SPECIFIC BINDING OF THE HUMAN MONOCYTIC CELL LINE U937 TO THE ALTERNATIVELY SPliced CONNECTING SEGMENT (IIICS) OF FIBRONECTIN

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Mononuclear phagocyte function and differentiation can be modulated by specific interactions of plasma membrane receptors with components of the extracellular matrix, in particular, fibronectin (Fn) (1, 2). Some of these receptors recognize the RGDS (Arg-Gly-Asp-Ser) sequence of the cell-binding domain (3). These receptors belong to the integrin family and have been recently isolated and partially characterized (4, 5). Other regions of Fn, particularly within the Hep II domain and the type III connecting segment (IIICS), contribute to the attachment and spreading of various cells, and may be correlated with different stages of cell differentiation (reviewed in reference 6).

Interaction of monocytes and macrophages with domains of Fn that do not contain RGDS has not been thoroughly investigated because these cells firmly adhere to plastic and glass surfaces in the absence of specific ligands. We have used the monocytoïd cell line U937, which does not adhere effectively to plastic surfaces, but adheres to substrata coated with Fn or with RGDS-containing fragments of Fn (4), as a model to study domain specificity of mononuclear phagocytes towards Fn. The present report shows that in addition to this prototype interaction, U937 cells bind efficiently to a 38-kD heparin-binding fragment derived from the A chain of Fn. It also shows that this cell-binding site is located within the IIICS region of Fn. Binding to IIICS is specific and independent of the RGDS-containing Fn domain.

Materials and Methods

Antibodies. mAbs N-288, N-294, and N-296 were purchased from Mallinckrodt (Maryland Heights, MO), and rabbit IgG anti-Fn was from CooperBiomedical, Inc. (Malvern, PA).

Synthetic Peptides. The peptides GRGDSPC and GRGESP, corresponding to sequences of the central cell-binding domain of Fn, were purchased from Peninsula Laboratories, Inc. (Belmont, CA) and from Peptides International, Inc. (Louisville, KY). Three overlapping peptides, CS-1, CS-2, and CS-3 (Fig. 1), were synthesized at the Microchemistry Laboratory of the New York Blood Center by Dr. Robert Fields, using a peptide synthesizer (9600; Bio-
search Inc., San Rafael, CA), and spanned the 67 amino acids of the IIICS region of the 38-kD domain (7, 8).

**Purification of Tryptic Fragments from Fn.** Human plasma Fn was the generous gift of Drs. B. Horowitz and R. Shulman (New York Blood Center, NY). Fragments of 80, 58, and 38 kD (Fig. 1) were prepared by trypsin digestion (1:200 [wt/wt], 90 min, 37°C) as described (4, 7). The 31- and 29-kD fragments were isolated by mild trypsin digestion (1:1,000 [wt/wt], 15 min, room temperature) and purified as described (9). The 58-kD fragment was isolated by affinity chromatography on heparin-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) and further purified on a DEAE-Sephacel column (Pharmacia Fine Chemicals) equilibrated with 10 mM Tris, 2 M urea, pH 7.0. The fragment was eluted by addition of 0.08 M NaCl to the starting buffer. Fn and fragments were dialyzed against PBS and stored at -70°C until use. The purified fragments migrated as a single band on SDS-PAGE (not shown).

**Cell Culture.** The human monocytoid cell line U937, obtained from the American Type Culture Collection (Rockville, MD), was maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (Irvine Scientific, Santa Ana, CA). After 3–4 d of incubation at 37°C in a 6% CO2 humidified atmosphere, cells were washed three times with RPMI and resuspended in RPMI with 1% BSA and 10 mM Hepes (attachment medium).

**Cell Attachment Assays.** Assays were performed on 96-well Linbro plates (Flow Laboratories, McLean, VA) with flat bottoms. Wells were coated with 100 µl of a dilution of Fn or of its fragments for 2 h at room temperature, as described (4). In some experiments, the amounts of protein bound to the plate were determined using 3H-labeled fragments. After coating, the plates were rinsed with PBS, bound proteins were solubilized with 0.5 N NaOH containing 1% SDS, mixed with scintillation fluid, and counted. For inhibition experiments, 5 x 10⁵ cells in 0.4 ml of attachment buffer were incubated with soluble Fn, Fn fragments, or synthetic peptides for 30 min at room temperature in a rotary mixer. The suspension was diluted to 1 ml, and 100 µl was added to each precoated well. Inhibition of cell attachment by antibodies to Fn was determined after incubation of plates coated with Fn or with the 38-kD fragment with 50 µl of the antibody dilution for 30 min at room temperature.

**Other Methods.** SDS-PAGE, Western blot analysis, and 3H-labeling of Fn fragments were carried out as described (4). NH₂-terminal amino acid sequences were determined at the Microchemistry Laboratory, using a liquid-phase sequencer (477A; Applied Biosystems, Inc., Foster City, CA).

**Results**

**Characteristics of the Fn Fragments.** Fig. 1 displays the position, ligand specificity, and NH₂-terminal amino acid sequence of the Fn fragments used in this study. Trypsin digestion of Fn releases two different fragments containing the Hep II domain, with molecular masses of 38 and 58 kD, respectively (7, 10, 11). The difference occurs because only the A chain of Fn contains the IIICS region (12). The 38-kD fragment comprises the first 67 amino acids of IIICS (7) and is derived from the A chain. The 38- and the 58-kD fragments have identical NH₂-terminal sequences. The 58-kD fragment is derived from the B chain, does not contain the trypsin-cleavage site present in the 38-kD fragment, and lacks the entire IIICS region (10–12). It contains the Fib II domain, has slower mobility on SDS-gels upon reduction, and reacts with N-296 mAb (data not shown).

**Attachment of U937 Cells to Fn and its Fragments.** U937 cells attached to plastic wells coated with Fn, and 80- and 38-kD fragments in a dose-dependent manner (Fig. 2). The calculated concentrations required for 50% cell attachment were 14 nM (Fn), 70 nM (80 kD), and 50 nM (38 kD), indicating that the two fragments had similar attachment activity, but were four- to fivefold less effective than intact Fn. Thus, U937 cells interact with two different domains of Fn. U937 did not bind to
uncoated wells or to wells coated with the 29-, 31-, or 58-kD fragments. When 2.0 g/cm² of 3H-labeled 80-, 58-, and 38-kD fragments were applied to the plates, 0.18, 0.25, and 0.31 g/cm², respectively, bound to the wells. This indicates that the lack of adhesion-promoting activity of the HAD fragment was not the result of poor adsorption to the plastic.

The 38-kD Fragment Contains an RGDS-independent Cell Attachment Domain. The following reagents were used in inhibition experiments to assess the specificity of U937 cell attachment to surfaces coated with 38-kD fragments: (a) anti-Fn antibodies; (b) RGD-containing synthetic peptides; and (c) Fn and Fn fragments.

The N-294 mAb, directed to the cell-binding domain of Fn, did not affect U937 adhesion to the 38-kD fragment, but inhibited cell attachment to Fn-coated plates (not shown). The mAb N-296, specific for the COOH-terminal domain, had no effect. Cell adhesion to 38-kD fragment- or to Fn-coated plates was completely abolished by small doses of a polyclonal rabbit IgG anti-Fn. Thus, U937 recognized 38-kD determinants that were distinct from those of the cell-binding domain. The amount of immobilized Fn (and therefore of antibody) was probably insufficient to promote Fc-mediated cell adhesion.

Preincubation of U937 cells with the synthetic peptide GRGDSPC inhibited cell attachment to Fn-coated substrata (Fig. 3 A), but had no effect on adhesion to 38-
kD-coated wells (Fig. 3B). The control peptide GRGESP had no effect in either case. Preincubation of U937 cells with the soluble 38-kD fragment abolished cell attachment to 38-kD-coated wells (Fig. 3C); preincubation with Fn or with the 80-kD fragment had no effect. These results indicated that U937 interaction with the 38-kD domain did not involve the RGDS sequence or the cell surface receptors that recognize this region.

The relative contribution of the fragments to U937 adhesion to Fn was evaluated using 38- and 80-kD fragments and intact Fn as soluble inhibitors. Preincubation of U937 with 1.2 mg/ml of the 80-kD fragment reduced cell attachment to Fn by 90% (Fig. 3D). The 38-kD fragment produced 55% inhibition at the same dose. The 80-kD fragment was 30-fold more effective in producing half-maximal inhibition than the 38-kD fragment (0.38 vs. 12 μM). Preincubation of the cells with soluble Fn had no effect on their subsequent adhesion to Fn-coated wells, in agreement with previously reported results (4).

Identification of the Region of the 38-kD Domain that Promotes Cell Adhesion. U937 cells bound to wells coated with the 38-kD fragment, but did not bind to wells coated with the 58-kD fragment, suggesting that cell attachment might be mediated by the unique IIICS region of the A chain, absent from the 58-kD fragment. To confirm this observation and locate the binding region, synthetic peptides representing three regions of IIICS were used as inhibitory probes in cell attachment assays. The CS-1 peptide (residues 1-25) inhibited attachment of U937 to 38-kD-coated substrata in a dose-dependent manner (Fig. 4A). This effect could be reversed by increasing the concentration of substratum-bound 38-kD fragments (not shown). The other two peptides, CS-2 (residues 22-46) and CS-3 (residues 43-67), had no effect. None of the peptides inhibited attachment of U937 cells to Fn-coated wells (Fig. 4B).

Discussion

The major conclusions of this report are: (a) a 38-kD heparin-binding fragment, derived from the A chain of Fn, supports adhesion of U937 cells as effectively as
the 80-kD fragment containing the RGDS cell-binding domain; (b) U937 cells interact independently with the 38-kD and with the 80-kD Fn fragments; (c) the cell attachment site of the 38-kD fragment is located within the first 25 amino acid residues of the IICRS region of the A chain of Fn.

U937 interaction with the 38-kD fragment did not involve recognition of RGDS, because the 80-kD fragment and the RGDS-containing peptide did not inhibit cell attachment to 38-kD fragment–coated wells. Consequently, the 38-kD fragment contains a second interacting site for U937 cells. The experiments described also suggest that the heparin-binding site of the 38-kD fragment is not involved in U937 adhesion, because two other fragments, the 29-kD fragment containing the Hep I domain, and the 58-kD fragment containing the Hep II domain, failed to support cell attachment.

The synthetic peptides CS-1, CS-2, and CS-3 allowed mapping of the U937 attachment site to the first 25 amino acid residues of the IICRS region. This region was previously shown to mediate attachment and spreading of murine melanoma and avian neural crest cells (6).

The presence of different cell-binding sites on Fn may have distinct functional significance. For example, cell interactions with a second Fn site may regulate the delivery of intracellular signals. In addition, the sequence of the CS-1 peptide can be present or absent from Fn subunits, due to alternative splicing mechanisms (12). Fibroblasts, the major producers of cellular Fn, can synthesize Fn molecules with and without CS-1. Therefore, fibroblasts could play a role in the regulation of monocyte interactions with extracellular matrices by varying the type of Fn deposited. Plasma Fn contains both cell-binding sites, the RGDS and the CS-1. Thus, extravasation of plasma Fn during inflammatory processes generates a uniform matrix with high attachment capacity for monocyte accumulation and differentiation. Future studies of the tissue distribution of Fn forms, and of the functional consequences of the interaction of monocytes with CS-1, may help in the elucidation of molecular mechanisms that modulate the behavior of mononuclear phagocytes.

**Summary**

U937 cells attach to the RGDS-containing 80-kD fragment of fibronectin (Fn). The present report examined whether these cells recognize other domains of Fn. U937 cells attach to a 38-kD fragment derived from the A chain of Fn, which includes the Hep II domain and most of the alternatively spliced IICRS region. U937
did not bind to a 58-kD fragment derived from the B chain (which lacks IIICS) and has the Hep II site. They also did not bind to a 31-kD COOH-terminal fibrin-binding fragment or to a 29-kD fragment containing the Hep I domain. Cell adhesion to the 38-kD fragment was not inhibited by the 80-kD fragment, by GRGDSPC synthetic peptides, or by a mAb directed to the RGDS-containing domain of Fn. Attachment was completely inhibited by the 38-kD fragment and by the synthetic peptide CS-1, comprising the first 25 amino acid residues of IIICS. These results indicate that U937 cells interact with two sites of Fn, the RGDS-containing region, and the IIICS region.

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