Immunology 1991 **72** 144–149 ADONIS 001928059100026K

Identification of an amino acid sequence in the laminin A chain mediating mast cell attachment and spreading

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Accepted for publication 19 September 1990

SUMMARY

PT18 mast cells and mouse bone marrow-derived mast cells have been shown to adhere and spread when in contact with a laminin substratum. Mouse bone marrow cells, however, first require activation with phorbol myristate acetate (PMA), ionophore, or antigen-specific IgE with antigen in order to exhibit these phenomena. Here, we have studied the interaction of these cells with three active synthetic peptides derived from different domains of laminin. PT18 cells and mouse bone marrow mast cells attached and spread on the 19 amino acid synthetic laminin A chain-derived peptide PA22-2, containing the active five amino acid sequence IKVAV, and this attachment did not require prior activation of the mouse bone marrow mast cells with PMA or IgE plus antigen. These cells did not adhere to the B1 chain peptide YIGSR-NH_2 or the RGD-containing peptide from the A chain. PT18 cell adherence to laminin was inhibited by soluble peptide PA22-2, but not by either YIGSR-NH_2, the RGD-containing, or control peptides. Antisera to the PA22-2 peptide completely abolished adherence to PA22-2, but only partially inhibited mast cell adherence to laminin. Antibody to the 67,000–32,000 MW laminin-binding protein receptor blocked cell adhesion to laminin and to the active A chain peptide. Thus, mast cell adhesion and spreading on laminin may be mediated by an interaction with the IKVAV sequence on the laminin A chain.

INTRODUCTION

Laminin is a large glycoprotein found exclusively in basement membranes,[1] where it binds other extracellular matrix constituents including collagen IV, heparan sulphate proteoglycan, and nidogen/entactin.[1] Laminin is composed of three chains, including the A chain (400,000 MW), B1 chain (210,000 MW),[1-3] and the B2 chain (200,000 MW), which are arranged in a cross structure.[4] Part of each chain forms a short arm, with the remainder of each chain projecting down the long arm.[2,5,6] Laminin mediates multiple biological activities, including cell attachment, migration, neurite outgrowth and cell spreading.[7] Several different active domains on laminin have been described which mediate these cellular responses. Synthetic peptide sequences from the B1 and A chain have been found to mimic the biological activity.[8-10] For example, a pentapeptide, YIGSR-NH_2, from the cysteine-rich region of the B1 chain, promotes B16-F10 mouse melanoma cell attachment and migration.[11-13] An RGD sequence from the short arm of the laminin A chain promotes adhesion and spreading (K. Tashiro, manuscript submitted for publication). In addition, a peptide designated PA22-2 (CSRARKQAASIKVAVSADR-NH_2), from the end of the long arm is active in the adhesion, spreading, migration, and neurite outgrowth of various epithelial and neuronal cells.[14] An active site of five amino acids comprising IKVAV has been identified within the PA22-2 peptide.[14] Thus, multiple distinct sites on laminin appear to mediate specific biological functions.

An understanding of cell-surface ligands responsible for mediating the cellular responses to laminin is beginning to be appreciated with the identification of several laminin binding proteins. A protein of 67,000 MW from tumour cells, muscle cells, and macrophages was initially described as a laminin receptor (reviewed by Von der Mark & Kuhl).[15] When cloned and sequenced, it was found that a message of 1·1 kb coded for a protein of 32,000 MW.[16,17] Antibody to the fusion protein identified at least three cross-reacting species of 32,000, 45,000 and 67,000 MW (B. Segui-Real, manuscript submitted for publication). These do not appear to be alternatively spliced molecules, but rather separate gene products. Antibodies to the fusion protein block cell attachment to laminin, but not neurite outgrowth (B. Segui-Real, manuscript submitted for publication).[18] Another class of laminin receptors is the integrin family (reviewed by Horowitz *et al.*).[19,20] These diverse molecules which were initially described as the fibronectin receptor and subse-

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quently were shown to recognize various adhesion molecules (fibronectin, thrombospondin, vitronectin, fibrinogen) containing the RGD sequence. Antibodies to integrin were first shown to block laminin-mediated neurite outgrowth[21] and, subsequently, various integrins have been isolated on laminin–Sepharose columns.[22] To date, at least four different integrins have been found to bind laminin.

Previous studies have shown that mast cells adhere and spread on a laminin substratum in a manner analogous to that seen for epithelial cells.[23] Total RNA isolated from both PT18 cells and mouse bone marrow mast cells was found to contain transcripts for the 32,000 MW laminin binding protein, and immunoblotting with antisera to this binding protein identified mast cell-surface ligands of 67,000, 45,000 and 32,000 MW. In this paper, we have identified an active laminin-derived peptide mediating mast cell adherence and spreading.

MATERIALS AND METHODS

Materials

Laminin (purified from the Englebreth–Holm–Swarm mouse tumour) and WEHI-3 culture supplement were obtained from Collaborative Research (Bedford, MA). Phorbol 13-myristate 12-acetate (PMA) was obtained from Sigma Chemical Co. (St Louis, MO). Peptides were all synthesized as the amide form as described elsewhere[9,24] using an automated Model 430A synthesizer (Applied Biosystems, Inc., foster City, CA). Purity was verified by both amino acid analyses and high-performance liquid chromatography. The peptide designated PA22-2 has a sequence of CSRARKQAASIKVAVSADR, and comprises residues 2091–2108 plus cysteine from the mouse laminin A chain.[14] The peptide designated PA21 has a sequence of CQAGTFALRGDNPQG, and comprises residues 1115–1129 plus cysteine from the mouse laminin A chain (K. Tashiro, manuscript submitted for publication). The control peptide PA22-10 has a sequence of CSRARKQAASGKVAVSADR, where the G (glycine) underlined was substituted for the I (isoleucine) found in PA22-2. The peptide from the B1 chain of laminin is YIGSR and has a sequence of Tyr-Ile-Gly-Ser-Arg and was not given a trivial name as described for the other peptides. Anti-H-2^k antibody (11.4.1) was obtained from Beckton-Dickinson (Parsippany, NJ). Antibodies to PA22-2 were raised in rabbits immunized with peptide which had been polymerized to a final molecular weight of 100,000.[14,24] After two injections, which were 4 weeks apart, the rabbit sera contained antibodies that reacted with the peptide, and with native and denatured laminin. Antibodies to the 67,000–32,000 MW laminin receptor were prepared by immunizing rabbits with the fusion protein obtained from a differentiated F9 cell library. Briefly a λgt11 cDNA library (from Dr Frank Gonzales, NCI, NIH, Bethesda, MD), prepared from human placental poly(A)$^+$ RNA, was screened with an oligonucleotide prepared from the published 3′ portion of the cDNA sequence of the human laminin receptor Lr 67.[16] A mouse cDNA library prepared from differentiated F9 cell poly(A)$^+$ RNA was screened with a cDNA probe obtained from the first prior screen of the human cDNA library (as described in ref. 17). A pEX vector was used containing the entire laminin binding protein FP0.9, for expression of a β-galactosidase fusion protein in bacteria. *Escherichia coli*, strain pop 2136 containing the pEX plasmid, was grown at 30° and the synthesis of fusion protein was induced by incubation of the bacteria at 42° for 2 hr. Subsequently, cell lysates were electrophoresed on a 5% acrylamide gel and the portion of the gel containing fusion protein was excised and the protein was electroeluted (B. Segui-Real, manuscript submitted for publication). Antisera made against a bacterial fusion protein coded for by the β-galactosidase gene plus the 0·9 kb cDNA sequence were found to recognize the several proteins, including the 32,000 MW, the 67,000 MW, and a 45,000 MW laminin-binding protein (B. Segui-Real, manuscript submitted for publication).[23,25] Antisera were raised against a 17-mer synthetic peptide from the NH_2-terminal region of the 32,000 MW cDNA (residues 25–41) and were found to recognize the 32,000 MW and the 45,000 MW laminin-binding proteins but not the 67,000 MW protein in M2 mouse melanoma cells (B. Segui-Real, manuscript submitted for publication) and mast cells.[23]

Cell culture

The interleukin-3 (IL-3)-dependent mouse PT18 mast cell line (as described in ref. 26) was grown in RPMI-1640 supplemented with 25 mM HEPES, 4 mM L-glutamine, 100 μg/ml penicillin/streptomycin, 0·1 mM non-essential amino acids, 1 mM sodium pyruvate (complete RPMI), 10% v/v heat-inactivated foetal calf serum (FCS) and 10% v/v WEHI-3 conditioned media.[27–29] Mouse bone marrow mast cells were obtained from primary bone marrow cultured for 3 weeks in complete RPMI supplemented with 10% v/v FCS and 25 U/ml of WEHI-3 conditioned media[30] and comprised approximately 90% mast cells by 3 weeks. Macrophages present within the cultures were removed prior to experiments by absorption to plastic surfaces of the tissue culture flask, resulting in cultures of >99% purity. Just prior to use in adhesion assays, cells were pelleted at 400 ***g*** for 10 min, resuspended in HBSS without calcium and magnesium, centrifuged at 400 ***g***, and finally resuspended at a concentration of 4×10^5 cells/ml in complete RPMI containing 0·02% bovine serum albumin (BSA).

Cell adhesion

Cell adhesion was performed in 96-well flat-bottomed microtitre plates as described previously[23] with some modifications. Laminin and synthetic peptides were added to the 96-well tissue culture plates in a final volume of 50 μl, and dried overnight. Although coating the plates with laminin in liquid suspension for 2 hr was found to produce identical results to drying overnight, it was found that effective coating of the plates with the peptides could only be achieved by the drying procedure, possibly due to the great difference in size of laminin and the synthetic peptides. The coated wells were washed gently one time with phosphate-buffered saline (PBS), and unbound surfaces were blocked subsequently with 100 μl of 3% BSA in complete RPMI for 30 min at 37°. Wells were washed twice with PBS, and 100 μl of complete RPMI containing 0·02% BSA were added to each well. Mast cells obtained as described above were added to each substrate-coated well (4×10^4 cells/100 μl complete RPMI with 0·02% BSA). Cells were also added to wells that had been coated with BSA alone. No adherence was noted in such wells. Immediately after the addition of mast cells, some of the wells received 4 μl of a solution of PMA, resulting in a final concentration of 50 ng/ml. Plates were incubated at 37° for 1 hr in a 5% CO_2 humidified atmosphere, after which the non-adherent cells were removed by gently washing twice with HBSS. To remove adherent cells from the wells, 200 μl of

trypsin-EDTA were added to each well. After 10 min at 37°, the 200 μl of trypsin-EDTA containing the detached cells were removed, and the cell number was determined using a cell counter (Coulter, ZBI model, Hialeah, FL). All cells were removed from the tissue culture well using this procedure as determined by subsequent microscopic analysis. Cell adhesion was expressed as a percentage value of the total number of input cells. Each peptide was tested at three or more concentrations in four separate experiments.

Inhibition of cell attachment

For the inhibition of attachment to laminin by the peptides, each well was coated with 5 μg of laminin in 50 μl, allowed to dry overnight, and treated with 3% BSA as described above. Prior to the addition of cells, varying concentrations of peptides solubilized in serum-free RPMI containing 0·02% BSA were added to each well in a volume of 50 μl. One-hundred microlitres of the above cell suspension were added, followed by 50 μl of complete RPMI containing 0·02% BSA, and the cells were incubated for 1 hr at 37° in a humidified atmosphere. Attached cells were then quantified as described above.

To examine the effect of anti-PA22-2 antibody[14] on mast cell adherence to laminin and to PA22-2, 96-well tissue culture plates were coated with 10 μg of laminin or 200 μg of peptide for 2 hr at 37°. Unbound surfaces were blocked with 0·1 ml of RPMI containing 3% BSA for 30 min at 37°. The wells were then rinsed with PBS and incubated at 37° for 30 min with varying concentrations (0–32 μl) of antisera in a final volume of 100 μl of complete RPMI containing 0·02% BSA which had been heated at 65° for 10 min to inactivate fibronectin. The antisera were removed and the wells were washed with PBS (100 μl). Mast cells, at 4×10^4/100 μl of complete RPMI containing 0·02% BSA, were added to the wells followed by the addition of 100 μl of complete RPMI containing 0·02% BSA, and plates were next incubated at 37° for 1 hr. Attached cells were quantitated as described above.

Mast cell adherence was also determined in the presence of antibody specific for the 32,000–67,000 MW laminin receptor. The effect of an affinity purified rabbit antisera produced against a murine laminin binding protein was determined by preincubating 4×10^4 cells with 0·05–1·0 μg/ml of this antibody preparation in a total volume of 200 μl of complete RPMI containing 0·02% BSA for 15 min at 4°. This preincubation was found subsequently to be unnecessary and direct addition of antibody and cells to laminin-coated wells was equally as effective in inhibiting adhesion. The cells were then placed in laminin- (10 μg) or peptide- (200 μg) coated wells (dried overnight), and adherence was assessed as described above. Preimmune serum was used as a negative control and was without effect. Anti-H-2^k antibody, which recognizes mast cell-surface proteins,[31] was used as a negative control and was also without effect.

Microscopy

To examine cell adhesion to the peptide PA22-2 and laminin by light microscopy, mouse bone marrow cells were added to laminin; (25 μg/ml) or PA22-2-coated (100 μg/ml) tissue culture chambers attached to glass slides (Labtek, Miles Scientific, IL) at a concentration of 4×10^4 cells per well in serum-free RPMI in the presence or absence of PMA (50 ng/ml). After incubation at 37° in 5% CO_2, 95% air humidified atmosphere for 1 hr, the wells were removed, the slides washed twice with HBSS and allowed to air dry. Slides were stained with Wright–Giemsa using an automatic slide stainer (Ames Hematek, Miles, Naperville, IL), and photographed under high power.

RESULTS

Mast cell adherence to laminin-derived synthetic peptides

We tested PA22-2, PA22-10, PA21, and YIGSR-NH_2 synthetic peptides derived from the A and B1 chains of laminin in an attempt to identify the active sites of laminin responsible for mast cell spreading and adhesion. A 19 mer peptide PA22-2, whose sequence is located at the carboxyl end of the helical region of the A chain, was found to promote significant adhesion (Fig. 1a) and spreading (not shown) of the IL-3-dependent mouse PT18 mast cells and exceeded the maximal level of adherence seen with 100 μg/ml of laminin. PT18 cell adhesion was minimal in response to the peptide YIGSR, whose sequence is located on the B1 chain of laminin. Cell spreading was not detectable. An RGD-containing peptide (PA21) from the amino half of the A chain and a control peptide PA22-10, in which the isoleucine of the active IKVAV sequence of PA22-2 was substituted for glycine, were inactive in promoting cell adhesion and spreading.

We next examined the biological effects of these peptides on primary mouse bone marrow-derived mast cells. Previously, we found that these cells did not adhere to laminin unless activated with PMA,[23] ionophore A23187, or antigen following passive

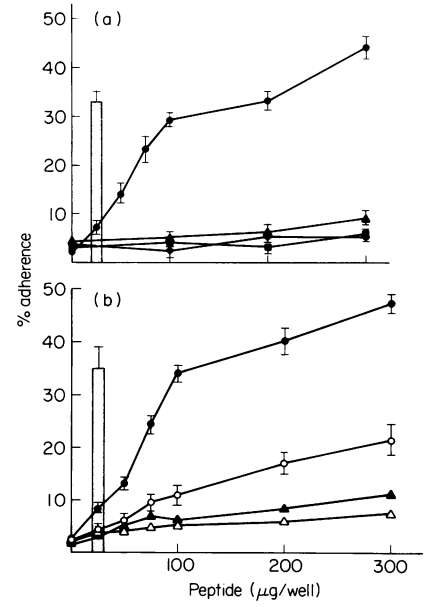

Figure 1. Mast cell adherence to laminin-derived synthetic peptides. (a) PT18 mast cells in the absence of PMA (closed symbols) or (b) mouse bone marrow-derived mast cells in the presence (open symbols) or absence (closed symbols) of PMA (50 ng/ml) were added to plates coated with varying amounts of peptide PA22-2 (circles), YIGSR-NH_2 (triangles), PA21 (diamonds) and PA22-10 (squares), which had been allowed to dry overnight. The maximal activity of these cells (activated with 50 ng/ml PMA in the case of mouse bone marrow mast cells) in response to laminin (100 μg/ml) is shown by the bar. Values represent mean ± SEM for four experiments performed in quadruplicate.

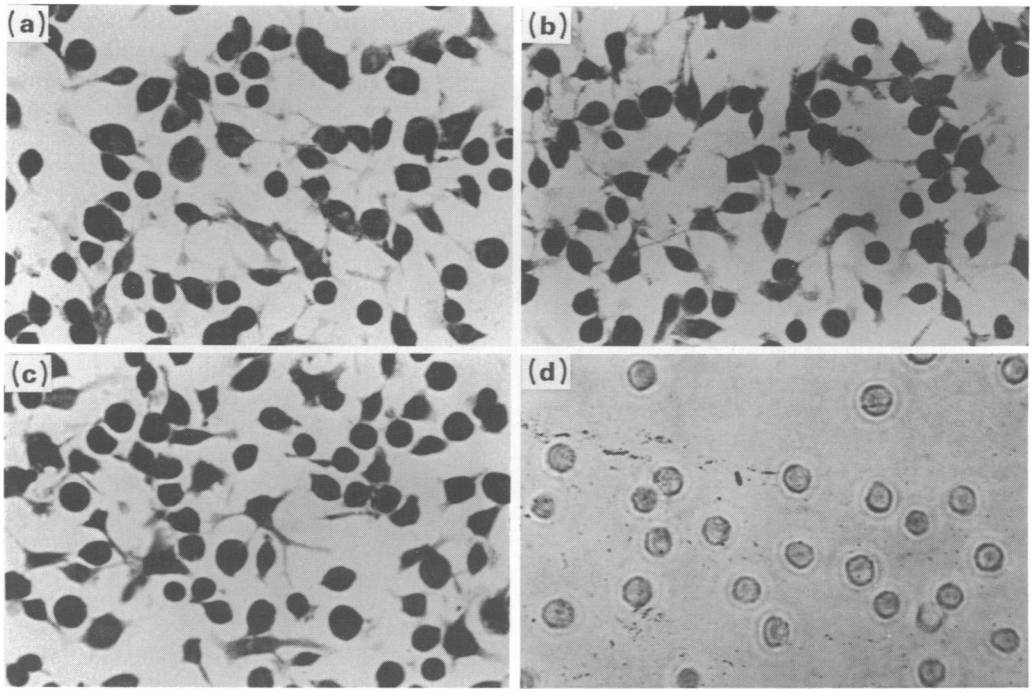

Figure 2. Adhesion and spreading of mouse bone marrow mast cells on both laminin and peptide PA22-2. Mouse bone marrow-derived mast cells were allowed to attach for 60 min to (a) laminin (10 μg) in the presence of PMA (50 ng/ml) and (b, c) peptide PA22-2 (200 μg) in the presence (b) or absence (c) of PMA and photographed at low power. These cells exist normally in tissue culture flasks as a non-adherent cell population (d).

sensitization with IgE.[32] Mouse bone marrow-derived mast cells were also found to adhere to peptide PA22-2 when activated with PMA (50 ng/ml) or in the absence of PMA (Figs 1b, 2). In the latter case, adherence was only 44% of that seen in the presence of PMA. The maximal level of adherence of activated mouse bone marrow mast cells to PA22-2 also exceeded the maximum levels of adherence to laminin. Mouse bone marrow mast cells showed a low level of adherence to the YIGSR peptide with and without activation with PMA. Cell spreading was not observed in response to this peptide. The RGD-containing peptides PA21 and PA22-10 were inactive in promoting cell adhesion or spreading (data not shown). PMA-activated mouse bone marrow mast cells adhered and spread on a laminin substratum (Fig. 2a) and on PA22-2 in either the presence or absence of PMA (Figs. 2b, c).

Inhibition of laminin-mediated cell attachment

We next sought to determine if the PA22-2 site is functional in laminin. The ability of PA22-2 and other peptides to block laminin-mediated adhesion was determined (Fig. 3). In these studies, PT18 cells and peptides were added to laminin-coated wells. Peptide PA22-2 was most active in inhibiting laminin-mediated cell adhesion, blocking attachment by more than 70%. As expected, significantly more peptide was required to compete against the activity of laminin than was required for attachment. Of the other peptides, YIGSR-NH_2 showed minimal inhibitory activity (approximately 10%), and PA22-10 and PA21 were inactive.

A specific antibody to the PA22-2 peptide was raised in rabbits and was assayed for its ability to block laminin-mediated

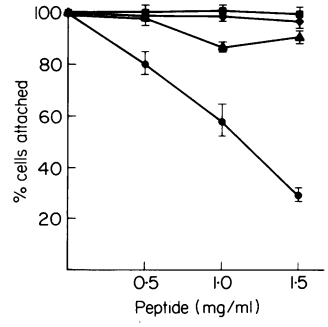

Figure 3. Effect of soluble peptides on PT18 mast cell attachment to a laminin substrate. Tissue culture wells were coated with 5 μg of laminin and allowed to dry overnight. Prior to the addition of cells, varying concentrations of peptides PA22-2 (●), YIGSR-NH_2 (▲), PA-21 (▲) and PA22-10 (■) solubilized in serum-free complete RPMI containing 0·02% BSA were added to the wells. Adhesion to 5 μg of laminin is taken as 100%. Values represent mean ± SEM for four separate experiments performed in quadruplicate.

attachment. Mouse bone marrow mast cell attachment to PA22-2 was inhibited by almost 95% in both the presence and absence of PMA (Fig. 4). Attachment to a laminin substratum was blocked by 60% in the presence of PMA by this peptide antibody. Identical results were observed when PT18 cells were employed in a similar experiment (data not shown). Pre-immune sera did not block attachment to either laminin or PA22-2 (data not shown). These data demonstrate that the PA22-2 site on laminin is a major active site for mast cells, but it is possible that another site(s) exists, since neither the free peptides nor the

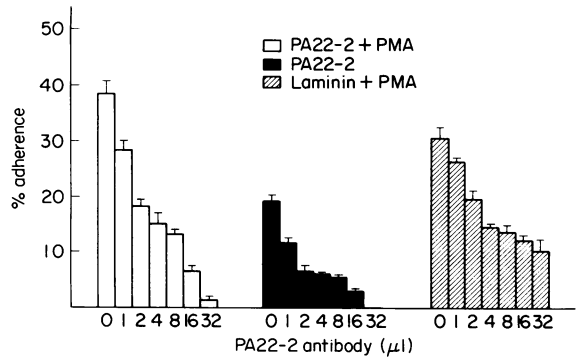

Figure 4. Inhibition of cell attachment by antisera against laminin-derived peptide PA22-2. Mouse bone marrow mast cells were added to tissue culture wells which had been previously coated for 2 hr with either laminin (10 μg) or PA22-2 (200 μg) and then varying concentrations of heat-inactivated antisera to PA22-2 were added. Results are expressed as mean ± SEM for four separate experiments performed in quadruplicate.

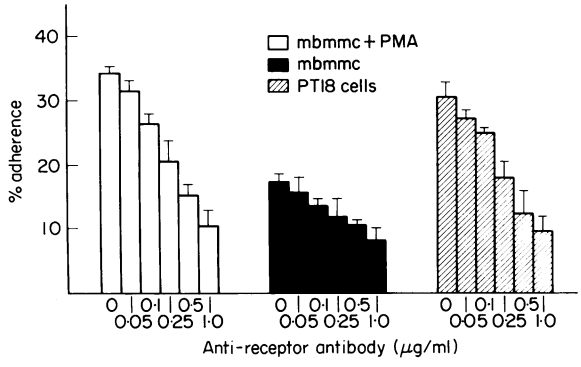

Figure 5. Inhibition of cell attachment by anti-receptor antibody. Mouse bone marrow mast cells (mbmmc) and PT18 mast cells were preincubated with affinity-purified antisera against a laminin binding protein for 15 min prior to addition to PA22-2-coated tissue culture wells in the presence or absence of PMA (50 ng/ml). Results are expressed as mean ± SEM for four separate experiments performed in quadruplicate.

peptide antisera were able to completely block cell adhesion to laminin.

We also examined the effect of an affinity-purified rabbit antisera produced against a murine 32,000–67,000 MW laminin binding protein (B. Segui-Real, manuscript submitted for publication)[14] on mast cell adherence to PA22-2. We have shown previously that this antibody is capable of inhibiting both PT18 cell and mouse bone marrow-derived mast cell adherence to laminin by greater than 95%.[23] When this antibody was preincubated with both PT18 cells and mouse bone marrow-derived mast cells, prior to the addition to peptide PA22-2-coated wells, the antibody inhibited PT18 cell and PMA-activated mouse bone marrow mast cells adhesion to PA22-2 by 60% and non-activated mouse bone marrow mast cell adhesion by 50% (Fig. 5).

DISCUSSION

Laminin is a large, multifunctional molecule that affects the biological activity of many cell types (reviewed by Kleinman *et al.*).[7] It appears to be a major adhesion glycoprotein for mast cells.[11-14] Various active sites on laminin have been defined with synthetic peptides.[12-16] We find that the peptide PA22-2 from the carboxyl end of the A chain is a potent promoter of mast cell adhesion and spreading, whereas the other peptides YIGSR-NH_2 from the B1 chain and the RGD-containing peptide from the A chain had no activity. The PA22-2 peptide can also compete laminin-mediated attachment by 70%, and antisera to the peptide can block laminin-mediated adhesion by 60%. These data define this site as a major active domain for mast cell binding. It is possible that other sites on laminin are also biologically active for mast cells in addition to the PA22-2 domain. For example, neither the PA22-2 peptide nor its antisera were able to completely block laminin-mediated attachment. The PA22-2 antisera did, however, completely block peptide-mediated attachment. Such multiple interactions have been observed with other cell types (K. Tashiro, manuscript submitted for publication)[14] and with other adhesive glycoproteins such as fibronectin, which also has four active sites defined by synthetic peptides.[33]

It was surprising that mouse bone marrow cells could adhere so well to the peptide PA22-2 in the absence of an activating stimulus. Since activation is required for adhesion to laminin,[25] it is likely that different mechanisms are employed by the cells adhering to this peptide and to laminin. It should be noted that recently PMA treatment was found to also induce laminin responsiveness in macrophages[25] and 3T3 cells.[34] The peptide PA22-2, unlike any of the other laminin peptides, is a potent promoter of collagenase IV synthesis and activity,[35] which is necessary for tumour metastasis.[36] The peptide itself may thus be an activator for mast cells, although this has not yet been tested.

In summary, mast cells interact directly with laminin and a major site on laminin has been identified. Other active sites probably exist and may be related to the different functions of mast cells. Although one major laminin binding protein has been identified, it is also probable that multiple receptors function either alone or together to mediate mast cell adhesion to laminin, as observed with hepatocyte adhesion to laminin.[37] Whether these receptors change in either affinity and/or amount after activation could likely influence mast cell behaviour.

ACKNOWLEDGMENTS

The authors would like to thank Brenda Rae Marshall for manuscript preparation.

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