Characterization of a 140-kD Avian Cell Surface Antigen As a Fibronectin-binding Molecule

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Abstract. A 140,000-D protein cell surface antigen (140k) complex has been implicated in fibronectin-mediated cell-substratum attachment. We have used three different experimental systems to evaluate the hypothesis that this 140k complex can function as a fibronectin receptor. A monoclonal antibody that binds to the 140k complex specifically inhibits the direct binding of 3H-labeled 75,000-D fibronectin cell-binding fragment (f75k) to chicken embryo fibroblasts in suspension. The 140k complex is retarded in its passage through an affinity column consisting of immobilized f75k, and this interaction is specifically inhibited by a synthetic peptide that contains the fibronectin cell-recognition signal sequence. Finally, exogenous purified 140k complex inhibits the attachment and spreading of chicken embryo fibroblasts on fibronectin-coated substrates. Thus, our results indicate that the 140k complex can bind directly to fibronectin and is likely to be a fibronectin receptor for chicken cells.

The attachment of cells to extracellular matrices is an important interaction that plays a role in such in vivo biological processes as wound healing, embryonic development, and disease (reviewed in references 11, 38, and 40). However, the molecular mechanisms involved in cell attachment are still poorly understood. One experimental strategy has been to analyze in detail the function of purified components of the extracellular matrix. As one of the best-characterized noncollagenous components of the extracellular matrix, fibronectin is a valuable model system with which to study cell–substratum interactions (19, 26, 29, 34, 41).

The interaction of fibronectin with fibroblastic cells has been examined using intact fibronectin (1, 13), defined cell-binding fragments of fibronectin (3), synthetic peptides derived from the fibronectin primary structure (2, 30–32, 42, 43), and fibronectin-coated beads (24). The direct binding of soluble fibronectin to cells occurs with only moderate affinity (Kd of 0.8 μM, reference 1). Such binding studies indicate the presence of a minimum of 10^5 fibronectin receptors per cell, suggesting that such a moleculcule is a major cell surface component. As judged by inhibition and cell attachment assays, the minimum cellular recognition signal on fibronectin for fibroblasts is a tetrapeptide with the sequence Arg-Gly-Asp-Ser (2, 30–32, 42, 43). This sequence is found in the fibronectin primary structure at a location that is very likely to be at a β-turn, suggesting that it is an exposed site (32, 43); this site may become even more exposed upon cleavage of the molecule, accounting for the higher apparent affinity of certain defined cell-binding fragments for cells (1, 20, 21).

Another approach towards understanding cell–extracellular matrix interactions has been to produce antibodies against cell surface antigens that have activity in altering the morphology and adhesion of cells cultured on extracellular materials (4–10, 15, 22, 27). One of the earliest such antibodies to be reported (10), which has subsequently been implicated in fibronectin-mediated cell adhesion, is a monoclonal antibody Greve and Gottlieb designated JG22; the hybridoma was later extensively subcloned to produce the line designated JG22E (6, 7, 15).

JG22E identifies a complex of integral membrane components designated as a 140,000-D protein cell surface antigen (140k). The 140k complex consists of three glycoproteins of apparent molecular size 155,000, 135,000, and 120,000 D (15). Both JG22E and polyclonal antibodies against purified 140k co-distribute in chicken embryo fibroblasts (CEF) with fibronectin fibrils at cell adhesion sites. Furthermore, JG22E inhibits the attachment and spreading of CEF on fibronectin substrates, and JG22E itself coated on substrates can mediate cell attachment and spreading, thus demonstrating direct, specific binding to a cell surface component (7).

Other monoclonal antibodies have been independently iso-

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1 Abbreviations used in this paper: 140k, glycoprotein cell surface antigen recognized by monoclonal antibody JG22E implicated in fibronectin cell-adhesion events; CEF, chicken embryo fibroblasts; f75k, 75,000-D fibronectin cell-binding fragment; GRGDS, synthetic peptide with the sequence Gly-Arg-Gly-Asp-Ser; GRGES, synthetic peptide with the sequence Gly-Arg-Gly-Glu-Ser; JG22E, monoclonal antibody that disrupts cell attachment and spreading on fibronectin substrates; Tris-saline, 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.0.
lated and characterized that recognize 140,000-D cell surface antigens that are probably related or identical to 140k (4, 5, 8, 9, 22, 27). By a nonimmunological approach, a glycoprotein of similar size that has several properties expected of a fibronectin receptor has been isolated from human cells by affinity chromatography with a 120,000-D fibronectin cell-binding fragment (33).

Although the data to date have been suggestive, there is as yet no complete, direct evidence that any of these 140k complexes function as fibronectin receptors. The antigens that were identified immunologically as being necessary for cell adhesion have never been shown to bind fibronectin. Conversely, there is no evidence as yet that the cell surface molecules that bind to fibronectin in vitro are required for cell adhesion by living cells. In this study we investigate the nature of the interaction between the 140k complex and the 75,000-D fibronectin cell-binding fragment (f75k) and provide evidence that 140k can serve as a fibroblast cell surface fibronectin receptor.

Materials and Methods

Materials

Monoclonal antibody JG22E and the 140k complex were purified as described (7, 15). The purity of the 140k complex was routinely analyzed by sodium dodecyl sulfate gel electrophoresis using 4% stacking and 7.5% resolving gels (7, 15). As we described previously, our 140k preparations stained by Coomassie Blue appear as three major bands of 120,000, 135,000, and 155,000 D under nonreducing conditions (7,15). Occasionally, a minor band of 167,000 D is also observed (7, 15). 140k and control proteins for spreading assays were dialyzed against Dulbecco's phosphate-buffered saline (PBS) (GIBCO, Grand Island, NY) without Ca** or Mg** for 2 d and then against PBS containing Ca** and Mg** for 1 d to remove detergent. The f75k was prepared from purified fibronectin and tritium labeled as described (1, 3, 17, 25, 36). The f75k and bovine serum albumin (BSA) (Calbiochem-Behring Corp., La Jolla, CA) were separated at a ratio of 10 mg protein to 1 g dry cyanogen bromide-activated Sepharose (Pharmacia, Uppala, Sweden). CEF were prepared from 9-d embryos as described (39). All cells were maintained in culture for no more than five passages. All synthetic peptides were custom synthesized to our specifications by Peninsula Laboratories, Inc. (Belmont, CA). The purity of the peptides was confirmed by amino acid analysis and by high performance liquid chromatography (HPLC) (>98% pure). The concentrations of all proteins were estimated by the method of Lowry et al. (23) using crystalline BSA as a standard.

Binding Assay

The direct binding assay for CEF was adapted from a protocol established earlier for baboon hamster kidney cells grown in suspension culture (1-3). Subconfluent CEF were collected from 175-cm² culture flasks by washing three times in Hanks' balanced salt solution (GIBCO), followed by brief treatment with 1 ml of 0.1 mg/ml crystalline trypsin (Worthington Biochemical Corp., Freehold, MA) in Hanks' balanced salt solution. After 1 min, trypsin activity was quenched by the addition of serum-containing growth medium (39). The CEF extract was prepared fresh by mixing 3 ml of cells with 1 ml ice-cold extraction buffer consisting of 200 mM β-octylglucopyranoside and 3 mM phenylmethylsulfonyl fluoride in PBS. The extraction buffer was freshly prepared for each flask of cells, and new flasks of cells were extracted for each pair of column runs. The CEF extract was clarified by centrifuging at 40,000 × g for 20 min at 4°C, diluted to 100 mM β-octylglucopyranoside with ice-cold PBS, and applied to the affinity column. Chromatography was performed at a flow rate of 0.9 ml/h, and 90 ml fractions were collected. Each fraction was assayed for 140k by spotting 3-μl aliquots on nitrocellulose grids prepared by drawing 1-cm squares on filter paper (0.45 μm B A85, Schleicher & Schuell, Inc., Keene, NH) with a soft pencil. The dotted nitrocellulose sheets were then incubated in 5% (wt/vol) BSA fraction (V, Miles Scientific Div., Naperville, IL) in 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.0 (Tris-saline) either overnight at room temperature or for 90 min at 37°C. All subsequent steps were performed at room temperature (22–24°C). The dot blots were incubated in a 1/200 dilution of polyclonal rabbit antibody to 140k in 5% BSA in Tris-saline for 90 min. The blot was then rinsed briefly in Tris-saline, incubated twice in Tris-saline for 10 min, rinsed in 0.1% Tween-80 (Sigma Chemical Co.) in Tris-saline, incubated four times in 0.1% Tween-80 in Tris-saline for 10 min each, and finally incubated two times in Tris-saline for 10 min each. The blot was then incubated in 5% BSA in Tris-saline for 5 min, then with 5 × 10⁵ cpm/ml [3H]-labeled protein A (New England Nuclear, Boston, MA) in 5% BSA and Tris-saline for 30 min. The blot was then washed as described above and dried. The 140k complex was then visualized by exposing Kodak X-Omat film to the blot using a Quanta III enhancing screen (DuPont Co., Wilmington, DE) for 12–16 h at ~80°C. Autoradiography was quantitated by exposing each spot along the pencil lines and counting using an LKB Wallac 1275 Mini-gamma counter (LKB Instruments, Inc., Gaithersburg, MD). For each blot, 4–10 squares were counted as blanks. In separate controls, the accuracy of the assay was periodically reexamined by spotting a dilution series of 140k complex or CEF extract onto nitrocellulose and measuring as described above.

Results

Inhibition of Direct Binding

[3H]f75k can be used as a probe of the fibronectin receptor on CEF by binding directly to these cells in suspension. The observed binding was greater than 80% specific as judged by competition experiments with 100-fold excess unlabeled f75k (Fig. 1, lane B). Monoclonal antibody JG22E inhibited 75% of the binding of [3H]f75k to these cells (Fig. 1, lane C). As JG22E does not bind to fibronectin (6, 7), this result indicates
that JG22E can inhibit direct fibronectin binding to CEF by blocking an active site on 140k. We saw no further decrease of [3H]f75k binding to CEF when both unlabeled f75k and JG22E were added simultaneously to the cells (Fig. 1, lane D). Therefore, the inhibitory effects of the monoclonal antibody and the fibronectin fragment were not additive, thus confirming the interpretation that both JG22E and [3H]f75k bind to the same cell surface component, i.e., a fibronectin receptor. In a separate control, normal mouse IgG had no effect on the binding of [3H]f75k to CEF.

**Interaction of 140k with Immobilized f75k**

Whereas detergent extracts of human cells (33) and hamster cells (Akiyama, S. K., and K. M. Yamada, unpublished data) contain a cell surface component(s) that appears to bind to immobilized fibronectin cell-binding fragments even after extensive washing, we could obtain no evidence for the existence of a similar membrane component from cultured CEF (not shown). We therefore compared the elution rates of 140k in CEF extracts through f75k-Sepharose columns and identical control BSA-Sepharose columns (Fig. 2). Under conditions where the 140k complex elutes from the BSA column in a nearly symmetrical peak spanning 17 fractions, the 140k complex eluted from a f75k column in a wide, trailing peak covering 46 fractions, indicating that even though tight binding was not occurring, a weak interaction between 140k and f75k was observed. This weak interaction produced a retardation of both the leading and trailing edges of the 140k peak and as well as a decrease in the absolute height of the peak. This result was reproducible in multiple runs and was not due to unequal loads applied to the two types of column: the total peak areas of the two elution profiles were equal with 2% error.

The specificity of the 140k–f75k interaction was assessed using two synthetic peptides. The peptide GRGDS contains the recognition signal for the mammalian fibronectin receptor (2,30–32,42,43), and a similar peptide has been used to elute a possible human fibronectin receptor from a fibronectin affinity column (33). As shown in Fig. 3a, equilibrating the column and the CEF extract with GRGDS and eluting in the presence of GRGDS abolished the retardation of both the leading and trailing edges of the 140k peak. The peak was more symmetrical and the peak height was increased, becoming very similar to the elution profile of 140k on the inert BSA affinity column.

The effect of GRGDS was specific. We compared the elution of 140k in the presence of 1 mg/ml GRGDS and in the presence of an equimolar concentration of the peptide GRGES; a related control peptide did not elute a proposed mammalian fibronectin receptor from fibronectin (33). As shown in Fig. 3b, the 140k peak trailed noticeably in the presence of GRGES, indicating that this control peptide has no effect on the interaction of 140k with f75k. The peak was quite asymmetrical and very similar to the peak in the f75k column in Fig. 2.

To exclude possible effects of other cellular components in the CEF homogenates on the interaction of 140k with fibronectin, the column retardation assay was repeated using 3 µg of purified 140k in the presence of GRGDS and GRGES. As shown in Fig. 4, purified 140k was retarded in a broad asymmetrical peak on immobilized f75k in the presence of GRGES. However, in the presence of GRGDS, the 140k–f75k interaction was inhibited, and the peak was narrower, taller, and more symmetrical. Thus, the results shown in Fig. 3 were due only to the direct interaction of 140k contained in the CEF extract with f75k coupled to Sepharose.

Figure 2. Elution profile of 140k on f75k- and BSA-Sepharose columns. A CEF detergent extract was chromatographed on columns of immobilized f75k (●) or BSA (○). Column conditions and quantitation are described in Materials and Methods. The stippled area indicates the relative loss of 140k in the leading peak on the f75k column compared with the BSA column. The hatched area indicates the degree of retardation of 140k observed on the f75k column compared with the BSA column, indicating an interaction between 140k and f75k.
Inhibition of Cell Spreading by Purified 140k

CEF can attach and spread on fibronectin-coated substrates. As shown in Fig. 5, ~70% of the cells spread on plastic coated with 1 μg/ml fibronectin. This cell spreading could be inhibited in a concentration-dependent manner by purified 140k. At 20 μg/ml, 140k inhibited ~30% of the fibronectin-mediated cell spreading as measured by counting the percentage of spread cells (Fig. 5b). At 90 μg/ml 140k, this inhibition increased to ~50%. The inhibition appeared to be specific for 140k, since neither equimolar concentrations of BSA nor fibrinogen showed any inhibitory activity (Fig. 5b).

Spreading as quantitated by measuring the areas of cells rather than as the percentage of spread cells appeared to be a more sensitive indicator of inhibition by purified 140k. In Fig. 5, A, treatment with 20 μg/ml purified 140k resulted in a decrease in the area covered by the cells shown in B to only 21% that of the control (data in Fig. 5 legend). Fibronectin-mediated cell spreading quantitated by measuring cell areas was also not significantly inhibited by BSA (C) or fibrinogen (D).

The data shown in Fig. 5, A and B, represent the results of a typical inhibition experiment. In multiple experiments using different preparations of 140k, we could usually obtain approximately this level of inhibition. However, in a small number of experiments, >50% inhibition was observed by the criterion of percentage of cells spread, and in one experiment >90% inhibition was observed. As might be expected for a system involving competitive inhibition (see references 3 and 42), the inhibition by 140k was substantially less if higher quantities of fibronectin were adsorbed onto the substrate (not shown).

Discussion

We have shown by the three following independent criteria that the 140k complex can act as a fibronectin receptor. (a) JG22E inhibited the binding of [3H]f75k to CEF in suspension to approximately the same extent as a 100-fold excess of unlabeled f75k. A mixture of unlabeled f75k and JG22E did not inhibit binding to a greater extent than either protein alone, indicating that they were both binding to the same cell surface component. As fibronectin cell-binding domains presumably bind to fibronectin receptors (1–3, 33), our results suggest that 140k is also a fibronectin receptor. (b) The 140k
Figure 5. (A) Inhibition of CEF attachment and spreading on fibronectin substrates by purified exogenously added 140k. CEF were allowed to attach and spread on substrates precoated with 1 μg/ml fibronectin in the presence of purified 140k or the control proteins albumin or fibrinogen. The phase contrast micrographs show CEF assayed in the presence of control buffer (a), 20 μg/ml dialyzed 140k (b), 90 μg/ml BSA from which detergent was removed by dialysis in parallel with the 140k sample (c), control buffer duplicate (d), 90 μg/ml dialyzed 140k (e), and 90 μg/ml dialyzed human fibrinogen (f). The average (± SEM) surface area of the cells is 1,280 ± 200 μm² (a), 270 ± 80 μm² (b), 990 ± 120 μm² (c), 1,240 ± 190 μm² (d), 310 ± 90 μm² (e), and 1,090 ± 130 μm² (f). Inhibition at both 20 μg/ml (b) and 90 μg/ml 140k (e) is statistically significant compared with controls (P < 0.001), whereas the difference between the inhibition at 20 μg/ml and 90 μg/ml 140k is not statistically significant (P > 0.5). Bar, 50 μm. (B) Quantitation of inhibition of CEF spreading by purified 140k by counting the percentage of spread cells. Cells on substrates precoated with 1 μg/ml fibronectin were incubated in the presence of purified, dialyzed 140k at 3, 20, or 90 μg/ml, or with controls consisting of control PBS buffer (Con), 90 μg/ml BSA, or 90 μg/ml human fibrinogen (Fbgn). Bars indicate the mean ± SEM of eight random phase contrast microscopy fields per point; a total of 800 cells were scored for each point. Quantitation of cell spreading was performed as described in references 12 and 14. There is no significant inhibition of cell spreading in the presence of 3 μg/ml 140k. The inhibition of cell spreading is significant at both 20 μg/ml (P < 0.005) and 90 μg/ml (P < 0.001).

complex could interact directly with 75k immobilized on Sepharose as judged by affinity column retardation experiments. This interaction could be inhibited specifically by the addition of the synthetic peptide GRGDS, which contains the fibronectin cell surface recognition signal. (c) The purified 140k complex, as might be expected for a putative receptor, competed with cell attachment to fibronectin adsorbed onto plastic, as judged by inhibition in cell spreading assays.

Our study demonstrates the direct binding of a previously identified and characterized membrane antigen to fibronectin. Earlier studies reported the localization of the 140k complex at the edges of focal contacts, in close contacts and extracel-
lular matrix contacts, and co-distributed with fibronectin fibrils (6–8). Such previous data suggested either of two models for the function of 140k: (a) that 140k is a fibronectin receptor or (b) that 140k is a structural molecule required for the proper placement or function of the fibronectin receptor. Our results permit the conclusion that 140k is a possible fibronectin receptor rather than only a simple structural complex.

The inhibition of direct binding of [3H]75k to CEF by JG22E is consistent with its inhibition of CEF cell spreading on fibronectin substrates (7), and it provides the first direct evidence for inhibition of fibronectin-binding activity by such an antibody. Taken together, these results strongly suggest that the 140k complex plays a role in both the binding of soluble fibronectin and in the spreading of CEF on immobilized fibronectin, consistent with its putative role as a fibronectin receptor.

That the apparent affinity of 140k for fibronectin is relatively low is not entirely unexpected. The fibronectin receptor on baby hamster kidney cells is of only moderate affinity (Kd ≈ 10^−7 M, references 1 and 3). A high affinity receptor might be inconsistent with the biological role of a ligand such as fibronectin that is involved with cellular processes such as migration, which requires the repeated making and breaking of bonds. The experiments reported here do not permit a direct determination of the affinity constant of 140k for fibronectin. However, from data reported here and elsewhere (1, 3, 33), we can roughly estimate that the 140k complex binds to fibronectin with an even lower affinity (Kd > 10^-6 M). Another 140,000-D complex termed CSAT has been isolated from chick cells with a monoclonal antibody, and its characteristics appear to be identical to those of the complex isolated by JG22E (compare references 5–10, 15, 22, 27). Using a different column interaction assay and intact fibronectin, that group has also obtained evidence for a weak, readily reversible interaction with the similar antigen CSAT and fibronectin (18a).

To summarize, we have been able to demonstrate that JG22E binds to a fibronectin-binding complex. The interaction of this 140k complex and fibronectin is specifically inhibited by both JG22E and by synthetic peptides modeled from the fibronectin sequence. Furthermore, the interaction of cells with immobilized fibronectin can also be inhibited by purified exogenous 140k.

A significant question that we are unable to answer at present is the relationship between the different cell surface proteins in the size range of 120,000–140,000 D, all of which have been implicated in fibronectin-mediated cell adhesion. So far, such molecules have been identified on chick cells (5–10, 15, 22, 27), hamster cells (4), and human cells (33). In all but one case, the approach has been to identify monoclonal antibodies that apparently disrupt cell attachment to fibronectin substrates and then to identify the antigen recognized by the antibody. The question is presently unanswerable due to the lack of cross-reactivity between different monoclonal antibodies. Concerning fibronectin receptor activity, in the most direct study to date, a human cell surface protein of 140,000 D was shown to bind relatively tightly to a fibronectin cell-binding fragment (33), although the functional importance of this protein to living cells has not yet been demonstrated. We have been able to confirm these observations with baby hamster kidney cells, but all attempts to isolate a comparable molecule from chick cells by fibronectin affinity chromatography have failed because of the lower apparent affinity (Figs. 2–4 and unpublished data). Since interaction of the chick 140k complex with fibronectin is characterized by retardation rather than by the firm binding seen with mammalian cells, we suggest that these molecules are in fact functionally different, at least in terms of apparent affinities. It will be important to determine in the near future the details of the differences and similarities between the biological and biochemical characteristics of these membrane components of similar size.

Our conclusion that the 140k complex has specificity as a fibronectin receptor does not rule out the possibility that the 140k complex can also act as a receptor for other cell adhesion molecules. Synthetic peptides from the fibronectin sequence have been found to inhibit cellular interactions with other adhesion molecules (16, 18, 35; Kennedy, D. W., and K. M. Yamada, unpublished data). Other studies have indicated that cells may have the potential to use more than one molecule in similar adhesion-related functions depending on which are available (reviewed in reference 38). It will be of considerable interest to determine which of the 140k complexes and other cell surface receptors are of high specificity and which have the ability to interact with a wider spectrum of adhesive molecules.

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