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Localization of the Ganglioside-binding Site of Fibronectin*

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It has been demonstrated via biological assays that fibronectin possesses a receptor for gangliosides that is involved in cell adhesion and restoration of the normal morphology of transformed cells. In this study, fluorescence polarization has been employed to monitor the binding of ganglioside oligosaccharide to fibronectin. Parameters involved in ganglioside oligosaccharide binding to fibronectin are described and compared to the interaction of heparin with fibronectin. A K_d of 1.4×10^{-8} mol/liter has been calculated, and it is demonstrated that labeled ganglioside oligosaccharides can be eluted from fibronectin with either unlabeled ganglioside oligosaccharides or 4 M urea.

Using the fluorescence polarization assay developed in this study for measurement of ganglioside binding to fibronectin, it is demonstrated that gangliosides bind to the 31,000-dalton amino terminal heparin-binding domain of fibronectin. A ganglioside-Sepharose affinity column has been constructed which specifically binds the 31,000-dalton amino terminal fragment of fibronectin. The localization of the ganglioside receptor to the amino terminal domain of fibronectin indicates that the ganglioside receptor is distinct from the putative fibronectin cell surface receptor which is located near the center of the fibronectin molecule.

Fibronectin, a key component of the extracellular matrix, has been shown to be involved in the adhesion of cells to collagen, artificial substrata, and cells (1, 2). In addition, fibronectin has also been demonstrated to play a part in several other biological phenomena including nonimmune opsonic activity, wound healing, cell migration, and organization of the extracellular matrix (1, 2). Fibronectin possesses binding sites for gangliosides, collagen, heparin, hyaluronic acid, fibrin, actin, DNA, a cell surface receptor, and several bacterial species (1, 2). Cooperative interactions between binding sites have been demonstrated in several of the biological phenomena mediated by fibronectin (3–6). For example, the fibronectin-binding sites for a 140,000-dalton cell surface receptor (7), collagen (8), and gangliosides (9, 10) have been shown to play a role in cell adhesion. At present, the parameters governing the binding of gangliosides and the 140,000-dalton cell surface receptor are not well understood. In this study, fluorescence polarization has been employed as a means to characterize the biochemical events involved in the binding of gangliosides to fibronectin.

Several lines of evidence indicate that gangliosides play a role in fibronectin-mediated cell adhesion. Initially, gangliosides were found to inhibit attachment of Chinese hamster ovary cells to a fibronectin-collagen complex (9). Inhibition of cell adhesion was also observed with the oligosaccharide portion of the gangliosides but not with the ceramide moiety or with a variety of simple carbohydrates (9). In addition, oxidation of the sialic acid residues of gangliosides destroyed their capacity to inhibit cell attachment to collagen (9). The observations of Kleinman and co-workers (9) were confirmed by the finding that mixed brain gangliosides inhibit binding of fibronectin to human fibroblasts (11). Yamada *et al.* (12) found that gangliosides inhibited fibronectin-mediated hemagglutination, cell spreading, and restoration of a normal morphologic phenotype to transformed cells. Fluorescent gangliosides have been shown to co-distribute on the cell surface with fibronectin (13–15), and gangliosides GD_2 and GD_3 have been shown to be specifically associated with the adhesion plaques of melanoma cells (16). The finding that purified gangliosides potentiated the interaction of fibronectin with a ganglioside deficient cell line (10) provides clear evidence that gangliosides are important in fibronectin-mediated functions.

The observations cited above support the hypothesis that gangliosides serve as cell surface receptors for fibronectin. While in previous studies, the interaction of fibronectin with gangliosides has been inferred from biological assays, the present study demonstrates the binding of gangliosides to fibronectin by biochemical means. In addition, it is shown that the fibronectin-binding site for gangliosides resides on a different region of the molecule than the receptor for the 140,000-dalton cell surface receptor protein.

EXPERIMENTAL PROCEDURES

Reagents—Fibronectin was obtained from human plasma by gelatin-Sepharose affinity chromatography (17). After elution with 4.0 M urea + 0.02 M sodium citrate, pH 5.5, fibronectin was dialyzed against 0.15 M NaCl + 0.05 M PBS,[1] pH 7.4, aliquoted, and stored at −80 °C. Fibronectin concentration was determined by the Bradford protein assay (18) with bovine serum albumin as a standard. GM_1 gangliosides were obtained from Supelco Inc. (Bellefonte, PA); mixed brain gangliosides were either prepared as described below or obtained from Supelco Inc. The fluorescent label, 5-([2-aminoethyl]thioureidyl) fluorescein (referred to as fluorescein ethylene diamine hereafter) was obtained from Molecular Probes, Inc. (Junction City, OR). Galactose oxidase and neuraminidase were obtained from Sigma. All other chemicals were of reagent grade.

Preparation of Gangliosides—Gangliosides were extracted from bovine brain by the procedure described by Trams and Lauter (19). Approximately 60 mg of ganglioside were obtained per 100 g of bovine

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[1] The abbreviation used is: PBS, phosphate-buffered saline.

brain. The ceramide portion of the gangliosides was removed to yield free oligosaccharides by the procedure described by MacDonald *et al.* (20). Using mixed brain gangliosides (Sigma) as a standard, ganglioside concentration was determined by the resorcinol method for sialic acid as described by Svennerholm (21).

Preparation of Fluorescein Ethylene Diamine-Labeled Gangliosides and Oligosaccharide Moiety of Gangliosides—Fluorescent gangliosides and oligosaccharides used in polarization experiments reported here were prepared by a modification of the procedure described by Spiegel *et al.* (22). In brief, galactose oxidase treatment yielded an aldehyde from terminal galactose residues which then reacted with the amino group of the fluorescein label to yield a Schiff base. Sodium cyanoborohydride was used to reduce the Schiff base to a nonhydrolyzable product. The specific procedures employed were as follows. Approximately 3–4 mg of oligosaccharide was suspended in 0.9 ml of PBS, pH 7.4 to which was added 5 units of galactose oxidase in 0.1 ml of PBS. After 1-h incubation at 37 °C, 1 ml of 1 mg/ml fluorescein ethylene diamine in PBS was added to the mixture and incubated at room temperature for 15 min in the dark. Reduction was carried out by addition of 1 ml of 12.4 mg/ml freshly prepared $NaCNBH_4$ followed by incubation for 30 min at room temperature in the dark. The sample was dialyzed for 16–18 h against PBS in 2000 MW cut off Spectrapor dialysis tubing. Assuming that mixed bovine brain ganglioside oligosaccharides have an average molecular mass of 2000 daltons, approximately 0.02 mol of label/mol of ganglioside was incorporated. Affinity chromatography with fibronectin-Sepharose was used to separate the fibronectin-bindable ganglioside fraction from the nonbindable fraction. Binding of labeled oligosaccharides to the fibronectin-Sepharose column was monitored by use of a black light. Labeled oligosaccharides were eluted from the column with 4 M deionized urea. Approximately 50% of the labeled oligosaccharide preparation was fibronectin bindable.

The affinity fractionated, labeled probe was inactivated by treatment of 50 μg probe with 0.1 units of *Clostridium perfringens* neuraminidase in 0.1 M potassium acetate, pH 4.5, at 37 °C for 15 min (23). The reaction was terminated by heating for 1.5 min in a boiling water bath (23).

Determination of the Critical Micelle Concentration of Mixed Bovine Brain Gangliosides—Micelle formation was accessed according to the method of Horowitz (24). Briefly, quantities of mixed bovine brain gangliosides indicated in the text were added to 2.0 ml of a solution of 5.5×10^{-5} M 2-*p*-toluidinylnaphthalene-6-sulfonic acid. Fluorescence measurements were made after thorough mixing. Using an excitation wavelength of 360 nm and an emission wavelength of 460 nm, intensity measurements were read for 1 min to ensure that steady-state values were achieved. The critical micelle concentration was taken to be the ganglioside concentration corresponding to the intersection of the two linear portions of the fluorescence response curve.

Purification of Fibronectin Domains—The various fibronectin domains used in this study were isolated as described by Hayashi and Yamada (25). In brief, 3.0 mg/ml fibronectin in 50 mM Tris, pH 7.0 + 1.0 mM $CaCl_2$ was warmed to 30 °C, and then trypsin was added at 0.2% (w/w) of the fibronectin concentration. After 30 min at 30 °C, the reaction was quenched with 1.0 mM phenylmethylsulfonyl fluoride. The digest was dialyzed against 10 mM Tris-HCl, pH 7.0, + 0.1 mM phenylmethylsulfonyl fluoride and applied to a 1.5 × 30-cm column of DEAE-cellulose. The effluent contained the *N*-terminal heparin-binding domain fragment (25) (which was found in this study to also bind ganglioside oligosaccharides). Bound fragments were eluted with 500 mM NaCl in 10 mM Tris, pH 7.0, and then dialyzed in 10 mM Tris, pH 7.0, containing 1 mM $MgCl_2$. The samples were chromatographed on a 1.5 × 30-cm gelatin-Sepharose column. Nonadherent material was pooled and adjusted to 2 mM EDTA then chromatographed on a 1.5 × 30-cm heparin-agarose column (Bio-Rad). The cell-binding domain bound to the column and was eluted with 0.13 M NaCl + 10 mM Tris, pH 7.0. The gelatin-binding domain was isolated after digestion of human plasma fibronectin with 1% thermolysin (Sigma) for 30 min at 30 °C. The thermolysin-treated fibronectin solution was adjusted to 50 mM EDTA prior to chromatography on a 1.5 × 30-cm gelatin-agarose column. Adherent polypeptides were eluted with 4 M urea + 10 mM Tris, pH 7.0. The protein was precipitated by the addition of solid ammonium sulfate to 70% saturation and then was dialysed against 10 mM Tris, pH 7.0. These fragments have been mapped as to their location in the intact molecule (25). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (26) was used to verify the presence of the heparin II-binding domain.

A ganglioside oligosaccharide-Sepharose column was prepared by (*a*) adding a 1,12-diaminododecane spacer arm to CNBr-activated Sepharose (27) followed by (*b*) coupling oligosaccharides to the amino group of the spacer arm as described above for the preparation of fluorescein ethylene diamine-labeled oligosaccharide. In brief, 40 ml of Sepharose was washed with 100 ml of 0.75 M Na_2CO_3, pH 11.6. The Sepharose was incubated in 100 ml of 0.75 M Na_2CO_3, and then 10 ml of 50% CNBr in acetonitrile was added for 5 min at 4 °C. Following activation, the Sepharose was washed at 4 °C with 100 ml of 0.2 M $NaHCO_3$, pH 8.5, then 100 ml 0.2 M borate, pH 8.5. The activated Sepharose was stirred for 30 min with 100 ml of 10 mg/ml 1,12-diaminododecane then allowed to stand overnight. (1,12-diaminododecane was dissolved by the addition of a minimal amount of HCl and then brought to 0.2 M borate, pH 8.5. The 1,12-diaminododecane-Sepharose was washed with 200 ml of 5% acetic acid, pH 3.0, then 120 ml of 1.0 M NaCl, followed by 120 ml of PBS, pH 7.4. Galactose oxidase-treated oligosaccharides, prepared as described in the fluorescent labeling procedure, were added to the 1,12-diaminododecane-Sepharose at a concentration of 2 mg oligosaccharide/g of Sepharose. The oligosaccharides were reduced with 12.4 mg/ml $NaCNBH_3$ for 30 min at room temperature. The oligosaccharide-Sepharose was then washed with PBS, pH 7.4.

Instrumentation—The principles of fluorescence polarization have recently been reviewed (28). In this study, an L-format spectrofluorometer (SLM Instruments, Inc., Urbana, IL) equipped with Jarrell Ash (Jarrell Ash, Waltham, MA) quarter-meter monochromators at the excitation and emission ports were used for the fluorescence measurements. Wavelengths were set at 495 nm for excitation and 540 nm for emission with slit widths maintained at 8 nm. Fluorescence was excited and viewed through rotatable Glan-Thompson polarizers. The detection system consisted of a Spex digital photometer (Spex Industries, Inc., Metuchen, NJ) which interfaced with a Franklin Ace 1200 personal computer (Franklin Computers, Pennsauken, NJ) via an Adalab data acquisition system (IMI, State College, PA). The cuvette chamber was equipped with a magnetic stirrer and had both temperature and dehumidifying capabilities.

Measurements were performed in 3-ml, 1-cm path length plastic cuvettes (Evergreen Scientific, Los Angeles, CA), containing 1.0 ml of 0.10 M NaCl + 0.05 M NaH_2PO_4, pH 7.4, and a variable amount of fluorescein-labeled oligosaccharide as described in the text. Measurements of ganglioside binding to fibronectin was assessed by first determining the polarization readings for free (unbound) fluorescein-labeled oligosaccharides (P_F). Increase in fluorescence polarization values (P_M) upon addition of varying quantities of fibronectin indicated binding of ganglioside oligosaccharide to fibronectin. In binding studies, either the fibronectin or oligosaccharide concentration was held constant while the concentration of the other component was varied as specified in the text. Likewise, dissociation was studied by observing the decrease in fluorescence polarization over time following the addition of agents which cause dissociation of gangliosides from fibronectin. Environmental parameters such as temperature, pH, and ionic strength were evaluated for their effect on ganglioside-oligosaccharide binding to fibronectin.

Determination of ionic strength effects on binding of the oligosaccharide moiety of bovine brain gangliosides to fibronectin was determined by dialyzing fibronectin in 0.05 M NaH_2PO_4, pH 7.4, with varying concentrations of NaCl to obtain the ionic strengths desired. The same ionic strength solutions were used as the sample buffer in the cuvette during the fluorescence polarization measurements. Effect of pH on oligosaccharide-fibronectin binding was determined by using the constant ionic strength buffer system of Miller and Golder (29) at 0.1 M while varying the pH from 5.0 to 12.0 at 2 °C. Temperature was monitored with a thermistor probe (Extech, Boston, MA).

Data Analysis—Assessment of fluorescein-labeled oligosaccharide binding to fibronectin was carried out directly in terms of the measured polarization (P_M) (28). An equation based on the law of addition of polarization values for the fraction of added oligosaccharide that was bound was used for quantitative analysis of fluorescein-labeled oligosaccharide binding to fibronectin (28). The fraction bound (f_B) is given by:

$$f_B = \frac{P_M - P_F}{P_B - P_F} \quad (1)$$

where P_M is the measured polarization value for a given mixture of fluorescein-labeled oligosaccharide and fibronectin; P_F is the measured polarization value of the fluorescein-labeled oligosaccharide in

the absence of fibronectin; P_B is the polarization of fluorescein-labeled oligosaccharide when completely bound to fibronectin (obtained by extrapolation to infinite fibronectin concentration using a double reciprocal plot derived from polarization data as a function of fibronectin concentration). Equation 1 is valid if there is no change in fluorescence intensity of the fluorescein-labeled oligosaccharide upon binding, a condition observed in the present study. The amount of oligosaccharide bound (B) is given by:

$$B = f_B(A) \quad (2)$$

where f_B is defined by Equation 1 and A is the mass of the oligosaccharide added.

RESULTS

In the present study, binding of the oligosaccharide moiety of gangliosides (referred to as oligosaccharides hereafter) to fibronectin is reported in terms of measured polarization values (P_M). Note that an increase in polarization value over the polarization of free-labeled oligosaccharide (P_F) is an indication of an increase in oligosaccharide binding to fibronectin (28).

Polarization experiments were initially conducted with labeled mixed bovine brain gangliosides. The polarization value of free gangliosides (P_F) was found to increase as the concentration of labeled gangliosides was increased. This observation indicates that the apparent molecular weight of gangliosides increases as their concentration is increased. Since micelle formation of gangliosides has been previously reported (30, 31), we infer from our fluorescence polarization findings that micelle formation occurs at relatively low ganglioside concentration. With the aid of the 2-*p*-toluidinylnaphthalene-6-sulfonic acid assay for micelles (24), a critical micelle concentration for mixed brain ganglioside of 5×10^{-3} μg/ml was determined (Fig. 1). Since micelle formation interfered with polarization readings and the active region of the molecule appears to be the oligosaccharide moiety (9), oligosaccharides were separated from the ceramide moiety of gangliosides as described under "Experimental Procedures" and used in subsequent studies.

Initially, polarization experiments were conducted using fluorescein-labeled oligosaccharides from mixed bovine brain gangliosides that had not been prepared by fibronectin-Sepharose affinity chromatography. Only a small increase in polarization was noted upon addition of fibronectin to unfractionated oligosaccharides which indicates that only a small fraction of the oligosaccharides bound. Since the oligosaccharide moieties of the mixed brain gangliosides are heterogenous (32), a fibronectin-Sepharose column was used to separate the fibronectin-bindable fraction of fluorescein-labeled oligosaccharide. Upon addition of fibronectin to the bindable fraction of fluorescein-labeled oligosaccharides, a large increase in P_M was observed indicating that the bindable fluorescein-labeled oligosaccharides had bound to fibronectin (Fig. 2). In the studies reported here, the fibronectin-bindable fluorescein-labeled oligosaccharide fraction of bovine brain gangliosides was employed.

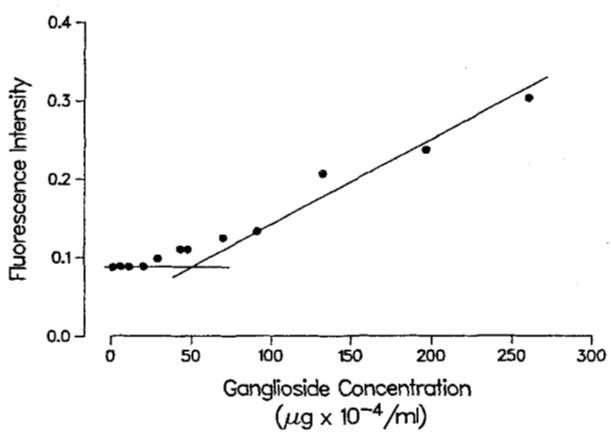

FIG. 1. **Determination of critical micelle concentration of bovine brain gangliosides with 2-*p*-toluidinylnaphthalene-6-sulfonate.** The fluorescence of 50 μM 2-*p*-toluidinylnaphthalene-6-sulfonate was determined at increasing concentrations of gangliosides. The critical micelle concentration is taken to be the ganglioside concentration corresponding to the intersection of the two distinct linear portions of the fluorescence response curve.

Several lines of evidence indicate that the fluorescence polarization assay described measures the binding of gangliosides to fibronectin. First, it can shown that fluorescein-labeled oligosaccharides prepared from homogeneous GM_1 gangliosides are bound by fibronectin (Fig. 2). Yamada, Kleinman, and their co-workers (9, 12) have demonstrated that the order of effectiveness of ganglioside species is $GT_{1b} > GD_{1a} > GM_1 > GM_2 > GM_3$. The finding that increasing fibronectin concentration results in an increase in the polarization value of labeled GM_1 oligosaccharides indicates that the population of free GM_1 oligosaccharides becomes increasingly bound as the concentration of fibronectin is increased (Fig. 2). Thus, a dose-response relationship can be established in the binding of GM_1 oligosaccharides to fibronectin. Second, it can be demonstrated that heat-denatured fibronectin and other proteins, such as bovine serum albumin and many fibronectin fragments, do not bind the ganglioside oligosaccharide probe described here. It can also be demonstrated that the fluorescent tag itself (fluorescein ethylene diamine) does not bind to fibronectin. Thus, the fluorescence polarization assay presented measures ganglioside oligosaccharide binding to fibronectin.

The derivation of the fluorescein-labeled mixed brain ganglioside oligosaccharide probe from gangliosides can be established from the following evidence. The affinity-fractionated, fluorescein-labeled oligosaccharide probe prepared from mixed brain gangliosides was found to contain sialic acid (*a*) by the resorcinol method (21) and (*b*) by the neuraminidase sensitivity of the probe. Under conditions described under the "Experimental Procedures" section, it could be demonstrated that the fibronectin bindability of the labeled probe was destroyed by neuraminidase treatment (25) but not by treatment with a heat-inactivated neuraminidase. The probe can also be shown to contain a terminal galactose due to the reactivity of the oligosaccharides with galactose oxidase during the fluorescein-coupling reaction described under "Experimental Procedures." The partitioning of oligosaccharides into the aqueous phase of a chloroform:methanol:water mixture following periodate cleavage of the ceramide portion of gangliosides is again an expected reaction of gangliosides (20). Thus, compositional and enzyme sensitivity data indicate that the mixed brain ganglioside probe is of ganglioside origin.

Time Course of Ganglioside Oligosaccharide Binding to Fibronectin—Upon addition of fibronectin to the fluorescein-labeled oligosaccharide moiety, an immediate increase in the measured polarization (P_M) was observed over the polarization of free, fluorescein-labeled oligosaccharide (P_F) (Fig. 3). The polarization had increased to 78% of the maximum observed polarization value within 15 s of the addition of fibronectin (Fig. 3). Polarization values indicated that the oligosaccharide moiety binding to fibronectin reached a plateau within 2 min and remained constant for at least 30 min.

Fibronectin-bound oligosaccharide was found to be readily exchangeable with free oligosaccharide since addition of excess, unlabeled oligosaccharide moiety to the fibronectin/

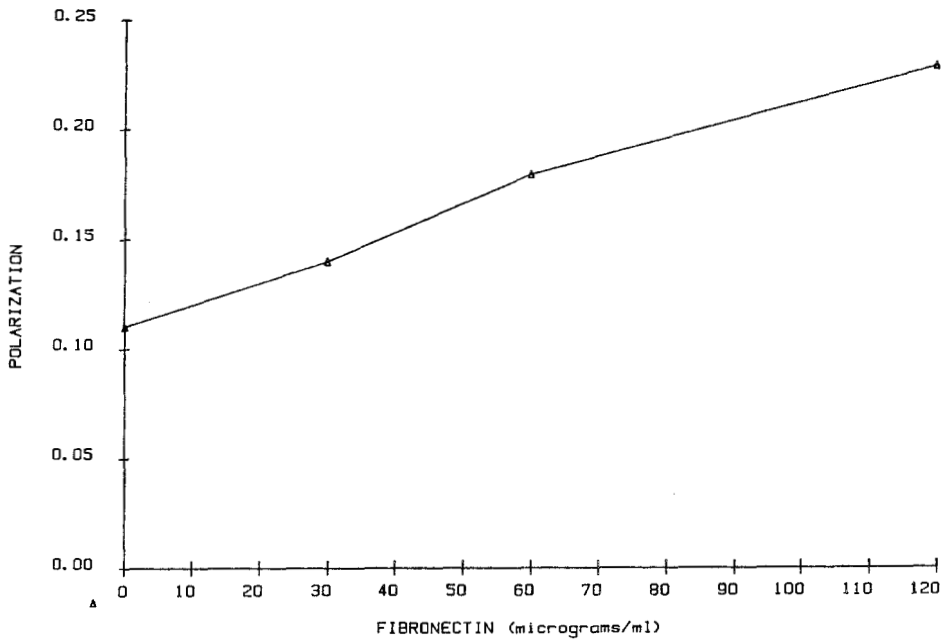

FIG. 2. **Binding of GM_1 ganglioside oligosaccharides to fibronectin.** The oligosaccharide moiety of GM_1 gangliosides was isolated and labeled with fluorescein ethylene diamine as described under "Experimental Procedures." 10 μg of labeled GM_1 oligosaccharide was titrated with increasing concentrations of fibronectin. The increase in polarization values observed with increasing fibronectin concentration indicates that increasing numbers of free GM_1 molecules become bound as increasing amounts of fibronectin are added.

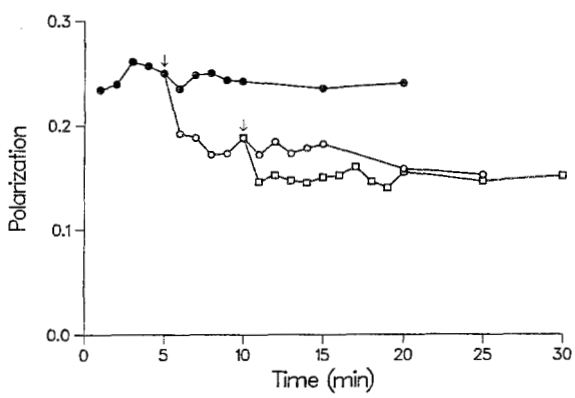

FIG. 3. **Time course of oligosaccharide binding to fibronectin.** Oligosaccharide binding was monitored by observing an increase in polarization upon addition of 30 μg/ml of fibronectin to 10 μg fluorescently labeled oligosaccharides at 2 °C (*closed circles*). P_f, the polarization of fluorescein-labeled oligosaccharide in the absence of fibronectin, did not change during the course of the experiment. A time course of elution of fluorescein-labeled oligosaccharides from fibronectin upon the addition of 68 μg (*open circles*) and (*open squares*) 135 μg of unlabeled oligosaccharide was monitored by observing a decrease in polarization values. The *arrows* indicate the time at which unlabeled oligosaccharides were added to a preformed fluorescein-labeled oligosaccharide-fibronectin complex. The results indicate that oligosaccharides bind rapidly to fibronectin and rapidly exchange with unlabeled oligosaccharide.

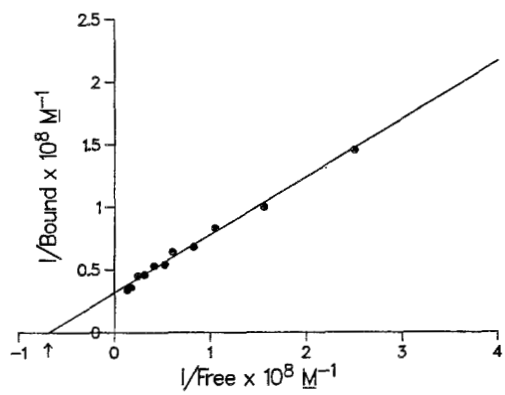

FIG. 4. **Determination of K_D for ganglioside oligosaccharide-fibronectin interaction.** As described under "Experimental Procedures," polarization values were analyzed in order to determine the amount of bound and free ganglioside oligosaccharide as the concentration of fibronectin was varied. Scatchard analysis indicates a K_D of 1.4×10^{-8} M (*arrow*) for the interaction between gangliosides and fibronectin.

fluorescein-labeled oligosaccharide complex resulted in an immediate decrease in P_M (Fig. 3), fibronectin-bound oligosaccharide appears to be easily eluted by free oligosaccharide. That P_M did not decrease to P_F upon addition of excess unlabeled probe is a consequence of Equation 1 which indicates that over a 100 excess of bindable probe would be required to completely reduce polarization readings to P_F.

Effect of Oligosaccharide Concentration—Titration of fibronectin with aliquots of fluorescein-labeled oligosaccharide showed high initial polarization values which decreased as further fluorescein-labeled oligosaccharide was added (Fig. 4). The decrease in polarization values with increase in oligosaccharide concentration is expected since the ratio of free to bound oligosaccharide increases beyond the point at which fibronectin is saturated with ligand. Polarization data was converted to the amount (in μg) of bound oligosaccharide as described under the "Experimental Procedures" section. The amount of oligosaccharide bound to fibronectin approached a plateau with increasing fluorescein-labeled oligosaccharide which indicated a saturation of the ganglioside-binding site. This data was used to determine the K_d which equaled 1.4×10^{-8} mol/liter (Fig. 4).

Effect of Temperature, Ionic Strength, and pH on Oligosaccharide Binding to Fibronectin—Oligosaccharide binding to fibronectin occurred over a wide range of temperatures (2–40 °C) (data not shown). A decrease in P_M from 0.25 to 0.185 as the temperature was increased from 2 °–40 °C is probably due to temperature-dependent changes in either the rotational mobility or flexibility of the fluorescein-labeled oligosaccharide probe or the ganglioside-binding domain of fibronectin. Fluorescence polarization analysis revealed that the binding of fluorescein-labeled oligosaccharides from bovine brain gangliosides were relatively uneffected by ionic strengths ranging from 0.1 to 1 M. Thus, in contrast to heparin binding to fibronectin which is abolished at 0.5 M NaCl (33), oligosaccharide binding does not appear to depend on an appreciable

electrostatic component. Polarization measurements were recorded over the pH range from 6.0 to 12.0. Measurements below pH 6.0 were not possible due to quenching of the fluorescent probe at low pH. Oligosaccharide binding to fibronectin decreased with increasing pH from a maximum at pH 7.0. Appreciable binding was observed at all pH values studied.

Localization of the Ganglioside-Binding Site of Fibronectin—To observe the effect of fibronectin concentration on ganglioside oligosaccharide binding, fluorescein-labeled oligosaccharide moieties from bovine brain gangliosides were titrated with 10-μg aliquots of fibronectin (Fig. 5). Increases in P_M were noted immediately upon addition of each aliquot of fibronectin. Each increase in P_M was progressively smaller until a plateau was reached at which point the labeled oligosaccharide available for binding was limiting (Fig. 5). Using the increase in polarization induced by intact fibronectin as an assay, the ganglioside-binding site of fibronectin was localized as described below.

In an initial study, fibronectin fragments obtained from a trypsin digest of human serum fibronectin were used to locate the ganglioside-binding region of fibronectin. In this study the gelatin-binding domain (#2), the cell-binding domain (#4), and the heparin II domain (#5) as described by Hayashi and Yamada (25) were employed. The results indicate that the 31,000-dalton amino terminal fragment of fibronectin, containing the heparin II-binding site (domain #5) bound fluorescein-labeled oligosaccharides in a dose-dependent fashion (Fig. 5). The fragments of fibronectin containing the cell-binding site (domain #4) and the gelatin-binding site (domain #2) had little if any effect on polarization values. Likewise, laminin was not effective in binding the fibronectin-bindable ganglioside oligosaccharides. Thus, the 31,000-dalton fragment at the amino terminal region of the molecule appears to contain the ganglioside-binding site of fibronectin.

In order to critically test the assignment of the ganglioside-binding site to the amino terminal 31,000-dalton fragment of fibronectin, several additional studies were performed. First, the 31,000-dalton fragment has been reported to contain a heparin-binding site (25). Thus, a fluorescence polarization assay for heparin binding to fibronectin (33) was used to determine whether the ganglioside-binding site fragment of fibronectin also contained a heparin-binding site. Fluorescence polarization revealed that the ganglioside-binding domain did indeed bind heparin (Fig. 6). Second, a ganglioside-Sepharose affinity column, constructed as described under "Experimental Procedures," was used to purify the ganglioside-binding fragment from a tryptic digest of fibronectin (Fig. 7). Material eluted from ganglioside-Sepharose by 8 M urea was found to migrate on sodium dodecyl sulfate-polyacrylamide gel electrophoresis at the same position as the 31,000-dalton fragment prepared as described by Hayashi and Yamada (25). Thus, the ganglioside-binding site of fibronectin can be assigned to a 31,000-dalton fragment of fibronectin which contains the amino terminus of the molecule.

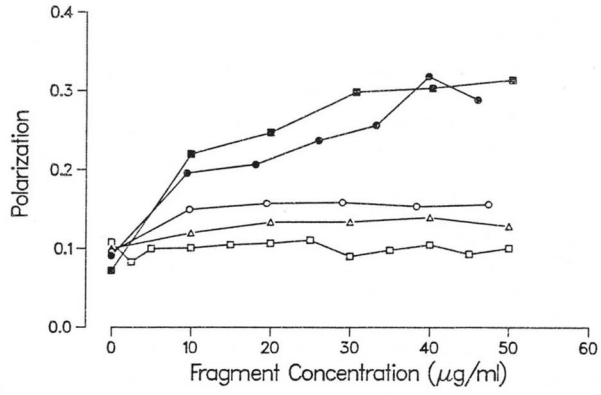

FIG. 5. **Effect of fibronectin or trypsin digestion fragments concentration on oligosaccharide binding.** 10 μg of fluorescein-labeled oligosaccharide was titrated with either aliquots of intact fibronectin (*closed squares*), the heparin II-binding domain fragment of fibronectin (*closed circles*), the cell-binding domain fragment of fibronectin (*open circles*), or the gelatin-binding domain (*open triangles*) isolated by affinity chromatography on gelatin-Sepharose. 10 μg of fluorescein-labeled oligosaccharide was also titrated with aliquots of laminin (*open squares*).

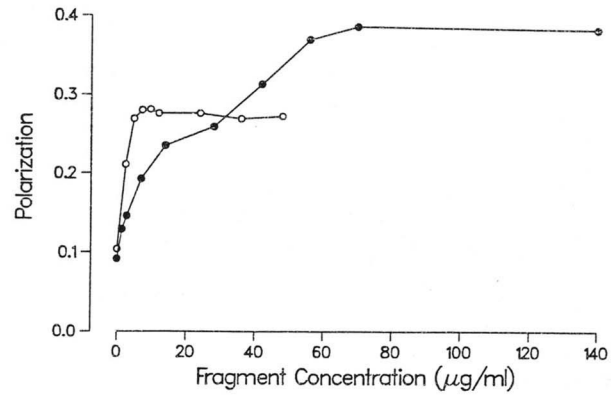

FIG. 6. **Heparin binding to the 31,000 dalton ganglioside-binding site fragment of fibronectin.** 0.35 μg of fluorescein-labeled heparin was titrated with either aliquots of fibronectin (*closed circles*) or heparin II-binding domain fragment of fibronectin (*open circles*) isolated by affinity chromatography on ganglioside-Sepharose. The results indicate that the ganglioside-binding fragment of fibronectin also contains a heparin-binding site.

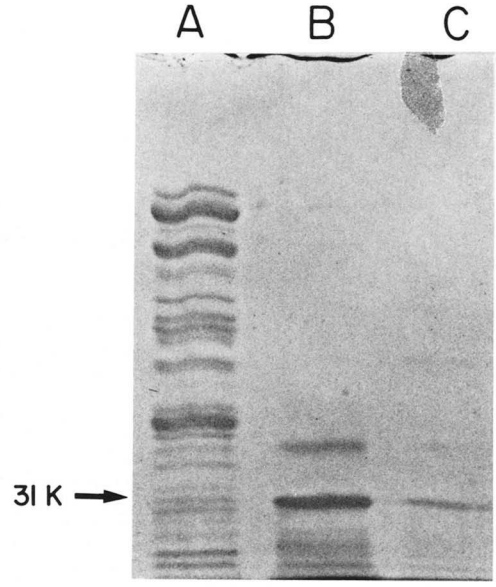

FIG. 7. **Isolation of the ganglioside-binding site of fibronectin.** Trypsin-digested human serum fibronectin was run on a DEAE-cellulose column as described under the "Experimental Procedures" section. *Lane A* is the trypsin-digested fibronectin. *Lane B* displays the ganglioside- and heparin-binding fraction from a DEAE-cellulose column. *Lane C* contains the bound fraction from a ganglioside-Sepharose column.

DISCUSSION

While several prior studies (9–16) employing biological assays have convincingly demonstrated that gangliosides influence fibronectin-mediated phenomena, biochemical evi-

dence has been lacking which clearly demonstrates a direct interaction between fibronectin and gangliosides. In this study, fluorescence polarization has been employed to quantitatively assess the parameters governing the binding of gangliosides to fibronectin. The evidence provided clearly establishes the existence of a specific interaction between gangliosides and fibronectin. With the aid of the biochemical assay for ganglioside binding to fibronectin described here, tryptic peptides derived from fibronectin have been analyzed for their ganglioside binding ability. It is demonstrated that a unique amino terminal heparin-binding peptide contains the ganglioside-binding site of fibronectin.

In order to devise a biochemical assay for ganglioside binding to fibronectin, the fluorescence polarization technique was chosen for several reasons. Fluorescence polarization, which has recently been reviewed (28), is an ideal method for measuring binding events involving a wide variety of molecules. The chief advantage of fluorescence polarization over many other binding assays (*e.g.* equilibrium dialysis, gel permeation chromatography, etc.) is the fact that bound and free ligands do not have to be physically separated in order to obtain information concerning binding events (28). Thus, binding events can be observed in real time with a sensitive, yet technically facile, methodology. The principle upon which fluorescence polarization depends to measure binding events involves the decrease in rotational Brownian motion of a small fluorescent ligand upon binding to a larger molecule (28). Since fibronectin is an unusually large molecule, the perturbation in fluorescence polarization values obtained upon binding of many molecules to fibronectin is also quite high and, hence, fluorescence polarization is ideally suited to studies involving fibronectin (33, 34).

With the aid of fluorescence polarization, it has been possible to demonstrate the binding of gangliosides to fibronectin and quantitatively measure parameters governing the binding of gangliosides to fibronectin. It can be shown that gangliosides bind rapidly and reversibly to fibronectin (Fig. 3). While free oligosaccharide moieties of gangliosides and 8 M urea elute bound gangliosides from fibronectin, many other agents fail to elute bound gangliosides. For example, while heparin is rapidly eluted from fibronectin under conditions of high ionic strength (33), 1 M NaCl fails to block even the initial binding of gangliosides to fibronectin. Since ganglioside binding to fibronectin can be observed both at high ionic strength and over a range of pH values, one can conclude that the binding event observed in this study is not primarily electrostatic in nature. That low concentrations of free oligosaccharides rapidly elute gangliosides from fibronectin (Fig. 3) indicates the specificity of the binding event studied here. A dissociation constant of $K_d = 1.4 \times 10^{-8}$ mol/liter can be calculated from data obtained by fluorescence polarization (28). Hence, the binding of gangliosides to fibronectin is a strong interaction.

Using the fluorescence polarization assay described here to detect binding of gangliosides to fibronectin, it has been possible to localize the ganglioside-binding site of fibronectin to a peptide containing the amino terminus and one heparin-binding site of the molecule (Figs. 5–7). Several lines of evidence can be cited which establish the position of the ganglioside-binding site within the fibronectin molecule. First, a peptide, with demonstrable heparin binding activity (Fig. 6), purified according to the procedure of Hayashi and Yamada (25) displays ganglioside binding activity (Fig. 5). Second, an identical peptide can be obtained from tryptic digests via chromatography on ganglioside-Sepharose. Since gangliosides have been shown to be involved in the cell adhesive activity of fibronectin (9–16), it is of interest that the cell surface binding site of fibronectin which binds a 140,000-dalton cell surface protein (7) does not bind gangliosides (Fig. 5). It is interesting to note that McKeown-Longo and Mosher (35) have identified a 70,000-dalton amino terminal peptide of fibronectin which binds to the surface of fibroblasts and blocks incorporation of intact fibronectin into the extracellular matrix. Based on the evidence provided here, the matrix-assembly receptor of McKeown-Longo and Mosher (35) contains the ganglioside-binding site of fibronectin. Since fibronectin clearly binds gangliosides (Figs. 2–7) and biological assays have demonstrated that gangliosides are involved in cell adhesion (9–16), the ganglioside-binding site of fibronectin as well as the cell surface binding site of fibronectin (7) may both be involved in the biochemical mechanism responsible for cell adhesion.

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