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Suppression of cell adhesion through specific integrin crosstalk on mixed peptide-polysaccharide matrices

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ABSTRACT

Crosstalk of different integrins, which bind to distinct types of extracellular matrix proteins, promotes specific functions. This crosstalk has not been investigated in depth. Previously, we demonstrated that integrin-syndecan crosstalk accelerated cell adhesion. Here, we evaluated the crosstalk of two different integrins using mixed peptide-polysaccharide (chitosan or alginate) matrices. Two different integrin binding peptides, FIB1 (integrin αvβ3), EF1zz (integrin α2β1), and 531 (integrin α3β1), were mixed in various molar ratios (9:1, 4:1, 1:1) and conjugated on a polysaccharide matrix. The mixture of FIB1/EF1zz- and FIB1/531-polysaccharide matrices did not show any difference in human dermal fibroblast (HDF) adhesion against the mono polysaccharide matrices. Interestingly, the EF1zz/531-polysaccharide matrix (molar ratio = 1:4) exhibited significantly decreased cell adhesion, but other EF1zz/531-polysaccharide matrices did not show any difference. When we examined the signal transduction of the EF1zz/531(1:4), Y397 phosphorylation of FAK significantly decreased but Y514 phosphorylation of Src did not exhibit any differences. Further investigation revealed that this suppression was mediated by PI3K signaling through the activation of integrin, and PKA signaling modulated suppression of HDF attachment. These findings suggest that a mixed peptide-polysaccharide matrix using receptor specific ligands can regulate cellular functions through receptor-specific crosstalk and is a useful approach to understand receptor specific crosstalk.

1. Introduction

Integrins are important cell surface receptors that interact with extracellular matrix (ECM) components, such as fibronectin, collagens, and laminins [1–3]. Integrins are transmembrane heterodimeric molecules, which are composed of α and β subunits. Integrin binding to ECM proteins is involved in the modulation of various biological functions, including cell adhesion, motility, survival, and proliferation. So far, eighteen α and eight β subunits have been identified and 24 different heterodimeric complexes have been determined as members of the integrin family. Cell adhesion to the ECMs lead to integrin activation, and this activation promotes intracellular signaling (outside-in) [2]. The outside-in integrin signaling controls many cellular events, such as proliferation, differentiation, migration, adhesion, and de-adhesion, and these processes are strictly related to the affinity of integrin to the ECMs from a low affinity state to a high affinity state (inside-out) [1–3]. Changes in integrin affinity take place during cell migration, ECM reorganization, platelet activation, and mechanotransduction. This outside-in or inside-out signaling is promoted not only by the same subtype of integrin but also by different types of cell surface receptors and different subtypes of integrins [4].

Various cell surface receptors, which influence integrin-mediated cell attachment, have been identified, such as growth factor receptors, GPI anchored receptors, and syndecans, a transmembrane proteoglycan [5–7]. These different types of crosstalk regulate many cellular events through integrin-mediated cellular signaling, including focal adhesion kinase (FAK), Src family kinase, phosphatidylinositol 3-kinase (PI3K), Akt, and Rho GTPases [6–8]. In contrast, evaluation of integrin–integrin crosstalk is relatively difficult to compare with that of the non-integrin–integrin

Abbreviations: ECM, extracellular matrix; Fmoc, 9-fluorenylmethoxycarbonyl; MBS, *N*-(*m*-maleimidobenzoyloxy) succinimide; DMF, *N,N′*-dimethylformamide; DMEM, dulbecco's modified essential medium; FBS, fetal bovine serum; BSA, bovine serum albumin; HDF, human dermal fibroblast; FAK, focal adhesion kinase.

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crosstalk, because many ECM-binding integrins share the β1 subunits [1,9]. The integrin β1 subunit is the most common integrin subunit forming various combination of heterodimers with α subunits. Mice lacking integrin β1 showed E 5.5 natal lethality with a failure of blastocyst development [10]. Additionally, a knock-down study using the RNAi method cannot be used to evaluate the crosstalk between same β subunits of integrins. The components and ratios of the mixtures of ECM proteins vary depending on the tissue type, tumor, and developmental stage. Thus, it is very difficulties to study integrin–integrin crosstalk resulting in a poor understanding of those mechanisms.

Many integrin-binding sequences have been identified from various ECM proteins [11–13]. The Arg-Gly-Asp (RGD) sequence originally from fibronectin is the most well characterized sequence, and RGD-containing short peptides mainly interact with either integrin αvβ3 or αvβ5 [14,15]. Previously, we identified integrin α2β1-binding peptides from the laminin α1 chain by systematic peptide screening of the laminin chains [16]. Recently, we developed easy-handling peptide-polysaccharide matrices, such as chitosan, alginate, and hyaluronic acid, and examined potential biomaterial applications using in vitro and in vivo assays [17–19]. We conjugated laminin-derived cell adhesive peptides to the polysaccharide matrices as a scaffold to better understand adhesive mechanisms. For example, we conjugated laminin α1 chain active peptides, AG73 (RKRLQVQLSIRT, mouse laminin α1 chain) and A99 (AGTFALRGDNPQG, mouse laminin α1 chain) on various polysaccharide matrices [17–19]. The AG73-polysaccharide matrix binds to syndecan and promotes cell adhesion with membrane ruffling and neurite outgrowth. The A99-polysaccharide matrix binds to integrin αvβ3 and promotes cell spreading with well-organized actin stress fibers. When AG73 and A99 were mixed together in various molar ratios and conjugated on polysaccharide matrices, the mixed-peptide polysaccharide matrices exhibited different biological activities depending on the mixture ratio of the two different biologically active peptides. When AG73 and A99 were mixed at a molar ratio AG73:A99 = 1:9, the AG73/A99 (1:9)-chitosan matrix showed the strongest cell attachment and neurite outgrowth activity by induction of the synergistic interaction between integrin and syndecan [17,19]. These findings indicate that the mixed-peptide polysaccharide matrix approach has potential as both a tool to investigate the molecular mechanisms of different cell surface receptors and as multifunctional biomaterial for mimicking the ECM molecules/matrices.

In this study, we focused on integrin–integrin crosstalk between different subtypes using peptide-polysaccharide matrices. Integrin subtype-specific binding peptides were conjugated on the polysaccharide in various ratios and cell attachment activities were assessed to identify the effect on the integrin–integrin crosstalk.

2. Experimental procedures

2.1. Materials

Specific antibodies directed against integrins α1 (FB12), α2 (P1E6), α3 (P1B5), α4 (P1H4), α6 (GoH3), β1 (6S6 or TS2/6), αvβ3 (CD51/61), FAK, p397FAK, the phosphorylated FAK at tyrosine 397, Src, p416Src, phosphorylated tyrosine at 416, and mouse purified polyclonal IgG (PP54) were used as described previously [20,21] and were purchased from AMAC (Westbrook, ME), Cell Signaling Technology (Beverly, MA), Santa Cruz Biotechnology (Santa Cruz, CA) and/or EMD Millipore (Billerica, MA). Signal transduction inhibited or activated reagents of 14–22 Amide myristoylated; PKA inhibitor, Gö 6976; PKC inhibitor, and Wortmannin and LY294002; PI3K inhibitor were purchased from EMD Millipore and 12-O-tetradecanoylphorbol-13-acetate (TPA); an integrin β1 primer was purchased from Cell Signaling Technology.

2.2. Synthetic peptides

All peptides were manually synthesized using the 9-fluorenylmethoxycarbonyl (Fmoc) strategy and prepared in the C-terminal amide form as previously described [18,22].Briefly, for conjugation to either a chitosan or an alginate matrix, a cysteine residue was added at the N-terminus and two glycine residues were used as a spacer between the cysteine and the active peptide sequence. Purity and identity of the peptides were confirmed by an analytical HPLC and an electrospray ionization mass spectrometer at the Central Analysis Center, Tokyo University of Pharmacy and Life Sciences.

2.3. Peptide-chitosan or –alginate matrices

Peptide-chitosan and -alginate matrices were prepared as described previously [17,18,23]. The MB content in both the MB-chitosan and MB-alginate are 1% (chitosan sugar unit) and 1.2% (alginate sugar unit), respectively [18,23]. Briefly, the MB-chitosan (1% MB) was dissolved in 4% AcOH and 50 μl of the solution was added to the 96-well plates (30 ng/mm^2, MB: 1.7 pmol/mm^2). After drying at room temperature for 24 h, the plates were washed with 1% $NaHCO_3$ (100 μl) and then washed with PBS (100 μl × 2 times). For conjugation of the peptides to MB-chitosan, the peptide solutions in 0.1% TFA aq. (50 μl, 0.049–50 nmol/well = 1.47 pmol/mm^2–1.5 nmol/mm^2) and an equal amount of 1% $NaHCO_3$ aq. were added into the wells and incubated for 2 h. Peptide-alginate matrices were prepared as follows. MB-alginate (1.2% MB) was dissolved in 0.1 M MES buffer containing 0.3 M NaCl. Then 50 μl of MB-alginate solution was added to the 96-well plates and dried at room temperature for 2 days (30 ng/mm^2, MB: 1.8 pmol/mm^2). After washing with PBS (100 μl × 2 times), peptide solutions in 0.1% trifluoroacetic acid (TFA) aq. were prepared. For conjugation to alginate, the peptide solutions (50 μl, 0.049–50 nmol/well = 1.47 pmol/mm^2–1.5 nmol/mm^2) and an equal amount of 1% $NaHCO_3$ aq. were added into the wells and incubated for 2 h. The relative amount of conjugated peptides on the polysaccharide matrices were analyzed using fluorescamine (Sigma–Aldrich). Obtained peptide-chitosan and -alginate matrices were washed with PBS and used for the biological assays.

2.4. Cell attachment assay

Human dermal fibroblasts (HDFs; Cell Applications Inc., San Diego, CA) were maintained in DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Peptide-chitosan or -alginate matrices coated on 96-well plates were blocked by the addition of 1% bovine serum albumin (BSA, Sigma–Aldrich, St Louis, MO) in DMEM (150 μl) for 1 h. HDFs were detached with 0.02% trypsin-EDTA solution and resuspended in DMEM containing 0.1% BSA. Then, the cells were added (100 μl, 2 × 10^4 cells/well) to each well and incubated at 37 °C for 90 min in 5% CO_2. After washing off the unattached cells, attached cells were stained with a 0.2% crystal violet aqueous solution in 20% methanol for 15 min and washed with Milli-Q water. The attached cells were photographed using a microscope and images were captured (Olympus, Tokyo, Japan). The attached cells in the central field of the well (9.8 mm^2) were counted and the areas of the attached cells were measured using Image J software. All assays were carried out in triplicate and each experiment was repeated at least three times. For inhibition or activation of cell attachment, HDFs were treated with either 10 μg/ml integrin antibodies, 1% DMSO, 20 μM 14-22 Amide myristoylated, 1 μM Gö 6976, 5 nM Wortmannin, 5 μM LY294002 or 100 nM TPA for 20 min or 60 min at room temperature. Then, the cells were added to the wells and incubated for 60 min or 90 min at 37 °C. The attached cells were counted as described above.

2.5. Immunoblotting of FAK and Src signaling

After incubation of the cells on the various peptide-coated plates (50 μl, 5 × 10^4 cells/well) for 90 min, the cells were lysed with 50 μl of SDS sample buffer, resolved by 7.5% SDA-PAGE, and transferred to PVDF membranes [21]. The membranes were blocked with 3% BSA/PBS, incubated with either anti-phospho-FAK (Tyr397), anti-FAK, anti-phosphor-Src (Tyr416), or anti-Src antibodies (1:1000) in 1% BSA/PBS overnight at 4 °C, and the proteins were detected with an HRP-conjugated secondary antibody (1:2000) using an ECL kit (GE Healthcare Bio-Sciences Corp, Piscataway, NJ).

Table 1
Peptide characteristics.

Peptide	Sequence	Derivation	Integrin subtype	References
FIB1	CGGYAVTGRGDSPAS	Fibronectin	αvβ3	Pankov et al., *J. Cell Sci.*, 2002 [15]
EF1zz	CGGATLQLQEGRLHFXFDLGKGR, X = Nle	Laminin α1	α2β1	Mochizuki et al., *Biopolymers*, 2007 [25]
531	CGGGEFYFDLRLKGDKY	Collagen VI α1	α3β1	Miles et al., *J. Biol. Chem.*, 1995 [24]