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INTERACTION BETWEEN FLUORESCENCE-LABELED FIBRONECTIN FRAGMENTS STUDIED BY GEL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

GENE A. HOMANDBERG*, DAVID B. EVANS, JEANETTE KRAMER and JOHN W. ERICKSON
The Department of Medicine, Hemostasis Research Laboratory, The University of Wisconsin Medical School, Milwaukee Clinical Campus, Mt. Sinai Medical Center, Milwaukee, WI 53233 (U.S.A.)

SUMMARY

Fibronectin is a large, adhesive glycoprotein which self-associates on many cell surfaces. We have begun to study this reaction by determining the domains of fibronectin which interact with each other. To avoid possible solid-phase artifacts of affinity chromatography, we have devised a solution-phase assay in which the smallest fibronectin fragment is labeled with fluorescamine, mixed with unlabeled fibronectin, and complexation is observed by the appearance of a new higher-molecular-weight peak on gel high-performance liquid chromatography columns. The assay allowed use of excess unlabeled reactant, high-sensitivity, low background without removal of reagent, and fast analysis. Our results show that the amino- and carboxyl-terminal fibronectin fragments bind the native molecule in solution.

INTRODUCTION

Protein–protein interaction can be studied by various methods, based on differences in properties between the reactants and the resultant complexes. One common method is gel chromatography, in which the resultant complex is separated from the reactants by size. Such a method can be time-consuming on conventional columns and may lack sensitivity if the elution is monitored by absorbance. Analysis time can be shortened by using high-performance liquid chromatography (HPLC); sensitivity can be improved by labeling the proteins with fluorescent reagents, such as fluorescamine.

We were interested in studying the interactions between several different fragments of human plasma fibronectin. Fibronectin is a 450-kD (kilodalton)[1] dimeric glycoprotein, which binds glycosylaminoglycans, fibrin, collagen, DNA and various cell surfaces, and which self-associates[1–5]. The multifunctionality of fibronectin derives from the multidomain structure of the molecule. Various active and functionally distinct fragments can be isolated by limited proteolysis of fibronectin[1–5]. Initially, we studied interactions between these fragments by affinity chromatography. However, some non-specific adsorption of fragments was observed. We therefore devised a system where one fragment was fluorescence-labeled and allowed to bind an unla-

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beled fragment in solution, and the resultant complex was then subjected to gel filtration by HPLC. Interaction was viewed as a function of incorporation of fluorescent label into a new higher-molecular-weight peak. With this approach, we were able to measure, with high sensitivity, and by using excess unlabeled reactants, interactions occurring to a limited extent between fragments of n*M* concentrations. In addition, the problem of resolving higher-molecular weight complexes from reactants with similar retention times was overcome, since only the fluorescence-labelled complex was detected.

EXPERIMENTAL

All buffers and common reagents were from Fisher (Pittsburgh, PA, U.S.A.). TSK gel filtration columns, 3000 SW (50 cm), 4000 SW (30 cm) and 6000 PW (30 cm), were purchased from Varian (Palo Alto, CA, U.S.A.). Cathepsin D and conventional chromatography resins were purchased from Sigma (St. Louis, MO, U.S.A.).

Human plasma fibronectin was purified by adsorption on gelatin-Sepharose[6]. An amino-terminal 72-kD fragment, carboxyl-terminal 140-kD fragments and 40-kD fragments were generated by cathepsin D digestion of fibronectin[7]. The 72-kD fragment was isolated by adsorption to gelatin-Sepharose. The 140-kD fragment was separated from the 40-kD fragment by chromatography on Sephadex G-200; the 40-kD fragment was purified further by adsorption on heparin-Sepharose. The 72-kD fragment was digested further with thrombin to generate the amino-terminal 29-kD fragment and the 50-kD gelatin-binding domain[8]. These fragments were separated by adsorption of the 50-kD fragment on gelatin-Sepharose. Carboxyl-terminal 190-kD fragments were generated by digestion of fibronectin with thrombin[9]; 29-kD fragment was the other major product.

Fluorescence-labeling of peptides was accomplished by the addition of 0.2 vols. of fluorescamine (0.15 mg/ml in acetone) to 1–500 μg of peptide in 25–250 μl of 0.1 *M* borate buffer (pH 8.5)[10]. The reaction was allowed to proceed at 37°C for 5 min. The pH was then lowered to 7.4 by the addition of dilute hydrochloric acid. The fluorescence intensity of samples decreased after 48 h. Amino acid analysis of lysine content before and after labeling of most fragments showed that between 10 and 20% of the total lysyl residues were modified.

HPLC of peptides was performed on a Varian Vista 54 system controlled by a Varian Model 401 microprocessor. Samples of 50–500 μl were injected into TSK columns, equilibrated in 150 m*M* sodium chloride, 20 m*M* Tris buffer (pH 7.4) at 22°C. The flow-rate was 0.5 ml/min. The detector was a Varian Fluorochrom with an excitation filter of 340–380 nm and emission filters of 460 nm or a Varian UV-5 selectable-wavelength monochrometor set at 240, 254 or 280 nm.

RESULTS AND DISCUSSION

TSK 3000 SW and 4000 SW columns have exclusion ranges for typical globular proteins of 30 000 to 500 000 dalton and 50 000 to 1 000 000 dalton, respectively[11–17]. The retention times of various fragments in TSK 3000 SW column chromatography are shown in Fig. 1. The retention time of the fragments deviated from the standard

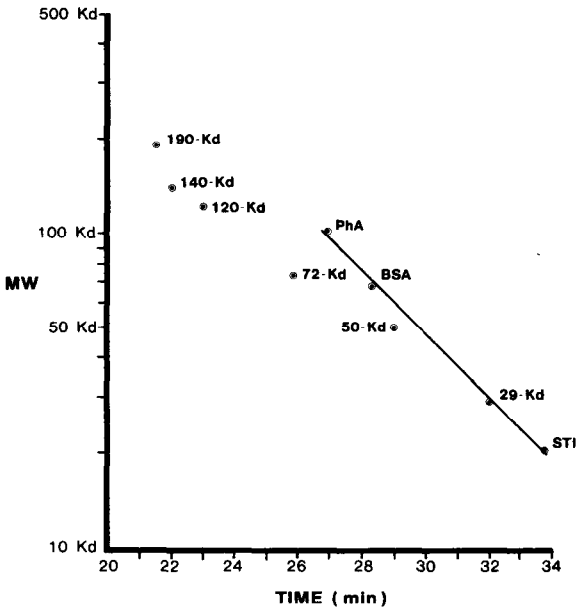

Fig. 1. Elution times of fragments on a TSK 3000 SW column *vs.* log molecular weight (MW). Fragment identified and molecular weight standards, phosphorylase A (PhA); bovine serum albumin (BSA), ovalbumin (Oval), and soybean trypsin inhibitor (STI) in 50–500 μl of 150 m*M* NaCl, 20 m*M* Tris buffer, (pH 7.4) were applied to a 50-cm TSK 3000 SW column at room temperature. Flow-rate was 0.5 ml/min. Detection was either by adsorbance at 240 nm or by fluorescence detection (excitation: 340–380 nm: emission: 460 nm). Amount of protein injected varied from 1 μg to 500 μg. Fibronectin (not shown) and 190-kD fragment co-eluted.

curve constructed with three glubular proteins, suggesting that the fragments are behaving asymmetrically with apparent Stokes radii nearly double that of globular proteins with similar molecular weights. The native fibronectin molecule behaves as a highly asymmetrical molecule with a Stokes radius of around 96 Å[18]. The apparent asymmetry of the fibronectin fragments was increased in the presence of 1 *M* potassium bromide, a chaotropic agent, which caused the 29-kD, 72-kD and 140-kD fragments to be eluted 1, 1.3, 0.8 and 3 min earlier, respectively than when 150 m*M* sodium chloride, 20 m*M* Tris (pH 7.4) was the eluent. A 50 m*M* phosphate buffer (pH 7.4) also caused the fragments to be eluted earlier, suggesting that phosphate confers a more compact structure on the fragments than does the Tris buffer.

Since the 190-kD fragment and PFn were eluted in the same positions on the TSK 3000 column, we tested the retention time of the fragments on a TSK 4000 SW column. The separation of the smaller fragments on the TSK 4000 SW column was poorer than on the TSK 3000-SW column (data not shown), and the separation between the 190-kD fragment and native fibronectin was similar to that on the TSK 3000 SW column.

In order to improve the resolution of both lower- and higher-molecular-weight fragments, we connected the TSK 3000 SW column (50 cm) ahead of the TSK 6000 PW column (30 cm). The sieving range for glubular proteins on a 6000 PW has not been described; the range on a 4000 PW column is over one million for a globular

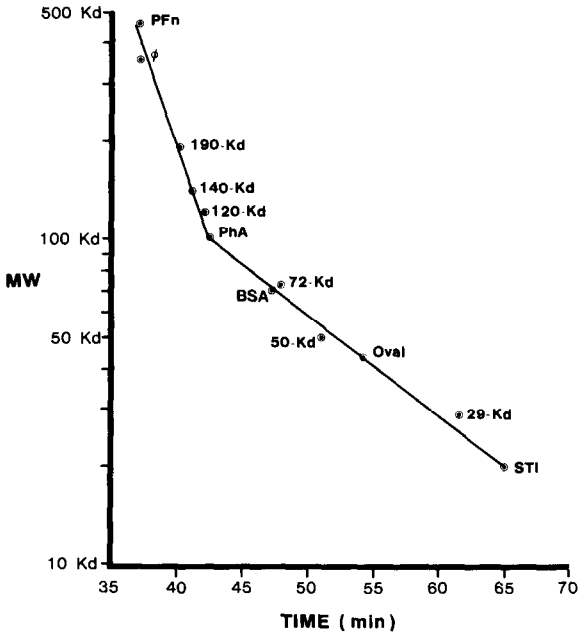

Fig. 2. Separation of fragments on a TSK 3000 SW column (50 cm), connected to a TSK 6000 PW column (30 cm). Samples and conditions were as in Fig. 1. Bovine fibrinogen (Ø) was also chromatographed. Fibronectin (PFn) and 190-kD fragment were separated.

protein[11]. Fig. 2 shows that the separation of fibronectin and the 190-kD fragment was improved over that by TSK 3000 SW column chromatography alone. However, the resulting separation was still not enough to allow the resolution of all the fragments in a mixture; chromatography resulted in three broad peaks. We were unable to test separation in a system of two TSK 3000 SW columns, so we cannot argue that we used the most optimal tandem system. We then fluorescence-labeled the fragments and determined their retention times. In all cases, the retention time was unchanged, suggesting that no major structural alteration had occurred upon fluorescent modification. Fig. 3 shows chromatograms in which small peaks, probably due to buffer salt, could have been mistaken for peptides when the elution was monitored by absorbance. Fluorescence detection of identical samples, labeled with fluorescamine did not show the 40- to 45-min peaks, suggesting that the contaminants were not peptides. These UV-absorbing contaminant peaks were also observed in samples of the 29-kD and 40-kD fragments, making quantitative analysis of the interaction with native fibronectin difficult.

Our objective was to determine which domains of fibronectin are responsible for fibronectin aggregation. Earlier work[21,22] had suggested that these self-association domains are in the amino- and carboxyl-terminal regions. We therefore examined the binding activity of the amino-terminal 29-kD fragment and of the carboxyl-terminal 40-kD fragments to native fibronectin. Each fragment was labeled with fluorescamine and mixed with fibronectin. Since fluorescamine quickly decays in aqueous buffer[19], the samples could be analyzed without removal of reagent. After

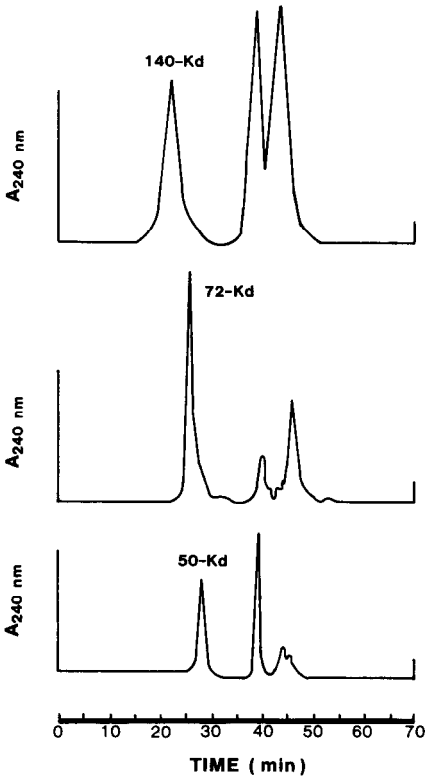

Fig. 3. Gel chromatography of selected fragments on the TSK 3000 SW column. Conditions as in Fig. 1. Detection by absorbance at 240 nm shows peaks at 40–45 min, which were not detected by fluorescence-monitoring of labeled fragments.

24 h, the solutions were subjected to gel chromatography. Only unbound labeled fragments and the complexes of fragment with fibronectin were observed by fluorescence detection. Thus, the problem of lack of separation of higher-molecular-weight unlabeled fragments and complexes was circumvented. Chromatography patterns of the 29-kD fragment, the 40-kD fragment, and of mixtures of each with native fibronectin are shown in Fig. 4. Interaction of each fragment with fibronectin occurred, but the retention time was not significantly shorter than that of fibronectin alone (see Fig. 2). Even with a 5- to 10-fold molar excess of fibronectin over the 29-kD fragment, the same degree of interaction was observed as with equimolar ratios. If the excess fibronectin had been visualized, detection of the complex would have been impossible.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis[20] of the 30- to 45-min fractions verified that the complexes contained either the 29-kD or 40-kD fragment, in addition to fibronectin. Further, these fragments bound fibronectin-Sepharose, consistent with the activities shown here for solution-phase fibronectin.

The objective of our efforts is to determine which of the domains of fibronectin interact. Our preliminary data show that the amino-terminal 29-kD and carboxyl-terminal 40-kD fragments interact with native fibronectin. These results are consist-

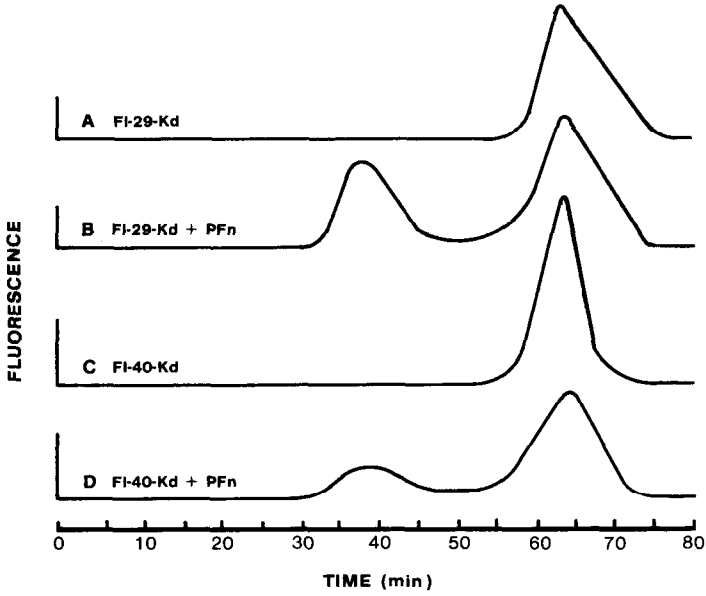

Fig. 4. Gel chromatograms of amino-terminal 29-kD fragment (A) of carboxyl-terminal 40-kD fragment (C) and of mixtures (B and D) of each with plasma fibronectin (PFn). Fragments were labeled with fluorescamine in 0.1 *M* borate buffer, (pH 8.5), the solution was adjusted to pH 7.4 after 5 min at 37°C, and one volume of 150 m*M* NaCl, 20 m*M* Tris buffer (pH 7.4) was added or a volume of PFn in Tris buffer was added. Chromatographic conditions were as in Fig. 1.

ent with reports that an amino-terminal 60-kD fragment and a 160-kD carboxyl-terminal fragment of equine fibronectin interact with Sepharose-bound native equine fibronectin[21,22]. Moreover, using our solution phase assay for studying self-association reactions, possible solid-phase artifacts, such as non-specifc binding, were avoided. Further studies will be presented elsewhere[23].

In summary, we have used tandem gel chromatography on a TSK 3000 SW followed by a TSK 6000 PW column to increase the resolution of fibronectin fragments over that seen with a 3000 SW column alone. Use of fluorescence-labeling of fibronectin fragments for studying interactions by gel HPLC gave us the following advantages oiver UV detection of conventional gel chromatography: (1) excess unlabeled reactants could be used without interfering with detection of the complexes, (2) sensitivity was enhanced, (3) UV-absorbing contaminants did not interfere, (4) reagents did not have to be removed prior to chromatography, and (5) the speed of HPLC was realized. These advantages should obtain in any protein/protein interaction system where activity is not abolished by reaction with a label.

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