Location of a Fibronectin Domain Involved in Newt Epidermal Cell Migration

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ABSTRACT The interaction of migrating newt epidermal cells with the extracellular matrix protein, fibronectin, was studied. Pieces of nitrocellulose coated with intact human plasma fibronectin or proteolytically derived fragments were implanted into wounded limbs so that the coated nitrocellulose served as wound bed for migrating epidermal cells as they attempted to form a wound epithelium. Epidermal cells migrated very poorly on nitrocellulose pieces coated with (*a*) a 27-kD amino-terminal heparin-binding fragment, (*b*) a 46-kD gelatin-binding fragment, (c) a combined 33- and 66-kD carboxy-terminal heparin-binding preparation representing peptide sequences in the A and B chains, respectively, or (*d*) a 31-kD carboxy-terminal fragment from the A chain, containing a free sulfhydryl group. In contrast, epidermal cells readily migrated onto nitrocellulose coated with a mixture of fragments from the middle of the molecule (80–125kD) that bind neither heparin nor gelatin. Attempts to block migration on fibronectin-coated nitrocellulose using IB10, a monoclonal antibody that blocks Chinese hamster ovary cell attachment to fibronectin, were unsuccessful despite saturation of the epitope against which IB10 is directed. In contrast, a polyclonal anti-fibronectin antibody did inhibit migration. These results show that the ability of fibronectin to support newt epidermal cell migration is not shared equally by all regions of the molecule, but is restricted to a domain in the middle third. They also suggest that the site supporting migration is separate and distinct from the site mediating Chinese hamster ovary cell attachment.

Fibronectin is a complex blood–plasma glycoprotein which is also found in the pericellular matrix on the surface of certain cell types. It is composed of two similar but perhaps not totally identical high molecular mass polypeptides (21), interconnected by disulfide bonds near the carboxy-terminal end (30). Distributed along the molecule is a cross-linking site for transglutaminase (18), as well as binding sites for collagen (1, 11), several glycosaminoglycans (24, 26, 32), Staphylococci (18, 29), and certain types of eucaryotic cells (10, 22, 25).[1] Through this organization into a series of structural domains which bind specific ligands, fibronectin participates in various biological activities. In wounds, plasma fibronectin is cross-linked to fibrin during clot formation (9, 16, 17). This immobilized fibronectin apparently is then used as a migration substrate by fibroblasts as they invade the clot (10).

Until recently it appeared that fibronectin might not support attachment and spreading of epithelial cells (6, 19, 28). But there have now been several reports showing that fibronectin can mediate the adhesion of epithelial cells to tissue culture plastic (8, 15, 33) and two reports that epithelial cell migration is enhanced in the presence of fibronectin (3, 20). These observations and the fact that the same fibrin–fibronectin clot that provides a matrix for fibroblast migration into a wound also serves as a substrate for epithelial cells as they migrate to cover the wound (2) suggest that epithelial-fibronectin interactions may play an important role in restoration of epithelial integrity following its loss.

We have recently developed a method for studying the interaction of epidermal cells with various macromolecules in wounded amphibian limbs which have been amputated and explanted into a dish of saline solution (5). In this system, pieces of polycarbonate filter, coverslip glass, or nitrocellulose, coated with various substances, are implanted under one edge

[1] Hasty, D. L., H. S. Courtney, W. A. Simpson, J. A. McDonald, and E. H. Beachey, manuscript submitted for publication.

THE JOURNAL OF CELL BIOLOGY · VOLUME 101 JULY 1985 73-78

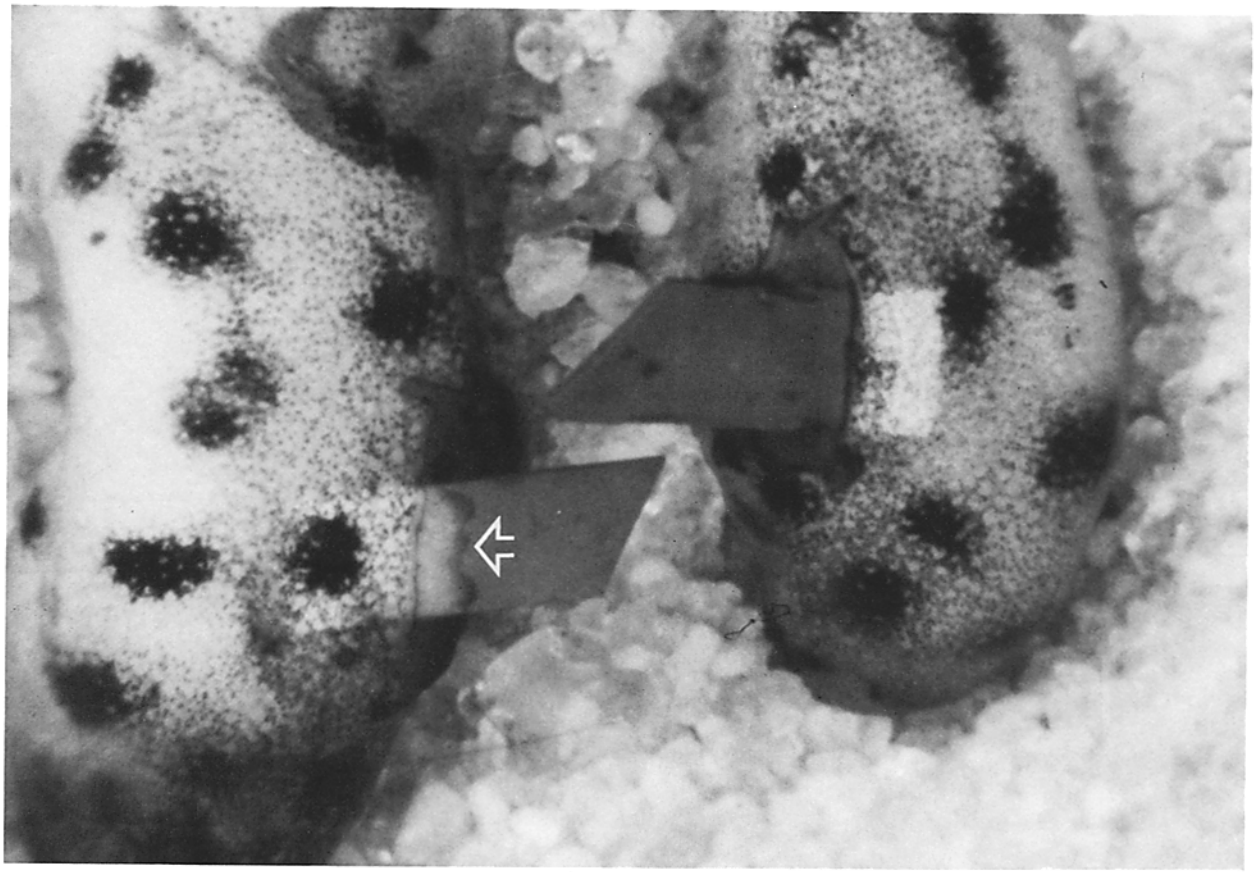

FIGURE 1 Test system for analyzing the ability of protein-coated nitrocellulose to support epidermal cell migration. 1- × 2-mm pieces of nitrocellulose were inserted (one per wound) under the anterior edge of a rectangular skin wound on newt limbs explanted to a dish of HS (amphibian saline). 10 h later, the limbs were fixed, stained briefly in 0.1% crystal violet, and the implant and any wound epithelium on it were drawn with the aid of a drawing tube-equipped microscope. Using a planimeter, the area covered by wound epithelium was then determined from a standardized region of each implant (relative distance migrated). In the figure, the lower implant (fibronectin coated) carries a substantial wound epithelium (arrow), whereas the upper one (PBS coated) has allowed no epidermal migration. (For photographic purposes the limbs in this figure were stained with eosin and methylene blue.) ×~17.

of a fresh skin wound so that epidermal cells attempting to form a wound epithelium must encounter the implant. Using this model, we recently demonstrated that epidermal cells will readily migrate over fibronectin-coated implants (3). In the present study we have attempted to localize the domain of fibronectin that mediates this migration.

MATERIALS AND METHODS

Animals: Adult male newts (*Notophthalmus viridescens*) were obtained from Connecticut Valley Biological, Southampton, MA. Details of animal maintenance have been described previously (3).

Wounding, Nitrocellulose Implantation, and Migration Measurement: Rectangular wounds (1.5 × 3.0 mm) were made by removing a piece of skin from the dorsal surface of each hind limb between the knee and ankle. Wounded limbs were then amputated through the thigh and explanted into Holtfreter solution (HS)[2] (0.06 M sodium chloride, 0.6 mM potassium chloride, 0.9 mM calcium chloride, 2.3 mM sodium bicarbonate, 0.005% [wt/vol] streptomycin sulfate). After the clotted blood was cleaned from each wound, the limbs were transferred to fresh HS and one end of a 1- × 2-mm piece of nitrocellulose was then inserted under the skin at the anterior wound margin (Fig. 1). The limbs were then incubated for 10 h at 23°C. After an overnight fixation in 10% formalin, the epithelial cells that had migrated onto the implanted nitrocellulose were stained by immersing the limb in 0.1% crystal violet for 10 s. Using a dissecting microscope equipped with a drawing tube, the magnified image of the nitrocellulose implant and its wound epithelium were drawn on a sheet of paper. The area occupied by a standardized width of wound epithelium was determined with a planimeter, and these values (relative distance migrated) were used to compare the ability of various fragments of fibronectin to mediate epithelial migration.

Preparation of Fibronectin Fragments: The generation and purification of proteolytic fragments of fibronectin has been described in detail elsewhere (see 23, 26, 27; see also Fig. 2). Briefly, gentle trypsinization with TPCK-trypsin (Cooper Diagnostics, Freehold, NJ) (1% enzyme/substrate by weight, pH 7.2 at 37°C for 2 min) of intact human plasma fibronectin generates four main fragment populations: one fragment with a molecular mass of 27 kD and a weak affinity for heparin, arises from the amino terminal end of both chains of the molecule. A second low molecular mass fragment (31 kD) containing a free sulfhydryl (27) originates from the A chain (12). Two high molecular mass fragments (190 and 200 kD) with collagen, heparin, and cell binding activity are produced from the A and B chains, respectively. Digestion of these last two fragments with cathepsin D (Sigma Chemical Co., St. Louis, MO) (1% enzyme to substrate, pH 3.1 at 37°C for 15 min) followed by affinity chromatography of the neutralized digest over gelatin-agarose followed by heparin-sepharose, yields four main fragment populations: a 46-kD gelatin-binding fragment (from both chains), a combined 33 and 66 kD heparin-binding preparation from the carboxy third of the A and B chains, respectively, and finally, a mixture of fragments from the center of both chains with molecular masses ranging from 80–125*kD. The fragments in this mixture do not bind gelatin or heparin, but do promote attachment, spreading, and migration of metastatic tumor cells (McCarthy, J. B., S. T. Hagen, and L. T.

[2] *Abbreviations used in this paper:* anti-FN, polyclonal antibodies against human plasma fibronectin; CHO, Chinese hamster ovary; HS, Holtfreter solution (0.06 M sodium chloride, 0.6 mM potassium chloride, 0.9 mM calcium chloride, 2.3 mM sodium bicarbonate, 0.005% [wt/vol] streptomycin sulfate); NRK, normal rat kidney.

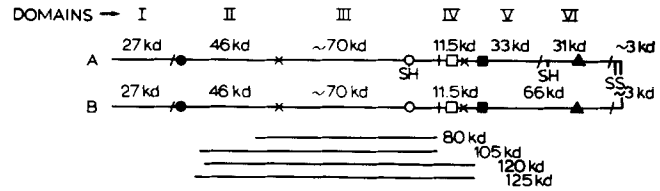

FIGURE 2 Model of fibronectin A and B chains (amino-terminal to the left) showing relative positions and biological activities of fragments used in this study (7, 12, 23). /, tryptic cleavage site; ×, catheptic cleavage site; |, peptic cleavage site; SH, free sulfhydryl group. Domain I (*27 kd*) has an affinity for *S. aureus*, a weak affinity for heparin, and contains the cross-linking site for collagen/fibrin. Domain II (*46 kd*) has a noncovalent binding site for gelatin (●). Domain III (*70 kd*) binds the monoclonal antibody, 180-8 (○). Domain IV (*11.5 kd*) contains a binding site for the monoclonal antibody, 3E3 (□), and a cell attachment site. Domain V (*33 kd*) has a strong affinity for heparin (■), whereas Domain VI (*31 kd*) binds the monoclonal antibody, 2-8 (▲). We tested each of the following: domain I; domain II; an 80-125-kD mixture from the middle of the molecule as shown below the model; a combined preparation of domain V from the A chain and V and VI from the B chain (the tryptic site between domains V and VI in the A chain is missing from the B chain [12], thus in the 33/66-kD preparation, the 33-kD component presumably originates from the A chain and the 66-kD from the B chain); and lastly, domain VI from the A chain.

Furcht, manuscript in preparation). In addition, Western transfer immunoblot analysis indicates that there is a peptide sequence in the mixture that binds the monoclonal antibody, 3E3 (23), an antibody that blocks normal rat kidney (NRK) cell attachment to fibronectin (22).

Coating of Nitrocellulose with Fibronectin or Fibronectin Fragments: 1- × 2-mm pieces of pure nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA) were coated with 10 μl of either fibronectin or fibronectin fragments diluted to the desired concentrations in phosphate-buffered saline (PBS), and air dried overnight at 23°C. To eliminate nonspecific interactions between the test cells and nitrocellulose, we treated all implants for 30–40 min in HS containing 0.5% wt/vol bovine serum albumin (BSA), followed by 15 min in HS.

Binding of Fibronectin and Fibronectin Fragments to Nitrocellulose: Fibronectin and its fragments were tritiated by reductive methylation (14). 1- × 2-mm pieces of nitrocellulose were coated with 10 μl (400 μg/ml) of these radiolabeled materials as described above. After a 30-min wash in HS containing 0.5% wt/vol BSA, the pieces were washed for 4 h in HS (five changes) by which time radioactivity leaching from them became negligible. After allowing the pieces to dry overnight, bound radioactivity was determined by adding the pieces individually to Scintiverse (Fisher Scientific Co., Pittsburgh, PA) and counting them in a Beckman LS 230 liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). PBS-coated pieces of nitrocellulose, processed in the same manner as the labeled pieces, were counted to determine the level of background radiation.

Antibodies: Polyclonal antibodies against human plasma fibronectin (anti-FN) were generated in rabbits (13). The monoclonal anti-fibronectin antibody, IB10, which blocks Chinese hamster ovary (CHO) cell attachment to fibronectin, has been described.[1] IgG from rabbits immunized with goat IgG was purchased from Sigma Chemical Co. The polyclonal antibody, anti-FN, and the monoclonal antibody, IB10, were affinity-purified before use, and along with the rabbit IgG, were diluted in PBS containing 0.5% wt/vol BSA and 0.025 M HEPES.

Binding of Antibodies to Fibronectin-coated Nitrocellulose: Pieces of nitrocellulose (1 × 2 mm) coated with fibronectin (177 μg/ml) were washed three times in PBS containing 0.5% wt/vol BSA and were then incubated in IB10 or anti-FN for 2 h at room temperature. They were next washed four times in 0.154 M NaCl, three times in 0.154 M NaCl/0.05% vol/vol Tween 20 (including an overnight wash at 4°C), and were then incubated in the appropriate second antibody conjugated to horseradish peroxidase (1:1,000 dilution in PBS containing 0.5% wt/vol BSA and 0.05% Tween 20) for 30 min at 37°C. After extensive washing (eight times, including an overnight wash at 4°C), in NaCl/Tween, an o-phenylenediamine-H_2O_2 substrate was added (0.024 M citric acid, 0.053 M sodium phosphate, 0.04% vol/vol 30% H_2O_2, 0.04% wt/vol o-phenylenediamine), and the reaction was allowed to proceed for ~10 min after which it was stopped with 2.5 M H_2SO_4, and the absorbance was read at 449 nm. Pieces of PBS-coated nitrocellulose included as controls were treated in an identical manner.

Migration on Fibronectin-coated Nitrocellulose Treated with Antibodies: Nitrocellulose pieces coated with fibronectin (177 μg/ml) were washed three times in PBS containing 0.5% wt/vol BSA, and were then incubated for 2 h in IgG, anti-FN, or IB10 (see Results for antibody concentrations). After four 0.154 M NaCl washes, the pieces were placed into wounds, and 10 h later the amount of wound epithelium on anti-FN- and IB10-treated pieces was compared to the amount on those treated with IgG.

RESULTS

Migration on Nitrocellulose Coated with Fibronectin or Fibronectin Fragments

In a preliminary experiment we found that a 2-min trypsin digest of fibronectin supported as much migration as the intact molecule, indicating that further studies comparing the ability of fibronectin fragments to support migration were feasible. We therefore tested five proteolytically derived preparations of fibronectin (see Fig. 2) isolated as described in Materials and Methods. We tested the following: a 27-kD amino-terminal heparin-binding fragment (domain I, from both A and B chains of the molecule); a 46-kD gelatin-binding fragment (domain II, also from both chains); a mixture of fragments originating from the middle of both chains (80–125 kD) with neither gelatin nor heparin affinity; a combined 33- and 66-kD carboxy-terminal heparin-binding preparation representing the peptide sequences of domain V of the A chain and domains V and VI of the B chain, respectively; and a 31-kD free sulfhydryl-containing fragment derived from the carboxy-terminal end of the A chain (domain VI).

When the above fragments and intact fibronectin were coated onto nitrocellulose at three different concentrations (400, 100, and 25 μg/ml), the 80–125-kD mixture supported considerably more migration than any other fragment (Fig. 3). For example, at 400 μg/ml, the 80–125-kD mixture produced 50 units of migration above the amount occurring on control (PBS-coated) nitrocellulose (shaded area in Fig. 3). The most migration produced by any other fragment was only 10 units above the control mean, a fivefold difference compared to the 80–125-kD mixture. At 100 and 25 μg/ml, the 80–125 kD mixture was even more effective, whereas migration on the other fragments remained at or near control levels. Not only was the 80–125 kD mixture clearly more effective than the other fragments, but it was also fully as effective as the intact molecule. (Statistical analysis of the data presented in this paragraph appears in the legend accompanying Fig. 3.)

Binding of Fibronectin and Fibronectin Fragments to Nitrocellulose

To be sure that the difference in activity between the 80–125-kD fragments and the others was not simply a reflection of fragment affinity for nitrocellulose, we radiolabeled the various fragments, applied them to nitrocellulose at 400 μg/ml, and then, after extensive washing, assayed the amount of bound radioactivity. The percent of applied counts that bound ranged from 21 to 51% (Table I), with three of the inactive preparations showing a binding efficiency greater than the 80–125 kD mixture, which had the biological activity. The remaining inactive fragment (31 kD) bound with an efficiency comparable to the active mixture. Thus, the inactive fragments were not inactive because of insufficient protein on the

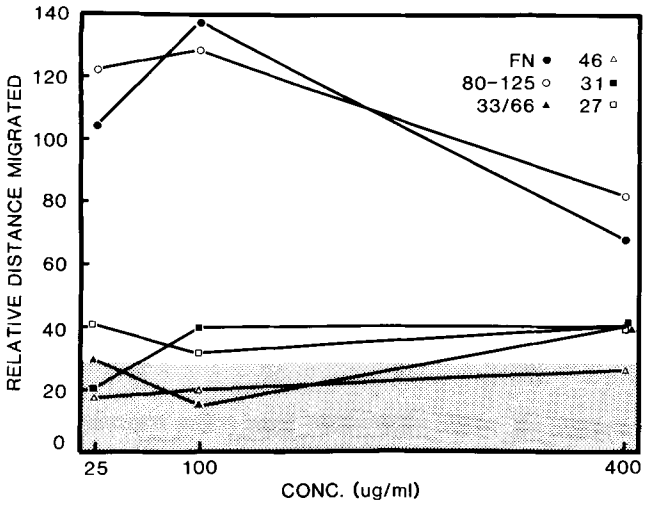

FIGURE 3 Epidermal cell migration on nitrocellulose coated with intact fibronectin (*FN*) or one of the fragment preparations described in Materials and Methods. Pieces of nitrocellulose were coated with the indicated materials by allowing 10 μl of a 400-, 100-, or 25-μg/ml solution to dry on them overnight. After washing in HS that contained 5 mg/ml of BSA, the ability of the coated nitrocellulose to support epidermal migration was tested as described in Fig. 1. Each point represents, at 400 μg/ml, the mean for at least 16 implants; at 100 μg/ml, at least 6; and at 25 μg/ml, at least 8. The shaded area represents the mean for control (PBS coated) nitrocellulose (n = 66). At each concentration tested, the means for the 80–125-kD mixture and intact fibronectin were significantly higher than the control mean ($p < 0.001$ in all cases). At each concentration, the mean for the 80–125 kD mixture was significantly higher than the corresponding point for any other fragment ($p < 0.005$ in all cases), but was not significantly different from the corresponding mean for intact fibronectin ($p = 0.4$ or higher). No point on the four lower curves was significantly higher than the control mean ($p = 0.2$ or higher). All p values were determined with an unpaired t test.

TABLE I. *Binding of Fibronectin Fragments to Nitrocellulose*

Protein applied	Counts applied	Counts recovered	Percent bound
Fibronectin	22,600	6,200	27
80–125 kD	22,900	5,100	22
33/66 kD	16,000	6,600	41
46 kD	19,000	7,900	42
31 kD	50,400	10,700	21
27 kD	27,400	14,000	51

10 μl of tritium-labeled intact fibronectin or fibronectin fragments (400 μg/ml) was dried onto 1- x 2-mm rectangles of nitrocellulose. Later, after the pieces of nitrocellulose were washed extensively, bound radioactivity was determined by scintillation counting. Counts applied and counts recovered represent the mean of triplicate samples.

implants.

The ability of fibronectin-coated nitrocellulose to support migration was better when 100 μg/ml of fibronectin was applied to the implant than when 400 μg/ml was used (Fig. 3). This behavior on nitrocellulose may be related to its porosity. Thus, small amounts of unbound fibronectin leaching into the medium from the depths of the implant may have inhibited migration by competing with bound fibronectin for the epidermal fibronectin receptor. A precedent for this explanation has recently been provided by Yamada and Kennedy (31), who showed that soluble fibronectin and certain synthetic fibronectin peptides could inhibit cell spreading on fibronectin-coated dishes. Indeed, our binding studies using radiolabeled materials showed that small amounts of label (1–6% of the amount bound) did leach into the medium from intact fibronectin and all fragments for several hours after the implants would normally have been placed in the wound. Thus, after the initial BSA treatment and a 30-min wash in HS, the amount of protein lost from each implant over the next 3.5 h was (for the 33/66-kD fragments) 0.07 μg, (for the 80–125 kD mixture) 0.05 μg, (for the 31-kD fragment) 0.04 μg, (for the 27-kD fragment) 0.03 μg, (for the 46-kD fragment) 0.03 μg, (for intact fibronectin) 0.01 μg, most of this label coming off in the first hour. Though this observation may account for the diminished effectiveness of the 80–125-kD active mixture and intact fibronectin at higher concentrations, the fact that these two forms of the molecule were at opposite ends of the range shows that there is no correlation between the amount of protein lost and assignment of a given fragment as active or inactive.

Antibody Blocking Studies

Since the position of the active sequence is between the 46-kD gelatin-binding fragment and a 66-kD carboxy-terminal end piece with an affinity for heparin (Fig. 2), the region of fibronectin with the greatest ability to support migration is in approximately the middle third of the molecule. To investigate the relationship between the fibronectin domain that supports epidermal cell migration and that which mediates the attachment of CHO cells, we used IB10, a monoclonal antibody that blocks CHO cell attachment to fibronectin.[1] Attempts to block epidermal migration on fibronectin-coated nitrocellulose using 55 μg/ml of IB10 were unsuccessful (Fig. 4*a*), despite the fact that this amount of antibody protein saturated the epitope it is directed against (Fig. 4*b*). In contrast, a polyclonal anti-fibronectin which did not saturate implant-bound fibronectin at 55 μg/ml of antibody protein (Fig. 4*b*) inhibited migration by 34% when used at this concentration, and by 80% when used at 400 μg/ml (Fig. 4*a*). Thus, the region of the fibronectin molecule involved in CHO cell attachment may not be required for newt epidermal cell migration.

DISCUSSION

In previous studies we found that the extracellular matrix proteins (fibrin[ogen], collagen, fibronectin, and to a lesser extent, laminin) all support amphibian epidermal cell migration, whereas the nonmatrix proteins (serum albumin, fetuin, casein, and myoglobin) do not (3, 4, 5). Newt epidermal cells therefore, show a distinct specificity in their response to purified proteins as potential migration substrates. The present study now extends this specificity to fragments of fibronectin, in which the ability to support epidermal migration appears to be located primarily in an 80–125-kD sequence in the middle third of the molecule (Fig. 2). By contrast, a 27-kD amino-terminal peptide that binds heparin and *Staphylococcus aureus*, a 46-kD gelatin-binding peptide, a 33/66 kD carboxy-terminal heparin-binding preparation, and a carboxy-terminal 31-kD peptide were all unable or only marginally able to support epidermal cell migration. Since the 46-kD fragment (domain II) was found to be inactive, the active site is probably somewhere in domain III or IV, as depicted in Fig. 2.

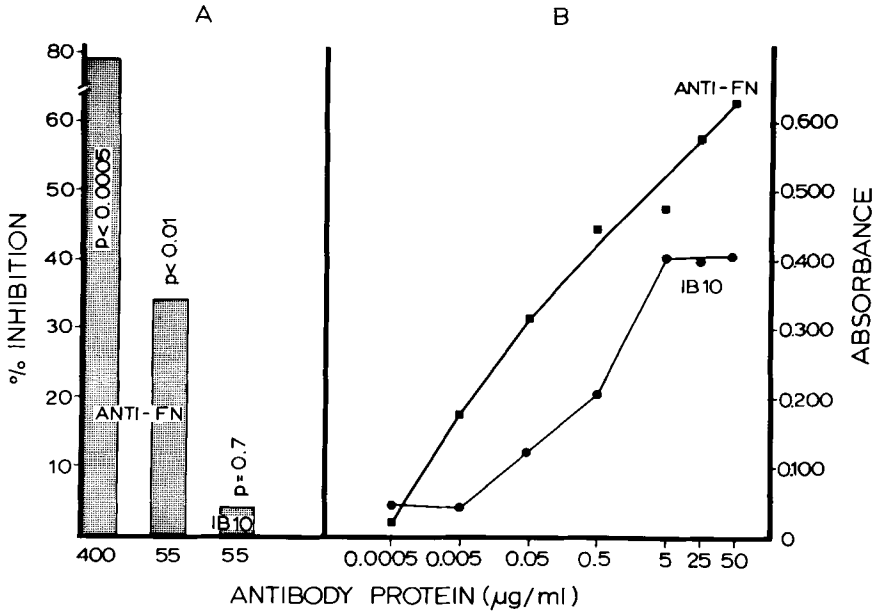

FIGURE 4 (*A*) Epidermal cell migration on fibronectin-coated nitrocellulose exposed to a polyclonal anti-fibronectin antibody (*anti-FN*), or IB10, a monoclonal anti-fibronectin antibody that blocks attachment of CHO cells to fibronectin. After pieces of nitrocellulose were individually exposed for 2 h to the indicated amounts of affinity-purified antibody protein, they were tested for their ability to support epidermal migration as described in Materials and Methods and the legend to Fig. 1. The mean values obtained for the relative distance migrated in each group was used to determine % inhibition according to the following formula:

$$1 - \frac{\text{mean for polyclonal- or monoclonal-treated}}{\text{mean for IgG-treated (400 } \mu\text{g/ml)}} \times 100.$$

In all cases, $n = 8$ or 9. P values were derived from unpaired t tests of the migration means for each group compared to the mean for IgG. (*B*) Binding curve of polyclonal anti-fibronectin (*anti-FN*) and the anti-fibronectin monoclonal, IB10, to fibronectin-coated pieces of nitrocellulose. After a 2-h exposure to anti-FN or IB10, the nitrocellulose was treated with a second antibody conjugated to horseradish peroxidase. Binding was visualized by exposure to an o-phenylenediamine-hydrogen peroxide substrate and the absorbance was read at 449 nm. Each point represents the mean of duplicate or triplicate samples minus the corresponding values for anti-FN- or IB10-treated nitrocellulose not coated with fibronectin.

Though the active domain may also contain the attachment site for CHO cells, our antibody blocking studies suggest that the site supporting epidermal cell migration may be separate and distinct from the CHO binding site. This possibility is based on experiments in which IB10, a monoclonal antibody that potently inhibits CHO cell attachment to fibronectin-coated dishes when used at the same concentration as in the present study,[1] failed to inhibit epidermal migration. Sensitivity of the migration system to antibody blockade was demonstrated when implants pretreated with a polyclonal anti-fibronectin showed a diminished ability to support migration. Thus, at 55 μg/ml, the polyclonal antibody produced a 34% inhibition of migration, and at 400 μg/ml, an 80% inhibition, suggesting near-saturation of the active site at the higher concentration. In contrast, treatment of fibronectin-coated implants with 55 μg/ml of IB10, a concentration well above the minimum amount needed for saturation of the fibronectin epitope against which this monoclonal antibody is directed, had no effect on migration.

The epitope that binds IB10 is not the same as that binding 3E3, a monoclonal antibody derived by Pierschbacher et al. (22) which blocks NRK cell attachment to fibronectin. This was shown when immunoelectroblots of thermolysin digests of fibronectin revealed similar but not identical binding patterns with IB10 and 3E3.[1] Furthermore, IB10 does not react with the 11.5-kD NRK cell attachment peptide purified by Pierschbacher et al.[1] (22). This could mean that IB10 blocks one attachment site and 3E3 blocks another. Alternatively, these monoclonal antibodies may block the same site, one or both acting by steric hindrance. If they block different attachment sites, then the site which supports epidermal cell migration could be the same one that mediates NRK cell attachment. If both monoclonal antibodies block the same site, as seems likely, and the epidermal-IB10 results are accepted at face value, then the epidermal migration site would be unrelated to either CHO or NRK cell attachment. Since embryonic chick neurons will attach and extend neurites on tissue culture plastic coated with the 33/66-kD heparin-binding fragments of fibronectin (23), this would not be the first instance of a cell-binding region unrelated to the attachment sequence described by Pierschbacher et al. (22). We should point out however, that in the epidermal migration system described in the present study, certain modifying molecules (proteins, glycoproteins, glycosaminoglycans, etc.) that are normal components of the wound environment may be involved in the interaction of newt epidermal cells with the fibronectin molecule. Despite the fact that the known binding sites for heparin, collagen, and fibrin(ogen) are excluded from the 80–125 kD sequence (Fig. 2), a wound-derived mediator may yet prove to be involved in newt epidermal cell migration over fibronectin-coated substrates. Should this be the case, the active site which promotes epidermal cell migration might simply represent an amino acid sequence that binds a migration-supporting wound macromolecule. Whatever the mechanism involved, we have shown that within domain III or IV of human plasma fibronectin, there is a sequence which not only mediates the initial attachment of epidermal cells in a semi-in vivo system, but allows those cells to engage in the

multiple cycles of adhesion and de-adhesion necessary for orderly cell movement.

This work was supported by National Institutes of Health grant AM 27940 (awarded to D. J. Donaldson) and National Institutes of Health grants AM 32660, CA 29995, CA 21463, and Leukemia Task Force funds (awarded to L. T. Furcht). L. T. Furcht is a Stone Professor of Pathology and a recipient of Research Career Development award K04-CA 00651 from the National Cancer Institute/National Institutes of Health.

Received for publication 13 August 1984, and in revised form 20 March 1985.

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