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Identification of the Peptide Sequences within the EIIIA (EDA) Segment of Fibronectin That Mediate Integrin $\alpha9\beta1$-dependent Cellular Activities*[S]

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Alternative splicing of the fibronectin (FN) gene transcript provides an efficient mechanism for generating functionally appropriate forms of this adhesive glycoprotein *in situ*. Cellular FNs that include the EIIIA and/or EIIIB FN-III segments are prominently expressed during embryogenesis, wound healing, tumor progression, and inflammation. However, the roles of this domain in altering overall FN protein structure and regulating cellular function remain unclear. We previously reported that two integrins, $\alpha9\beta1$ and $\alpha4\beta1$, ligate the EIIIA segment (Liao, Y. F., Gotwals, P. J., Koteliansky, V. E., Sheppard, D., and Van De Water, L. (2002) *J. Biol. Chem.* 277, 14467–14474) and that the epitopes for function-blocking monoclonal antibodies lie within the C-C′ loop of EIIIA (Liao, Y. F., Wieder, K. G., Classen, J. M., and Van De Water, L. (1999) *J. Biol. Chem.* 274, 17876–17884). We have now performed site-directed mutagenesis within the EIIIA segment and carried out cell adhesion assays on these mutant EIIIAs. We find that the Asp[41] and Gly[42] residues within the C-C′ loop of EIIIA are necessary for integrin $\alpha9\beta1$ binding. Synthetic peptides based on the predicted important amino acid sequence from the C-C′ loop encode sufficient information to completely inhibit $\alpha9\beta1$-mediated cell adhesion. We also report that EIIIA promotes filopodial formation in $\alpha9\beta1$-expressing cells accompanied by Cdc42 activation. Our data provide a cellular activity for the EIIIA segment, evidence for conformational lability, and peptide sequences for probing EIIIA functions *in vitro* and *in vivo*.

Guidance of cell function during adult tissue repair, the tumor microenvironment, and embryogenesis is highly orchestrated and involves the concerted action of extracellular matrix (ECM),[3] growth factors, and mechanical forces. Although it is now clear that the ECM serves both structural and instructional roles, the mechanisms that regulate the presentation of this "instruction set" to cells remain unclear. Potential mechanisms include alternative splicing to enhance the sequence complexity for an ECM gene product at a needed site. In addition, once expressed, ECM proteins can harbor cryptic sites that are exposed by specific proteolysis or by conformation changes induced by mechanical forces (3–6). The fibronectins (FNs) comprise a family of ECM proteins in which all three potential mechanisms have been implicated in its function.

FNs are high molecular weight, multifunctional adhesive glycoproteins present in the ECM, connective tissues, basement membrane, and various body fluids. They provide excellent substrates for cell adhesion and spreading, thereby promoting cell migration during embryonic development, wound healing, and tumor progression and interact with other ECM proteins and cellular ligands, such as glycosaminoglycans, collagen, fibrin, and integrins (7). FNs are disulfide-bonded dimers of two closely related subunits, each consisting of three types of homologous repeating modules termed types I, II, and III (8). FN molecules have multiple isoforms generated from a single gene by alternative splicing of combinations of three exons: extra domain A (EDA/EIIIA), extra domain B (EDB/EIIIB), and connecting segment III (V). Both EIIIA and EIIIB exons are type III repeating units (7). Plasma fibronectin (pFN), produced by hepatocytes and abundant in plasma, lacks both the EIIIA and EIIIB domains. However, cellular FNs, produced by fibroblasts and epithelial and other cell types, are insoluble, and incorporated into the pericellular matrix, they contain the EIIIA and EIIIB segments in various combinations (9).

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This paper is dedicated to the memory of Dr. Charles Lowry, a gifted teacher and generous colleague.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

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[3] The abbreviations used are: ECM, extracellular matrix; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EIIIA, fibronectin extra domain A; EIIIB, fibronectin extra domain B; FN, fibronectin; FN-III, fibronectin type III repeat; GST, glutathione *S*-transferase; III4, fibronectin type III repeat 4; mAb, monoclonal antibody; PBS, phosphate-buffered saline; pFN, plasma fibronectin; rFN, recombinant FN fragment; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; HPLC, high pressure liquid chromatography; DPBS, Dulbecco's PBS; CAPS, 3-(cyclohexylamino)propanesulfonic acid; RK, rat keratinocyte; ELISA, enzyme-linked immunosorbent assay.

The FN type III (FN-III) repeats exhibit high structural homology (10, 11) despite only 20–40% identity in amino acid sequence (12) (see Fig. 1*A*). The canonical FN type III repeat is a conserved β-sandwich conformation consisting of two β-sheets comprising four strands (G, F, C, C′) and three strands (A, B, and E) (10). Considerable interest has arisen with the observation that these FN-IIIs are conformationally labile (13, 14). Alterations in FN-III conformation can occur as a result of mechanical perturbation or proteolysis and may reveal novel "cryptic" sites (6, 15). Alternative splicing may also influence the function of adjacent domains. For example, the inclusion of the EIIIB segment into cellular FN reveals a novel epitope for monoclonal antibody (mAb) BC.1 (16). Inclusion of the EIIIA segment into FN may promote cell spreading via $\alpha5\beta1$ (17). This alteration might affect the strength of the central cell-binding domain interaction with integrins.

In normal adults, the EIIIA and EIIIB segments are expressed at relatively low levels; expression is elevated markedly in pathological settings. EIIIA is prominently expressed during disease states, such as rheumatoid arthritis (18), wound healing (19–21), vascular intimal proliferation (22, 23), cardiac transplantation (24), and in invasive tumors (25). The temporal expression of the EIIIA and EIIIB segments differ, suggesting an active role for these extra domains (21, 24).

Although the precise functions of the EIIIA and EIIIB segments have been elusive, the EIIIA segment has been observed to regulate cell adhesion and proliferation *in vitro* (17, 26, 27). Some functions that have been attributed to EIIIA are regulation of matrix assembly (28), dimer formation (29), myofibroblast differentiation (30, 31), inflammation (32), and cell cycle progression and mitogenic signal transduction (33). Mice lacking normal EIIIA splicing regulation ($EIIIA^{-/-}$) are viable and phenotypically similar to the wild-type mice ($EIIIA^{+/+}$) (34), although some alterations in cutaneous skin wound healing as well as a significantly shorter life span than wild-type mice have been reported in one model but not another (34, 35). No definitive impact of either the EIIIA or EIIIB segments alone in tumor progression has been observed (36). Importantly, Astrof *et al.* (37) recently demonstrated that an embryonic lethal phenotype (day 10.5) occurs in conditional null mice in which FNs are expressed at normal levels during development, albeit without *both* the EIIIA and EIIIB segments, thereby demonstrating biological relevance for these segments.

Integrins are heterodimeric receptors for ECM and cell surface counterreceptors that play important roles in embryonic development, inflammation, wound healing, and tumorigenesis (38–40). As a result of their dual roles in adhesion and signaling and because of their close association with the actin cytoskeleton, integrins play important roles in regulating cell shape and cell migration (41). Ligand binding specificity depends on the specific α and β subunit present in each heterodimer. In addition to the ligand specificity, sequence homologies between the α-subunits determine the classification of integrins into distinct subfamilies. Of these integrin subfamilies, the $\alpha4/\alpha9$ integrins ($\alpha4\beta1$, $\alpha9\beta1$, and $\alpha4\beta7$) recognize short peptide sequences that include an Asp residue (39). Integrin $\alpha9\beta1$ binds a variety of ligands in an RGD-independent manner (Table 2). These ligands, with the exception of tenascin-C, ADAM-12, ADAM-15, VEGF-C, and VEGF-D, also bind to integrin $\alpha4\beta1$, the closest relative of $\alpha9\beta1$, with which it shares 39% amino acid identity (42–44). The biological significance of the interactions of $\alpha9\beta1$ with most of these ligands remains to be determined.

This laboratory previously reported that the loop between the C and C′ β-strands of the EIIIA segment within FN encompasses the epitope for a function-blocking mAb (IST-9) and for two other antibodies, DH1 and 3E2, all three of which react with the EIIIA segment of human, rat, and chicken FN (2). We reported subsequently that EIIIA serves as a ligand for two integrins, $\alpha9\beta1$ and $\alpha4\beta1$ (1). Moreover, adhesion of cells expressing either $\alpha9\beta1$ or $\alpha4\beta1$ was reduced when cultured on two of the shorter deletion mutants with intact C-C′ loop of EIIIA (EIIIA-(24–66) and EIIIA-(30–57)). These data suggested that the optimal ligand binding site for $\alpha9\beta1$ and $\alpha4\beta1$ in the EIIIA segment could require both the C-C′ loop and additional flanking sequences (1). In addition, mAbs IST-9 or 3E2 inhibited $\alpha9$- and $\alpha4$-mediated cell adhesion to the EIIIA segment, suggesting that the ligand binding site includes the C-C′ loop (1). Yokosaki *et al.* (45) reported that a synthetic peptide, AEIDGIEL, specifically inhibited $\alpha9\beta1$-mediated cell adhesion in tenascin-C, and a homologous sequence (PEDGIHE) is present within the C-C′ loop region in the EIIIA segment.

In the present study, we report that the amino acids Asp^{41} and Gly^{42} in the C-C′ loop region participate in the specific binding of the EIIIA segment to integrin $\alpha9\beta1$. Our studies employed alanine-scanning mutagenesis and synthetic peptides to precisely delineate the critical residues involved in cell adhesion and filopodial extension via integrin $\alpha9\beta1$. Our data establish key amino acid residues that participate in integrin binding but also suggest that the conformation of the epitope plays a role in recognition of integrin ligands by the EIIIA segment.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Restriction enzymes, DNA ligase were obtained from New England Biolabs® Inc. (Ipswich, MA). PCR supermix was obtained from Invitrogen. The anti-EIIIA mAb, IST-9, was purchased from Harlan Bioproducts (Indianapolis, IN). The HUTS-4 antibody against the active conformation of the $\beta1$ integrin was purchased from Chemicon (Temecula, CA). Secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG, was purchased from Bio-Rad. Alexa Fluor 594-labeled goat anti-mouse antibody was obtained from Molecular Probes, Inc. (Eugene, OR). The BCA protein assay kit, Gel Code Blue, BPer® bacterial protein extraction reagent, inclusion body solubilization reagent, SuperBlock Super Signal, Super Signal West Pico and West Femto, EZ-Detect™ Cdc42 activation kit, and horseradish peroxidase-conjugated goat anti-mouse IgG were from Pierce. The GST gene fusion vectors were obtained from Amersham Biosciences. The prokaryotic expression vector pQE30, Qiagen Plasmid Maxi kit, and QIAquick Gel Extraction kit were from Qiagen Inc. (Valencia, CA). The QuikChange® II site-directed mutagenesis kit was from Stratagene (La Jolla, CA). The MERmaid® kit was from Qbiogene Inc. (Irvine, CA). The glutathione (reduced form), glutathione-immobilized agarose, and AEBSF hydrochloride

TABLE 1
Synthesized oligonucleotide sequences

Sequence	Description
For III4-EIIIA-III4 chimera	
Sense strand (5′–3′): G ↓ GTACCGATGATGTTCCCGCTCCAAAGGACCTACAGTTTGTGGA AGTGACCGACGTGAAAGTCACCA-TCATGTGGACACCTCCTAATAGCGCAG ↓ TCGAC	Full-length III4 oligonucleotide (insert 1) restriction sites KpnI/Acc65 and Sal I
Antisense strand (5′–3′): G ↓ TCGACTGCGCTATTAGGAGGTGTCCACATGATGGTGACTTT CACGTCGGTCACTTCCACAAACTGTAGGTCCTTTGGAGCGGGAACATCATCGGTACC	
Sense strand primer (5′–3′): CCTCCTGTCGACGTTTCCAGGTACAGGGTG	Encoding EIIIA sequence (insert 2)
Antisense strand primer (5′–3′): CCTCCTAAGCTTGGTGGTCTGTTGTGCTGTCAGAGGCT TGCTTTCCCTGCCAGATCT-ACCGTGCAAGGCAACCACACTGAC	Encoding EIIIA and III4 sequence restriction sites SalI and HindIII (the underlined sequence in the antisense primer is for strand G from III4).
For double mutant D41A/G42A	
Sense strand primer (5′–3′): TAC TCA AGC CCT GAG GCG GCAATC CAT GAG CTT	
Antisense strand primer (5′–3′): AAG CTC ATG GAT TGC CGCCTC AGG GCT TGA GTA	

were obtained from Sigma. All synthetic peptides and oligonucleotides were synthesized by Sigma. All of the peptides were analyzed by mass spectrometry and HPLC analyses and were >95% pure. Methanol and acetonitrile (HPLC grade) were purchased from Fisher and Mallinckrodt Baker (Phillipsburg, NJ), respectively. All other reagents were at least reagent grade and were obtained from standard suppliers.

PCR and Cloning of the III4-EIIIA-III4 Chimera—A His-tagged chimeric FN-III fusion protein that contained the β-strands A, B, and G from type III repeat 4 (III4) and β-strands C, C′, E, and F from EIIIA was prepared in pQE30 (see Fig. 1). A two-step strategy was used in which, first, DNA encoding the N-terminal 28 amino acids of FN-III4 was synthesized as long oligonucleotide sense and antisense fragments that included a 5′ KpnI site and a 3′ SalI sites. Next, DNA encoding the central portion of EIIIA (amino acids 29–77) was amplified using primers listed in Table 1 by PCR with a 5′ SalI site and a 3′ HindIII site. In addition to the HindIII site, this long antisense primer also included DNA encoding the C-terminal 13 amino acids from III4.

This insert was amplified by PCR for one cycle at 94 °C (5 min), 50 °C (2.5 min), and 72 °C (5 min) followed by 24 cycles each at 94 °C (1.5 min), 50 °C (2.5 min), and 72 °C (5 min) (50-μl final volume), and the amplified products were run on 0.8% agarose gels, extracted, purified, and restriction-digested with the appropriate enzymes. The addition of restriction sites on the primers resulted in the addition of four amino acids in the chimera at positions 38, 39, 89, and 90. The long oligonucleotide fragments containing the N-terminal region from III4 were then complemented and annealed by mixing 75–100 μg of each oligonucleotide with sodium chloride (20 mM final concentration) and subsequently boiled for 3 min. The mixture was then cooled and stored at −20 °C until ligation. The PCR-amplified, enzyme-digested, and annealed fragments were ligated and cloned into the KpnI/Acc65 and HindIII sites of the vector PQE30, which was then transformed into *Escherichia coli* (DH10B). Transformants were picked and expanded, and protein expression was induced (4 h, 37 °C) by the addition of isopropyl-β-D-thiogalactoside (Sigma) to a final concentration of 1 mM.

Bacteria were then centrifuged (7000 × *g* for 10 min), and the resulting cell pellets were washed with Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen). The pellet was either immediately used for protein purification or stored at −80 °C. For protein purification, cell pellets were resuspended in 10 ml of BPer lysis solution with 1 mM AEBSF (PBS/AEBSF), 0.2 mg/ml lysozyme (Sigma), 10 mM $MgCl_2$, and (880 units of) RNase-free DNase I (Invitrogen), thoroughly mixed, and centrifuged at 12,000 × *g* for 20 min. The clarified supernatant was collected and gently mixed with Ni^{2+}-nitrilotriacetic acid resin by inversion (1.5 h, room temperature). This Ni^{2+}-nitrilotriacetic acid resin was washed batchwise with DPBS until all unbound protein was removed, poured into a column, and then treated with 1 ml of elution buffer (50 mM NaH_2PO_4, 250 mM imidazole, and 300 mM NaCl, pH 8.0). Eluted protein was dialyzed against DPBS, further purified using a DEAE-Sepharose column, and dialyzed again. Protein concentrations, purity, and molecular mass were verified by SDS-PAGE on a 10% polyacrylamide gel and analysis by BCA protein assay. Immunological protein reactivity was investigated by Western blotting with mAbs IST-9, 5C11F3, and 4D6E8. The protein was stored at −80 °C until future use.

Site-directed Mutagenesis of the EIIIA Expression Plasmid—Point mutations were selectively introduced into the wild-type rat EIIIA expression construct, EIIIA-pGEX-2T, using the QuikChange II site- directed mutagenesis kit. Reactions (50 μl final) included synthesized complementary oligonucleotide primers with the desired mutations (100 ng/reaction; see Table 1), 5–50 ng of double-stranded DNA template EIIIA-pGEX-2T, 1 μl of dNTP mix, and *PfuUltra* HF DNA polymerase (2.5 units) in the buffer supplied. These reaction mixtures were overlaid with mineral oil (30 μl) and subjected to PCR (one cycle at 95 °C (30 s); 12 cycles each at 95 °C (30 s), 55 °C (10 min), and 68 °C (4 min)) (50 μl final). The reactions were then placed on ice for 2 min, DpnI restriction enzyme (10 units/μl) was added, and the mixture was gently agitated and incubated (37 °C, 1 h) to digest the parental supercoiled double-stranded DNA. *E. coli* (XL1-Blue supercompetent cells) were transformed as described by the manufacturer and incubated in 0.5 ml of NZY^+ broth at 37 °C for 1 h with shaking at 250 rpm, dispersed onto LB plates containing 100 μg/ml ampicillin, and incubated at 37 °C overnight. Individual transformant colonies were selected to prepare plasmids for sequencing to verify the presence of the desired point mutations. DNA sequencing was performed by Northwoods DNA Inc. (Solway, MN), and the results were analyzed by Chromas software (version 2.31; Technelysium Pty. Ltd.).

Production and Purification of the Bacterially Expressed Proteins—Circle Grow medium (Bio 101 Systems, Morgan Irvine, CA) containing ampicillin (100 μg/ml) was inoculated with an overnight culture (50 ml) of transformants, protein

expression was then induced, and cells were harvested as described above. The cell pellets were washed with DPBS and used for protein purification immediately or stored at −80 °C. For protein purification, the procedure implemented was similar to that of the chimera (see above) with the following difference; the clarified supernatant obtained from disruption of cell pellet was collected and gently mixed with glutathione-agarose (1 ml, 50% slurry pre-equilibrated with DPBS) (1.5 h, room temperature). Protein-bound agarose beads were washed batchwise with DPBS until all unbound proteins were removed and then poured into a column and treated with 1 ml of 25 mM glutathione, 120 mM NaCl, and 100 mM Tris-HCl, pH 8.0. Eluted protein was dialyzed against DPBS, purified proteins were quantitated using the Bio-Rad protein assay reagent (Bio-Rad), purity was determined (≥95%) by SDS-PAGE, and proteins were stored at −80 °C.

The recombinant fragments of FN (rFNs) were cloned as described previously (46). They were purified as follows. FN fusion proteins were expressed in *E. coli* grown to late log phase and induced with 1 mM isopropyl-β-D-thiogalactoside for 4 h. Cells were harvested by centrifugation and stored at −80 °C in PBS containing a mixture of protease inhibitors (Sigma). Bacterial pellets were thawed, and the buffer was adjusted to 50 mM sodium phosphate, pH 8.0, 10 mM imidazole, 300 mM NaCl. The pellets were incubated with 2 mg/ml lysozyme and DNase I for 30 min at 4 °C, lysed using a French press, brought to 1% Triton X-100, and centrifuged at 20,000 × *g* for 20 min. Supernatants were incubated with TALON resin (Clontech) and washed in 50 volumes of wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole), and proteins were eluted with 2 volumes of elution buffer (wash buffer adjusted to 250 mM imidazole). Proteins were dialyzed against CAPS buffer (20 mM CAPS, pH 11.0, 150 mM NaCl). The final protein concentrations were determined by UV absorption, and protein sizes were confirmed by SDS-PAGE.

Thrombin Cleavage of the GST-tagged EIIIA Segment—Recombinant GST-tagged wild-type and point mutants of rat EIIIA were purified as described above, and 1 ml of the purified proteins (500 μg/ml) were incubated with 1 ml of glutathione-agarose slurry (50%) for 1 h at room temperature on a rotator, followed by washes with DPBS. The beads were then treated with bovine thrombin (20 units final volume) (Calbiochem) for 1 h at room temperature. Cleaved EIIIA mutant proteins were then separated from the beads by centrifugation, and thrombin was inhibited by the addition of 10 μl of AEBSF (100 mM). The purity of the cleaved proteins was then detected by SDS-PAGE (data not shown). Protein concentrations of the cleaved products were then determined by the Bio-Rad assay protein reagent kit.

Cell Culture—SW480, two human colon carcinoma cell lines, stably transfected with either integrin α9 or vector alone, were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% penicillin and streptomycin, and 2% Geneticin (Invitrogen) as described previously (1, 47). Cells were incubated in a humidified incubator (Thermo Electron Corp., Waltham, MA) at 37 °C in 5% CO_2.

Cell Adhesion Assays—Graded concentrations of either chimeric, wild-type, or mutant recombinant FN-III repeats, as indicated in the figure legends, were coated on the wells of 96-well flat-bottomed microtiter plates (Nalge Nunc International, Rochester, NY) at 37 °C for 1 h. Wells were washed with DPBS and blocked by 1% BSA in DMEM at 37 °C for 1 h. Cells (either SW 480 α9 or mock-transfected cells) were detached using trypsin-EDTA solution (Invitrogen), washed, and resuspended in serum-free DMEM.

To quantitate adhesion, cells were prestained with Hoechst dye (33342; Molecular Probes) for 6 min, recentrifuged, and counted before pipetting into wells. Cell suspensions (50,000 cells/well) were then pipetted directly into wells. Plates were centrifuged (top side up) at 10 × *g* for 5 min followed by incubation for 1 h at 37 °C in a humidified incubator with 5% CO_2. Nonadherent cells were removed by centrifugation (top side down) at 50 × *g* for 5 min. The wells were then filled with 150 μl of PBS, and the plates were read in the fluorescent plate reader (Molecular Devices, Sunnyvale, CA).

In some experiments, cells were fixed and stained with crystal violet, and the absorbance (595 nm) of adherent cells was determined in a microplate reader as described previously (1). Experiments were conducted in either triplicates or quadruplicates and included BSA-coated wells as blank.

For blocking experiments using either EIIIA-specific (data not shown) or anti-integrin antibodies, the cells were either left untreated or preincubated with Y9A2 (10 μg/ml) for 30 min prior to plating. Cell suspensions (50,000 cells/well) either with or without pretreatments were then plated onto the wells.

Quantitation and Statistical Analysis—Values for cell adhesion were obtained at different coating concentrations for each point mutation (see Fig. 3). Values at each serial dilution were normalized to binding at the lowest concentration of coated protein for each specific mutant. Thus, cell adhesion to increasing coating concentrations is represented as -fold increase above binding at the lowest coating concentration. Cell adhesion to the highest coating concentration of the various EIIIA point mutants is shown in Fig. 3*A* (mean ± S.E.). A one-way analysis of variance was performed on the data in Fig. 3*A*, and significant differences ($p < 0.05$) between the mutants were determined using Newman-Keuls *post hoc* analysis.

Peptide Inhibition Assays—Either soluble recombinant His-tagged EIIIA or FN-III4 (1 μM) was coated on the wells of 96-well flat-bottomed microtiter plates at 37 °C for 1 h. Wells were washed with DPBS, blocked with 1% BSA in DMEM for 1 h. Hoechst-stained cells (either SW 480 α9 or mock-transfected cells) were prepared and resuspended in serum-free DMEM as described above. Cells were either left untreated or preincubated with either the cyclized or linear versions of EIIIA peptides (2 mM) for 30 min prior to plating. Cell suspensions (50,000 cells/well), either with or without pretreatments, were then plated onto the wells. The plates were then treated as described above (see "Cell Adhesion Assays"). Experiments were conducted in either triplicates or quadruplicates and included BSA-coated wells as blank.

Reduction and Alkylation of Cyclic EIIIA Peptide—Iodoacetic acid was dissolved in petroleum ether (100 ml) at room temperature, stirred for 2 h in complete darkness, and then set on ice (1 h), and the clear liquid was decanted, followed by desiccation overnight to obtain pure iodoacetic acid crystals. Cyclic EIIIA

peptide (CTYSSPEDGIHEC) (10 mg) along with 1.0 ml of Tris buffer, pH 8.6 (5.23 g of Tris and 9 ml of 1.0 N HCl diluted to 30 ml with water), and 0.1 ml of mercaptoethanol were mixed in a screw-capped vial under nitrogen barrier and incubated at room temperature for 4 h. 0.268 g of iodoacetic acid dissolved in 1 ml of 1 N NaOH (freshly prepared) was added to the peptide and incubated for 15 min. A Sep-Pak column (Waters, Milford, MA) was prepared by washing it successively with water (10 ml), 100% acetonitrile. The peptide sample was then applied to the column, washed with distilled water (10 ml), and eluted with 10% acetonitrile in water. The eluate was then shell-coated onto a round-bottomed flask on an acetone-dry ice bath and lyophilized overnight to obtain pure linear lyophilized peptide.

NMR Characterization of the EIIIA Peptides—Either disulfide bond-linked cyclic or linear reduced CTYSSPEDGIHEC peptide was dissolved to 5.6 mM in 50 mM $NaHCO_3$ at pH 6.7. NMR resonance assignment was carried out using two-dimensional NOESY and TOCSY experiments. Three two-dimensional NOESY experiments were carried out at 600 MHz using mixing times of 80, 300, and 500 ms. Two-dimensional TOCSY was carried out at 600 MHz using a mixing time of 70 ms. The spectra were processed using nmrPipe and analyzed using Sparky. Few long range nuclear Overhauser effects were observed except those across the disulfide bridge, indicating the cyclic and linear peptides had flexible conformations.

Integrin β1 Activation Assays—SW 480 α9 cells were grown to confluence in 48-well plates and treated with peptides as designated in the figure legend. To detect activated β1 integrins, cells were incubated with 1 μg/ml HUTS-4 monoclonal antibody for 1 h. Parallel experiments were carried out with 1 μg/ml P5D2 antibody to detect total integrin β1 levels. Cells were then washed three times with DPBS, fixed with 3% paraformaldehyde, and blocked with 2% BSA for 1 h. Bound antibody was detected by incubating cells for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG. Cell layers were washed three times with DPBS and incubated with Sigma *FAST*™ *o*-phenylenediamine soluble substrate solution for 0.5 h. After the color was allowed to develop, optical density was measured at 440 nm.

Fluorescence Microscopy—Lab-Tek chambers (Nunc, Inc., Naperville, IL) were coated with mixtures of various proteins as indicated. SW 480 α9 cells were then added to the wells in complete medium and allowed to adhere for 20 h. Cells were then fixed (20 min in 4% paraformaldehyde) and permeabilized (0.5% Triton X-100 in PBS) for 10 min. F-actin was visualized with Alexa Fluor 594-conjugated phalloidin (0.3 units/well) (Molecular Probes). Cell layers were examined using an inverted microscope (TE2000-E; Nikon) equipped with phase-contrast and epifluorescence optics, a digital camera (CoolSNAP HQ; Roper Scientific), and MetaVue software (Molecular Devices). The statistical analyses were done using Statistica software (StatSoft, Inc.).

Cdc42 Activation Assay—Cdc42 activation was assessed using a Cdc42 activation kit (Pierce) according to the manufacturer's instructions. Briefly, cells (10-cm diameter dish, 80–90% confluent, serum-free medium) were treated as described and lysed (400 μl of lysis buffer containing phosphatase and protease inhibitors). Protein concentration was determined, and equal amounts of cell lysate were incubated (1.5 h, 4 °C) with the p21-binding domain of GST-PAK1 (GST-PAK1-PBD) and glutathione beads. GST-PAK beads were separated by centrifugation, and the amount of GTP-bound Cdc42 was determined by immunoblotting sequentially with supplied Cdc42-specific antibody, goat anti-mouse horseradish peroxidase, and West Femto substrate. Immunoreactivity was quantified using a Fluor-S multi-imager and Quantity-One software (Bio-Rad). The amount of GTP-bound Cdc42 was normalized to the total amount of Cdc42 detected in each cell lysate.

RESULTS

A III4-EIIIA-III4 Chimera Retains Adhesive Capacities Similar to the Wild-type Rat EIIIA—Individual FN-IIIs show variations in protein sequence identity (20–40%) but exhibit a high degree of structural homology (10–12, 48). EIIIA, like the other FN-III repeats, is a β-sandwich composed of seven anti-parallel β strands with exposed loops between these strands (Fig. 1*A*). Previously published data from our laboratory identified two integrins α9β1 and α4β1 that bound the EIIIA segment of FN. Interestingly, two small deletion mutants of the EIIIA segment with intact C-C′ loops retained some adhesive activity for both integrins, although their activities were reduced relative to wild-type EIIIA protein (1). This suggested that the optimal ligand binding site for integrins α9β1 and α4β1 could require both the C-C′ loop and the flanking sequences. Flanking sequences could participate directly in integrin binding or contribute to the conformation of this type III repeat. We tested the possibility that all of the necessary sequence information required for binding to the integrin α9β1 could lie within amino acids 30–57 of the EIIIA segment. To do so, we subcloned the portion of the EIIIA segment spanning amino acids 28–76 that we found earlier had reduced activity between corresponding flanking sequences from the fourth type III repeat, III4, previously observed to have no adhesive activity (III4-EIIIA-III4 chimera) (1). Our strategy was built on earlier findings that the intact FN-IIIs all have very similar structures despite wide sequence divergence (10–12, 48). A recombinant chimeric protein composed of III4-EIIIA-III4 was prepared containing the 6× His tag and purified to homogeneity (Fig. 1*D*).

We conducted cell adhesion assays with SW480 cells that had been stably transfected with either an α9 expression plasmid (SW-α9) or empty vector (SW-mock) (47). Wells were coated with recombinant chimera, His-tagged wild-type rat EIIIA, or FN-III4 alone. Cells were then allowed to adhere to coated wells, and the unbound cells were removed by centrifugation. We found that the SW-α9 cells exhibited significant adhesion to the EIIIA segment but not to FN-III4 and that the chimera supported adhesion of the cells at levels comparable with those observed with the wild-type EIIIA (Fig. 2*A*). Mock-transfected SW480 cells did not bind to either chimera or EIIIA (Fig. 2*A*). The α9-mediated adhesion to EIIIA was dependent on the concentration of EIIIA used to coat the wells (Fig. 2*A*, *i*). The binding of SW480 α9 cells to EIIIA was specific, since the binding could be abolished by function-blocking mAb, Y9A2 (Fig. 2*A*, *ii*). These data suggested that the critical sequences mediating integrin binding were within the C, C′, E, and F regions of EIIIA.

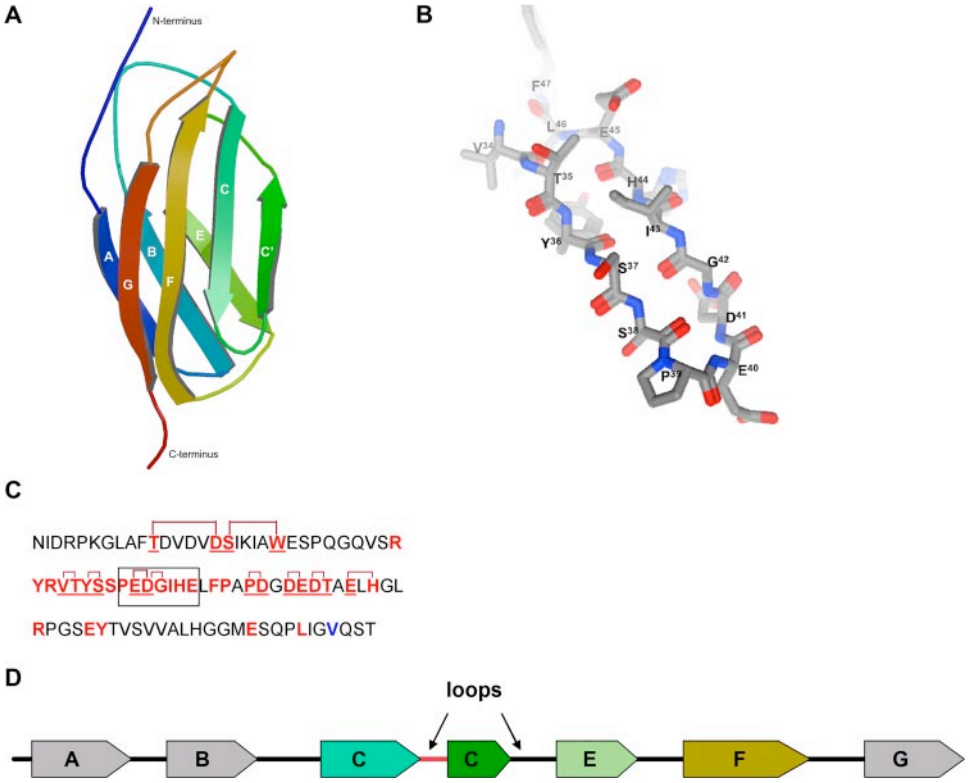

FIGURE 1. **Structure of wild-type EIIIA and schematic of chimeric EIIIA.** *A*, *ribbon structure* of EIIIA, a canonical FN-III, with two β-sheets: one composed of four β-strands (C, C′, F, and G) and the other composed of three strands (A, B, and E). The structure is displayed in MOLMOL; accession numbers are as follows: MMDB, 18401; Protein Data Bank, 1J8K (49). *B*, key amino acids within the C-C′ loop are highlighted. *C*, mutants of rat EIIIA generated by alanine-scanning mutagenesis. Single amino acid substitutions to alanine (*red letters*) and single amino acid substitution to isoleucine (*blue*) are shown. The *letters* that are *underlined* and *connected with braces* represent double point mutations to alanine. *D*, schematic of the III4-EIIIA-III4 chimera in which the coding sequences for β-strands A, B, and G are from FN type III repeat 4 (*gray*) and strands C, C′, E, and F are from EIIIA (*colored*). These were fused to obtain an intact type III repeat.

Rat Keratinocytes That Express Endogenous α9β1 Adhere to Rat EIIIA but Not a Nonspecific Type III Repeat III4—These studies and our earlier ones with full-length EIIIA took advantage of transfected cell lines. To verify the biological significance of our earlier results as well as those below, we employed rat keratinocytes (RKs), which express endogenous integrin $\alpha 9$ in adhesion assays. Integrin $\alpha 9$ expression on RK cells was detected by Western blotting.[4] Fig. 2*B* shows the adhesion of RK cells to wild-type His-tagged rat EIIIA and his-tagged III4. The results demonstrate that the cells expressing endogenous integrin $\alpha 9$ show adhesive behavior similar to the cells transfected with exogenous integrin $\alpha 9$, albeit to a lesser extent.

The Asp (D41A) and Gly (G42A) within the C-C′ Loop Are Critical Residues Involved in Binding α9β1-expressing Cells—Integrin binding motifs in the FN type III or Ig-like repeats in ECM proteins and cell adhesion molecules are commonly situated on exposed loops or turns between β-strands (10). Our adhesion assay data with the III4-EIIIA-III4 chimera (Fig. 2*A*) suggested that the region encompassing the C-C′ loop is critical to binding integrin $\alpha 9\beta 1$. Inspection of the sequence within the C, C′, E, and F strands and loops revealed that the PEDGIHE sequence within the C-C′ loop of the EIIIA segment resembled the ligand binding site for integrin $\alpha 9\beta 1$ in the third FN-III repeat of tenascin-C (AEIDGIEL) (45). The C-C′ loop also included the epitope for blocking mAbs (2). We therefore decided to map the specific sequences within this portion of EIIIA that mediate binding to integrin $\alpha 9\beta 1$.

A total of 45 different single and double point mutants spanning much of the EIIIA segment were prepared by alanine-scanning mutagenesis (Fig. 1*C*). These fusion proteins with an amino-terminal GST tag were expressed in reasonable yields (1–2 mg/liter culture), were soluble, and were purified to homogeneity. We performed an ELISA using an anti-GST mAb and determined that all recombinant EIIIA point mutants adsorbed to plastic surface to a similar extent as wild-type EIIIA protein (data not shown). We also verified by CD spectral analyses that the mutant proteins had an appropriate secondary structure (supplemental Fig. 1).

Next, we tested whether and to what extent integrin $\alpha 9\beta 1$ mediated cell adhesion to a panel of EIIIA single and double point mutants. All mutants targeting amino acids outside of the C-C′ loop were fully active (data not shown). By contrast, mutation of specific residues within the C-C′ loop reduced cell adhesion to $\alpha 9\beta 1$. Fig. 3*A* shows the cell adhesion of SW-$\alpha 9$ that occurred for each of the mutants in the C-C′ loop of the EIIIA segment at the highest coating concentration of that point mutant. The double mutant D41A/G42A was found to reduce cell adhesion to background levels (Fig. 3*A*). At the highest dose, the mutants containing single mutations of these two amino acids, D41A and G42A, did not alter the adhesion of SW-$\alpha 9$ cells, although the G42A response was highly variable (Fig. 3*A*). Closer analysis of these two point mutants over a range of different coating concentrations showed that lower coating concentrations (0.12 and 0.25 μM) of the G42A and D41A mutants supported less cell adhesion than EIIIA, but adhesion at higher concentrations (0.5 and 1 μM) was similar to EIIIA (Fig. 3*B*, *ii*). By contrast, the D41A/G42A double mutant was inactive at all concentrations tested (Fig. 3*B*, *i*). Taken together, these data underscore the importance of the involvement of these residues in mediating binding to integrin $\alpha 9\beta 1$. As noted in the literature, the RGD binding and the $\alpha 4/\alpha 9$ integrins both recognize Asp residues in their ligands. It therefore was of interest that the recognition sequence for integrin $\alpha 9$ in the EIIIA segment again included a "conserved" Asp residue.

We considered the possibility that the GST moiety in the purified EIIIA fusion proteins could alter the conformation or

[4] P. Singh and L. Van De Water, unpublished observations.

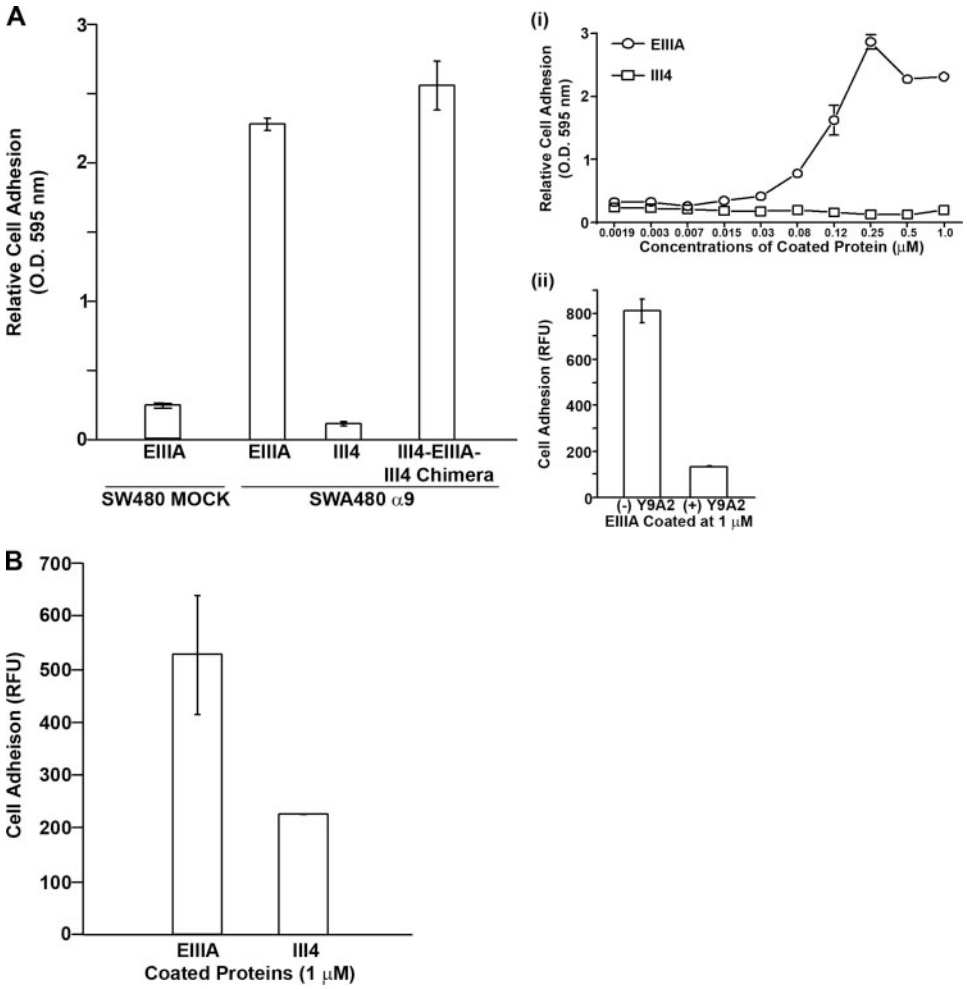

FIGURE 2. **Cells expressing either exogenous or endogenous integrin $\alpha 9\beta 1$ adhere to EIIIA.** *A*, SW480 α9-expressing cells adhere to the chimeric protein. Recombinant histidine-tagged III4-EIIIA-III4 chimera was cloned as described under "Experimental Procedures." Wells of 96-well flat-bottomed microtiter plates were coated with soluble chimeric protein (1 μM, 37 °C, 1 h), washed, and blocked with BSA. SW480 α9 cells were detached, resuspended in serum-free DMEM, added to wells (50,000 cells/well), and incubated (1 h, 37 °C). Nonadherent cells were removed by centrifugation (50 × *g*), adherent cells were stained with crystal violet solution, and the absorbance was measured (595 nm). *Inset i* shows adhesion of SW480 cells, stably transfected with integrin α9, to soluble, recombinant His-tagged EIIIA and III4 repeats at graded concentrations. III-4 is a nonadhesive homologous FN-III and serves as a negative control. Controls also include SW480 α9 cells pretreated with blocking antibody Y9A2 (*inset ii*). Data are representative of four independent experiments; *error bars*, S.E. from quadruplet wells. *B*, rat keratinocytes that express endogenous α9 adhere to recombinant rat EIIIA. Wells were incubated with either soluble recombinant His-tagged EIIIA or III4 repeats (1 μM), washed, blocked and charged with RKs. RK cells were prestained with Hoechst (33342) dye (10 ng/μl), and an adhesion assay was carried out as described above. The wells containing adherent cells were then filled with 150 μl of DPBS, and the plates were read in the fluorescent plate reader and the relative fluorescence units (*RFU*) determined (excitation, 360 nm; emission, 460 nm). Data are representative of three independent experiments; *error bars*, S.E. from duplicate wells.

adhesive capacities of rat EIIIA. However, comparable cell adhesion to the double mutant D41A/G42A was obtained when assays were carried out with SW-α9 cells after the GST was removed by thrombin cleavage followed by repurification (Fig. 3*C*).

Sufficient Sequence Information Is Present within Peptides Mimicking the C-C′ Loop to Completely Block α9-dependent Cell Adhesion to the EIIIA Segment—A complementary approach was also taken to determine further whether sequences within the C-C′ loop were critical for $\alpha 9\beta 1$-mediated adhesion to EIIIA. We designed a series of synthetic peptides corresponding to the amino acid sequence of the C-C′ loop of rat and human EIIIA and evaluated the ability of these peptides to inhibit adhesion of α9-transfected SW480 cells to 1 μM His-tagged rat EIIIA (Fig. 4*A*). The peptide was designed with flanking cysteine residues, which when oxidized form a cysteine-bonded cyclic peptide. Our goal was to emulate the type II β-turn of the native C-C′ loop observed in the NMR structure of EIIIA (49) (Fig. 1*B*). The cyclic peptide CTYSSPEDGIHEC (oxidized) caused complete inhibition of adhesion of SW-α9 cells to EIIIA at 2 mM (Fig. 4*B*). This effect was concentration-dependent, and no effect was observed on adhesion of the mock-transfected control cells (data not shown). In contrast, the scrambled versions of the cyclic peptide (Cys-SEDIHYTEGPS-Cys) as well as a linear irrelevant peptide (RKENEDSWDWVQKNQ) were without effect. These data demonstrated that the sequence of the amino acids within the C-C′ loop contains all of the necessary and sufficient sequence information to mediate as well as inhibit ligand binding.

EIIIA Peptides May Bind to Their Integrins by a "Selected Fit" Mechanism—Our data identify the C-C′ loop as both necessary and sufficient for cell adhesion. However, we also inferred from our data that the conformation of the intact EIIIA was important. To determine the extent to which the cyclic peptide adopted the conformation of the C-C′ loop, we performed NMR studies on the peptide. We observed that although the peptide does have a cyclic structure, it does not adopt a fixed conformation.[5] We then linearized the cyclic CTYSSPEDGIHEC peptide by reducing and alkylating the cysteine residues. Cell adhesion experiments were performed using SW480 α9 cells; we observed that both the cyclic and linear peptides blocked cell adhesion (Fig. 5*A*). These data suggest that both the linear and cyclic peptides can adopt functionally appropriate conformations once they encounter the appropriate residues involved in ligand binding. Taken together, our data demonstrate that the sequences within the C-C′ loop are sufficient to inhibit binding and suggest that the relatively flexible peptides

[5] A. V. Shinde, C. Bystroff, C. Wang, and L. Van De Water, unpublished observations.

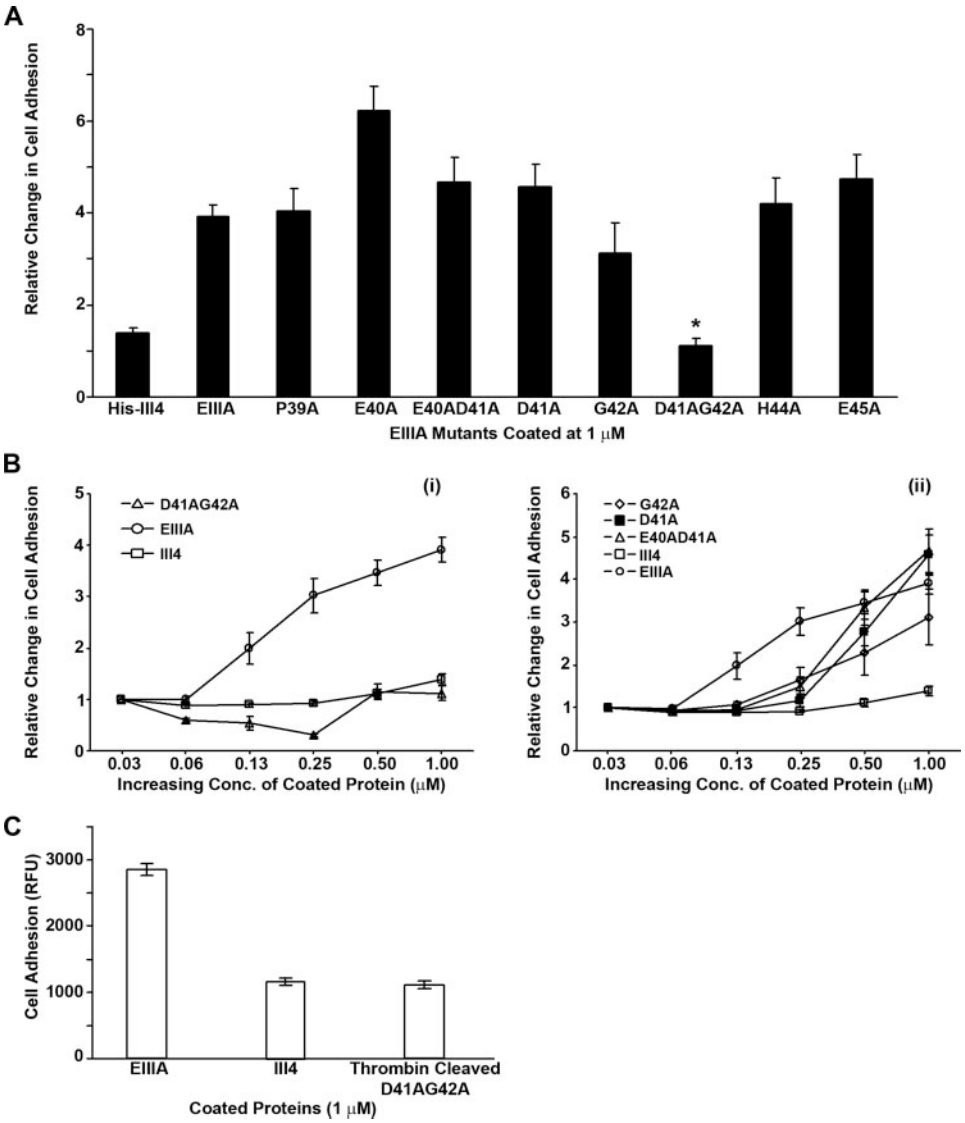

FIGURE 3. **Differential adhesion of cells expressing integrin $\alpha9\beta1$ to various site-directed mutants of EIIIA segment.** *A*, mutants were prepared by site-directed mutagenesis, expressed in bacteria as GST fusion proteins, and affinity-purified on glutathione-agarose. Wells were coated with the proteins (1 μM), and a cell adhesion assay was performed using SW480 $\alpha9$ cells as described in the legend to Fig. 2. Controls include a nonadhesive homologous FN-III and mock-transfected cells (not shown). The Asp (D41A) and Gly (G42A) are critical residues involved in binding $\alpha9\beta1$-expressing cells. Amino acids in the EIIIA protein are indicated by single-letter code followed by the residue number representing the mutated position relative to the native protein. *B*, *i*, line graph representing the binding activities of recombinant EIIIA and III4 to SW480 $\alpha9$ cells at increasing concentrations. The double mutant D41A/G42A does not promote cell adhesion at any concentration. *ii*, similarly, the binding activities of the mutants E40A, E40AD41A, D41A, and G42A at increasing concentrations are shown. *, difference from wild-type EIIIA ($p < 0.05$). *Error bars*, S.E. For details, see "Statistical Analysis." *C*, the differential adhesion of SW480 cells stably transfected with integrin $\alpha9$ to thrombin cleaved mutant fusion protein of EIIIA segment. Recombinant GST-tagged wild-type rat EIIIA, III4, and the D41A/G42A point mutants were digested with bovine thrombin (100 μl of 200 units/ml) and repurified on glutathione-agarose. The thrombin-cleaved proteins were then coated onto the wells of a 96-well flat-bottomed plates at a concentration of 1 μM, and a cell adhesion assay was performed using SW480 $\alpha9$ cells as described in the legend to Fig. 2. Thrombin-cleaved D41A/G42A shows adhesive capacities comparable with those of the GST-tagged fusion proteins. Data are representative of three independent experiments; *error bars*, S.E. from duplicate wells.

used here can adopt the needed conformation as they bind integrins, perhaps by a "selected fit" mechanism.

We also determined if the peptides blocked adhesion to integrin $\alpha9\beta1$ by directly interacting with the integrin. We tested this by performing receptor activation assay for integrin $\beta1$. Using the antibody, HUTS-4, which is specific for the active conformation of human $\beta1$ integrins, we found that the EIIIA peptide specifically activates the integrin $\alpha9\beta1$ in solution, whereas the irrelevant peptide does not (Fig. 5*B*). The total levels of integrin $\beta1$, however, were similar in all conditions as detected by antibody P5D2 (data not shown).

The Adhesive Activity of EIIIA to Its Receptor $\alpha9\beta1$ Is Not Detected in Longer FNs, and EIIIA Does Not Alter or Affect RGD-dependent Cell Adhesion—Earlier work demonstrated that inclusion of the EIIIA segment in full-length FNs increased cell spreading through the $\alpha5\beta1$ integrin (17). Given our work demonstrating that integrin $\alpha9\beta1$ binds the EIIIA segment, we next determined whether or not the presence of the EIIIA segment modulated the affinity of RGD tripeptide in the 10th type III repeat of FN. We employed several rFNs, which spanned FN type III repeats 7–15 but excluded the alternatively spliced V region (Fig. 6). These fragments were characterized by either the presence or absence of either the EIIIA segment or the RGD motif in III-10. The "RGD" moiety was removed by specific deletion of these residues. We then confirmed that the recombinant proteins were properly folded by two independent approaches. In addition to the CD spectral analysis (Supplemental Fig. 1), we also performed ELISAs with two different mAbs, IST-9 and 5C11F3, both directed against the EIIIA segment and one of which reacts preferentially with native EIIIA (IST-9; see Ref. 2 and supplemental Fig. 2). We found that indeed, the recombinant proteins showed similar secondary structure and retained the reactivity for the antibodies, suggesting that they are appropriately folded.

First, we determined whether or not the EIIIA segment binds to integrin $\alpha9$ in the context of the longer fragments of FN but in the absence of the RGD motif. Cell adhesion assays were performed by coating plates with graded but equimolar concentrations of rFN (A+ RGD−), rFN (A− RGD−), His-tagged rat EIIIA, or His-tagged III4. Wells were charged with SW-$\alpha9$ cells, incubated, and centrifuged to remove unbound cells. Essentially no $\alpha9$-mediated adhesion occurred to rFNs that included the EIIIA segment but excluded

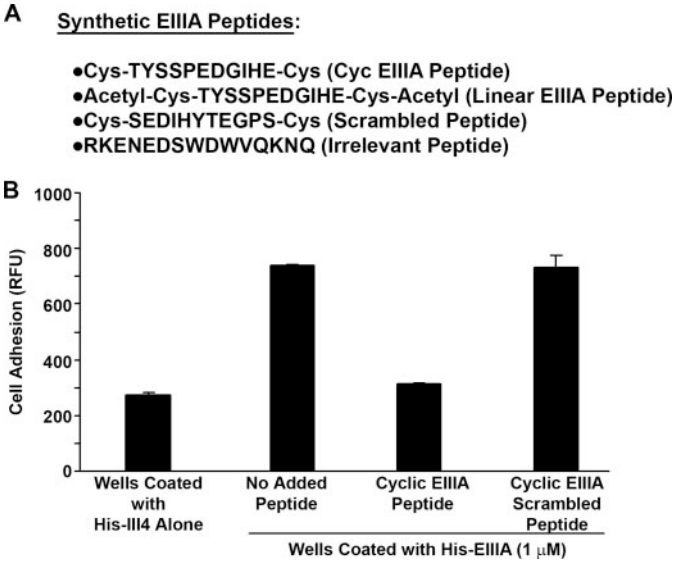

FIGURE 4. **Soluble, cyclized EIIIA peptide inhibits $\alpha9\beta1$-mediated adhesion to EIIIA.** *A*, sequences of synthetic peptides modeled after the sequence of C-C′ loop of EIIIA and controls. *B*, soluble, cyclized EIIIA peptide but not the control peptide inhibits SW480 $\alpha9$ cell adhesion to EIIIA. Either soluble, cyclized EIIIA peptide (2 mM) or control peptide (2 mM; a cyclized scrambled version of the EIIIA peptide) was preincubated with SW480 $\alpha9$ cells, and this mixture was transferred to wells (50,000 cells/well in serum-free DMEM) coated with either His-EIIIA or His-III4, a negative control. Plates were incubated, and adherent cells (prelabeled with Hoechst-33342) were quantitated as described in the legend to Fig. 2. Sufficient sequence information is present within the C-C′ loop to completely block $\alpha9$-dependent cell adhesion to the EIIIA segment. Data are representative of five independent experiments; *error bars*, S.E. from triplicate wells.

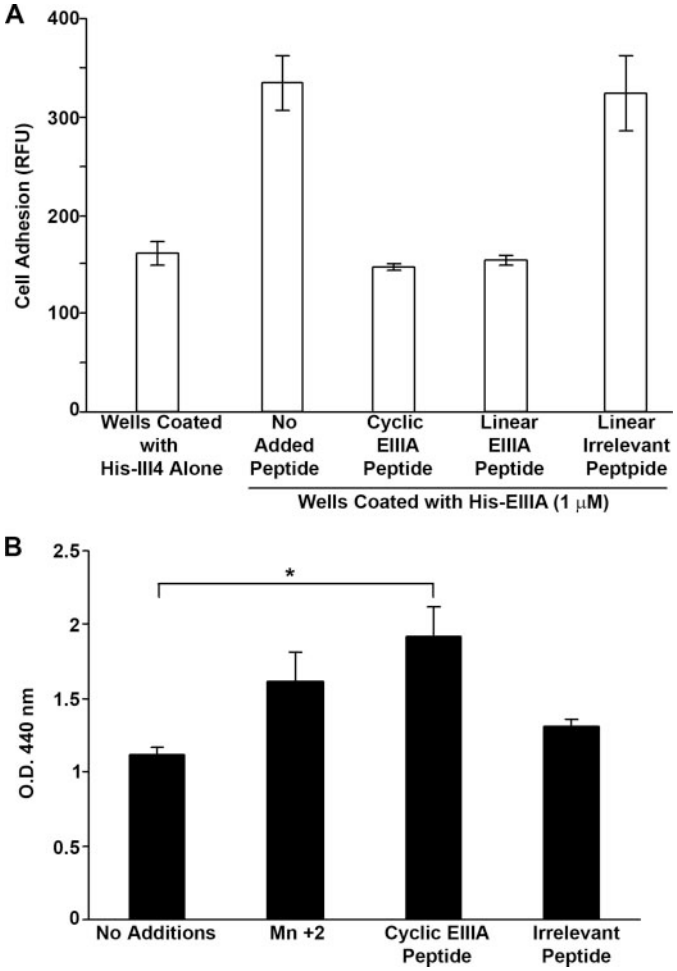

FIGURE 5. **The linear EIIIA peptide inhibits SW480 $\alpha9$ cell adhesion to EIIIA.** *A*, the cyclized EIIIA peptide (oxidized) was reduced and alkylated (linearized) and repurified as described under "Experimental Procedures" and then used in the cell adhesion inhibition assay (See Fig. 4). Similar to the cyclized peptide, the linear peptide at a concentration of 2 mM inhibited the adhesion of SW480 $\alpha9$ cells to recombinant His-tagged EIIIA. Controls include a 15-mer linear irrelevant peptide used at the same concentration (2 mM). This demonstrates that both cyclized and linear peptides are equally active. Data are representative of three independent experiments; *error bars*, S.E. from triplicate wells. *B*, confluent monolayers of SW480 $\alpha9$ cells were incubated with either the cyclic EIIIA peptide or control irrelevant peptide (2 mM) in complete DMEM for 1 h. The integrin activation was measured by an ELISA using the HUTS-4 antibody (1 μg/ml) specific for activated $\beta1$ integrin. Mn^{2+} (2 mM) was used as a positive control. *, significant difference from no treatment as determined by a *t* test, $p < 0.05$.

the RGD (Fig. 7*A*). The EIIIA segment within the rFNs was detectable by Western blotting and ELISA (data not shown). In addition, EIIIA, when present as an individual type III repeat, showed concentration-dependent adhesion as expected (Fig. 7*A*). These data suggest that the adhesive activity of EIIIA for $\alpha9\beta1$ is silenced when flanked by other type III repeats, but when present alone, EIIIA binds to its cognate integrin receptor $\alpha9\beta1$.

Next, we studied the adhesive activities of EIIIA to $\alpha9\beta1$ in the presence of RGD to see if the presence or absence of EIIIA affects RGD binding to its receptor $\alpha5\beta1$. Adhesion assays were performed using SW-$\alpha9$ cells plated on surfaces coated either with rFN (A+ RGD+), rFN (A− RGD+), or EIIIA or III4 controls. SW480 cells express $\alpha5\beta1$ integrin that could bind RGD sequence (47). We observed that EIIIA does not significantly alter the binding of RGD with its receptor $\alpha5\beta1$ (Fig. 7*B*). These results are in substantial agreement with Manabe *et al.* (17), who observed that augmentation of integrin binding by the EIIIA segment occurred only in the context of the full-length, intact FN molecule. When binding of integrin $\alpha5\beta1$ to a shorter rFN fragment, as we use here, consisting of the central cell-binding domain and the adjacent heparin-binding domain Hep2, was studied, no effect of insertion of the EIIIA segment was seen (17). In order to rule out the possibility that there might be a change in receptor levels of $\alpha5\beta1$ due to stable transfection of integrin $\alpha9$ in SW-$\alpha9$ cells, we performed flow cytometry, finding no difference in $\alpha5\beta1$ as have others (data not shown) (47). We also performed adhesion assays with SW mock-transfected cells that express $\alpha5\beta1$ and obtained no EIIIA-dependent differences in adhesion to longer rFNs (Fig. 7*C*).

The EIIIA Segment Affects SW-$\alpha9$ Cell Behavior Accompanied by the Activation of Cdc42—We next determined whether or not the EIIIA segment would affect SW-$\alpha9$ cell behavior. Chambered glass slides were coated with a mixture of pFN and either EIIIA or III4 segments. Cells were then incubated on the treated surfaces for 20 h. We found that cells incubated on the mixture of pFN and EIIIA stimulated the formation of extensive filopodia on the cell periphery (Fig. 8, *A* and *B*). This effect, however, was not significant in cells plated with pFN alone or a mixture of pFN and III4 (Fig. 8, *A* and *B*; data not shown). This effect was also integrin $\alpha9$-specific, since parallel experiments with SW mock-transfected cells did not result in filopodia formation when plated on any of the above protein mixtures (data

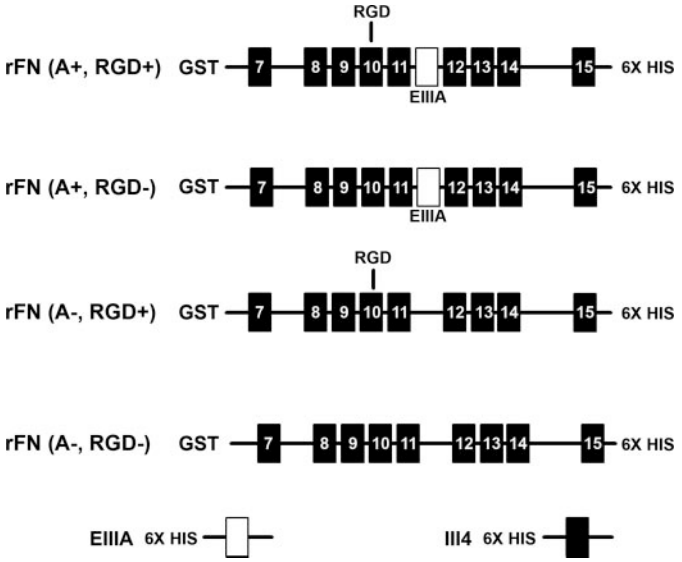

FIGURE 6. **Diagrammatic representation of the rFNs used in subsequent cell adhesion assays.** The *shaded boxes* denote individual FN type III repeats; the presence (*A*+) or absence (*A*−) of the EIIIA segment are noted, as is the presence (*RGD*+) or absence (*RGD*−) of the RGD tripeptide in the 10th type III repeat.

not shown). The Rho family GTPase, Cdc42, is required for the formation of filopodia. We next investigated whether the filopodial extensions stimulated by EIIIA were accompanied by the activation of Cdc42. SW-α9 cells were cultured (20 h) on tissue culture dishes coated with either pFN alone or with a mixture of pFN and either EIIIA or III4 segments. The active, GTP-loaded, form of Cdc42 was quantitated by affinity for PAK, a downstream effector (Fig. 8*C*). We observed a 1.8-fold ($n = 3$) increase in active Cdc42 in cells adhering to surfaces coated with pFN and EIIIA relative to cells adhering to pFN alone or pFN and III4 (1.1-fold relative to pFN alone).

DISCUSSION

In this study, we identify a novel integrin binding motif within the C-C′ loop of the EIIIA segment, which mediates adhesion to integrin $\alpha9\beta1$. Mutagenesis studies demonstrate that the amino acid residues in the C-C′ loop of EIIIA are of primary importance for integrin recognition. We also demonstrate that a synthetic cyclic peptide contains sufficient sequence information to completely inhibit cell adhesion of integrin $\alpha9\beta$l to the EIIIA segment. We further report that β-strands flanking the C-C′ loop confer additional binding activity and that inclusion of flanking FN-IIIs in rFNs suppresses EIIIA cell adhesion. Finally, we observe that EIIIA, in the presence of pFN, promotes filopodia formation and activation of GTPase Cdc42.

This laboratory reported previously that two key amino acids, Ile[43] and His[44], in the C-C′ loop region of the EIIIA segment are a necessary part of the epitope for three mAbs that react with the EIIIA segment of human, rat, and chicken FN. Two of these mAbs, DH1, and IST-9 were reported to block function (30, 31, 50). Reduction in binding of mAbs IST-9, 3E2, and DH1 was also observed when Asp[41] and Gly[42] were mutagenized to Ala (2). All of these mAbs were found not to react with deletion mutants within the EIIIA segment. Subse-

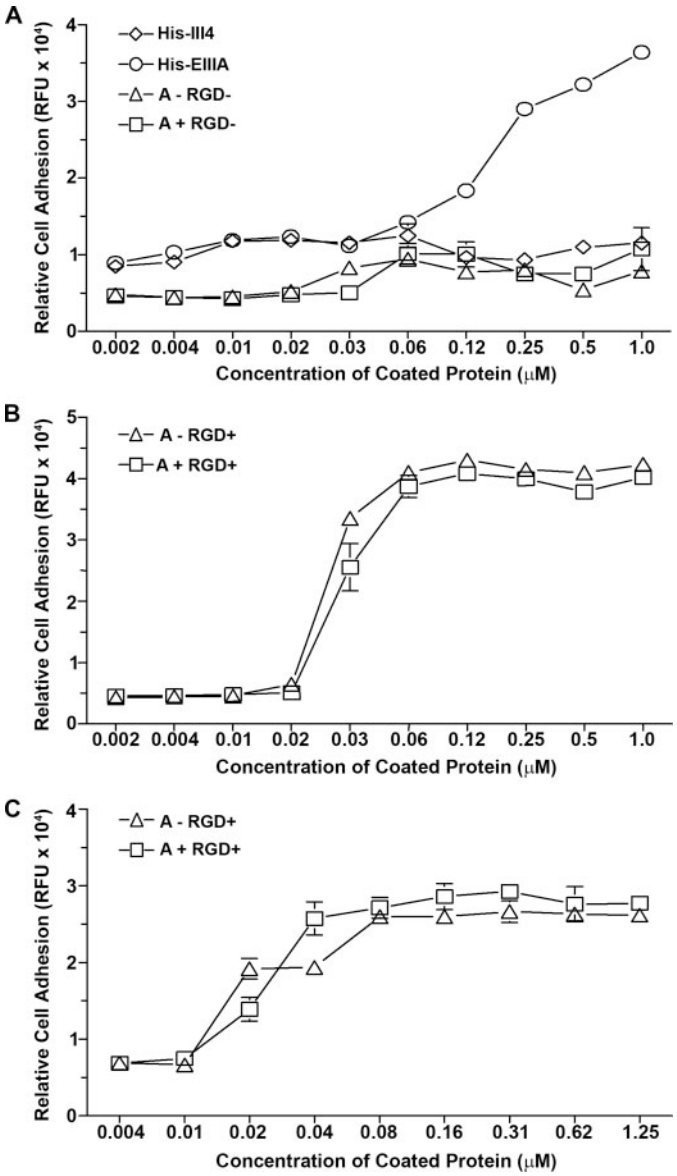

FIGURE 7. **The $\alpha9\beta1$-dependent adhesion of the SW480 $\alpha9$ cells does not occur on longer rFNs that include the EIIIA segment.** *A*, EIIIA does not bind $\alpha9\beta1$ when flanked by other FN-IIIs. Wells were incubated with soluble recombinant His-tagged EIIIA, III4 repeats, rFN (*A*+ *RGD*−), and rFN (*A*− *RGD*−) (1 μM), washed, blocked, and charged with SW480 $\alpha9$ cells (50,000 cells/well), and an adhesion assay using Hoechst-labeled cells was carried out as described in the legend to Fig. 2. These data demonstrate that EIIIA does not bind $\alpha9\beta1$ when flanked by neighboring FN-IIIs. Data are representative of three independent experiments; *error bars*, S.E. from quadruplet wells. *B*, EIIIA segment within cellular FN does not alter RGD binding to cells bearing $\alpha9\beta1$. Wells were incubated with soluble recombinant His-tagged EIIIA, III4 repeats, rFN (*A*+ *RGD*+), and rFN (*A*− *RGD*+) (1 μM), washed, blocked, and charged with SW480 $\alpha9$ cells, and an adhesion assay was performed as in *A*. Data are representative of three independent experiments; *error bars*, S.E. from quadruplet wells. *C*, the binding of SW480 cells without $\alpha9\beta1$ to rFNs is $\alpha5$-dependent. Wells were incubated with soluble recombinant His-tagged EIIIA, III4 repeats, rFN (*A*+ *RGD*+), and rFN (*A*− *RGD*+) (1 μM), washed, blocked, and charged with SW480 mock-transfected cells, and an adhesion assay was performed as in *A*. These data demonstrate that the binding to the rFNs containing the tripeptide RGD is not influenced by the presence of EIIIA within rFN and is $\alpha9$-independent. Data are representative of three independent experiments; *error bars*, S.E. from quadruplet wells.

quently, we identified two integrins, $\alpha9\beta1$ and $\alpha4\beta1$, that bind to a recombinant EIIIA segment and proposed that the ligand binding sites within EIIIA probably include the C-C′ loop and

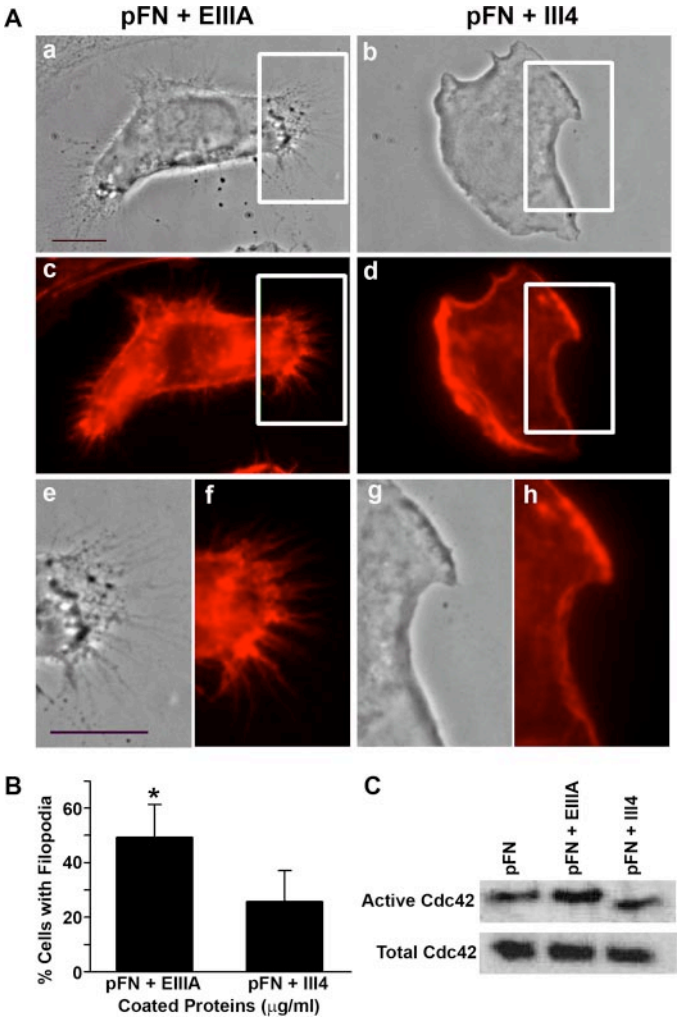

FIGURE 8. **Mixtures of pFN and EIIIA stimulate formation of filopodial protrusions at the cell periphery and activate Cdc42.** *A*, Lab-tek chambers were coated with mixtures of pFN (2.5 μg/ml) with either EIIIA (10 μg/ml) (*a*, *c*, *e*, and *f*) or III4 (10 μg/ml) (*b*, *d*, *g*, and *h*) segments. Cells were then seeded onto wells in complete medium for 20 h, fixed, and stained to visualized phalloidin (*c*, *d*, *f*, and *h*). Corresponding phase-contrast images are also shown (*a*, *b*, *e*, and *g*). The *boxed regions* in *a* and *b* are *zoomed out* in *e*–*h*. All images were taken at ×100 magnification. *Scale bar*, 10 μm. *B*, the morphological data in *A* were quantified from three different experiments. *Error bars*, S.E. *, significant difference of EIIIA from III4 treatment group by a *t* test for dependent samples; $p < 0.005$. *C*, tissue culture coated dishes were coated with mixtures of pFN (2.5 μg/ml) without or with either EIIIA (10 μg/ml) or III4 segments. Cells were cultured (serum-free medium; 20 h), lysates were prepared, and amounts of total and activated Cdc42 were measured by Western blotting.

flanking sequences (1). We now advance these findings by identifying amino acids within the C-C′ loop that mediate integrin binding. Our data indicate that the region encompassed by the C-C′ loop is important, since it not only forms epitopes for function-blocking mAbs but also is a ligand for integrins $\alpha9\beta1$ and $\alpha4\beta1$.

Integrin $\alpha9\beta1$ binds to a number of diverse ligands in an RGD-independent manner (Table 2). Most of these recognition sequences recognize either short peptide sequences that include an Asp residue or a homologous tripeptide composed of a leucine or isoleucine followed by an acidic residue and a neutral amino acid with a small side chain. It is worth noting that in the ligands for which the structure is known, the Asp residues (Asp[40] in vascular cell adhesion molecule-1 (51) and

TABLE 2
Ligands for integrin $\alpha9\beta1$

Ligands	Sequence within which $\alpha9\beta1$ binds	References
A disintegrin and metalloproteinase domain-12 (ADAM-12) (human and mouse)	SNSCD	65
ADAM-15 (human)	RGDCD	65
ADAM-15 (mouse)	TDDCD	65
ADAM-15 (mutated form of human)	SGACD	65
ADAM-2	MLDG	66
Blood coagulation factor XIII (FXIII)		42
EIIIA	PEDGIHE	This paper
L1 cell adhesion molecule (L1-CAM)		67
Osteopontin	SVVYGLR	68, 69
Propolypeptide of von Willebrand factor (pp-vWF)	DCQDHSFSIVIETVQ	42
Tenascin-C	EIDGIEL	45
Thrombospondin-1	N-terminal domain (NoC1)	70
Tissue transglutaminase (tTG)		42
Vascular cell adhesion molecule-1 (VCAM-1)	DCQDHSFSIVIETVQ	42
Vascular endothelial growth factor-C (VEGF-C)		71
VEGF-D		71

Asp[42] in mucosal addressin cell adhesion molecule-1 (52)) are presented on protruding loops, similar to the presentation of the RGD tripeptide in FN (10). Moreover, structural studies with integrin-ligand pairs demonstrate direct binding of Asp residue in the ligand with MIDAS (invariant ligand-binding site) in the integrins (53, 54). We report that, as in other $\alpha9\beta1$ integrin ligands, Asp[41] and Gly[42] in EIIIA participate in binding (Fig. 3*A*). In our experiments, the GST moiety in the purified EIIIA fusion proteins does not appear to alter the conformation of rat EIIIA, because comparable reactivities are obtained when the GST and EIIIA moieties are separated by thrombin cleavage (Fig. 3*C*).

The markedly high expression of FNs that include both the EIIIA and EIIIB segments during development suggests important roles for these segments (19, 55, 56). Mouse embryos null for the entire FN gene show severe defects by day 8 during gastrulation and die by 10–11 days, whereas mice that express FNs lacking either EIIIA or EIIIB develop normally (34, 35, 57, 58). By contrast, Astrof *et al.* (37) recently demonstrated that an embryonic lethal phenotype (day 10.5) occurs in conditional null mice in which FNs are expressed at normal levels during development, albeit without *both* the EIIIA and EIIIB segments. These mice exhibit severe cardiovascular defects, demonstrating biological relevance and some overlapping functions for these two alternatively spliced segments.

Alternative splicing of EIIIA plus FNs, *in vivo*, provides a means of creating both structural and functional diversity in the FN molecule. Blocking mAbs to EIIIA inhibited the condensation events that occur during chondrogenesis in chick embryos (50). Moreover, treatment with the EIIIA segment alone facilitated cartilage catabolism and markedly induced expression of matrix metalloproteinase in chondrocytes and synovial cells in an interleukin-1-dependent manner (32). Importantly, matrix metalloproteinase-1 production is not as pronounced when EIIIA is flanked by an adjacent FN-III segment (FN-III 11 or 12; see Ref. 32). Our data demonstrating differential adhesive activ-

ity (Fig. 7) together with these data are consistent with a model in which proteolysis of FN during the arthritic process releases EIIIA activity (4).

However, in other settings in which the EIIIA segment is prominently expressed, mutant mice lacking this segment appear quite normal. For example, the absence of either the EIIIA or the EIIIB segment does not significantly affect either the generation of myofibroblasts in tumor stroma, tumor angiogenesis, or tumor growth in Rip1-Tag2 transgenic mice (36). EIIIA-null mice are viable, fertile, and phenotypically similar to wild-type mice (34, 35). Although small differences in atherosclerosis, wound healing, and life span are evident, these animals appear normal. Moreover, the role of EIIIA in myofibroblast generation has also been controversial. EIIIA-containing polymerized FN has been reported necessary for the induction of the myofibroblastic phenotype, *in vitro* (30, 31, 59). Interestingly, recombinant EIIIA but not longer rFNs that include EIIIA were incorporated into the fibroblast ECM (59). Given the apparent "cryptic" adhesive activity of EIIIA-containing FNs shown here, we speculate that some of the discordant data reported may be clarified by understanding the mechanisms regulating the presentation of EIIIA to cells.

The type III repeat structure is highly conserved despite relatively low levels of sequence identity among FN-III repeats and yet shows plasticity in conformation under mechanical stress (6, 13, 14). The overall β sandwich structure of FN-III is highly conserved, whereas the sequences of the loops between the β-strands are quite distinct and mediate functions (*e.g.* integrin binding) (11). Indeed, we find that that C-C′ loop contains sufficient sequence information in the peptide to mediate binding. It is important to note that both linear and cyclic EIIIA peptides were effective as soluble antagonists, apparently suggesting that a preformed loop conformation optimal for binding was not important (Figs. 4*B* and 5). Moreover, the binding of the peptides is specific to integrin $\alpha9\beta1$, as shown by the integrin activation assay (Fig. 5*B*). However, we also find using deletion mutants within EIIIA, a chimeric EIIIA (Fig. 2*A*), and long rFNs that the conformation of the EIIIA segment is influenced by neighboring repeats (Fig. 7). Furthermore, mutation G42A predicted to only have a local conformational effect has a significant impact on cell adhesion (Fig. 3*B*, *ii*) (60). Our working hypothesis is currently focused on a "selected fit" mechanism in which a subset of the peptide is in the proper conformation and is bound by integrin $\alpha9\beta1$. Once the optimal conformation for binding is selected from the equilibrium ensemble, the equilibrium shifts toward generating more peptide in the optimal conformation for integrin binding (61). Although the cyclic peptide is more conformationally constrained than the linear peptide, the data suggest that neither peptide is enriched in the optimal conformation (Fig. 5*A*).

Our data using longer rFNs (Fig. 7) also suggest that the conformation of FN-IIIs generally, and EIIIA specifically, is quite labile. Crystallographic data demonstrated that adjacent FN-IIIs are often related in a 2-fold axis of symmetry (~180°). Insertion of an alternatively spliced segment, such as EIIIA, has been postulated to alter the presentation of the RGD to cells (33). Conversely, we find that the $\alpha9\beta1$-dependent adhesive activity of EIIIA is suppressed in rFNs lacking the RGD (Fig. 7). We also find that a recombinant 11-EIIIA-12 shows $\alpha9\beta1$-dependent adhesive activity comparable with that of EIIIA alone.[6] These data suggest that the adhesive suppressive effect of neighboring FN-IIIs on EIIIA must require more than FN-IIIs 11 and 12.

Our data also demonstrate that the EIIIA segment exerts a biological effect on cells, namely the formation of extensive filopodia by SW-$\alpha9$ cells, but not by mock-transfected cells, on surfaces coated with pFN and EIIIA, but not III4 (Fig. 8; data not shown). The filopodia formation is accompanied by GTPase Cdc42 activation. Interestingly, the treatment of FN matrix with III1-C, a fragment from the C-terminal two-thirds of first type III repeat of FN, was also shown to inhibit Rho functions and activate Cdc42, resulting in formation of filopodia in human fibroblastic and umbilical vein endothelial cells (62). We find that SW-$\alpha9$ cells also extend filopodia when cultured on surfaces co-coated with pFN and longer rFNs that include the EIIIA segment (see map in Fig. 6) but not on pFNs and rFNs without EIIIA. Thus, although EIIIA adhesive activity is silenced by flanking FNIIIs, filopodial activity apparently is not.[6]

Several FN-III domains of FN have been elucidated that contain sites that become active only after exposure through partial unfolding (63, 64). Such sites, when exposed during repair or remodeling, offer an additional level of control of function by providing hitherto suppressed instructional cues that are expressed when required (5). Interestingly, NMR studies performed on human EIIIA demonstrated that the EIIIA segment when alone is mostly unfolded at 37 °C but exists in dynamic equilibrium between the folded and the unfolded states at 20 °C (49). Although the conformation of the EIIIA segment within FN is unknown, we speculate that EIIIA could be transformed to the unfolded state as a result of mechanical stress or proteolysis. Both processes occur during, and are critical to, successful tissue repair. The detailed characterization of the structure of the EIIIA segment and the generation of synthetic peptides reported herein provide a new basis for studying EIIIA function *in vivo* and may lead to new therapeutic strategies for various pathological diseases in which there is excessive production of EIIIA.

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