Bioorganic & Medicinal Chemistry 20 (2012) 4608–4613

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

The cell adhesion and proliferation activities of a peptide derived from human tenascin-C are dependent on two Ile residues

Ryo Hayashi [a], Shogo Miura [b], Yohei Saito [b], Satoshi Osada [a], Takuya Iyoda [b], Fumio Fukai [b], Hiroaki Kodama [a,*]

[a] Department of Chemistry, Graduate School of Science and Engineering, Saga University, 1 Honjo-machi, Saga 840-8502, Japan
[b] Department of Molecular Patho-Physiology, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Chiba 278-8510, Japan

ARTICLE INFO

Article history:
Received 31 May 2012
Revised 19 June 2012
Accepted 19 June 2012
Available online 27 June 2012

Keywords:
Extracellular matrix
Tenascin-C
Structure–activity relationship
Cell adhesion

ABSTRACT

A tenascin-C derived peptide (TNIIIA2 peptide, **1**) stimulated β1 integrin-mediated cell adhesion via binding to syndecan-4. Ala-substituted peptides were synthesized to understand the structure–activity relationship. Peptides in which basic amino acids were substituted showed reduced cell adhesion activity, but their proliferation activities were similar to or higher than those mediated by peptide **1**. In contrast, peptides in which the Ile residues of peptide **1** were replaced were inactive, indicating that the Ile residues are critical for the peptide's activity. CD analysis suggested that the Ile residues are necessary for the formation of a specific conformation required for binding to syndecan-4.

1. Introduction

Cells and extracellular matrix (ECM) form tissues, and collections of tissues form organs. The interactions between receptors expressed on cell surfaces and ECM components such as collagen, fibronectin and tenascin play an important role in the regulation of cell shape, proliferation and differentiation.[1–3] Therefore, these interactions are critical events in the normal development of organs and the maintenance of tissue architecture.[4] Tenascin-C is a large, hexameric extracellular proteoglycan that was independently identified by various laboratories.[5–7] Little or no tenascin-C is detected in healthy adult tissues, but it is transiently re-expressed upon tissue injury. Upon tissue damage, tenascin-C plays a multitude of different roles that mediate both inflammatory and fibrotic processes to enable effective tissue repair, following which it is down-regulated.[8,9] The structure of tenascin-C includes fibronectin type III domains and epidermal growth factor-like domains, and the protein can bind to other extracellular matrix proteins and receptors on cell surfaces. Matrix metallo-proteases and serine proteases cleave tenascin-C, thus potentially releasing cryptic sites that may create adhesive sites for cell adhesion receptors.[8]

Fibronectin is a cell adhesive glycoprotein present in a soluble form in blood plasma and in an insoluble fibrillar form inextracellular matrix.[1,10–13] While fibronectin contains domains recognized by receptors such as integrins, we found that a fragment from the C-terminal heparin-binding (Hep2) domain of fibronectin suppressed cell adhesion to fibronectin.[14] The cryptic site in the Hep2 domain was determined to be TEATITGLEPGTEYTITYVIAL, termed FNIII14 peptide, and the core sequence for the anti-adhesive activity appeared to be the hydrophobic sequence, YTITYVIAL.[15] Interestingly, the activity of FNIII14 was dependent on the presence of a membrane protein with a molecular mass of 50-kDa, but not β1 integrins.[16] A sequence homology search using BLAST indicated that tenascin-C also contained a similar hydrophobic sequence, YTITI, suggesting that it is a biologically active cryptic site.

Interestingly, in contrast to FNIII14 peptide, the synthetic peptide derived from tenascin-C, TNIIIA2 peptide **1** (Table 1), stimulated β1 integrin-mediated cell adhesion of adherent and non-adherent cell types via binding to syndecan-4.[17] In the present study, to understand the structure–activity relationship of TNIIIA2 peptide **1**, TNIIIA2 peptide analogs in which the YTITI sequence and basic amino acids were substituted with Ala residues were synthesized, and the effects of the substitutions on cell adhesion and proliferation activities were determined in normal NIH-3T3 fibroblasts.

2. Results

2.1. A low substitution resin is suitable for the synthesis of TNIIIA2 peptides

Solid phase synthesis is a powerful tool for the preparation of long peptides and small proteins. However, it is known that peptides

* Corresponding author. Tel.: +81 952 28 8562; fax: +81 952 28 8548.
E-mail address: hiroaki@cc.saga-u.ac.jp (H. Kodama).

0968-0896/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2012.06.036

Table 1
The structures of synthetic peptides derived from human tenascin-C

	Peptide	Sequence
1	TNIIIA2	RSTDLPGLKAATHYTITIRGVKC
2	[R1A]TNIIIA2	**A**STDLPGLKAATHYTITIRGVKC
3	[K9A]TNIIIA2	RSTDLPGL**A**AATHYTITIRGVKC
4	[R19A]TNIIIA2	RSTDLPGLKAATHYTITI**A**GVKC
5	[Y14A]TNIIIA2	RSTDLPGLKAATH**A**TITIRGVKC
6	[T15A]TNIIIA2	RSTDLPGLKAATHY**A**ITIRGVKC
7	[I16A]TNIIIA2	RSTDLPGLKAATHYT**A**TIRGVKC
8	[T17A]TNIIIA2	RSTDLPGLKAATHYTI**A**IRGVKC
9	[I18A]TNIIIA2	RSTDLPGLKAATHYTIT**A**RGVKC

Bold characters indicate the substituted amino acid in each peptide.

consisting of repeating β-branch residues and hydrophobic resides such as Ile, Val, and Thr are difficult to synthesize because of the decreased reaction kinetics for amino acylation due peptide aggregation on solid supports, and/or the formation of secondary structures. For example, Oliveira et al.[18] chose a very hydrophobic peptide derived from the transmembrane domain of rat bradykinin B2 receptor as a model for studying the problems associated with the synthesis and purification of hydrophobic peptides. Recently, an amyloid-β peptide has also been shown to be a difficult peptide.[19] TNIIIA2 peptide **1** contains 6 hydrophobic residues and 6 β-branch residues within its 22 amino acids (Table 1). In particular, the 4 β-branch residues C-terminal to the TITI sequence are indicative of a difficult peptide. Figure 1A shows the HPLC profile of TNIIIA2 peptide **1** elongated on Wang resin with a substitution of 0.68 mmol/g. Broad peaks were observed in the HPLC profiles. In addition, the broad peaks fractionated under the analytical conditions used indicated the absence of a mass spectrum in the range between 500 and 3000. This suggested that the crude peptide did not contain TNIIIA2 peptide **1**, while broad peaks were observed at a retention time of around 13 min. An efficient method for preventing peptide aggregation on a solid support is the solvation of a peptide-polymer matrix. Thus, TNIIIA2 peptide **1** was synthesized using the polyethyleneglycol containing PAL-PEG resin (substitution, 0.21 mmol/g). Figure 1B shows the HPLC profile of TNIIIA2 peptide **1** synthesized using the low substitution resin. Sharp peaks were observed compared to the HPLC profile shown in Figure 1A. The calculated m/z value corresponding to TNIIIA2 peptide **1** was observed at the peak indicated by the asterisk in Figure 1C. Figure 2 depicts the HPLC profiles of purified Ala-substituted TNIIIA2 analogs **2**–**9**. All peptides were obtained with good purity. These results indicate that the polyethylene-glycol containing low substitution resin is suitable for the synthesis of TNIIIA2 peptide **1** and its analogs.

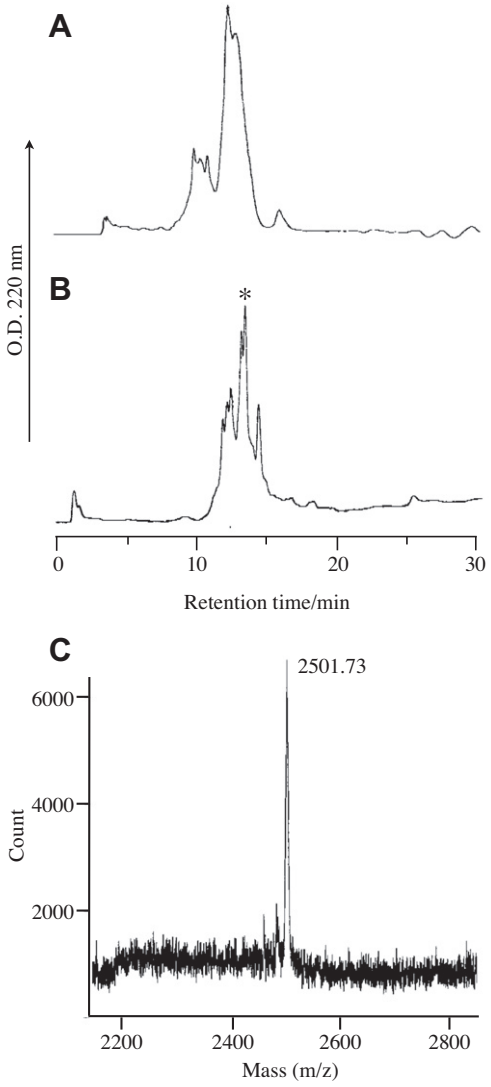

Figure 1. Low-substitution resin is suitable for the synthesis of TNIIIA2 peptide **1**. HPLC profiles of crude TNIIIA2 peptide **1** synthesized utilizing Wang resin (A) and PAL-PEG resin (B) on a C4 analytical column. The peak indicated by an asterisk (*) in panel B is peptide **1**. HPLC conditions: column, Wakosil 5C4 200 (2.0 × 150 mm); eluents, water/acetonitrile/trifluoroacetic acid, 95/5/0.05, v/v (solution A), and water/acetonitrile/trifluoroacetic acid, 5/95/0.04, v/v (solution B); flow rate, 1.0 ml/min; detection, 220 nm; gradient, 0–100% B over 30 min. (C) The mass spectrum of the peak indicated by the asterisk (*) in Figure 1B. MALDI-TOF *m*/*z*: 2501.73 ([M+H]$^+$ Calcd 2501.37).

2.2. Substitution of basic amino acids in the TNIIIA2 peptide affects the adhesion, but not the proliferation, of NIH-3T3 cells

Previously, we reported that TNIIIA2 peptide **1** activated β1 integrin via binding to syndecan-4.[17] Syndecan-4 is a ubiquitous transmembrane proteoglycan that localizes to the focal adhesions of adherent cells, and its external glycosaminoglycan chains bind to a range of extracellular ligands including growth factors and extracellular matrix proteins such as fibronectin and tenascin-C.[20–25] TNIIIA2 peptide **1** has three basic residues, Arg1, Lys9, and Arg19 (Table 1). To investigate the effects of these basic residues on the cell adhesion and proliferation activities mediated by TNIIIA2 peptide **1**, Ala-substituted peptides **2**–**4** were synthesized. Figure 3A and B show the number of adhered and spreading cells in the presence of TNIIIA2 peptide **1** or the Ala-substituted peptides **2**–**4**, respectively, after incubation for 2 h. The substituted peptides showed reduced activities compared to TNIIIA2 peptide **1**. However, the cell proliferation activities mediated by the substituted peptides were similar to or greater than that of TNIIIA2 peptide (Fig. 3C). In particular, peptides **2** and **3** exhibited approximately 2-fold increased activity. These results suggested that a hydrophobic amino acid, and/or an amino acid containing a small side chain, may be preferred for cell proliferation activity, and that the basic residues correlate with the initial binding affinity of TNIIIA2 peptide **1** to the heparan sulfate of syndecan-4. In addition, TNIIIA2 peptide **1** seems to contain two domains for inducing binding to syndecan-4 and evoking proliferation activity of TNIIIA2 peptide **1**.

2.3. Two Ile resides are essential for both the cell adhesion and proliferation activities of the TNIIIA2 peptide

Previously, we found that the FNIII14 peptide derived from a fibronectin type III-like repeat in human plasma fibronectin inhibited cell adhesion to fibronectin without binding to β1 integrin.[26] The peptide contains a C-terminal YTIYVIAL sequence, and the activity of the FNIII14 peptide was remarkably reduced by substi-

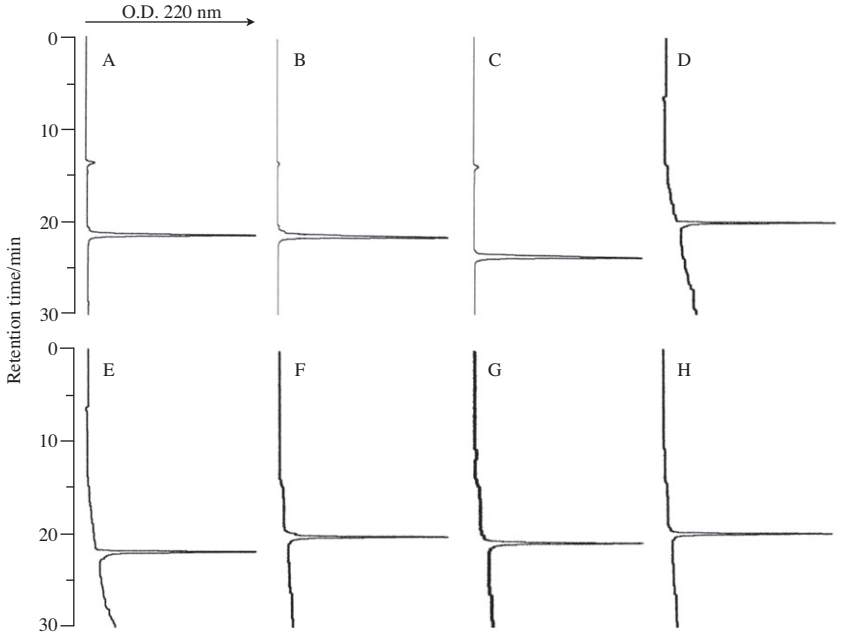

Figure 2. HPLC elution profiles of purified Ala-substituted TNIIIA2 peptides **2** (A), **3** (B), **4** (C), **5** (D), **6** (E), **7** (F), **8** (G), and **9** (H). The conditions were the same as those described in Figure 1.

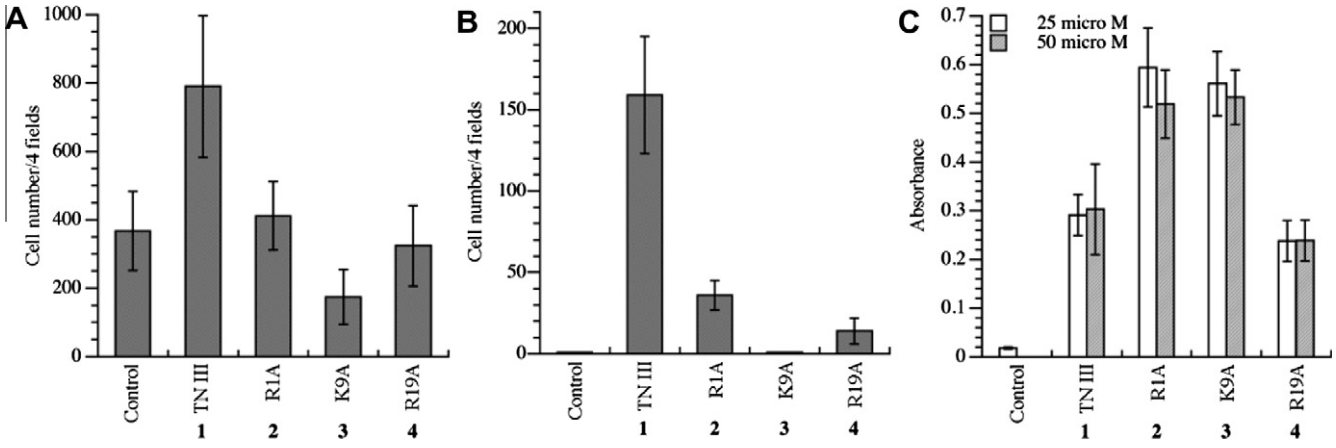

Figure 3. Substitution of basic residues with an Ala residue reduced cell adhesion activity, but did not affect proliferation activity, in NIH-3T3 cells. The cell adhesion activities of peptides **1**, **2**, **3**, and **4** were estimated from the number of cells either attached (A) or spread (B) counted in 4 independent fields after incubation for 1 h at 37 °C in the presence or absence of the peptides (50 μg/ml). The activities are presented as the mean ± S.E. of triplicate measurements. (C) The proliferation activities of peptides **1**, **2**, **3** and **4** were evaluated using MTT assay. NIH-3T3 cells were incubated for 2 days at 37 °C with 25 μg/ml (white bar) and 50 μg/ml (closed bar) of the peptides.

tution of the Ala residue within the sequence. TNIIIA2 peptide **1** also has a similar C-terminal sequence, YTITI. Thus, to investigate the importance of the sequence to the cell adhesion and proliferation activities mediated by the peptide, Ala-substituted peptides **5**–**9** (Table 1) were synthesized and their activities were determined. While the substitutions in the YTITI sequence did not affect the number of adhered cells (Fig. 4A), peptides **5**, **7** and **9** showed dramatically reduced cell spreading activity (Fig. 4B). In contrast to peptides **2**–**4**, peptides **7** and **9**, in which the Ile residues were replaced, showed remarkably reduced cell proliferation activity (Fig. 4C). These results indicate that the two Ile residues are critical for the biological activity of TNIIIA2 peptide **1**.

2.4. The specific solution conformation of TNIIIA2 peptide is affected by substitution of the Ile residues in the YTITI sequence

We predicted that substitution of the Ile residues in the YTITI sequence changed the specific conformation of TNIIIA2 peptide **1**, resulting in dramatically reduced activity. Figure 5 shows circular dichroism (CD) spectra of TNIIIA2 peptide **1** and substituted peptides **7** and **9** in phosphate buffer at pH 7.0. The CD spectrum of TNIIIA2 peptide **1** included two negative bands near 200 and 220 nm. The CD spectrum of peptide **2** also was similar to that of peptide **1** while the negative band around 220 nm was weak. In contrast, the CD spectra of peptides **7** and **9** indicated a random-coil structure in neutral conditions. The sequence of the TNIIIA2 peptide **1** contains three basic amino acids and only one acidic amino acid, Asp4 (Table 1), indicating that it is a basic peptide. Figure 6A shows CD spectra of TNIIIA2 peptide **1** in different pH conditions. The CD spectra of TNIIIA2 peptide **1** in phosphate buffers of pH 5.7 and 7.7 consist of a negative band near 198 nm and a low ellipticity band from 210 to 230 nm, and a broad negative band around 218 nm, respectively. These results indicate that the solution conformation of TNIIIA2 peptide **1** strongly depends on pH. In contrast, the CD spectra of peptides **7** and **9** were similar at both acidic and basic pH (Fig. 6B and C). These results suggest that Ile16

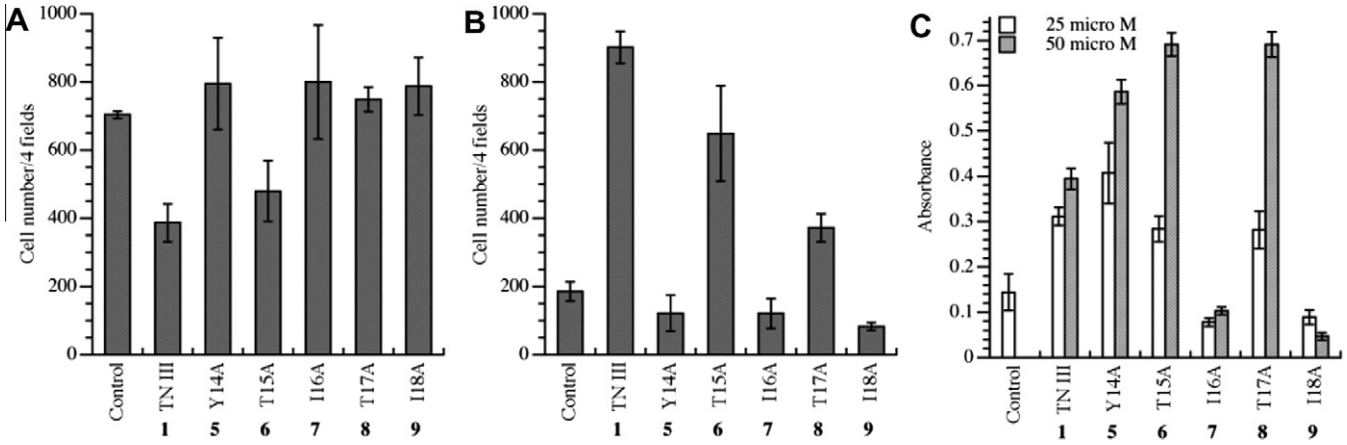

Figure 4. Ile16 and Ile18 are critical to both cell adhesion and proliferation activities in NIH-3T3 cells. The numbers of attached cells (A) and spreading cells (B) were counted after incubation for 1 h at 37 °C with peptides **1**, **5**, **6**, **7**, **8** and **9** (50 μg/ml) or PBS as a control. (C) The effect of the peptides on cell proliferation activity was measured at peptide concentrations of 25 μg/ml (white bar) and 50 μg/ml (closed bar). The assay conditions were the same as those described in Figure 3.

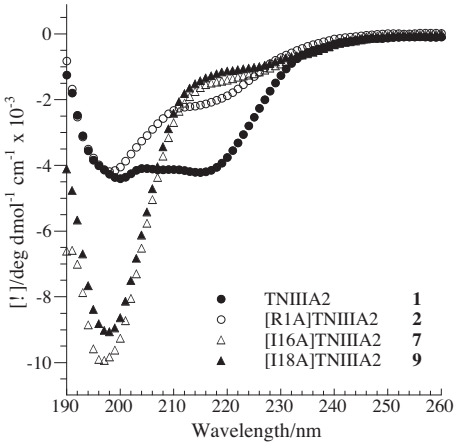

Figure 5. TNIIIA2 peptide **1** formed a specific structure under neutral conditions. The CD spectra of peptides **1** (closed circle), 2 (open circle), **7** (open triangle) and **9** (closed triangle) were measured in phosphate buffer (pH 7.0). The peptide solution (25 μM) was incubated for 2 h at room temperature prior to measurement.

and Ile18 are necessary for the formation of a specific conformation required for binding to syndecan-4.

3. Discussion

In this study, to investigate the effects of amino acid residues in TNIIIA2 peptide **1**, which activates β1 integrin via binding to syndecan-4,[17] on cell adhesion and proliferation activities in NIH-3T3 cells, Ala-substituted peptides **2**–**9** were synthesized and their activities were determined. Although TNIIIA2 peptide **1** and its analogs consist of hydrophobic amino acids (Table 1), their synthesis was accomplished by utilizing a low substitution resin for 9-fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis (Figs. 1 and 2).

While peptides **2**–**4** showed decreased cell adhesion activity (Fig. 3A and B), similar cell proliferation activity was observed for all peptides (Fig. 3C). These differences appear to be a result of the incubation times used in both assays. The incubation times for the cell adhesion assay and the cell proliferation assay were 1 and 24 h, respectively. The electrostatic interaction of peptides **2**–**4** with syndecan-4 may be reduced by the amino acid substitutions, resulting in low activity during short term incubation. Peptides **5**–**9** also showed reduced cell spreading activity in the cell adhesion assay (Fig. 4A and B). However, peptides **5**, **6** and **8** exhibited similar cell proliferation activity to TNIIIA2 peptide **1** (Fig. 4C). In contrast, it was interesting that peptides **7** and **9** were inactive (Fig. 4C). These results indicate that the two Ile residues, Ile16 and Ile18, are critical amino acids for the biological activity of TNIIIA2 peptide **1**. Previously, we reported that fibronectin-derived

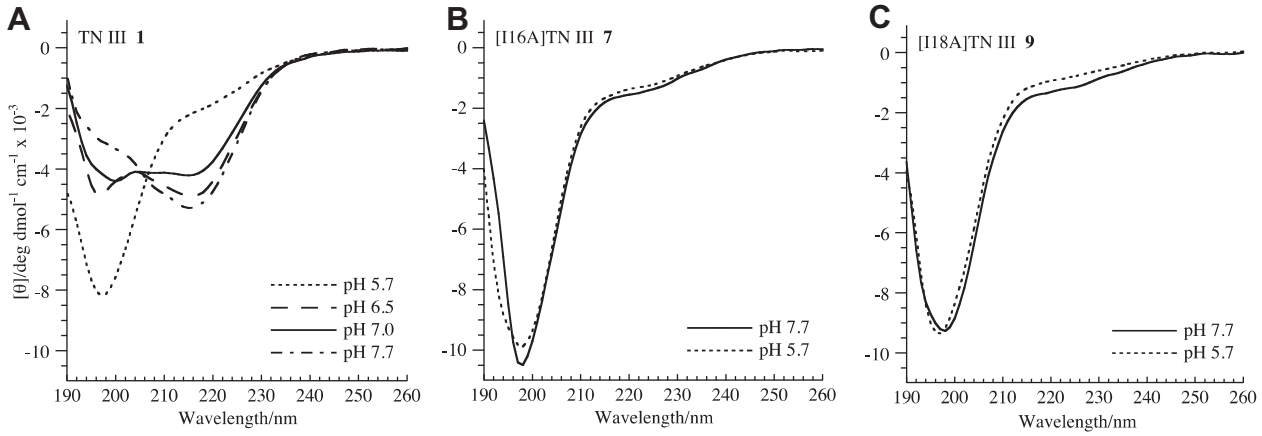

Figure 6. Peptides **7** and **9** exhibited a random-coil structure in both neutral and basic pH conditions. The conformation of peptides **1** (A), **7** (B) and **9** (C) were evaluated at pH 5.7 (dotted line), 6.5 (broken line), 7.0 (solid line) and 7.7 (dashed line). The peptide solution was prepared at 25 μM in 10 mM phosphate buffers with different pH values, and incubated for 2 h at room temperature.

21-mer peptides containing C-terminal YTIYVI or YTITVY sequences, called FNIII10 (STATISGLKPGVDYTIT-VYAV) and FNIII14 (TEATITGLEPGTEYTITYVIAL), exhibited anti-adhesion activity and accelerated anoikis-like apoptosis in normal fibroblasts via binding to a 50-kDa membrane protein.[26] In contrast, the Coll XII-1 (SVVLQKLKP-DTPYTITVSSL) peptide derived from fibronectin did not show any cell adhesion activity despite it containing the YTITV sequence. In addition, the membrane proteins that bind to TNIIIA2 peptide **1** and FNIII10 are different.[17,26] These results seem to indicate disparate roles for the hydrophobic sequence, Tyr-Thr-Ile-Xaa-Yaa (Xaa = Thr or Tyr, Yaa = Ile or Val), and that these roles depend on sequences coupled to the hydrophobic sequence. To explore why substitution of the Ile residues resulted in dramatically reduced activity, we analyzed the structures of these peptides. The CD spectra of TNIIIA2 peptide **1** showed that peptide **1** possessed a specific conformation in phosphate buffer (Fig. 5), and its conformation depended on pH (Fig. 6A). In contrast, peptides **7** and **9** did not possess a distinct structure (Figs.5 and 6B,C). These results suggest that a specific conformation is required for the binding to or the activation of syndecan-4, and that the Ile resides are required for the formation of the specific conformation.

4. Experimental

4.1. Materials

Wang Resin, *N*-α-9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, and *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino) methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) were purchased from Novabiochem (Tokyo, Japan). PAL-PEG resin was purchased from Applied Biosystems (Foster City, CA, USA). *N*-hydroxybenzotriazole (HOBt), trifluoroacetic acid (TFA), and *N*-ethyldiisopropylamine (DIEA) were purchased from Peptide Institute Inc. (Osaka, Japan). Piperidine and organic solvents were obtained from Wako Pure Chemicals Industries (Tokyo, Japan). Triisopropylsilane was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

4.2. Peptide synthesis

TNIIIA2 peptide analogs were synthesized with a stepwise solid phase method utilizing Fmoc/*tert*-butyl chemistry. Peptides were assembled on Wang resin or PAL-PEG resin. The coupling reaction was performed with the HBTU-HOBt method using a 10-fold molar excess of Fmoc-amino acid. Fmoc-amino acid was dissolved in 0.45 M HBTU-HOBt solution in *N*,*N*-dimethylformamide, and then 2.0 M DIEA in 1-methyl-2-pyrrolidone was added to the solution. After preactivation for 5 min, the active ester was reacted with amines on the solid support. The Fmoc group was deprotected by treatment with 20% piperidine for 10 min. Cleavage of the peptide from the resin was achieved with a trifluoroacetic acid (TFA)/water/triisopropylsilane mixture (92.5/5/2.5, v/v) for 2 h. After removing the resin by filtration, the filtrate was concentrated by flushing with nitrogen gas and crude peptides were precipitated with diethyl ether. Crude peptides were purified using reversed-phase high-performance liquid chromatography (RP-HPLC) on a preparative C4 column with a water/acetonitrile solvent system containing trifluoroacetic acid. Purified peptides were characterized by matrix-associated laser desorption ionization time-of-flight mass spectrometry and RP-HPLC on an analytical C4 column. The purity of all peptides was found to be >95%.

4.3. HPLC analysis

Chromatography was performed using a JASCO HPLC system with a Wakosil 5C4-analytical column (2.0 × 250 mm). The HPLC apparatus was equipped with a PU-980 Intelligent HPLC pump, a UV-970 Intelligent UV/VIS detector, and an 807-IT Integrator. Registration of the retention time value and the peak area integration was performed using the built-in data system of the integrator. The column was equilibrated with solution A (water/acetonitrile/TFA, 95/5/0.05, v/v) and eluted with a gradient of solution B (water/acetonitrile/TFA, 5/95/0.04, v/v) from 0 to 100% over 30 min. The flow rate was 0.5 mL/min.

4.4. Cell adhesion assay

Peptide-mediated cell adhesion assays were performed as described previously.[17,26] Ninety-six-well tissue culture plates were coated with fibronectin at 37 °C and then blocked with bovine serum albumin (BSA). An aliquot of cell suspension and an aliquot of peptide were added to each well, and the plates were incubated at 37 °C for 1 h. After removing non-adhered cells by washing, the numbers of attached and spreading cells were determined by counting under a microscope.

4.5. Cell proliferation assay

Ninety-six-well tissue culture plates were coated with fibronectin at 37 °C and then blocked with BSA. An aliquot of cell suspension and an aliquot of peptide were added to each well and the plates were incubated at 37 °C for 2 days. MTT solution (0.5 mg/ml MTT in RPMI-1640 without phenol red) was then added and the cells were incubated for an additional 3 h. The converted dye was solubilized with 1 ml acidic isopropanol (0.04 M HCl in absolute isopropanol). The absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 650 nm.

4.6. Circular dichroism (CD) spectroscopy

The CD spectra of TNIIIA2 peptides were recorded at room temperature on a JASCO J-720 spectropolarimeter (Tokyo, Japan) with a cylindrical cell of 2 mm path length. The CD cell was washed with an aqueous NaOH solution before each measurement to remove any peptide adhering to the inner surface. Intermolecular disulfide bond formation was performed using the air oxidation method. The reaction was monitored by the Ellman test. Peptide concentrations were determined by amino acid composition analysis using the phenylthiocarbamyl method. Sample solutions were prepared 2 h before measurement. The results were expressed as the mean residue ellipticity.

References and notes

1. Yamada, K. M. In *Fibronectin domains and receptors*; Mosher, D. F., Ed.; Academic Press: New York, 1989; p 47.
2. Hynes, R. O. *Cell* **1992**, *69*, 11.
3. Ruoslahti, E.; Engvall, E. *J. Clin. Invest.* **1997**, *100*, S53.
4. Ekblom, P.; Vestweber, D.; Kemler, R. *Annu. Rev. Cell Biol.* **1986**, *2*, 27.
5. Chiquet-Ehrismann, R.; Hagios, C.; Matsumoto, K. *Perspect. Dev. Neurobiol.* **1994**, *2*, 3.
6. Chiquet-Ehrismann, R.; Chiquet, M. *J. Pathol.* **2003**, *200*, 488.
7. Tucker, R. P.; Chiquet-Ehrismann, R. *Biochim. Biophys. Acta* **2009**, *1793*, 888.
8. Midwood, K. S.; Orend, G. *J. Cell Commun. Signal.* **2009**, *3*, 287.
9. Midwood, K. S.; Hussenet, T.; Langlois, B.; Orend, G. *Cell. Mol. Life Sci.* **2011**, *68*, 3175.
10. Yamada, K. M. *Curr. Opin. Cell Biol.* **1989**, *1*, 956.
11. Mosher, D. F.; Sottile, J.; Wu, C.; McDonald, J. A. *Curr. Opin. Cell Biol.* **1992**, *4*, 810.
12. Wu, C. *Histol. Histopathol.* **1997**, *12*, 233.
13. Schwarzbauer, J. E.; Sechler, J. L. *Curr. Opin. Cell Biol.* **1999**, *11*, 622.
14. Fukai, F.; Takahashi, H.; Habu, Y.; Kubushiro, N.; Katayama, T. *Biochem. Biophys. Res. Commun.* **1996**, *220*, 394.
15. Fukai, F.; Hasebe, S.; Ueki, M.; Mutoh, M.; Ohgi, C.; Takahashi, H.; Takeda, K.; Katayama, T. *J. Biochem.* **1997**, *121*, 189.
16. Kamiya, S.; Kato, R.; Wakabayashi, M.; Tohyama, T.; Enami, I.; Ueki, M.; Yajima, H.; Ishii, T.; Nakamura, H.; Katayama, T.; Takagi, J.; Fukai, F. *Biochemistry* **2002**, *41*, 3270.

17. Saito, Y.; Imazeki, H.; Miura, S.; Yoshimura, T.; Okutsu, H.; Harada, Y.; Ohwaki, T.; Nagao, O.; Kamiya, S.; Hayashi, R.; Kodama, H.; Handa, H.; Yoshida, T.; Fukai, F. *J. Biol. Chem.* **2007**, *282*, 34929.
18. Oliveira, E.; Miranda, A.; Albericio, F.; Andreu, D.; Paiva, A. C.; Nakaie, C. R.; Tominaga, M. *J. Pept. Res.* **1997**, *49*, 300.
19. Tickler, A. K.; Clippingdale, A. B.; Wade, J. D. *Protein Pept. Lett.* **2004**, *11*, 377.
20. Woods, A.; Couchman, J. R. *Trends Cell Biol.* **1998**, *8*, 189.
21. Rapraeger, A. C.; Ott, V. L. *Curr. Opin. Cell Biol.* **1998**, *10*, 620.
22. Couchman, J. R.; Woods, A. *J. Cell Sci.* **1999**, *112*, 3415.
23. Woods, A.; Couchman, J. R. *Curr. Opin. Cell Biol.* **2001**, *13*, 578.
24. Simons, M.; Horowitz, A. *Cell. Signal.* **2001**, *13*, 855.
25. Bass, M. D.; Humphries, M. J. *Biochem. J.* **2002**, *368*, 1.
26. Miura, S.; Kamiya, S.; Saito, Y.; Wada, S.; Hayashi, R.; Taira, J.; Kodama, H.; Yajima, H.; Ueki, M.; Fukai, F. *Biol. Pharm. Bull.* **2007**, *30*, 891.