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TWO-DIMENSIONAL PEPTIDE MAPPING OF FIBRONECTINS FROM BOVINE AORTIC ENDOTHELIAL CELLS AND BOVINE PLASMA

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SUMMARY

We have made a comparison between plasma and endothelial cell fibronectin, since these cells are in intimate contact with plasma in vivo. Cellular and secreted fibronectins were purified from cloned lines of adult bovine aortic endothelial cells, and compared to purified bovine plasma fibronectin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and two-dimensional peptide mapping. When unreduced, all three fibronectins migrated on gels as single bands with M_r 440,000. After reduction, cellular and secreted fibronectins migrated on gels as single bands with M_r 220,000, but plasma fibronectin migrated as two bands with M_r 220,000 and 210,000. All three fibronectins, including the two subunits of plasma fibronectin, had identical structures by peptide mapping analysis.

INTRODUCTION

Fibronectin is a major cell surface glycoprotein of many untransformed cell lines grown in vitro (1 - 3). The cellular form of fibronectin is a very insoluble protein associated with an extracellular matrix in vitro (4) and basement membranes and connective tissue in vivo (5, 6). Soluble or secreted fibronectin is released by cells into the culture medium in vitro and into the plasma in vivo as a protein previously known as cold-insoluble globulin (7). The structure of cell surface, secreted and plasma fibronectins is very similar, as judged by immunological cross-reactivity, amino acid and carbohydrate compositions and one-dimensional peptide maps (1 - 3, 8, 9). All forms of fibronectin exist as dimers of approximate M_r 440,000 whose subunits are linked by disulfide bonds (10 - 12), and the cellular

ABBREVIATIONS: ABAE - adult bovine aortic endothelial; SDS - sodium dodecyl sulfate

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and secreted fibronectins have identical subunits of approximate M_r 220,000. However, many workers have now shown that plasma fibronectin from many species contains nonidentical subunits of approximate M_r 220,000 and 210,000 (13 - 20). At present it is not clear if secreted fibronectin is structurally different from cellular fibronectin, and it is not known how the subunits of plasma fibronectin differ from each other.

We and others have shown that cultured bovine (21, 22) and human endothelial cells (23) synthesize fibronectin which is incorporated into the extracellular matrix and is secreted into the culture medium. In order to learn more about the relationship between cell surface, secreted and plasma fibronectins, in this paper we report on two-dimensional peptide mapping studies on cellular and secreted fibronectins from cultured bovine aortic endothelial cells, and on fibronectin from bovine plasma.

METHODS

Growth of cells. The isolation, cloning and growth of adult bovine aortic endothelial (ABAE) cells has been previously described (24). For some experiments, ABAE cells were grown in medium supplemented with fetal calf serum which was depleted of fibronectin by successive passages through a gelatin-Sepharose (25) and an anti-fibronectin IgG-Sepharose column.

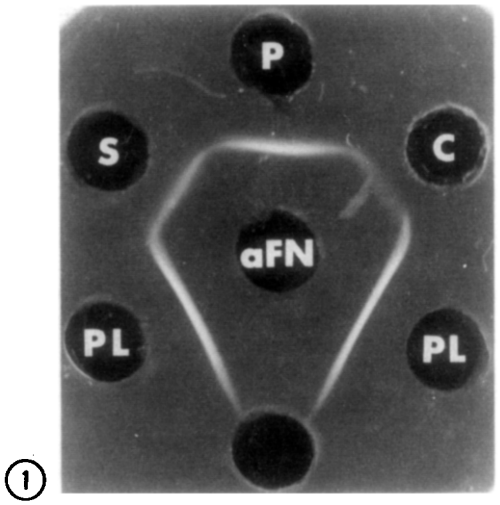
Purification of fibronectin. All buffers and reagents contained phenylmethyl sulfonyl fluoride and Trasylol at 10^{-4} M and 100 Units/ml, respectively, to inhibit proteolysis. Extracellular matrix from ABAE cells was isolated as previously described (21) and then extracted with 6 M Urea, 0.05 M Tris, pH 7.5. The extract was diluted to give 0.5 M Urea, 0.05 M Tris, pH 7.5, and the matrix fibronectin was then purified on a gelatin-Sepharose column as previously described (25).

Secreted fibronectin was purified from the media of ABAE cells grown in the presence of fibronectin - depleted fetal calf serum. Media from confluent monolayers were harvested and centrifuged at 1000 xg for 15 minutes to remove cell debris. Fibronectin was purified on a gelatin-Sepharose column as previously described (25).

Bovine plasma fibronectin was purified as previously described on a gelatin-Sepharose column (25). Because of recent evidence that fibronectin binds to glycosaminoglycans (26, 27), each of the different fibronectins was further purified by DEAE-cellulose chromatography, which has been shown to remove glycosaminoglycans from fibronectin (28).

Preparation of antisera. Antisera were prepared in rabbits against purified bovine plasma fibronectin by subcutaneous injections every two weeks in complete Freund's adjuvant. Antisera were adsorbed by successive passages through a gelatin-Sepharose column and a Sepharose column to which fibronectin-depleted bovine plasma had been covalently coupled. Antibodies specific for fibronectin were then affinity-purified from the adsorbed antisera on a fibronectin-Sepharose column.

Radiolabeling, SDS-polyacrylamide gel electrophoresis and peptide mapping. Purified matrix, secreted and plasma fibronectins were radiolabeled with $Na^{125}I$ in 4.5 M urea, 0.05 M Tris, pH 7.5, by the chloramine T method (29) and then immunoprecipitated



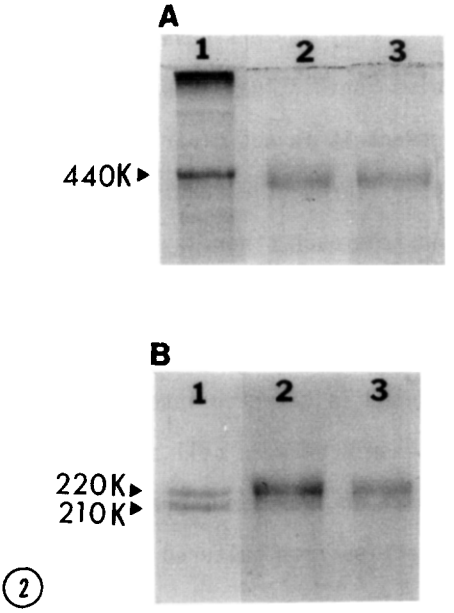


Figure 1. Double immunodiffusion of fibronectin. Cellular (C) and secreted (S) fibronectin were purified from ABAE cells; P (fibronectin purified from bovine plasma); PL (whole bovine plasma); a-FN (affinity-purified IgG specific for bovine plasma fibronectin).

Figure 2. SDS-polyacrylamide gel electrophoresis of fibronectins. A, unreduced samples; B, reduced samples. Lane 1, bovine plasma fibronectin; lane 2 cellular fibronectin from ABAE cells; lane 3, secreted fibronectin from ABAE cells. See Methods for molecular weight determination.

with affinity-purified anti-fibronectin IgG using published procedures (30). The immunoprecipitates were analyzed on 4% SDS-polyacrylamide slab gels as previosly descri (31), in the presence and absence of 2% 2-mercaptoethanol. The following proteins were used as molecular weight standards: ovalbumin (43,000), bovine serum albumin (68,000), phosphorylase B (94,000), B-galactosidase (116,500) and myosin (200,000). Fibronectin bands were carefully sliced from the gel and subjected to two-dimensional peptide mapping as previously described (32).

RESULTS

When purified cellular (matrix) and secreted fibronectins from ABAE cells were compared to purified fibronectin from bovine plasma by immunodiffusion assay, a continous precipitin line was observed, indicating antigenic identity of all three fibronectins (Fig. 1). Similar observations have been made with fibronectins from other cell types (1 - 3).

Analysis of the different fibronectins by SDS - polyacrylamide gel electrophoresis is shown in Fig. 2. When unreduced all three fibronectins migrated as a single

major band of approximate M_r 440,000 (Fig. 2A). The plasma fibronectin (Fig. 2A, lane 1) contained some high molecular weight aggregates which barely entered the resolving gel; this type of aggregation is not unusual for unreduced plasma fibronectin (17, 33). After reduction, plasma fibronectin migrated as two bands of nearly equal amounts with approximate M_r 220,000 (upper band, or band A) and 210,000 (lower band, or band B), respectively (Fig. 2B, lane 1). The cellular and secreted fibronectins migrated as single major bands of approximate M_r 220,000, and very faint bands of approximate M_r 210,000 (Fig. 2B, lanes 2 and 3).

In order to further characterize the different fibronectins, we decided to conduct two-dimensional peptide mapping studies on reduced and unreduced fibronectins, using the technique of Elder et al., (32). This method was chosen because it is a more sensitive technique than CnBr-cleavage and one-dimensional peptide mapping, both of which have been used to study the different fibronectins (89, 16, 20), and also bands can be cut directly from SDS-polyacrylamide gels for analysis. The results of these studies are shown in Fig 3. The peptide map of a tryptic digest of unreduced iodinated plasma fibronectin (Fig. 3A) was identical to the maps of band A (Fig. 3E) and band B (Fig. 3I) isolated from reduced plasma FN. These maps were also identical to those produced by tryptic digests of unreduced secreted fibronectin (Fig. 3C), reduced secreted fibronectin (Fig. 3G) and reduced cellular (matrix) fibronectin (Fig. 3K). Because only a few peptides were produced by tryptic digestion, chymotryptic digests were also performed on reduced and unreduced fibronectins to produce more detailed maps (Fig. 3B, D, F, H, J and L); these maps were also identical to each other. Thus, the cellular and secreted fibronectins have identical structures, at least within the limits of sensitivity of this particular peptide mapping technique. Furthermore, the structure of bands A and B from reduced plasma fibronectin are identical to each other and to the cellular and secreted fibronectins.

DISCUSSION

There has been considerable disagreement over the relationship of cellular, secreted (from cultured cells) and plasma fibronectins to each other. Both

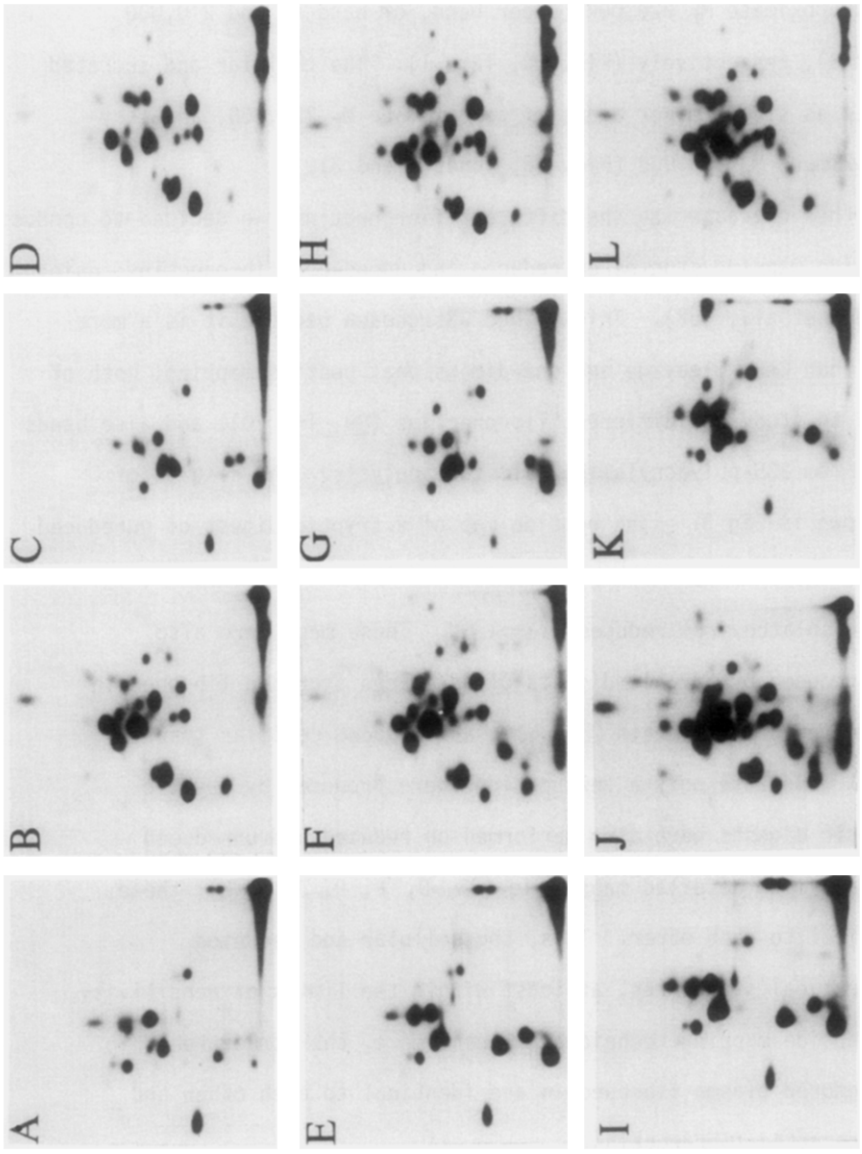


Figure 3. Two-dimensional peptide maps of tyrosine - containing tryptic and chymotryptic peptides of fibronectins. Tryptic maps: A, E, I; C, G, K. Chymotryptic maps: B, F, J; D, H, L. Bovine plasma fibronectin, unreduced - A, B; band A, bovine plasma fibronectin - E, F; band B, bovine plasma fibronectin - I, J. Secreted fibronectin, ABAE cells, unreduced - C, D; secreted fibronectin, ABAE cells, reduced - G, H. Cellular fibronectin, ABAE cells, reduced - K, L. The origin was at the lower right hand corner of each map, with electrophoresis from right to left and chromatography from bottom to top.

similarities (8) and differences (16, 20) between these fibronectins have recently been reported, based on amino acid and carbohydrate composition, migration on SDS-gels, one-dimensional peptide mapping and biological activity. The present study shows that cellular and secreted fibronectins from ABAE cells comigrate as single bands when reduced on SDS-polyacrylamide gels, but bovine plasma fibronectin when reduced migrates as two closely spaced bands on similar gels. Using a sensitive two-dimensional peptide mapping technique, the structures of these fibronectins, including bands A and B of bovine plasma fibronectin, are identical. As far as we know, this is the first report on a comparison between the structures of fibronectin from endothelial cells and from plasma. We reasoned that using cultured endothelial cells would be more appropriate than using fibroblasts for these studies, since endothelial cells probably make a major contribution to the pool of plasma fibronectin.

We do not know why our results indicate no structural differences between cellular, secreted and plasma fibronectins (bands A and B), whereas others have reported differences, but there are several possible explanations: 1) different gel systems with varying degrees of resolution have been used; 2) most of the reported differences are differential susceptibility to various proteases, which may depend on conformational changes that could be undetected by our peptide mapping techniques; 3) most of the previous studies have employed fibroblasts as a source of fibronectin, and perhaps the structural differences are greater between fibroblast and plasma fibronectin than between endothelial and plasma fibronectin; however, there is no evidence at present that there are structural differences between fibronectin from different cell types of the same species.

One theory concerning the secretion of fibronectin from cells is that the insoluble matrix fibronectin is somehow modified, perhaps by enzymatic cleavage of a hydrophobic region in the plasma membrane, to release a soluble fibronectin with a different structure. However, our results have not revealed any differences in structure between these fibronectins in endothelial cells, and thus argue against this proposed mode of fibronectin secretion. Recent results have shown that soluble fibronectin in the culture medium can be incorporated into the

insoluble matrix of fibronectin on the cell surface (34), implying that cellular and secreted fibronectin are in equilbrium and that no structural modification is required for the conversion of insoluble to soluble fibronectin.

The subunits of bovine plasma fibronectin clearly migrated differently on SDS-polyacrylamide gels when reduced, but again we found no differences in structure by peptide mapping analysis. The nature of the differential migration of the two subunits on SDS gels is unknown, but differences in glycosylation may be responsible. It has been shown recently that the subunits of human plasma fibronectin have different susceptibilities to plasmin but not to S. aureus proteinase (20), suggesting another explanation for the difference between the subunits. A difference in protease sensitivity may reflect differences in conformation, which may in turn result from a modification such as glycosylation rather than from differences in the primary structure. More data on amino acid and carbohydrate composition, and amino acid sequence, of the two subunits of plasma fibronectin are needed to resolve this question.

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