Characterization of a Membrane-Associated Glycoprotein Complex Implicated in Cell Adhesion to Fibronectin

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We have characterized a 140-kDa glycoprotein complex purified by a monoclonal antibody and implicated in cell adhesion to the extracellular molecule fibronectin. Three major polypeptide components were purified by monoclonal antibody JG22E, which had apparent molecular weights of 155,000 (band 1), 135,000 (band 2), and 120,000 (band 3). In two-dimensional gel electrophoresis, each subunit migrated as either a broad band or a series of spots at acidic isoelectric points. After treatment with neuraminidase, the spots became focused around pH 6.2 (band 1), pH 5.6 (band 2), and pH 5.3 (band 3). These three major bands were compared by two-dimensional peptide mapping in a series of pairwise combinations and were found to be distinct proteins. In sucrose gradients, these proteins co-migrated as a complex sedimenting at approximately 8.4 S either before or after affinity purification, whereas separated subunits migrated at 4.7 to 5.8 S. Amino acid analysis revealed no detectable hydroxyproline and a composition characterized by a substantial number of cysteine residues compared to the average protein. Our results suggest that a noncovalent complex of structurally distinct glycoproteins is involved in adhesive interactions of fibronectin with cells.

Cell adhesion is a complex process involving a variety of possible molecular mechanisms [1,2]. One approach to analyzing adhesion has been to characterize isolated extracellular proteins such as fibronectin that mediate cell-to-substrate adhesion [3–9]. A complementary approach is to identify membrane proteins essential for adhesion by the use of inhibitory monoclonal antibodies [10–14]. The JG22E antibody inhibits fibroblast attachment and spreading on fibronectin [35]. In immunofluorescence assays, the antigen co-localizes with fibronectin in attachment sites of fibroblasts. In analogy to fibronectin itself, this otherwise inhibitory monoclonal antibody can also serve as an adhesive molecule if attached to substrates [35]. The JG22E

antigen has therefore been suggested to be involved in the mechanisms of cell interaction with fibronectin, eg, possibly as a transmembrane mechanism of interaction with this adhesion protein.

In this study, we have characterized the proteins purified by the JG22E antibody. Our results indicate the existence of a noncovalent complex of distinct polypeptides involved in cell adhesive interactions with fibronectin.

MATERIALS AND METHODS

Preparation of Monoclonal Antibody Affinity Columns

The hybridoma line JG22E was derived from the original cell line JG22 [12] by subcloning twice and adapting to growth in serum-free medium. JG22E hybridomas were cultured in serum-free HB101 medium (Hana Biologics, Berkeley, CA) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, penicillin (50 U/ml), and streptomycin (50 μg/ml), all purchased from Gibco Laboratories (Grand Island, NY). Monoclonal antibody JG22E was prepared from culture supernatants by ammonium sulfate precipitation (50% saturated solution) and DEAE-cellulose chromatography. Approximately 300 mg of purified antibody was obtained from 5 liters of culture supernatant. The antibody was coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) at a ratio of 10 mg antibody per 1 ml of packed beads.

We generated a new hybridoma line, ET22, which secretes monoclonal antibodies specific for band 1a (see text for nomenclature). The ET22 line was produced by hybridization of the Y3 rat myeloma cell line with spleen cells from a Sprague-Dawley rat immunized with purified JG22E antigen, following the methods of Furth et al [15]. Specificity of the ET22 monoclonal antibody was determined by Western immunoblotting (data not shown). ET22 hybridomas were cultured in serum-free medium consisting of a 1:1 mixture of Dulbecco's minimal essential medium (MEM) and Ham's F12 medium supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml) (CR-ITS Premix), and charcoal extracted bovine serum albumin (0.4 mg/ml), obtained from Collaborative Research (Lexington, MA), plus 2 mM glutamine, 1 mM sodium pyruvate, penicillin (50 U/ml), and streptomycin (50 μg/ ml), obtained from Gibco. Purification of the ET22 monoclonal antibody and coupling to CNBr-activated Sepharose 4B were performed as described for JG22E.

Preparation of JG22E Antigen

The JG22E antigen was isolated by a protocol modified from previous methods [12,13,35]. After removal of heads and viscera, 36 13-day-old chick embryos were homogenized in a Waring blender at maximum speed at 4°C for 2 min in 400 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 0.25 sucrose, 0.5 mM $CaCl_2$, 1 mM PMSF, and 1 mM benzethonium chloride. The homogenate was filtered through three layers of cheesecloth, and the filtrate was further homogenized in a Dounce homogenizer in ice with 20 strokes of the tight pestle. A crude membrane fraction was obtained after serial centrifugations at 1,900g for 10 min (discarding the pellet), and 150,000g for 60 min. The membrane pellet was extracted for 60 min at 4°C with 50 ml of a 10 mM Tris-HCl buffer, pH 7.5, containing 40 mM octyl-β-glucopyranoside (OGP; Calbiochem, San Diego, CA), 0.5 mM $CaCl_2$, and 1 mM phenylmethyl-sulfonylfluoride (PMSF), followed by centrifugation at 150,000g for 30 min.

Sodium acetate buffer, pH 5.5, was added to the supernatant solution to 30 mM, and the pH was corrected to pH 5.5 with 5 M acetic acid. The resultant acid precipitate was removed by centrifugation at 12,000g for 20 min. After neutralization by addition of 1 M Tris-HCl, pH 7.5, to a final concentration of 30 mM, and adjusting the pH to 7.5 with 2 N HCl, the supernatant solution was incubated with JG22E-Sepharose 4B (5-ml packed bead volume) overnight at 4°C with gentle agitation. The beads were packed in a column and washed with 200 ml of the extraction buffer. The antigen was then eluted with 50 mM diethylamine, pH 11.3, containing 40 mM OGP, 0.5 mM CaCl$_2$, and 1 mM PMSF. Fractions containing antigen were identified by absorbance at 280 nm. After repeating the acid precipitation step at pH 5.5, the final yield of purified antigen was approximately 400 μg. In some cases, the antigen was further purified by re-chromatography on JG22E-Sepharose 4B or by chromatography on ET22-Sepharose 4B. For sugar analysis, the antigen was purified using 1% Nonidet P-40 instead of OGP.

Sucrose Density Gradient Centrifugation

Samples were applied to density gradients containing 20.0–29.1% sucrose in 0.1 M NaCl, 40 mM OGP, 1 mM CaCl$_2$, 3 mM NaN$_3$, and 10 mM Tris-HCl, pH 7.5. The gradient was formed by an LKB Ultrograd gradient maker and was designed to maintain the parameter rx $(\rho_p - \rho_s)/\eta_s$ constant, where r = distance from the center of the rotor, ρ_p = density of the solute (assumed to be 1.3 g/cm^3), ρ_s = density of the solution, and η_s = viscosity of the solution. Centrifugation was performed in a Beckman SW-40 swinging bucket rotor at 40,000 rpm for 22 hr at 15°C. After centrifugation, 0.5-ml fractions were collected. Each fraction was analyzed by sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis or was subjected to analytical affinity chromatography on 0.8 × 2.0-cm columns of JG22E-Sepharose 4B. Sedimentation coefficients for samples were estimated by using human transferrin (5.5 S), rat IgG (7.0 S), aldolase (7.6 S), and glucose oxidase (7.9 S) as standards.

Amino Acid Analysis

Samples were lyophilized after exhaustive dialysis against H$_2$O. They were hydrolyzed in 6 N HCl at 110°C for 24, 48, and 72 hr. Each hydrolysate was analyzed in duplicate with a Beckman Labotron Liquimat 4 amino acid analyzer. For analysis of cysteine, material was oxidized with a 1:9 mixture of 30% hydrogen peroxide and 90% formic acid before hydrolysis. Samples for amino acid analysis are shown in Figure 1, lane 1.

Western Immunoblotting

Blotting was performed as described by Towbin et al [16] with slight modifications. After SDS gel electrophoresis in nonreducing conditions, proteins were transferred onto nitrocellulose sheets in 5 mM sodium borate, pH 9.2, in a Trans-blot apparatus (Bio-Rad, Richmond, CA) for 2 hr at 4°C. The nitrocellulose sheets were incubated overnight in phosphate-buffered saline containing 50 mg/ml bovine serum albumin, and then stained with 3 × 10^6 cpm/ml ^{125}I-JG22E (2.8 × 10^5 cpm/μg). JG22E monoclonal antibody was iodinated by the chloramine T method [17].

Other Procedures

For treatment with neuraminidase, purified JG22E antigen (200 μg) was incubated with 0.2 U of Clostridium perfringens neuraminidase (type X, Sigma, St.
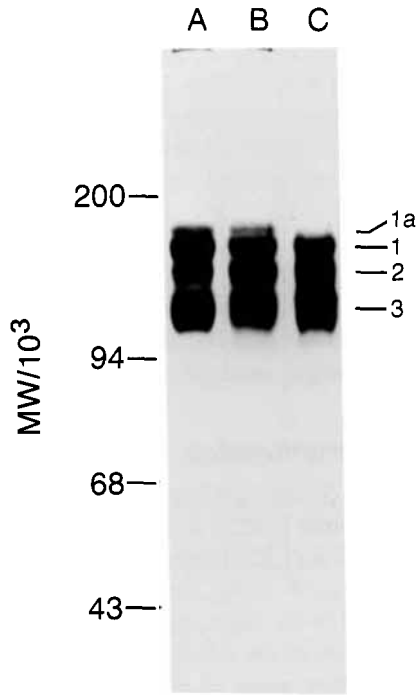
Fig. 1. SDS gel electrophoresis of affinity-purified JG22E antigen. The antigen was purified from chick embryos as described in Materials and Methods. Samples (40 μg/lane) were subjected to SDS gel electrophoresis under nonreducing conditions. Lane A) The antigen purified by affinity chromatography on JG22E-Sepharose 4B. Lane B) The antigen re-chromatographed on JG22E-Sepharose 4B. Lane C) Flow-through fraction from affinity chromatography of the sample shown in lane B on ET22-Sepharose 4B.

Louis, MO) at 25°C for 14 hr in 0.1 M sodium acetate, pH 5.0, 40 mM OGP, 1 mM EDTA, and 1% Aprotinin (Sigma).

Neutral sugars were analyzed by the method of Dubois et al [18] using mannose as a standard.

Two-dimensional gel electrophoresis was performed by the method of O'Farrell [19], except that samples were treated with 1% SDS before the addition of urea, Ampholine (LKB, Gaithersburg, MD), and NP-40 [20]. Two-dimensional tryptic peptide mapping was performed exactly as described previously [21,22]. SDS polyacrylamide gel electrophoresis was performed in 1-mm-thick slab gels with 4% stacking and 7.5% resolving gels by the method of Laemmli [23]. Molecular weights were estimated by using myosin heavy chain (200,000), phosphorylase b (94,000), bovine serum albumin (68,000), and ovalbumin (43,000) as standards. Protein concentrations were determined by the method of Lowry et al [24] using bovine serum albumin as a standard.

RESULTS

As shown in Figure 1, the JG22E monoclonal antibody purifies a set of three major polypeptides (bands 1, 2, and 3) and one minor polypeptide (band 1a) from the crude membrane fraction of chick embryo homogenates. After re-chromatography on immobilized JG22E, the three major polypeptides were still co-purified. However, the ratio of band intensities often changed, with decreased intensities of bands 1 and 3 compared to band 2 (Fig. 1, lane B). The amount of band 1a was also significantly reduced after re-chromatography. The three major polypeptides could be completely separated from band 1a by passage through an affinity column containing an anti-band 1a monoclonal antibody, ET22, (Fig. 1, lane C), suggesting the absence of strong interactions between band 1a and bands 1-3.

We characterized the three major bands by two-dimensional gel electrophoresis and two-dimensional peptide mapping. In two-dimensional gels, the three major polypeptides migrated as broad bands with isoelectric points ranging from 4.4 to 6.0 (Fig. 2A). After treatment with neuraminidase, these polypeptides were much more focused at distinctive values of pH 6.2 (band 1), pH 5.6 (band 2), and pH 5.3 (band 3) (Fig. 2B).

Although they were distinct in size and isoelectric point, these polypeptides could theoretically still have been related, eg, by proteolytic processing from one to another. Bands 1-3 were excised from SDS polyacrylamide gels, iodinated, and subjected to tryptic two-dimensional peptide mapping. In pairwise comparisons of the maps of each band, most tryptic peptides were unique, with few in common between the different bands (Fig. 3). Mixing experiments confirmed the absence of overlap between most spots in the maps of different bands (Fig. 3D-I). These polypeptide bands therefore appear to be distinct moieties, without evidence for proteolytic interconversion.

Three major polypeptides could have been co-purified either because they form a noncovalent complex or because the antibody recognizes an epitope on each individual polypeptide. To examine for a complex, the affinity-purified antigen was analyzed on sucrose density gradients (Fig. 4A). At least half of the molecules co-sedimented in a putative complex at 8.4 S as estimated by comparisons with protein standards. Individual subunits sedimented more slowly at approximately 4.7 to 5.8 S (Fig. 4A). Similar results were obtained in three other repetitions of this experiment.

One possible explanation for the existence of free subunits after the initial preparative affinity chromatography step (and the alteration in band ratios in re-chromatographed material shown in Fig. 1) was that a fraction of the subunits became dissociated from purified complexes during treatment at the strongly alkaline pH of 11.3, which was required for elution of the antigen from affinity columns. To test this hypothesis, the sequence of affinity chromatography and sucrose gradient analysis steps were reversed, so that complexes and possible free subunits would be separated on sucrose gradients before isolation of the JG22E antigen.

Affinity chromatography of each fraction following a sucrose gradient analysis of detergent extracts revealed only the putative complex of bands 1, 2, and 3 migrating at 8.2 S, a rate virtually identical to that of the putative complex from purified material (Fig. 4B). No evidence of binding of excess free subunits to the antibody was detected, either as individual bands of free subunit, or as altered ratios of bands at different points in the gradient (Fig. 4B).
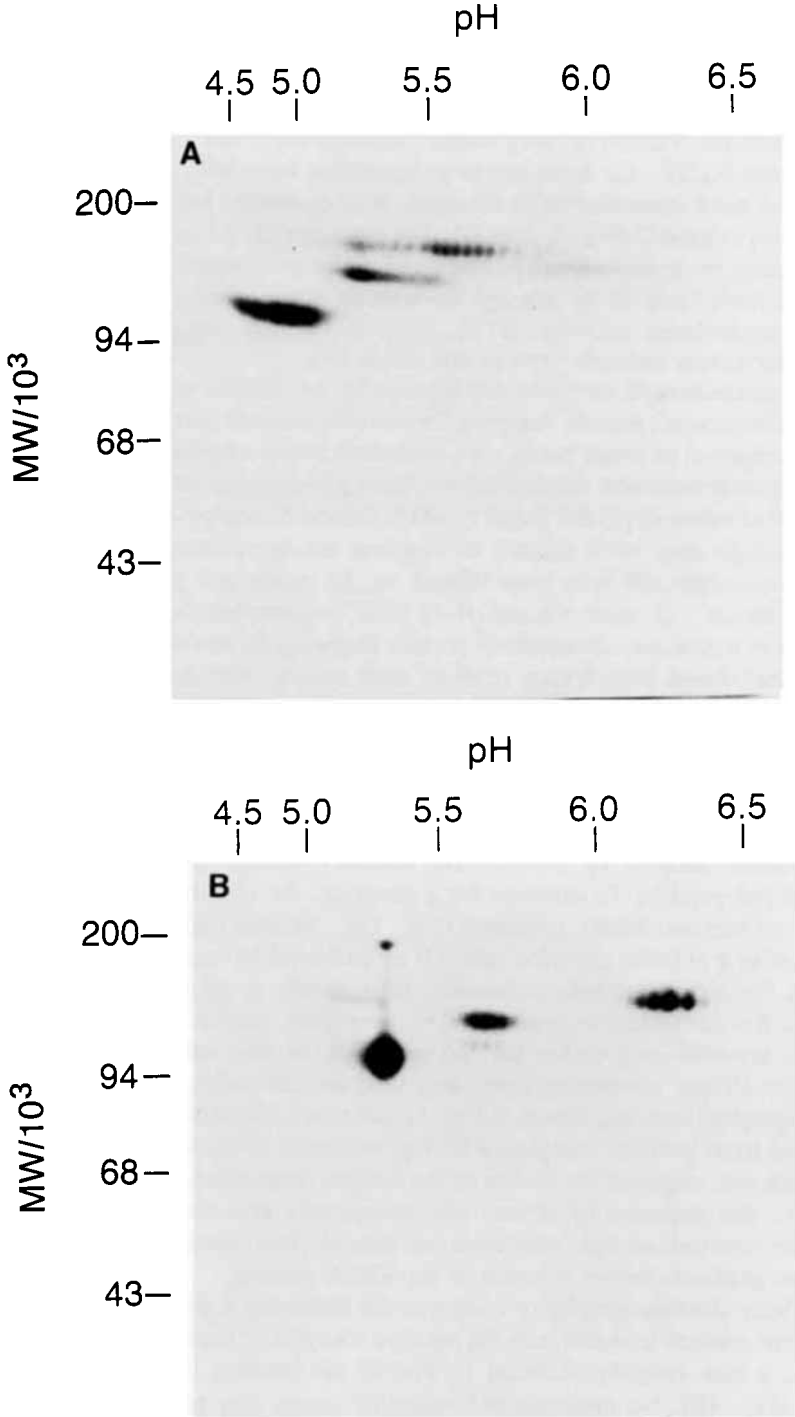
Fig. 2. Two-dimensional gel electrophoresis of purified JG22E antigen. Samples (80 μg) were subjected to 2-D gel electrophoresis under nonreducing conditions. A) the antigen re-chromatographed on JG22E-Sepharose 4B. B) the same sample as A treated with neuraminidase as described in Materials and Methods.
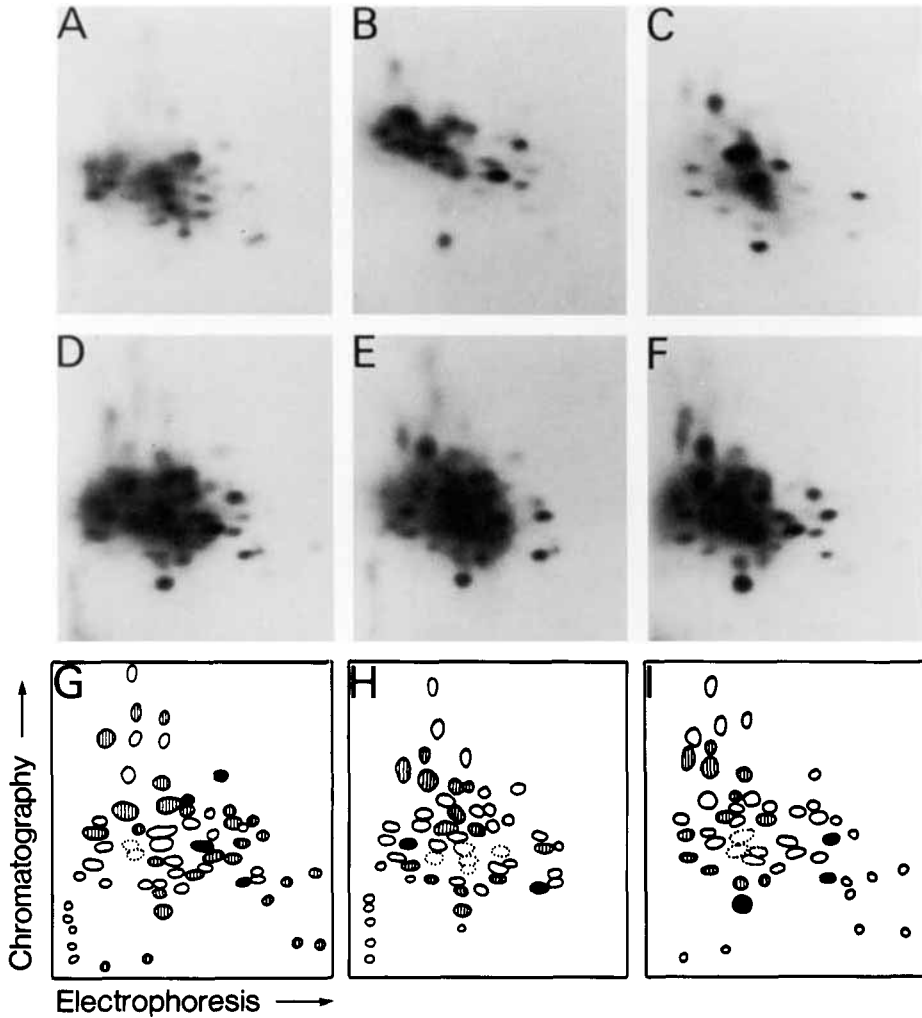
Fig. 3. Tryptic peptide maps of JG22E antigen. After SDS gel electrophoresis, each band of the antigen was excised, iodinated, and trypsinized. Samples of 8×10^5 cpm (A-C) and 1.6×10^6 cpm (D-F) were subjected to two-dimensional peptide mapping, followed by autoradiography with exposure for 25 hr. A) band 1; B) band 2; C) band 3; D) 1:1 mixture of band 1 and band 2; E) 1:1 mixture of band 1 and band 3; F) 1:1 mixture of band 2 and band 3; G) schematic diagram of D, where open spots enclosed by a continuous line are from band 1, shaded spots are from band 2, filled spots are superimposable spots from both bands, and open spots enclosed by a dotted line are of uncertain origin; H) schematic diagram of E, where open spots enclosed by a continuous line are from band 1, shaded spots are from band 3, filled spots are superimposable spots, and open spots enclosed by a dotted line are of uncertain origin; I) schematic diagram of F, where open spots enclosed by a continuous line are from band 2, shaded spots are from band 3, filled spots are superimposable spots, and open spots enclosed by a dotted line are of undetermined origin.

In attempts to identify the antigenic site for JG22E, Western immunoblotting was performed as described in Materials and Methods; no binding of antibody to blots was detected after the denaturation in SDS and electroblot transfer. We therefore
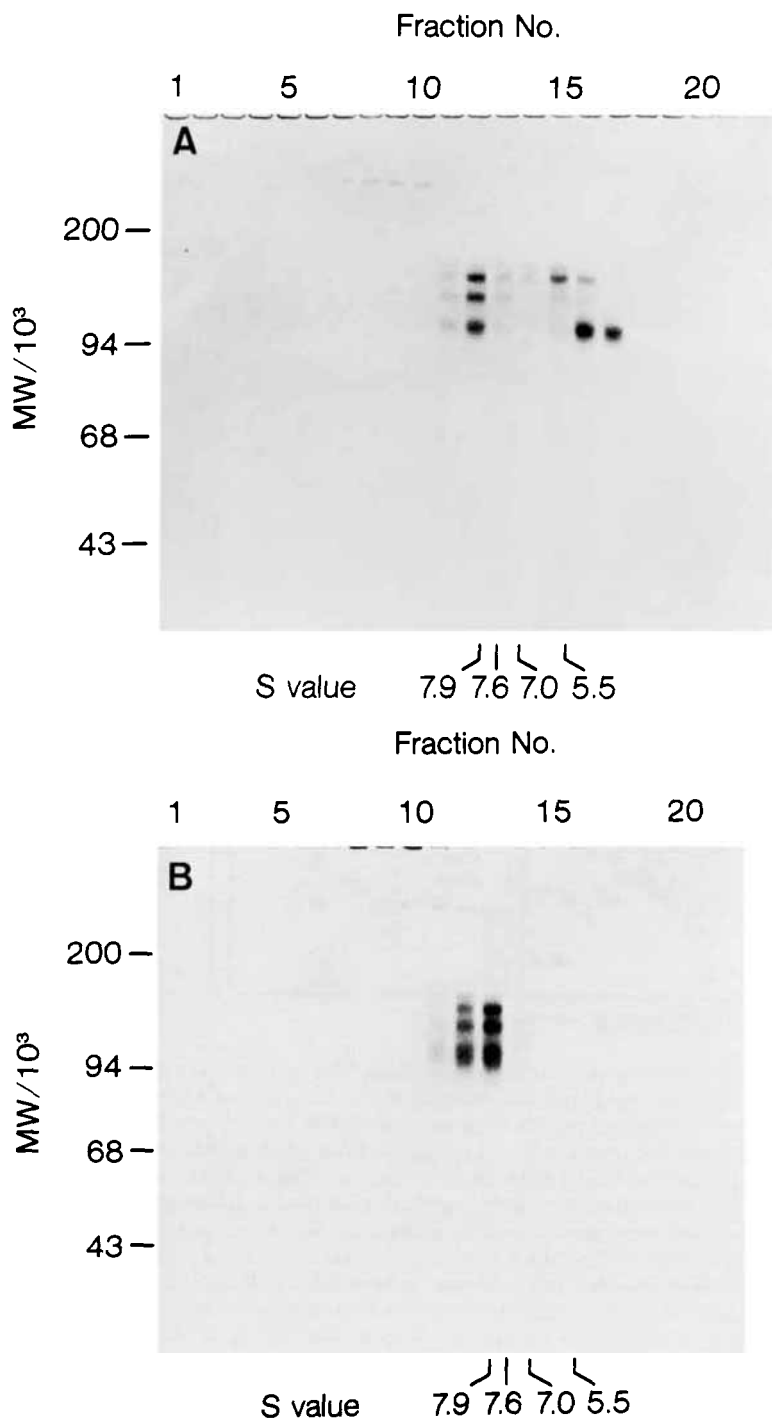
Fraction No.

Fig. 4. Sucrose density gradient centrifugation analysis of JG22E antigen. A) affinity-purified JG22E antigen (shown in Fig. 1, lane A) was subjected to sucrose density gradient centrifugation as described in Materials and Methods. After centrifugation, an equal volume of each fraction was analyzed by SDS gel electrophoresis. B) crude membrane extract was first subjected to sucrose density gradient centrifugation, and then each fraction was subjected to affinity chromatography on JG22E-Sepharose 4B as described in Materials and Methods. Material bound to each affinity column was analyzed by SDS gel electrophoresis.
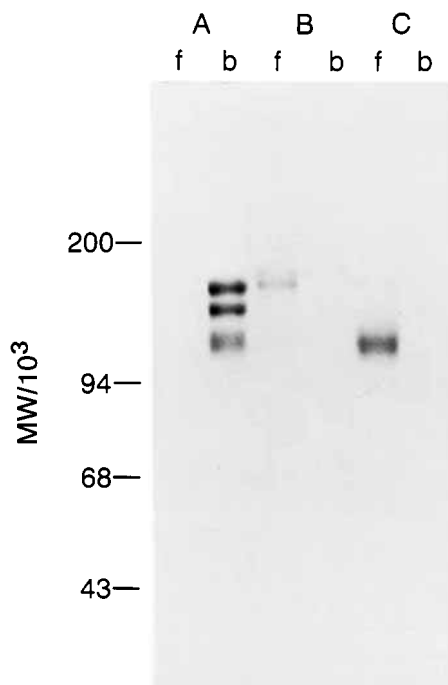
Fig. 5. Analysis of antigenic sites of JG22E. Affinity-purified JG22E antigen was fractionated by sucrose density gradient centrifugation as shown in Figure 4A. Three fractions containing bands 1-3 (A), bands 1a and 1 (B), and band 3 (C) were subjected to analytical affinity chromatography on JG22E-Sepharose 4B. Both flow-through (f) and bound (b) fractions from each affinity column were analyzed by SDS gel electrophoresis. See Figure 1 for identifications of bands.

examined the binding of free subunits to JG22E in comparison to binding of the putative intact complex by analytical affinity chromatography of fractions from sucrose gradients of affinity-purified antigen treated at pH 11.3 (Fig. 4). The separated components, ie, bands 1a, 1, and 3, did not bind to immobilized JG22E, whereas the intact complexes containing bands, 1, 2, and 3 readily re-bound to the JG22E affinity matrix (Fig. 5). These results suggested that the monoclonal antibody does not recognize free bands 1 or 3, but binds to band 2, or to a protein complex containing band 2.

The protein complex purified by JG22E was subjected to three-point amino acid analysis (Table I). There was an average of 49 cysteine residues per mol of 140-kDa glycoprotein complex, which is a relatively high value consistent with the marked alterations in migration of bands after reduction by dithiothreitol [25,26,35]. No hydroxyproline could be detected, a finding consistent with the absence from these preparations of collagen and the collagen-related 140-kDa glycoprotein described by Carter [27].

Neutral sugar analysis of the isolated complex indicated the presence of 5.6% carbohydrate.

TABLE I. Amino Acid Composition of JG22E Antigen*

Amino acid	Mol %	Residues per mol of 140-kDa glycoprotein complex
Asx	10.5	134
Thr	5.4	69
Ser	6.7	86
Glx	11.7	150
Pro	5.1	65
Gly	8.4	108
Ala	7.4	95
Val	6.0	76
Met	2.7	34
Ile	4.6	59
Leu	8.2	104
Tyr	3.2	41
Phe	4.0	52
Lys	5.7	73
His	2.0	26
Arg	4.6	58
Cys	3.8	49
Hyp	ND[a]	ND

*Amino acid analysis was performed as described in Materials and Methods. Tryptophan was not determined.
[a]ND, None detected.

DISCUSSION

The JG22E monoclonal antibody was previously shown to inhibit fibronectin-mediated adhesion, yet was also found to be capable of mediating cell adhesion if attached to substrates [35]. This dualistic activity is also displayed by fibronectin itself, which is an adhesive protein that also inhibits cell adhesion if present in excess in solution [28–30], presumably because of its binding to a fibroblast receptor of moderate affinity [31]. Because it also co-localizes with fibronectin in fibroblast adhesion sites, this monoclonal antibody was suggested to recognize an antigen closely associated with fibronectin receptor function in fibroblasts [35]. This study further characterizes this interesting antigen.

Our new conclusions are: (1) The polypeptides purified by the JG22E monoclonal antibody are distinct by two-dimensional peptide mapping criteria, and cannot be accounted for by simple proteolytic processing of one band to another. (2) The isoelectric points of the bands also differ, and are acidic; all show heterogeneity of charge that is reduced substantially after treatment with neuraminidase, indicating the presence of sialic acid-containing carbohydrates on all three components. (3) The three major components purified by JG22E migrate as a large complex on sucrose gradients, whereas separated subunits migrate as apparent monomers. (4) Amino acid analyses rule out a relationship to collagenous proteins; they reveal a large amount of cysteine residues, consistent with known substantial alterations in electrophoretic mobility after reduction of disulfide bonds [25,26,35]. Neutral sugar analysis reveals a moderate amount of glycosylation (5–6% by weight).

The demonstration that the JG22E antigen exists as a complex of unique polypeptides suggests the existence of considerable complexity in the cell surface molecules associated with fibronectin binding or function. Heterogeneity in SDS gel patterns of components purified by monoclonal antibodies CSAT, JG9, and JG22, which have similar effects as JG22E on myoblast attachment to gelatin, has been reported previously [25,26,32]. Chapman [32] has recently described complementary studies of this antigen using the parental JG22 and another hybridoma line JG9. Both antibodies were reported to interact with a single 140-kDa protein [12], which may correspond to the broad band that we observed after reduction that we believe to be comprised of two proteins, bands 2 and 3. She also describes a 170-kDa band that could correspond to either band 1 or 1a in our study. The 140-kDa and 170-kDa bands were shown to be on the cell surface, glucosamine-labeled, and unrelated to fibronectin or each other by peptide mapping [32].

After completion of this manuscript, we learned of a study in press by Knudsen et al [33] that contains data in general agreement with our independent studies. They show that bands 1, 2, and 3 are distinct by one-dimensional rather than two-dimensional peptide mapping, and they suggest that the bands form a complex. A sucrose density gradient analysis similar to ours is shown in a symposium chapter in press [34] by Buck et al that is in general agreement with our results, although only one free band appears to be present in that study. The only major discrepancy in results concerns the amount of cysteine present in the proteins of this complex. We found 38 residues per 1,000 (3.8 mol %), whereas they found only 2.9 residues of 1/2 cysteine and none of cysteic acid per 1,000 residues. One possible explanation is that we took precautions to avoid destruction of cysteine residues by the use of a performic acid oxidation step. The moderately high number of cysteine residues that we find is more consistent with the observed substantial alterations in electrophoretic mobility of all bands after reduction of disulfide bonds than is the unusually low estimate in reference [33]. In general, though, there is excellent agreement between the results from our two different laboratories using two different monoclonal antibodies, strongly supporting the notion that these membrane-associated glycoproteins exist as a complex.

Our findings suggest that further analyses of the function of this glycoprotein complex will require immunological reagents and isolation/reconstitution procedures that can dissect the function of each component of a multisubunit, potentially multifunctional complex.

ACKNOWLEDGMENTS

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