Journal of Supramolecular Structure
and Cellular Biochemistry 17:153–161 (1981)
Cellular Recognition 377–385

Structural Analysis of Fibronectin With Monoclonal Antibodies

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The reactivity of six monoclonal antibodies with fragments of fibronectin produced with trypsin, chymotrypsin, and Staphylococcus aureus V8 protease is described. All these antibodies reacted with fragments derived from the C-terminal one-third of fibronectin. This region probably contains sites for the binding of fibronectin to cells, and to heparin and may also contain active sites for the reattachment, spreading, and alignment of transformed cells. Analysis of the reactivities of different sets of proteolytic fragments with the antibodies and with other ligands (eg. heparin) allows one to determine overlaps between the fragments and to locate the positions of the different binding sites for antibodies and ligands. One of the antibodies has allowed us to identify a site of structural difference between cellular and plasma fibronectins from hamsters. The site recognized by this antibody is located near to, but not at, the C-terminal end and does not involve carbohydrate groups. Because of its internal location in fibronectin, this difference suggests that there are probably different genes for cellular and plasma fibronectin. These monoclonal antibodies should be useful for further probing the functions present in the C-terminal regions of fibronectin and for determining their locations.

Key words: difference between cellular and plasma forms, fibronectin, monoclonal antibodies, structure and function

Fibronectin is a major extracellular matrix protein found in the glycocalyx of many cells and in basement membranes [1, 2]. A slightly different form of fibronectin is found in plasma [3, 4]. Fibronectins interact with other matrix materials via specific binding sites for collagen, heparin, and hyaluronic acid [2, 5]. Fibronectins also have binding sites for certain blood plasma proteins, such as Factor XIII, fibrinogen, and fibrin [4]. Fibronectins stimulate the attachment and spreading of certain normal and transformed cells, enhance migratory activity, restore a more normal morphology to transformed cells, and act as opsonins for the endocytosis of certain particles [1, 2, 4]. Some of these activities are retained in proteolytic fragments of fibronectin [6]. Fragments containing the binding sites for Factor XIII, collagen and fibrin have been shown to be located in the N-terminal 70 kilodaltons (kd) of intact fibronectin [7–9]. Other active sites have

Received April 16, 1981; accepted July 21, 1981.

0275-3723/81/1702-0153$03.00

been positioned in the intact molecule only roughly. For example, the cell binding site and a heparin binding site are both somewhere in a 160-kd stretch of chicken fibronectin [5, 6], but the precise location of these active sites within the fragment and the position of the fragment in the intact molecule have not been determined.

We are using monoclonal antibodies to identify, map, and isolate fragments of fibronectin which contain functional sites of the molecule and to identify structural differences between fibronectins from different sources. By virtue of their specificity for single determinants, monoclonal antibodies to fibronectin serve as markers for determining the relatedness and overlap of fragments produced by a variety of proteases. These antibodies are also able to detect structural differences between fibronectins from different species and differences between cellular and plasma fibronectin from the same organism [10].

In this paper we describe results obtained with several monoclonal antibodies to hamster cellular fibronectin and which demonstrate the potential of this approach for analyzing the structure and function of fibronectin.

METHODS

Cell Culture and Preparation of Hybridomas

Hamster NIL8 cells were cultured and metabolically labeled with ^{35}S-methionine as previously described [11]. The mouse myeloma cell lines used in cell fusions were SP2/O-Ag (provided to us by Dr. Malcolm Gefler, Massachusetts Institute of Technology) and NSI/1-Ag4-1 (a gift from Dr. Thomas Linsenmeyer, Massachusetts General Hospital). Cell fusion and maintenance of myeloma cell lines were as described by Oi and Herzenberg [12]. Hybridomas were cloned twice by limiting dilution in the presence of 2×10^6 thymocytes/ml and were injected intraperitoneally into pristane-primed Balb/c mice for the production of ascites fluids. Screening of hybridoma cultures and ascites fluids for antibodies to fibronectin was by the ELISA technique [13].

Purification of Fibronectin

Metabolically labeled fibronectin secreted by NIL8 cells was purified from conditioned medium as described [11]. Labeled fibronectin was digested in 0.05 M Tris (pH 7.2) containing 1 mM $CaCl_2$ at room temperature with one of the following enzymes: (a) 1 μg/ml trypsin (Koch-Light) for variable times up to 15 min; (b) 5 μg/ml α-chymotrypsin (Miles) for 3 hr; (c) 100 μg/ml Staphylococcus aureus V8 protease (Miles) for 15 min.

Immunoprecipitation and Polyacrylamide Gel Electrophoresis

Immunoprecipitation of proteolytic fragments of fibronectin with monoclonal antibodies was by the double antibody method as described previously [10]. Immunoprecipitates were analyzed on 10% SDS-polyacrylamide slab gels by the methods of Laemmli [14]. Gels were impregnated with Enhance (NEN) for fluorography.

ELISA Competition Binding Assay

The ELISA [13] was modified for competition experiments as previously described [10]. Hamster fibronectin was purified from NIL8 cell culture medium

or from golden hamster plasma as described [11]. Purified cellular or plasma fibronectin was tested for its competiton for the binding of monoclonal antibodies in solution versus cellular fibronectin bound to plastic tubes.

RESULTS

Monoclonal Antibodies Provide Markers for Structural Analyses

Ascites fluids produced by hybridoma clones grown in mice were used for immunoprecipitation of proteolytic fragments of ^{35}S-methionine-labeled fibronectin. Initially, fragments produced by limited cleavage with trypsin or extended digestion with chymotrypsin were used in immunoprecipitations because they provide a starting point for establishing the lineage of smaller fragments produced by other enzymes. Since much is already known about the functional activities contained in these tryptic and chymotryptic fragments [6, 15], they also provide guidance as to which activities are likely to be found in novel small fragments derived from them. Table I shows the fragment sets produced by each enzyme and the reaction of six monoclonal antibodies with them. Figure 1 shows the results of an immunoprecipitation of a limited tryptic digest with antibodies SP/1, SP/4, and NS/7. Under these conditions 190-, 40-, and 25-kd fragments are produced [11]. Both SP/1 and SP/4 precipitated the 40-kd fragment, but not the 190-kd or 25-kd fragments (Fig. 1 track 2 and 4). The 190-kd fragment was precipitated by NS/7, but the 40- and 25-kd fragments were not (Fig. 1 track 3). Table I shows that the two SP antibodies gave identical reactions with tryptic and chymotryptic fragments, whereas the NS antibodies gave a reaction pattern different from SP, but several of the NS clones in the group precipitated the same subset of fragments. The results with these fragments provided information regarding the domain specificities of the antibodies, which was useful in determining their relationship with smaller fragments produced by other enzymes and also provided a rationale for deciding which functional activities were likely to be present in related small fragments. The results in Table I do not distinguish whether there is

TABLE I. Immunoprecipitation of Proteolytic Fragments of Fibronectin by Monoclonal Antibodies

	Tryptic fragments					Chymotryptic fragments			
Antibody	(220 kd)	(215 kd)	(190 kd)[b]	(40 kd-SH)[c]	(25 kd)	180-130 kd)	(40 kd)[d]	(40 kd)[e]	(25 kd)
SP/1	+	–	–	+	–	–	–	+	–
SP/4	+	–	–	+	–	–	–	+	–
NS/6	ND[a]	ND	+	–	–	+	–	–	–
NS/7	ND	ND	+	–	–	+	–	–	–
NS/16	ND	ND	+	–	–	+	–	–	–
NS/23	ND	ND	+	–	–	–	–	–	–

[a]ND, not done.
[b]Previous publications from this laboratory have used a molecular weight of 200 kd for this fragment.
[c]Contains a free sulfhydryl group and is not bound by gelatin-sepharose.
[d]Bound by gelatin-sepharose.
[e]Not bound by gelatin-sepharose [10].

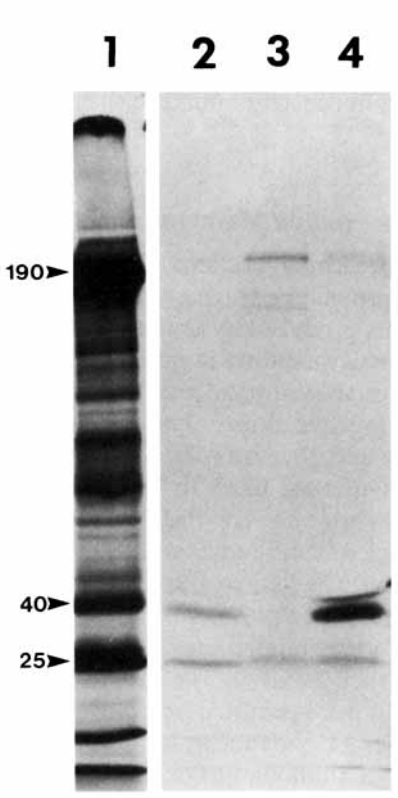

Fig 1 Immunoprecipitation of a tryptic digest of fibronectin by monoclonal antibodies Fluorograph of a 10% SDS-polyacrylamide gel Track (1) total tryptic digest Tracks 2–4 immunoprecipitates of tryptic digest. (2) SP/4; (3) NS/7, (4)) SP/1 The 25-kd material in tracks 2–4 represents nonspecific background binding as shown by control precipitations

redundancy in the specificity of the monoclonal antibodies tested, or whether each of the fragments tested contains multiple determinants recognized by different antibodies. The latter seems more likely and is in fact suggested by the failure of SP/1 and SP/4 to compete for binding in preliminary competition ELISA assays (data not shown).

In order to investigate this possibility further, we determined the reactivity of these antibodies with smaller fragments produced by more extensive digestions. Fig. 2 shows the reactivity of the antibodies listed in Table I with fragments produced by cleavage of fibronectin with S aureus V8 protease (V8). With this preparation, differences were seen in the array of fragments bound by most of the antibodies. Antibodies SP/1 and SP/4 differed in the fragments precipitated in the size range of 130–185 kd and in small fragments of 30 and 33 kd (Fig. 2 lanes 3 and 4). The differences between antibodies of the NS group are primarily in the array of small fragments they precipitate (Fig. 2 lanes 5–8). Further analyses along these lines, on the one hand, should allow precise localization of the binding sites of each of these antibodies and, on the other, should provide information on overlaps between different proteolytic fragments.

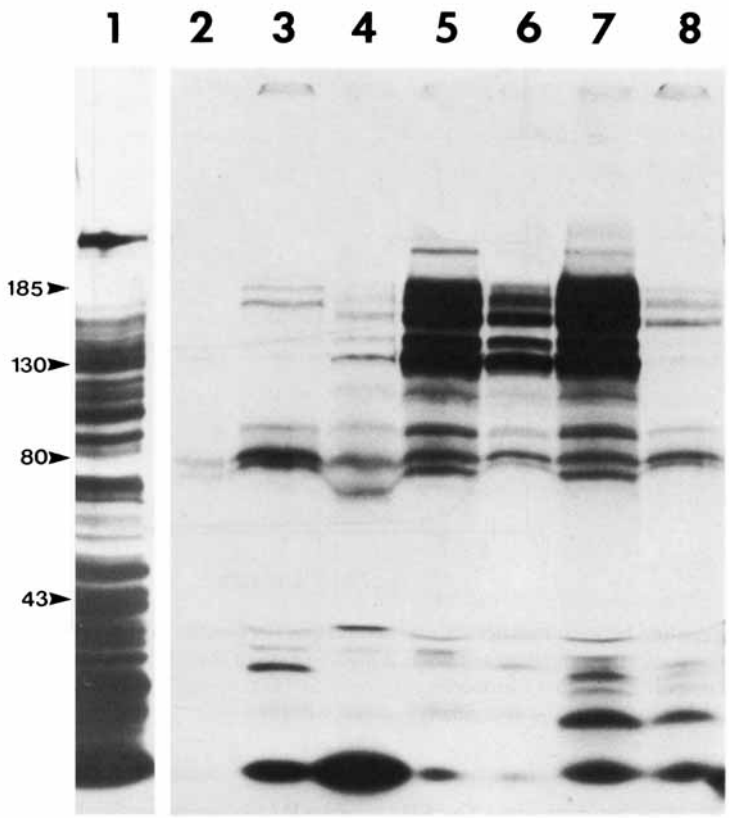

Fig. 2. Immunoprecipitation of a Staphylococcus aureas V8 protease digest by monoclonal antibodies. Fluorograph of a 10% SDS-polyacrylamide gel. (1) Total digest; (2) negative control (first antibody omitted); (3) SP/1; (4) SP/4 (5) NS/6; (6) NS/7; (7) NS/16; (8) NS/23.

Monoclonal Antibodies Distinguish Different Fibronectins

In addition to their utility for establishing lineages of proteolytic fragments, the monoclonal antibodies have proven useful in confirming and locating structural differences between the cellular and plasma forms of fibronectin from hamsters. Figure 3 shows the results of competition binding experiments in which purified cellular and plasma fibronectins were used as competitors for the binding of SP/1 or SP/4 antibodies. As expected, cellular fibronectin competed effectively for the binding of both antibodies. Plasma fibronectin was also an effective competitor for the binding of SP/1, but competed only slightly for the binding of SP/4. These results indicated that SP/4 recognized a structural difference between cellular and plasma fibronectins, whereas SP/1 did not distinguish between the two forms of the molecule.

DISCUSSION

In the set of monoclonals described here, none recognizes the two domains (25 kd and 40 kd gelatin binding) which comprise the N-terminal 70 kd of fibro-

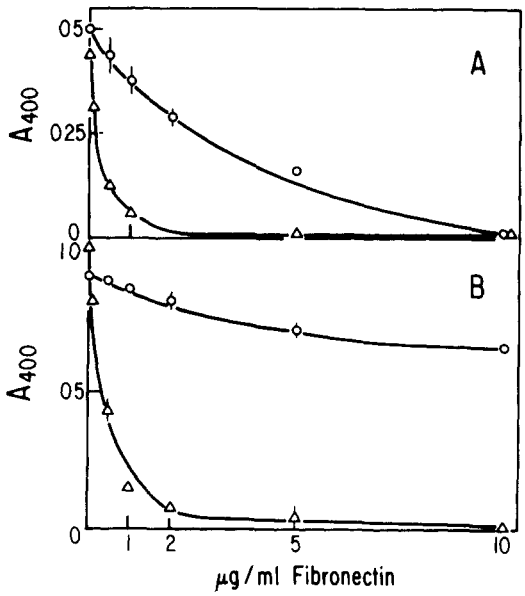

Fig. 3. Competition between hamster cellular and plasma fibronectins for the binding of monoclonal antibodies. The competition binding assay was a modification of the ELISA as described [10]. (A) Competition by purified cellular fibronectin; ○ – – ○ SP/4, △ – – △ SP/1. (B) Competition by purified plasma fibronectin; ○ – – ○ SP/4, △ – – △ SP/1.

nectin [7, 8]. The binding sites for SP/1 and SP/4 have been fairly precisely located near to, but not at, the C-terminal end of fibronectin [10]. The argument is as follows. Previous work indicates that (a) fibronectin contains a single, free sulfhydryl group located approximately 170 kd from the N-terminal end. There may be another free sulfhydryl further toward the C-terminal end. (b) The 190kd and 40kd tryptic fragments probably overlap and each contains a free sulfhydryl group (c) 220, 215, 190 and 40kd tryptic fragments do not contain interchain disulfide bonds [11, 15]. Monoclonal antibodies SP/1 and SP/4 bind to intact fibronectin and to the 220kd and 40kd tryptic fragments, but not to tryptic fragments of 190kd or 25 kd [10]. Both the 220kd and the 40kd fragments contain free sulfhydryls [15]. Figure 4 shows where we believe the binding sites for SP/1 and SP/4 are in fibronectin and two alternative arrangements of the tryptic fragments. Although the two models differ in details of the proposed number and/or placement of free sulfhydryl groups and in the exact arrangement of large tryptic fragments, the location of the antibody binding sites is the same in both models. It is clear from Figure 4 that the binding sites for SP/1 and SP/4 are near to, but not at, the C-terminal end of fibronectin. Since neither the 220kd nor 40kd tryptic fragments contains interchain disulfide bonds, the interchain disulfides of the fibronectin dimer must be closer to the C-terminal than the antibody binding sites.

Given the foregoing, any fragment which reacts with SP/1 or SP/4 extends close to the C-terminal. The V8 digest contains a set of nested fragments of 30kd and 130–185kd which have this property (Fig. 2). The set of NS monoclonals

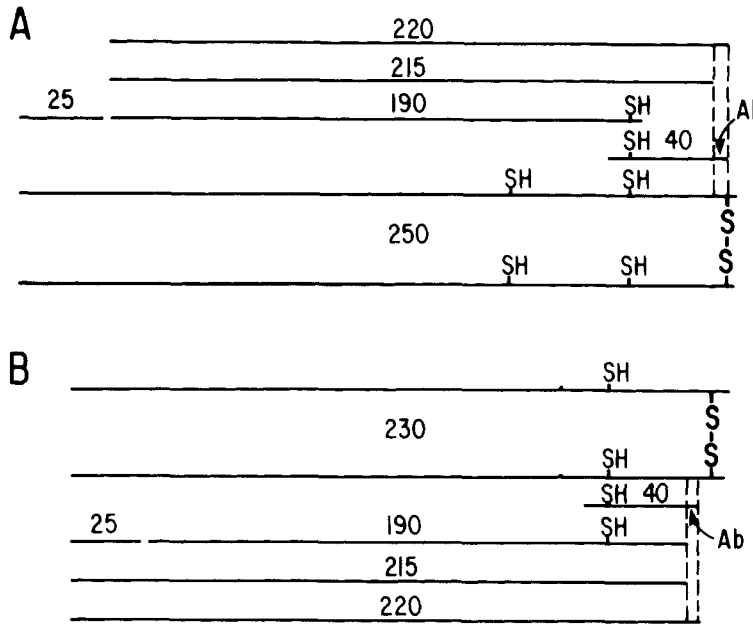

Fig. 4. Proposed arrangement of tryptic fragments of fibronectin and location of binding sites for monoclonal antibodies SP/1 and SP/4. (A) and (B) represent two alternative arrangements of the tryptic fragments that are consistent with available data (see Discussion). Conclusions regarding the location of the antibody binding sites are the same in both models. In model (A) it is assumed that : (1) the large tryptic fragments arise by cleavages at both ends of the molecule; (2) the molecular weight of fibronectin is 250-kd in order to accommodate the 220 and 25-kd fragments arranged in tandem; (3) there are two free sulfhydryl groups. In model (B) it is assumed that : (1) cleavage occurs only at the C-terminal end of 215- and 220-kd fragments; (2) the placement of the single free sulfhydryl group on the basis of previous data [14] is in error by 10%. Certain aspects of the two models could also be combined. The invariant features are that the 40-kd SH fragment must extend beyond the C-terminal end of the 190-kd fragment and that part of the same extension must be present in the 220-kd fragment. The antibodies must bind in the region shared by the 40- and 220-kd fragments.

reacts with these fragments; in particular, several react with the 30kd fragment (Fig. 2). Therefore, three of the four antibodies in this set have binding sites in the C-terminal ¼ of fibronectin. This region of fibronectin is interesting because it contains one or two free sulfhydryl groups [11], a site of structural difference between cellular and plasma fibronectin [10], and quite possibly the sites for binding of fibronectin to cells, heparin, and hyaluronic acid [5, 6]. Active sites for the stimulation of reattachment, spreading, and alignment of transformed cells by fibronectin may also be in this region. These monoclonal antibodies will be useful as probes for localizing more precisely these active sites of fibronectin.

The results of immunoprecipitations with the monoclonal antibodies have also provided futher preliminary information about the fragments produced by trypsin, chymotrypsin, and V8 protease. Using the binding of SP/1 and SP/4 as indicators for the C-terminal region, one can conclude that the 220kd but none of the other large tryptic or chymotryptic fragments, extends into this region (see

Table I). Small V8 fragments of 30 and 33kd recognized by SP/1 and SP/4 must also be from the C-terminal region (Fig.2). Furthermore, one can place the binding sites for several of the NS antibodies in the C-terminal part of these large fragments, which must therefore be reasonably close to the C-terminal region recognized by SP/1 and SP/4. The evidence for this is as follows: (a) the binding sites for the NS/6, NS/16 and NS/23 antibodies are within 30kd of those for SP/1 and SP/4; (b) NS/6, NS/7, and NS/16 recognize the chymotryptic 130-to 180-kd fragments and (c) these three and NS/23 also recognize the tryptic 190-kd fragment. The tryptic 190-kd and chymotryptic 130- to 180-kd fragments all bind to heparin (our unpublished data and ref. 5). Since these fragments bind our NS antibodies but not the SP antibodies, the heparin-binding site must also be further from the C-terminal end than the SP antibody-binding sites.Thus, one can construct two topological models of fibronectin: NH_2–25kd–40kd gelatin–NS–SP–CO_2H & NH_2–25kd–40kd gelatin–heparin–SP–CO_2H, where NS and SP represent regions of fibronectin where the antibodies of the two groups bind. Further work along these lines should allow a more precise definition of the locations of the binding sites for heparin and the NS antibodies.

Having obtained five or six monoclonal antibodies with independent binding sites clustered in the C-terminal region of fibronectin, we can begin to probe the function(s) of this region via antibody blocking experiments. Furthermore, the antibodies have identified a set of small fragments of fibronectin derived from this region which range in size around 30 kd. If a function maps to this region, it is possible that one of the small fragments will contain the active site.

We have also used monoclonal antibodies to locate a site of structural difference between cellular and plasma fibronectins from hamsters. Competition experiments showed that SP/4 distinguishes between cellular and plasma fibronectin. Prior to this finding, there was already indirect evidence suggesting that structural differences exist between the two forms of fibronectin. Although both forms of fibronectin share all known biological functions, they differ quantitatively in their specific activities in certain biological assays [3]. Cellular fibronectin runs as a single band on SDS-PAGE with an apparent molecular weight slightly higher than that of plasma fibronectin which runs as a doublet. These differences are retained by 200-kd tryptic fragments [3]. There is also evidence suggesting that the two forms of fibronectin may differ in their carbohydrate groups [4]. Given that there are structural differences between plasma and cellular fibronectin, a number of questions arise: (a) How many sites of difference are there? (b) Where are they located? (c) Do the differences involve the polypeptide chain, the carbohydrate groups, or other post-translational modifications? (d) Do any of the structural differences relate to differences in biological activity?

Because monoclonal antibody SP/4 recognizes structural differences between the two forms of fibronectin, its binding site on cellular fibronectin pinpoints one region of structural difference. Consequently, it was important to localize, as precisely as possible, where the antibody binds to fibronectin. We have shown elsewhere [10] that the site of difference is near to, but not at, the C-terminal end of fibronectin and that SP/4 does not merely recognize a difference in carbohydrate residues. Other difference(s) are present in the 200-kd fragments, as mentioned previously. These latter differences could be either in

the polypeptide backbone or in the carbohydrate or other post-translational modifications.

The existence of noncarbohydrate difference(s) in internal position(s), as detected by SP/4, raises the question of the origin of different forms of fibronectin. Since they do not appear related either by proteolytic cleavages or by carbohydrate modifications, it seems likely that there are two different structural genes for cellular and plasma fibronectin. A less likely but intriguing possibility is that the two forms might be coded on different RNAs derived from a single structural gene by differential splicing, as has been shown for IgM and the T antigens of papova viruses [16–18]. Antibodies distinguishing the two forms of fibronectin should aid in further analyses of these possibilites and also in the investigation of the distributions and sources of the two forms.

ACKNOWLEDGMENTS

This research was supported by a grant from the USPHS, National Cancer Institute (PO1CA20172). BTA was supported by a USPHS postdoctoral fellowship and ROH by a USPHS Research Career Development Award.

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