Antiseras against a M, = 60,000 peptide containing the gelatin-binding domain of human plasma fibronectin (McDonald, J. A., and Kelley, D. G. (1980) J. Biol. Chem. 255, 8848–8858) bound the M, = 60,000 peptide and intact fibronectin but not three other fragments released by leukocyte elastase proteinolysis (the M, = 25,000 amino-terminal sequence, M, = 140,000 sequence containing cell adhesive activity, and a M, = 31,000 fragment). Affinity-purified Fab' blocked M, = 60,000 peptide binding to gelatin and inhibited plasma and cellular fibronectin gelatin binding without affecting fibronectin-mediated cell spreading. In contrast, anti-fibronectin Fab' absorbed with the gelatin-binding domain completely blocked fibronectin-mediated cell spreading. These data indicate that the gelatin-binding domain of fibronectin is immunogenic, and antiseras against this domain recognize cellular fibronectin gelatin-binding sites. Inhibition of gelatin binding but not cell spreading by anti-gelatin binding domain Fab' confirms the hypothesis that fibronectin has separate sites mediating these activities. Selective inhibition of fibronectin-collagen binding by domain-specific antiseras may help elucidate the role of fibronectin in organization of the extracellular matrix.

The adhesive glycoprotein fibronectin distributed in plasma, connective tissues, and basement membranes in vitro (1) is implicated in extracellular matrix organization. In vitro, fibronectin is a major component of fibroblast extracellular matrix co-distributed with procollagen types I and III (2, 3), and fibronectin binds to two major components of the extracellular matrix, collagen and hyaluronic acid (4–6). Addition of fibronectin to differentiated chondrocytes results in altered patterns of collagen synthesis (7). Although specific antiserum inhibits fibronectin-mediated cell adherence (8, 9), selective inhibition of binding to individual matrix components by polyclonal antiseras is unlikely since fibronectin has separate binding domains for collagen, glycosaminoglycans, and cells (4, 5, 10–15). We have utilized proteolytic cleavage to isolate the gelatin-binding domain of fibronectin, and in this report we describe the effects of antiserum specific for the gelatin-binding domain of human plasma fibronectin (10) upon fibronectin-mediated cell spreading and gelatin binding.

### EXPERIMENTAL PROCEDURES

**Gelatin-binding Fragment Antibody—** Purified 60 k fragment (10) (1.0 mg) in 1.0 ml of complete Freund’s adjuvant was injected into two New Zealand rabbits at multiple intradermal sites in the back. Rabbits were boosted intradermally with 1 mg of 60 k fragment in incomplete adjuvant at 6-week intervals. Antiserum was titered by radioimmunoassay. Lactoperoxidase-glucose oxidase 125I-labeled 60 fragment (10) (20,000 cpm, approximately 10 ng) was incubated with antiserum in polystyrene tubes (11 × 75 mm) in a total volume of 0.2 ml of buffer A (17.3 mm Tris-HCl-150 mm NaCl-0.5% Tween 20-0.1 mm Na3EDTA-0.1 mm phenylmethylsulfonyl fluoride, pH 7.4, at 25 °C). After 16 h at 4 °C, 50 μl of a 10% suspension of IgSORB (The Enzyme Center, Boston, MA) was added and incubated at 37 °C for 30 min. Bound 125I-probe was separated from free by adding 3 ml of buffer A, vortexing, and sedimenting (2,500 × g, 10 min) the IgSORB, and aspirating the supernatant. Bound 125I was determined by γ counting.

Specificity of anti-60 k antiserum was assessed by incubating 125I-labeled 60 k with an antiserum dilution giving 50% of maximal binding plus varying amounts of fibronectin or the 60 k, 25 k (previously designated 29 k fragment in Ref. 10), 140 k, and 31 k fragments (10).

**Isolation of Preimmune and Anti-60 k IgG and Fab’—** Pure 60 k fragment was coupled to CNBr-activated Sepharose CL-4B (16) (Pharmacia Fine Chemicals, Piscataway, NJ) using 2 mg of fragment/ml of gel. Before use, columns were washed with 8 mM urea in 50 mM Tris-HCl, pH 7.4, 0.2 μM glycine-HCl, pH 2.3, and PBS. Antiserum (50 μl/10 ml of gel) containing 0.02% NaN3 was circulated over the column overnight at 25 °C. The column was washed with PBS until the A280 nm was base-line, then with 0.5 M NaCl in 50 mM Tris-HCl, pH 7.4, and bound proteins were eluted with 0.2 M glycine-HCl, pH 2.3. Identity of the eluted fraction as IgG was verified by SDS-polyacrylamide gel electrophoresis (10, 17) with and without reduction.

**Preimmune and Anti-60 k IgG and Fab’—** Rabbit anti-FN IgG, affinity purified on FN-Sepharose CL-4B, was used to test the effect of Fab’ fragments on FN-Sepharose CL-4B as described above. Preimmune Fab’ was obtained from rabbit IgG (18) as described above and analyzed by SDS-gel electrophoresis.

**Isolation of Anti-fibronectin Fab’ Absorbed with 60 k Fragment—** Rabbit anti-FN IgG, affinity purified on FN-Sepharose CL-4B as described above, was cyclically oxidized with 60 k fragment-Sepharose CL-4B. Fab’ obtained from the IgG fraction not binding to the 60 k fragment column (designated anti-FN-minus 60 k) was used to test the effect of anti-FN Fab’ without 60 k binding activity in biological assays.

**Gelatin-binding Assay—** Binding of 125I-labeled fibronectin and 60 k fragment to heat-denatured rabbit skin type I collagen was determined as previously described (10).

**Cell-spreadability Assay—** Tissue culture plates (Falcon 3001) were

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1. The abbreviations used are: 25, 31 k, 60 k, 140 k, the M, = 25,000, 31,000, 60,000, and 140,000 fragments from limited digestion of human plasma FN by leukocyte elastase (see Ref. 10 for details); FN, fibronectin; PBS, Dulbecco’s phosphate-buffered saline without Ca2+ or Mg2+; CHO, Chinese hamster ovary K-1 cells; DMEM, Dulbecco’s modified Eagle’s medium (MEM); SDS, sodium dodecyl sulfate.

2. This basic peptide stacks poorly in discontinuous gel electrophoresis, and recent M, determinations of the transglutaminase reactive amino-terminal FN fragment (10) have given values closer to 25,000 than the published value of 29,000.

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coated with 25 μg/ml of gelatin, rinsed with PBS, and incubated with 25 μg/ml of fibronectin alone or after incubation with Fab’ (1 h, 37 °C). Trypsinized CHO cells washed with MEM plus soybean trypsin inhibitor were then plated in the presence of Fab’ preparations or medium alone. After incubation for 1 h at 37 °C, the plates were rinsed, and cell spreading was determined by morphologic scoring (9, 10). Cell spreading assays were blinded to avoid observer bias. Cell spreading on fibronectin-coated plastic substrate was carried out similarly, as described previously (10).

Effect of Anti-60 k Fab’ upon Fibroblast Fibronectin Gelatin Binding—Human lung fibroblasts (IMR-90), passage 12, were seeded at a 1:3 subcultivation ratio in DMEM + 5% fetal bovine serum depleted of fibronectin-gelatin binding activity by gelatin-Sepharose chromatography (10) and cultured 5 days. On day 5, culture medium was replaced with DMEM + 5 μCl of [14C]proline/ml (specific activity, 260 μCi/μmol) and 100 μg/ml of Fab’, and the cells were incubated for an additional 40 h. After labeling, the medium was removed, proteinase inhibitors (10 mM Na3EDTA, 1 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride) were added, and the medium was dialyzed for 4 h against 3 changes of buffer B (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-150 mM NaCl-10 mM Na3EDTA-1 mM phenylmethylsulfonyl fluoride-1 mM N-ethylmaleimide, pH 7.5) at 4 °C. The cell layers were extracted with 1.0 ml of 2× denaturing buffer B for 1 h at 37 °C, the extract was centrifuged (15,000 × g, 15 min, 0 °C), and the supernatant was dialyzed as described above. Total nongelatin binding and gelatin binding labeled polypeptides were displayed as previously described (10, 17, 19). Quantitative densitometry of autoradiograms was performed using a Ziehen soft laser scanning densitometer. Fibronectin-gelatin binding was quantified by summing the density of the fibronectin band in nonbound and bound fractions and calculating the percentage bound.

RESULTS

Specificity of Antiserum and Fab’—Titers of 1/1,200 to 1/8,000 (antiserum dilution giving 50% specific binding of probe) of anti-60 k antiserum developed by 2–3 months after initial injection. About 0.5 mg of IgG/ml of antiserum was obtained by affinity chromatography. SDS-polyacrylamide gel electrophoresis of affinity-purified and preimmune IgG gave one band of M, = 150,000 (unreduced) and bands at M, = 50,000 and 25,000 (reduced), while Fab’ preparations had one band at M, = 50,000 (unreduced) and at 25,000 (reduced).

As shown in Fig. 1, anti-60 k antiserum bound both 60 k fragment and intact fibronectin identically using a monomeric M, = 220,000 for intact fibronectin. No other elastase-released FN fragment competed with 60 k antiserum for 60 k binding even at 50-fold excess molar concentration. Identical results were obtained with affinity-purified anti-60 k IgG. Human plasma depleted of fibronectin by sequential gelatin- and anti-fibronectin-IgG affinity chromatography did not compete for fibronectin or 60 k probe binding by anti-60 k-IgG, demonstrating lack of cross-reaction of anti-60 k-IgG with other plasma proteins. Anti-60 k Fab’ inhibited binding of 125I-labeled 60 k probe by anti-60 k antiserum, while anti-FN-minus 60 k Fab’ did not compete for binding of 60 k fragment by anti-60 k serum (Fig. 2), demonstrating the removal of 60 k binding activity by affinity chromatography.

Effect of Anti-60 k Fab’ Upon Fibronectin and 60 k Gelatin Binding—Anti-60 k Fab’ competitively blocked both 60 k and fibronectin binding to gelatin, while preimmune Fab’ had no effect (Fig. 3). Anti-60 k Fab’ appeared to block 60 k fragment binding more effectively than fibronectin binding. Affinity-purified anti-FN-minus 60 k Fab’ had no effect upon 60 k fragment binding to gelatin but did block fibronectin-gelatin binding (Fig. 3).

Effect of Preimmune, Anti-60 k, and Anti-FN-minus 60 k Fab’ Upon Fibronectin-mediated Cell Spreading—Fab’ preparations were incubated with gelatin-coated substrate, with fibronectin before addition to substrate, and with CHO cells during attachment and spreading on fibronectin-gelatin. Cell adhesion and spreading was only inhibited when anti-60 k Fab’ was incubated with fibronectin, and the mixture was added to the gelatin-coated substrate prior to the addition of cells, demonstrating selective inhibition of fibronectin-gelatin binding, without affecting cell spreading (Table I, Fig. 4). Identical results were obtained with IMR-90 fibroblasts. To verify these results, similar experiments were carried out on substrates coated with fibronectin alone. Preimmune and anti-60 k Fab’ had no effect upon CHO attachment or spreading.
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FIG. 4. Effect of preimmune anti-60 k fragment and anti-FN-minus 60 k Fab' upon fibronectin-mediated CHO cell spreading on gelatin-coated culture plates. A. fibronectin-gelatin substrate; B. gelatin alone; C. fibronectin incubated with 100 μg/ml of anti-60 k Fab' before addition to gelatin plate; D. identical with C, except fibronectin incubated with preimmune Fab'; E. gelatin plate incubated with 100 μg/ml of anti-60 k Fab' and rinsed before addition of fibronectin; F. cells plated on fibronectin-gelatin in the presence of 100 μg/ml of anti-60 k Fab'. Note the marked inhibition of spreading when fibronectin is incubated with anti-60 k Fab' before addition to gelatin substrate and the lack of effect when incubated with gelatin before fibronectin or with cells. These photographs are taken from the experiment in Table I.

TABLE I

Effect of Fab' upon CHO cell spreading on gelatin-coated or fibronectin-coated tissue culture plastic dishes

| Additions* | Cell spreading* on gelatin substrate at Fab' (μg/ml) of: | Additions | Cell spreading* on fibronectin substrate at Fab' (μg/ml) of: |
|------------|---------------------------------------------------------|-----------|---------------------------------------------------------|
| A. fibronectin | 4.0 | Preimmune Fab' | 3.5 | 3.2 | 3.5 |
| B. buffer | 0 | anti-60 k Fab' | 3.5 | 3.5 | 3.5 |
| C. anti-60 k Fab' + fibronectin | 3.0 | 1.5 | 0.5 | anti-FN-minus 60 k Fab' | 1.8 | 0 | 0 |
| D. preimmune Fab' + fibronectin | 4.0 | 4.0 | 4.0 | |
| E. anti-60 k Fab' + gelatin, fibronectin | 4.0 | |
| F. fibronectin, CHO + anti-60 k Fab' | 4.0 | |

* Gelatin-coated dishes (25 μg/ml) were rinsed and incubated (30 min, 37 °C) with the indicated additions, rinsed again, and CHO cells plated. Thus, anti-60 k Fab' inhibited CHO attachment and spreading when incubated with fibronectin prior to addition to gelatin-coated plates (C), but not when incubated with gelatin-coated plates prior to addition of fibronectin (E), nor when incubated with CHO cells added to fibronectin-gelatin substrate (F).

Spreading was scored by published criteria (10) by observation of three microscopic fields, and the scale 1, 2, 3, 4 equals 0-25, 25-50, 50-75, and 75-100% of cells spread, respectively.

CHO cells were plated on fibronectin-coated dishes in medium containing the indicated amount of Fab'.

Inhibition of Newly Synthesized Fibronectin-Gelatin Binding by Anti-60 k Fab' in Vitro—Anti-60 k Fab' did not grossly alter fibroblast protein synthesis and secretion as the labeled polypeptides secreted into medium by preimmune and anti-60 k Fab'-treated fibroblasts were similar. However, anti-60 k Fab' inhibited both medium and cell fibronectin-gelatin binding (Fig. 5). We cannot comment upon the relative amounts of fibronectin and procollagens synthesized in the presence of anti-60 k Fab' in the absence of more definitive biochemical analysis, although clearly both were synthesized and secreted. Quantitative densitometry (Table II) revealed that maximal inhibition of medium fibronectin-gelatin binding was achieved by 25 μg/ml of anti-60 k Fab', and urea-extracted fibronectin-gelatin binding was also decreased at this level. IMR-90 maintained normal morphology and parallel alignment when labeled in DMEM in the presence of preimmune or anti-60 k Fab'.

DISCUSSION

Unlike previous investigators (11), we obtained useful antisera in rabbits immunized with a fibronectin-gelatin binding fragment. Balb/c mice also developed antibody titers to 1/12,800, so human plasma fibronectin 60 k fragment is immunogenic in two mammalian species. Anti-60 k antiserum gave no precipitin lines on immunodiffusion; thus monitoring of antibody production by radioimmunoassay was essential. Only intact fibronectin and the 60 k fragment were recognized by anti-60 k antiserum, supporting the hypothesis that elastase fragments represent discrete domains of fibronectin (10). Antisera...
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**Table II**

| Fab'     | FN-gelatin binding of |
|----------|-----------------------|
|          | Control | Anti-60 k | Medium | Urea extract |
| µg/ml    |         |           |        |             |
| 100      | 0       | 100       | 67     |
| 75       | 25      | 14        | 41     |
| 0        | 100     | 27        | 0      |

60 k Fab' inhibition of gelatin binding without affecting cell spreading corroborates the presence of separate fibronectin domains mediating gelatin binding and cell spreading (5, 10, 11, 20). Since anti-FN-minus 60 k Fab' also inhibited gelatin binding, we cannot state that the 60 k domain is the only region of fibronectin-mediating gelatin binding. It is possible that Fab’ binding to regions closely adjacent to the 60 k domain could cause steric hinderance of gelatin binding mediated by the 60 k domain.

The significance of inhibiting fibronectin-gelatin binding in vitro by anti-60 k Fab' may be questioned. However, fibronectin has less avidity for native than denatured collagens in vitro (3). Thus, our assay for inhibition of high affinity gelatin binding may well underestimate the potential for inhibition of fibronectin binding to newly synthesized native collagens. Indeed, in preliminary studies, fibroblasts cultured with anti-60 k Fab' maintained normal growth but exhibited marked alterations in organization of fibrillar extracellular fibronectin and collagens (21).

The demonstration that domain-specific anti-plasma FN recognizes and inhibits specific functions of cellular FN is not surprising in view of similar recognition of plasma and cellular FN by monoclonal antibodies (22). However, polyvalent domain-specific antisera to easily obtained fragments of plasma FN should prove useful for selective inhibition of cellular FN activities. In addition, such domain-specific antisera allow the quantification of FN in the presence of other FN fragments capable of competing with FN binding.

**Fig. 5.** Inhibition of newly synthesized cellular fibronectin-gelatin binding by anti-60 k Fab'. Upper, effect of preimmune and anti-60 k fragment Fab' upon fibroblast fibronectin-gelatin binding. IMR-90 fibroblasts were labeled with [14C]proline in the presence of 100 µg/ml of preimmune or anti-60 k Fab', and the total (T), nongelatin binding (NB), and gelatin binding (B) fractions of medium and urea extracts were displayed by SDS-7.5% polyacrylamide gel electrophoresis and autoradiography. Note the similarity of total labeled polypeptides in preimmune and anti-60 k Fab' cultures and the inhibition of fibronectin binding in both medium and cell fractions from cultures containing anti-60 k Fab'. Lower, densitometric scans of total, nongelatin, and gelatin-bound fractions of labeled medium polypeptides from preimmune and anti-60 k fragment Fab' cultures. The position of fibronectin is indicated by the arrow, and the following two prominent bands represent procollagens. Note that all medium fibronectin from preimmune Fab' cultures bound to gelatin-Sepharose, while anti-60 k Fab' inhibited medium fibronectin binding (100 versus 27% binding). These scans were taken from the autoradiogram shown in the upper panel.
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