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A Synthetic Peptide, FN-C/H-V, from the C-Terminal Heparin-Binding Domain of Fibronectin Promotes Adhesion of PMA Stimulated U937 Cells

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Hematopoietic cells differentially bind to the C-terminal heparin-binding domain of fibronectin depending on the activation state of integrin $\alpha_4\beta_1$. In this study, we have identified a synthetic peptide derived from the C-terminal heparin-binding domain of fibronectin that promotes adhesion of PMA-treated U937 cells (a monocytic cell line) in a dose-dependent manner. A peptide (FN-C/H-V; residues Gln[1892] to Gly[1910]) was active to inhibit adhesion of PMA-treated U937 cells to the 29-kDa fragment comprising the C-terminal heparin-binding domain of fibronectin. A peptide with scrambled version of FN-C/H-V lost the inhibitory activity on the adhesion. Furthermore, the IgG-conjugated FN-C/H-V promoted the adhesion of PMA-treated U937 cells to an extent comparable to that of the 29-kDa fragment. The adhesion of PMA-treated U937 cells on IgG-conjugated FN-C/H-V was inhibited both by anti-$\alpha_4\beta_1$ antibody and by glycosaminoglycans including chondroitin sulfate and heparan sulfate. The other peptide, FN-C/H-II, was also a weak adhesion-promoting domain. These results suggest that the amino acid sequence defined by peptide FN-C/H-V contributes to the main adhesion-promoting activity of the 29-kDa fragment of fibronectin to stimulated U937 cells. The regulation of interactions of $\alpha_4\beta_1$ integrin and glycosaminoglycans with ligands in fibronectin may have important implications for the migration and function of U937 cells.

Fibronectin is a large dimeric glycoprotein composed of similar, but not identical polypeptide subunits, termed the A and B chains, that are linked disulfide bonds.[1] It has a capacity to interact with cell surface integrin family[2-6] and with the cell surface heparan sulfate proteoglycans.[6-10] Several regions of the fibronectin molecule support cell adhesion, spreading and migration.[6,11] The Arg-Gly-Asp (RGD) sequence in the central cell-binding domain of fibronectin has been identified as an important cell recognition site[12-14], and its receptor has been shown to be the integrin $\alpha_5\beta_1$.[12,15] Second cell-adhesion promoting domains are located within the alternative spliced type III connecting segment (IIICS)[6], recognized by a limited number of cell types including neural crest cells, lymphocytes and monocytes. Two active sites for adhesion have been identified in the IIICS, represented by peptides CS1 (residues 1-25 of the IIICS) and CS5 (residues 90-109).[16] The minimal active sites within CS1 and CS5 have been narrowed down to the sequences of Leu-Asp-Val (LDV)[18,19] and Arg-Glu-Asp-Val (REDV)[16,17], respectively. These sites are recognized by the integrin $\alpha_4\beta_1$.[18,20,21,22] The RGDS- and LDV-containing peptides can also inhibit monocytic U937 cell adhesion to intact fibronectin.[23]

The C-terminal heparin-binding domain of fibronectin has also been implicated in these events by an RGD-independent mechanism.[7,24-28] Previous studies have identified a number of peptide sequences within this domain that promote the adhesion of various cell types. Two of these active peptides are FN-C/H-I (residues 1906-1924) and FN-C/H-II (residues 1946-1960),[24,29,30] which promote melanoma and neuroblastoma cell adhesion. Previous evidence suggests that melanoma cells may adhere to these sequences vis a cell surface proteoglycans.[31] Additional sequence was found in the residues from Tyr[1721] to Glu[1736] (FN-C/H-III) near the amino-terminal end of the C-terminal heparin-binding domain which was necessary for heparin-binding, melanoma cell adhesion and rabbit corneal epithelial cell adhesion.[32,33] This interaction was completely resistant

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Abbreviations: PMA, phorbol-12-myristate 113-acetate; IIICS, alternative spliced type III connecting segment; SPDP, *N*-succinimidyl-3(2-pyridyldithio) propionate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ECM, extracellular matrix.

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to anti-$\alpha_4\beta_1$ antibodies, but sensitive to specific inhibitors of proteoglycan function. However, these three sequences can only promote adhesion formation when presented as multicopy complexes. Further studies have shown that two other peptides of Ser[1784]-Pro-Pro-Arg-Arg-Ala-Arg-Val-Thr[1792] (SPPRRARVT; FN-C/H-IV) and Trp[1892]-Gln-Pro-Pro-Arg-Ala-Arg-Ile[1899] (WQPPRARI; FN-C/H-V) promote cell adhesion as free peptide.[34,35] These studies suggest the importance of the C-terminal heparin-binding domain of fibronectin in promoting the adhesion of normal and malignant cells, although inhibitory activity in this region is relatively weak.

The interaction of monocytes with extracellular matrix (ECM) proteins including fibronectin can also result in monocytic cell adhesion and spreading.[36] Monocytic U937 cells have shown to bind to fibronectin.[37,38] The contribution of the C-terminal heparin-binding domain of fibronectin to cell adhesion may vary among different cell types.[18,22] Hematopoietic cells may differentially bind to the C-terminal heparin-binding domain of fibronectin depending on the activation state of integrin $\alpha_4\beta_1$, a fact that may be relevant for the migration and function of leukocytes.[39] Indeed, U937 cells without activation was inactive to bind to the C-terminal heparin-binding domain of fibronectin.[23,40]

In this paper, we extended our previous study[23] to investigate the molecular mechanisms and regulation of interaction of U937 cells with the C-terminal heparin-binding domain of fibronectin. We provide the evidence that the one peptide sequence, FN-C/H-V, mainly contributes to the adhesion of PMA-treated U937 cells to the C-terminal heparin-binding domain of fibronectin.

MATERIALS AND METHODS

Human plasma fibronectin was purchased from Bachem Chemical Co. (Torrance, CA). Heparin (porcine intestinal mucosa), heparan sulfate (bovine intestinal mucosa) and chondroitin sulfate B (porcine intestinal mucosa) were purchased from Sigma (St. Louis, MO).

The 29-kDa fragment of fibronectin was generated and purified as previously described.[23,28,41] This fragment is derived from the A chain of fibronectin and located in the C-terminal heparin-binding domain with the first three amino acids of IIICS and without the RGDS or CS1 sequence.

Synthetic peptides. Peptides were synthesized on a peptide synthesizer at TANA Bio-Systems L.C. (Houston, TX). Lyophilized crude peptides were purified by reversed-phase HPLC on a C18 column, using a linear elution gradient of 0-60% acetonitrile with 0.1% trifluoroacetic acid in water. The compositions of the peptides were confirmed by quantitative amino acid analyses. Peptide solutions were then neutralized with acylizer (Asahi Chemical, Tokyo, Japan), lyophilized, and stored at 4°C before use. The sequences and selected properties of the synthetic peptides used in this study are shown in *Table 1.* Hydropathy indices were calculated using the method of Kyte and Doolittle.[42]

Cell culture. U937 monocytic cells were purchased from the Japanese Cancer Research Resources Bank (JCRB)-Cell (Tokyo, Japan).

Table 1. Synthetic Peptides from the C-terminal Heparin-Binding Domain of Fibronectin and effects of these peptides on PMA stimulated U937 cell adhesion to the 29-kDa fragment-coated substra

Peptide Nomenclature	*Primary Sequence* *	*Hydropathy ndex #*	*Net Charge +*	*IC_{50} (mg/ml)*
FN-C/H-I	YEKPGSPPREVVPRPRPGV (residue 1906-1924)	**-24.3**	**+ 2**	**> 3.0**
FN-C/H-II	KNNQKSEPLIGRKKT (residue 1946-1960)	**-29.3**	**+ 4**	**2.1 ± 0.5**
FN-C/H-III	YRVRVTPKEKTGPMKE (residue 1721-1736)	**-23.7**	**+ 3**	**> 3.0**
FN-C/H-IV	TLENVSPPRRARVTD (residue 1779-1793)	**-15.4**	**+ 1**	**> 3.0**
FN-C/H-V	QPPRARITGYIIKYEKPG (resiude 1892-1910)	**-17.4**	**+ 3**	**0.05± 0.01**
Scrambled FN-C/H-V	IPKRGPPIQITKRAYEGY			**> 3.0**

* The sequence was shown with one-letter abbreviations for individual amino acids.
\# Hydropathy index was calculated by the method described elsewhere (45).
\+ The sum of all charged residues where lysine (K) and arginine (R) residues are positively charged (+1) and glutamic acid (E) and aspartic acid (D) are negatively charged (-1) at neutral pH.

Cells were cultured in tissue culture dishes (Falcon, Oxnard, CA) containing RPMI-1640 (Flow Laboratories, McLean, VA) supplemented with 10 % fetal bovine serum (HyClone, Logan, UT), 10 mM Hepes, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C under a humidified 95 % air and 5 % CO_2 atmosphere. For induction of activation and differentiation, U937 cells (5×10^5/ml) were transferred to Teflon vials (Norton Performance Plastic Co., Akron, OH) and incubated with 10 ng/ml of PMA (Sigma Chemical Co., St. Louis, MO) in a 5 % CO_2 incubator at 37°C for 48 hours. Monocytic differentiation was confirmed by morphological changes and by increased expression of the CD11c antigens (*Figure 1*). Cell viability was examined by trypan blue exclusion. Cell viability always exceeded 98%.

Preparation of peptide-IgG conjugates. Covalent coupling of the peptides with an N-terminal cysteine residue to IgG via their N-terminal cysteine residues was accomplished by cross-linking with a heterobifunctional agent, N-succinimidyl-3 (2-pyridyldithio)propionate (SPDP), as described elsewhere.[16] The activated IgG was then added to the peptides at a peptide:IgG weight ratio of 0.3 - 0.45. After mixing overnight on a rotator at 22°C, free peptides were removed by dialysis against Dulbecco's phosphate-buffered saline.

Monoclonal antibodies. Monoclonal antibodies, P1D6, P4G9 and P1B5, with specificities for the α chains of $\alpha_5\beta_1$, $\alpha_4\beta_1$, and $\alpha_3\beta_1$ integrin, respectively, were purchased from Dako Corp. (Carpinteria, CA). The characterizations of these monoclonal antibodies have previously been described in detail.[18,43-47]

Cell adhesion assay. These assays were performed with some modifications.[23,40] In brief, flat-bottomed 96-well plates (Nunc, Copenhagen, Denmark) were coated with 100 μl of 29-kDa fragment at appropriate dilutions with 0.05 M sodium bicarbonate, pH 9.6, for 2 hours at 22°C. The plates were washed with PBS (phosphate-buffered saline) and incubated at 22°C with 1% BSA. After a rinse with PBS, 50 μl of a 1×10^6/ml cell suspension was added to each well. Adhesion was assessed after incubation for 90 min at 37°C. Nonadherent cells were removed by two gentle washes with RPMI 1640 and attached cells were fixed for 20 min with 2% glutaraldehyde, washed, and stained with 0.5% crystal violet. After 30 min at 22°C, the absorbance at 590 nm was determined using an automatic microplate reader (Model 3550-UV, Bio-Rad). For inhibition assays, cells were incubated with monoclonal antibodies or synthetic peptides at various concentrations for 1 hr at 22°C before being added to the coated wells.

Immunofluorescence analyses. After activation and differentiation by PMA, cells (1×10^6) were incubated for 30 min at 4°C with 100 μl of culture supernatant containing the appropriate monoclonal antibody. Cells were washed twice with cold PBS containing 1% BSA

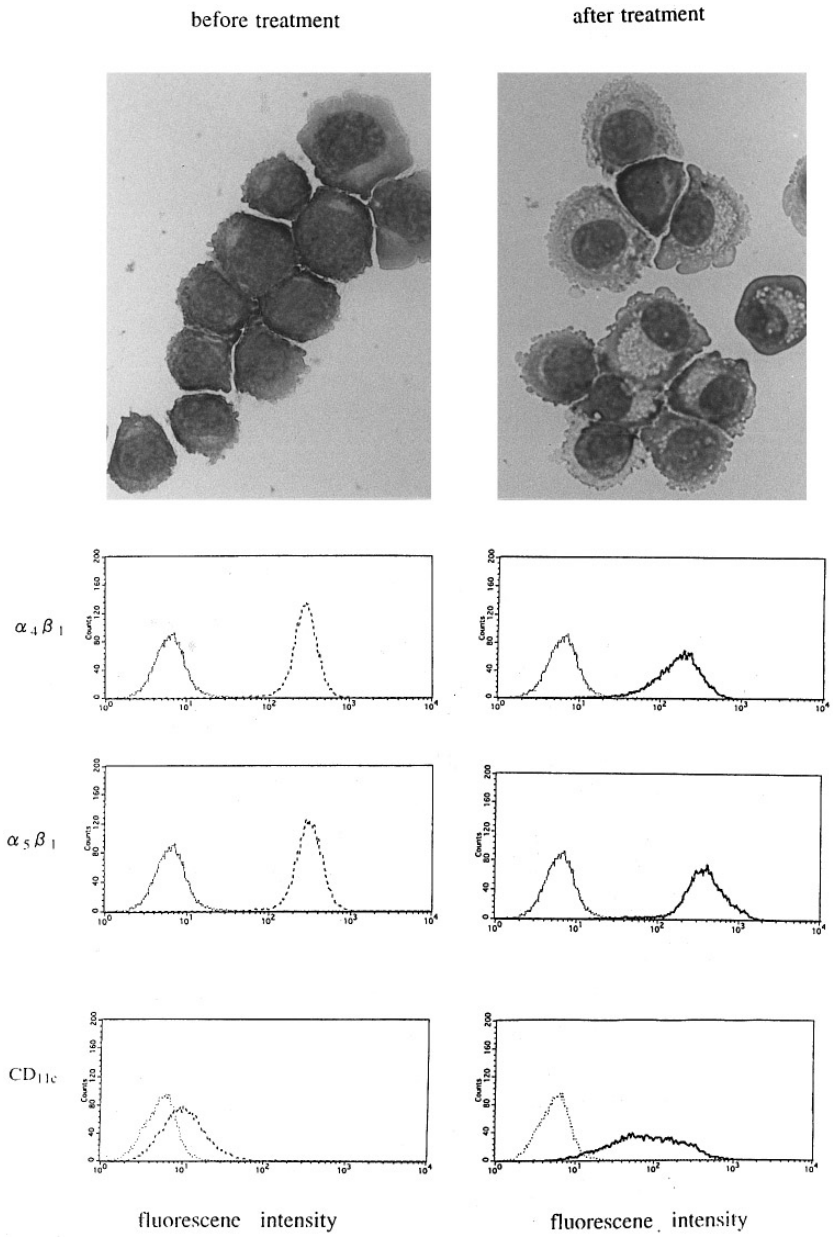

FIG. 1. Morphological changes and expression of integrins of U937 cells by PMA treatment. (*Upper panel*) U937 cells were untreated (A) or treated with 10 ng/ml of PMA (B) in a 5% CO_2 incubator at 37°C for 48 hours. (*Lower panel*) Expression of the integrins on resting and PMA-treated U937 cells. Expression of CD11c after PMA treatment was significantly increased, suggesting that U937 cells were activated and differentiated. Monocytic differentiation was confirmed by the morphological changes and by increased expression of the CD11c antigens.

and resuspended in 100 μl of a 1/10 dilution of fluorescein-conjugated $F(ab')_2$ fragments of goat antibody against mouse IgG (Dako, Copenhagen, Denmark). After 30 minutes at 4°C the cells were washed twice, resuspended in PBS, and analyzed by flow cytometry on an EPICS-ELITE (Coulter Cientifica, Mostoles, Spain). The mean fluorescence intensities of the positive cells corresponded to values on a linear scale.

Protein concentrations. Concentrations of purified IgGs were calculated from absorbance at 280 nm, assuming $E^{280}_{1\%} = 14.3$. Concentrations of other proteins were measured with a Bradford assay using BSA as a standard.[48] Concentrations of peptides were determined by the bicinchonic acid assay (Pierce Chemical, IL) with BSA as a standard.

RESULTS

Adhesion of PMA-Treated U937 Cells to the 29-kDa Fragment of Fibronectin

U937 cells without PMA treatment did not attach to the C-terminal heparin-binding domain of fibronectin-

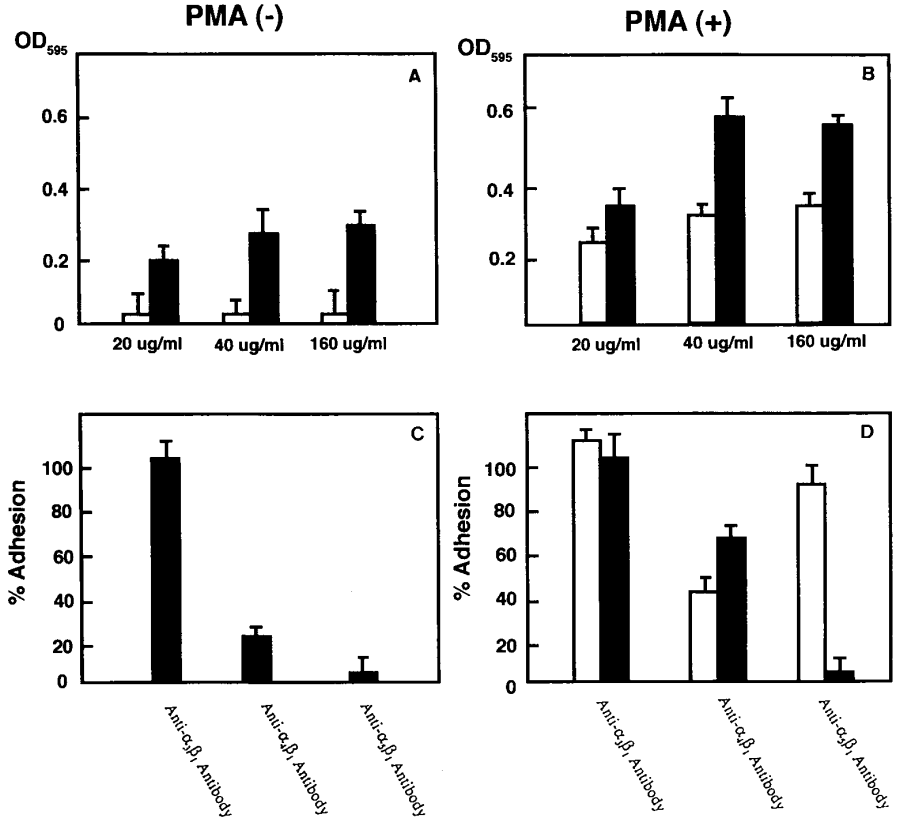

FIG. 2. (A, B) Adhesion of Resting and PMA-treated U937 cells on the 29-kDa fragment and fibronectin. Ninety-six-well plates were coated with the 29-kDa fragment (open bar) or native fibronectin (solid bar). The coated wells were incubated with U937 cells with or without PMA treatment. The 29-kDa fragment or fibronectin was coated at the concentrations indicated on the *abscissa.* After incubation, the wells were washed to remove unattached cells. Absorbance was read in an ELISA reader at 595 nm. Each value represents the mean ± SD from three separate determinations. (C, D) Inhibition of PMA-treated U937 cell adhesion to the 29-kDa fragment of fibronectin by anti-integrin monoclonal antibodies. PMA-untreated and -treated U937 cells were preincubated with the following monoclonal antibodies before being added to the 29-kDa fragment-coated wells: $\alpha_3\beta_1$ (P1B5), $\alpha_4\beta_1$ (P4C2), and $\alpha_5\beta_1$ (P5D1). Monoclonal antibodies were used at a concentration of 10 μg/ml. Results are expressed as the percentage of adherent cells in controls (wells without monoclonal antibodies).

coated plastic wells (*Figure 2A*) in agreement with previous reports.[23,39,40] After PMA treatment, the 29-kDa fragment (the C-terminal heparin-binding domain of fibronectin) promoted adhesion of U937 cells, but the maximal level of adhesion was less than that for native fibronectin (*Figure 2B*). Preincubation with excess soluble fibronectin inhibited cell adhesion to the 29-kDa fragment-coated substrates. More than 70% of U937 cells were unattached in the presence of 1 mg/ml fibronectin (*data not shown*).

Effects of Anti-integrin Monoclonal Antibodies on Adhesion of PMA-Treated U937 Cells to the 29-kDa Fragment-Coated Substrates

The adhesion of untreated U937 cells to native fibronectin-coated wells was inhibited by both the anti-$\alpha_4\beta_1$ and the anti-$\alpha_5\beta_1$ monoclonal antibodies. It should be noted that the inhibition of anti-$\alpha_4\beta_1$ antibody was less potent than that of anti-$\alpha_5\beta_1$ antibody *(Figure 2C).* Moreover, the adhesion of PMA-treated U937 cells onto fibronectin was also inhibited by the anti-$\alpha_5\beta_1$ monoclonal antibody and was not inhibited by the anti-$\alpha_4\beta_1$ monoclonal antibody (*Figure 3D*).

The adhesion of PMA-treated U937 cells to the 29-kDa fragment-coated wells showed approximately 60% inhibition by the anti-$\alpha_4\beta_1$ monoclonal antibody (P4C2) at a concentration of 10 μg/ml. The monoclonal antibodies against other integrin receptors ($\alpha_5\beta_1$, P1D6; $\alpha_3\beta_1$, P1B5) did not affect this adhesion (*Figure 3D*). These results suggest that the treatment of U937 cells by PMA should be accompanied by the conversion of $\alpha_4\beta_1$ receptor to an active form to interact with the C-terminal heparin-binding domain of fibronectin.

To establish whether PMA treatment affected the relative pattern of expression of $\alpha_4\beta_1$, untreated- and PMA-treated U937 cells were analyzed by immunofluorescence using anti-$\alpha_4\beta_1$ monoclonal antibody. As shown in *Figure 1,* treatment with PMA showed slight

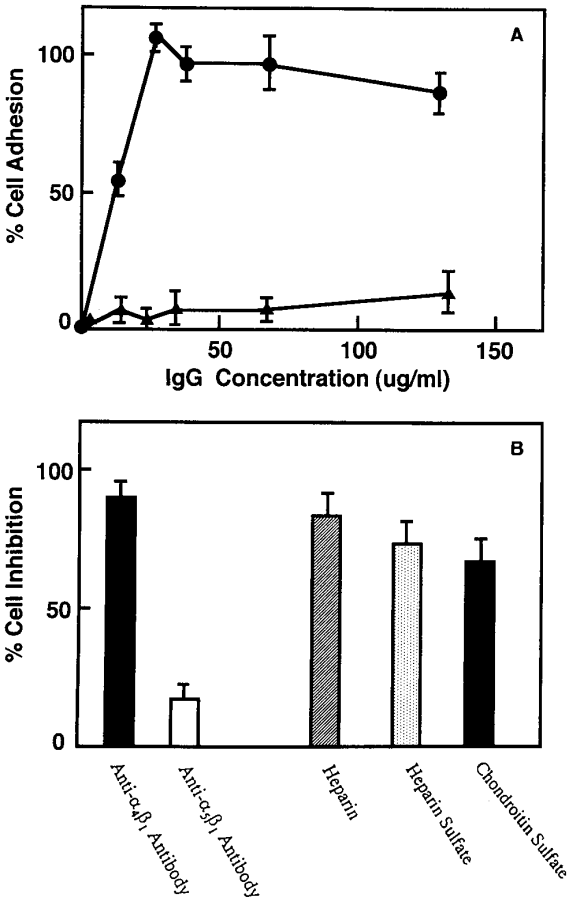

FIG. 3. (A) Adhesion of PMA-treated U937 cells on peptide-IgG. The abscissa shows the coating concentration of the conjugates. After incubation, the wells were washed to remove unattached cells. Absorbance was read in an ELISA reader at 595 nm. Each value represents the mean of three separate determinations, and the standard error of the mean was less than 5% in each case. Three separate experiments gave similar results. (B) Effects of anti-integrin antibodies and glycosaminoglycans on the adhesion of peptide FN-C/H-V-IgG to PMA treated U937 cells. Results are expressed as percentage inhibition, calculated as 100 minus the residual percent adhesion relative to a control. The coating concentration of peptide-IgG was 30 μg/ml. Anti-integrin monoclonal antibodies were used at a concentration of 10 μg/ml. Glycosaminoglycans were used at a concentration of 1 mg/ml. Each value represents the mean of three separate determinations, and the standard error of the mean was less than 5% in each case. Three separate experiments gave similar results.

down-regulation in cell surface expression of $\alpha_4\beta_1$ receptor in agreement with a previous report.[39]

Effects of Synthetic Peptides on the Adhesion of PMA-Treated U937 Cells to the 29-kDa Fragment-Coated Substrates

PMA-treated U937 cells bound to the 29-kDa fragment. To identify a crucial sequence in the C-terminal heparin-binding domain of fibronectin that participates in the recognition of activated U937 cells, five peptides (FN-C/H-I ~ V) were synthesized (*Table 1*). These peptides were based on the sequences described elsewhere and promoted cell adhesion.[24,29-33,35] In competitive binding assays, one peptide (FN-C/H-V) showed a strong inhibitory effect on the adhesion of PMA-treated U937 cells to the 29-kDa fragment-coated substrates with IC_{50}s of 0.05 ± 0.01 mg/ml. FN-C/H-II only partially blocked this adhesion. Other three peptides did not substantially block the adhesion of PMA-treated U937 cells to the 29-kDa fragment-coated substrates. To verify the specificity of FN-C/H-V, we then synthesized scrambled versions of this peptide, which was composed of Ile-Pro-Lys-Arg-Gly-Pro-Pro-Ile-Gln-Ile-Thr-Lys-Arg-Ala-Tyr-Glu-Gly-Tyr (IPKRGPPIQITKR-AYEGY, scrambled FN-C/H-V). Scramble FN-C/H-V did not inhibit the adhesion of PMA-treated U937 cells to the 29-kDa fragment-coated substrates up to 3.0 mg/ml (*Table 1*).

Direct Adhesion of Peptide-IgG Conjugate to PMA-Treated U937 Cells

Our results strongly indicate that FN-C/H-V is a crucial binding site to PMA-treated U937 cells. PMA-treated U937 cells adhered equally well to surfaces coated with IgG-conjugated FN-C/H-V while IgG-conjugated scrambled FN-C/H-V did not. Cell adhesion was concentration-dependent (*Figure 3A*). Furthermore, the adhesion of PMA-treated U937 cells onto IgG-conjugated FN-C/H-V was completely inhibited by anti-$\alpha_4\beta_1$ antibody at a concentration of 10 μg/ml and glycosaminoglycans including heparin, heparan sulfate and chondroitin sulfate at a concentration of 1 mg/ml. Anti-$\alpha_5\beta_1$ antibody did not inhibit this adhesion (*Figure 3B*). These inhibitory effects were dose-dependent (*data not shown*).

DISCUSSION

Although monocytic U937 cells have shown to bind to fibronectin,[37,38] hematopoietic cells including U937 cells may differentially bind to the C-terminal heparin-binding domain of fibronectin depending on the activation state of integrin $\alpha_4\beta_1$.[39] We have studied the adhesion of PMA-treated U937 cells to the 29-kDa fragment that in the C-terminal heparin binding domain of fibronectin.[28,41] There were three key findings: (1) U937 cells adhered to the 29-kDa fragment in an activation-dependent manner through the $\alpha_4\beta_1$ integrin and cell surface glycosaminoglycans; (2) a novel peptide sequence, FN-C/H-V, directly promoted PMA-treated U937 cell adhesion and bound to integrin $\alpha_4\beta_1$ and glycosaminoglycans; (3) a second peptide, FN-C/H-II, also promoted the weak PMA-treated U937 cell adhesion, suggesting that FN-C/H-II may be of low affinity.

Apparently, U937 cells do not bind to the 29-kDa fragment without activation.[23,39] It is now well established that the functional activity of integrins can be modulated by several agents including PMA.[49,50] Previous study has demonstrated that in hematopoietic cells the LDV sequence of the CS1 within IIICS has only been shown to be as an activation-dependent recognition site.[19] In this report, we demonstrated that PMA-treated U937 cells indeed bind to the C-terminal heparin-binding domain of fibronectin. This is the first report of a recognition site in the C-terminal heparin-binding domain of fibronectin to activated hematopoietic cells. However, the maximal extent of adhesion was less than that for native fibronectin, suggesting that interaction of U937 cells with the C-terminal heparin-binding domain may be of low affinity. Furthermore, this correlates with a slight down-regulation of surface expression of integrin $\alpha_4\beta_1$, consistent with a previous study from an independent laboratory.[39] The treatment of U937 cells by PMA may be accompanied by the conversion of $\alpha_4\beta_1$ to a fully active form in order to interact with the C-terminal heparin-binding domain of fibronectin. Indeed, U937 cell adhesion to the 29-kDa fragment was mediated by $\alpha_4\beta_1$ integrin, since it was inhibited by the monoclonal antibody to $\alpha_4\beta_1$ integrin (P4C2).

We identified one peptide derived from the C-terminal heparin-binding domain of fibronectin (FN-C/H-V) that promote PMA-treated U937 cell adhesion. The other peptide, FN-C/H-II, is a weak adhesion-promoting domain. These peptides are chemically synthesized peptide and promoted the adhesion of PMA-treated U937 cells in a concentration dependent manner. Adhesion of PMA-treated U937 cells to FN-C/H-V was peptide-specific because other peptides, also hydrophilic and cationic peptides derived from the 29-kDa fragment of fibronectin, did not promote PMA-treated U937 cell adhesion. Importantly, FN-C/H-I, FN-C/H-III and FN-C/H-IV do promote the adhesion of a variety of other cell types,[24,29,30,32--34,51] suggesting that cell adhesion to peptides from the C-terminal heparin-binding domain of fibronectin also cell-type specific and that the activity of these peptides is not simply the results of net charge or net hydropathy.[35] Furthermore, a scrambled version of FN-C/H-V did not promote this adhesion, also supporting that PMA-treated U937 cell adhesion to FN-C/H-V was specific.

Cell adhesion to the C-terminal heparin-binding domain of fibronectin has a complex molecular basis, likely involving multiple cell adhesion-promoting determinants that interact with both cell surface proteoglycans and $\alpha_4\beta_1$ integrin. Although much is known about the structure and function of fibronectin, the details of many of the interactions remain to be analyzed. The present results, therefore, present additional binding site(s) in the C-terminal heparin-binding domain of fibronectin to differentiated and activated U937 cells. The regulation of strong or weak interactions of $\alpha_4\beta_1$ and glycosaminoglycans with ligands in fibronectin may have important implications for the migration and function of hematopoietic cells. In addition, it is important to identify novel sites in fibronectin for the interaction of hematopoietic malignant cells in order to understand the pathophysiological mechanism of cell invasion in hematological malignant cells.

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