ECRG2 Regulates Cell Migration/Invasion through Urokinase-type Plasmin Activator Receptor (uPAR)/β1 Integrin Pathway**†‡.

Xiaolong Cheng‡, Zheng Shen‡, Litian Yin‡, Shih-Hsin Lu†, and Yongping Cui**‡‡†

From the ‡Department of Anatomy, Shanxi Medical University, Taiyuan, Shanxi 030001, China, the ‡Department of Molecular Pharmacology and Physiology, University of South Florida Health Sciences Center, Tampa, Florida 33612, the ‡Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Institute of Biotechnology, Shanxi University, Taiyuan, Shanxi 030006, China, the §Department of Etiology and Carcinogenesis, Cancer Institute, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100021, China, and the **Key Laboratory of Cellular Physiology, Ministry of Education, and the ††Department of Cell Biology and Genetics, Shanxi Medical University, Taiyuan, Shanxi 030001, China

ECRG2 is a novel gene that shows sequence similarity to KAZAL-type serine protease inhibitor. We have previously demonstrated that ECRG2 inhibits migration/invasion of lung cancer PG cells. However, the mechanism by which ECRG2 performs these activities is a compelling question. Urokinase-type plasmin activator (uPA) binding to uPAR induces migration/invasion through multiple interactors including integrins. In this study, we found that ECRG2 binds specifically to the kringle domain of uPA. Moreover, we demonstrated that ECRG2 forms a complex with uPA-uPAR, that such a complex modifies the dynamical association of uPAR with β1 integrins, and that disruption inhibits Src/MAP (mitogen-activated protein) kinase pathway, resulting in suppression of cell migration/invasion in an in vitro Matrigel migration/invasion assay. Conversely, depletion of ECRG2 markedly enhanced the association of uPAR with β1 integrins, elevated basal Src/MAP kinase activation, and stimulated HT1080, MDA-MB-231, and MCF-7 cell migration/invasion. Together, our results provide evidence that ECRG2 is involved in the regulation of migration/invasion through uPA/uPAR/β1 integrins/Src/MAP kinase pathway and may represent a novel therapeutic target for cancer.

Tumor cells must invade through the adjacent basement membrane into surrounding tissues and then migrate to and invade the vasculature to metastasize to distant sites (1). The processes of tumor cell migration and invasion involve a dynamic interaction between the tumor cells and the extracellular matrix and are regulated by multiple cytokines and growth factors, integrins, cell-cell adhesion molecules/communication, matrix-degrading enzymes, and loss of activity of degradative enzyme inhibitors (2). The esophageal cancer-related gene 2 (ECRG2) is a novel gene that is highly expressed in adult esophageal mucosa, brain, thyroid, mouth epithelia, fetal skin, and thymus and poorly expressed in the fetal esophagus, brain, lung, heart, stomach, liver, spleen, colon, kidney, testis, and muscle tissue. Reverse transcription-PCR and Northern blot results showed that the ECRG2 gene was expressed in normal esophagus, liver, colon, and lung tissues but was down-regulated in the adjacent and cancerous tissues, especially with low frequency in esophageal cancer (3). The ECRG2 gene contains a characteristic secondary structure of a KAZAL-type conserved domain and is a novel member of the KAZAL-type-related serine protease inhibitor family (4). The serine protease inhibitor (serpin) superfamily includes inhibitors of a number of serine proteases with roles in a variety of cellular process, including cell migration and adhesion, preventing tumor metastasis (5). Some serpins have biological activities independent of protease inhibition. For example, PAI-1 is a specific inhibitor and regulator of the serine proteases urokinase-type and tissue-type plasminogen activator, modulating cell adhesion and migration (6). Other serpins lack intrinsic inhibitory activity. Examples of this are ovalbumin, thyroid-binding globulin (SERPINA6), angiotensinogen (SERPINB8), and pigment epithelium-derived factor (SERPINF1), which has neurotrophic and antiangiogenic activity (7, 8). Maspin (SERPINB5) is thought to be another non-inhibitory serpin and inhibits cell migration in the absence of detectable protease inhibitory activity (9). In our previous experiments, the ECRG2 gene was shown to reduce the migration/invasion of PG cancer cells and suppress metastases in nude mice (10). In addition, we showed that there is a direct interaction between ECRG2 and the urokinase-type plasminogen activator (uPA) (10). Thus, our previous studies provide evidences that the ECRG2 gene plays an important role in the prevention of tumor cell migration and invasion, possibly through the uPA. However, little is known about how ECRG2 regulates the cellular response to uPA binding.

The uPA-uPA receptor (uPAR) system has been shown to play a critical role in the regulation of cancer cell migration, extracellular matrix invasion, and metastasis (11). uPA binds with high affinity to a cell surface uPAR (12). uPAR is a heavily

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** This work was supported by National Nature Science Foundation of China Grants 30971518 and 30500588 and by the Program for the Top Young Academic Leaders of Higher Learning Institutions of Shanxi (to Y. C.).
† The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–6.
‡ To whom correspondence should be addressed: Dept. of Cell Biology and Genetics, Shanxi Medical University, Taiyuan, Shanxi 030001, China. E-mail: cuiy0922@yahoo.com.

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* The abbreviations used are: uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; FAK, focal adhesion kinase; PTK2, phosphatidylinositol 3-kinase; DMSO, dimethyl sulfoxide; PP2, protein phosphatase 2A; siRNA, small interfering RNA; shRNA, short hairpin RNA; UTR, untranslated region; P, phosphorylated; SCR, scrambled.
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glycosylated glycosylphosphatidylinositol-anchored protein formed by three cysteine-rich LY6-like extracellular domains (LU domains D1, D2, and D3) (13). There are three basic steps involved in migration/invasion and intracellular signaling. (a) uPAR-uPAR promotes extracellular proteolysis by regulating plasminogen activation; (b) uPAR-uPAR regulates cell-extracellular matrix interactions as an adhesion receptor for vitronectin and through its capacity to modulate integrin function; and (c) uPAR-uPAR regulates cell migration as a signal transduction molecule and by its intrinsic chemotactic activity (14). One important mechanism through which uPAR directs these actions is by complexing with other membrane proteins (e.g. integrins) for signal transduction (15, 16). It has also become clear that uPAR complexes can transduce intracellular signals (17). Several groups have reported that the binding of uPAR to uPAR stimulates intracellular signaling, and much of this signaling is consistent with an integrin-mediated pathway (18). Integrins are known to activate the PI3K, Src, and extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, and recent studies have shown that the higher activation of FAK is associated with increased cell motility and cytoskeletal changes (19–21).

In this study, we have investigated the mechanism by which ECRG2 regulates the fibrosarcoma HT1080, breast cancer MDA-MB-231, and MCF-7 cell migration/invasion. We demonstrate that the direct binding of ECRG2 to uPAR present in the uPAR-uPAR complex disrupts the association of uPAR with β1 integrins, leading to reduced activation of the Src/MAP kinase pathway, resulting in abatement of uPAR signaling through the uPAR-β1 integrins complex. In contrast, depletion of ECRG2 enhanced association of uPAR-β1 integrins, increased Src/MAP kinase activity, and promoted cell migration/invasion. Thus, our results reveal an important role of the ECRG2 gene in suppression of cancer cell migration/invasion and shed new light on how ECRG2 participates in the regulation of cancer cell migration/invasion.

EXPERIMENTAL PROCEDURES

Reagents—Monoclonal anti-ECRG2 antibody was produced by our laboratory (22, 23). Human uPA and uPAR monoclonal antibodies were purchased from American Diagnostica (Greenwich, CT). Monoclonal antibodies to integrin α3 (P1B5), α5β1 (HA5), αvβ3 (LM609), and polyclonal anti-β1 (AB1937) integrin were obtained from Chemicon International (Temecula, CA). The monoclonal antibodies to FAK kinase and phospho-FAK were obtained from Transduction Laboratories (Lexington, KY). Polyclonal anti-phospho-ERK, anti-phospho-Src, and total Src antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rabbit P13K and p-P13K antibody were purchased from Abcam (Cambridge, MA). The phospho-ERK inhibitor PD98059 and P13K inhibitor wortmannin were purchased from Sigma-Aldrich. Inhibitor to Src family tyrosine kinases PP2 was from Calbiochem. Peptide α325 (PRHRHM-GAVFLLSQEAG) was synthesized by Quality Controlled Biochemicals (Framingham, MA).

Generation of Inducible ECRG2-Tet-on, ECRG2-Tet-on-3’UTR-si, ECRG2-Tet-on-3’UTR-si-uPA-KD, and ECRG2-Tet-
orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 
76 
50 
411
5 4 158 131 50 4
10 K D 25 K D 50 K D
Flag-uPA D1
Flag-uPA D2
Flag-uPA D3
Flag-uPA FL
GFD
GFD
Krinkle domain
Serine-protease domain

FIGURE 1. Identification of ECRG2-binding sequence in uPA. A, schematic representation of the human urokinase structure shows the N-terminal growth factor-like domain (GFD, residues 1–49), the kringle domain (residues 50–131), the CP region (residues 132–158), and the catalytic domain (residues 159–411). FLAG-tagged variant deletion mutants of human uPA are shown. B, FLAG-uPA full-length and variant deletion mutants were transiently transfected into HT1080 ECRG2-Tet-On cells. Cells were incubated in the presence of doxycycline (0.1 
75 kD
75 kD
50 kD
25 kD
10 kD
Flag-uPA FL
Flag-uPA D1
Flag-uPA D2
Flag-uPA D3
Flag-uPA FL
Flag-uPA D3
Flag-uPA D2
Flag-uPA D1
Flag-uPA FL
IgG ctrl
Flag-uPA IP
ECRG2 WB
ECRG2 (9KD)
IgG ctrl
Flag-uPA IP
ECRG2 WB
Flag-ubPA-FL
Flag-uPA D2
Flag-uPA D3
Flag-uPA FL

RESULTS
Identification of ECRG2-binding Sequence in uPA—Previous
observations have demonstrated that ECRG2 physically inter-
acts with uPA (10). uPA consists of two disulfide bridge-linked
polypeptide chains: the N-terminal polypeptide containing a
GFD, a kringle domain, and a linked or connecting peptide region
and a large C-terminal, serine protease polypeptide (21). In an effort
to characterize the binding domain of ECRG2 in uPA, several uPA
variants were generated as shown in Fig. 1A. FLAG-tagged full-
length human uPA (FLAG-uPA-FL) and three variants carrying
the uPAR-binding region growth factor-like domain (amino
acids 1–50), the kringle domain, and a linked or connecting peptide region and a large C-terminal, serine protease polypeptide (21). In an effort to charac-
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**FIGURE 2. The binding of ECRG2 to uPA forms an ECRG2-uPA-uPAR complex.** A, cell lysates from ECRG2-Tet-on-3' UTR-si cells in the absence of doxycycline were subjected to immunoprecipitation (IP) with anti-uPAR antibody or IgG (control (ctrl)) and Western blotting for analysis for uPA. B, cell lysates from ECRG2-Tet-on-3' UTR-si cells in the absence of doxycycline were subjected to immunoprecipitation with anti-uPAR antibody or IgG (control) and Western blotting for analysis for uPA and uPAR. C, cell lysates from ECRG2-Tet-on-3' UTR-si cells in the presence of doxycycline were subjected to immunoprecipitation with anti-uPAR antibody or IgG (control) and Western blotting for analysis for uPAR and uPA. D, cell lysates from ECRG2-Tet-on-3' UTR-si cells in the presence of doxycycline were subjected to immunoprecipitation with anti-uPAR antibody or IgG (control) and Western blotting for analysis for uPA and uPAR. E, cell lysates from ECRG2-Tet-on-3' UTR-si cells in the presence of doxycycline were subjected to immunoprecipitation with anti-uPA antibody or IgG (control) and Western blotting for analysis for ECRG2 and uPAR.

ECRG2 Regulates uPAR/β1 Integron Pathway—Having demonstrated that ECRG2 binds to uPA present in the uPA-uPAR complex on the cell surface, we further tested whether ECRG2 was likely to affect the direct interaction of uPA with integrins as they are well characterized transmembrane adaptor proteins for uPAR (2). Tet-On empty mutants (Fig. 1B). Immunoprecipitations were performed using anti-FLAG antibody. As shown in Fig. 1C, ECRG2 was observed in the FLAG-uPA-D2 deletion mutant and full-length uPA expression cells. No ECRG2 was seen in FLAG-uPA-D1 and D3 deletion mutant expression cells, although equivalent amounts of ECRG2 were expressed. In parallel experiments, FLAG-uPA-D2 and FLAG-uPA-FL were specifically detected in the ECRG2 immunoprecipitates (Fig. 1D). Similar results were observed in breast cancer cell lines MDA-MB-231 and MCF-7 (supplemental Fig. 2). These data suggest that the kringle and CP regions (50–158) are required for the association of uPA with ECRG2 in vivo.

**ECRG2 Does Not Impair the uPA-uPAR Complex Formation—** To explore whether ECRG2, which physically associates with uPA as described above, affects the direct binding of uPA to uPAR, ECRG2-Tet-on-3' UTR-si cells were cultured in the absence or presence of doxycycline for 24 h and subjected to immunoprecipitation. As expected, uPA-uPAR forms a complex in absence of doxycycline in ECRG2-Tet-on-3' UTR-si cells (Fig. 2A and B). We then examined the binding of uPA to uPAR in the presence of doxycycline in ECRG2-Tet-on-3' UTR-si cells. Cell lysates were immunoprecipitated with anti-ECRG2 antibody, and the resultant ECRG2-immunoprecipitate was probed for uPA and uPAR (Fig. 2C). Similar experiments were performed using either immunoprecipitation with anti-uPAR antibody and Western blotting with anti-ECRG2 and anti-uPA antibodies (Fig. 2D) or immunoprecipitation with uPA antibody and Western blotting with anti-ECRG2 and anti-uPAR antibodies (Fig. 2E). uPA and uPAR were detected in ECRG2 immunoprecipitate (Fig. 2C). Similar patterns were obtained from uPAR immunoprecipitation (Fig. 2D) and uPA immunoprecipitation (Fig. 2E) experiments. We obtained very similar results in MDA-MB-231 and MCF-7 cells (supplemental Fig. 3), indicating that ECRG2 does not disrupt the association of uPA with uPAR and forms an ECRG2-uPA-uPAR complex in culture cells.

uPAR Fails to Complex with α3β1 and α5β1 Integrins in ECRG2 Expression Cells—Having demonstrated that ECRG2 binds to uPA present in the uPA-uPAR complex on the cell surface, we further tested whether ECRG2 was likely to affect the direct interaction of uPA with integrins as they are well characterized transmembrane adaptor proteins for uPAR (2). Tet-On empty
formation of the ECRG2-uPA-uPAR complex impairs the binding ability of uPAR to α3β1 and α5β1 integrins but not αvβ5 integrin.

ECRG2 Regulates the Association of uPAR with β1 Integrins through uPA—The above data indicate that binding of ECRG2 to uPA present in the uPA-uPAR complex on the cell surface alters uPAR conformation and changes its matrix ligand binding properties. To probe this idea further, ECRG2-Tet-On-3’UTR-si cells were infected with pSUPER.retro.puro-uPA containing a small siRNA targeting uPA previously shown to suppress uPA mRNA (ECRG2-Tet-On-3’UTR-si-uPA-KD) (24) or pSUPER.retro.puro-LacZ (scramble control) and incubated in the presence or absence of doxycycline. The suppression or expression of uPA and ECRG2 was verified 24 h later by Western blotting (Fig. 4A). Cell lysates were immunoprecipitated by anti-uPAR antibody and Western blotting by anti-ECRG2, anti-α3β1, or anti-α5β1 antibody, respectively. Consistent with our data described above, ECRG2 was detected in uPAR immunoprecipitates in scramble control cells (Fig. 4B, right panel, second lane). However, the association of ECRG2 with uPAR was completely blocked in uPA depletion cells (Fig. 4B, right panel, third lane), confirming that ECRG2 associated with uPAR through uPA. Moreover, further analyses of interaction between uPAR and integrins show that ECRG2 expression compromises the interaction of uPAR with α3β1 (Fig. 4C, right panel, second lane) and α5β1 (Fig. 4D, right panel, second lane) in scramble control cells, consistent with the results described above. However, α3β1 (Fig. 4C, right panel, third lane) and α5β1 (Fig. 4D, right panel, third lane) were found in uPAR immunoprecipitates in uPA-si cells even when ECRG2 was expressed. These results confirm that uPAR forms complexes with β1 integrins when ECRG2 was depleted, and these associations were completely blocked when ECRG2 was expressed in the presence of uPA. However, the inhibition function of ECRG2 on interaction of uPAR with β1 integrins was compromised when uPA was depleted. Together, these data confirm our finding that ECRG2, through its direct interaction with uPA, disrupts the binding ability of uPAR to β1 integrins.

Src/MAP Kinase, but Not FAK and PI3K, Is Involved in ECRG2-regulated, uPA-dependent Cell Migration/Invasion—uPA-uPAR regulates cell migration as a signal transduction molecule and by its intrinsic chemotactic activity (13–15). To explore a molecular pathway by which ECRG2 influences migration and invasion through disrupting the physical association between uPAR and β1 integrins, HT1080 Tet-On empty vector and shRNA control cells or ECRG2-Tet-On-3’UTR-si cells were cultured in the presence or absence of doxycycline for 24 h. Cells were collected and subjected to Western blotting to probe the normal and phosphorylation forms of second messenger pathways known to be activated by integrin signaling.
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**FIGURE 4.** ECRG2 disrupts the interaction of uPAR with integrins through uPA. A, ECRG2-Tet-on-3′ UTR-si cells were transfected with either an siRNA previously shown to suppress uPA mRNA or a scramble control siRNA and incubated in the presence or absence of doxycycline for 24 h. Suppression of uPA expression and induction of ECRG2 expression were verified by Western blotting. B–D, cells were then immunoprecipitated (IP) with anti-uPAR antibody or IgG (control (ctrl)) and subjected to Western blotting (WB) using anti-ECRG2 (B), anti-α3β1 (C), or anti-α5β1 (D) antibodies. The efficiencies of immunoprecipitations were also shown by Western blotting with anti-uPAR antibody.

ECRG2 Regulates uPA-dependent Cell Migration/Invasion through β1 Integrin/Src/MAP Kinase Pathway—Our results indicate that the signal response to ECRG2 in cell migration/invasion is achieved through the α3β1 or α5β1 integrin pathway, and Src/MAP kinase is involved in ECRG2-regulated cell migration/invasion. We hypothesize that ECRG2 regulates cell migration/invasion mainly through the uPA/uPAR/α3β1 or α5β1 integrins/Src/MAP kinase pathway. To test this possibility, we depleted β1 integrin in ECRG2-Tet-on-3′ UTR-si cells by using the siRNA retrovirus infection approach (ECRG2-Tet-on-3′ UTR-si-β1-KD). Cells were cultured in the presence or absence of doxycycline for 24 h, and the depletion of β1 integrin was confirmed by Western blotting analysis (Fig. 6A). As shown in Fig. 6B, ECRG2 depletion significantly increased, whereas overexpression decreased the phosphorylation forms of Src and MAP kinase in scramble control cells. However, the phosphorylation forms of these kinases remained more or less unchanged in β1 integrin-depleted cells, indicating that ECRG2 loses the ability to regulate Src/MAP kinase activity when β1 integrin was depleted. To evaluate the effect of β1 integrin depletion on ECRG2-regulated cell migration, Transwell migration assays were performed. We used a synthetic peptide α325, a 17-mer derived from the α3 integrin sequence shown to block uPAR-α3β1 and uPAR-α5β1 interaction (2), as a positive control. Scramble control or ECRG2-Tet-on-3′ UTR-si-β1-KD cells were analyzed for their ability to migrate. As expected, cells showed a substantially higher basal migration when ECRG2 was depleted in scramble control cells, and the addition of α325 strongly suppressed the migration. No migration was observed in the presence of doxycycline in scramble control cells. However, the cell migration remained unchanged no matter with or without ECRG2 expression in β1-depleted cells (Fig. 6C), indicating that β1 integrin is necessary for ECRG2-regulated cell migration. Similar results were observed in a cell invasion assay as shown in Fig. 6D. Thus, the complex of ECRG2-uPA-uPAR suppresses cell migration/invasion by abolishing the interaction of uPAR with α3β1 and α5β1 integrins and subsequently inhibiting the downstream Src/MAP kinase pathway. To further confirm this, we overexpressed FLAG-uPA D2 to disrupt the effect of ECRG2 on the uPA-uPAR-integrin complex and then examined the p-Src and p-ERK levels and cell migration/invasion. As shown in Fig. 7, the expression of ECRG2 inhibited the p-Src and p-ERK levels as described above (third lane). However, overexpression of

(Src, MAP kinase, PI3K, and FAK) (19–21). As shown in Fig. 5A, total Src expression remained unchanged, whereas the level of p-Src increased in the absence of doxycycline and decreased in the presence of doxycycline in ECRG2-Tet-on-3′ UTR-si cells when compared with that of controls. Similar results were obtained for the expression of ERK and p-ERK. In the case of PI3K and FAK, total and phosphorylation forms of PI3K and FAK showed similar levels with or without ECRG2 expression, indicating that the ECRG2-uPA-uPAR complex retards the phosphorylation of Src and MAP kinase but not PI3K and FAK. To further confirm this, ECRG2 depletion cells were exposed to various inhibitors of kinases or the same volume of DMSO for 6 h before being subjected to the cell migration assay. Fig. 5B confirmed that all the inhibitors effectively inhibited the kinase activity. As shown in Fig. 5C, cells exposed to inhibitors of PI3K (wortmannin, 100 nM) or FAK inhibitor (TAE226, 100 nM) maintained a similar migration with ECRG2-depleted cells, indicating that their activity did not account for the enhanced migration caused by ECRG2 depletion. In contrast, a specific inhibitor of Src family kinases (PP2, 1 μM) and a specific inhibitor of MAP kinase (PD98059, 10 μM) were found to reverse the enhanced migration of ECRG2 depletion cells, indicating that Src/MAP kinase was involved in cell migration regulated by ECRG2. Similar patterns were observed in cell invasion assays, as shown in Fig. 5D. We observed similar results in MDA-MB-231 and MCF-7 cells (supplemental Fig. 5).
FIGURE 5. Involvement of Src/MAP kinases in ECRG2-regulated, uPA-induced cell migration. A, ECRG2-Tet-On-3’ UTR-si and Tet-On empty and shRNA control cells were cultured in the presence or absence of doxycycline (Dox) for 24 h and treated with uPA (10 nM) for 1 h before being collected, and then total cell lysates were prepared, fractionated by SDS-PAGE, and subjected to Western blotting for normal and phosphorylated forms of Src, ERK, PI3K, and FAK. Actin level served as a loading control. B, ECRG2–3’ UTR-si cells were incubated with src kinase inhibitor PP2, MAP kinase inhibitor PD98059, PI3K kinase inhibitor wortmannin, or FAK kinase inhibitor TAE226 in the medium contained uPA (10 nM), respectively. The same volume of DMSO was used as control. The inhibitory activity of inhibitors was verified by Western blotting. C, ECRG2–3’ UTR-si cells and scrambled (SCR) control (Tet-on empty and shRNA control) cells were induced to migrate in the presence of inhibitors or same volume of DMSO. Random cell migration of non-pretreated cells is referred to as 100% of migration. Results are representative of three independent experiments. D, A Transwell invasion assay was done in ECRG2–3’ UTR-si cells and SCR control (Tet-on empty and shRNA control) cells in the presence of inhibitors or the same volume of DMSO. An equal volume (0.5 ml/chamber) of cell suspensions (2 × 10^4 cells/ml) was seeded in Matrigel invasion chambers and incubated at 37 °C for 48 h. The number of cells that invaded through the Matrigel was quantified by counting cells from 10 microscopic fields. Columns, means of 20 high power fields from two independent experiments; error bars, S.D.
brane protein have now been identified as mediators of uPAR, and they are involved in signaling in response to uPA: integrins, G-protein-coupled receptors, and caveolin. Several different lines of evidence indicate that uPAR can dynamically and functionally interact with multiple integrins including $\alpha_\beta_1$, $\alpha_\beta_2$, $\alpha_\beta_3$, and $\alpha_\beta_5$ integrins and control their activation, which contributes to migration and invasion (28).

Among $\beta_1$ integrins, uPAR directly associates with $\alpha_3\beta_1$ via a surface loop within the $\beta$-propeller (29). uPAR is also able to associate with $\alpha_5\beta_1$ and to regulate $\alpha_5\beta_1$-mediated cell migration (30). Other integrins such as $\alpha_\omega\beta_3$ were also involved in the association with uPAR (2, 21). The interaction of integrins to uPAR is not constitutive, but it is dynamically regulated by conformational changes in integrin extracellular domains.

FIGURE 6. $\beta_1$ integrin is necessary for ECRG2-dependent promotion or suppression of cell migration. A. ECRG2-Tet-on-3′ UTR-si cells were infected with $\beta_1$-integrin siRNA retrovirus (ECRG2-Tet-On-3′ UTR-si-$\beta_1$-KD) or scramble control siRNA (SCR control). After 48 h, cells were subjected to Western blotting for suppression of $\beta_1$-integrin. Actin was used as loading control. shRNA ctrl, shRNA control. B. ECRG2-Tet-On-3′ UTR-si-$\beta_1$-KD cells or SCR control cells were incubated with or without doxycycline (Dox) for 24 h and subjected to Western blotting for phosphorylation and total forms of Src and ERK. The expression of ECRG2 and depletion of $\beta_1$-integrin were also shown. C. 48 h after infection, SCR control (Tet-on empty and shRNA control) cells or ECRG2-Tet-On-3′ UTR-si-$\beta_1$-KD cells were seeded in Matrigel migration chambers cultured with or without doxycycline. $\alpha_325$, a positive control, was added to ECRG2-Tet-On-3′ UTR-si cells in the absence of doxycycline for 20 min at 37 °C. Migrated cells were counted. Data are expressed as mean ± S.D. from three independent experiments, each in triplicate. Random cell migration of non-pretreated cells is referred to as 100% of migration. D. A Transwell invasion assay was done in SCR control (Tet-on empty and shRNA control) cells or ECRG2-Tet-On-3′ UTR-si-$\beta_1$-KD cells in the presence or absence of doxycycline. $\alpha_325$, a positive control, was added to ECRG2-Tet-On-3′ UTR-si cells in the absence of doxycycline for 20 min at 37 °C. Equal volume (0.5 ml/chamber) of cell suspensions ($2 \times 10^5$ cells/ml) was seeded in Matrigel invasion chambers and incubated at 37 °C for 48 h. The number of cells invaded through the Matrigel was quantified by counting cells from 10 microscopic fields. Columns, means of 20 high power fields from two independent experiments; error bars, S.D.
lular domains, by intracellular signal transduction pathways, and by the interaction of integrins with neighboring membrane-associated proteins, including integrin-associated protein, caveolin, and uPAR (16, 31). Our present studies have focused on the regulation of integrin function by uPAR. Surprisingly, the α3β1 and α5β1 integrins were completely lost in uPAR immunoprecipitates when ECRG2 was overexpressed. In contrast, the protein levels of α3β1 and α5β1 integrins in uPAR immunoprecipitate were significantly increased in ECRG2 depletion cells (Fig. 3 and supplemental Fig. 4), indicating that the binding of ECRG2 to uPA disrupts the association of the uPA-uPAR complex with α3β1 and α5β1 integrins. The block function of uPAR-α3β1 or uPAR-α5β1 interaction disappeared no matter whether ECRG2 was depleted or overexpressed when uPA was knocked down using the siRNA approach (Fig. 4), confirming that ECRG2 regulates the association of uPAR with integrins through uPA. The binding of uPA to uPAR induces conformational changes in uPAR, which promotes its interaction with a variety of integrins (25). The active molecular complex seems to be a trimeric uPA-uPAR-integrin complex. Our results indicate that ECRG2 modifies uPAR through binding to the uPA-uPAR-integrin complex, and such modification blocks the dynamical association of uPAR with integrins through uPA. The binding of uPA to uPAR induces conformational changes in uPAR, which promotes its interaction with a variety of integrins (25). The active molecular complex seems to be a trimeric uPA-uPAR-integrin complex. Our results indicate that ECRG2 modifies uPAR through binding to the uPA-uPAR-integrin complex, and such modification blocks the dynamical association of uPAR with integrins through uPA. The binding of uPA to uPAR induces conformational changes in uPAR, which promotes its interaction with a variety of integrins (25). The active molecular complex seems to be a trimeric uPA-uPAR-integrin complex. Our results indicate that ECRG2 modifies uPAR through binding to the uPA-uPAR-integrin complex, and such modification blocks the dynamical association of uPAR with integrins through uPA.

The uPAR-integrin interaction has been shown to transduce intracellular signals and plays critical roles in cell invasion and migration. Integrins are known to activate the Src, ERK/MAPK, FAK, and PI3K pathways (13). Our results demonstrated that depletion of ECRG2 causes the high activation of p-Src and p-ERK, whereas overexpression of ECRG2 leads to dephosphorylation of Src and ERK. An \textit{in vitro} migration/invasion assay showed

![FIGURE 7. ECRG2 regulates cell migration through uPA/uPAR/integrin/Src/ERK pathway. ECRG2-Tet-on-3 UTR-si cells were transiently transfected with or without FLAG-uPA-D2 plasmid and incubated in the absence or presence of doxycycline (Dox) for 24 h. A, cell lysates were subjected to Western blotting analysis for phospho-Src and -ERK. B, cell lysates were subjected to immunoprecipitation (IP) with anti-uPAR antibody and Western blotting for ECRG2 and uPA. C, cells were subjected to the \textit{in vitro} migration assay as described above. D, cells were subjected to the \textit{in vitro} Transwell invasion assay as described above.](http://www.jbc.org/)

![FIGURE 8. Schematic representation showing the signaling and functional consequences from uPA-uPAR-integrin interactions and model of the plausible signaling events regulated by ECRG2. A, at the leading edge of migrating cells, the uPAR binds inactive urokinase (pro-uPA), which is then converted to active uPA. uPAR lacks a cytosolic domain but transmits intracellular signals through its association with transmembrane integrins. uPA-bound uPAR frequently interacts with α3β1 or α5β1 integrin, causing its activation. This leads to integrin-dependent recruitment of Src, FAK, or PI3K, which results in activation of MAPK signaling. B, model of ECRG2 regulating cell migration and invasion. The binding of ECRG2 to the uPA-uPAR complex blocks the interaction of uPAR with α3β1 or α5β1 integrin and results in integrin inactivation, disassembly of the uPA-uPAR-integrin complex, inactivation of its intracellular signaling components, and reduced Src/MAP kinase activation.](http://www.jbc.org/)
that depletion of ECRG2 markedly increased the cell migration/invasion when compared with control cells. However, cell migration/invasion was not increased in the presence of Src inhibitor (PP2) or MAP kinase inhibitor (PD98059) in ECRG2 depletion cells (Fig. 5 and supplemental Fig. 5), indicating that Src and MAP kinases are a downstream pathway stimulated by the uPAR-integrin complex response to the binding of ECRG2 to uPA and are required for ECRG2 to regulate cell migration/invasion. Based on our results, we hypothesize that ECRG2 regulates cell migration/invasion through uPAR/α3β1 or α5β1 integrin/Src/MAP kinase pathway. To test this possibility, β1 integrin was depleted using the siRNA approach. Similar patterns of p-Src and p-ERK were observed in scramble control cells. However, the phospho-kinases remained more or less unchanged with or without ECRG2 expression in cells. Moreover, the depletion of ECRG2 markedly increases, whereas the expression of ECRG2 marked-down integrin depletion cells (Fig. 6B). Furthermore, disrupting the effect of ECRG2 on uPA-uPAR-integrin complexes by overexpression FLAG-uPA D2 reverted the inhibition of ECRG2 on Src/Erk pathway, promoting cell migration and invasion (Fig. 7 and supplemental Fig. 6). Our results suggest that the interaction of ECRG2 with uPA-uPAR-β1 integrin complexes is required for ECRG2 to regulate the Src/MAP kinase pathway and cell migration/invasion.

We obtained very similar results for MDA-MB-231 and MCF-7 breast cancer cells (supplemental Figs. 2–6). Taken together, we propose a model in which ECRG2 regulates cell migration and invasion (Fig. 8). The binding of ECRG2 to the uPA-uPAR complex blocks the interaction of uPAR with α3β1 or α5β1 integrin and results in inactivation of integrin intracellular signaling components (Src/MAP kinase). High levels of uPA and uPAR often correlate with poor prognosis of cancer patients. Therefore, the specific inhibition of uPA-uPAR with small molecule active site inhibitors is one strategy to decrease the invasive and metastatic activity of tumor cells. Our data show that ECRG2 can regulate cell migration and invasion through several mechanisms. Understanding the detail of this model will provide more direct and efficient tools for the control of uPA-uPAR-mediated migration in tumor metastasis.

Acknowledgment—We thank the Medical Experiment Center of Shanxi Medical University for Microscopy analysis.

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ECRG2 Regulates Cell Migration/Invasion through Urokinase-type Plasmin Activator Receptor (uPAR)/ β1 Integrin Pathway
Xiaolong Cheng, Zheng Shen, Litian Yin, Shih-Hsin Lu and Yongping Cui

doi: 10.1074/jbc.M109.011213 originally published online August 28, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M109.011213

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