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Structure–Activity Relationships of RGD-Containing Peptides in Integrin αvβ5-Mediated Cell Adhesion

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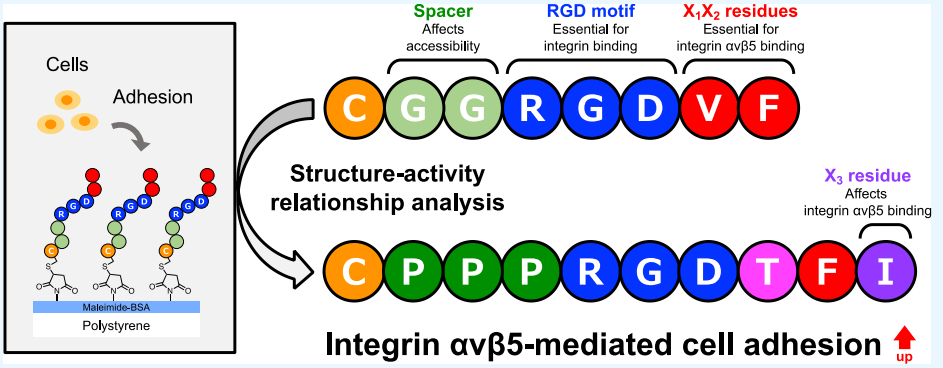

ABSTRACT: The RGD motif is a cell adhesion sequence that binds to integrins, a receptor family for extracellular matrix proteins. We previously reported that the $RGDX_1X_2$ sequence, where X_1X_2 is VF or NY, is required for integrin $\alpha v\beta 5$-mediated cell adhesion. However, the importance and applications of the X_1X_2 combinations and their surrounding sequences of integrin $\alpha v\beta 5$-binding $RGDX_1X_2$-containing peptides have not been comprehensively elucidated. Therefore, we aimed to identify an RGD-containing peptide with enhanced integrin $\alpha v\beta 5$ binding activity. We synthesized various peptides based on the RGDVF and RGDNY peptides to optimize the N-terminal, C-terminal, and X_1X_2 combinations of the $RGDX_1X_2$ sequence. These peptides were immobilized on maleimide-functionalized bovine serum albumin-coated plates via a thiol-maleimide reaction, and cell adhesion was evaluated using HeLa cells and human dermal fibroblasts. Consequently, CPPP-RGDTF and CPPP-RGDTFI were identified as highly active peptides for integrin $\alpha v\beta 5$-mediated cell adhesion. CPPP-RGDTF and CPPP-RGDTFI are expected to serve as cell adhesion molecules for developing culture substrates and biomaterials. Furthermore, these findings provide important novel insights into the interaction between the RGD motifs and integrins.

INTRODUCTION

Integrins are receptors of extracellular matrix proteins and control diverse biological functions, such as cell adhesion, migration, proliferation, differentiation, and survival.[1−4] Integrins are heterodimers composed of an α- and β-subunit, with 24 subtypes identified thus far.[1−4] Since its discovery in 1984, the Arg-Gly-Asp (RGD) motif has been used in a wide range of studies as a standard of integrin-binding cell adhesion peptides.[5−7] The integrin subtypes to which RGD binds include $\alpha 5\beta 1$, $\alpha 8\beta 1$, $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha v\beta 8$, and $\alpha IIb\beta 3$.[8−10] Notably, the affinity and selectivity for various integrin subtypes vary depending on the RGD-neighboring sequences[9,11−13] and conformation of peptides.[14−17] For example, the cyclic peptide c(RGDf(*NMe*)V), named cilengitide, exhibits a much higher affinity for integrin $\alpha v\beta 3$ and $\alpha v\beta 5$ than linear RGD peptides.[15,18] RGD-based compounds are expected to be used as integrin ligands for drug development,[19,20] drug delivery,[6,21,22] and tissue engineering.[6,7] However, RGD-based compounds have failed to pass clinical trials for applications other than as antithrombotic agents. This is probably due to the lack of understanding about the pharmacological properties of the RGD motif and the heterogeneity and redundancies of various integrin subtypes.[23] Further understanding of the interactions between the RGD motif and integrins is required to realize their clinical applications.

We previously reported that the RGD sequence alone has a low affinity for integrin $\alpha v\beta 5$; however, the presence of certain two amino acid residues (X_1X_2 = VF, NY) at the C-terminus of RGD increases its affinity for integrin $\alpha v\beta 5$.[24] Therefore,

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$RGDX_1X_2$-containing peptides can mediate cell adhesion through integrin $\alpha v\beta 5$. However, integrin $\alpha v\beta 3$-mediated cell adhesion is not affected by the X_1X_2 sequence. Thus, the $RGDX_1X_2$ motif enables the culture of cells that express integrin $\alpha v\beta 5$ but not those expressing integrin $\alpha v\beta 3$, such as induced pluripotent stem cells (iPSCs), HeLa cells, and A549 cells. The $RGDX_1X_2$ motif has the potential as a cell adhesion factor for a wide range of cells. However, the importance of the X_1X_2 combination and surrounding sequences of integrin $\alpha v\beta 5$-binding $RGDX_1X_2$-containing peptides requires further investigation. Optimization of $RGDX_1X_2$-containing peptides will lead to the development of more potent integrin $\alpha v\beta 5$ binding ligands for further applications.

The purpose of this study was to determine the structure−activity relationship of the peptides with the CGG-$RGDX_1X_2$ sequence, which were identified in our previous study as being essential for the adhesion via integrin $\alpha v\beta 5$. This would further clarify the specific sequence arrangement of amino acids required for integrin $\alpha v\beta 5$ binding and for obtaining peptides with more potent binding affinity. Integrin $\alpha v\beta 5$ binding peptides are expected to be applied as cell adhesion factors to diverse cells including iPSCs. To achieve this, we optimized the combination of X_1X_2 residues, the N-terminal spacer, and the C-terminal amino acid of $RGDX_1X_2$-containing peptides. The resultant peptides were immobilized on maleimide-functionalized bovine serum albumin (Mal-BSA)-coated plates via a thiol-maleimide reaction. Their cell adhesion activity was evaluated using HeLa cells and human dermal fibroblasts (HDFs).

MATERIALS AND METHODS

Peptide Synthesis. All peptides were manually synthesized with a C-terminal amide form using the 9-fluorenylmethoxycarbonyl strategy. The resulting protected peptides were exposed and cleaved from the resin using trifluoroacetic acid (TFA)−1,3-dimethoxybenzene−thioanisole−m-cresol−ethanedithiol−H_2O (85:3:3:3:3:3, v/v) for 3 h. Crude peptides were purified by reverse-phase high-performance liquid chromatography (HPLC) on a COSMOSIL 5C18-AR-II column (Nacalai Tesque, Kyoto, Japan) using gradient elution with water/acetonitrile containing 0.1% TFA. The purity and mass of peptides were confirmed by analytical high-performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry at the Central Analysis Center, Tokyo University of Pharmacy and Life Sciences (Tokyo, Japan).

Preparation of Mal-BSA. Mal-BSA was synthesized as previously described.[25] BSA (FUJIFILM Wako, Osaka, Japan) was dissolved in phosphate-buffered saline (PBS, 500 mg/50 mL) and mixed with N-succinimidyl 4-(*N*-maleimidomethyl)-cyclohexanecarboxylate (Tokyo Chemical Industry, Tokyo, Japan) in dimethyl sulfoxide (25.3 mg/5 mL). After incubation for 30 min at 25 °C, the resulting Mal-BSA was purified by dialysis (10 kDa MWCO) against water containing 0.1% TFA for three days and then lyophilized. The maleimide content of Mal-BSA was quantified from the mass difference between BSA and Mal-BSA, as measured by matrix-assisted laser desorption/ionization-mass spectroscopy (MALDI-TOF-MS) (Bruker, Billerica, MA, USA). Each BSA molecule was determined to have 8.33 maleimide groups.

Preparation of Peptide-BSA-Coated Plates. Peptide-BSA-coated plates were prepared as previously described.[25] Briefly, Mal-BSA was dissolved in water at 10 μg/mL and added to untreated plates (AGC Techno Glass, Shizuoka, Japan; 100 μL/well for 96-well plates, 2 mL/well for six-well plates). After incubation for 30 min at 37 °C, wells were washed twice with PBS. Then, cysteine-containing peptides in 100 mM HEPES buffer at pH 7 (100 μL/well for 96-well plates, 2 mL/well for six-well plates) were added, and wells were incubated for 2 h. The resulting peptide−BSA coatings were washed twice with PBS and used for cell adhesion experiments.

Cell Culture. HeLa cells (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan) and human dermal fibroblasts (Kurabo, Tokyo, Japan)) were maintained in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific).

Antibodies. Normal mouse IgG (isotype control) was purchased from Fujifilm Wako. Mouse monoclonal antibodies against human integrin $\alpha v\beta 3$ (LM609) and $\alpha v\beta 5$ (P1F6) were purchased from Merck (Kenilworth, NJ, USA). Alexa Fluor 488 goat anti-mouse IgG (H + L) antibody was purchased from Thermo Fisher Scientific.

Cell Adhesion Assay. HeLa cells were detached with 1 mM ethylenediaminetetraacetic acid (EDTA)/1 mM ethylene glycol tetraacetic acid (EGTA) in PBS. HDFs were detached using a 0.05% trypsin−EDTA solution. Detached cells were suspended in 0.1% BSA in DMEM. Cells were then seeded into peptide-BSA-coated 96-well plates (2×10^4 cells/100 μL/well for HeLa cells; 5×10^3 cells/100 μL/well for HDFs). For the inhibition assay, cells were seeded in the absence or presence of 10 μg/mL anti-integrin antibodies. After incubation for 1 h, attached cells were fixed, stained with 0.2% crystal violet aqueous solution containing 20% methanol, and photographed using a BZ-X810 microscope (Keyence, Osaka, Japan). The numbers of attached cells in nine central fields (0.77 mm^2 each) were counted, and their averages were calculated. All values in the figures are represented as the mean ± standard error (SE) of three independent experiments.

Statistical Analysis. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test.

RESULTS AND DISCUSSION

Cell Adhesion Activity of $RGDX_1X_2$-Containing Peptides against HeLa Cells and HDFs. In this study, we synthesized all peptides with a cysteine residue at the N-terminus via a spacer and immobilized them on maleimide-BSA-coated plates. We evaluated the cell adhesion activity of peptides using HeLa cells and HDFs. We have previously analyzed the expression levels of integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ in these cells by flow cytometry.[24] HeLa cells express integrin $\alpha v\beta 5$ but not $\alpha v\beta 3$, whereas HDFs express both integrins.[24] Therefore, evaluation of the cell adhesion activity of RGD-containing peptides listed in Table 1 revealed that HeLa cells only adhered to CGG-vnRGD, CGG-RGDVF, CGG-bspRGD, and CGG-RGDNY, all of which had the $RGDX_1X_2$ motif (X_1X_2 = VF, NY), but not to CGG-RGDAA and CGG-RGD (Figure 1A). Conversely, HDFs expressing integrin $\alpha v\beta 3$ adhered to all peptides, including CGG-RGDAA and CGG-RGD, indicating that the X_1X_2 residues were not required for binding to integrin $\alpha v\beta 3$. We did not detect any peptide-dependent differences in the morphology of adhered HeLa cells and HDFs (Figure 1B,C). Notably, we observed that

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Table 1. Sequences of RGD-Containing Peptides

peptide	sequence[a]	protein
CGG-vnRGD	CGGPQVTRGDVFT**n**P	vitronectin
CGG-RGDVF	CGGRGDVF	vitronectin
CGG-bspRGD	CGGNGEPRGDNYRAY	bone sialoprotein
CGG-RGDNY	CGGRGDNY	bone sialoprotein
CGG-RGDAA	CGGRGDAA	not applicable
CGG-RGD	CGGRGD	not applicable

[a]All peptides were synthesized with a C-terminal amide form. **n** = norleucine.

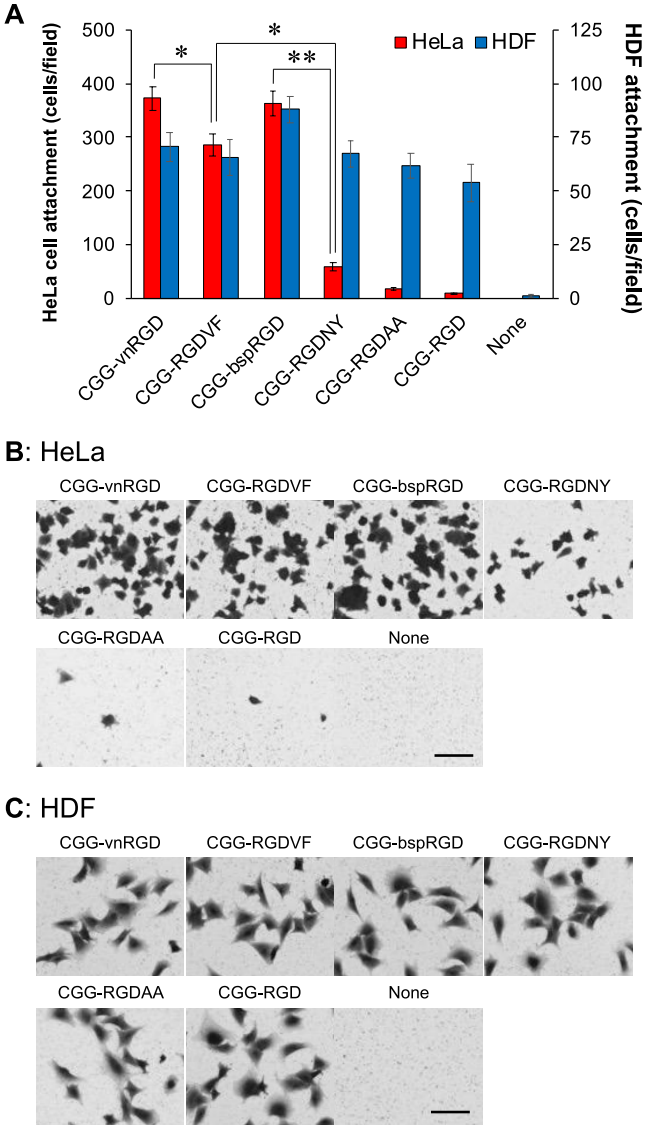

Figure 1. Cell adhesion activity of RGD-containing peptides. Peptides were conjugated to Mal-BSA-coated plates at a concentration of 1 μM. HeLa cells (2×10^4 cells/well) and HDFs (5×10^3 cells/well) in 0.1% BSA/DMEM were seeded into wells and incubated for 1 h. (A) Number of attached cells per 0.77 mm^2 was counted. Values are shown as the mean ± standard error of three independent experiments. *P < 0.05, **P < 0.0001. (B,C) Morphology of HeLa cells (B) and HDFs (C) attached to peptides. Scale bar = 100 μm.

HDFs exhibited the typical integrin-mediated elongated morphology on all tested peptides.

Effects of X_1X_2 Combinations on Cell Adhesion Activity of $RGDX_1X_2$ Peptides. In the case of HeLa cells, we observed that CGG-RGDVF, the core peptide of CGG-vnRGD, exhibited stronger activity than CGG-RGDNY, the core peptide of CGG-bspRGD. Notably, the affinity of CGG-RGDVF for integrin $\alpha v\beta 5$ is considered to be higher than that of CGG-RGDNY. This is because the X_1X_2 combination of the $RGDX_1X_2$ sequence critically affects the affinity for integrin $\alpha v\beta 5$. Therefore, to optimize X_1X_2 combinations, we synthesized peptides in which the VF residues of CGG-RGDVF were replaced sequentially with other amino acids (CGG-$RGDX_1F$ and CGG-$RGDVX_2$) and evaluated their cell adhesion activity using HeLa cells and HDFs (Figure 2). We detected that the adhesion of HeLa cells was high when X_1 = V, S, and T, indicating that the presence of a methyl and hydroxyl group on the β carbon of X_1 might be necessary for integrin $\alpha v\beta 5$ binding. Among them, CGG-RGDTF exhibited significantly higher activity than CGG-RGDVF. Conversely, we observed that the adhesion of HeLa cells was decreased when the other CGG-$RGDX_1F$ peptides were used, especially when X_1 = L, E, P, F, Y, and W. This trend suggested that amino acids with small side chains at the X_1 position are optimal for integrin $\alpha v\beta 5$-binding. Furthermore, we noted that most CGG-RGDVX2 peptides led to reduced adhesion of HeLa cells compared with that of CGG-$RGDX_1F$ peptides, indicating the importance of the X_2 amino acid in integrin $\alpha v\beta 5$ binding. Notably, the CGG-$RGDVX_2$ peptides were highly active when X_2 = F, P, Y, or W, with CGG-RGDVF exhibiting the highest activity. The enhanced activity of peptides with X_2 = F, Y, and W suggested that the aromaticity of the X_2 position contributes to integrin $\alpha v\beta 5$ binding. These results were consistent with previous studies reporting that integrin $\alpha v\beta 5$-binding RGD-containing cyclic peptides identified from the phage display peptide library mainly had S or T at X_1 and F or P at X_2 of their $RGDX_1X_2$ sequence.[13] Our study suggested that TF is the best X_1X_2 combination for binding to integrin $\alpha v\beta 5$. Evaluating the cell adhesion activity of CGG-$RGDX_1F$ and CGG-$RGDVX_2$ using HDFs revealed that no peptides except CGG-RGDPF demonstrated significantly decreased activity compared with that of CGG-RGDVF. This might be because HDFs express $\alpha v\beta 3$, which does not require X_1X_2 for its binding. We further noticed that CGG-RGDSF, CGG-RGDTF, and CGG-RGDVP, which exhibited high activity in HeLa cells, also demonstrated relatively high activity in HDFs, although not significantly increased compared with that of CGG-RGDVF. As HDFs also express integrin $\alpha v\beta 5$, the X_1X_2 combination might also slightly affect their adhesion. Among the CGG-$RGDX_1F$ and CGG-$RGDVX_2$ peptides, we detected that only CGG-RGDPF indicated no cell adhesion activity to either HeLa cells or HDFs, whereas CGG-RGDVP exhibited high activity. These results suggested that P differentially affects the integrin binding of $RGDX_1X_2$ motifs depending on its position, owing to the considerable effect that a proline residue generally exerts on the structure of peptides.[26]

Effect of Spacers on Cell Adhesion Activity of $RGDX_1X_2$ Peptides. As depicted in Figure 1, CGG-RGDVF and CGG-RGDNY led to significantly weaker cell adhesion of HeLa cells than their parent peptides, CGG-vnRGD and CGG-bspRGD. This suggested that sequences other than the $RGDX_1X_2$ motif in parent peptides also affect the activity. Therefore, we focused on CGG-RGDNY, which largely differed from the parent peptide, and analyzed the effect of RGDNY-neighboring sequences on the adhesion of HeLa cells. Since RGDNY was less active than RGDVF, it was easy to evaluate the effects of RGDNY-neighboring sequences on the

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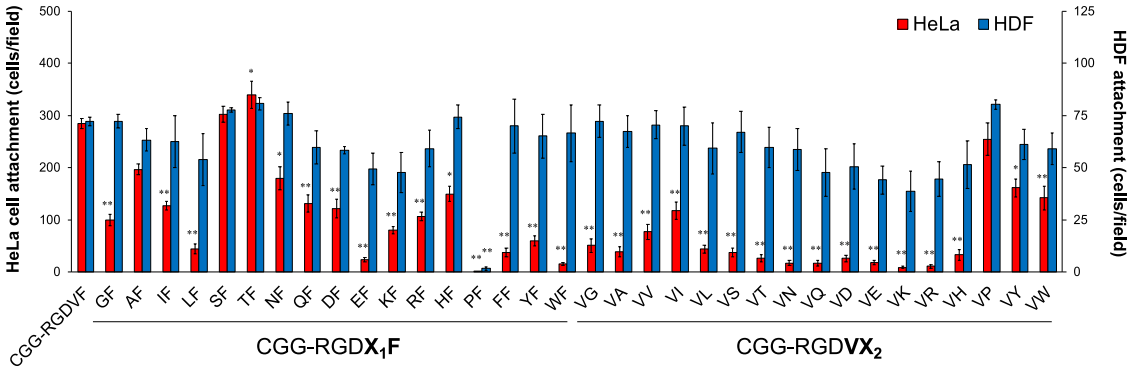

Figure 2. Effect of the X_1X_2 combination on cell adhesion activity of CGG-RGDX$_1$X$_2$ peptides. CGG-RGDX$_1$F and CGG-RGDVX$_2$ peptides were conjugated to Mal-BSA-coated plates at a concentration of 1 μM. HeLa cells (2×10^4 cells/well) and HDFs (5×10^3 cells/well) in 0.1% BSA/DMEM were seeded into wells and incubated for 1 h. The number of attached cells per 0.77 mm^2 was counted. Values are shown as the mean ± standard error of three independent experiments. *P < 0.05, **P < 0.0001 vs CGG-RGDVF.

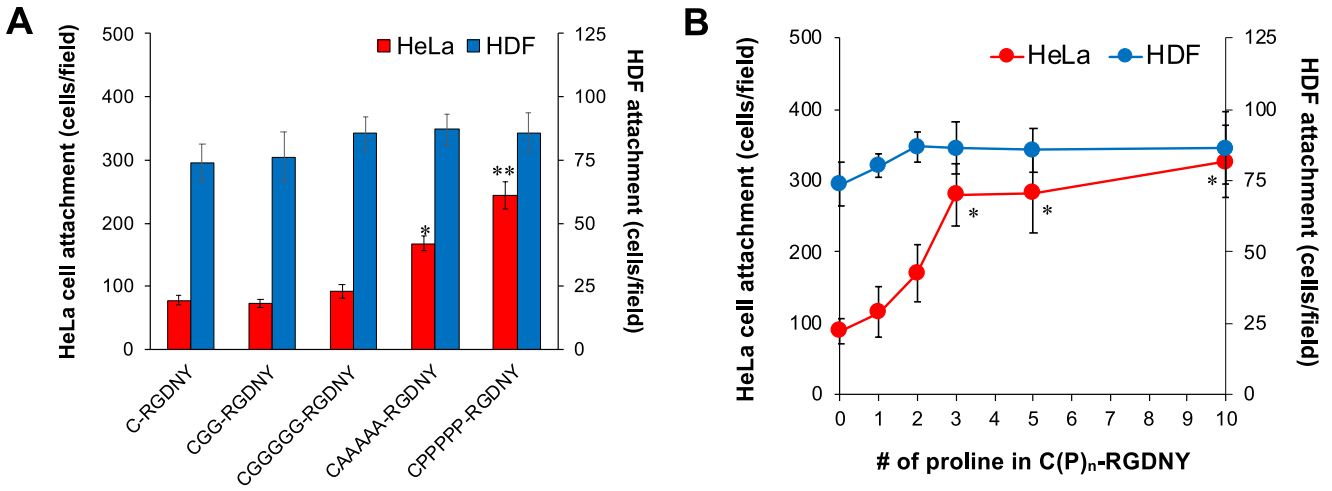

Figure 3. Effect of spacers on cell adhesion activity of RGDNY peptides. RGDNY peptides with various or no spacers (C-RGDNY) were conjugated to Mal-BSA-coated plates at a concentration of 1 μM. HeLa cells (2×10^4 cells/well) and HDFs (5×10^3 cells/well) in 0.1% BSA/DMEM were seeded into wells and incubated for 1 h. The number of attached cells per 0.77 mm^2 was counted. Values are shown as the mean ± standard error of 3 independent experiments. (A) Effects of di-glycine, penta-glycine, penta-alanine, and penta-proline spacers on adhesion activity. *P < 0.05, **P < 0.0001 vs C-RGDNY. (B) Cell adhesion activity as a function of proline spacer length (n = 0, 1, 2, 3, 5, or 10 in C$(P)_n$-RGDNY). *P < 0.05 vs C-RGDNY (n = 0).

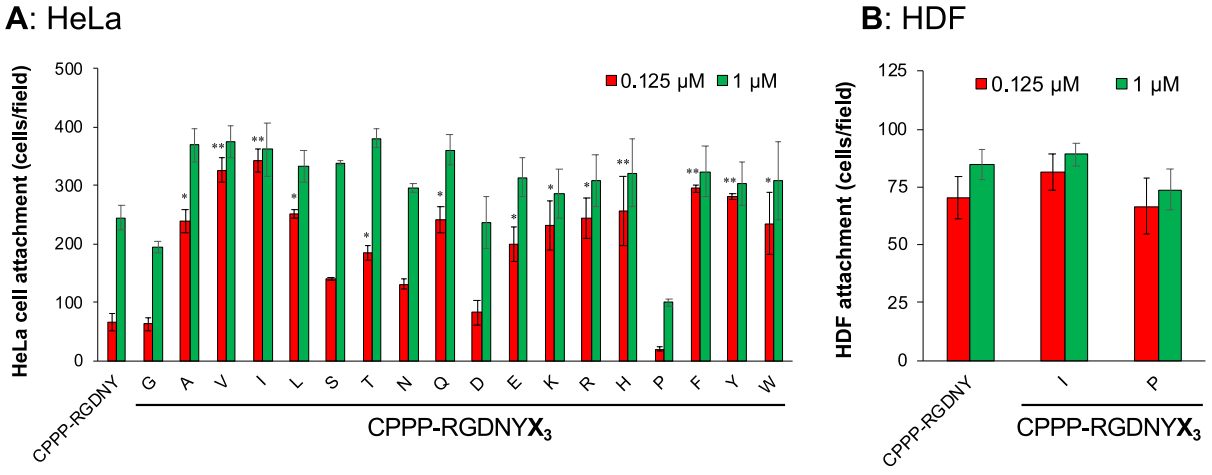

Figure 4. Effect of the X_3 residue on the cell adhesion activity of CPPP-RGDNYX$_3$ peptides. CPPP-RGDNY and CPPP-RGDNYX$_3$ peptides with various X_3 amino acids were conjugated to Mal-BSA-coated plates at concentrations of 0.125 μM and 1 μM. (A) HeLa cells (2×10^4 cells/well) and (B) HDFs (5×10^3 cells/well) in 0.1% BSA/DMEM were seeded into wells and incubated for 1 h. The number of attached cells per 0.77 mm^2 was counted. Values are shown as the mean ± standard error of three independent experiments. *P < 0.05, **P < 0.0001 vs CPPP-RGDNY.

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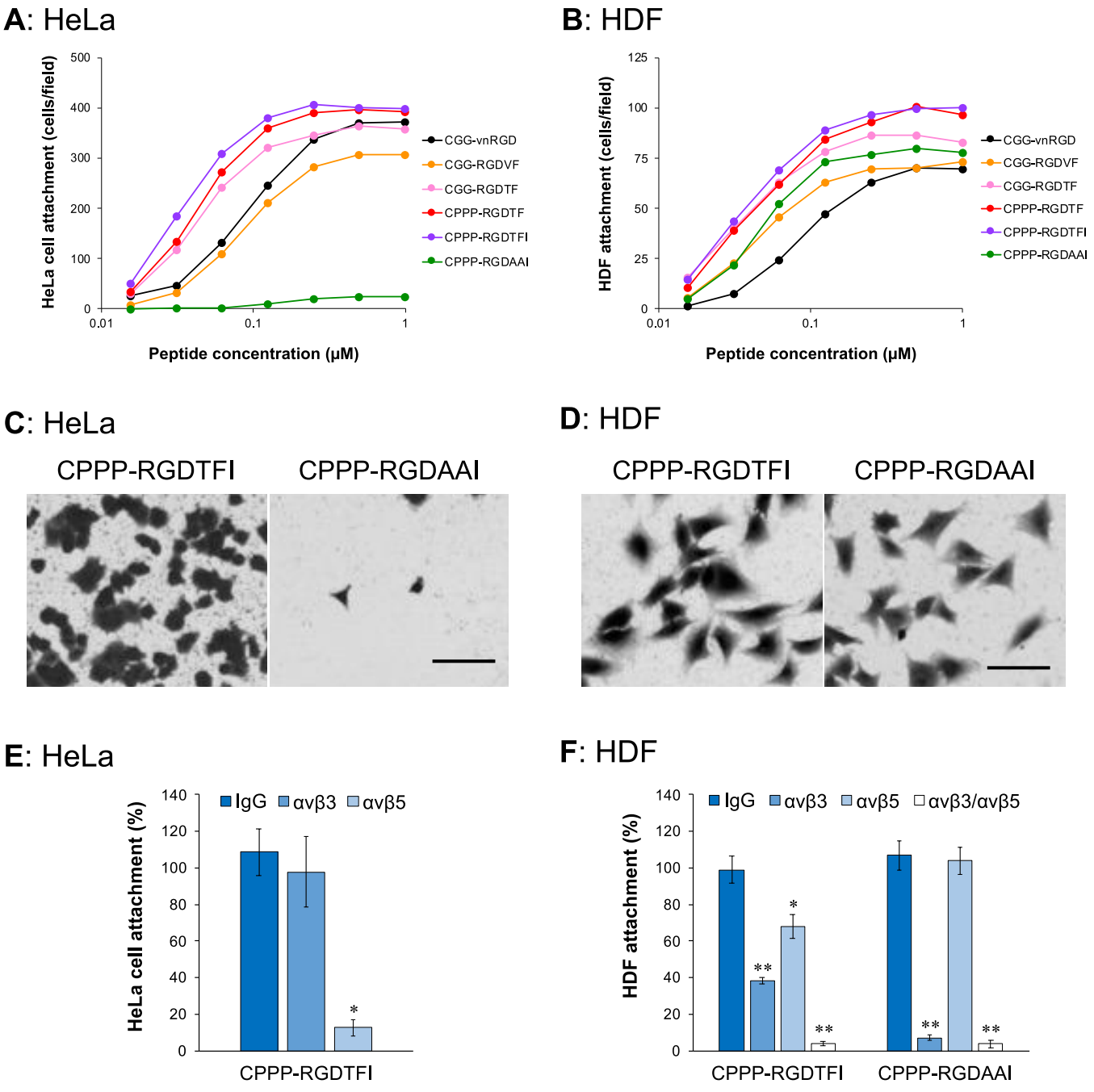

Figure 5. Cell adhesion activity of CPPP-RGDTFI. (A,B) Dose-dependent cell adhesion to CGG-vnRGD, CGG-RGDVF, CGG-RGDTF, CPPP-RGDTF, CPPP-RGDTFI, and CPPP-RGDAAI. (A) HeLa cells (2×10^4 cells/well) and (B) HDFs (5×10^3 cells/well) in 0.1% BSA/DMEM were seeded into wells and incubated for 1 h. The number of attached cells per 0.77 mm^2 was counted. Values are shown as the mean ± standard error of three independent experiments. (C,D) Morphology of (C) HeLa cells and(D) HDFs attached to CPPP-RGDTFI and CPPP-RGDAAI. Peptides were conjugated to plates at a concentration of 1 μM. Scale bar = 100 μm. (E,F) Effect of anti-integrin antibodies on cell adhesion to CPPP-RGDTFI and CPPP-RGDAAI. Peptides were conjugated to Mal-BSA-coated plates at a concentration of 0.1 μM. Cells in the absence or presence of 10 μg/mL anti-integrin antibodies (IgG isotype control, $\alpha v\beta 3$, or $\alpha v\beta 5$, or both $\alpha v\beta 3$ and $\alpha v\beta 5$) were added to wells and incubated for 1 h. The number of attached cells and relative cell attachment were calculated. Cell attachment in the absence of the antibody was set to 100%. Values are shown as the mean ± standard error of three independent experiments. *$P < 0.05$, **$P < 0.0001$ vs IgG.

activity. We hypothesized that the N-terminal sequence of RGDNY acts as a spacer between RGDNY and the cysteine that binds to the scaffold, Mal-BSA. Spacers between RGD motifs and scaffolds have been reported to affect cell adhesion activity.[7,27] Therefore, we used di-glycine, penta-glycine, -alanine, and -proline as the N-terminal spacer of the RGDNY motif and compared their effects on cell adhesion (Figure 3A). Glycine is the smallest amino acid, and its oligomers are highly flexible.[28] Alanine is another small amino acid with a methyl group as a side chain and is often used to design rigid spacers. In contrast, proline oligomers form polyproline helices and act as highly rigid spacers.[28,29] In Figures 1 and 2, in which di-glycine was used as a spacer for peptides, we did not observe any difference in activity between

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the RGDNY motif with no spacer and that with di-glycine. Likewise, we did not detect any increase in activity when the penta-glycine spacer was used. However, when we used the penta-alanine or penta-proline spacer, we observed that both increased activity, with penta-proline demonstrating the highest activity. This indicated that spacers with high rigidity are suitable for the activity of RGDNY. Subsequently, we compared the length of the proline spacer (Figure 3B). We observed that the activity of the motif reached a plateau at tri-proline, indicating that tri-proline was the shortest spacer with an optimal effect. In the case of HDFs, the observed differences in activity due to spacers were relatively small. This might be because RGDNY alone exhibits sufficiently potent activity in integrin $\alpha v\beta 3$-mediated adhesion. Based on these results, we decided to insert the tri-proline spacer between the $RGDX_1X_2$ sequence and the cysteine residue in subsequent experiments.

Effect of the X_3 Residue on Cell Adhesion Activity of $RGDX_1X_2$ Peptides. The C-terminal side of RGDNY might also be essential for cell adhesion via integrin $\alpha v\beta 5$. In a previous study, an amino acid on the C-terminus of RGDNY, the X_3 residue of $RGDNYX_3$, affected cell adhesion.[24] Therefore, we synthesized various CPPP-$RGDNYX_3$ peptides and evaluated their cell adhesion activity (Figure 4). Figure 4A displays the adhesion of HeLa cells in plates coated with 1 and 0.125 μM peptides. We observed that at 1 μM peptides, the differences in adhesion activity were minimal, but at 0.125 μM, significant differences were observed depending on the X_3 amino acid of the motif. Notably, we observed that the presence of amino acids other than P, G, and D at the X_3 position resulted in higher activity than that of CPPP-RGDNY. The activity was exceptionally high when X_3 was a hydrophobic amino acid, such as V or I. Conversely, we noticed that CPPP-RGDNYP exhibited lower activity compared with that of CPPP-RGDNY, indicating that P is not suitable at the X_3 position. When we evaluated the cell adhesion activity of CPPP-RGDNYI and CPPP-RGDNYP in HDFs, the effect of the X_3 amino acid was negligible (Figure 4B). This was probably attributed to the inclusion of integrin $\alpha v\beta 3$-mediated adhesion in HDFs. These results indicated that adding a single amino acid residue, especially a hydrophobic amino acid such as I, to the C-terminus of $RGDX_1X_2$ could enhance integrin $\alpha v\beta 5$-mediated cell adhesion.

Cell Adhesion Activity of CPPP-RGDTFI. Our study indicated that in terms of integrin $\alpha v\beta 5$-mediated cell adhesion, tri-proline served as an effective spacer at the N-terminus of $RGDX_1X_2$. TF was the optimal X_1X_2 combination, and the presence of I at the X_3 position increased the adhesion activity of the motif. Therefore, we synthesized CPPP-RGDTFI and compared its cell adhesion activity with that of CGG-vnRGD, CGG-RGDVF, CGG-RGDTF, CPPP-RGDTF, and CPPP-RGDAAI (Figure 5A,B). We here evaluated the dose-dependent cell adhesion to compare the activity of peptides in more detail. We found that CPPP-RGDTFI showed the strongest cell adhesion activity among the peptides assessed, although the effects of the tri-proline spacer and I at the X_3 position were much smaller than those in RGDNY. In particular, we detected almost no difference between the activity of CPPP-RGDTF and CPPP-RGDTFI. We assumed that X_1X_2 contributes more to integrin $\alpha v\beta 5$-mediated cell adhesion than the spacer and X_3 residue, with RGDTF itself having a sufficiently high affinity to integrin $\alpha v\beta 5$. We also found that the activity of CPPP-RGDTF and CPPP-RGDTFI was higher than that of the parent peptide CGG-vnRGD. The lack of HeLa cell adhesion with CPPP-RGDAAI indicated that the TF residues in CPPP-RGDTFI are essential for integrin $\alpha v\beta 5$-mediated adhesion. In addition, we observed that CPPP-RGDTF and CPPP-RGDTFI also exhibited high activity when HDFs were used, indicating that the optimization of the $RGDX_1X_2$-containing peptides improved adhesion even in cells expressing both $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins. The morphology of cells exposed to CPPP-RGDTFI did not differ from that of cells exposed to other active peptides shown in Figures 1B,C and 5C,D. Inhibition experiments using anti-integrin antibodies showed that CPPP-RGDTFI mediated the adhesion of HeLa cells via integrin $\alpha v\beta 5$ (Figure 5E). HDF adhesion to CPPP-RGDTFI was inhibited by both anti-integrin $\alpha v\beta 3$ and $\alpha v\beta 5$ antibodies, confirming binding to both integrin $\alpha v\beta 3$ and $\alpha v\beta 5$ (Figure 5F). These results were consistent with what we have previously reported for CGG-RGDVF,[24] confirming that the conversion of the structure to CPPP-RGDTFI did not change the interacting integrin subtype. It has been reported that HeLa cells express integrin $\alpha 5\beta 1$, $\alpha v\beta 6$, and $\alpha v\beta 8$ as well as $\alpha v\beta 5$.[30,31] However, the adhesion of HeLa cells to CPPP-RGDTFI was inhibited by the anti-integrin $\alpha v\beta 5$ antibody by about 90%, suggesting that the adhesion was mostly mediated by $\alpha v\beta 5$.

CONCLUSIONS

In this study, we optimized the combination of X_1X_2 residues, the N-terminal spacer, and the C-terminal X_3 amino acid of $RGDX_1X_2$ peptides from the aspect of integrin $\alpha v\beta 5$-mediated cell adhesion activity. Consequently, CPPP-RGDTF and CPPP-RGDTFI were identified as peptides that led to the considerably high cell adhesion of HeLa cells and HDFs. For the X_1 position, amino acids with relatively small side chains were found to be suitable; V, S, and T were particularly excellent, suggesting that the methyl and hydroxyl groups on the β-carbon are important for binding to integrin $\beta 5$. Aromatic amino acids were superior with respect to the X_2 position. This suggests that π interactions may be involved in the binding between the X_2 residue and integrin $\beta 5$. Hydrophobic amino acids were found to be more suitable at position X_3. The reason for this is not clear, but the hydrophobic side chains may contribute directly to binding to integrin $\beta 5$, or hydrophobicity at the C-terminus of the peptides may increase the hydrophobicity of the plate surface and improve cell affinity. This is probably the reason why F was superior to the other aromatic amino acids at the X_2 position. These findings are crucial for understanding the interaction between RGD motifs and integrin $\alpha v\beta 5$. As integrin $\alpha v\beta 5$ is expressed in a wide range of tissues and cells, CPPP-RGDTF and CPPP-RGDTFI are expected to be applied as cell adhesion molecules for the development of various cell culture substrates and biomaterials.

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Notes

The authors declare no competing financial interest.

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