The EIIIA Segment of Fibronectin Is a Ligand for Integrins $\alpha_9\beta_1$ and $\alpha_4\beta_1$ Providing a Novel Mechanism for Regulating Cell Adhesion by Alternative Splicing*

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Alternative splicing of the fibronectin gene transcript gives rise to forms that include the EIIIA (or ED-A) segment. EIIIA-containing fibronectins are prominently expressed during embryogenesis and wound healing and appear to mediate changes in cell adhesion and gene expression. Nonetheless, integrins that bind the EIIIA segment have not been identified. We previously mapped the epitope for two function-blocking monoclonal antibodies to the C-C' loop region of the EIIIA segment (Liao, Y.-F., Wiede, K. G., Classen, J. M., and Van De Water, L. (1999) J. Biol. Chem. 274, 17876–17884). The sequence of this epitope (PEDGHHELFFK) resembles the sequence within tenascin-C to which the integrin $\alpha_9\beta_1$ binds. We now report that either integrin $\alpha_9\beta_1$ or $\alpha_4\beta_1$ can mediate cell adhesion to the EIIIA segment. Moreover, this interaction is blocked both by epitope-mapped EIIIA antibodies as well as by the respective anti-integrins. Deletion mutants of the EIIIA segment that include the C-C' loop and flanking sequence bind cells expressing either $\alpha_9\beta_1$ or $\alpha_4\beta_1$. Adhesion of $\alpha_4\beta_1$-containing MOLT-3 cells to the EIIIA segment stimulates phosphorylation of p44/42 MAP kinase. Our observation that two integrins bind the EIIIA segment establishes a novel mechanism by which cell adhesion to fibronectin is regulated by alternative splicing.

Although it has been clear for many years that fibronectin (FN) is alternatively spliced, the functions of, and receptors for, two alternatively spliced segments termed EIIIA (or ED-A) and EIIIB (or ED-B) segments have remained elusive. More is known about a non-homologous IIICS repeat encoding the CS-1 and EIIIB (or ED-B) segments than about the CS-2 (or ED-A) segment. FN type III repeats and are prominently expressed during embryogenesis; homozygous mutations in FN are embryonic lethal (2–7). During wound healing (5, 8), lung, liver, and kidney fibrosis (9–11), vascular intimal proliferation (12, 13), vascular hypertension (14), and cardiac transplantation (15), the expression of FNs containing the EIIIA and EIIIB domains is significantly increased. A ~170-kDa species of EIIIA-containing FNs is found in synovial fluid from patients with rheumatoid arthritis but not osteoarthritis (16). The EIIIB segment has been postulated to have a role in angiogenesis (17). The EIIIA segment has been observed to regulate cell adhesion and proliferation (18–21). Liver lipocytes and skin fibroblasts differentiate into myofibroblasts when adhering to FNs that include the EIIIA segment (10, 22). One monocalonal antibody (IST-9) to the EIIIA segment has been shown to inhibit myofibroblast differentiation, whereas another (DH1) blocks chondrogenesis during chick development (10, 22, 23). Moreover, the expression of MMP-9 is regulated by the EIIIA segment in chondrocytes and myelomonocytic cells potentially through toll-like receptors (24, 25).

We recently reported detailed epitope maps for function-blocking monoclonal antibodies that bind to the C-C' loop of the EIIIA segment (26). The FN type III (FN-III) repeats, of which the EIIIA segment is one, exhibit high structural homology (27–31) despite only 20–40% identity in amino acid sequence (32). The canonical FN type III repeat is a conserved $\beta$-sandwich conformation consisting of two $\beta$ sheets comprising four strands (G, F, C', C) and three strands (A, B, and E) (27). Epitope mapping of the EIIIA segment reveals that function-blocking mAbs interact with the loop between the C and C'-strands and the adjacent Ile43 and His44 residues are critical to the epitope (26). Given that these monoclonal antibodies blocked EIIIA function we reasoned that the peptide comprising the C-C' loop region (EDGHIHEL) could encode a sequence that bound cell surface receptors, possibly integrins.

The integrins are a family of heterodimeric transmembrane receptors that mediate cell-extracellular matrix and cell-cell interactions (33). One integrin, $\alpha_9\beta_1$, binds to a peptide sequence within the B-C loop of tenascin-C (34). This sequence (AEIDGIEL) is similar to the EDGHIHEL sequence that we identified in the EIIIA segment (26). The $\alpha_9$ subunit binds unrelated sequences in other ligands including the vascular cell adhesion molecule-1 (VCAM-1) (35), osteopontin (36), the propolyptide of von Willebrand factor (pp-vWF) (37), tissue transglutaminase (tTG) (37), blood coagulation factor XIII (FXIII) (37), and L1-CAM (38). These ligands, with the exception of tenascin-C, also bind to integrin $\alpha_4\beta_1$, the closest relative of $\alpha_9\beta_1$ with which it shares 39% amino acid identity (37, 38).
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MATERIALS AND METHODS

Reagents—The anti-EIIIA mAbs, IST-9 (43) and 3E2, were purchased from Harlan Bioproducts (Indianapolis, IN) and Sigma, respectively. Anti-human integrin αβ1 (clone Y9A2) was prepared as previously described (44). Mouse mAbs against human integrins α1 (clone P4C2) and α2 (clone P1D6) were purchased from Invitrogen. Another mouse mAb against human integrin α1 (clone HP2/1) was purchased from Beckman Coulter, Inc. (Fullerton, CA). FITC-conjugated goat anti-mouse IgG and mouse IgG were purchased from Zymed Laboratories Inc. Mouse anti-β1, integrin mAb (clone P4C10) was a gift from Dr. Donald Senger (Beth Israel Deaconess Medical Center). Histidine-tagged recombinant FN III repeats were prepared and purified as previously described (45). Mouse thymus and AEBSF (4-(aminomethyl)benzenesulfonyl fluoride) hydrochloride were from Calbiochem. BCA protein assay reagent kit and Reacti-Bind maleic anhydride-activated polystyrene 96-well plates were purchased from Pierce. Tissue culture media and fetal bovine serum were purchased from Invitrogen. Neomycin analog, G418, glutathione-agarose, and serum replacement medium SITE-3 were from Sigma. Complete protease inhibitor mixture was from Roche Molecular Biochemicals. Rabbit anti-phospho-p44/p42 MAP kinase antibody, anti-p44/p42 MAP kinase antibody, and horseradish peroxidase-conjugated anti-rabbit IgG were from Cell Signaling Technology, Inc. (Beverly, MA). All other reagents were at least reagent grade and obtained from standard suppliers.

Cell Culture—Integrin α1 or mock-transfected SW480 human colon cancer cells (SW-α1 or SW-mock) were generated as described (46) and maintained in DMEM supplemented with 1 ng/ml G418, 10% fetal bovine serum, and 0.1 mg/ml penicillin and streptomycin. MOLT-3 human acute lymphoblastic leukemia cells were purchased from American Type Culture Collection and cultured in RPMI 1640 containing 10% fetal bovine serum. Cells were incubated in a humidified incubator at 37 °C in 10% CO2.

Adhesion Assays—Cell adhesion assays were performed as previously described (47). Soluble recombinant FN III repeats (10 μg/ml in PBS) were coated on the wells of 96-well flat-bottomed microtiter plates (Corning-Costar) at 4 °C overnight. Wells were washed with PBS and blocked by 1% BSA in DMEM (for SW480) or RPMI 1640 (for MOLT-3) at 37 °C for 1 h. SW480 cells were detached using EDTA (20 mM in PBS), washed, and resuspended in serum-free DMEM. MOLT-3 cells were detached by centrifugation and resuspended in serum-free RPMI 1640 containing 250 μM MnCl2. For blocking experiments, cells were preincubated either with Y9A2 (10 μg/ml) or P4C2 (10 μg/ml) at 4 °C for 15 min or with various concentrations of synthetic peptides for 30 min. Labeled/mouse mAb (HP2/1, 2 μg/ml) and histidine-tagged EIIIA segment (100 μg/ml) were coated onto 6-well plates (1 ml/well) at 4 °C overnight. On the day of the experiment, MOLT-3 cells were washed with RPMI 1640 and resuspended in RPMI 1640,0.5% BSA at 2 x 106 cells/ml. This was followed by pretreatment with 250 μM MnCl2 at 4 °C for 30 min prior to plating. Protein-coated wells were blocked with 1% BSA/PBS at 37 °C for 1 h and then coated with PBS. Molt-3 cells were layered onto the protein-coated wells, allowed to settle at 4 °C for 30 min, and then brought to 37 °C for the time specified before being placed on ice. After the reactions were terminated, the medium was aspirated and nonadherent cells were removed by brief centrifugation (plate inverted) at 45 x g. Adherent cells were lysed in ice-cold cell extraction buffer containing 50 mM Tris-HCl (pH 5.0), 1% Triton X-100, 150 mM NaCl, 5 mM dithiothreitol, and 0.5% NP-40. Aliquots of cell lysates were then processed with the Molecular Image System GS-525 using Multi-Analyst software version 1.1 (Bio-Rad).

RESULTS

Integrin αβ1 Mediates Cell Adhesion to the EIIIA Segment of FN—Sequence comparisons revealed that the C-C′ loop region within the EIIIA segment resembled the ligand binding site for integrin αβ1 in the third FN-III repeat of tenasin-C. This finding prompted us to determine whether or not the EIIIA segment could be a novel ligand for integrin αβ1. We conducted cell adhesion assays with SW480 cells that had been stably transfected with either an αβ1-expression plasmid (SW-αβ1) or empty vector (SW-mock) (46). Wells were coated with recombinant fusion proteins representing either the EIIIA segment alone or the fourth type III repeat, FN-III4, alone. Cells were then allowed to adhere to coated wells either in the presence or absence of Mn2+, and the specificity of αβ1-medi-
Fig. 1. Adhesion assays using either α9- (SW-α9) or mock-transfected (SW-mock) SW480 cells. A, integrin αβ1 specifically adheres to the EIIIA segment. Recombinant histidine-tagged EIIIA and FN-III4 (10 μg/ml in PBS) were coated onto 96-well microtiter plates overnight at 4°C. Transfected SW480 cells were preincubated with or without anti-α9 blocking antibody Y9A2 (10 μg/ml) for 30 min at 4°C before plating. Solid bar, the adhesion to EIIIA-coated wells; shaded bar, the adhesion to FN-III4 coated wells; +, pretreatment with Y9A2; −, no treatment. Inset, an independent experiment using blocking antibodies to integrins α9 (Y9A2, 10 μg/ml), αv (P1D6, 10 μg/ml), or αvβ3 (P1F6, 10 μg/ml) shows the adhesion of SW-α9 to EIIIA does not involve other β1-associated integrins or non-β1 integrins. Mouse IgG (mIgG, 10 μg/ml) was used as a control. B, Mn2+ is not required for the adhesion of SW-α9 cells to the EIIIA segment. Recombinant histidine-tagged EIIIA and FN-III4 were coated onto 96-well microtiter plates overnight at 4°C. Cells were pretreated with or without Mn2+ (250 μM) at 4°C for 30 min before plating. Inset, a separate experiment indicating that the presence of Mn2+ does not promote the adhesion of SW-α9 cells to FN-III4 (shaded bar). +, pretreatment with Mn2+; −, no treatment. C, SW-α9 cells adhere to the EIIIA segment in a dose-dependent manner. Various concentrations of recombinant histidine-tagged EIIIA and FN-III4 were coated onto 96-well microtiter plates overnight at 4°C. SW480 cells were preincubated with or without Y9A2 (10 μg/ml) for 30 min at 4°C before plating. Key at right shows different permutations of coated proteins, cell lines, and treatments. For all these experiments, cells were allowed to attach to protein-coated wells at 37°C for 1 h, and nonadherent cells were removed by centrifugation as described under "Materials and Methods." Adherent cells were stained with crystal violet and quantified by measurement of absorbance at 570 nm. Results from a representative experiment are expressed as the mean (± S.D.) of triplicate measurements.

Integrin αβ1 Mediates Adhesion of MOLT-3 Cells to EIIIA Segment—A number of ligands for αβ1, including osteopontin, vascular cell adhesion molecule-1 (VCAM-1), the propolypeptide of von Willebrand factor (pp-vWF), tissue transglutaminase (tTG), and blood coagulation factor XIII (FXIII), have also been observed to bind the closely related integrin αβ1. Because the EIIIA segment bound αβ1-transfected cells, we sought to determine whether or not αβ1 also served as a receptor for the EIIIA segment. MOLT-3 cells were used because of their significant expression of αβ1 and their lack of αβ1 (37). Mn2+-pretreated MOLT-3 cells significantly adhered to the EIIIA segment but only minimally to FN-III4. MOLT-3 cells without the pretreatment of Mn2+ did not adhere to either FN segment (Fig. 2A). The adhesion of Mn2+-treated MOLT-3 cells to the EIIIA segment was blocked by an anti-αβ1 mAb, P4C2 (Fig. 2A). Complete inhibition of MOLT-3 cells adhesion to the EIIIA segment was observed with either anti-α4 or anti-β1 blocking antibodies or with CS-1 peptide, a specific ligand for integrin α4 (Fig. 2B), indicating that this adhesion was specifically mediated by integrin αβ1 rather than other β1 integrins. Function-blocking Anti-EIIIA Antibodies Block αβ1- and αβ1-mediated Binding—Several mAbs to EIIIA have been shown to block the differentiation of fibroblasts into myofibro-
blasts as well as the process of chondrogenesis (see Introduction). The epitopes for these EIIIA-specific mAbs reside in the C-C' loop and flanking sequences. Whereas Mn²⁺ is not required for α₄β₁-mediated binding to EIIIA, it is required for α₄β₁-EIIIA interactions. These data identify a novel ligand for both receptors and a new adhesive site within FN that is alternatively spliced. That this alternative splicing is functionally important is indicated by a

We have identified two integrins, αβ₁, and αβ₁, that bind to the EIIIA segment of FN. Both integrin-EIIIA interactions are blocked by EIIIA-specific mAbs and by their respective anti-integrin mAbs. For both αβ₁ and αβ₁, the ligand binding sites within EIIIA likely include the C-C' loop and flanking sequences. Whereas Mn²⁺ is not required for αβ₁ interaction with EIIIA, it is required for αβ₁-EIIIA interactions. These data identify a novel ligand for both receptors and a new adhesive site within FN that is alternatively spliced. That this alternative splicing is functionally important is indicated by a...
Our understanding of EIIIA function has long been hampered by the lack of information on specific cell receptors. The current studies provide the first direct evidence of EIIIA-specific integrins. FN in its most prevalent form, plasma FN which lacks EIIIA, has been recognized as a key ligand for a number of integrins, and its function in mediating cell-ECM (extracellular matrix) interactions has been well established (2). Among these integrins, αβ₁ binds to the alternatively spliced CS-1 and CS-5 in the IIICS segment (49, 50), as well as the constitutively present sites H₁ in FN-III₁₄ (49) and H₂ in FN-III₅ (51). Whereas the IIICS segment within plasma FN is a ligand for αβ₁, no segment within plasma FN is a ligand for αβ₄

**Fig. 3.** EIIIA-specific mAbs block α₉α₄ and α₄α₉-mediated adhesion to EIIIA. Recombinant EIIIA segment was coated on 96-well microtiter plates at 4 °C overnight. EIIIA-specific mAbs (IST-9 and 3E2) at various dilutions (1:100, 1:1000, and 1:10,000) were preincubated with EIIIA-coated wells at room temperature for 30 min. Unbound antibodies were removed prior to the addition of α₉-transfected SW480 cells (A) or MOLT-3 cells (B). Cells were incubated at 37 °C for 60 min (SW480) or 90 min (MOLT-3) followed by removal of nonadherent cells by centrifugation. The adhesion of SW-α₉ and mock-transfected SW480 cells (SW-mock) to EIIIA was also determined in the presence (open bar, A) or absence (dotted bar, A) of anti-α₉, mAb Y9A2 (10 μg/ml). MOLT-3 cells were pretreated with Mn²⁺ for 30 min at 4 °C prior to plating. Adherent cells were stained with crystal violet and quantified by the measurement of absorbance at 570 nm. Data are shown as the mean (± S.D.) of triplicate measurements from a representative experiment. Experimental conditions are indicated at the right of each panel.

**Fig. 4.** Schematic representation of rat EIIIA deletion mutants. Map of various deletion mutants (arrows) derived from wild type rat EIIIA (rEIIIAwt, 90 amino acids). Deletion constructs were generated by PCR and subcloned into the pGEX-2T vector as described previously (26). Arrows indicate the length of individual deletion constructs relative to the wild type sequence that is shown at the top of the figure. Solid boxes in the wild type sequence represent the conserved β-strands denoted A, B, C, C’, E, F, and G. The amino acids included in truncated mutant rat EIIIA proteins are numbered.
Thus, the process of splicing the EIIIA segment into new FN transcripts would generate a novel adhesive motif for α6β1 and an additional site for α4β1. Expression patterns for EIIIA-containing FNs suggest that the interactions of these integrins with EIIIA-containing FNs may subserve different functions. Recent data suggesting a role for EIIIA in cell differentiation, ligation of α6β1 or α4β1 could signal key changes in cell phenotype without altering cell adhesion.

Expression patterns for α6β1 and α4β1 as well as EIIIA-containing FNs suggest that the integrations of these integrins with EIIIA-containing FNs may subserve different functions. Integrin α6β1 is expressed in adult squamous epithelium, airway epithelium, visceral smooth muscle, skeletal muscle, hepatocytes, and neutrophils (35, 39). During embryogenesis α6β1 is expressed in developing airway, visceral, and vascular smooth muscle at a time closely associated with the appearance of α-smooth muscle cell actin (52). Following vascular injury α6β1 expression is increased in forming neointima.2 Several of the ligands that bind α6β1, including EIIIA-containing FNs, tenascin, and osteopontin, are also expressed in the neointima (12, 13, 53, 54). Integrin α4β1 is expressed by a narrow spectrum of tissues found predominately in the leukocyte lineage (55). It has been known for many years that most normal adult tissues contain FNs that are largely missing the EIIIA segment (56). However, following injury the expression of EIIIA-FNs is strikingly up-regulated (see Introduction). Recent findings in adult mice depleted of plasma FN demonstrate that plasma FN is not required for normal skin wound healing (57). Importantly, these data suggest a role for locally expressed EIIIA- or EIIIB-variants of FN in healing wounds. Coupling regulated temporal and spatial expression of EIIIA+FNs with ligation by integrin α6β1 or α4β1 could provide a powerful combinatorial approach to generating a regulated response to tissue injury.

The interaction of α6β1 with EIIIA is observed in Mn2+-activated MOLT-3 cells, and α4β1 is the only major integrin on MOLT-3 cells. Our results demonstrating Mn2+ dependence are in concordance with a previous report that α6β1-mediated adhesion of leukocytes requires activation of β1 integrins by divalent cations, stimulatory antibodies, or both (58). It has been shown that 250 μM Mn2+ is sufficient to activate α6β1-mediated adhesion of MOLT-3 (37). Mn2+ is believed to be a physiological activator of β1 integrins (45) distinct from other β1 activators such as phorbol-12-myristate-13-acetate (PMA) and mAb TS2/16. Although the estimated concentration of Mn2+ in tissue is 1–14 μM and can be as high as 50 μM in bone or 30 μM in liver (59, 60), higher concentrations of Mn2+ (1 mM) have also been used to activate α6β1 (58). The possibility also exists that various concentrations of Mn2+ are required for

2 D. Sheppard, unpublished data.
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suggest that the C-C' loop of the EIIIA segment is important to αβ1 and αδβ1 integrin binding, it is possible that this region supports adhesion by sustaining an optimal conformation of another ligand binding site.

The appearance of greatly increased levels of EIIIA-containing FN following tissue injury suggests a functional role for the EIIIA segment in wound healing (5). The role of EIIIA in healing wounds and other pathological settings has remained enigmatic for many years. A recent study suggests that either IST-9 or soluble recombinant EIIIA segment can inhibit the TGF-β-induced expression of smooth muscle cell α-actin (α-SMA) in fibroblasts (22). We and others find that αβ1 is a prominent integrin in primary cultured fibroblasts, consistent with the idea that αβ1 could play a functional role during wound healing (61).3 Therefore it is of interest that we observe (Fig. 6) that the interaction of EIIIA segment with αβ1 in MOLT-3 cells increases phosphorylation of p44/42 MAP kinase. Whereas αδβ1 is not present on the fibroblasts that we tested, it is present on neutrophils and keratinocytes. Our current results that αδβ1 and αβ1 serve as cell surface receptors for the EIIIA segment suggest novel mechanisms for the regulation of EIIIA-containing FN function during wound healing.

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3 Y. Liao, unpublished data.
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