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Dimerization is essential for HAb18G/CD147 promoting tumor invasion via MAPK pathway

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ABSTRACT

HAb18G/CD147 is a transmembrane glycoprotein of the immunoglobulin superfamily (IgSF) and is reported to be correlated with invasion and metastasis of many cancers. The crystal structure of HAb18G/CD147 ectodomain has shown that it can form homodimers in crystal. However, the functional significance of HAb18G/CD147 dimerization remains unclear. In the present study, guided by the crystal structure, we performed extensive mutational and functional studies to identify residues critical for dimerization and molecular function of HAb18G/CD147. Fourteen mutants were purified and evaluated for their ability to form dimers in solution and in living cells. Subsequent functional validation revealed that K63E and S193A mutants, which disrupted CD147 dimerization both in solution and in living cells, showed clearly dominant-negative effects on MAPK activation, MMP2 induction and invasiveness in tumor cells. Taken together, the present study provides mutational and functional evidences demonstrating for the first time the functional importance of CD147 dimerization and its direct correlation with invasion and metastasis of tumor cells.

1. Introduction

CD147 plays fundamental roles in many normal and pathologic processes, with its important functions in tumor progression being most documented. It is ubiquitously expressed in various cell types, especially at high levels in tumor cells [1]. Different groups have found that CD147 stimulates the elevated expression of several matrix metalloproteinases (MMPs) including MMP1 [2], MMP2, MMP3, MMP9 and MMP11 [3–5] by surrounding stromal cells and tumor cells themselves [6–8], and by which promotes the invasion and metastasis of tumors [9]. We previously identified a hepatocellular carcinoma (HCC)-associated antigen named HAb18G by screening the HCC cDNA library using anti-HCC monoclonal antibody HAb18 [10]. The amino acid sequence of HAb18G is identical to that of CD147. Extensive functional studies have indicated that HAb18G/CD147 plays a key role in tumor cell motility and metastasis, viral infection, rheumatoid arthritis and T cell activation [11–15]. The underlying mechanisms involve comprehensive intermolecular interactions between HAb18G/CD147 and many associated molecules, such as integrins [16,17], annexin II [15] and cyclophilin A [11,18]. More significantly, HAb18G/CD147 has been shown to be a novel universal cancer biomarker for diagnosis and prognostic assessment of a wide range of cancers [19]. Based on these findings, the bivalent fragment of HAb18G/CD147-specific mAb HAb18, HAb18F(ab′)$_2$ (Metuximab), has been developed as a radioimmunotherapeutic agent, named LICARTIN [generic name, Iodine ^{131}I Metuximab Injection], and has been proved to be safe and effective in the treatment of HCC in clinical trials [20].

Besides in-depth biological and clinical studies of HAb18G/CD147 and its antibodies, we also determined the crystal structure of the soluble recombinant ectodomain (ECD) of HAb18G/CD147 [21]. Four homophilic dimers exist in the crystal and these dimers further adhere to each other by sharing some conserved β-strands at either edge of the β-barrels. Later, a crystal structure of CD147-ECD1 (residues 1–103) bearing double mutations of A19D and N44D also revealed a dimeric structure [22].

However, it remains to be confirmed whether the dimerization observed in crystal structure is biologically relevant, especially in stimulating MMPs production and promoting tumor invasion and metastasis. In the present study, we first systematically mutated dimer interface residues observed in the crystal structure, generated a series of mutants, and examined their dimerization both in solution and in living cells. We chose three representative mutants, V30A, K63E and S193A, to assess their abilities to induce MMPs secretion and promote invasion of tumor cells. Our results

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showed that K63E and S193A significantly decreased MMPs production and invasiveness of tumor cells due to disrupted dimerization and hence MAPK signaling pathway. Collectively, our data suggest that dimers constitute the functional components and are essential to molecular functions of HAb18G/CD147.

2. Materials and methods

2.1. Cell lines, antibodies and reagents

Human SMMC-7721 hepatoma cell line, human cervical carcinoma cell line CCL-2 (HeLa), normal human lung fibroblasts (MRC-5) and human embryonic kidney cell line (HEK-293) were obtained from Chinese Academy of Medical Science. For stably transfected cells, G418 (300 μg/ml) was added into the medium. For co-culture experiments, SMMC-7721 cells and MRC-5 cells were mixed in proportion of 1:1 and cultured with RPMI 1640 medium supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2. The vectors used for bimolecular fluorescence complementation (BiFC) assay were obtained from Addgene (Purdue University, US). HAb18G/CD147-specific mAb HAb18 was produced by our lab. Antibodies against His_6, MAPK and Phospho-MAPK were from Cell Signaling Technology. Anti-FLAG tag and anti-HA tag antibodies were from Invitrogen. Antibody against MMP2 was from Santa Cruz Laboratories. Anti-tubulin antibody and goat anti-mouse horseradish peroxidase (HRP)-conjugated IgG were obtained from Sino-American Biotechnology Co.

2.2. Site-directed mutagenesis of the dimer interface residues

HAb18G-pEGFP-N1 plasmid containing full-length HAb18G/CD147 (residues 1–269) previously constructed in our lab was used as template for mutagenesis. Totally fourteen mutations (G24A, T25A, V26A, T28A, V30A, K63A, K63E, L67A, E73A/K75A, F74A, R184A, K191A, S193A, Q195A) were generated by site-directed mutagenesis with the QuikChange multi-sited mutagenesis kit (Stratagene) according to the manufacturer's instructions.

2.3. Western blot analysis

Cell pellets were lysed with RIPA buffer (Beyotime, Inc., Nantong, China) containing 1 mM PMSF. Blots were probed with the appropriate antibodies and developed using ECL kit (Pierce, US).

2.4. Bimolecular fluorescence complementation (BiFC) assay

A BiFC assay developed by Hu et al. [23] to study protein–protein interaction in living cells was used in this study. cDNAs encoding the rWT or rMTs HAb18G/CD147ECD were amplified and inserted into pBiFC-VC155 vector and pBiFC-VN173 vectors separately. HEK-293 cells were transfected either individually or simultaneously with selected pairs of BiFC construct (0.2 μg of each plasmid). 24 h after transfection, cells were resuspended and analyzed with a flow cytometry (FACS Calibur, BD biosciences corp., US). Cells transfected with single BiFC plasmid were used to set the baseline for fluorescence detection.

2.5. Quantitative real-time PCR analysis

Total RNA was extracted using the TRIzol reagents (OMEGA Bio-Tek). Reversetranscription was performed using the PrimeScript RT reagent Kit (TaKaRa Biotechnology). All primers were synthesized by Shanghai Sangon Co. (Sangon, Shanghai, China) as follows: MMP2, forward primer 5′-GGCAGTGCAATACCTGAACACC-3′, reverse primer 5′-GTCTGGGGCAGTCCAAAGAACT-3′; MMP9, forward primer 5′-TTCCCCTTCACTTTCCTGGGTA-3′, reverse primer 5′-CGCCACGAGG AACAAACTGTAT-3′; GAPDH, forward primer 5′-GCACCGTCAAGGCT-GAGAAC-3′ and reverse primer 5′-TGGTGAAGACGCCAGTGGA-3′. Real-time PCR was performed using the SYBR Premix Ex Taq II Kit (TaKaRa Biotechnology).

2.6. In vitro invasion assay

The effect of dimerization mutation on SMMC-7721 cells invasion ability *in vitro* was analyzed in Transwell invasion chambers (Millipore) containing polycarbonate filters (pore size, 8 μm) as previously described [13].

3. Results

3.1. The key residues involved in HAb18G/CD147 dimerization

To pinpoint the residues critical for HAb18G/CD147 dimerization, we generated a series of single-/multi-point mutations of dimer interface residues based on the crystal structure of HAb18G/CD147 ectodomain [21] (Fig. 1A), and evaluated their dimerization state. All recombinant proteins were expressed in *E. coli* OrigamiB DE3 (Novagen) strain and purified to high homogeneity (Fig. 1B). When proteins were pretreated with denaturing loading buffer, a single band at molecular weight ~20 kDa corresponding to monomeric HAb18G/CD147ECD was detected for all proteins (Fig. 1C). In sharp contrast, when proteins were pretreated with non-denaturing loading buffer, Western blot using the anti-His_6 antibody revealed two major bands for the wild type (WT) protein at ~20 kDa and 40 kDa, indicating the existence of dimeric HAb18G/CD147ECD in solution (Fig. 1D, upper panel). Furthermore, the densitometry analysis (Fig. 1D, lower panel) suggested that the dimer/total protein ratio shifted from ~0.49 for rWT to ~0.01 for S193A as well as K63E, while the rest of the mutants showed a dimer/total protein ratio of below 0.08 (T28A) except V30A, K63A and F74A, whose dimer/total protein ratio is comparable to that of rWT. Although all the mutated residues were derived from the dimer-interface of four potential dimers observed in the crystal structure, they did not contribute equally to the dimerization. Noticeably, two mutations involving the same residue Lys-63 (K63E and K63A) led to different outcomes: K63E is completely monomeric while K63A retains as a dimer. The reason for causing this result is likely that K63A mutation is not strong enough to dissociate the dimer (e.g. the neighboring residues can still hold the dimer); while K63E causes dramatic electrostatic change to repel the binding partner and abolish dimerization. Based on these results, three representative mutants, V30A, K63E and S193A, were selected for subsequent functional validation, according to their dimeric state and their location on different domains.

3.2. Homophilic interaction of rWT or rMT HAb18G/CD147ECD in living cells

To further explore whether the WT HAb18G/CD147ECD could homo-dimerize and whether mutations introduced into the ectodomain would eliminate the protein–protein interactions in living cells, we used a BiFC assay. Since the Venus-expressing cells give rise to fluorescence due to fluorescence complementation of N-terminal and C-terminal fragments (VN and VC), the presence of homophilic interactions of HAb18G/CD147ECD can be readily detected with a flow cytometry. As illustrated in Fig. 2A, four pairs of fusion constructs were designed and designated as WT-VN/WT-VC, V30A-VN/V30A-VC, K63E-VN/K63E-VC, and S193A-VN/S193A-VC. Then, HEK-293 cells were transfected with equal amounts of pairwise BiFC plasmids. 24 h after transfection, Western blot of cell