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Domain 5 of high molecular weight kininogen inhibits collagen-mediated cancer cell adhesion and invasion in association with α-actinin-4

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ABSTRACT

High molecular weight kininogen (HK) is a plasma glycoprotein with multiple functions, including the regulation of coagulation. We previously demonstrated that domain 5 ($D5_H$), a functional domain of HK, and its derived peptides played an important role in the vitronectin-mediated suppression of cancer cell adhesion and invasion. However, the underlying mechanisms of the $D5_H$-mediated suppressive effects remain to be elucidated. Here, we showed that $D5_H$ and its derivatives inhibited the collagen-mediated cell adhesion and invasion of human osteosarcoma MG63 cells. Using purified $D5_H$ fused to glutathione-*S*-transferase (GST) and $D5_H$-derived peptides for column chromatography, an actin-binding protein, α-actinin-4, was identified as a binding protein of $D5_H$ with high-affinity for P-5m, a core octapeptide of $D5_H$. Immunofluorescence microscopy demonstrated that $D5_H$ co-localized with α-actinin-4 inside MG63 cells. In addition, exogenous GST-$D5_H$ added to the culture media was transported into MG63 cells, although GST alone as a control was not. As α-actinin-4 regulates actin polymerization necessary for cell adhesion and is related to the integrin-dependent attachment of cells to the extracellular matrix, our results suggest that $D5_H$ may modulate cell adhesion and invasion together with actinin-4.

1. Introduction

Cell adhesion is a fundamental cellular function, and is indispensable for the organization of cell morphology, leading to normal tissue formation and maintenance [1]. In addition to normal cells, the regulation of cell adhesion activity is an important factor for the invasiveness of cancer cells and progression of the tumor itself [2,3]. Thus, investigation of the molecular mechanisms of cell adhesion and invasion may increase our understanding of the properties of cancer cells, and suggest new approaches for the development of anti-cancer therapeutics.

High molecular weight kininogen (HK) is a plasma glycoprotein and is involved in the regulation of coagulation [4]. It initiates the intrinsic pathway of the blood coagulation cascade by promoting the conversion of factor XII to factor XIIa in cooperation with kallikrein and a negatively charged surface [5]. We previously reported that HK also functions as an inhibitor of cell adhesion [6]. Following this, several studies focused on the effect of HK or its derivatives on cellular functions [7,8], and demonstrated that domain 5 ($D5_H$), one of the six functional domains of HK, inhibited angiogenesis by inducing apoptosis and down-regulating proliferation and migration of endothelial cells [9–11].

We also observed that $D5_H$ impaired the vitronectin-mediated cell adhesion and invasion in different types of cancer cells by *in vitro* assay, and that it dramatically suppressed cancer metastasis in an *in vivo* assay [12,13]. It was further elucidated that the core amino acid sequence of $D5_H$ necessary for such inhibitory effects was Gly^{484} through Lys^{491}, designated P-5m [12]. However, little is known about the mechanism by which $D5_H$ induces the

Abbreviations: HK, high molecular weight kininogen; $D5_H$, domain 5 of high molecular weight kininogen; MEME, Minimum essential medium Eagle; GST, glutathione-*S*-transferase; Col-I, collagen type-I; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.

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inhibition of cell adhesion and invasion of cancer cells. In addition, since the role of $D5_H$ in cell adhesion and invasion was mainly examined using materials coated with vitronectin, it is unclear whether $D5_H$ exerts similar functions in other extracellular matrices, such as collagen. Therefore, this study examined the effects of $D5_H$ on the collagen-mediated cell adhesion and invasion in human osteosarcoma MG-63 cells, and identified a $D5_H$-binding protein, α-actinin-4, that shows high-affinity binding to P-5m, suggesting that this protein may participate in $D5_H$-induced functions.

2. Materials and methods

2.1. Cell culture

Human osteosarcoma MG63 cells were obtained from Riken Cell Bank (Tsukuba, Japan) and were cultured and maintained as monolayers in Minimum essential medium Eagle (MEME, Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum, non-essential amino acids solution (Wako, Osaka, Japan) and antibiotics solution (100 unit/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B; Wako) in a humidified atmosphere of 5% CO_2 in air at 37 °C.

2.2. Glutathione-S-transferase-fusion protein and peptides

Glutathione-*S*-transferase (GST)-fusion protein containing human $D5_H$ (GST-$D5_H$; Gly^{402}–Asn^{495}) was prepared as previously described [13]. All peptides derived from $D5_H$, P-5 (Lys^{480}–Asn^{495}), P-5c (His^{488}–Asn^{495}) and P-5m (Gly^{484}–Lys^{491}), were synthesized with an automated peptide synthesizer, Pioneer Peptide Synthesis System (PerSeptive Biosystems, Framingham, MA, USA) as previously described [12]. Peptides were purified to >90% purity by high-performance liquid chromatography using a BioCAD Sprint Perfusion Chromatography System (PerSeptive Biosystems). Peptide synthesis and purification was performed at the Central Research Laboratory in Shiga University of Medical Science, Japan.

2.3. Cell adhesion assay

Cell adhesion to bovine collagen type-I (Col-I; Millipore Japan, Tokyo, Japan) was performed as previously described with minor modifications. Briefly, ninety six-well microtiter plates (Maxisorp; Nunc, Roskille, Denmark) were coated overnight with 100 μl per well of 5 μg/ml Col-I at 4 °C. The plates were washed with phosphate-buffered saline (PBS), and non-specific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. MG63 cells (100 μl of 2×10^4 suspended cells) were placed in each well with or without various concentrations of GST, GST-$D5_H$, P-5, P-5c or P-5m. After 1 h incubation at 37 °C, non-adherent cells were removed by rinsing twice with PBS and adherent cells were fixed with 4% formaldehyde for 15 min, and then stained with Diff-Quik staining solution. Cells in four non-overlapping microscopic fields of each well were counted in at least three independent experiments. Statistical significance of the data was determined using the two-tailed Student's *t* test.

2.4. Cell invasion assay

The modified Boyden chamber assay was applied to assess the effects of $D5_H$ and its derived peptides on the Col-I-mediated invasive activity of MG63 cells [12–14]. The lower surfaces of polyvinyl 8-μm pore membranes were coated with 25 μl of 10 μg/ml Col-I, and the upper surfaces of the membranes were coated with 100 μl of 0.15 mg/ml Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA), and dried overnight at 37 °C. MG63 cells (100 μl of 1×10^5 suspended cells) in MEME with 0.1% BSA were added to each of the upper wells with or without various concentrations of GST, GST-$D5_H$, P-5, P-5c or P-5m, and incubated for 6 h at 37 °C. After incubation, cells on the upper surface of the membrane were removed by wiping with a cotton swab. Cells that had passed through the membrane and attached to the lower surface of the membrane were fixed with 4% formaldehyde and stained with Diff-Quik staining solution. The cell counting and statistical analysis were performed as described in cell adhesion assay.

2.5. Detection of $D5_H$-binding proteins from the membrane fractions of MG63 cells

Collection of membrane fractions of MG63 cells was performed as previously described, with several modifications [15]. Briefly, approximately 1×10^8 cultured MG63 cells were scraped and harvested in 0.02 M HEPES buffer, pH 7.2, containing 0.25 M sucrose. The cells were homogenized using a Dounce grinder (Wheaton, Millville, NJ, USA) and centrifuged at 1000*g* for 7 min at 4 °C. The supernatant was additionally centrifuged at 12,000*g* for 30 min at 4 °C. The pellets were used as membrane fractions. These samples were solubilized in lysis buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1% Triton X-100, 11 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 μM phenylmethanesulfonyl fluoride [PMSF]). After ultracentrifugation, the supernatant was loaded on the GST-$D5_H$-Shepharose affinity column, in which GST-$D5_H$ was coupled to CNB-activated Sepharose 4B as manufacturer's instructions. To obtain specific proteins that show a high-affinity to P-5m, but not P-5c, among the proteins that bound to $D5_H$, the $D5_H$-binding proteins were first eluted with 0.5 mM P-5c peptide solution, and then eluted with 0.5 mM P-5m peptide solution. The primary and secondary eluted proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE), followed by staining with Bio-safe-Coomassie Brilliant Blue (BioRad, Hercules, CA, USA). Only protein bands observed in the secondary eluate were isolated from the gel, and digested with trypsin. The digested peptides were analyzed by liquid chromatography tandem mass spectrometry.

2.6. Immunofluorescence microscopy

This experiment was performed as previously described [16]. Briefly, cells incubated with GST-$D5_H$ were washed three times with PBS, and fixed with 4% formaldehyde. Cells were permeabilized with 0.2% Triton X-100/PBS for 10 min at room temperature or they were untreated, and blocked with 1% BSA for 1 h at room temperature. The samples were incubated with primary antibodies for α-actinin-4 (1:200; Thermo Scientific, Rockford, IL, USA) and GST (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight, and then with Alexa488- and Alexa555-labeled secondary antibodies (1:1000; Invitrogen, Carlsbad, CA, USA) at room temperature for 1 h. Cell images were taken using an Olympus IX-71 microscope (Olympus, Tokyo, Japan) equipped with a cooled CCD camera CoolSNAP HQ (Nippon Roper, Tokyo, Japan). The overlay images were produced using MetaMorph software (Molecular Device Japan, Tokyo, Japan).

2.7. Western blot analysis

This analysis was performed as previously described [17]. Briefly, cells incubated with GST or GST-$D5_H$ were washed three times with PBS, and were harvested. Cells were then solubilized in RIPA buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% deoxycholate, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μM PMSF). The lysate was subjected to SDS–PAGE, and then electro-transferred to PVDF

membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk for 1 h, and incubated overnight in the same solution containing an rabbit anti-GST antibody (1:1000; Santa Cruz Biotechnology), followed by incubation with a secondary anti-rabbit IgG antibody (1:1000) conjugated to peroxidase (Beckman Coulter, Brea, CA, USA). Protein detection was performed using ECL Prime Western blotting detection reagent (GE Healthcare Life Sciences, Piscataway, NJ, USA), and imaged on an LAS-4000 luminescent image analyzer (Fujifilm Life Science, Stamford, CT, USA).

3. Results

3.1. $D5_H$ inhibits the collagen-mediated cell adhesion and invasion of MG63 cells

We previously reported that GST-$D5_H$ inhibited the vitronectin-mediated cell adhesion and invasion of cancer cells [12,13]. In this study, we examined whether the inhibitory effects of $D5_H$ are also observed in MG63 cells using a different extracellular matrix, collagen. GST-$D5_H$ significantly inhibited cell adhesion of MG63 cells on the Col-I-coated plates in a concentration-dependent manner, compared with GST (Fig. 1A). Similar results were obtained from the cell invasion assay, where GST-$D5_H$ significantly inhibited cell invasion of MG63 cells (Fig. 1B), although the degree of inhibition was less than that in our previous study [13]. These results indicate that the inhibitory effects of $D5_H$ might be common to extracellular matrices, but that the degree of inhibition appears to be different among the matrices.

3.2. Peptides derived from $D5_H$ inhibit the collagen-mediated cell adhesion and invasion of MG63 cells

As P-5 (Lys^{480}–Asn^{495}) and its core octapeptide P-5m (Gly^{484}–Lys^{491}) (Fig. 2A) inhibited the vitronectin-mediated cell adhesion and invasion of cancer cells similar to $D5_H$ [12,13], we next examined the inhibitory effects of these peptides on the collagen-mediated cell adhesion and invasion of MG63 cells. Both P-5 and P-5m inhibited the Col-I-mediated cell adhesion and invasion in a concentration-dependent manner (Fig. 2 B and C). Similar to $D5_H$, the degree of inhibition was less than that in our previous study [13]. In contrast, P-5c (His^{488}–Asn^{495}), which displayed no inhibitory activity for the vitronectin-mediated cell adhesion and invasion [12], had no effects on the Col-I-mediated cell adhesion and invasion. These results suggest that the basic properties of $D5_H$-derived peptides resembles those of $D5_H$.

3.3. Identification of $D5_H$-binding proteins with high-affinity for P-5m

To identify $D5_H$-binding proteins with high-affinity for P-5m, but not P-5c, membrane fractions of MG63 cells were prepared and loaded onto a GST-$D5_H$-bound Sepharose column. The binding proteins were first eluted using P-5c solution and then by P-5m solution. When the primary and secondary eluates were separated by SDS–PAGE and stained with Coomassie Brilliant Blue, two bands (~90 kDa and ~40 kDa) were specifically observed in the secondary eluates (Fig. 3). Mass spectrometry with protein database searching identified the 90 kDa and 40 kDa proteins as α-actinin-4 (Accession No. BAA24447) and isovaleryl-CoA dehydrogenase (Accession No. NP_002216), respectively. Since α-actinin-4 is an F-actin-binding peripheral membrane protein and is involved in cell adhesion [18], we focused on the association of $D5_H$ with α-actinin-4 in the regulatory mechanism of cell adhesion and invasion in the following experiments.

3.4. Intracellular localization and association of $D5_H$ with α-actinin-4

Although the above experiment suggested that $D5_H$ might bind to α-actinin-4, the localization of these proteins is different, as α-actinin-4 is in the cellular cytoplasm, and exogenous $D5_H$ was added to the medium. Thus, we hypothesized that $D5_H$ is transported into cells. To prove this hypothesis, GST-$D5_H$ and α-actinin-4 were double immunostained with anti-GST and anti-α-actinin-4 antibodies with or without cell permeabilization and analyzed by immunofluorescence microscopy. In the absence of cell permeabilization, neither GST-$D5_H$ nor α-actinin-4 was stained

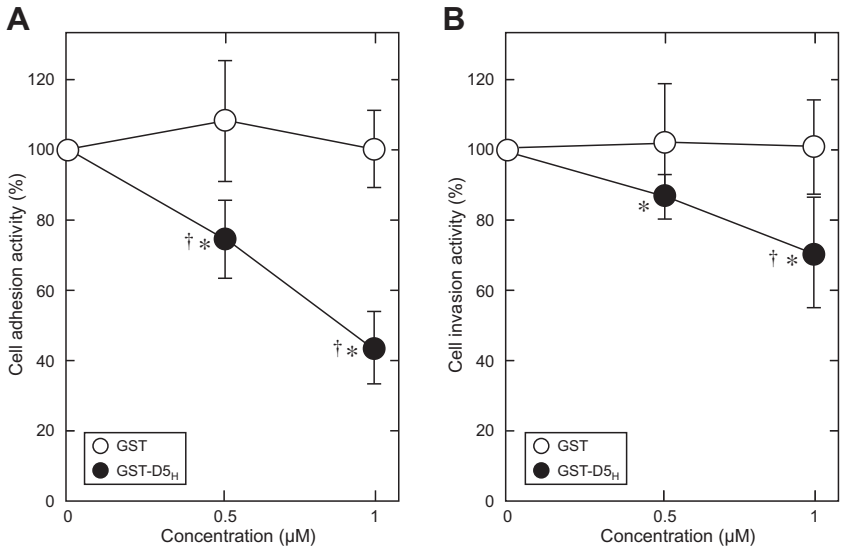

Fig. 1. Inhibition of the collagen-mediated cell adhesion and invasion of MG63 cells by $D5_H$. (A) Impaired cell adhesion induced by $D5_H$. MG63 cells were seeded on Col-I-coated 96-well microtiter plates in the presence of the indicated concentrations of GST-$D5_H$ or GST as a control, and incubated for 1 h. Cell adhesion activity was expressed as the percentage of adhesive cells at each concentration of GST-$D5_H$ or GST, of those in the absence of GST-$D5_H$ or GST. (B) Impaired cell invasion induced by $D5_H$. MG63 cells were seeded on Col-I-coated and Matrigel-filled chambers at the indicated concentrations of GST-$D5_H$ or GST, and incubated for 6 h. Cell invasion activity was expressed as the percentage of cells that crossed the membrane at each concentration of GST-$D5_H$ or GST, of those in the absence of GST-$D5_H$ or GST. Data are represented as mean ± S.D. $^*p < 0.05$ vs absence of GST-$D5_H$. $^\dagger p < 0.05$ vs GST.

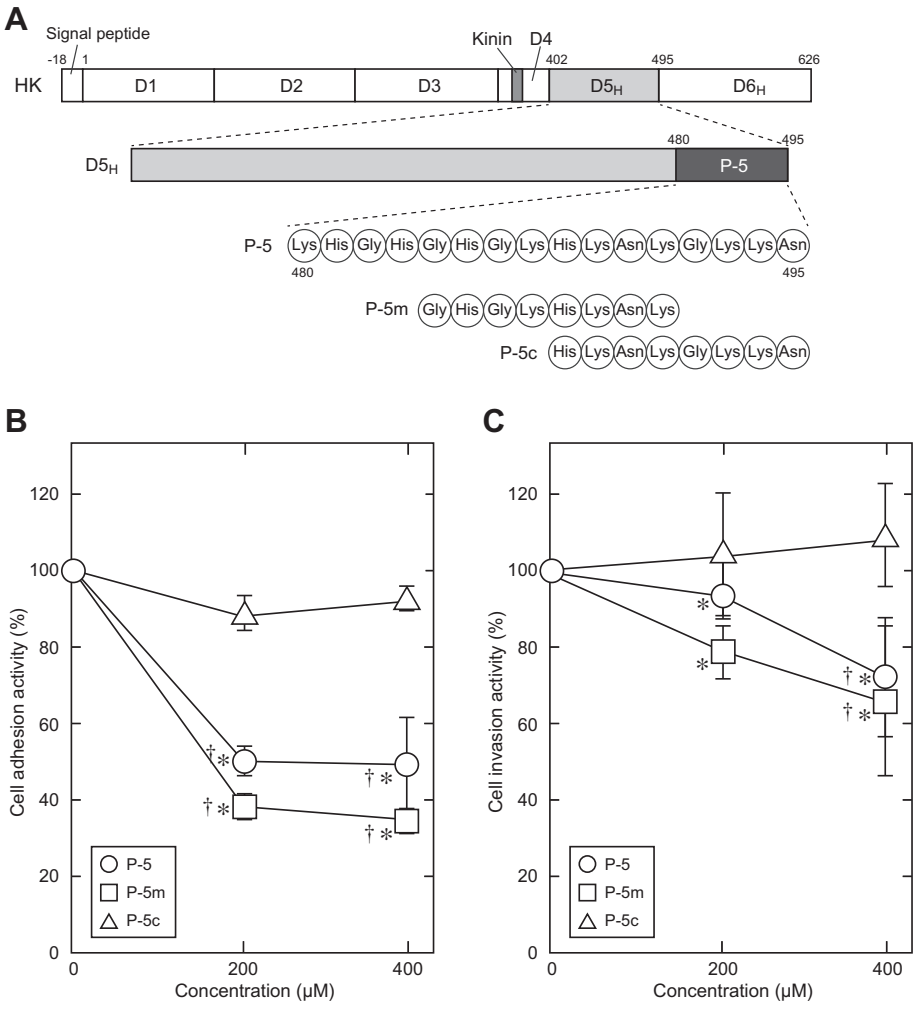

Fig. 2. Inhibition of the collagen-mediated cell adhesion and invasion of MG63 cells using peptides derived from $D5_H$. (A) Schematic representation of HK, D5 and its derived peptides (P-5, P-5c, P-5m). (B) Impaired cell adhesion induced by $D5_H$-derived peptides. (C) Impaired cell invasion induced by $D5_H$-derived peptides. These assays were performed as described in Fig. 1. Data are represented as mean ± S.D. $^*p < 0.05$ vs absence of each peptide. $^\dagger p < 0.05$ vs P-5c.

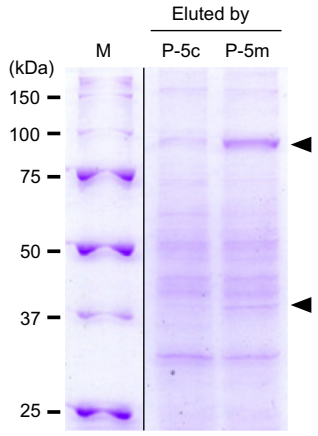

Fig. 3. Identification of $D5_H$-binding proteins with high-affinity for P-5m. After the membrane fractions of MG63 cells were loaded onto a GST-$D5_H$-bound Sepharose column, proteins eluted by P-5c or P-5m solution were separated by SDS–PAGE and stained with Coomassie Brilliant Blue. Arrowheads (~90 kDa and ~40 kDa proteins) indicate the specific bands observed from eluates using the P-5m solution.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4A, upper panels). However, both GST-$D5_H$ and α-actinin-4 were stained and these proteins were co-localized when the cells were permeabilized (Fig. 4A, lower panels), suggesting that $D5_H$ is transported into MG63 cells and associates with α-actinin-4. In addition, when lysates of MG63 cells were incubated with GST-$D5_H$ or GST as a control and were subjected to SDS–PAGE, the band for GST-$D5_H$, but not GST, was detected by Western blotting with an anti-GST antibody (Fig. 4B). The molecular weight of GST-$D5_H$ was estimated to be ~37 kDa, and the bands visible around 25 – 30 kDa were assumed to be the degraded products of GST-$D5_H$.

4. Discussion

Previously we demonstrated the inhibitory effects of $D5_H$ and its derived peptides on the vitronectin-mediated cell adhesion and invasion of cancer cells [12,13]. Here, we showed similar effects of $D5_H$ and its derived peptides using a different extracellular matrix, collagen, although the degree of the inhibition was slightly different between the matrices. Vitronectin is a ligand for most αv integrins, such as αvβ3 and αvβ5 [19], whereas collagen is a ligand for β1 integrins [20,21]. Our findings suggest that $D5_H$ may control the function of each type of integrins by distinct mechanisms and/or signaling pathways.

Furthermore, we identified two binding proteins of $D5_H$ that had a high-affinity for P-5m, the core octapeptide of $D5_H$. One of the proteins was α-actinin-4. This may be surprising, because the primary localization of $D5_H$ and α-actinin-4 is completely different.

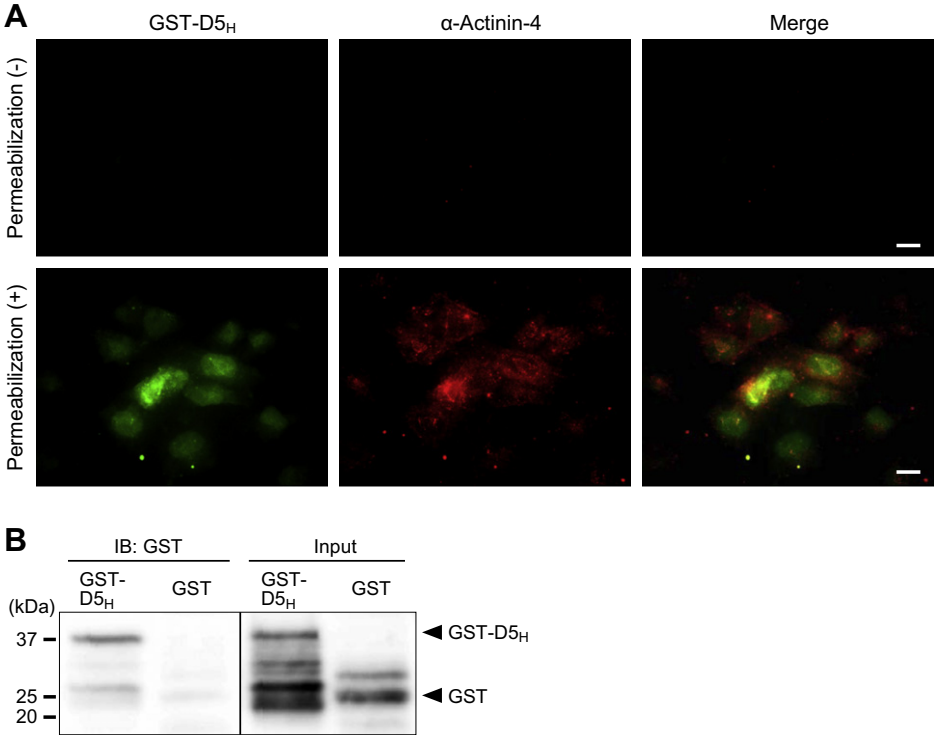

Fig. 4. Association of $D5_H$ with α-actinin-4. (A) Co-localization of $D5_H$ with α-actinin-4. MG63 cells incubated with GST-$D5_H$ were immunostained with anti-GST and anti-α-actinin-4 antibodies with or without permeabilization by Triton X-100. Scale bars: 20 μm. (B) Transportation of GST-$D5_H$, but not GST, into MG63 cells. MG63 cells incubated with GST-$D5_H$ or GST as a control were lysed, and the lysates were subjected to SDS–PAGE, followed by Western blotting with an anti-GST antibody.

$D5_H$ was added to the medium and was extracellular, whereas α-actinin-4 is intracellular. We, therefore, speculated that $D5_H$ might be transported into cells and would then associate with α-actinin-4. Indeed, we could detect GST-$D5_H$ in the cell lysate and the co-localization of GST-$D5_H$ with α-actinin-4. As a review article has shown that α-actinin-4 is involved in the regulation of cell adhesion together with actin and integrins [18], the association of $D5_H$ with α-actinin-4 may modulate the function of these molecules to impair cell adhesion and invasion. Future studies are required to reveal the molecular mechanisms of these functions.

P-5m is a short bioactive peptide consisting of 8 amino acids, is easy to synthesize, and has a significant suppressive effect on cancer cell invasion. Thus, P-5m may be a candidate for clinical use, especially to avoid or reduce cancer metastasis, which is a main cause of cancer death. Since the peptide is derived from a protein produced in the human body, the safety of administration of the peptide might be higher than other artificially produced anti-cancer drugs. The selective delivery to cancer cells, but not normal cells, should be resolved at the next step before testing this peptide as an anti-cancer therapy.

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