Extracellular Membrane-proximal Domain of HAb18G/CD147 Binds to Metal Ion-dependent Adhesion Site (MIDAS) Motif of Integrin β1 to Modulate Malignant Properties of Hepatoma Cells*

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Background: HAb18G/CD147 interacts with integrin β1 subunit.
Results: Extracellular membrane-proximal domain of HAb18G/CD147 (I-type domain) binds at the metal ion-dependent adhesion site (MIDAS) in the βA domain of the integrin β1 subunit. Interaction of HAb18G/CD147 with integrin α1 activates the downstream FAK signaling pathway, enhancing the malignant properties of hepatocellular carcinoma cells.
Significance: This is the first time binding sites of CD147 and integrin β1 are revealed.

Several lines of evidence suggest that HAb18G/CD147 interacts with the integrin variants α3β1 and α6β1. However, the mechanism of the interaction remains largely unknown. In this study, mammalian protein-protein interaction trap (MAPPIT), a mammalian two-hybrid method, was used to study the CD147-integrin β1 subunit interaction. CD147 in human hepatocellular carcinoma (HCC) was interfered with by small hairpin RNA. Nude mouse xenograft model and metastatic model of HCC were used to detect the role of CD147 in carcinogenesis and metastasis. We found that the extracellular membrane-proximal domain of HAb18G/CD147 (I-type domain) binds at the metal ion-dependent adhesion site in the βA domain of the integrin β1 subunit, and Asp179 in the I-type domain of HAb18G/CD147 plays an important role in the interaction. The levels of the proteins that act downstream of integrin, including focal adhesion kinase (FAK) and phospho-FAK, were decreased, and the cytoskeletal structures of HCC cells were rearranged bearing the HAb18G/CD147 deletion. Simultaneously, the migration and invasion capacities, secretion of matrix metalloproteinases, colony formation rate in vitro, and tumor growth and metastatic potential in vivo were decreased. These results indicate that the interaction of HAb18G/CD147 extracellular I-type domain with the integrin β1 metal ion-dependent adhesion site motif activates the downstream FAK signaling pathway, subsequently enhancing the malignant properties of HCC cells.

CD147 is a transmembrane glycoprotein that is categorized as a member of the immunoglobulin superfamily and is broadly expressed on the surfaces of many kinds of tumor cells. Previous studies in our laboratory have demonstrated that HAb18G/CD147, a member of the CD147 family that is highly expressed in HCC4 cells, can promote the invasive and metastatic potentials of HCC cells by stimulating fibroblasts and tumor cells to produce matrix metalloproteinases (MMPs) (1). Specifically, the promotion of invasion and metastasis is mediated by the integrin-mediated FAK-paxillin and FAK-PI3K-Ca2+ signaling pathways (2).

Integrins are composed of two type I transmembrane subunits, α and β. Because there are integrin variants that can bind a variety of extracellular matrix molecules and cell-surface receptors (3), integrins can serve as bidirectional transducers of extracellular and intracellular signals in the processes of cell adhesion, cell-cell interactions, proliferation, differentiation, apoptosis, and tumor progression.

CD147 both oligomerizes with itself (4) and interacts with the α3β1 and α6β1 integrin variants at points of cell-cell contact (2, 5, 6). Published reports support a crucial role for CD147 in the migration and metastatic potential of tumor progression through its interactions with integrin (5, 7, 8). However, the fundamental mechanism of the interaction between HAb18G/CD147 and integrin is not well understood; specifically, the binding domains of HAb18G/CD147 and integrin during interaction have not been thoroughly characterized.

Previous results suggest that the RGD motif, which binds to integrin through the metal ion-dependent adhesion site (MIDAS) of the integrin β1 subunit, is found in many extracellular matrix proteins, including vitronectin, fibronectin, fibrinogen, laminin, collagen, Von Willebrand’s factor, osteoponin, and adenovirus particles (9). Because the RGD motif can inhibit the interaction between HAb18G/CD147 and integrin (8), the

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4 The abbreviations used are: HCC, hepatocellular carcinoma; MMP, matrix metalloproteinase; FAK, focal adhesion kinase; MIDAS, metal ion-dependent adhesion site; MAPPIT, mammalian protein-protein interaction trap; FN, fibronectin.
question arises of whether MIDAS is also the binding site of HAb18G/CD147 on integrin.

In the present study, we demonstrate that the I-type domain of HAb18G/CD147 can bind at the MIDAS pocket of the integrin-β1 subunit and that Asp179 in the I-type domain may play an important role in this binding interaction. The FAK signaling pathway and its downstream signals are activated by the interaction of HAb18G/CD147 with the integrin-β1 subunit. Subsequently, tumor growth potential and the invasive and metastatic potentials of HCC cells are enhanced.

EXPERIMENTAL PROCEDURES

Cell Culture—HCC SMMC-7721 cells and human embryonic kidney (HEK) 293T cells were obtained from the Institute of Cell Biology, Academic Sinica, Shanghai, China. SMMC-7721 cells were cultured in RPMI 1640 medium (Invitrogen), whereas HEK 293T cells were cultured in DMEM (Invitrogen). Both media were supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in 5% CO2.

Construction of Plasmids for Chimeric Proteins in MAPPIT System—MAPPIT, a mammalian two-hybrid method (10, 11), was used to study the CD147-integrin-β1 subunit interaction (Fig. 1). To detect the interaction between the integrin-β1 subunit and HAb18G/CD147, human extracellular residues of the integrin-β1 subunit may play an important role in this binding interaction. The FAK signaling pathway and its downstream signals are activated by the interaction of HAb18G/CD147 with the integrin-β1 subunit. Subsequently, tumor growth potential and the invasive and metastatic potentials of HCC cells are enhanced.

CD147 Binds to MIDAS Motif of Integrin β1

FIGURE 1. Principle of MAPPIT, adapted from Ref. 10. A, MAPPIT employed the leptin receptor, which signals through the JAK-STAT pathway. B, bait constructs (cloned into pCLL vector) were designed as chimeric receptors consisting of the transmembrane and intracellular regions of a STAT3 recruitment-deficient leptin receptor and the extracellular portion of the normal leptin receptor with a bait attached to the C terminus. Prey constructs (cloned into pMG1 vector) were composed of a prey polypeptide with a section of the gp130 chain carrying four STAT3 recruitment sites. The ITGB1bait represents the bait construct containing the integrin-β1 propeller domain gene, and the CD147/FNprey represents the prey construct containing the CD147 extracellular domain gene or the fibronectin F33-10F3 domain gene. If the bait and prey interacted, co-expression would lead to functional complementation of STAT3 activity, which could be measured by luciferase expression driven by the STAT3-responsive rat pancreatitis-associated protein I (rPAPI) promoter.
fibronectin, which contain the N-terminal FLAG tag from the pcdNA3.1-HAb18G/CD147 plasmid and SMMC-7721 cDNA, respectively. We cloned both PCR products into the pMET7 vector using Apal-Xbal, and the final constructs were named pMET7-FLAG-CD147 and pMET7-FLAG-9F3-10F3. All constructs were verified by DNA sequence analysis.

**Luciferase Reporter Assays for MAPPIT**—Transfection procedures and luciferase assays were performed as described previously (10). HEK 293T cells were seeded in 6-well plates overnight and transfected with the desired constructs together with the luciferase reporter gene. Forty eight hours after transfection, cells were left untreated or stimulated overnight with 100 ng/ml G418. Both shCD147-1 and shCD147-2 decrease expression of CD147 significantly (Fig. 4A). In the following experiments, shCD147-1 and snc-1 were used. The stably transfected SMMC-7721 cells were named 7721-snc (transfected with pGenesil-2.1-snc) and 7721-shCD147 (transfected with pGenesil-2.1-CD147), respectively.

**RNA Interference**—Transfection of small interfering RNAs was performed using Lipofectamine 2000 (Invitrogen). si-CD147-1 (5'-GGTTCTTCGTAGGTTCCT-3') and si-CD147-2 (5'-GTACAAGATCTAGCTGCT-3') were synthesized by Shanghai GenePharma Co., Ltd.

**Western Blot Analysis and Co-immunoprecipitation**—The peptides GRGDS, containing the RGD motif, and GRGES, containing the RGE motif, were synthesized by CL Xi'an Bio-Scientific Co., Ltd. Cells with different treatments were lysed with RIPA cell lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. Co-immunoprecipitation was then carried out to detect the interaction of HAb18G/CD147 with the integrin β1 subunit. 7721 cells were seeded into culture dishes and incubated with the peptides for 1 week and 300 μg/ml for 2 weeks. Both shCD147-1 and shCD147-2 decrease expression of CD147 significantly (Fig. 4A). In the following experiments, shCD147-1 and snc-1 were used. The stably transfected SMMC-7721 cells were named 7721-snc (transfected with pGenesil-2.1-snc) and 7721-shCD147 (transfected with pGenesil-2.1-CD147), respectively.

**TABLE 1**

Sequences of primers for plasmids construction

| No. | Description | Primer sequences |
|-----|-------------|------------------|
| 1   | Integrin β1 (residues 1–441) | Forward: 5'-TTTGAAGCTCTTCAAGCTGGAATAG-3' | Reverse: 5'-TATGGCGGCCGTCATTAGCTGGAATAG-3' |
| 2   | pCLL-ITGB1M-A150D (A150D and A154S) | Forward: 5'-GAATTCTTCAAGCTGGAATAG-3' | Reverse: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' |
| 3   | pCLL-ITGB1M-A150D (A279D) | Forward: 5'-GAATTCTTCAAGCTGGAATAG-3' | Reverse: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' |
| 4   | shCD147-1 ( Santa Cruz Biotechnology, Santa Cruz, CA), anti-p-FAK (BD Biosciences), anti-inhibitor of activated protein kinase (IAP) (Santa Cruz Biotechnology) | Forward: 5'-GAATTCTTCAAGCTGGAATAG-3' | Reverse: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' |
| 5   | FLAG-CD147 | Forward: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' | Reverse: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' |
| 6   | pGenesil-2.1-CD147 | Forward: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' | Reverse: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' |
| 7   | 9F3-10F3 domain of fibronectin | Forward: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' | Reverse: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' |
| 8   | HAb18G/CD147 | Forward: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' | Reverse: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' |
| 9   | HAb18G/CD147 1-type domain (residues 101–206) | Forward: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' | Reverse: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' |
| 10  | pMG1-CD147-A136 | Forward: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' | Reverse: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' |
| 11  | pMG1-CD147-A144 | Forward: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' | Reverse: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' |
| 12  | pMG1-CD147-A147 | Forward: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' | Reverse: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' |
| 13  | pMG1-CD147-A179 | Forward: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' | Reverse: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' |
| 14  | pMG1-CD147-A194 | Forward: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' | Reverse: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' |
| 15  | FLAG-CD147 | Forward: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' | Reverse: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' |
| 16  | 9F3-10F3 | Forward: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' | Reverse: 5'-TTTGAAGCTTTCAAGCTGGAATAG-3' |

(Sigma) for 1 week and 300 μg/ml for 2 weeks. Both shCD147-1 and shCD147-2 decrease expression of CD147 significantly (Fig. 4A). In the following experiments, shCD147-1 and snc-1 were used. The stably transfected SMMC-7721 cells were named 7721-snc (transfected with pGenesil-2.1-snc) and 7721-shCD147 (transfected with pGenesil-2.1-CD147), respectively.

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GRGDS (100 μg/ml) and GRGES (100 μg/ml), respectively. Cells were lysed with the M-PER Reagent from the mammalian co-immunoprecipitation kit (Pierce) after adding or omitting the designated peptides (100 μg/ml). The lysates were then collected onto a coupling gel that was pre-bound with HAb18 overnight at 4 °C. To detect interaction of the integrin β1 subunit with Asp-mutated CD147 prey, lysates of HEK 293T cells co-transfected with the plasmids encoding ITGB1 and Asp-mutated CD147 prey were immunoprecipitated with anti-FLAG antibody. Immunocomplexes were washed four times with the co-immunoprecipitation buffer, analyzed by immunoblotting with the indicated antibodies.

Enzyme Linked Immunosorbent Assay (ELISA)—The extra-cellular portion of HAb18G/CD147, produced and purified as described previously (13), was added to the wells of a 96-well ELISA plate at a concentration of 50 μg/100 μl. Plates were incubated overnight at 4 °C, and wells were blocked for 1 h with 200 μl of 5% fat-free milk. The pellet of 7721-shCD147 cells (1 × 10⁹) was extracted at 4 °C with 10 ml of 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 2% (w/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, 2 mg/ml bovine serum albumin and protease inhibitor mixture, EDTA-free tablet (Roche Applied Science). The extract was centrifuged at 12,000 × g for 10 min. and the supernatant was concentrated by passing it through an Amicon ultrafiltration filter (50 kDa, Millipore). The concentrated extract was diluted as indicated and added to wells (100 μl/well). After incubation overnight at 4 °C, wells were washed with 200 μl of PBST three times and aliquots (100 μl each) of integrin β1 pAb (Santa Cruz Biotechnology, 1:50) with different concentrations of cations or EDTA. To analyze the effect of a range of Mn²⁺, Mg²⁺, and Ca²⁺ concentrations on the binding of CD147 to integrin β1, Mn²⁺, Mg²⁺, or Ca²⁺ was added to a final concentration of 0.25–4 mM. The plate was then incubated at 37 °C for 2 h, and the wells were washed three times with PBST. After incubation with horseradish peroxidase-conjugated goat anti-mouse IgG for 20 min at room temperature, wells were then washed, and color was developed using 3,3′,5,5′-tetramethybenzidine solution. After incubation for 10 min, an equal volume of stopping solution (2 M H₂SO₄) was added, and the absorbance was read at 450 nm.

Immunofluorescence—7721-shCD147 cells and 7721-snc cells were grown on coverslips and incubated with GRGDS or GRGES peptides (100 μg/ml) for 24 h and fixed with 4% paraformaldehyde. The human HCC tissues for frozen sections were supplied by the General Hospital of the People’s Liberation Army. The coverslips or frozen sections were first blocked with 10% bovine serum albumin in phosphate-buffered saline (PBS), pH 7.0, and then incubated with primary antibodies, including HAb18 against HAb18G/CD147 (prepared by our laboratory), anti-integrin β1 (Santa Cruz Biotechnology), and anti-α-tubulin antibodies (Santa Cruz Biotechnology). Alexa Fluor 594 goat anti-mouse IgG (H+L) (Molecular Probes, Eugene, OR), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Pierce), and FITC-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) were used as secondary antibodies. To detect F-actin, the cells were probed with Alexa Fluor 488-phalloidin (Molecular Probes) at 1:40 for 20 min. Cell nuclei were stained with DAPI. Images were obtained with an FV1000 laser scanning confocal microscope (Olympus, Japan).

Gelatin Zymography—7721-shCD147 cells and 7721-snc cells were incubated with serum-free medium in the presence of the GRGDS or GRGES peptides (100 μg/ml). The conditioned medium was collected, and the volume from each cell line was adjusted to provide a predetermined quantity of protein. Media were separated by 10% acrylamide gels containing 0.1% gelatin. The gels were incubated overnight in a reaction buffer (0.05 mol/liter Tris-HCl, pH 7.5, 0.2 mol/liter NaCl, and 0.01 mol/liter CaCl₂) at 37 °C after the SDS was removed by washing with buffer containing 2.5% Triton X-100 with gentle agitation. After the reaction, the gels were stained with Coomasie Brilliant Blue R-250 for 6 h and destained for ~0.5 h. The zones of gelatinolytic activity were indicated by negative staining.

In Vitro Wound Healing Assay—7721-shCD147 cells and 7721-snc cells were seeded in 6-well plates and cultured to 100% confluence. After a cell-free area was created by scraping the monolayer with a pipette tip, the cells were incubated with medium supplemented with 1% FBS or medium supplemented with 1% FBS and 100 μg/ml GRGDS or GRGES peptides. Digital images were taken with an inverted phase-contrast microscope (BX60, Olympus) at time points up to 20 h.

Invasion Assay—An invasion assay was performed in 24-well Transwell units with an 8-mm pore size polycarbonate (Millipore), according to the manufacturer’s instructions. Briefly, filters were coated with Matrigel to form a continuous thin layer. The cells were then seeded in 0.5% fetal calf serum in RPMI 1640 medium in the upper chamber, and the lower chamber was filled with 10% fetal calf serum in RPMI 1640 medium. GRGDS or GRGES (100 μg/ml) peptides were added to the upper chambers at the same time. Following 24 h of incubation at 37 °C, cells remaining in the upper compartment were completely removed using cotton swabs. The cells that invaded through the filter into the lower compartment were fixed with 4% paraformaldehyde/HBS-Ca²⁺/, stained with crystal violet (0.5% in 20% methanol), and counted.

Colony Formation Assay—7721-shCD147 cells and 7721-snc cells were plated on a 0.6% agarose base in a 24-well plate (1.0 × 10⁴ cells per well) in 1 ml of DMEM containing 10% FBS, 0.3% agarose, GRGDS, or GRGES peptides (100 μg/ml). At day 15 after plating, colonies of >50 cells were counted. The colony formation rate was calculated using the following formula: colony formation rate = number of colonies/10⁴ cells.

Nude Mouse Xenograft Model of HCC—Four-week-old nude mice were divided randomly into three groups (six mice per group). An identical number of 7721-shCD147 cells and 7721-snc cells were subcutaneously injected into the right and left flanks of every nude mouse. From the 6th day after inoculation, GRGDS (100 μg), GRGES (100 μg) or 1× PBS were injected into the growing tumors of groups 1–3 mice, respectively. The tumors were monitored with a caliper every 2 days. The tumor volume was determined for each mouse (in cubic millimeters) by measuring the tumor in two directions and was calculated as tumor volume = length × (width)²/2. All animal procedures
were performed in accordance with Laboratory Animal Ethics Committee of Fourth Military Medical University.

**Immunohistochemistry**—After the tumor model experiment, half of the human tumor xenografts in the nude mice were fixed with 10% formalin and embedded in paraffin. Sections were deparaffinized and incubated with primary antibodies, including HAb18 against HAb18G/CD147, anti-MMP-2 (Santa Cruz Biotechnology), and anti-integrin β1 (Santa Cruz Biotechnology), followed by visualization with the Histostain®-Plus kit (Invitrogen).

**Real Time PCR Amplification**—Total RNA was isolated from the tumor tissues of the nude mouse xenograft model of HCC using TRIzol reagants (Invitrogen), according to the manufacturer’s instructions. After RNA isolation, total RNA was reverse-transcribed into cDNA with the ReverTra Ace-a kit (Toyobo, Japan). SYBR Green real time RT-PCR was performed as described in the product instructions using SYBR Premix EX Taq II (2×) (TaKaRa, Japan) with the sequence detection system Stratagene Mx3005P (Agilent Technologies, Germany). In all PCRs, a negative control corresponding to an RT reaction without the reverse transcriptase enzyme and a blank sample were carried out; they exhibited no PCR product amplification. Amplification of GAPDH cDNA was used as an internal control to quantify the expression of a given gene. For screening expression and quantification studies, PCR analysis was performed using specific primers made by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., listed in Table 2.

**Metastatic Model of Human Hepatocellular Carcinoma in Nude Mice via Orthotopic Implantation of HCC Cells**—Four-week-old nude mice were divided randomly into four groups (7721-snc group, 7721-shCD147 group, 7721-snc-RGE group and 7721-snc-RGD group). Viable HCC cells (2.5 × 10⁶) mixed with Matrigel (BD Biosciences) were injected into left lobe of liver in nude mice. From the 7th day after the injection, RGE were performed; they exhibited no PCR product amplification. PRGs also displayed a strong interaction with the integrin β1 subunit, although the interactions of mutations of the β1 subunit with GRGD5 were significantly attenuated. The expression of the FLAG-tagged HAb18G/CD147 extracellular fragment prey was revealed by immunoblotting using an anti-FLAG antibody (Fig. 2B). Identical expression levels of various preys confirmed that the elevated MAPPIT signal was not due to unequal prey levels.

**Fibronectin 9F3-10F3 Domain Inhibits the Interaction of HAb18G/CD147 and Integrin β1 Subunit**—The Arg-Gly-Asp (RGD) motif in the central cell-binding domain of many ligands was an important site of cell recognition for integrins. The fibronectin (FN) central cell-binding 9F3-10F3 domain also contained the RGD sequence. The α5β1 integrin variant binds to the RGD motif of FN at the interface between the α and β subunits of integrin, and the aspartic acid residues of the RGD motif contacts MIDAS at the βA directly, with the mediation of a divalent cation, forming the Mn²⁺-bound integrin-RGD complex (14, 15). In this study, the sequences encoding 9F3-10F3 domain of FN was inserted into a prey construct. In HEK 293T cells, the co-expression of the FN prey and integrin bait elevated the MAPPIT signal, and integrin mutants abolished the MAPPIT signal (Fig. 2A). To gain further insight into whether the MIDAS of the integrin β1 subunit is the CD147 integrin-binding site, the CD147 prey was co-expressed with the wild type fibronectin 9F3-10F3 domain. Fibronectin 9F3-10F3 domain expression markedly reduced the CD147 prey signal (Fig. 2C). Co-expression of the extracellular CD147 fragment also repressed the fibronectin 9F3-10F3 prey signal (Fig. 2C). The expression levels of the FLAG-tagged proteins were confirmed by immunoblotting using an anti-FLAG antibody (Fig. 2D).

**RESULTS**

**Extracellular Portion of HAb18G/CD147 Interacts with the βA Domain of Integrin β1 Subunit**—We fused a leucine receptor with a bait protein, the fragment of the integrin β1 subunit’s ovoid head containing residues 1–387 (wild type or two different mutants of MIDAS). To determine the interaction, the integrin β1 fusion protein (pCLL-ITGB1) was transiently co-expressed with the extracellular fragment of the HAb18G/CD147 prey. As shown in Fig. 2A, the two bait protein mutants of MIDAS (pCLL-ITGB1M145) and pCLL-ITGB1M120) of the β1 subunit were sufficient to abrogate MAPPIT signals. GRGD3 also displayed a strong interaction with the integrin β1 subunit, although the interactions of mutations of the β1 subunit with GRGD5 were significantly attenuated. The expression of the FLAG-tagged HAb18G/CD147 extracellular fragment prey prey was revealed by immunoblotting using an anti-FLAG antibody (Fig. 2B). Identical expression levels of various preys confirmed that the elevated MAPPIT signal was not due to unequal prey levels.

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**HAb18G/CD147 I Domain, but Not the C2 Domain, Interacts with the Integrin β1 Subunit**—We fused a leucine receptor with a bait protein fragment containing the C2 domain of CD147 (residues 22–107) and a fragment containing the I domain of
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**FIGURE 2. Interaction of HAb18G/CD147 with the integrin β1 subunit analyzed by MAPPIT.** A, HEK 293T cells were transiently co-transfected with plasmids, including pMET7-LR, various pCLL-β1 variants for the integrin β1 subunit (ITGB1, ITGB1M135, or ITGB1M145), and prey constructs of pMG1-9F3-10F3 (FN prey), pMG1-CD147 (CD147 prey), pMG1-CD147-C2 (CD147-C2 prey), and pMG1-CD147-I (CD147-I prey) or mock vectors combined with pXP2d2-rPAP1-luci. LR, leptin receptor. B, Western blot analysis of FN prey, CD147 prey, CD147-C2 prey, and CD147-I prey expression levels. C, HEK 293T cells were transiently co-transfected with plasmids encoding ITGB1, the wild type fibronectin 9F3-10F3 domain (pMET7-FLAG-9F3-10F3, FN), and CD147-prey constructs or the extracellular CD147 fragment (pMET7-FLAG-CD147, CD147) and the FN prey combined with pXP2d2-rPAP1-luci. D, Western blot analysis of CD147 prey, FN, FN prey, and CD147 expression levels. E, HEK 293T cells were transiently co-transfected with plasmids expressing ITGB1, CD147-prey, or Asp-mutated CD147 prey (marked as CD147A136prey, CD147A144prey, CD147A147prey, CD147A179prey, and CD147A194prey) combined with pXP2d2-rPAP1-luci. F, Western blot analysis of CD147 wild type prey or Asp-mutated CD147 prey expression levels. G, interaction of the integrin β1 subunit with Asp-mutated CD147 prey, analyzed by co-immunoprecipitation. Lysates of HEK 293T cells co-transfected with the plasmids encoding ITGB1 (pCLL-ITGB1) and Asp-mutated CD147 prey were immunoprecipitated with anti-FLAG antibody. The resulting immunoprecipitates (IP) or whole cell lysates were analyzed by immunoblotting (IB) with anti-leptin receptor antibody or anti-FLAG antibody. A, C, and E, transfected cells were either stimulated for 24 h with leptin or were untreated. Luciferase measurements were performed in triplicate. Data are expressed as the mean-fold induction (leptin-stimulated/NS ± S.D. B, D, F, and G, expression of the FLAG-tagged fusion prey proteins in the same transfected cells was verified in lysates using an anti-FLAG antibody.
CD147 (residues 101–206). The prey proteins, including a fragment of the integrin β1 subunit’s ovoid head that contained residues 1–387, and two MIDAS-targeted mutants or the fibronectin 9F3–10F3 domain, which contains the RGD sequence, were linked to the gp130 chain. Preys containing the C2 domain of CD147 (residues 22–107) or the I domain of CD147 (residues 101–206) were co-transfected with the integrin β1 fusion protein. As shown in Fig. 2A, the fragment containing the I domain exhibited a novel interaction with the integrin β1 subunit, unlike the fragment containing the C2 domain of CD147. Mutants of the β1 subunit showed a weak interaction with the fragment containing the I domain. These results indicated that the I domain of CD147, rather than the C2 domain, might play a central role in the interaction with the integrin β1 subunit. Similar prey expression levels confirmed that the reduced signal was not caused by variations in prey levels (Fig. 2B).

Asp179-targeted Mutation in HAb18G/CD147 I Domain Inhibits the CD147-Integrin Interaction—To further indicate whether aspartic acid-based sequences such as RGD, LDV, KGD, RTD, and KQAGD containing Asp (16) in HAb18G/CD147 might play an important role in the interaction between integrin and CD147, each Asp of the I domain was mutated to Ala by site-directed mutagenesis, represented as pMG1-CD147-A136, pMG1-CD147-A144, pMG1-CD147-A147, pMG1-CD147-A179, and pMG1-CD147-A194, and each construct was co-transfected with the integrin β1 fusion protein. As shown in Fig. 2F, MAPK and MAPK signals were reduced markedly in an experiment using pMG1-CD147-A179 as the prey, compared with the signals obtained using a wild type HAb18G/CD147 extracellular fragment or other mutants of HAb18G/CD147 extracellular fragment. Equal prey expression levels confirmed that the reduced signal was not caused by differences in prey levels (Fig. 2F). As shown in Fig. 2G, Asp179 mutation inhibited binding of CD147 to integrin β1, although other mutations did not inhibit the interaction significantly.

Co-immunoprecipitation between HAb18G/CD147 and the integrin β1 subunit is decreased by GRGDS Treatment—A previous study indicated that HAb18G/CD147 co-immunoprecipitates with integrin β1 in HCC cells (2). The decreased co-immunoprecipitation (by 71.1%) in GRGDS-treated 7721 cells compared with control 7721 cells demonstrates that incubation with the GRGDS peptide could inhibit the interaction of HAb18G/CD147 and the integrin β1 subunit (Fig. 3A).

Bivalent Cations Modulate the Apparent Affinity of Binding of HAb18G/CD147 to Integrin β1—Although it is clear that bivalent-cation occupancy and integrin-ligand binding are intimately linked, the precise role of bivalent cations in CD147–integrin interaction remains uncertain. We tested the effect of a range of cation concentrations on the binding of CD147 to integrin β1. As shown in Fig. 3B, CD147 binding was increased by Mn2+ in a dose-dependent manner and to a less extent by Mg2+ and Ca2+. Different concentrations of cell extract influenced the CD147-binding activity of integrin β1 in the presence of Mn2+. EDTA inhibited CD147 binding to integrin β1 in a dose-dependent manner (Fig. 3C). Replacing Mn2+ with EDTA led to reduced binding of CD147 to integrin β1 (Fig. 3D).

HAb18G/CD147 Regulates Expression and Phosphorylation of FAK and Mediates Cytoskeletal Rearrangements—FAK is a cytoplasmic tyrosine kinase that plays a major role in integrin signaling. To identify whether the FAK pathway is involved in the HAb18G/CD147-mediated integrin signaling pathway, we tested the levels of HAb18G/CD147, integrin, FAK, and p-FAK in 7721-shCD147 cells or snc-transfected 7721 cells, with or without GRGDS or GRGES treatment. The expression levels of both FAK and p-FAK were significantly diminished, by 65.5 and 71.4%, respectively, in 7721-shCD147 cells as compared with that in 7721-snc cells (Fig. 4B). However, in 7721-shCD147 cells, the levels of FAK and p-FAK were not further diminished after treatment with the GRGDS peptide (Fig. 4B). As shown in Fig. 4C, the expression level of phosphorylation of Akt (p-Akt) was decreased in 7721-shCD147 cells and GRGDS-treated 7721-snc cells, indicating the diminished activation of PI3K/Akt signaling. The level of p-Akt was not decreased further in 7721-shCD147 cells after treatment with the GRGDS peptide. To explore the mechanism responsible for FAK pathway activation, 7721 cells were transfected with si-RNAs and collected at the indicated times. mRNA levels of CD147 and FAK and protein levels of CD147, FAK, p-FAK, and p-Akt decreased in a time-dependent manner (Fig. 4, D and E). As shown in Fig. 4F, CD147 extracellular domain increased protein levels of p-Akt.
and p-FAK in 7721 cells in a dose-dependent manner. However, FAK expression levels were not affected. Both RGD peptides and integrin subunit attenuated activation of Akt and FAK pathways, although expression of FAK was not significantly changed (Fig. 4G).

There are less co-localizations of HAb18G/CD147 with integrin subunit on the cell membrane of 7721-shCD147 cells compared with 7721-snc cells (Fig. 5A). Compared with 7721-snc cells, cells treated with GRGDS acquired a round-shaped morphology and had decreased co-localizations of HAb18G/CD147 with integrin B1 at cell-cell junctions. As shown in Fig. 5B, HAb18G/CD147 co-localized with integrin B1 on HCC cells but not on mesenchymal cells (white arrows) of human HCC tissues.

The integrin downstream signaling pathways involve many cytoskeletal proteins and enzymes. Activation of integrin signaling pathways ordinarily results in cytoskeletal reorganization (17). We tried to determine whether GRGDS and HAb18G/CD147 deletion could cause a cytoskeletal rearrangement in HCC cells. To quantify the changes in cytoskeletal arrangements, we labeled F-actin stress fibers with rhodamine-phalloidin, with or without GRGDS treatment, in 7721-snc or 7721-shCD147 cells.

FIGURE 4. Activation of downstream FAK signaling pathway induced by HAb18G/CD147-integrin subunit interaction. A, design of the shRNA. Top, structure of small hairpin RNAs targeting CD147 mRNA. Middle, structure of small hairpin RNAs acting as a negative control. Bottom, protein levels of HAb18G/CD147 in 7721 cells stably transfected with snc-shRNA vectors or shRNA vectors targeting CD147 mRNA, examined by Western blotting. In the following experiments, shCD147-1 and snc-1 were used. B, expression levels of HAb18G/CD147, integrin, FAK, and p-FAK (pY397) in 7721-snc cells or 7721-shCD147 cells incubated with the GRGDS or GREGS peptides. C, p-Akt levels in 7721-snc cells or 7721-shCD147 cells incubated with or without the GRGDS peptides. D, 7721 cells transiently transfected with siRNAs targeting different sequences of CD147 mRNA for the indicated times prior to cell lysis. Cells were then subjected to real time RT-PCR (D) and Western blot analysis (E) to detect mRNA levels of CD147 and FAK, and protein levels of CD147, p-Akt, p-FAK, and FAK. F, 7721 cells were treated with increasing amounts of the extracellular domain of CD147 for 36 h. G, 7721 cells were treated with 20 μg/ml of the extracellular domain of CD147 for 36 h and were simultaneously treated with GRGDS, GREGS (100 μg/ml), or integrin subunit antibody (10 μM). F and G, protein levels of p-Akt, FAK, and p-FAK were detected by Western blot analysis. Protein expression levels were normalized by human α-tubulin expression.
and were reorganized in GRGDS-treated 7721-snc cells compared with those observed in GRGES-treated 7721-snc cells (Fig. 5C). F-actin stress fibers in 7721-shCD147 cells and GRGDS-treated 7721-snc cells indicated changes from polarization throughout the cytoplasm toward polarization under the cell membrane. Meanwhile, the treatment of 7721-shCD147 cells with GRGDS did not result in further changes in F-actin stress fiber polarization.

**GRGDS Attenuates HAb18G/CD147-mediated Invasion, Migration, Growth, and Metastasis Potentials**—To confirm the involvement of an HAb18G/CD147-integrin interaction in the invasion, migration, and metastasis potentials of HCC cells, 7721-shCD147 cells and 7721-snc cells were subjected to wound healing assays, invasion assays, and gelatin zymography, with or without treatment with GRGDS or GRGES peptides. As shown in Fig. 6, A–C, both sh-CD147 and GRGDS treatments inhibited cell migration, MMP secretion, and the invasion potentials of 7721 cells. The levels of pro-MMP-9, MMP-9, pro-MMP-2, and MMP-2 examined by gelatin zymography were decreased by 60.0, 53.5, 77.6, and 46.8%, respectively, in 7721-shCD147 cells, and they were diminished by 56.8, 59.4, 84.7, and 43.2%, respectively, in GRGDS-treated 7721-snc cells, compared in both cases with the levels observed in 7721-snc cells (p < 0.01, Fig. 6B). As shown in Fig. 6D, the colony formation ability was significantly decreased in 7721-shCD147 cells and GRGDS-treated 7721-snc cells (p < 0.01). However, the co-treatment of sh-CD147 and GRGDS did not result in further inhibition of malignant properties compared with the single treatments (p > 0.05, Fig. 6, A–D).

Next, we inoculated the same number of 7721-shCD147 cells and 7721-snc cells subcutaneously into the flanks of nude mice to determine the role of HAb18G/CD147 in tumor formation in vivo. 7721-shCD147 cells showed decreased capacity of tumor growth compared with 7721-snc cells, and GRGDS treatment to tumors generated by 7721-snc cells decelerated tumor growth compared with the effects of PBS treatment. However, the treatment of 7721-shCD147 cells with GRGDS did not further decelerate tumor growth (Fig. 7A). The mRNA levels of CD147, ITGB1, MMP-2, and MMP-9 and the protein expression levels of HAb18G/CD147, integrin β1, FAK, and p-FAK in tumor tissues examined using real time RT-PCR (Fig. 7B), Western blot analysis (Fig. 7C), and immunohistochemistry (Fig. 7D) were consistent with the results obtained by the Western blot analysis and the immunofluorescent double staining in vitro (Figs. 4B and 5, A and C). Tumors generated by 7721-shCD147 cells or treated with GRGDS showed decreased levels of MMP-2 in mesenchymal cells adjacent to tumor cells, compared with the level in tumors generated by 7721-snc cells (Fig. 7D).
We further tested the potential of HAb18G/CD147 and integrin β1 in tumor metastasis. The incidence of metastasis after intrahepatic injection of HCC cells into nude mice was shown in Table 3 and Fig. 7E. Eight of eight mice in the 7721-snc cell group and six of seven mice in the RGE-treated 7721-snc cell group developed visible intrahepatic metastasis, whereas only three of seven mice in the 7721-shCD147 cell group and one of seven mice in the RGD-treated 7721-snc cell group developed visible intrahepatic metastasis. These results showed that both shCD147 and RGD treatments significantly decreased incidences of experimental intrahepatic metastasis of 7721 cells.

DISCUSSION

As an HCC-associated antigen, HAb18G/CD147 has been shown to play crucial roles in the intercellular interactions involved in tumor metastasis (18). Previous studies have found that CD147 interacts with the integrins α3β1 and α6β1 in HCC cells and activates the downstream FAK-PI3K-Ca²⁺ pathway, thus contributing to the enhanced invasion and metastatic potentials of HCC cells (2, 6). Although the intracellular molecules interacting with HAb18G/CD147 and

| Table 3 | Tumor metastasis of HCC cell lines in nude mice |
|---------|-----------------------------------------------|
| Cell lines | No. of mice with liver metastatic tumor/total mice | No. of mice with spleen metastatic tumor/total mice | No. of mice with lung metastatic tumor/total mice |
| 7721-snc | 8/8 | 3/8 | 0/8 |
| 7721-shCD147 | 3/7* | 2/7 | 0/7 |
| 7721-snc-RGE | 1/7a | 0/7 | 0/7 |
| 7721-snc-RGD | 1/7b | 0/7 | 0/7 |

*7721-snc versus 7721-shCD147, p = 0.026.

a 7721-snc-RGE versus 7721-snc-RGD, p = 0.0291.
CD147 Binds to MIDAS Motif of Integrin β1

A

B

C

D

E
CD147 Binds to MIDAS Motif of Integrin β1

their signal transduction pathways have been partially revealed, the fundamental mechanism of the interaction between HAb18G/CD147 and integrin was not well understood. This study attempted to accurately reveal the binding sites of HAb18G/CD147 and the integrin β1 subunit and the mechanism regulating the CD147-integrin β1 interaction and its downstream pathways.

In this study, we found that the extracellular head of the bind ligands through a putative MIDAS-like motif (14, 21, 22). Many studies have suggested that mechanism regulating the CD147-integrin tumor formation, as determined by the calculation of tumor volumes (19, 20). In this study, we found that the extracellular head of the β1 subunit (the βa domain) interacts with the extracellular portion of HAb18G/CD147. The mutated MIDAS motif of the βa domain in the β1 subunit abrogates the interaction of CD147 and the integrin β1 subunit, indicating a crucial role for MIDAS in the CD147-integrin β1 interaction.

As an important cell recognition site with the integrin, the Arg-Gly-Asp (RGD) sequence exists in various ligands, including FN. The Asp of the RGD motif has previously been proven to bind to the MIDAS motif in the β subunit (14), and β1 also contains a MIDAS motif (21). In this study, FN interacted with the β1 subunit and competitively repressed the CD147-integrin β1 interaction. The results of the co-immunoprecipitation experiments showed that the CD147-integrin β1 interaction is mediated by the MIDAS motif. It has been reported that Mn$^{2+}$ is effective at inducing the conformational changes associated with integrin MIDAS activation (14). Analysis of the binding of CD147 to integrin β1 over a range of Mn$^{2+}$, Mg$^{2+}$, or Ca$^{2+}$ concentrations demonstrated the affinity of cations (especially Mn$^{2+}$) to elicit conformational changes and to modulate the CD147-binding potential of integrin β1 MIDAS. These results imply two possibilities. First, the CD147-binding site on the integrin β1 subunit is a MIDAS motif. Second, occupation of the MIDAS site might help to expose/reorient the synergy site to create an optimal and stable complementary interface between the integrin and CD147.

Our laboratory has found that the crystal structure of the extracellular portion of HAb18G/CD147 comprises a membrane-distal N-terminal Ig C2 domain and a membrane-proximal C-terminal Ig I domain, which are connected by a five-residue flexible linker (13). Here, we showed that the I domain, but not the C2 domain, could bind the βA domain of integrin β1. Because the interactions of the MIDAS motif with the βA domain and various ligands are mediated by aspartic acid-based sequences such as RGD, LDV, KGD, RTD, and KQAGD in the ligands, we wondered whether aspartic acid-based sequences in HAb18G/CD147 also play an important role in the interaction of the βA domain and CD147. Asps in the I domain of CD147 were mutated by site-directed mutagenesis, and of five targeted Asp mutations, only the fourth Asp (Asp$^{179}$) mutation partially abolished the interaction with the βA domain compared with the other mutations.

The MIDAS motif in the βA domain of the integrin β1 subunit interacts with the I domain of CD147; moreover, the exposed Asp$^{179}$ residue in CD147 is critical for recognition by the integrin β1 subunit. A structural model of the integrin α3β1

FIGURE 7. Effects of interaction of HAb18G/CD147 with the integrin β1 subunit on the tumor growth of HCC cells in a nude mouse model. A, kinetics of tumor formation, as determined by the calculation of tumor volumes (n = 6). Tumors were generated in BALB/c nude mice by the subcutaneous injection of 7721-snc cells or 7721-shCD147 cells. Beginning on the 6th day (d) after inoculation, the same dose of GRGDS, GRGES, or PBS was injected into the growing tumors, and the tumors were monitored with a caliper every 2 days. B, expression levels of the mRNAs encoding CD147, the integrin β1 subunit, MMP-2, and MMP-9 in experimental tumors. GAPDH mRNA was used to normalize the variability in template loading. The data are reported as the mean ± S.D., *p < 0.01 versus 7721-snc cells with no treatment. C, expression of HAb18G/CD147, the integrin β1 subunit, p-FAK, and FAK in tumors determined by Western blot analysis. D, expression and localization of HAb18G/CD147; the integrin β1 subunit and MMP-2 in tumors were determined by immunohistochemistry. Scale bars, 20 μm. N indicates negative control group without treatment of peptides. E, metastasis assay by intrahepatic injection of HCC cells in nude mice. Liver and spleen were excised for examination. Yellow arrows, intrahepatic metastases.

FIGURE 8. Potential model of the interaction between CD147 and integrin α3β1 on different cells. The β subunit is blue; the β subunit is red, and the CD147 is green. Inset, a structural model of the integrin α3β1 variant in a complex with CD147. Only a portion of the integrin head region is shown, although the propeller is shown as blue, and the metal ion occupying the MIDAS of βA (red) is shown as gold. The integrin contacts CD147 at the I domain, and the loop (residues 142–147) of the I domain points to the MIDAS. The α3β1 structure in the model was generated using Modeller 8.0 software, based on the crystal structure of αvβ3 extracellular domain (Protein Data Bank code 1M1X). The CD147 structure in the model was the crystal structure of CD147 extracellular domain (Protein Data Bank code 3BH5). The model was docked using HADDOCK 2.0 software with the restrictions listed in the text. Extracellular interaction of CD147 and integrin α3β1 triggers FAK signaling, which regulates Akt and paxillin downstream pathways affecting cytoskeleton reorganization, cell growth, migration, and invasion.
in a complex with CD147 is shown in Fig. 8. The ovoid head of the integrin αβ3 containing the βα domain cannot approach a position adjacent to the cell membrane in the activated state, similar to the condition of β1 (15). Because Asp179 of the I domain is adjacent to the cell membrane, we presume that the interaction of CD147 and the integrin β1 subunit does not occur in the same HCC cell, but instead it occurs between neighboring HCC cells (Fig. 8). The resulting interactions between HCC cells also provide a possible explanation for enhanced cell junction and adhesion capacity.

The integrin signaling pathways consist of many cytoskeletal proteins and enzymes, including FAK, paxillin, and PI3K. We previously discovered that the activated FAK-paxillin and FAK-PI3K-Ca\(^{2+}\) signaling pathways, which enhance the metastatic potentials of HCC cells, closely correlate with HAb18G/CD147 expression (2, 6). The interaction of CD147 with the integrin β1 subunit could be competitively blocked by both the GRGDS peptide and the transfection of a CD147-specific shRNA (sh-CD147). Because of an attenuated interaction, the downstream FAK pathway failed to be activated, which resulted in a rearranged actin cytoskeleton. Simultaneously, HCC cells exhibited attenuated malignant properties, including MMP release, invasion, and migration potential and tumor formation capability. Co-treatment of sh-CD147 and GRGDS did not result in further inhibition to FAK pathway activation and malignant properties of the cells, indicating that HAb18G/CD147 interacts with integrin at the RGD-binding site (MIDAS pocket) to activate the FAK pathway.

A previous study in our laboratory has found down-regulation of FAK in 7721 cells transfected with si-RNA targeting CD147 mRNA (23). Our study showed that RNA interference (either by si-RNA or sh-RNA) resulted in decreased levels of both FAK and phosphorylated FAK. However, treatment with CD147 extracellular domain did not alter the FAK protein levels, and neither did treatments with GRGDS peptides or integrin β1 antibody. These results confirmed that attenuated interaction of HAb18G/CD147 with integrin decreased the phosphorylation level of FAK. Meanwhile, HAb18G/CD147 seemed to be capable of affecting the protein level of FAK by some other pathways. Besides CD147-integrin interaction, new mechanisms downstream of CD147 are being explored in our laboratory. As a transmembrane glycoprotein, CD147 localizes to both the cell membrane and the endomembrane system in HCC cells. A recent study in our laboratory has proved that CD147 localizing to endomembrane system inhibits the RhoA/ROCK signaling pathway and amoeboid movement via depression of annexin II phosphorylation (24). Although a previous study claimed that the ERK pathway was involved in FAK regulation by CD147 (23), further investigation is needed to study the responsible mechanism.

These data demonstrate that the CD147-integrin interaction executes an accelerative function of CD147 in the metastatic process of HCC cells. When the interaction was disrupted, CD147 failed to ensure the transduction of outside-in signals across integrins and proper intracellular functions.

RGD peptides have been proven to competitively interact with integrin, thus blocking the interaction of ligands with integrin in various tumor types. Additional RGD peptides could induce apoptosis of tumor cells through the activation of pro-caspase 3 (25). The radiolabeled RGD peptides could be used as a potent tumor imaging reagent, and recombinant adenoviral vectors containing RGD peptides are very effective in targeted cancer therapies, including those of breast cancer, melanoma, osteosarcoma, glioma, ovarian cancer, and pancreatic carcinoma (26–32).

It is significant to consider CD147 as a target for the development of diagnostic and treatment methods for cancers. Licartin (a \(^{131}\)I-labeled mAb specific for HAb18G/CD147) was developed in our laboratory and has been used safely and effectively in treating hepatocellular carcinoma patients (33, 34). Our results show that the RGD motif inhibits the CD147-integrin β1 interaction, blocking the outside-in signals of CD147 and attenuating the malignant properties of tumor cells that are induced by CD147. The regulatory binding site of the CD147-integrin β1 interaction might be a novel potential target for tumor therapy.

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