The High Affinity Heparin-binding Domain and the V Region of Fibronectin Mediate Invasion of Human Oral Squamous Cell Carcinoma Cells in Vitro*

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Fibronectin is an extracellular matrix molecule composed of repeating subunits that create functional domains. These domains contain multiple binding sites for heparin and for various cell-surface receptors that modulate cell function. To examine the role that the high affinity heparin-binding region and the alternatively spliced V region of fibronectin play in tumor invasion, we expressed and purified four complementary recombinant fibronectin proteins. These proteins either included or excluded the alternatively spliced V region and contained either a mutated, non-functional high affinity heparin-binding domain (Hep⁺) or an unmutated heparin-binding domain (Hep⁻). Cultured oral squamous cell carcinoma cells were assayed for invasion into a Matrigel/collagen matrix supplemented with these four purified recombinant proteins, and for spreading and motility on plastic. Increased invasion was observed in gels supplemented with the V Hep⁺ protein when compared with the V Hep⁻ protein. Inclusion of the V region in the proteins enhanced the invasion and migration associated with both Hep⁺ and Hep⁻ proteins, whereas cell spreading was enhanced with the Hep⁺ recombinant proteins. These data demonstrate that both the high affinity heparin-binding domain and the V region of fibronectin play important roles in invasion, motility, and spreading of oral squamous cell carcinoma cells.

Squamous cell carcinomas are the most common type of malignant oral neoplasm and account for a major portion of deaths related to oral cancer. These tumors represent 4% of all cancers of males in the United States, but in some Asian countries they are the most common malignant tumor. At present the survival rate approximates 50% and has not improved significantly in patients treated over the past several decades (1). Much of the morbidity and mortality associated with these tumors is related to their invasive characteristics. Studies relating to mechanisms used by squamous cell carcinomas for adhesion, motility, and invasion are, therefore, an important aspect of cancer therapy.

Previous studies have implicated cell-surface receptors belonging to the integrin and the proteoglycan families in the invasion and metastasis of tumors (2–4). Furthermore, coordinate interactions among these receptors have been implicated in tumor cell adhesion to extracellular matrix (ECM) components (5, 6). Since some of these adhesive interactions can be blocked by small synthetic peptides derived from ECM molecules, peptide blocking experiments have also identified specific molecules that are potentially useful for therapy (7).

One such molecule which has been studied for its potential therapeutic use is fibronectin (FN), an ECM adhesion molecule composed of multiple functional domains that interact with multiple cell-surface receptors. The domains are composed of repeating structural units, as evidenced by sequence homology, and are designated as type I, type II, and type III repeats. Repeats are numbered from the amino terminus of the molecule. The functional regions include a cell-binding domain, which contains an arginine-glycine-aspartic acid (RGD) adhesive sequence, and a carboxyl-terminal heparin-binding domain (8, 9). The translated protein also includes additional regions that arise through alternative splicing. In rat FN these domains are designated IIIIIB, IIIIIA, and the V region (see Fig. 1).

Many cell-surface receptors interact with specific portions of the FN molecule. The RGD site in the 10th type III repeat (III-10) has been shown to interact with both the αβ1 integrin (10) and αβ1 integrins (11). The αβ1 integrin binds to the V region as well as to sequences in the adjacent 14th type III repeat (III-14) (12). FN also contains numerous heparin-binding sites within the carboxy-terminal portion of the molecule, including a high affinity binding site in the 13th type III (III-13) repeat and multiple low affinity binding sites within III-14 (5, 6). Many of these FN domains retain biological activity when isolated as purified proteolytic or recombinant protein fragments (13). Fragments from the RGD cell-binding, heparin-binding, and alternatively spliced V regions of FN have been used extensively to examine interactions between cells and FN in general (14) and to better understand tumor cell adhesion, motility, and invasion (5, 6, 15–20).

Although many domains of FN have been implicated in mediating tumor cell functions, the contributions of each domain and the relative importance of each family of receptors to these processes are difficult to assess. However, these evaluations are important since they may suggest potential therapeutic interventions by targeting the portions of the FN molecule that play the greatest role in the invasion process. These inquiries are often best made using recombinant proteins that exhibit...
Fibronectin and Carcinoma Invasion

altered function because of specific point mutations rather than deletions of large protein segments, which may alter protein function nonspecifically. In the present study, we used four different purified recombinant FN proteins, with or without function-perturbing point mutations in the high affinity heparin-binding region and with or without the alternatively spliced V region, to evaluate the relative contributions of these domains to the process of invasion by oral squamous cell carcinoma cells. Our results showed that the high affinity heparin-binding domain and the alternatively spliced V region of FN both contribute to the invasive behavior of these cells.

EXPERIMENTAL PROCEDURES

Materials—Growth factor reduced Matrigel basement membrane matrix was purchased from Collaborative Biochemical Products, Bedford, MA. Vitrogen 100-purified collagen was purchased from the Col-lagen Corp., Palo Alto, CA. Nutridoma-HU, a serum-free medium supplement, was purchased from Boehringer Mannheim. Transwell porous cell culture inserts, polycarbonate membranes in 24-well plates (6.5 mm diameter, 8.0 mm pore size), were purchased from Costar, Cambridge, MA.

Cells and Cell Culture—Human oral squamous cell carcinoma cells (HepII) and cells derived from fibrosarcoma (Hep) were a gift from Dr. Randall Krane (University of California, San Francisco) and have been described previously (21). These cells were maintained in α-minimum essential medium containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin.

Recombinant Fibronectin Proteins—Rat fibronectin cDNA was engineered between type III repeats 10 and 15 (Fig. 1A) to form four constructs, which were expressed as recombinant FN proteins and subsequently purified. The proteins spanned repeats III-10 to III-15, and all contained the RGD cell-binding region in III-10, the alternatively spliced EIIIA type III repeat, and the low affinity heparin-binding sequences in the carboxyl-terminal heparin-binding domain. However, the proteins either included (V+) or excluded (V−) the alternatively spliced V region and differed in the high affinity heparin-binding region of III-13 (22). In the latter region, two point mutations were introduced into the cDNA to replace adjacent arginines with threonines (Fig. 1B). Since the threonine mutations have subsequently been shown to virtually abrogate heparin-binding function, these recombinant proteins have been designated as either Hep+ for the unmutated protein or Hep− for the mutated protein. The four proteins are therefore identified as V Hep+, V Hep−, V Hep+, and V Hep−.

The CDNAs used to generate the four recombinant proteins were engineered in two parts as follows. The CDNAs corresponding to repeats III-10 to 15 (Fig. 1A) to form four constructs, which were expressed as recombinant FN proteins and subsequently purified. The proteins spanned repeats III-10 to III-15, and all contained the RGD cell-binding region in III-10, the alternatively spliced EIIIA type III repeat, and the low affinity heparin-binding sequences in the carboxyl-terminal heparin-binding domain. However, the proteins either included (V+) or excluded (V−) the alternatively spliced V region and differed in the high affinity heparin-binding region of III-13 (22). In the latter region, two point mutations were introduced into the cDNA to replace adjacent arginines with threonines (Fig. 1B). Since the threonine mutations have subsequently been shown to virtually abrogate heparin-binding function, these recombinant proteins have been designated as either Hep+ for the unmutated protein or Hep− for the mutated protein. The four proteins are therefore identified as V Hep+, V Hep−, V Hep+, and V Hep−.

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transwell membranes were coated with 1.6 mg/ml collagen, and subsequently incubated at 37 °C in a humidified 5% CO2 incubator. The number of cells from five high power fields (400 magnification) was counted. Each experimental condition was tested in triplicate chambers, with cell counts averaged between the triplicate samples for each experiment.

Cell Adhesion and Spreading—HSC-3 cells were trypsinized, pelleted under centrifugation, washed twice with PBS, and suspended in control medium (serum-free a-minimum essential medium supplemented with 0.2% lactalbumin hydrolysate (Life Technologies, Inc.) and 1% penicillin/streptomycin) at a density of 3 × 10^5 cells/ml. 100 μl of this cell suspension was aliquoted per well into 96-well plates and subsequently incubated at 37 °C in a humidified 5% CO2 incubator. The four recombinant FN proteins were added to the wells immediately before plating cells, yielding a final protein concentration of 100 μg/ml per well. Cells were photographed after 2 h of incubation with the recombinant proteins at 200 × magnification (26).

Cell Migration—Cells were prepared as described for the cell adhesion assay, except that they were suspended at a density of 1 × 10^6 cells/ml; 100 μl of this cell suspension was aliquoted per well into a 96-well plate. After 2 h of incubation, a scratch was made down the center of the well with a sterile needle, removing cells from this area and creating a clear zone into which cells could migrate (27). Photographs of the cell-free scratched area were taken, and then the 96-well plates were incubated for another 7 h. After this time, photographs were again taken to determine if cells had migrated into the scratched area in the presence of the recombinant test proteins.
RESULTS

The diagrams in Fig. 1A depict the III-10 to III-15 repeats of FN, which were expressed as fusion proteins in bacteria. These complementary proteins either included (V⁺) or excluded (V⁻) the V region and contained either the wild-type high affinity heparin-binding region (Hep⁺) or the mutated sequence (Hep⁻). The high affinity heparin-binding consensus sequence (22) was mutated in III-13 to create the Hep⁺ proteins (Fig. 1B). Point mutations were incorporated into the FN cDNA such that in the resultant proteins, adjacent arginines were replaced with threonines. These mutations effectively abrogated heparin-binding function.

Electrophoresis of the four purified recombinant FN proteins produced two bands at approximately 70 kDa, which correspond to the V⁺ Hep⁺ and V⁺ Hep⁻ proteins (Fig. 2A, lanes 1 and 2, respectively), and two bands at approximately 80 kDa, which correspond to the V⁻ Hep⁺ and V⁻ Hep⁻ proteins (Fig. 2A, lanes 3 and 4, respectively). Proteins were detected by staining with Coomassie Blue to demonstrate efficacy of purification. The four recombinant proteins all reacted with a mouse monoclonal antibody to rat FN (24) (Fig. 2B).

We examined the functional significance of the heparin-binding mutation by determining the ability of the V⁻ proteins to bind to columns of heparin-Sepharose. The recombinant proteins were applied to the column and eluted in 3-ml fractions with continuous salt gradients from 0 to 0.5 M NaCl. Recombinant protein was detected in the 3-ml fractions by Western immunoblots. The mutated V⁻ Hep⁻ protein bound tightly to the heparin-Sepharose column, and all protein eluted at 0.5 M NaCl (fractions 39 and 40). The starting material (SM) and flow-through (FT) are shown in lanes 1 and 2 of the Western immunoblot. Molecular mass standards are indicated in kilodaltons (kDa) on the left of the Western immunoblots.

Having purified and functionally characterized the recombinant FN proteins, we next examined the invasive properties of HSC-3 squamous carcinoma cells in response to these FN proteins in a collagen/Matrigel matrix. Initial assays performed with only the V⁻ Hep⁺ and V⁻ Hep⁻ proteins demonstrated that both stimulated cell invasion, but that the Hep⁺ protein was more effective than the Hep⁻ protein in facilitating this process (Fig. 4, B and C). There was at least a 3-fold increase in invasion in gels supplemented with the Hep⁺ protein as compared with gels supplemented with the Hep⁻ protein (Table I). Additional experiments were performed with all four recombinant proteins (Table II). Both V⁺ proteins induced significantly elevated levels of invasion when compared with gels containing either the V⁻ Hep⁻ protein or Matrigel alone. The V⁻ Hep⁺ protein was also more effective than the V⁻ Hep⁻ protein in facilitating invasion. The V⁻ Hep⁻ protein was marginally better at inducing invasion than was Matrigel alone.

These results were confirmed in assays with HOC313, a second oral squamous cell carcinoma cell isolate. These cells invaded the gels with the same order of efficacy as the HSC-3 cells (Fig. 5, Table III). The relative magnitude of invasion induced by the FN proteins was as follows: V⁻ Hep⁻ < V⁻ Hep⁺ < V⁺ Hep⁻ < V⁺ Hep⁺.

Since adhesion and migration are components of invasion, these processes were evaluated separately for HSC-3 cells in the presence of the recombinant FN proteins. Migration assays
showed a 3-fold increase in invasion in gels supplemented with V.

Invasion in cells exposed to the V region was maximized in wells supplemented with the V region. At 7 h of migration, differences in migration were readily demonstrated in assays measuring cell spreading. Cell spreading was maximized in wells supplemented with medium plus V Hep' protein when compared with gels containing the V Hep' protein. Inclusion of the V region of FN enhanced the invasion associated with both Hep' and Hep proteins. Similar results were obtained with HOC313, another oral squamous cell carcinoma isolate. Assays for cell migration demonstrated that inclusion of the V region substantially increased motility of HSC-3 cells. However, cell spreading was most enhanced when HSC-3 cells were incubated with recombinant FN proteins in which the high affinity heparin-binding domain was functional. These data demonstrate that both the high affinity heparin-binding domain and the V region of FN mediate invasion by human oral squamous cell carcinoma cells. However, cell spreading is more associated with the heparin-binding function.

The FN constructs used in this study contain all known low affinity heparin-binding sequences within the carboxyl-terminal type III repeats, yet these sequences did not impart significant functional heparin binding or in vitro invasion. Inclusion of functional high affinity heparin-binding sites did, however, increase invasion. These experiments therefore reveal a structure-function correlation between high affinity heparin binding and important cellular responses. We demonstrated that the V Hep' protein induced an increase in cell invasion of HSC-3 cells when compared with the V Hep' protein. The invasion induced by the V Hep' protein may be mediated in part through heparan-sulfate and chondroitin-sulfate proteoglycan receptors (5, 6). However, since the V region, which contains the principal binding site for the αVβ3 integrin, is not present, these responses are probably not mediated by this integrin (28). Since the constructs do contain theIII-10 repeat and the RGD cell-binding sequence of FN, any interactions with known members of the integrin family probably involve the α5β1 integrin (13).

The V region of FN enhanced the invasive phenotype of both HSC-3 and HOC313 cells. In fact, its presence may predict a

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**Table I**

| Fragment | Experiments* |
|----------|--------------|
| None     | 21 ± 9       |
| V Hep    | 90 ± 15      |
| V Hep*   | 287 ± 22*    |

* The total number of invading cells in 5 high power (400 ×) fields was counted. Data represent the mean ± the standard error of the mean derived from triplicate invasion chambers. Data were analyzed using an ANOVA followed by the Scheffe′ post hoc test.

**Table II**

| Fragment | Experiments* |
|----------|--------------|
| None     | 42 ± 6       |
| V Hep    | 232 ± 56     |
| V Hep*   | 492 ± 83*    |
| V Hep**  | 702 ± 35*    |

* The total number of invading cells in 5 high power (400 ×) fields was counted. Data represent the mean ± the standard error of the mean derived from triplicate invasion chambers. Data were analyzed using an ANOVA followed by the Fisher′ post hoc test.

**Table III**

| Fragment | Invasion* |
|----------|----------|
| None     | 0        |
| V Hep    | 163 ± 4* |
| V Hep*   | 240 ± 5* |

* The total number of invading cells in 5 high power (400 ×) fields was counted. Data represent the mean ± the standard error of the mean derived from triplicate invasion chambers. Data were analyzed using an ANOVA followed by the Scheffe′ post hoc test.

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**DISCUSSION**

In this study, invading HSC-3 squamous cell carcinoma cells showed a 3-fold increase in invasion in gels supplemented with the V Hep− recombinant FN protein when compared with gels containing the V Hep' protein. Inclusion of the V region of FN enhanced the invasion associated with both Hep' and Hep proteins. Similar results were obtained with HOC313, another oral squamous cell carcinoma isolate. Assays for cell migration demonstrated that inclusion of the V region substantially increased motility of HSC-3 cells. However, cell spreading was most enhanced when HSC-3 cells were incubated with recombinant FN proteins in which the high affinity heparin-binding domain was functional. These data demonstrate that both the high affinity heparin-binding domain and the V region of FN mediate invasion by human oral squamous cell carcinoma cells. However, cell spreading is more associated with the heparin-binding function.

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The V region of FN enhanced the invasive phenotype of both HSC-3 and HOC313 cells. In fact, its presence may predict a
change toward a more invasive phenotype. The increase in invasion may be assisted by the high affinity heparin-binding region, but the V region appears to play the dominant role in this process. Other portions of the FN molecule such as low affinity heparin-binding sequences may also be important factors in the malignant process but were not examined in this study.

The high affinity heparin-binding sequence likely mediates interactions with other FN domains and receptors over large spans of FN. This sequence is separated from the binding sites for the α5β1 integrin in the V region by approximately two type III repeats, and from the RGD binding site for the α5β1 integrin by either two or three type III repeats (depending on whether the alternatively spliced EIIIA domain is excluded or included). Analysis of HSC-3 cells using fluorescence-activated cell sorting has in fact shown that these cells express β1, α4, and α5 integrin subunits.2 Interactions between cell-surface receptors such as proteoglycans, which may bind the high affinity heparin-binding domain, and either of these integrins must, therefore, occur between separated portions of the FN molecule. In contrast, virtually all other known heparin-binding sequences in the type III repeats of FN are clustered in the alternatively spliced V region (14). In fact, all three of these low affinity heparin-binding sites and the LDV sequence are located within a stretch of 91 amino acids that encompasses the carboxyl-terminal portion of III-14, the amino portion of the V region, and the intervening hinge region between these repeats. This entire span is the approximate size of one type III repeat. Thus, it seems likely that the low affinity heparin-binding sites mediate cellular interactions coordinately with the α4β1 integrin over a short distance.

Other studies have investigated cellular responses to heparin-binding regions of FN by using either of two approaches. In one series of multiple studies (7, 26, 29, 30), synthetic peptides were constructed from short heparin-binding sequences, and cellular responses to these peptides were evaluated in vitro and in vivo. The results demonstrated that some of the peptides were highly effective in modulating functions such as migration and invasion in many different cell types. Most of these peptides were clustered in the 91-amino acid sequence within III-14 and the V region. Interestingly, the synthetic peptide corresponding to sequences within the high affinity heparin-binding domain was often ineffective in modulating cellular functions (7).

The second approach was to express and purify recombinant FN type III repeats (12, 15). The intervening sequences between the repeats of interest were often deleted in an attempt to eliminate amino acids believed to be nonessential to the biologic responses being measured. These studies have shown that repeats containing either the RGD cell-binding sequence or the high affinity heparin-binding segment were marginally effective at promoting invasion of HT1080 fibrosarcoma cells (14). Mixtures of these two individual repeats were not appreciably better. The recombinant proteins were, however, highly effective in promoting invasion when repeats containing the high affinity heparin-binding segment were linked in tandem with repeats containing the high affinity heparin-binding domain (14).

2 D. Ramos, personal communication.

Fig. 6. Migration of HSC-3 carcinoma cells in the presence of recombinant FN proteins. Images show migration of HSC-3 tumor cells into denuded “scratch” areas in 7-h migration assays. Margins of the denuded areas are indicated by arrows. Panels are from cultures tested with medium alone (panel A), medium plus V Hep⁺ protein (panel B), medium plus V Hep⁻ protein (panel C), medium plus V⁺ Hep⁻ protein (panel D), and medium plus V⁻ Hep⁺ protein (panel E).

Fig. 7. Attachment and spreading of HSC-3 carcinoma cells in the presence of recombinant FN proteins. Images demonstrate attachment and spreading of cells after 2-h incubations. Panels are from cultures tested with medium alone (panel A), medium plus V Hep⁺ protein (panel B), medium plus V Hep⁺ protein (panel C), medium plus V Hep⁻ protein (panel D), and medium plus V⁻ Hep⁺ protein (panel E).
On the basis of these studies, the carboxyl-terminal heparin-binding sites of FN can be classified into two groups. One group is the low affinity heparin-binding sequences located on repeat III-14 immediately adjacent to the V region, which contains the LDV binding site. The second group is the high affinity heparin-binding domain located on repeat III-13, which may require other, nonadjacent cell adhesion sequences to modulate cell function. Previous studies have indicated that small synthetic peptides corresponding to sequences in the high affinity heparin-binding domain are often ineffective in blocking biological activity in vivo. This region, in isolation, may also be ineffective in inhibiting tumor metastasis. Our results would indicate that the high affinity heparin-binding domain plays an important role in tumor invasion. Other studies in which type III repeats containing cell-binding sequences were joined to larger fragments are effective in inhibiting tumor metastasis. However, our results would indicate that the high affinity heparin-binding domain plays an important role in tumor invasion. Other studies in which type III repeats containing cell-binding sequences were joined to larger fragments are effective in inhibiting tumor metastasis.

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