Cell Attachment on Replicas of SDS Polyacrylamide Gels Reveals Two Adhesive Plasma Proteins

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ABSTRACT A novel procedure that detects adhesive proteins in complex mixtures was used to characterize such proteins in plasma. The proteins are separated by SDS PAGE and transferred to nitrocellulose filters. Cells incubated on these filters attach to those proteins that have adhesive properties. When applied to human plasma proteins this procedure reveals, in addition to fibronectin, a cell-attachment protein with a polypeptide molecular weight of 70,000. Using a monoclonal antibody that inhibits attachment of cells to fibronectin, we show that this polypeptide is not a fragment of fibronectin and we present evidence that it is a component of the serum spreading factor. Therefore, as defined by our assay, this protein and fibronectin are the major attachment proteins for fibroblastic cells in plasma or serum.

Many important biological phenomena—including morphogenetic migration of cells, wound closure, and tumor metastasis—involve the ability of cells to establish and to break adhesive interactions with the extracellular substratum or with other cells. That many normal cells must adhere and spread to survive in vitro reflects the importance of these interactions. Serum contains factors that are required for cell adhesion and spreading (1). Fibronectin (2, 3), the best characterized adhesive serum protein, promotes attachment of many types of cells (4–6). However, several observations suggest that fibronectin is not the only adhesive protein in serum or plasma (7–11). Serum has been shown to promote the attachment of chondrocytes by a fibronectin-independent mechanism (7, 8). In addition, 60,000–80,000-dalton serum proteins that promote cell attachment and spreading have been identified (4, 9–12).

To analyze the occurrence and relationships of adhesive proteins in plasma, we studied the attachment of fibroblastic cells to plasma proteins that have been separated by SDS PAGE and transferred to nitrocellulose filters. We show here that this technique reveals in plasma, in addition to fibronectin, another major attachment protein with a molecular weight of 70,000.

MATERIALS AND METHODS

Cell Culture

Normal rat kidney (NRK) cells (13) were grown in Dulbecco's minimal essential medium (DME) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Flow Laboratories, Inc. Rockville, MD).

Isolation of Fibronectin and Serum Spreading Factor

Fibronectin was purified from human plasma using affinity chromatography on gelatin-Sepharose (14). Serum spreading factor was isolated from human plasma or serum by chromatography on glass bead columns (15).

Depletion of Albumin and IgG from Human Plasma

Human plasma was depleted of most of its albumin and IgG by passing it through Affi-gel Blue (Bio-Rad Laboratories, Richmond, CA) and staphylococcal protein A-Sepharose (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) columns according to the manufacturers' instructions.

Electrophoresis and Transfer of Proteins

Proteins were separated on a slab gel of 7% polyacrylamide in the presence of SDS and 2-mercaptoethanol (16), and the polypeptides were transferred to nitrocellulose following the procedure of Bowen et al. (17) as described (18). Protein bands on the gel were detected by staining with Coomassie Blue and on the filter by staining with amido black (17, 18). The molecular weight standards used were: myosin, 200,000; phosphorylase b, 94,000; and albumin, 67,000.

Cell Attachment to Filter

A filter with transferred protein was rinsed in phosphate buffered saline (PBS) and soaked for 16 h in 500 ml of PBS containing 5 mg/ml bovine serum albumin (BSA), to prevent nonspecific cell attachment to uncoated filter paper. The filter was then washed with PBS and placed in a flat-bottom dish, blotted side up. A single-cell suspension in DME (10^7 cells/50 ml) of NRK cells, which had been prepared using crystalline trypsin (Sigma type III, Sigma Chemical Co., St. Louis, MO) and washed with soybean trypsin inhibitor (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) as described (13), was added to the filter and incubated for 1 h at 37°C. The filter was washed with PBS, fixed with

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3% paraformaldehyde, stained for 1–2 min with 0.1% amido black (Sigma Chemical Co.) in 45% methanol, 10% glacial acetic acid, 45% deionized water, and destained in 90% methanol, 2% acetic acid, and 8% water. With this staining procedure, cells that are attached to the protein bands give dark blue bands on a pale blue background.

Cell Attachment in Microtiter Wells

The cell attachment in microtiter wells has been described in detail previously (13). Briefly, the wells were coated with 10 μg/ml of human plasma fibronectin or a preparation of serum spreading factor. The nonbound protein was removed by washing and some of the wells were incubated with either rabbit anti-human fibronectin serum (13) diluted 1:100 or purified monoclonal antibodies (100 μg/ml). One of the monoclonal antibodies used here, 3E3, is directed against the cell attachment site of fibronectin; the other, 4B2, is directed against a site near the gelatin-binding domain of fibronectin (19). After washing, NRK cells prepared as described above were added to the wells. Nonattached cells were removed after 1 h by washing with PBS, and attached cells were fixed, stained, and counted.

RESULTS

Human plasma was first depleted of most of the albumin and IgG by chromatography on Affi-gel Blue and staphylococcal protein A-Sepharose, thereby increasing the relative amounts of the other plasma proteins in the samples to be analyzed. The nonbound proteins were separated by SDS PAGE and the polypeptides were transferred from the gel to cellulose nitrate filters as described in Materials and Methods. The protein bands revealed by Coomassie Blue staining of the original gel were identical to those revealed by amido black in the blotted filter. Thus, only the original gel pattern is shown (Fig. 1 *a*). Two bands were visualized by staining with amido black after allowing suspended NRK cells to attach to the filter. One band corresponded to a molecular weight of 220,000 and the other to 70,000 slightly larger than albumin (Fig. 1 *b*). Microscopy showed that these bands contained cells that were attached and spread, whereas the unstained areas were mostly devoid of cells (Fig. 1 *c–e*). Staining of individual protein bands was obscured by the uniform staining of the BSA that coats the filter.

The 220,000-dalton band detected by cell attachment to the filters corresponded to the position of fibronectin. Plasma or serum samples depleted of fibronectin by chromatography on gelatin-Sepharose lacked this band. The lower band active in our attachment assay is of a similar size as serum spreading factor (11, 12), an adhesive glycoprotein. Serum spreading factor can be isolated from serum or plasma by chromatography on glass bead columns (12, 15) and can be recovered in an active form after electrophoresis in the presence of SDS (20). Consequently, we wanted to investigate whether the 70,000-dalton adhesive component we detect in plasma with the present technique is related to serum spreading factor. The factor was isolated on columns of glass beads (15) and analyzed

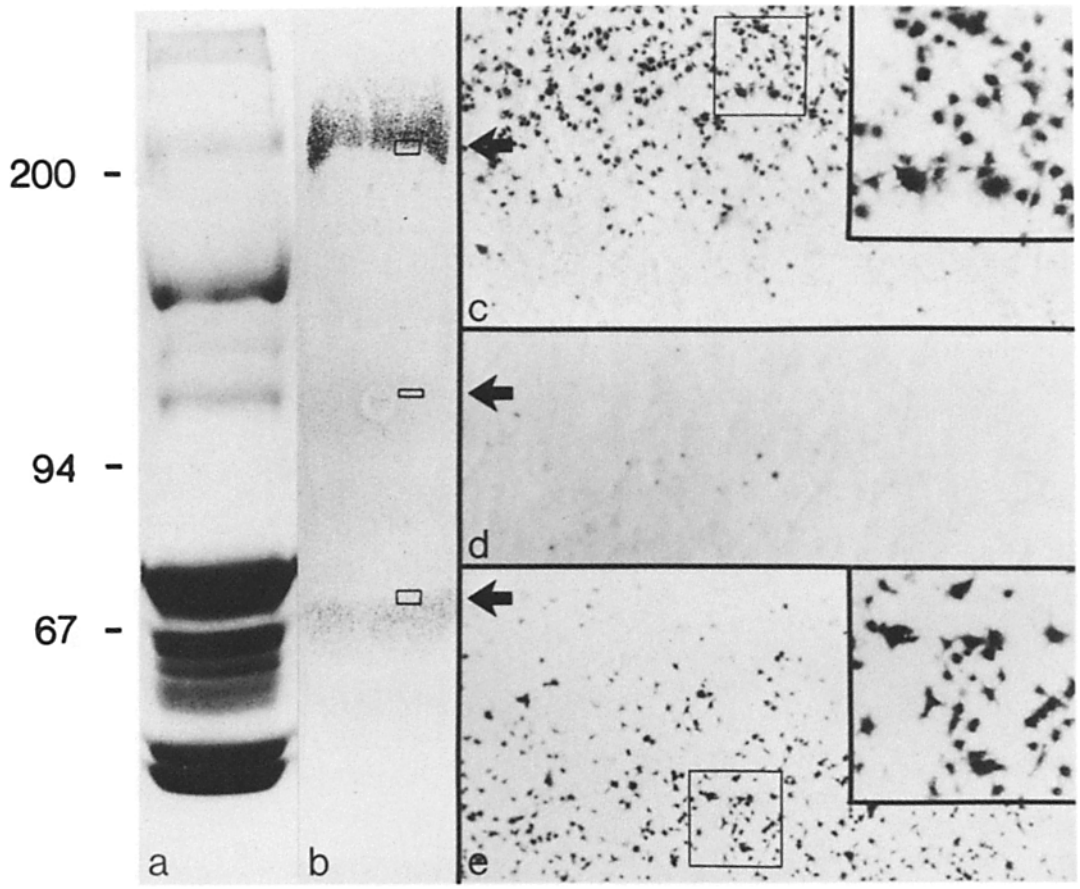

FIGURE 1 Attachment of NRK cells to plasma proteins separated by gel electrophoresis and transferred to nitrocellulose filters. Human plasma proteins (120 μg), after chromatography on Affi-gel Blue and protein A-Sepharose, were separated on a slab gel in the presence of SDS and 2-mercaptoethanol, and the polypeptides were transferred to nitrocellulose filters. (*a*) A gel stained with Coomassie Blue. (*b*) The corresponding filter after attachment of cells to proteins on the filter and staining of the attached cells. 200, mol wt 200,000; 94, mol wt 94,000; 67, mol wt 67,000. (*c*, *d*, and *e*) Photomicrographs of cells attached to the protein bands in (*b*). × 33. Areas from the margins of the bands are shown. The two active bands (*c* and *e*) are covered with a carpet of attached and spread cells, whereas the areas outside these bands (*d*) are mostly devoid of cells. The *insets* show the morphology of the attached cells at a higher magnification × 66.

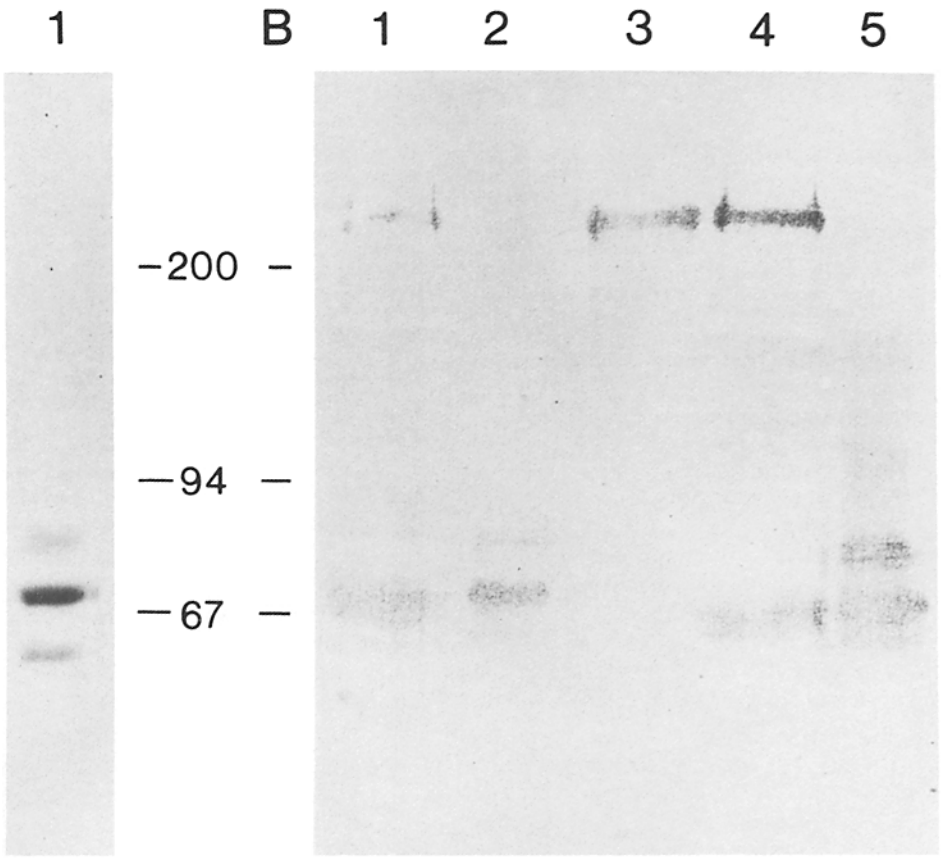

FIGURE 2 Attachment of cells to proteins separated by SDS PAGE and transferred to nitrocellulose filters. The procedure and molecular weight markers were the same as in Fig. 1. 200, mol wt 200,000; 94, mol wt 94,000; 67, mol wt 67,000. (*A*) A gel strip stained with Coomassie Blue showing a preparation of serum spreading factor (60 μg) isolated on a glass bead column. (*B*) Cell attachment on a nitrocellulose filter blotted from a gel similar to that in *A*. Lanes *1* and *4*, plasma proteins (120 μg and 240 μg/ml, respectively) after chromatography on Affi-gel Blue and protein A-Sepharose; lanes *2* and *5*, isolated serum spreading factor, 30 and 60 μg, respectively; lane *3*, purified human plasma fibronectin (5 μg).

by gel electrophoresis. In accordance with earlier results (11), a major polypeptide at 70,000 and minor components at ~80,000, 62,000, and 47,000 daltons, as well as some minor low molecular weight bands, were seen in such preparations by staining of the polyacrylamide gel for protein (Fig. 2*A*). The major component at 70,000 and the minor component at 80,000 daltons supported cell attachment (Fig. 2*B*). In addition, another minor band to which cells attached was visible at 65,000 daltons. The main component in the eluate from the glass bead column coincided with the 70,000-dalton cell adhesive band found in plasma. The minor active components seen in isolated serum spreading factor preparations were not seen in plasma using this technique, but they may be present in amounts below the detection limit of the assay.

Previous studies have shown that serum spreading factor does not react with rabbit antibodies to fibronectin when tested in immunodiffusion (11). However, the cell-attachment function is retained in fibronectin fragments of different sizes ranging from the nearly intact 220,000-dalton subunit down to 15,000 daltons whereas antigenicity is diminished (19). It was, therefore, deemed necessary to confirm that the 70,000-dalton band is not a fragment of fibronectin. To study this, we used a recently developed monoclonal antibody that specifically inhibits the attachment of cells to fibronectin (19).

The cell attachment-inhibiting monoclonal antibody, as well as polyclonal antibodies to fibronectin, inhibited the attachment of cells to fibronectin, but not to the isolated serum spreading factor (Fig. 3). Since these antibodies also prevent attachment of cells to fibronectin fragments (unpublished results), this confirms that serum spreading factor is not derived from or immunologically related to the cell-attachment domain of fibronectin.

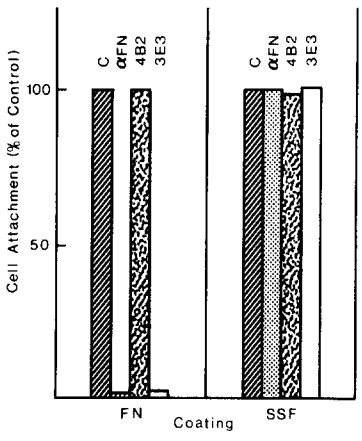

FIGURE 3 Cell-attachment assay in microtiter wells. The wells were coated with 10 μg/ml of fibronectin (*FN*) from human plasma or a preparation of serum spreading factor (*SSF*). Normal rabbit serum (*C*), rabbit antiserum to human fibronectin (*αFN*), *4B2* monoclonal antibody which reacts near the collagen-binding domain of fibronectin and has been shown previously not to inhibit attachment of cells, and 3E3 antibody which reacts at the cell attachment site of fibronectin and inhibits cell attachment were incubated in the coated wells prior to cell attachment.

DISCUSSION

Of all the plasma proteins separated by the gel electrophoresis, fibronectin and serum spreading factor were the only ones that attracted cells to attach and spread. This reveals the specificity of our method for cell-attachment proteins. Klebe et al. (21) have used an assay somewhat similar to this to detect collagen-dependent cell attachment proteins after electrophoresis on cellulose acetate. The method we have developed allows detection of adhesive proteins whether or not they bind to collagen, and it also has the higher resolution of SDS PAGE. A possible limitation of the technique is that not all adhesive proteins may recover sufficiently from the treatment with SDS (and reducing agent, if employed). That we can detect the cell attachment activity of two plasma glycoproteins as well as that of collagen (unpublished results) with this method suggests that this may not be a frequently encountered problem. It is likely that the analytical resolution of the procedure could be further improved by using two-dimensional gel electrophoresis.

It appears from our studies that fibronectin and serum spreading factor are the two main plasma proteins adhesive for fibroblastic cells. The biological role of these proteins is not known but both fibronectin and serum spreading factor have been shown to affect morphology, proliferation rate, and differentiation capacity of a variety of cultured cells (2, 3, 7, 11, 15, 20, 22–24). In some cases serum spreading factor can mediate biological effects that are distinct from those of fibronectin (11, 20). Whereas the molecular characteristics of fibronectin have been extensively studied (2, 3), serum spreading factor has only been partially purified. Our results provide a functional identification of this protein as a 70,000-dalton polypeptide with some size heterogeneity that becomes apparent after isolation. The identification of its polypeptides should facilitate the characterization of this potentially important protein. The application of our method to biological samples other than plasma and the use of several cell types may allow detection of new proteins involved in cell-type specific adhesion and recognition.

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