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Amino Acids and Peptides. Part 39: A Bivalent Poly(ethylene glycol) Hybrid Containing an Active Site (RGD) and Its Synergistic Site (PHSRN) of Fibronectin

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Abstract—Fibronectin contains the active sequence Arg-Gly-Asp (RGD), along with its synergic site Pro-His-Ser-Arg-Asn (PHSRN). However, the PHSRN peptide does not show synergic activity when it is mixed with the RGD peptide, indicating that a spatial array between RGD and PHSRN in fibronectin may be necessary for synergic activity. Here, we have used an amino acid type poly(ethylene glycol) derivative (aaPEG) to design a bivalent PEG hybrid of fibronectin active peptides. We prepared the aaPEG hybrid peptides PHSRN–aaPEG, aaPEG–RGD, and PHSRN–aaPEG–RGD, and tested their biological activity. Whereas aaPEG–RGD promoted cell spreading activity, PHSRN–aaPEG had no activity. The PHSRN–aaPEG–RGD hybrid strongly promoted cell spreading compared with aaPEG–RGD. These results suggest that the PHSRN sequence in the PHSRN–aaPEG–RGD molecule synergistically enhances the cell spreading activity of the RGD sequence, and that the bivalent aaPEG hybrid method may be useful for conjugating functionally active peptides.

Fibronectin, a well-characterized multi-functional extracellular matrix protein, plays an important biological role in many cell–surface interactions,[1] mediating cell adhesion, embryonic cell migration, and wound healing.[1] Among the many active sites in extracellular matrix proteins, the cell-adhesive domain of fibronectin has been well studied.[2] An Arg-Gly-Asp (RGD) sequence located in the 10th type III repeating unit is a critical cell-adhesive site for the cell-surface receptor integrin,[2,3] and a Pro-His-Ser-Arg-Asn (PHSRN) sequence in the 9th type III repeating unit, although itself not biologically active, enhances the cell-adhesive activity of RGD.[4,5] However, the cell-adhesive activity of a recombinant protein corresponding to the 10th type III repeating unit of fibronectin is not stimulated by a PHSRN peptide, and thus the spatial array between PHSRN and RGD has been reported to be important for the synergistic interaction.[5]

As PEG has low toxicity and immunogenicity, and has good solubility in both aqueous and organic solvents, it has been used as a carrier for various drugs. The pegylation (hybrid formation with PEG) of many proteins (such as PEG–asparaginase[6] and PEG–tumor necrosis factor[7]) has been reported. Furthermore, a pegylated adenosine deamidase[8] is commercially available for clinical use. Relatively few studies have focused on the pegylation of small peptides,[9,10] possibly because it is assumed that the modification of a small bioactive peptide with a large molecule such as PEG would cause a loss of activity. When we conjugated laminin-related peptides with PEG, however, these PEG–peptides strongly inhibited tumor metastasis activity in vivo.[9] In addition, Lu and Felix were successful in the site-specific pegylation of an interleukin-related peptide.[10] These results indicate that PEG is a promising candidate for a drug carrier, although its simple structure limits the types of hybrid that can be produced. Usually PEG is converted to a carboxyl derivative and introduced onto a protein as a simple acyl moiety. In this study, we planned to prepare a multi-functional

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PEG–peptide hybrid using an amino-acid-type PEG (aaPEG).

We report the preparation of a bivalent PEG hybrid, PHSRN–aaPEG–RGD, in which aaPEG functions as a spacer between two functional peptides. The flexible conformation of PEG allows the two functional groups to bind properly. To bind two different peptides on each end of a PEG, we generated aaPEG from poly(oxyethylene) diglycolic acid 3000 (carboxymethylated PEG, cmPEG, MW 2400–3500, Wako Chemical Ind. Ltd., Osaka, Japan). We previously reported[11] the preparation of aaPEG, which was purified by Sephadex column chromatography and ion-exchange column chromatography at the final step. In this study, we established a more effective purification procedure for generating high-purity aaPEG. We then compared the cell spreading activity of synthetic PHSRN–aaPEG–RGD with that of RGD and fibronectin.

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cmPEG was allowed to react with ethylenediamine in the presence of 1.1 equivalents of diisopropylcarbodiimide (DIC) to form aaPEG.[12] The crude aaPEG was converted to fluorenylmethyloxycarbonyl-aaPEG (Fmoc-aaPEG-OH) and then purified successively by Sephadex LH-20 column chromatography and HPLC. The PEG hybrid, PHSRN–PEG–RGD, was prepared on a Rink amide resin[13] using an Fmoc-based solid-phase strategy.[14] The Fmoc groups were removed by treatment with 20% piperidine/dimethylformamide (DMF) for 20 min. Coupling reactions were performed by the DIC/1-hydroxybenzotriazole (HOBt) method.[15] We protected the side chains of amino acids as follows: His and Asn by a trityl (Tri) group; Ser and Asp by a *t*-butyl (Bu^t) group; and Arg by a 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) group.[16]

Fmoc-aaPEG-Arg(Pmc)-Gly-Asp(OBu^t)-Rink amide resin was treated with 20% piperidine/DMF and the resulting H-aaPEG-Arg(Pmc)-Gly-Asp(OBu^t)-Rink amide resin was treated with trifluoroacetic acid (TFA)/thioanisole/ethanedithiol (94:3:3). The aaPEG–RGD product was purified by HPLC on a Daisopak SP-120-5-ODS-B column (Daiso Co., Ltd., Osaka, Japan). Synthetic Fmoc-Pro-His(Tri)-Ser(Bu^t)-Arg(Pmc)-Asn(Tri)-aaPEG-Arg(Pmc)-Gly-Asp(OBu^t)-Rink amide resin was treated first with 20% piperidine/DMF and then with TFA/thioanisole/ethanedithiol (94:3:3) at room temperature for 3 h. The resulting product was purified by HPLC using Daisopak SP-120-5-ODS-B followed by Asahipak GS-320P (Asahi Chemical Industry Co., Ltd., Tokyo, Japan) columns. PHSRN–PEG was prepared on TentaGel-NH_2 resin (Rapp Polymer GmbH, Tübingen, Germany). The resin contains PEG and liberates a peptide–PEG hybrid following TFA treatment after the completion of peptide synthesis. According to the manufacturer, the average molecular weight of the PEG portion of TentaGel-NH_2 is approximately 3000. The hybrid was purified by HPLC on a Daisopak SP-120-5-ODS-B column.

The cell spreading activity of the PEG hybrids was examined using baby hamster kidney (BHK) cells as described previously.[4] Ninety-six-well plastic tissue culture plates were coated with various amounts of PEG–peptides or fibronectin, and dried overnight. The plates were subsequently blocked by 3% bovine serum

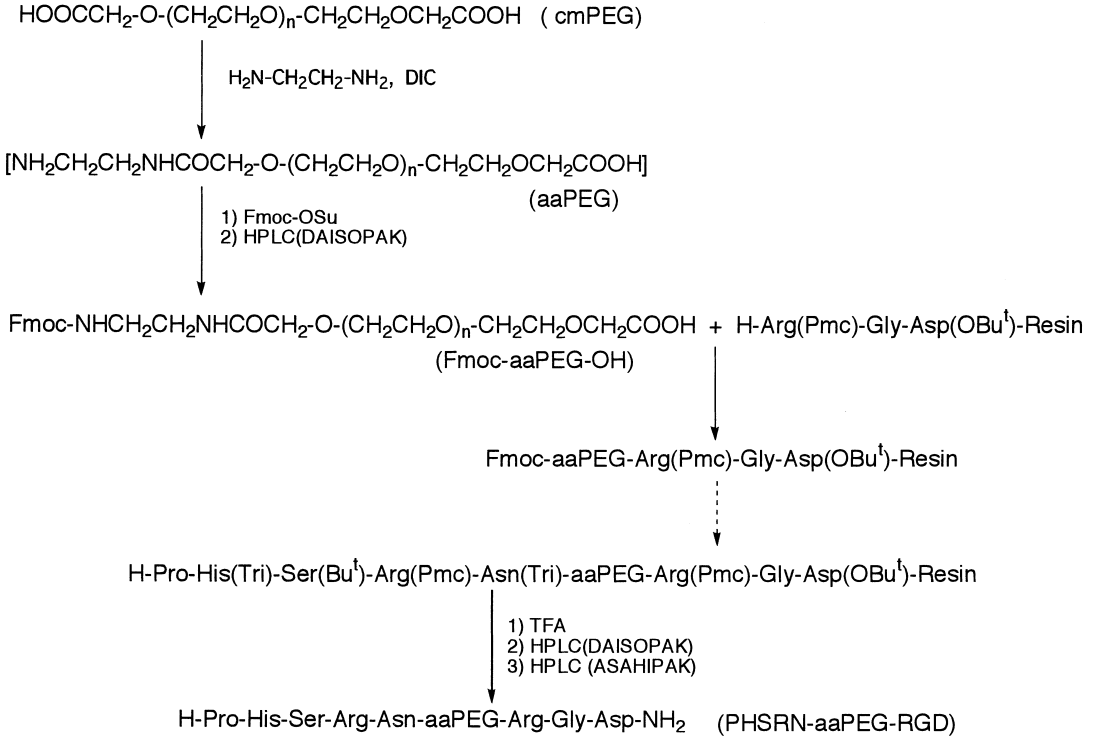

Figure 1. Synthetic scheme for PHSRN–aaPEG–RGD.

albumin (BSA, Sigma) in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) for 1 h at room temperature. BHK cells were detached with trypsin/EDTA and recovered in the presence of 10% fetal bovine serum (Life Technologies, Inc.) for 20 min at 37 °C. After washing twice with 0.1% BSA in DMEM, cells were placed in the coated wells at 5×10^3 cells/well. After a 45-min incubation at 37 °C, the cells were fixed with 3% formaldehyde and the percentage of spreading BHK cells was counted under a phase contrast microscopy (Fig. 1).

Results and Discussion

Previously, aaPEG was prepared using dicyclohexylcarbodiimide (DCC) as a coupling reagent,[11] however, the low solubility of the reaction product dicyclohexylurea caused problems in Sephadex LH-20 column chromatography. Here, DIC was used instead of DCC, because its reaction product diisopropylurea has good solubility. In the previous paper,[11] Sephadex LH-20 and ion-exchange column chromatographies were used successively for product purification but, as described above, this yielded aaPEG with low purity. This time, we converted crude aaPEG to Fmoc-aaPEG-OH and then purified it by Sephadex LH-20 column chromatography and HPLC on a Daisopak SP-120-5-ODS-B column.

Because aaPEG has no UV absorbance it was detected previously by the ninhydrin test,[11] whereas in this study Fmoc-aaPEG-OH was detected easily by a UV spectrometer. Introduction of Fmoc-aaPEG-OH on Arg(Pmc)-Gly-Asp(OBu^t)-Rink amide resin was slow and the reaction was repeated twice using 5 equivalents of Fmoc-aaPEG-OH. Removal of the Fmoc group from the Fmoc-aaPEG-Arg(Pmc)-Gly-Asp(OBu^t)-Rink amide resin was also slow, and the reaction was performed twice with 20% piperidine/DMF. The H-aaPEG-Arg(Pmc)-Gly-Asp(OBu^t)-Rink amide resin was treated with TFA/thioanisole/ethanedithiol (94:3:3), and the hybrid aaPEG–RGD was purified by HPLC on a Daisopak SP-120-5-ODS-B column. Synthetic H-Pro-His(Tri)-Ser(Bu^t)-Arg(Pmc)-Asn(Tri)-aaPEG-Arg(Pmc)-Gly-Asp(OBu^t)-Rink amide resin was treated with TFA/thioanisole/ethanedithiol (94:3:3). The crude hybrid PHSRN–aaPEG–RGD was purified by LH-20 column chromatography followed by two HPLC steps using Daisopak SP-120-5-ODS-B and Asahipak GS-320P columns. The average molecular weight of the purified PHSRN–aaPEG–RGD (measured by TOF-MS) was 4007. The amino acid ratios in the acid hydrolysate were: Pro, 0.92; His, 1.10; Ser, 0.83; Arg, 2.03; Gly, 1.01; Asp, 2.00 (average recovery 92%).

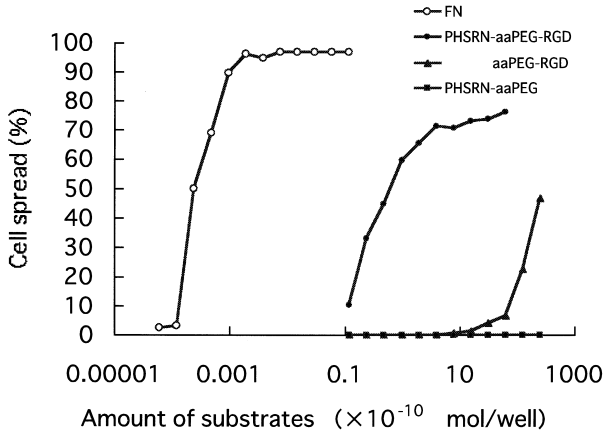

Figure 2. Cell spreading activity of synthetic PEG hybrids. Various amounts of synthetic PEG hybrids and fibronectin were coated on 96-well dishes and dried overnight. After blocking with BSA, BHK cells (5×10^3/well) were added and incubated for 45 min. The percentage of cells that had spread (%) was counted under a microscope. Each value represents the mean of five separate determinations. Triplicate experiments gave similar results.

The cell spreading activity of the PEG hybrids (aaPEG–RGD, PHSRN–PEG, and PHSRN–aaPEG–RGD) was examined, using fibronectin as a positive control (Fig. 2). Fibronectin showed the strongest cell spreading activity (Fig. 2). The aaPEG–RGD hybrid showed cell spreading activity but PHSRN–PEG did not have any activity. These findings are comparable to previous results obtained using synthetic peptides.[5] The PHSRN–aaPEG–RGD peptide showed stronger cell spreading activity than aaPEG–RGD, suggesting that PHSRN synergistically enhances the cellular activity of RGD in the PEG-bridged compounds. These observations are comparable to previous results obtained using recombinant proteins.[5]

Thus, aaPEG is potentially useful as a spacer and support matrix that allows the binding of functional peptides. As RGD peptide and PEG–RGD hybrid inhibit tumor metastasis,[7,8] PHSRN–aaPEG–RGD may be useful for testing the inhibition of tumor metastasis.

Recently, many biologically active sequences have been identified in extracellular matrix proteins.[16] Some of the active peptides may synergistically interact with cellular receptors;[17] thus, the bivalent aaPEG hybrid method may be useful for enhancing the synergistic interactions of these peptides.

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