Involvement of Hypoxia-inducing Factor-1α-dependent Plasminogen Activator Inhibitor-1 Up-regulation in Cyr61/CCN1-induced Gastric Cancer Cell Invasion

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This article has been withdrawn by authors Ming-Tsai Lin, I-Hsin Kuo, Cheng-Chi Chang, Chia-Yu Chu, Been-Ren Lin, and Min-Liang Kuo. The same images were used to represent different experimental conditions. In Fig. 1A, lanes 2 and 4 of the HIF-1α DNA gel were duplicated. The HIF-1α DNA gel from Fig. 1A was reused in Fig. 1E in the HIF-1α rCyr61 panel. The GAPDH DNA gel from Fig. 1A was reused in Fig. 1E as GAPDH rCyr61 and IGF-1 panels, Fig. 5A as GAPDH, and Fig. 6B as input, left panel. The HIF-1β immunoblot from Fig. 1A was reused in Fig. 1B as HIF-1β, AGS and TSGH panels, Fig. 1D as HIF-1β, N87 panel, Fig. 1F as HIF-1β, rCyr61 panel, and Fig. 3D as HIF-1β. The tubulin immunoblot from Fig. 1A was reused in Fig. 5B as tubulin, lower panel, and reused in Fig. 5E as tubulin, left panel. In Fig. 1C, lanes 1 and 2 of the HIF-1α immunoblot were reused in lanes 5 and 6. In Fig. 1D, the HIF-1α immunoblot from the N87 panel was reused in Fig. 1F in the HIF-1α IGF-1 panel. In Fig. 1E, lanes 2 and 3 of the HIF-1α DNA gel from the rCyr61 panel were duplicated in lanes 5 and 6 of the same panel. Also in Fig. 1E, the HIF-1α DNA gel from the CoCl2 panel was reused in the IGF-1 panel as HIF-1α. In Fig. 1F, lanes 4 and 5 were duplicated in the HIF-1β immunoblot from the CoCl2 panel. The HIF-1β immunoblot from the IGF-1 panel in Fig. 1F was reused in Fig. 3A as tubulin. In Fig. 1G, lanes 1 and 2 of the tubulin immunoblot, left panel, was reused in lanes 3 and 4 of the same panel. In Fig. 2A, lanes 2 and 4 of the HIF-1α immunoblot and lanes 3 and 4 of the HIF-1β immunoblot from the CoCl2 panel were duplicated. In Fig. 2C, lanes 1 and 2 of the HIF-1β immunoblot were duplicated in lanes 4 and 5, lanes 7 and 8, lanes 9 and 10, and lanes 11 and 12. Also, in the same panel, lanes 3 and 6 were duplicated. In Fig. 3A, lanes 4 and 5 of the HIF-1β immunoblot were duplicated. Also in the same figure, lane 1 of the p-AKT immunoblot was reused in lanes 3 and 5, and lane 2 of the AKT immunoblot was reused in lane 5. The AKT immunoblot from Fig. 3A was also reused in Fig. 3D as 4E-BP1. In Fig. 3B, lane 1 of the p-AKT immunoblot was reused in lanes 5 and 6, and lane 1 of the AKT immunoblot was reused in lane 6. In Fig. 3D, lane 1 of the HIF-1α immunoblot was reused in lane 6, and lane 1 of the p-p70S6K immunoblot was reused in lane 5. The graphs in Fig. 4A were duplicated. In Fig. 5A, lane 1 of the c-MET DNA gel was reused in lanes 5 and 6, and lane 2 of the same gel was reused in lane 4. Also in Fig. 5A, lanes 1–3 of the AMF gel were reused in lanes 4–6. In Fig. 5C, lane 1 of the PAI-1 DNA gel was reused in lane 2, and lane 1 of the GAPDH DNA gel was reused in lane 2. In Fig. 6A, lanes 1 and 4 of the tubulin immunoblot were duplicated. Lane 2 of the PAI-1 DNA gel from Fig. 6B, left panel, was reused in lanes 2 and 3 of the PAI-1 DNA gel, right panel. In Fig. 6B, lanes 1 and 4 of the input DNA gel, right panel, were duplicated.

An emerging family of proteins with secreted and cell-surface functions. The CCN proteins, also known as CYR61, CTGF, and WISP-3, can be induced in a number of cell types upon growth factor stimulation. Among the CCN family, CYR61, a cysteine-rich 61-kDa protein, has been identified as an inducer of angiogenesis and invasion in various tumor cell lines (1–3). The mechanism by which CYR61 exerts its effects on angiogenesis remains unknown, and it is thought to function as an autocrine growth factor or as a paracrine factor acting through slow-acting mechanisms (4–6).

Cytokines or growth factors can induce angiogenesis and cancer cell invasion by up-regulating CYR61 expression (7, 8). This CYR61 protein, in turn, activates a number of downstream signaling pathways, including PI3K/AKT, ERK, RhoA, and Rac1 (9, 10). These downstream pathways have been shown to play a role in the promotion of angiogenesis and invasion (11). CYR61 has also been shown to bind adhesion molecules such as selectins and vitronectin and to bind to cell surfaces, integrins, and ECM proteins (12–14). These findings suggest that CYR61 functions as an autocrine growth factor as well as a paracrine factor that is responsible for proangiogenesis. It regulates cell adhesion, migration, and plastic transformation (4–6).

Cytoskeletal rearrangement is an essential step in the process of cell invasion (15, 16). Cytoskeletal organization is mediated by numerous proteins, including cytoskeletal proteins, growth factors, and their receptors. The function of CYR61 is to up-regulate the expression of CYR61 in a number of tumor cell lines (1–3). CYR61 binds to ECM proteins such as fibronectin, laminin, and collagen, which are expressed in various tumor tissues (17, 18). CYR61 also binds to integrins, such as αvβ3 and αvβ5, which are expressed in various tumor cell lines (19). CYR61 binds to integrins, such as αvβ3 and αvβ5, which are expressed in various tumor cell lines (19).

In conclusion, our data provide a novel insight into the mechanism underlying Cyr61-mediated cancer cell invasion. Insight into the mechanism underlying Cyr61-mediated cancer cell invasion is necessary for the development of new therapeutic approaches for the treatment of cancer.
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hypoxic conditions, HIF-1α is stabilized, dimerizes with HIF-1β, translocates into the nucleus, and transactivates a broad array of downstream genes, including activation mitogenic, pro-inflammatory, pro-angiogenic, and pro-metastatic genes (17, 18). Interestingly, it has been demonstrated that HIF-1α is also regulated by oxygen-independent mechanisms. Oncogenic genes (e.g. Ha-ras, myc, or src), tumor suppressor genes (e.g. p53, PTEN, or VHL), and a variety of growth factors (EGF, IGF-1, insulin, interleukin-1β, HGF, TNF-α, thrombin) are associated with HIF-1-mediated tumor progression (19). To date, numerous reports have proven that HIF-1α is overexpressed in many human cancers. HIF-1α-transactivated genes, such as iNOS, IGF, and VEGF, playing important roles in tumor metastasis and invasion (20), are overexpressed in a majority of metastatic tumors and cell lines (21). However, the detailed role of HIF-1α in tumor invasion is still very obscure in most of the metastatic tumor cells.

In this study, we investigated the possible involvement of HIF-1α in Cyr61-enhanced invasion ability in gastric cancer cells. We found that HIF-1α protein was elevated and constitutively activated in Cyr61-overexpressing gastric cancer cells. Dominant-negative (DN)-HIF-1α mutant could abolish the invasive ability of Cyr61-overexpressing cells, suggesting a critical role of HIF-1α in such an invasion mechanism. Plasminogen activator inhibitor-1 (PAI-1), a HIF-1α-dependent invasion gene, was significantly upregulated in Cyr61-overexpressing cells. Our results further indicate a novel mechanism for HIF-1α-dependent up-regulation of PAI-1, which contributes to the invasion activity.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Reagents were purchased from Abnova (Woburn, MA), Calbiochem (La Jolla, CA), Bio-Rad (Hercules, CA), Thermo Fisher Scientific (Waltham, MA), or HyCult Biotechnology (Uden, The Netherlands). Human anti-Cyr61 polyclonal antibody, anti-PAI-1 monoclonal antibody, anti-p-ERK1/2 or total ERK1/2 antibodies, and anti-phosphorylated (Ser-209) p70S6K antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-α-tubulin monoclonal antibody was purchased from Sigma. Function-blocking antibody against PAI-1 (clone MA-33H1F7) was obtained from HyCult Biotechnology (Uden, The Netherlands).

Cell Cultures—Human gastric carcinoma cells (AGS, N87, and SNU16) were obtained from the American Type Culture Collection (Manassas, VA and Rockville, MD), and TSGH was obtained from Food Industry Research and Development Institute (Hsinchu, Taiwan). All gastric carcinoma cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 units/ml penicillin (all from Invitrogen). Cell cultures were maintained at 37 °C in a humidified 5% CO2 atmosphere. Adherent cells were detached from the culture dishes with trypsin/EDTA (Sigma).

Transient and Stable Transfections—Details are available as supplemental information.

RT-PCR and Western Blot Analysis—Details are available as supplemental information.

Nuclear and Cytosolic Protein Extraction—For nuclear and cytosolic protein extraction, the protein extracts were prepared from rCyr61-treated AGS cells or Cyr61-stably transfected-AGS cells using a modified procedure, as described previously.

HIF-1α Reporter Activity Assay—Details are available as supplemental information.

Immunofluorescence Staining—Details are available as supplemental information.

Boyden Chamber Assay—The details of the Boyden chamber assay are available as supplemental information.

PAI-1 Antisense Oligonucleotides—The sequence of the PAI-1-specific antisense oligonucleotides was 5′-AGGATCTGCATCCCTGAAGTT-3′, and that of control oligonucleotides was 5′-AAGTACAGGATGCAGAATTT-3′. For PAI-1 blocking studies, gastric cancer cells were cultured to 70% confluence, control and PAI-1 antisense oligonucleotides (25–100 nM) were transfected. After 48 h of transfection, the cells were washed and analyzed by Western blot analysis.

Chromatin Immunoprecipitation Assay—Briefly, cells were treated with 1 µg of specific antisense oligonucleotides (25–100 nM) and 1.5 µg of control oligonucleotides. Chromatin was immunoprecipitated with HIF-1α antibody and analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

RESULTS

We first examined whether Cyr61 expression was associated with HIF-1α expression in a series of human gastric adenocarcinoma cell lines. Among these four cell lines tested, we found a strong association between the expression levels of Cyr61 and HIF-1α protein (Fig. 1A, bottom) but not mRNA levels (Fig. 1A, top). Elevated expression of Cyr61 by transiently transfected with different doses of Cyr61 sense-oriented expression vector significantly induced HIF-1α protein level in AGS (Fig. 1B, upper) and N87 cells (Fig. 1B, middle) with a dose-dependent manner. In addition, TSGH cells were transiently transfected with Cyr61 antisense-oriented expression vector resulted in significantly decreased HIF-1α protein level (Fig. 1B, lower).

Cyr61 contains a signal peptide that is believed to be a secreted protein. To test this hypothesis, we examined the Cyr61 protein amount in conditioned medium from the cultured gastric adenocarcinoma cells. Western blot analysis demonstrated a higher Cyr61 protein content in conditioned medium from the cultured gastric adenocarcinoma cells.
FIGURE 1. Cyr61 stimulates HIF-1α accumulation and activation in human gastric carcinoma cells. A, the correlation between Cyr61 and HIF-1α protein levels in human gastric adenocarcinoma cell lines. Cells were cultured in the same condition and analyzed by RT-PCR and Western blot. B, AGS and N87 cells were transfected with different doses of empty vector or sense-oriented Cyr61 (S) plasmids followed by incubation for 48 h. Total proteins were isolated, and expression of Cyr61 and HIF-1α was analyzed by Western blot. TSGH cells were transfected with different doses of empty vector or antisense-oriented Cyr61 (AS) plasmids and used the same methods to analyze protein expression. C, upper: Cyr61 protein contents in conditioned medium from the gastric adenocarcinoma cells and Cyr61 stably transfected AGS cells by Western blot analysis. “50x”: 1-ml samples from condition medium were typically concentrated into 20 μl, using centrifugal filter devices (Amicon Inc., Beverly, MA). Lower: HIF-1α protein expression in TSGH and Cyr61 stably transfected AGS cells. Cyr61-neutralizing antibody (10 μg/ml) or control IgG was added in culture medium to deplete Cyr61 protein existence. D, duplicate plates of AGS and N87 cells were cultured in the absence of serum for 24 h, exposed to vehicle (lane 1), 10–100 ng/ml recombinant Cyr61 (lanes 2–6) for 16 h. Western blot analyses of HIF-1α expression. Kinetics of HIF-1α mRNA (E) and protein (F) induction. Serum-starved cells were exposed to vehicle (lane 1), 40 ng/ml recombinant Cyr61, or 100 μM CoCl2, or 100 ng/ml IGF-1 (lanes 2–7) prior to analysis of HIF-1α mRNA and protein. Data were quantified by Edit EZ-1D Software from EZLab Technology Co., Ltd. G, cytosolic and nuclear fractions were prepared from Cyr61 stably transfected AGS cells and AGS cells treated with Cyr61 or not. Cytosolic and nuclear fractions were subjected to Western blot analysis by using the indicated antibodies for HIF-1α, α-Tubulin and SP-1 acted as the internal loading control for cytosolic and nuclear fractions, respectively. H, nuclear localization of HIF-1α was then observed by fluorescence microscopy. Cyr61 stably transfected AGS cells (upper panel) or AGS cells, which were incubated with Cyr61 for 8 and 16 h (lower panel), were fixed and immunostained with a mouse monoclonal anti-HIF-1α antibody, detected by fluorescein isothiocyanate-conjugated antibody. I, HIF-1α transcriptional activity was measured by the application of a luciferase assay using the reporter constructs containing tandem copies of hypoxia response element (HRE). pGL2 was used as vector control. Upper: Cyr61 stably transfected AGS cells were transiently transfected with pCEP4, DN-HIF-1α, and then analyzed for luciferase activity. Lower: AGS cells transfected with various plasmids were treated with different doses of rCyr61 (20–80 ng/ml) for 16 h, after which samples were analyzed for luciferase activity. All data are the average of three independent experiments. Columns, mean of three experiments; bars, ± S.D. Statistical significance was determined with Student’s t test (p < 0.05).
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(Annually provided by Dr. Lester F. Lau (University of Illinois, Chicago, IL)) to the culture medium from TSGH and Cyr61 stably transfected AGS cells, and both cells secreted significant amounts of Cyr61 protein. As expected, decreasing Cyr61 protein concentration in culture medium significantly attenuated the endogenous HIF-1α protein level (Fig. 1C, bottom).

Next, to investigate whether the induction of HIF-1α directly depends on Cyr61, we added recombinant Cyr61 (rCyr61) into the cultures to examine the downstream signaling. Exposure of serum-starved AGS human gastric adenocarcinoma cells to rCyr61 for 16 h resulted in a concentration-dependent induction of HIF-1α protein expression and reached its maximal effect at 40 ng/ml rCyr61 (Fig. 1D, top). Similar results were obtained with N87 gastric adenocarcinoma cell lines (Fig. 1D, bottom). Moreover, we used CoCl2, a hypoxia mimetic, and IGF-1 as a comparison, because they are well known inducers of HIF-1α expression. We observed that exposure of serum-starved AGS cells to rCyr61, CoCl2, or IGF-1 did not significantly affect HIF-1α mRNA levels (Fig. 1E). However, the rCyr61 treatment to serum-starved AGS cells resulted in HIF-1α protein expression after 8 h (Fig. 1F, top). The effect of CoCl2 was extremely rapid (Fig. 1F, middle). We also found that CoCl2 is a more potent inducer of HIF-1α expression compared with Cyr61. These observations suggest that Cyr61 and CoCl2 play a distinct role in signaling pathways to increase the HIF-1α protein expression. Additionally, in the presence of IGF-1, 100 ng/ml, HIF-1α protein level increased at 2 h, suggesting differences between Cyr61 and classic growth factors in HIF-1α induction mechanism (Fig. 1F, bottom). The novel finding is that Cyr61 stimulates HIF-1α protein accumulation in N87 gastric adenocarcinoma cells with a different profile compared with CoCl2 and IGF-1.

HIF-1α subunit is a pivotal transcription factor and may stimulate the nuclear translocation of functional HIF-1α protein in response to recombinant Cyr61 treatment or an increase in translation of the HIF-1α protein. blockage of the binding of HIF1α-HIF1β complex with target HRE-responsive element sequence and the subsequent transcriptional activation (22, 25, 26). The results showed that the Cyr61-induced HRE luciferase activity was significantly attenuated by the DN-HIF-1α in AGS cells. Our data thus suggest that Cyr61 not only stimulates the expression of functional HIF-1α protein but also induces the HIF-1α-mediated HRE-dependent transcription activation.

Quantification of HIF-1α mRNA revealed that there was no significant increase in HIF-1α mRNA expression by rCyr61 in AGS human gastric carcinoma cells (Fig. 1E), indicating that Cyr61 was unable to regulate HIF-1α mRNA transcription. These results raised the question of whether increased amounts of HIF-1α protein were the result of reduced degradation of the protein in response to recombinant Cyr61 treatment or an increase in translation of the mRNA.

To analyze the possible effect of Cyr61 on HIF-1α protein synthesis, we performed a time-course analysis of HIF-1α turnover in the presence of the protein translation inhibitor, CHX. According to the Western blot analyses conducted and the results of quantitative data elicited (Fig. 2A; quantification by Edit EZ-1D Software from EZLab Technology Co., Ltd., Taipei,
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Taiwan), the half-life of HIF-1α was not longer than 60 min in CoCl2-treated cells but <30 min in recombinant Cyr61-treated cells. As expected, observation is consistent with previous studies showing that CoCl2 had no effect on HIF-1α synthesis but blocked its degradation. We also monitored that there was no significant change in HIF-1α protein half-life upon CHX treatment in the presence or absence of recombinant Cyr61 (both <30 min). Collectively, rCyr61 treatment seems to increase HIF-1α protein levels by increasing translation rather than by inhibiting degradation.

To corroborate these results, exposure of rCyr61 for 14 h in AGS cells and further treatment with or without the proteasome inhibitor MG132 (10 μM) for 2 h, HIF-1α protein was highly elevated in rCyr61-treated cells than in control cells (Fig. 2B, lane 2 and 6). HIF-1α protein expression, promoted by MG132, was significantly inhibited by treatment with both MG132 and CHX (Fig. 2B, lane 4 and 8). If Cyr61 induces HIF-1α expression by stimulating synthesis of the protein, then it can be expected to have an additive effect with CoCl2, which can act by increasing the stability of the protein. Exposure of AGS cells to rCyr61 combined with CoCl2 resulted in a greater increment of HIF-1α protein (Fig. 2C). The above results indicated that HIF-1α protein accumulation induced by Cyr61 is mainly through enhanced de novo protein synthesis.

To determine the signal transduction pathways mediating the effects of Cyr61 on HIF-1α protein expression, AGS cells were pre-treated with PD98059, LY294002, and rapamycin, which are selective inhibitors of MEK, PI3K, and FRAP/mTOR kinase activity, respectively. All these three agents inhibited the induction of HIF-1α protein expression in rCyr61-treated cells (Fig. 3A, top). To examine whether the MAPK and PI3K pathways were activated serially or independently in rCyr61-treated cells, the phosphorylation of ERK and AKT were also analyzed. The increased phosphorylation of ERK that was induced by rCyr61 was blocked by PD98059 but not by LY294002 or rapamycin (Fig. 3A, middle). The increased phosphorylation of AKT, which was induced by the rCyr61 treatment, was blocked by LY294002 but not by PD98059 or rapamycin (Fig. 3A, bottom). Moreover, the induction of HIF-1α by rCyr61 was inhibited in a dose-dependent manner by PD98059 (Fig. 3B, upper) and LY294002 (Fig. 3B, lower). Thus, both MAPK and PI3K activities are required for induction of Cyr61-enhanced HIF-1α protein expression. In contrast, PD98059 and LY294002 had no significant inhibitory effect on the expression of HIF-1α in CoCl2-treated AGS cells (Fig. 3C). These results confer further evidence that Cyr61 and CoCl2 act as a distinct molecular mechanisms.

The signal transduction pathway involving PI3K, AKT, and FRAP/mTOR has been shown to regulate protein translation via phosphorylation of p70^skc and 4E-BP1 (27). Therefore we asked whether Cyr61 stimulates HIF-1α expression through a translation-dependent mechanism, and we also evaluated the phosphorylation status of p70^skc and 4E-BP1 in response to rCyr61. In AGS cells, HIF-1α protein and the phosphorylation of both p70^skc and 4E-BP1, which were induced by rCyr61 stimulation, could be blocked by rapamycin in a dose-dependent manner (Fig. 3D). Our results demonstrate that the Cyr61 induces HIF-1α through PI3K/mTOR and the MAPK-dependent pathway.

We previously found that Cyr61 was highly expressed in more advanced gastric adenocarcinoma, and overexpression of Cyr61 in human gastric cancer cell lines significantly increased their invasion abilities (11). To better understand the molecular mechanism, we examined whether HIF-1α participated in...
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Cyr61-induced gastric cancer cell invasion. Co-transfection of DN-HIF-1α and sense-Cyr61 expression plasmids to AGS cells significantly abrogated the Cyr61-enhanced invasion ability (Fig. 4A, upper). Similar results were also observed in N87 cells (Fig. 4A, lower). In contrast, TSGH cells transiently transfected with Cyr61 antisense-oriented expression vector significantly abrogated cell invasion abilities and can be restored when co-transfected with WT- HIF-1α expression vector (Fig. 4B). Collectively, the Cyr61-induced invasion ability in gastric cancer cells is largely due to the increase in HIF-1α expression.

The question remains as to which gene is the possible downstream effector that contributes to the Cyr61-mediated HIF-1α-dependent cell invasion. It has been known that several invasion/metastasis-related genes such as PAI-1, VIM, c-MET, and ADM are the transcriptional targets of HIF-1α (27). To address this question, we analyzed the expression of these invasion/metastasis genes in rCyr61-treated cells by using RT-PCR. It appears that only the mRNA of PAI-1 was substantially increased in a dose-dependent manner in rCyr61-treated cells compared with control cells (Fig. 5A). Similar results of PAI-1 protein expression were also observed in Cyr61-overexpressing AGS cells (Fig. 5B).

In the previous investigation (11), we have found that Cyr61 is directly involved in invasion in human gastric cancer cell lines. In this study, we have demonstrated that Cyr61 up-regulates HIF-1α-dependent invasion protein PAI-1. Therefore, to examine whether PAI-1 is involved in Cyr61-mediated gastric cancer cell invasion, three monolayer gastric cancer cell lines, including AGS, N87, and TSGH, were examined for their basal expression levels of PAI-1 protein and in vitro invasion capacity. Our results show that the level of PAI-1 protein was highly elevated in TSGH cells but was moderately expressed in N87 cells. AGS cells displayed an extremely low level of PAI-1 protein compared with other gastric cancer cells (Fig. 5C, top). In Matrigel-coated chamber assay, TSGH cells exhibited the strongest invasive ability among these gastric cancer cell lines. In AGS cells, which expressed weak invasive potency (Fig. 5C, bottom), we hypothesize that PAI-1 plays a critical role in the malignant progression of gastric cancer cells.

A specific inhibition of PAI-1 expression was accomplished with antisense oligonucleotides. As anticipated, the PAI-1-specific antisense oligonucleotides used at 0.25 μM significantly blocked the invasion ability by 60% in TSGH cells compared with the sense oligonucleotide group (Fig. 5D, top). Western blot analysis was used to confirm the PAI-1-specific antisense oligonucleotides activity (Fig. 5D, bottom). Furthermore, treatment with PAI-1-specific antisense oligonucleotides 0–1 μM in Cyr61-overexpressing AGS cells also abolished Cyr61-enhanced cell invasion in a dose-dependent manner (Fig. 5E). Supportively, treatment of Cyr61-overexpressing AGS cells with PAI-1-neutralizing antibody effectively inhibited Cyr61-induced gastric cancer cell invasion (Fig. 5F). The above studies using genetic inhibition and pharmacological treatments all vividly demonstrated a critical role of PAI-1 in Cyr61-mediated up-regulation of the gastric cancer cell invasion.

Inhibition of HIF-1α by transfection with DN-HIF-1α significantly reduced the amount of PAI-1 mRNA expression in AGS/Cyr61 cells, suggesting that the HIF-1α pathway is required for the Cyr61-induced PAI-1 mRNA expression (Fig. 6A, upper). Western blot analysis further confirmed that the increase of PAI-1 protein present in AGS/Cyr61 cells was also effectively attenuated by transfection with DN-HIF-1α (Fig. 6A, lower). Finally, we examined whether HIF-1α would directly bind to the HRE (hypoxia response element) within the PAI-1 gene promoter by chromatin immunoprecipitation assay (Fig. 6B). As shown in Fig. 6B (upper panel), there were three HRE sites located between −687 and −411 bp of the PAI-1 pro-
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In the present study, we demonstrate a novel function of Cyr61 in gastric cancer cell invasion by molecular dissection. This study demonstrated, for the first time, that Cyr61 is able to up-regulate HIF-1α through de novo protein synthesis. This up-regulation of HIF-1α by Cyr61 commonly occurs in a variety of gastric cancer cell lines, involving PI3K/AKT/mTOR and ERK1/2 signaling pathways. Furthermore, we decipher the molecular mechanism for Cyr61-enhanced cell invasion, which is mediated by HIF-1α-dependent PAI-1 up-regulation.

The role of HIF-1α in tumor invasion/metastasis has been extensively exploited in different cell systems (29–34). Here we show the first time that Cyr61 enhances HIF-1α expression through a translation-dependent mechanism rather than inhibition of protein degradation. The underlying process is distinct from that observed during hypoxia. The hypoxia inhibits HIF-1α proteasomal degradation, leading to its accumulation (35). Previous studies supported that heregulin was found to activate HER2 tyrosine kinase receptor and stimulate HIF-1α translation (36). The stimulatory action on HIF-1α translation seems to be a general mechanism used by tyrosine kinase receptors, such as insulin and IGF-1 receptors, HER2, and by cytosolic tyrosine kinases such as Src (36, 37). Our finding revealed that Cyr61 regulates HIF-1α expression through both the PI3K

FIGURE 5. PAI-1 is involved in Cyr61-mediated gastric cancer cell invasion. A, Serum-starved AGS cells were incubated for indicated time in the presence of 40 ng/ml rCyr61, followed by chromatin immunoprecipitation assay, which was performed with an equal amount of RNA was used in this assay. B, PAI-1 expression was determined by Western blot and RT-PCR. GAPDH and α-tubulin were used as an internal loading control. C, Western blot analysis of the PAI-1 protein levels. The same methods were used in Cyr61-stably-transfected AGS cells, which were treated with 40 ng/ml rCyr61 and Cyr61 stably transfected AGS cells, respectively, were subjected to invasion assay. Each experiment was done in triplicate. Columns, mean of three experiments. *p < 0.05.

DISCUSSION

Members of the CCN family are multifunctional growth factors, and the nature of their regulation seems to be dependent on the cellular circumstance. Recent clinical evidence shows that Cyr61, one of CCN family members, is substantially involved in the development and progression of human malignancies. The significant importance of Cyr61 in human malignant alterations can be explained by its active regulation of multifaceted biological functions. For tumor invasiveness, Cyr61 can enhance motility of fibroblasts and microvascular endothelial cells. Recently, Nguyen et al. (28) have demonstrated that Cyr61 expression causes tumor cell migration and invasion in breast cancer cells, and their preliminary report indicated that gene silencing of Cyr61 in breast cancer cells suppressed matrix metalloproteinase-1 induction in stromal fibroblasts. However, the detailed mechanism by which Cyr61 enhances tumor invasiveness has not yet been characterized.

In the present study, we demonstrate a novel function of Cyr61 in gastric cancer cell invasion by molecular dissection. This study demonstrated, for the first time, that Cyr61 is able to up-regulate HIF-1α through de novo protein synthesis. This up-regulation of HIF-1α by Cyr61 commonly occurs in a variety of gastric cancer cell lines, involving PI3K/AKT/mTOR and ERK1/2 signaling pathways. Furthermore, we decipher the molecular mechanism for Cyr61-enhanced cell invasion, which is mediated by HIF-1α-dependent PAI-1 up-regulation.

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A

| AGS/Neo | AGS/Cyr61 |
|---------|-----------|
| DN-HIF-1α (μg) | 3 | 0 | 0 |
| pCEP4 (μg) | 3 | 3 | 0 |
| PAI-1 | 0 | 1 | 0 |
| GAPDH | 0 | 0 | 0 |

B

![Diagram of HIF-1α expression and PAI-1 up-regulation](Image)

FIGURE 6. Expression of Cyr61 increases the HIF-1α-dependent invasion protein PAI-1. A, Cyr61-regulated PAI-1 expression via the HIF-1α pathway. AGS/Neo and AGS/Cyr61 cells were transiently transfected with pCEP4-DN-HIF-1α, and analyzed by RT-PCR (upper panel) and Western blot (lower panel). B, coimmunoprecipitation of HIF-1α on the PAI-1 promoter was done in Cyr61 stably transfected AGS cells and AGS cells treated with 40 ng/ml rCyr61 for 16 h. Immunoprecipitation using HIF-1α-specific antibody. Primers for the upper panel were for the human PAI-1 promoter were used for PCR.

and the MAPK cascades. MAPK phosphorylation of p70S6K and 4E-BP1 is a critical step downstream of the PI3K/AKT pathway. The phosphorylation of p70S6K results in the release of the eukaryotic initiation factor 4E (eIF4E) and the phosphorylation of p70S6K results in the activation of the 40 S ribosomal protein. The ultimate outcome of all these events is the stimulation of protein synthesis. We found that MAPK is not involved in the phosphorylation of 4E-BP1 and p70S6K in response to Cyr61. This is in contrast to the previous study in which IGF-1 stimulates phosphorylation of p70S6K and 4E-BP1 through both PI3K- and MAPK-dependent pathways in colon cancer cells (38). We propose that MAPK regulates downstream of 4E-BP1 in response to Cyr61 in gastric cancer cells. Indeed, it has been shown that MnK1 (MAPK signal integrating kinase-1) phosphorylates eIF4E leading to increased protein synthesis (39).

Studies to uncover the signaling pathways involved in non-hypoxic HIF-1α activation revealed accumulation and transactivation of not only MAPKs and PI3K/AKT kinases but also NF-κB (40). Our previous studies suggest that the Cyr61 elevates functional COX-2 via an integrin αvβ3/NF-κB-dependent pathway in gastric cancer cells. Under such a scenario, we suggested that Cyr61 and IGF-1 influence HIF-1α up-regulation through quite distinct pathways. Effect of IGF-1 on HIF-1α accumulation is mediated directly through MAPKs or PI3K/AKT signaling pathways (37, 38, 41). However, the effect of Cyr61 on HIF-1α expression is very complex, and this regulation might involve not only MAPKs or PI3K/AKT pathways but also NF-κB/COX-2-related pathways. To support this, our preliminary data showed that celecoxib, a COX-2-specific inhibitor, inhibits Cyr61-induced HIF-1α elevation, indicating that COX-2 may have an intermediate role in regulating Cyr61-induced HIF-1α expression. This observation could account for the different kinetic induction patterns of HIF-1α in response to IGF-1 and rCyr61 treatments in AGS cells (Fig. 1F).

PAI-1, a member of the serpin family, has shown that its high expression in various cancers correlates with unfavorable prognosis (42–44). It has been initially suggested that increased PAI-1 expression in tumors may reflect a general up-regulation of the plasminogen activator system in proliferating cancer cells and a stromal reaction aimed at limiting excessive degradation of the extracellular matrix in more aggressive and invasive cancer forms (45). This issue is further strengthened in our current study where oligonucleotide antisense PAI-1 treatment not only reduced Cyr61-induced elevated PAI-1 protein but also significantly suppressed suppression of PAI-1 expression in AGS cells (46). As a result, PAI-1 expression in tumors could reflect a general up-regulation of PAI-1 in tumors (45). Concomitantly, these results suggested that PAI-1 may be a critical factor in regulating the invasion of human gastric cancer cells, which is up-regulated by Cyr61. We also investigated how the decreased cell invasion in human fibrosarcoma cancer cells (48). Concomitantly, these results also provided evidence that PAI-1 may be a critical factor in regulating human gastric cancer cells, which are up-regulated by Cyr61. We also investigated the expression and regulation of PAI-1 in these cells. Our results are consistent with the results of others that PAI-1 plays a critical role in Cyr61-enhanced gastric cancer invasion via HIF-1α.

It appears to be contradictory that PAI-1 is required for invasion, because elevated PAI-1 should reduce plasmin generation, whereas invasion requires cooperation between both proteolytic and inhibitory activities, probably to control and confine the areas of invasive growth. It has been suggested that the role of PAI-1 may be merely to shield the tumors from ongoing urokinase-type plasminogen activator-mediated proteolysis (52). More recent studies support the role of PAI-1 in promoting invasion and metastasis (48, 53–56). PAI-1 specifically binds to the somatotropin domain of vitronectin and competes with and inhibits urokinase-type plasminogen activator receptor-mediated cell attachment to vitronectin (15). Vitronectin-bound PAI-1 is still able to bind urokinase-type plasminogen activator, and the resulting complex has a highly reduced affinity for vitronectin and allows the migratory process to occur.

In conclusion, we have shown that Cyr61 activates the transcription factor HIF-1α protein synthesis through PI3K/AKT/mTOR and ERK1/2 signaling pathway in AGS cells. Aug-
Involvement of HIF-1α-dependent PAI-1 Up-regulation in Cyr61/CCN1

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Supplementary Information

Materials and Methods

Transient and Stable Transfections

The cloning process of cyr61 expression plasmid was described previously (10). Briefly, total RNA was extracted and Cyr61 complementary DNA was cloned and amplified by RT-PCR with the primer with cloning sites 5’-TATAGGATCCGAATTCATGAGCTCCCGCATCGCC-3’ (forward) and 5’-TATAGCGGCCGCTTAGTGGTGGTGGTGGTGGTGGTGGTCCCTAAATT TGTGAATG-3’ (reverse) subcloned into a pcDNA3 vector (Invitrogen, San Diego, CA) in forward (sense) or reverse (anti-sense) direction. pCMV-Cyr61, a constitutive expression vector, carries 1.14-kb full-length human Cyr61 cDNA under control of the CMV promoter. AGS cells were transfected with pCMV-Cyr61 or control pcDNA3 vector (GIBCO Invitrogen, Grand Island, NY, USA) containing a CMV promoter and a neomycin selection marker, using the TransFast™ transfection reagent (Promega, Madison, WI, USA). Twenty-four hours after transfection, the cells were serum-starved for 16 h and lysed for RT-PCR and Western blot. For stable cell population selection, twenty-four hours after transfection, cells were replated in RPMI-1640 (GibcoBRL, Rockville, MD, USA) with 10% (vol/vol) fetal calf serum (FCS) and 800 μg/ml G418 (Sigma, St. Louis, MO, USA). G418-resistant clones were
selected and expanded. The mRNA and protein levels of Cyr61 in these cells were checked by RT-PCR and Western blot analysis. AGS cells transfected with control vector (AGS/pcDNA3) served as control. AGS/Cyr61 and AGS/pcDNA3 cells were grown at 37°C and 5% CO₂ in RPMI-1640 medium supplemented with 10% FCS and 100 μg/ml G418.

**RNA Isolation and Reverse Transcriptase-PCR**

Total RNA was isolated by using Trizol reagent (Invitrogen Life Tech) according to the manufacturer's instructions. Total RNA (3 µg) was reverse transcribed into single-stranded cDNA using a Moloney murine leukemia-virus reverse transcriptase and random hexamers (Promega, Madison, WI). The cDNAs were amplified with the forward (F) and reverse (R) primers by PCR as described. The primer sequences for CYR61 were 5'- CAG GGT GGA GTT GAC GAG AAA C -3' (F) and 5'- AGG ACT GGA TAT CAT GAC GTT CT -3' (R). The primer sequences for HIF-1α were 5'- CAG CTA TTT GCG TGT GAG GA -3' (F) and 5'- CCA AGC AGG TCA TAG GTG GT -3' (R). The primer sequences for PAI-1 were 5'- TCG TCC AGC GGG ATC TGA -3' (F) and 5'- CCT GGT CAT GTT GCC TTT C -3' (R). The primer sequences for c-MET were 5'- GGA AAC ACC CAT CCA GAA TGT CAT T -3' (F) and 5'- TGA TAT CGA ATG CAA TGG ATG ATC T -3' (R). The primer sequences
for AMF were 5'- ATG GCC AGC ATG CTT TTT AC -3' (F) and 5'- GGT AGA AGC GTC GTG AGA GG -3'(R). The primer sequences for HGF were 5'- TGG ATG CAC AAT TCC TGA AA -3' (F) and 5'- TTG TAT TGG TGG GTG CTG CA -3' (R).

The primer sequences for ADM were 5'- GGG TAG CTG CTG GAC ATC CG -3' (F) and 5'- GTA GCC CTG GGG GCT GAT CT -3'(R). Primers were used at a final concentration of 0.5 µM. Reaction mixture was first denatured at 95°C for 10 min.

The PCR conditions applied were 30 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, followed by 72°C for 10 min. PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

**Western Blot Analysis**

Cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, 1 mM EGTA, 1 mM PMSF, 1 µg/ml leupeptin, and aprotinin), cleared by centrifugation for 20 min at 4 °C, and the supernatant collected. Protein (20–50 µg) was loaded onto 8–12% gradient SDS-PAGE gels, separated, and transferred onto polyvinylidene difluoride Immobilon membranes. The membranes were blocked with 5% milk and incubated with the appropriate primary antibody. After washing, the membranes were
stained with the correct secondary antibody. Protein bands were visualized by chemiluminescent detection (ECL) (Amersham Biosciences).

_HIF-1α reporter Activity Assay_

For transfections, cells were seeded in 6-well plates. After reaching about 70% confluence, the cells were transfected with pGL2 vector, Hypoxia response element (HRE), using TransFast™ (Promega). After transfection, the medium was replaced by fresh normal growth medium, and the cells were incubated for 24 h. After starvation in serum-free medium for 16 h, the cells were harvested, and the luciferase activity was measured with the Promega Dual-Luciferase Reporter Assay system (Promega) as described by the manufacturer, using a Turner Designs model TD-70/20 Luminometer (Jimenez et al., 1999).

_Immunofluorescence Staining_

Cells were cultured to 60 to 80% confluence on degreased glass coverslips in regular culture medium and then subjected to starvation for 16 h, after which cells were treated with Cyr61 (40µg/mL) for a period of 8 hr and 24 hr. Cells were then fixed in methanol/acetic acid [3:1 (v/v)] for 30 min at 4°C. After this, these cells were then rinsed and blocked for one hour in 5% fetal bovine serum at room temperature. The
cells were then incubated with anti-HIF-1α monoclonal antibody (BD Biosciences) at 4°C overnight, after which cells were washed in PBS and then incubated with a secondary fluorescein isothiocyanate-conjugate antibody (1:100, Sigma) and DAPI (1:10000) for 1h at room temperature. After extensive washing, the coverslips were inverted onto glass slides using Mowiol (Calbiochem) as a mounting medium. The slides were examined with a fluorescent microscope.

**Boyden chamber assay**

Invasion assays were done using modified Boyden chambers inserts for 24-well dishes containing 8-µm pores (Nucleopore Corp., Pleasanton, CA). Matrigel (30 µg, Collaborative Biomedical, Becton Dickinson Labware, San Jose, CA)–coated filters were used. Cells (2.5 x 10⁴) were plated into 100 µL of complete RPMI in the upper chamber, and the lower chamber was filled with 1 mL of RPMI. After 48 hours in culture, cells were fixed in methanol for 15 minutes and stained with 0.05% crystal violet in PBS for 15 minutes. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed in PBS. Cells on the underside of the filters were viewed and counted under a microscope (type 090-135.001, Leica Microsystems, Wetzlar, Germany). Each clone was plated in triplicate in each experiment and each experiment was repeated at least thrice.
Supplementary Figure 1

Proposed invasive molecular mechanisms of HIF-1α-mediated PAI-1 expression in Cyr61-overexpressing cells.
Supplementary Fig 2

In order to verify the specificity of reducing PAI-1 or Cyr61 activity, a siRNA approach was done as the following:

Fig 2A. the sequence of siRNA to PAI-1 or Cyr61

siRNA sequence

siCyr61
(RNA) UAA AGG GUU GUA UAG GAU GCG AGG C
(RNA) GCC UCG CAU CCU AUA CAA CCC UUU A

siPAI-1
(RNA) AUA GCU GCU UGA AUC UGC UGC UGG G
(RNA) CCC AGC AGA UGC AUC AAU CAG CUA U

Fig 2B. Transient transfection of scramble or siRNA to Cyr61 into TSGH gastric cancer cells, which constitutively expressing Cyr61. The protein expression of Cyr61 and its downstream target HIF-1α were specifically reduced by siRNA to Cyr61 (Lane 4).
Fig 2C. Transient transfection of scramble or siRNA to PAI-1 into TSGH gastric cancer cells, which constitutively expressing PAI-1. *(Upper)* The protein expression of PAI-1 was specifically reduced by SiRNA to PAI-1 (Lane 4) and *(lower)* the invasiveness of TSGH was also attenuated significantly (※ P values of <0.05).

Fig 2D. Transient transfection of scramble or siRNA to PAI-1 into AGS/Cyr61 gastric cancer cells, which constitutively expressing PAI-1. *(Upper)* The protein expression of PAI-1 was specifically reduced by SiRNA to PAI-1 (Lane 4) and *(lower)* the invasiveness of AGS/Cyr61 was also attenuated significantly (※ P values of <0.05).
Inhibitor-1 Up-regulation in Cyr61/CCN1-induced Gastric Cancer Cell Invasion-dependent Plasminogen Activator α Involvement of Hypoxia-inducing Factor-1α-dependent Plasminogen Activator Inhibitor-1 Up-regulation in Cyr61/CCN1-induced Gastric Cancer Cell Invasion

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J. Biol. Chem. 2008, 283:15807-15815.
doi: 10.1074/jbc.M708933200 originally published online April 1, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M708933200

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December 7, 2016