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Interaction of uteroglobin with lipocalin-1 receptor suppresses cancer cell motility and invasion

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Abstract

Cellular migration and invasion are critical for important biological processes including cancer metastasis. We previously reported that uteroglobin (UG), a multifunctional secreted protein, binds to several cell types inhibiting migration and invasion [G.C. Kundu, A.K. Z. Zhang Mandal, G. Mantile-Selvaggi, A.B. Mukherjee (1998) Uteroglobin (UG) suppresses extracellular matrix invasion by normal and cancer cells that express the high affinity UG-binding proteins. J Biol Chem. 273: 22819–22824]. More recently, we reported that HTB-81 adenocarcinoma cells, which do not bind UG, are refractory to UG-mediated inhibition of migration and invasion [Z. Zhang, G.C. Kundu, D. Panda, A.K. Mandal et al. (1999) Loss of transformed phenotype in cancer cells by overexpression of the uteroglobin gene. Proc Natl Acad Sci U S A. 96, 3963–3968]. Since UG shares several biological properties with lipocalin-1 that mediates some of its biological effects via its receptor (Lip-1R), we sought to determine whether UG might interact with Lip-1R and inhibit migration and invasion of HTB-81 cells. To address this question, we first transfected COS-1 cells, which do not bind UG, with a Lip-1R-cDNA construct and performed binding assays using ^{125}I-human UG (hUG). The results show that hUG binds Lip-1R on these cells with high specificity. Further, transfection of HTB-81 cells with the same construct yielded ^{125}I-hUG binding with high affinity (K_d = 18 nM) and specificity. The hUG-Lip-1R interaction was further confirmed by transfecting HTB-81, HTB-30 and HTB-174 cells, which are refractory to UG-binding, with a green fluorescent protein (GFP)-Lip-1R-cDNA construct and testing for Lip-1R-hUG colocalization by fluorescence microscopy. Finally, we demonstrate that Lip-1R-hUG interaction on Lip-1R transfected HTB-81 cells renders them fully responsive to hUG-mediated inhibition of migration and invasion. Taken together, these results suggest that Lip-1R is at least one of the UG-binding proteins through which UG exerts anti-motility and anti-invasive effects.
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Keywords: Clara cell 10 kDa protein; Secretoglobin; Extracellular matrix; Green fluorescent protein

Uteroglobin (UG), the founding member of the newly formed protein superfamily, Secretoglobin (Uteroglonin nomenclature report, 2000), is a steroid-inducible, multifunctional protein with potent anti-inflammatory and anti-chemotactic properties (reviewed in Mukherjee et al., 1999). It is an excellent substrate of transglutaminase (Manjunath et al., 1984). This protein was first discovered in the pregnant rabbit uterus and variously called as blastokinin (Krishnan and Daniel, 1967) or uteroglobin (Beier, 1968). While steroid hormones induce its expression, non-steroid hormones such as prolactin augments steroid-induced expression of UG (Chilton et al., 1988).

We previously reported that UG inhibits the chemotactic migration of several cell types including neutrophils and monocytes (Vasanthakumar et al., 1988). We also reported that UG binds to the surface of several cell types including NIH-3T3 and mastocytoma cells and suppresses their migration and extracellular matrix (ECM)-invasion (Kundu et al., 1998). Most interestingly, the results of our previous study showed that HTB-81 adenocarcinoma cells do not bind UG. As a result, UG

Abbreviations: UG, uteroglobin; cc10, Clara cell 10kDa; ECM, extracellular matrix; GFP, green fluorescent protein.

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has no inhibitory effect on the motility and ECM-invasion of these cells. However, the molecular mechanism(s) of UG action underlying these observations remains unclear (Zhang et al., 1999).

It has been reported that UG binds retinoids (Lopez de Haro et al., 1994), interacts with lipophilic compounds (Gillner et al., 1988; Mandal et al., 2004) and functions as an endogenous immunomodulatory agent in virtually all mammals (Mukherjee et al., 1999). These properties of UG are shared by lipocalins, a family of extracellular proteins that exert biological effects through their interactions with cell surface receptors (reviewed in Flower, 2000). Multiple molecular recognition properties of lipocalins including their ability to bind to cell surface receptors have been described. Indeed, a number of lipocalin-binding proteins (receptors) have been identified, which include those of α_1-microglobulin, insecticyanin, glycodelin, retinol-binding protein, α_1-acid glycoprotein, beta-lactoglobulin and odorant-binding protein (Flower, 2000). Recently, the cDNA encoding a receptor for lipocalin-1 (Lip-1R) has been characterized (Wojnar et al., 2001) and it has been demonstrated that antisense-mediated suppression of this receptor inhibits internalization of the ligand (lipocalin-1) by NT2 cells (Wojnar et al., 2003). Because of the shared characteristics between UG and the lipocalin family, we sought to determine whether UG interacts with Lip-1R and whether this interaction mediates at least in part UG's effects on cancer cell motility and invasion.

We report here that forced expression of Lip-1R in COS-1 cells facilitates UG-binding on these cells, whereas the untransfected or mock-transfected COS-1 cells do not interact with UG. We also demonstrate that transfection of Lip-1R-cDNA to HTB-81 adenocarcinoma cells, which are normally refractory to ^{125}I-hUG-binding and to UG-mediated action, facilitates specific binding of UG on these cells, and render them sensitive to UG-mediated inhibition of migration and invasion. Fluorescence microscopic analyses of GFP-Lip-1R-cDNA transfected cells treated with hUG show co-localization of Lip-1R with hUG providing strong physical evidence for Lip-1R-hUG interaction. In addition to HTB-81, we also tested two other cancer cell lines (e.g., HTB-30 and HTB-174), which do not bind hUG and transfection of these cells with GFP-Lip-1R-cDNA yielded results that are virtually identical to those obtained with HTB-81. We conclude that Lip-1R is at least one of the UG-binding proteins through which UG mediates its anti-motility and anti-invasive effects.

1. Experimental procedures

1.1. Cell culture

All cell lines used in this study were purchased from the American Type Culture Collection (Manassas, VA). COS-1 and HTB-81 cells were cultured in DMEM. HTB-30 cells were cultured in McCoy's 5A medium with 10% fetal bovine serum (FBS) and HTB-174 cells were cultured in RPMI-1640 with 5% FBS (Life Technologies, Gaithersburg, MD). Cell cultures were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% air. Recombinant human UG (hUG) was expressed in *E. coli* and purified to homogeneity according to the method described previously (Mantile et al., 1993). BioCoat Matrigel™ invasion chambers were from Becton Dickinson Labware (Bedford, MA).

1.2. Construction of GFP-Lip-1R expression vectors and transfection

A full length Lip-1R cDNA was generated by PCR using the following primers: Lip-1R-L: 5′-ATGGAAGCAG CTGAC-TACGA AGTG-3′, and Lip-1R-R: 5′-GAG GTC ATT G A TGC TGC TTC TTC C-3′. Human testicular Quick clone-cDNA (Clontech, Palo Alto, CA) served as the template. The amplified PCR fragment was subcloned into the PCR-II TOPO TA vector (Invitrogen). The fidelity and orientation of Lip-1R cDNA fragment was verified by DNA sequencing. This full-length Lip-1R cDNA fragment was excised from the TA vector by digestion with HindIII and NotI and then re-ligated into the pRC/RSV expression vector (Invitrogen) that was predigested with HindIII and NotI. To generate Lip-1R-GFP fusion-protein expression vector construct, the following primers were used: forward primer, Lip-1R-GFP-F: 5′-AAG CTT ATG GAA GCA GCT GAC TAC GAA GTG CTA-3′, and reverse primer, Lip-1R-GFP-R: 5′-CCG CGG TTG ATG CTG CTT CTT CCT AGA AGC CCG-3′. This PCR product was subcloned into the pCR-BluntII-TOPO vector (Invitrogen). It was then excised by HindIII and SacII, and re-ligated into the pEGFP-N1 vector (Invitrogen) that was predigested with HindIII and SacII. The vector constructs used for transfection were prepared by using a plasmid mini kit (Qiagen). The purified vector constructs were used to transfect cell lines using Lipofectamine 2000 reagent (Invitrogen). Cells transfected with pEGFP-N1 vector without the Lip-1R-cDNA served as mock-transfected control.

1.3. Quantitative mRNA analysis by real-time PCR

Total RNA was isolated from cells using TriZol reagent (Life Technologies) according to the manufacturer's instructions. RNA was further purified by RNeasy Mini Kit and DNAse treated (DNAse I, 30 U/μg total RNA) (Qiagen) then reverse transcribed using SuperScript III First-Strand Synthesis System (Invitrogen) according to the protocols supplied by the manufacturer. Quantitative gene expression analysis was performed using ABI Prism 7000 Sequence Detection System (Applied Biosystems) with cDNA equivalent to 10 ng of total RNA and SYBR Green PCR Master Mix (Applied Biosystem) following the manufacturer's protocol. The forward (F) and reverse (R) primers are Lip-1R (F) 5′-TGA CCT GCT GGG TGA CTT TG-3′; Lip-1R (R) 5′-GGC TGC GTT GTA GAG GAA CAC-3′; Internal control, mouse β-actin: (F) 5′-ACG GCC AAG TCA TCA CTA TTG-3′; (R) 5′-TGG AAA AGA GCC TCA GGG C-3′, human β-actin: (F) 5′-CGG CCA GGT CAT CAC CAT T-3′; (R) 5′-TGG AAG AGT GCC TCA GGG C-3′. Samples were heated for 10 min at 95 °C. Subsequently, 50 PCR cycles consisting of 15 s at 95 °C and 1 min at 60 °C were applied. The final data from each PCR run were analyzed using ABI Prism Software

version 1.01 (Applied Biosystems). These data were normalized to β-actin and presented as relative expression levels. Quantitation was performed using at least three independent total RNA samples for each group and the results are expressed as the mean±S.D.

1.4. Matrigel invasion assays

Cell migration and invasion were tested by Matrigel invasion assay as previously described (Kundu et al., 1998; Zhang et al., 1999). Briefly, the cells were trypsinized and washed twice with PBS containing 0.1% BSA. They were then resuspended in Optim-I serum-free medium containing 0.1% BSA and placed in the upper compartment of the Matrigel invasion chamber. The lower compartment of the chamber was filled with fibroblast conditioned medium, a chemoattractant for cell invasion, which was prepared from the supernatant of proliferating cultures of NIH 3T3 fibroblasts. The cells were incubated at 37 °C for 36–48 h. The cells in Matrigel invasion chamber were stained with Giemsa for 3 min and immediately washed twice with absolute ethanol for 5 min. The noninvaded cells along with the Matrigel were scraped from the upper surface of the filters with moist cotton swabs, and the chambers were washed three times with water. The invaded cells left on the filter were counted by using an inverted microscope.

1.5. UG-binding assay

Recombinant hUG was radioiodinated as previously described (Kundu et al., 1998). The specific activity of purified ^{125}I-hUG was 20 μCi/μg. ^{125}I-hUG-binding assay was performed according to the procedure described previously (Kundu et al., 1998). In binding assays for HTB-30 and HTB-174, ^{3}H-hUG was used. Briefly, the cells were cultured in 12-well plates. Semi-confluent cells were washed with PBS, pH 7.4, and incubated with ^{125}I-hUG (1.5 nM) in 1 ml of Hanks' balanced salt solution (HBSS), pH 7.6, containing 0.1% BSA in the absence or presence of varying concentrations of unlabeled reduced recombinant hUG at room temperature for 1 h. The cells were washed with PBS, pH 7.6, twice and then solubilized in 1N NaOH followed by addition of an equal volume of 1N HCl. The radioactivity was measured by a gamma scintillation counter. The specific binding was calculated by subtracting the nonspecific binding from the total binding.

1.6. Fluorescence microscopic analysis of UG-lipocalin-1R interaction

After transfection, the cells were incubated at 37 °C in an atmosphere of 5% of CO_2 and 95% air for 24 h. They were washed with PBS, pH 7.6 and incubated with hUG (100nM) in HBSS, pH 7.6 containing 0.1% BSA at 4 °C for 1 h. After incubation, the cells were washed and incubated in a 3.7% formaldehyde solution for 15 min. Following fixation, the cells were washed and sequentially incubated with rabbit-anti-hUG antibody and goat anti-rabbit-rhodamine conjugated antibody (Sigma) in PBS, pH 7.6, containing 2% BSA at room temperature for 1 h. Nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma). Fluorescence was visualized with the Axioskop 2 fluorescence microscope (Carl Zeiss), and the image was processed with the Photoshop 7.0 program (Adobe).

2. Results and discussion

To determine if hUG interacts with Lip-1R, we first transfected COS-1 cells with GFP-Lip-1R-cDNA construct. The results of real-time PCR show that compared with non-transfected (NT) COS-1 cells, those transfected with Lip-1R-cDNA construct express high levels of Lip-1R-mRNA (Fig. 1A). Due to the unavailability of reliable Lip-1R antibody, we performed Western blot analysis using GFP antibody. As expected, the results show that the cells transfected with GFP construct without Lip-1R-cDNA express only GFP (Fig. 1B, lane 1) while the GFP-Lip-1R-cDNA-transfected cells express a higher molecular weight GFP-Lip-1R fusion protein (Fig. 1B,

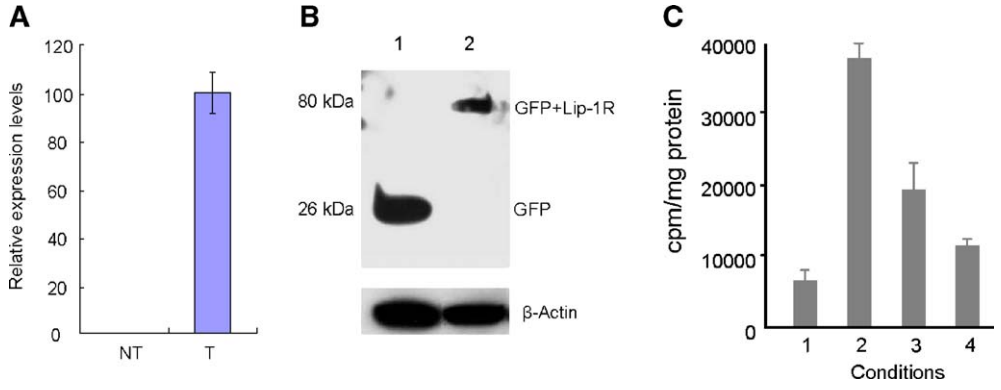

Fig. 1. Expression of Lip-1R in COS-1 cells promotes specific binding of hUG. (A) Relative expression of Lip-1R-mRNA non-transfected (NT) and cells transfected with Lip-1R-cDNA construct (T). (B) Western blot analysis using GFP-antibody. Lane 1: COS-1 cells transfected with GFP-construct; lane 2: COS-1 cells transfected with GFP-Lip-1R construct. Note that in lane 2, a high-molecular-weight GFP-Lip-1R fusion-protein band is clearly visible; (C) ^{125}I-hUG binding assay on non-transfected COS-1 cells (lane 1); Lip-1R transfected COS-1 cells incubated with ^{125}I-hUG in the absence of unlabeled UG for competition (lane 2) and in the presence of unlabeled hUG at varying concentrations (lane 3, 20 nM; lane 4 100 nM). Note a dose-dependent displacement of ^{125}I-UG by unlabeled hUG suggesting specific binding.

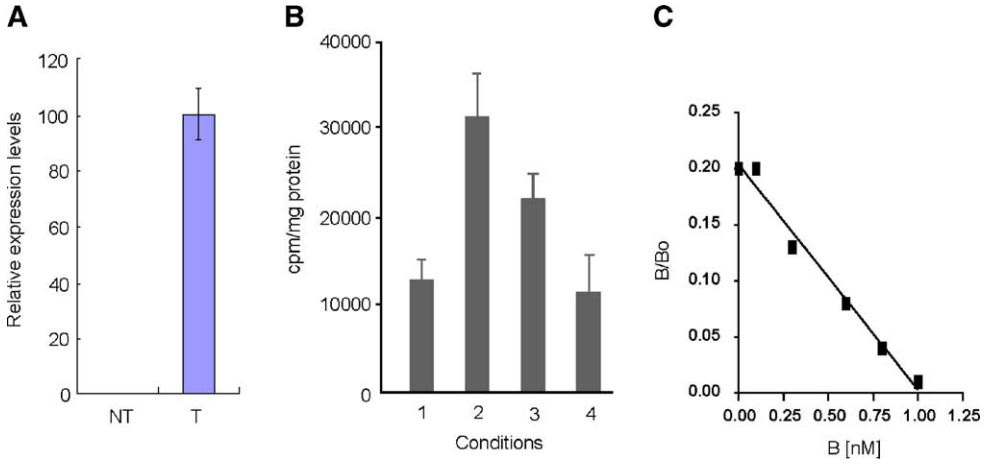

Fig. 2. Forced expression of Lip-1R in HTB-81 adenocarcinoma cells by transfection with Lip-1R cDNA and demonstration of specific binding of ^{125}I-hUG by transfected cells. (A) Relative expression of Lip-1R-mRNA by real-time PCR in non-transfected (NT) and Lip-1R cDNA transfected (T) HTB-81 cells. Note that while Lip-1R mRNA is virtually undetectable in NT cells abundant Lip-1R mRNA is present in the transfected cells; (B) ^{125}I-hUG binding assay on non-transfected (Bar 1) and Lip-1R cDNA-transfected HTB-81 cells (Bar 2). Note a clear displacement of ^{125}I-hUG by 20 nM (Bar 3) and 100 nM (Bar 4) of non-radioactive hUG in a competition assay indicating a dose-dependent displacement of ^{125}I-UG by unlabeled hUG suggesting specific binding. (C) Scatchard plot of the binding data.

lane 2). To delineate whether the expression of Lip-1R promotes specific binding of hUG on COS-1 cells, we used GFP-Lip-1R-cDNA-transfected cells to perform binding studies with ^{125}I-hUG. Unlabeled hUG in increasing concentrations was used to determine specific binding in a competition assay. The results show that Lip-1R-transfected cells bind ^{125}I-hUG with high specificity (Fig. 1C).

We previously reported that HTB-81 cancer cells do not bind hUG (Zhang et al., 1999). To test whether forced expression of Lip-1R could render these cells sensitive to UG-binding, we transfected HTB-81 cells with the Lip-1R construct and performed real-time PCR to quantitate the level of Lip-1R-mRNA expression. The results show that compared with non-transfected (NT) cells, Lip-1R cDNA-transfected (T) cells express high levels of Lip-1R mRNA (Fig. 2A). Subsequent ^{125}I-hUG binding assays performed with Lip-1R cDNA-transfected cells show that HTB-81 cells transfected with Lip-1R cDNA bind hUG and this binding is displaced by competition with unlabeled hUG suggesting that hUG binding to Lip-1R is specific (Fig. 2B). Further, Scatchard analysis of the binding data (Fig. 2C) show that hUG binds with Lip-1R transfected HTB-81 cells with high affinity (K_d=18 nM). To further confirm these results, we used two additional cell lines (HTB-30 and HTB-174) that did not manifest ^{3}H-hUG binding and transfected them with the Lip-1R cDNA construct. The results of hUG binding assays before and after transfection with

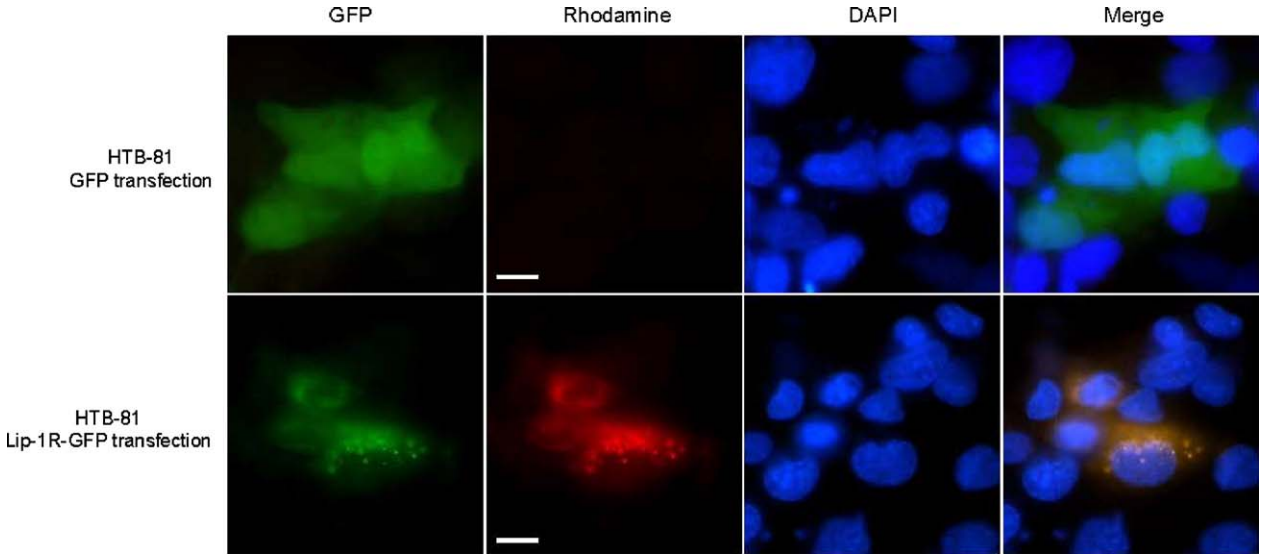

Fig. 3. Interaction of hUG with mock-transfected (pEGFP-N1) and GFP-Lip-1R-cDNA (pEGFP-N1-Lip-1R)-transfected HTB-81 cells. Expression of GFP-Lip-1R protein and its interaction with hUG were examined by using fluorescence microscopy. Green fluorescence represents GFP-Lip-1R expression, and red fluorescence indicates hUG protein. Nuclei were stained by DAPI. Scale bar, 20 μm. The merging of green fluorescence with red indicates an interaction between Lip-1R and hUG on the cell surface. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

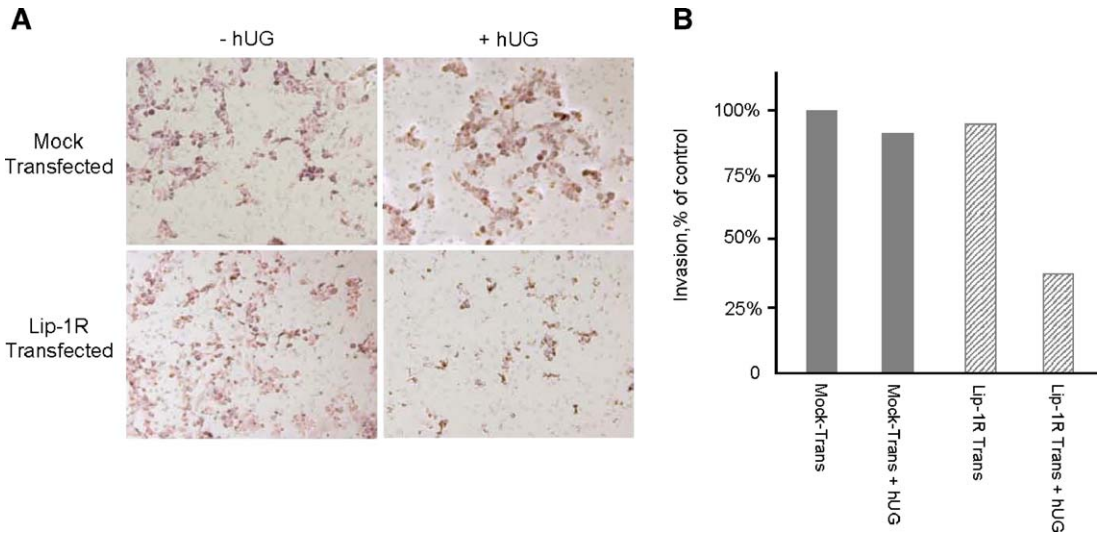

Fig. 4. Effects of hUG-treatment on migration and invasion of mock-transfected and Lip-1R cDNA transfected HTB-81 cells. (A) Upper panels: mock-transfected HTB-81 cells without (left) and with hUG-treatment (right). Lower panels: hUG-cDNA-transfected HTB-81 cells without hUG treatment (left) and the same cells treated with hUG (right). Note marked inhibition of migration and ECM-invasion in the lower panel. Magnification ×200. (B) Graphic quantitative representation of inhibition of migration and ECM invasion by HTB-81 cells.

Lip-1R cDNA construct show that while before transfection the cells do not show ^{3}H-hUG binding, the same cells after transfection bound ^{3}H-hUG with high-affinity and specificity (data not shown). Taken together, these results strongly suggest that forced expression of Lip-1R in these cells enable them to bind hUG, suggesting that Lip-1R is at least one of the UG-binding proteins.

In order to confirm hUG-Lip-1 interaction in further detail, we performed fluorescence microscopic analyses of GFP mock-transfected and GFP-Lip-1R-cDNA-transfected cells in the presence and absence of hUG. In this assay, we used unlabeled hUG as the ligand of Lip-1R and detected it by rabbit-hUG antisera as the primary antibody and rhodamine (red)-labeled goat anti-rabbit IgG as the second antibody. The results (Fig 3) show that while mock-transfected HTB-81 cells show only GFP fluorescence indicating no association of hUG (red) with these cells, the GFP-Lip-1R-cDNA transfected cells show expression of Lip-1R (green), which merges with hUG (red) suggesting an interaction of hUG with Lip-1R (Fig. 3). DAPI was used to detect the nuclei. To further confirm these results, we repeated the above study using HTB-30 and HTB-174 cell lines that do not manifest hUG binding and transfected them with GFP-Lip-1R cDNA-construct. The results are virtually identical to those obtained with HTB-81 cells (Supplemental Fig. S1). Taken together, these results provide visual confirmation that forced-expression of Lip-1R, in cells previously refractory to hUG binding, allows hUG to bind with high affinity and specificity. These results provide strong evidence that Lip-1R is a UG-binding protein.

Previous studies showed HTB-81 cells to be refractory to hUG-mediated inhibition of migration and ECM-invasion (Zhang et al., 1999). To delineate whether Lip-1R is capable of mediating hUG-induced inhibition of migration and ECM-invasion, we performed migration and invasion assays using HTB-81 cells that are either mock transfected or transfected with Lip-1R-cDNA construct. The results show that while mock-transfected HTB-81 cells are refractory to hUG-mediated effects (consistent with the results of previous studies), those cells transfected with Lip-1R-cDNA construct and treated with hUG show striking inhibition of migration and invasion (Fig. 4A). Quantitatively, hUG treatment inhibits migration and invasion of more than 50% of Lip-1R-cDNA-transfected cells, while such treatment has virtually no effect on mock-transfected HTB-81 cells (Fig 4B).Taken together, Our results provide insight into at least one mechanism by which UG may regulate cellular migration and invasion via its interaction with Lip-1R and raises the possibility that disruption of this regulatory process may lead to pathogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2005.10.027.

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