s modified Eagle’s medium, and incubated for 24 h. In all, 20 ml of serum-free conditioned medium was mixed with SDS sample buffer without heating or reduction and applied to 10% polyacrylamide gels copolymerized with 1 mg/ml gelatin. After electrophoresis, gels were washed in 2.5% (v/v) Triton X-100 for 2 h at room temperature to remove SDS, rinsed twice with water, and then incubated in developing buffer (50 mM Tris-HCl, pH 7.5, 0.2 mM NaCl, 5 mM CaCl₂, 0.1% Triton X-100) for 36 h at 37 °C. Subsequently, gels were fixed and stained with 10% acetic acid containing 0.5% Coomassie Blue R250. For destaining, gels were destained with 5% methanol and 8% acetic acid. Analysis of the gels by densitometry was inverted to reveal dark bands on white background (Bio-Rad Gel DocTM XR).

**Small Interfering RNA (siRNA) Transfection**—siRNA targeting of CLD1, PKCa, PKCβ, PKCδ, PKCζ, Fyn, Lyn, c-Src, and c-Abl were purchased from Ambion (Austin, TX). siRNA targets of PKCa, PKCβ, PKCζ, and PKCδ were purchased from Ambion (Austin, TX). The siRNA targets of CLD1, Fyn, Lyn, c-Src, and c-Abl were purchased from Samchully Pharmaceutical Co. Ltd. (Korea, Seoul). A control siRNA specific for green fluorescent protein (5′-CCACTACCTGAGCACCCAG-3′) was used as the negative control. Cells were plated on 100-mm dishes at 30% confluence, and siRNA duplexes (40 nM) were introduced into cells using Lipofectamine 2000 (Invitrogen) by following the procedure recommended by the manufacturer.

**Western Blot Analysis**—Cell lysates were prepared by extracting proteins with lysis buffer (40 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.1% Nonidet-P40) supplemented with protease inhibitors. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline and incubated with primary antibodies overnight at 4 °C. Blots were developed with a peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence (ECL) procedures (Amersham Biosciences), using the manufacturer’s protocol.
Immune Complex Kinase Assay—Proteins from cell extracts were immunoprecipitated with primary antibody at 4 °C for 4 h. The immunoprecipitates were washed twice with kinase reaction buffer (50 mM HEPES (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, 2.5 mM EGTA, 1 mM NaF, 0.1 mM Na₃VO₄, and 10 mM-glycerophosphate) and then resuspended in 20 μl of kinase reaction buffer. The kinase assay was initiated by adding 20 μl of kinase reaction buffer, containing 10 μg of substrate and 2 μCi of [γ-³²P]ATP (Valeant Pharmaceuticals International). The reactions were carried out at 30 °C for 30 min and terminated by adding SDS sample buffer, and the mixtures were boiled for 5 min. The reaction products were analyzed by SDS-PAGE and autoradiography.

RESULTS

CLD1 Has a Critical Role in HCC Cell Invasion—To investigate the involvement of CLD1 in human HCC cell invasion, we measured CLD1 expression levels in SNU-354, SNU-398, SNU-423, SNU-449, and SNU-475 HCC cells and used invasion assays to determine the invasive potential of these five cell lines. As shown in Fig. 1A, the CLD1-expressing HCC cell lines, SNU-354, SNU-423, and SNU-449, exhibited higher invasive behavior than those that did not express (or minimally expressed) CLD1. To confirm the role of CLD1 in HCC cell invasion, we used three different siRNAs against CLD1. The CLD1-targeting siRNAs clearly attenuated both CLD1 expression and cellular invasion in all three CLD1-expressing HCC cell lines (Fig. 1B). Conversely, overexpression of CLD1 in HCC cells that did not express CLD1 (SNU-398 and SNU-475) markedly increased levels of cellular invasion (Fig. 1C). In addition, overexpression of CLD1 clearly induced cellular invasion in Chang cells, a normal hepatocyte cell line (Fig. 1C). These results indicate that CLD1 may have a critical role in the cellular invasion potential of human HCC cells.

CLD1 Has a Causal Role in the Acquisition of Invasive Capacity in Normal Liver Cells—To confirm that CLD1 has a causal role in the acquisition of invasive capacity in normal liver cells, we examined the invasive potential of Chang cell line clones that stably overexpress CLD1. As shown in Fig. 2A, immunoblot analyses revealed that expression level was increased in several clones when compared with parental Chang
cells, and we chose clones 1, 11, and 13 for further analysis. Overexpression of CLD1 did not alter cell growth in these Chang cell clones (Fig. 2B). However, all three CLD1-overexpressing clones clearly exhibited a marked, time-dependent increase in cell invasion (Fig. 2C) and migration (Fig. 2D). In addition, siRNA-mediated CLD1 knockdown completely inhibited cell invasion in invasive Chang cell clone (supplemental Fig. S1). Importantly, all three clones clearly showed marked increases in the production and enzyme activity of MMP2 (Fig. 2E), characteristics of invasive cells. In addition, siRNA targeting of MMP-2 inhibited cell invasion in CLD1-overexpressing clones (Fig. 2F). These results indicate that CLD1 may have a causal role in the acquisition of invasive capacity in normal liver cells.

**PKCδ Activation Is Required for Invasion in CLD1-overexpressing Normal Liver Cells**—PKC has been shown to have an important role in mediating cellular invasion in response to diverse stimuli (24, 25). To determine the potential involvement of PKC in CLD1-dependent cellular invasion, we first examined the activation status of PKC isoforms in CLD1-overexpressing clones using an immune complex kinase assay. As shown in Fig. 3A, PKCδ activity was selectively increased in clones expressing CLD1. In contrast, PKCβ activity was decreased in all three clones, and the levels of PKCa and PKCζ...
activity were not altered. There was no change in any of the PKC isoforms at the total protein level. To further determine whether PKC activity is required for CLD1-dependent cellular invasion, we treated CLD1-overexpressing clones with specific inhibitors of PKC isoforms and analyzed changes in cellular invasion. Treatment with the broad spectrum PKC inhibitor Go6850 completely blocked cellular invasion in all three clones (Fig. 3B). Importantly, invasive behavior was completely suppressed by treatment with the specific PKC inhibitor Rottlerin (Fig. 3B), which also suppressed CLD1-dependent cell migration (supplemental Fig. S2). Moreover, siRNA targeting of PKCβ clearly attenuated cell invasion (Fig. 3C) and migration (supplemental Fig. S2) in CLD1-overexpressing clones. In contrast, Go6976, a specific inhibitor of PKCα and PKCβ, failed to inhibit cell invasion in CLD1-overexpressing clones (Fig. 3B), and siRNAs against other PKC isoymes (PKCα, PKCβ, and PKCζ) did not alter CLD1-dependent cell invasion (supplemental Fig. S3). The CLD1-dependent production and enzymatic activity of MMP-2 was also clearly attenuated by siRNA-mediated silencing of PKCβ (Fig. 3D). These results specifically implicate PKCβ in CLD1-dependent invasive behavior in normal liver cells.

Activation of c-Abl Kinase Is Required for CLD1-dependent Cellular Invasion—Non-receptor tyrosine kinases are well known upstream activators of PKC and have a role in cellular invasion and metastasis (21–25). To investigate the potential involvement of non-receptor tyrosine kinases in CLD1-dependent cell invasion, we performed immune complex kinase assays using antibodies against Src family kinases (c-Fyn, Lyn, and Src) and c-Abl kinase. As shown in Fig. 4A, the activities of Src family kinases were unchanged in CLD1-overexpressing clones. However, the level of c-Abl kinase activity was
increased in these clones without a corresponding change in the c-Abl protein expression level (Fig. 4B). Moreover, siRNA-mediated c-Abl knockdown clearly suppressed CLD1-dependent cell invasion (Fig. 4C and supplemental Fig. S4) and migration (Fig. 4D), as well as MMP-2 activation (Fig. 4E). In addition, siRNA targeting of c-Abl effectively inhibited PKCδ activation in CLD1-overexpressing clones (Fig. 4F and supplemental Fig. S4). These results indicate that c-Abl is required for CLD1-dependent invasive capacity and that PKCδ acts downstream of c-Abl activation.

**c-Abl-PKCδ Signaling Is Essential for Invasive Behavior in Human HCCs**—To confirm that the c-Abl-PKCδ signaling pathway acts downstream of CLD1 in the cellular invasion pathway, we analyzed c-Abl and PKCδ activation and cellular invasion in CLD1-expressing HCC cell lines (SNU-354, SNU-423, and SNU-449) after siRNA-mediated silencing of CLD1. siRNA targeting of CLD1 clearly inhibited c-Abl and PKCδ activities (Fig. 5A), as well as cell invasion (Fig. 5B), in all three HCC cell lines. To further determine whether c-Abl-PKCδ signaling is critical for cell invasion in CLD1-expressing HCC cell lines, we targeted c-Abl and PKCδ with specific siRNAs and examined cellular invasion. As shown in Fig. 5B, siRNA-mediated knockdown of c-Abl or PKCδ effectively inhibited cellular invasion in all three HCC cell lines. These results indicate that the c-Abl-PKCδ signaling pathway is essential for the maintenance of invasive potential in CLD1-expressing human HCC cells.
Claudin-1-induced Liver Cell Invasion Signaling Pathway

**FIGURE 5.** c-Abl-PKCζ signaling is essential for invasive behavior in human HCCs. A, CLD1-expressing HCC cell lines (SNU-354, SNU-423, and SNU-449) were transfected with control siRNA (si-cont) or si-CLD1. After 48 h, total cell extracts were immunoprecipitated (IP) with anti-PKCζ and -c-Abl antibodies, and an immune complex kinase assays was performed as described under “Experimental Procedures.” MBP was used as a substrate for PKCζ and c-Abl. Cell lysates transfected with the indicated siRNAs were analyzed by immunoblot analysis with anti-claudin-1 and -c-Abl antibodies. β-Actin was used as the loading control. B, CLD1-expressing HCC cell lines (SNU-354, SNU-423, and SNU-449) were transiently transfected with control siRNA, si-CLD1, si-PKCζ, or si-c-Abl. Left, after 24 h, the cells (2 × 10⁴) were seeded in each Matrigel chamber and cultured for 48 h. Invaded cells were fixed and stained with a Diff-Quick kit and photographed (magnification: ×200). Middle, protein levels of PKCζ and c-Abl were detected by immunoblot analysis with the specific-antibodies, respectively. β-Actin protein levels served as loading controls. Right, invasiveness was determined by counting cells in randomly selected five microscopic fields per well. Bars represent mean ± S.D. of triplicate samples.

**DISCUSSION**

The invasiveness of human cancers stands as a major obstacle to improved prognosis in patients undergoing cancer therapy; thus, understanding the molecular basis for the spread of cancer is one of the most important issues in cancer research. However, little is known about how cells acquire invasive properties during metastatic progression. In this study, we demonstrated for the first time that CLD1 has a causal role in the acquisition of invasive capacity in both human HCC and normal liver cells. Moreover, we showed that activation of c-Abl is required for CLD1-dependent invasion in human liver cells and that PKCζ is located downstream of c-Abl activation in this pathway.

Claudins are a family of integral membrane proteins that are important components of tight junctions, structures that are critical for the maintenance of cellular polarity and are involved in paracellular transport and cellular growth and differentiation (6–8). Recently, altered expression of individual claudins has been demonstrated in diverse types of human cancers and shown to be associated with malignant progression and cellular invasion. However, the role of claudins in tumor progression is controversial. Down-regulation of CLD2 has been implicated in the progression as well as the development of breast carcinomas (15, 18, 33). Moreover, decreased or abnormal expression of CLD4 or CLD7 is correlated with liver metastases (12–14).

However, other reports have demonstrated that increased expression of CLD2 participates in tumor colonization in non-small cell lung cancer (20) and that increased CLD4 expression is associated with poor prognosis and high tumor grade in breast cancer (15, 18). Similarly, overexpression of CLD1 has been linked with advanced stage disease in oral squamous cell carcinomas (16, 17). In this study, we provide evidence that CLD1 has a causal role in the acquisition of invasive capacity in human HCC and normal liver cells. We showed that siRNA targeting of CLD1 completely inhibited cellular invasion in CLD1-overexpressing, invasive HCCs. Conversely, overexpression of CLD1 clearly induced invasive behavior in normal liver cells as well as in CLD1-underepressing, non-invasive HCC cells. These results indicate that overexpression of CLD1 is both necessary and sufficient to induce cellular invasion in human HCC and normal liver cells.

Recent reports have demonstrated an association between PKC activity and the expression and/or subcellular distribution of claudins. For example, PKCζ was associated with shifts in the subcellular distributions of CLD1 and ZO-2 into the tight junctional complex (34). PKC regulates the increase in CLD1 expression in rat choroid plexus cells (35). Moreover, in melanoma, CLD1 overexpression is regulated by PKC and contributes to melanoma cell motility (30). Consistent with these findings, we found that PKC, particularly PKCζ, is involved in CLD1 expression in human HCC cells. PKCζ activity was greater in CLD1-expressing versus non-expressing human HCC cells, and siRNA-mediated knockdown of PKCζ significantly attenuated the expression of CLD1, indicating that PKCζ has a critical role in regulating the expression of CLD1 in human HCCs. Interestingly, we also found that there was cross-talk between CLD1 and PKCζ in CLD1-overexpressing normal liver cells. PKCζ activity was selectively increased in clones expressing CLD1, and chemical inhibition or siRNA-mediated silencing of PKCζ clearly suppressed the acquired invasive capacity of CLD1-overexpressing normal liver cells. Moreover, we found that inhibition of PKCζ effectively attenuated cellular invasion in three different human HCC cells expressing relatively high levels of CLD1. These results indicate that selective activation of PKCζ is required for CLD1-dependent acquisition of invasive capacity in both human HCC cells and normal liver cells.

The product of the c-abl protooncogene is a non-receptor tyrosine kinase activated by various growth factors, including
platelet-derived growth factor, epidermal growth factor, and transforming growth factor-β (30, 36, 37), that is involved in the regulation of cell growth (36, 38), survival (36, 39), and transformation (39). c-Ab1 tyrosine kinase was recently implicated in growth factor-induced cell migration and invasion (40). Indeed, c-Ab1 has been shown to regulate the invasive activity of aggressive breast cancers (41, 42) and thyroid cancer cells (43). Likewise, in this study, we found that c-Ab1 tyrosine kinase is activated in liver cells overexpressing CLD1 and is associated with the CLD1-dependent acquisition of cellular invasive capacity. We clearly showed that siRNA-mediated knockdown of c-Ab1 kinase inhibited CLD1-dependent MMP-2 activation, cell migration, and invasion in CLD1-overexpressing liver cell clones. In addition, inhibition of c-Ab1 effectively suppressed invasive activity in HCC cells expressing high levels of CLD1. These observations support the view that CLD1 acts through c-Ab1 kinase to promote cell migration and invasion.

In summary, we demonstrate here that CLD1 has a causal role in liver cell invasion. We found that CLD1 is both necessary and sufficient to induce cellular invasion in human HCC and normal liver cells. We also showed that activation of the c-Ab1-PKC8 signaling pathway is critical for the expression and activation of MMP-2 and the subsequent induction of cellular invasion in response to CLD1 expression. The present observations raise the possibility that CLD1 could be exploited as a potential diagnostic and prognostic biomarker for liver cancer progression and might provide an important target for therapeutic intervention.

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