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Synthetic Pentapeptide From the B1 Chain of Laminin Promotes B16F10 Melanoma Cell Migration

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Laminin is a basement membrane-specific glycoprotein that promotes cell adhesion, proliferation, differentiation, and tumor cell migration. Synthetic peptides from the amino acid sequence deduced from a cDNA clone of the B1 chain of laminin were tested for their ability to promote the migration of B16F10 melanoma cells. A peptide, CDPGYIGSR, that is able to mediate epithelial cell attachment to laminin was found to promote migration, and the constituent pentapeptide YIGSR was also active but to a lesser degree. This nine-amino acid peptide blocked migration of melanoma cells to laminin but had no effect on migration to fibronectin. These data suggest that the cell-binding site and migration site on laminin share a common sequence that is unique to laminin.

Laminin, a large cross-shaped glycoprotein (Mr = 800,000), is a major constituent of all basement membranes (Timpl et al., 1979; Engel et al., 1981). It is composed of A (M_r = 400,000), B1 (M_r = 210,000), and B2 (M_r = 200,000) chains that are held together by disulfide bonds (Howe and Dietzschold, 1983; Barlow et al., 1984; Paulsson et al., 1985). Laminin has diverse biological activities including promoting the attachment, differentiation, morphology, and hapotaxis of a variety of cells (Kleinman et al., 1985; Timpl and Dziadek, 1986; McCarthy and Furcht, 1985). Some progress has been made in identifying biologically active domains in laminin. A cell attachment domain has been localized to a large proteolytic fragment (Mr = 300,000–400,000), which includes those portions of the three short arms near the intersection of the cross (Terranova et al., 1983). In addition, a 67 kD cell surface adhesion receptor for laminin that mediates these biological activities has been identified (Rao et al., 1983; Lesot et al., 1983; Malinoff and Wicha, 1983).

Recently, the amino acid sequence of the B1 chain has been deduced from the nucleotide sequence of cDNA clones (Sasaki et al., 1987). These data show that the B1 chain, minus its signal peptide, is composed of some 1,786 amino acids that form six major domains. In order to identify biologically active sites, peptides to sequences in the domains were synthesized and were used to generate antisera. Antibodies to a peptide from domain III inhibited cell attachment although the peptide itself was inactive (Graf et al., 1987). Subsequent studies established that an adjacent peptide, CDPGYISGR, was directly active in cell attachment and in binding to the 67-kD adhesion receptor and represented a major cell attachment site in laminin (Graf et al., 1987).

Here we have found that the CDPGYIGSR peptide has migration-promoting activity for melanoma cells. Analysis of constituent peptides identified a pentapeptide, YIGSR, which directly competes with laminin for this activity.

MATERIALS AND METHODS

Materials

Peptides were synthesized using an automated synthesizer, Model 430A (Applied Biosystems, Inc.) as previously described (Graf et al., 1987). Deprotection and release of the peptide from the solid-phase support matrix was accomplished by treating the protected resin-attached peptide with anhydrous HF containing 10% thioanisole or 10% anisole for 1–3 hours at 0°C. The composition of the peptides was determined by amino acid analyses, and purity was established by high-performance liquid chromatography (HPLC). The peptides of nine amino acids or larger were purified by HPLC, whereas the peptides of six amino acids or less were found to be pure enough (greater than 90%). YIGSR was also tested for composition by fast atom bombardment mass spectroscopy (performed by R.J. Klebe, Univ. of Texas at San Antonio).

Mouse laminin was extracted and purified from the Engelbreth-Holm-Swarm (EHS) tumor using methods previously described (Timpl et al., 1979). Human fibronectin was purified from plasma by affinity chromatography on gelatin Sepharose (Hopper et al., 1976; Engvall and Ruoslahti, 1977).

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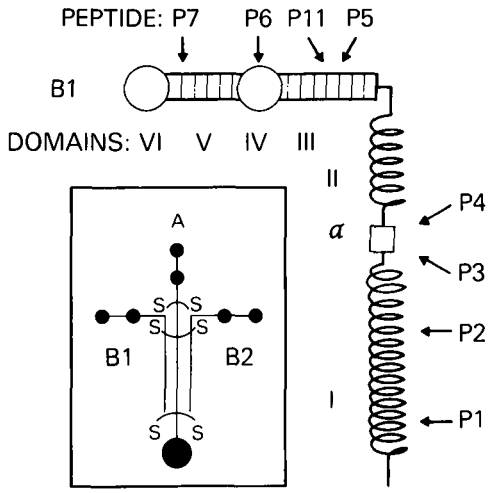

Fig. 1. Schematic model for the B1 chain of laminin. The roman numerals designate the domains. The arabic numbers designate the locations of synthetic peptides. The sequences and amino acid residue numbers (Sasaki et al., 1987) of the peptides are as follows: peptide 1 (residue 1593–1611) = KQADEDIQGTQNLLTSIES, peptide 2 (residue 1509–1529) = KSGNASTPQQLQNLTIDIRER, peptide 3 (residue 1395–1416) = CRTDEGEKKCGGPGCGGLVTVA, peptide 4 (residue 1363–1383) = KLQSLDLSAAAQMTCGTPPGA, peptide 5 (residue 960–978) = NIDTTDPEACDKDTGRCLK, peptide 6 (residue 615–634) = KIPASSRCGNTVPDDDNQVV, peptide 7 (residue 364–385) = PERDIRDPNLCEPCTCDPAGSE, peptide 11 (residue 925–933) = CDPGYIGSR. **Inset:** Schematic model for the entire laminin molecule.

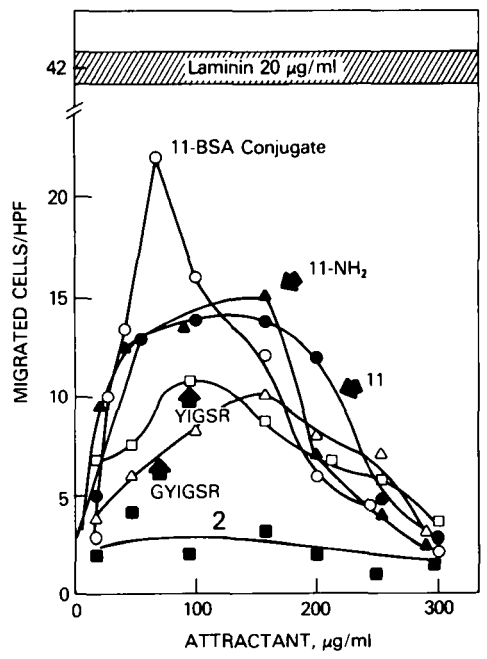

Fig. 2. Migration of melanoma cells to peptides from the B1 chain of laminin. In this assay, the number of cells migrated in the absence of attractant and in the presence of 20 μg/ml laminin were 3 and 42, respectively. The assay was repeated twice, and similar results were obtained. Data are expressed as cells migrated per high-power field. Each data point represents the average of quadruplicate samples, and values did not vary by greater than 15%.

Cell culture

B16F10 melanoma cells (Fidler, 1973) were obtained from I.J. Fidler, Houston, and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with glutamine (242 μg/ml), 1% penicillin (50 units/ml), streptomycin (50 μg/ml), nonessential amino acids, and 10% fetal calf serum (FCS).

Migration assay

The cells were harvested from tissue culture dishes by incubation for 2–3 minutes with 0.025% ethylenediamine tetraacetic acid (EDTA) (Sigma). After the addition of a tenfold excess of DMEM containing 10% FCS, the cells were washed twice in DMEM culture medium containing 0.1% bovine serum albumin (Miles Laboratories) and were suspended in the same medium at a concentration of 3×10^5 cells/ml. The peptides were diluted in the same medium, and various amounts of peptide in a final volume of 0.2 ml were placed in the lower compartment of the Boyden blind well chemotaxis chamber (Postlethwaite et al., 1976). Polyvinylpyrolidine-free polycarbonate filters (8 μm pore size, Nucleopore, CA) coated with type IV collagen (10 μg/filter) were placed above the attractant, and 0.8 ml of the cell suspension was added to the upper compartment. In the "checkerboard" assay, varying amounts of peptides were added to both the upper and the lower compartments (Zigmond and Hirsch, 1973). In some experiments, to determine whether the peptides bound to the filter, the filters were first preincubated for 1 hour with peptide, rinsed two times in medium, and then the assay was continued in the absence of added peptide. After incubation for 5 hours at 37°C in 5% CO_2 and 95% air, the cells on the upper surface of the filter were removed, and the cells that had migrated to the lower side of the filter were fixed with methanol and stained with hematoxylin and eosin. The cells in at least five microscopic fields (250×) per filter were counted.

Competition between synthetic peptides and laminin or fibronectin

The migration assay was carried out as described above with either laminin (20 μg/ml) or fibronectin (20 μg/ml) in the lower compartment of the Boyden chamber as an attractant. B16F10 melanoma cells were placed in the upper compartment of the Boyden chamber with 200 μg/ml of the peptide being tested. After 5 hours, those cells that had migrated to the lower surface of the filter were stained and counted.

RESULTS

Migration of melanoma cells to peptides from the laminin B1 chain

Various synthetic peptides from the B1 chain of laminin were tested for their ability to promote the migration of B16F10 melanoma cells (Figs. 1, 2). Peptides 1–7, which correspond to various domains in the molecule, did not stimulate the migration of the melanoma cells when placed in the lower compartment of the Boyden chamber (only peptide 2 is shown in the Fig. 2), whereas peptide 11 and its amide form stimulated cell migration with a maximal response occurring at 100–150 μg/ml. Peptide 11 conjugated to bovine serum albumin was somewhat more active than peptide 11 or 11-amide. Smaller peptides within the peptide 11 sequence, includ-

A

UPPER COMPARTMENT
μg/ml PEPTIDE

LOWER COMPARTMENT μg/ml PEPTIDE	0	10	50	100
0	4±2	4±2	2±1	5±4
10	7±3	8±2	4±1	4±3
50	12±4	10±1	13±1	6±3
100	21±1	10±3	12±1	13±1

B

UPPER COMPARTMENT
μg/ml PEPTIDE

LOWER COMPARTMENT μg/ml PEPTIDE	0	10	50	100
0	5±2	6±1	4±2	3±2
10	7±1	9±4	5±3	5±3
50	12±3	10±3	9±2	6±1
100	13±5	11±3	11±4	11±3

Fig. 3. Checkerboard analysis of the response melanoma cells to peptide 11 (A) and to peptide 11-amide (B). Different concentrations of the peptide in DMEM containing 0.1% BSA were added to the upper and lower compartments of the migration chamber. Tumor cells in DMEM containing 0.1% BSA were introduced into the upper compartment. The incubation was carried out for 5 hours at 37°C. Numbers within the inner boxes represent the number of migrated cells per field. Responses of cells to a positive gradient are shown below the diagonal, to a negative gradient above the diagonal, and in the absence of a gradient on the diagonal. Results are expressed as the averages of triplicate experiments (cells per high-power field) plus and minus the standard deviation.

ing the 5-mer YIGSR and a 6-mer GYIGSR, appeared to have a lower but a significant level of migration activity ($P < .01$, t-test) (Fig. 2). It should be noted that high levels of these peptides give a reduced response as seen with other attractants, presumably because the gradient of the attractant is lost.

To establish if a true chemotactic response was evoked, the "checkerboard" analysis was performed. In this assay, various concentration of peptides were placed in the upper and lower chambers (Zigmond ahd Hirsch, 1973) (Fig. 3). The response of melanoma cells to peptide 11 and to peptide 11-amide was tested. These studies showed that the cells migrated best in the presence of a positive gradient, i.e., a higher concentration of the peptide in the lower chamber than in the upper chamber. Some chemokinetic activity (along the diagonal) was also observed.

We also tested whether the cells were migrating to an adhesion gradient of peptide (i.e., haptotaxis) or were migrating directly to the peptide (i.e., chemotaxis). In the studies, the peptide YIGSR-NH2 was first incubated in the lower compartment of the Boyden chamber and then some of the filters were washed to remove unbound peptide. The washed and unwashed filters were then tested in the migration assay. Since the washed filters (mean ± S.D = 29.6 ± 8.3 for YIGSR-NH_2 vs. 51.1 ± 8.4 for laminin) promoted cell migration slightly better that the unwashed filters (mean ± S.D = 21.5 ± 4.0 for YIGSR-NH_2 vs. 36.7 ± 5.7 for laminin), we conclude that the peptide was bound to the filter and was acting as a haptotactic factor. The reason for the elevated migration after washing the filter relative to the unwashed filters could be due to removal of excess peptide in the solution, which came off the surface of the filter during the 1-hour incubation.

Effect of synthetic peptides from the B1 chain of laminin on the migration of cells to fibronectin and to laminin

To determine if the peptides were acting through the laminin receptor, we carried out studies using laminin as an attractant (lower well) and various peptides as inhibitors (upper well) (Fig. 4A). Parallel studies were carried out using fibronectin as an attractant (Fig. 4B). Peptides 1–7 did not inhibit chemotaxis when added to the upper chamber (only peptide 2 is shown in Fig. 4A). However, free and bovine serum albumin (BSA)-conjugated peptide 11, peptide 11-amide, GYIGSR, and YIGSR were able to compete with laminin and inhibit its migration activity. Similar studies were carried out using fibronectin as an attractant, but the peptides did not significantly inhibit migration to fibronectin.

DISCUSSION

A synthetic peptide with the sequence CDPGYIGSR from the amino acid sequence of the B1 chain of laminin is able to mediate the attachment of human fibrosarcoma HT-1080 and Chinese hamster ovary (CHO) cells (Graf et al., 1987). Further, this peptide has been found to interact with a high-affinity receptor for laminin (Mr = 67,000) on the surface of many cells. Here we demonstrate that this cell-binding sequence also promotes the migration of B16F10 melanoma cells. The maximal response observed with either peptide 11 or its amide form was only 30% of that observed with 20 μg/ml of laminin itself, suggesting that there may be additional active domains on laminin, or that the CDPGYIGSR peptide lacks important conformational features necessary for maximal migration activity. The 5-mer, YIGSR, and 6-mer, GYIGSR, show less activity than CDPGYIGSR.

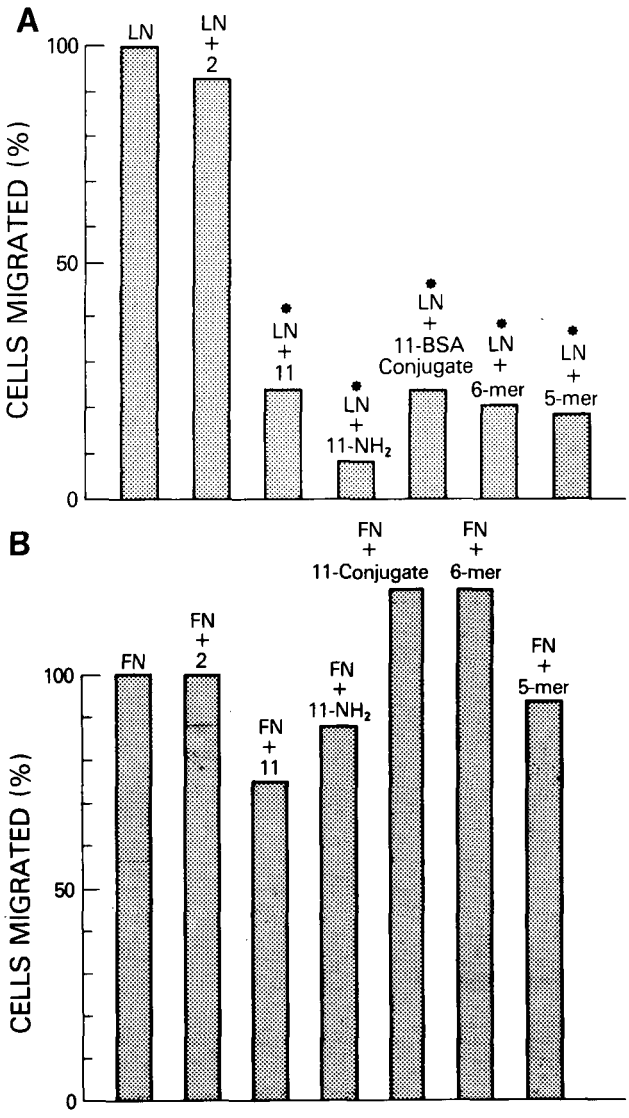

Fig. 4. Inhibition of migration activity to laminin or to fibronectin by synthetic peptides from the B1 chain of laminin. The ability of various peptides from the B1 chain of laminin to block laminin-mediated (A) and fibronectin-mediated (B) melanoma cell migration was tested. In this assay, the number of cells that migrated toward laminin (20 μg/ml) (A) and fibronectin (20 μg/ml) (B) in the absence of any peptides were 42 and 16, respectively. The assays were repeated twice, and similar results were obtained. The bars represent the average of triplicate samples.
*Designates $P = .01$ (t-test).

On a molar basis, more peptide is necessary to elicit a chemotactic response than is required with laminin. Similar loss of molar activity is observed with fibronectin peptides (Akiyama and Yamada, 1985). Since CDPGYIGSR and YIGSR are very effective (90% and 80%, respectively) in inhibiting haptotaxis to laminin, however, it is likely that the major migration site in laminin may be YIGSR.

CDPGYIGSR shows no significant competition for migration to fibronectin. The sequence RGDS in fibronectin is a major site for cell attachment (Pierschbacher and Ruoslahti, 1984) but not for fibroblast migration (McCarthy et al., 1986). Thus, in the case of both laminin and fibronectin, certain cells may recognize distinct sequences (Humphries et al., 1986).

It should be noted that the B16F10 melanoma cells have an overall migratory response to laminin that is low relative to the response observed by other cells to matrix molecules and by connective tissue cells for growth factors (Grotendorst et al., 1982). It is not clear why so few of these cells respond to laminin and to the peptides. It should be noted, however, that only a very small proportion of even the most responsive cells migrate to potent growth factors such as PDGF.

Laminin has been reported to be haptotactic for tumor cells, i.e., they migrate along an increasing concentration of the factor present in an insoluble form (McCarthy and Furcht, 1984). Thus, in vivo where laminin is localized almost entirely in the basement membrane, haptotaxis would be expected to be the principal migration mechanism. In situations where basement membrane and laminin are degraded, however, soluble fragments could serve a chemotactic function. Our data with both the checkerboard assay and the washed filters suggest that the peptide has chemoattractant activity.

Since YIGSR is a major binding site for the laminin receptor (Mr = 67,000), it probably inhibits migration by competing with laminin for binding to this receptor. These results indicate that migration and cell attachment are mediated via the same receptor. Another site on laminin located near the end of the long arm has been found also to promote the adhesion of some cells and to promote neurite outgrowth (Edgar et al., 1984; Goodman et al., 1987). Whether this site will promote migration is unclear. It may also be cell specific in its activity.

YIGSR exhibits the dual activities of cell adhesion and cell migration and could represent an important site on laminin in the developing embryo during tissue formation or in other dynamic tissue processes such as wound healing. The possibility exists for this peptide to be used clinically to accelerate the repair of certain epidermal or corneal wounds, events which may require cell attachment and migration to laminin.

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