A Mechanism for Regulation of Melanoma Invasion

LIGATION OF $\alpha_6\beta_1$ INTEGRIN BY LAMININ G PEPTIDES

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Invasion of LOX human melanoma cells involves extracellular matrix (ECM) degradation and formation of cell surface invadopodia. Here we show that the ligation of $\alpha_6\beta_1$ by two peptides derived from the COOH-terminal globular domain of laminin-1 $\alpha_1$ chain (laminin G peptides), designated AG-10 (NPWHISIYTRFG) and AG-32 (TWYKIAFQRNRK), and antibodies against $\alpha_6$ and $\beta_1$ integrins promoted invasiveness. AG-10 and AG-32 inhibited cell adhesion on laminin, and the antibodies blocked cell adhesion on immobilized AG-10 and AG-32, suggesting that the peptides interact primarily with $\alpha_6\beta_1$ integrin. These soluble peptides and integrin antibodies induced invasiveness by causing an 2–3-fold increase in ECM degradation and invadopodial activity independently of adhesion activity of integrins that were prebound to ECM. The induced ECM degradation and invasion was associated with an increased surface expression of the 170-kDa membrane-bound gelatinase, seprase, as well as its intense localization at invadopodia but not at focal adhesions. However, the total expression levels of seprase, gelatinase A and $\beta_1$ integrins were not altered. We suggest that laminin G peptides act on the $\alpha_6\beta_1$ integrin signaling of invasion by stimulating invadopodial activities, which is distinct from their direct effects on cell adhesion on immobilized ECM.

Cell invasion through the extracellular matrix (ECM) is an essential process driving tissue development and cancer metastasis (1, 2). Invading cells possess integrated surface activities of ECM degradation and adhesion that appear at unique surface structures, invadopodia (3). The factor that promotes cell invasiveness is not known. However, increasing evidence indicates that laminin, a major basement membrane glycoprotein, and its peptide fragments are able to promote cell invasion. One active peptide, IKVAV, is located on the COOH terminus of the long arm of the laminin $\alpha_1$ chain and promotes cell attachment, migration, tumor growth, metastasis (4–7), and production of gelatinase A/type IV collagenase (matrix metalloprotease-2) (4, 5, 8). A 140-kDa matrix protein, ladsin, which appears to be identical to the laminin $\beta_3$ chain, has been shown to promote scattering of carcinoma cells, stimulate cell migration, and bind to $\alpha_6\beta_1$ integrin (9, 10). Thus, it is possible that laminin/integrin interactions function not only in cell adhesion but also to transduce biochemical signals that modulate surface activities of degradation and invasion (11–13).

Previously, we showed that a membrane-bound, 170-kDa gelatinase, seprase, and secreted, metallo-type gelatinase A were localized on invadopodia of LOX human melanoma cells during degradation/invasion of fibronectin-coated cross-linked gelatin films (14, 15). Further analysis using fibronectin, laminin, type IV collagen, type I collagen, and Matrigel substrata demonstrated that degradation of these ECM components by transformed and tumor cells occurred at invadopodia, suggesting membrane association and activation of latent proteases during invasion (15, 16). However, no specific membrane-associated or secreted protease has yet been associated with an induced invasion. Recently, a systematic screening for cell binding sites with 113 overlapping synthetic peptide beads covering the laminin $\alpha_1$ chain carboxyl-terminal globular domain (G domain amino acid residues 2111–3060) resulted in the identification of 19 potential adhesion-active sequences (17). Here, we identified the peptides designated AG-10 and AG-32 that ligated $\alpha_6\beta_1$ integrin in a similar manner as anti-$\beta_1$ and $\alpha_6$ integrin antibodies promote melanoma invasiveness independently of the adhesion function of integrin receptors. These soluble peptides and antibodies could signal cell invasion at sites of ECM contact. We found that, accompanying the induced invasiveness, there was an increased organization of seprase on invadopodia, but the expression levels of seprase, gelatinase A, and $\beta_1$ integrins were not altered.

**EXPERIMENTAL PROCEDURES**

**Materials**—The human amelanotic melanoma cell line LOX was obtained from Professor Oystein Fodstad, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway. Mouse laminin-1 was prepared from the Engelbreth-Holm-Swarm tumor and all peptides, including AG-10 (NPWHISIYTRFG), AG-32 (TWYKIAFQRNRK), control AG-10-s (NYTTRFGPWSHI), control AG-32-s (TFQRR-KWYKIA), and fibronectin fragment RGD peptide (GRGDS), were synthesized as the COOH-terminal amide form as described (17). Rat mAbs D8, D28, E34, and 13 were described previously (15, 18). mAbs P1E6, an anti-$\alpha_6$ integrin antibody, and P1B5, an anti-$\alpha_5$ integrin antibody, and mouse preimmune IgG were purchased from Life Technologies, Inc. mAb GoH3, an anti-$\alpha_6$ integrin antibody and mouse mAb K20, a noninhibitory anti-$\beta_1$ integrin antibody were purchased from Immunotech (Westbrook, ME).

**Cell Attachment and Spreading Assays**—Attachment and spreading of melanoma cells were assayed as described (17).

**Cell Culture and in Vitro Degradation/Invasion Assays**—All cells were cultured as described (14). The in vitro degradation/invasion technique measures the ability of cells to locally degrade laminin- or fibronectin-coated cross-linked gelatin films as well as to form invadopodia and create surface indentations in cross-linked gelatin films was performed as described (15, 19). In the case of incubation with laminin G peptides (50 mg/ml), cells were allowed to attach to cross-linked gelatin films in the complete medium at 37 °C for 1 h, the medium was replaced by serum-free DMEM, peptides were added to the culture medium, and then cells were incubated for 5 h. Cells grown on the films were photo-

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‡ The abbreviations used are: ECM, extracellular matrix; ELISA, enzyme linked immunosorbent assay; mAb, monoclonal antibody; TBS, Tris-HCl-buffered saline; PBS, phosphate-buffered saline.
Adhesion of Melanoma Cells to Immobilized Matrix Involves α6β1 Integrin—LOX human melanoma cells can adhere and spread on laminin- or fibronectin-coated substrata (Fig. 1). Attachment and spreading on fibronectin substrata were blocked by RGDS peptides but not AG-10 and AG-32 (Fig. 1, A and B). In contrast attachment and spreading on laminin substrata were inhibited by AG-10 and AG-32 peptides but not RGDS (Fig. 1, C and D). Melanoma cells could, furthermore, adhere and spread on AG-10 or AG-32 substrate. Anti-β1 and anti-α6 antibodies, but not preimmune IgG or noninhibitory anti-β1 integrin antibody K20, inhibited both the attachment (Fig. 1E) and spreading (Fig. 1F) of LOX cells on AG-10- and AG-32-coated substrata, suggesting possible receptor α6β1 integrin for AG-10 and AG-32. However, cell attachment to the AG-32 substratum was partially inhibited by anti-α6 and α6 integrin antibodies, suggesting that AG-32 can bind α6 and α5 integrin in addition to α6. These data suggest that AG-10 and AG-32 fragments of laminin bind to the α6β1 integrin receptor and that AG-10 and AG-32 peptides could act on α6β1 integrin-mediated adhesion and spreading of LOX cells (17).

When cells adhere on laminin- or fibronectin-coated gelatin films, β1 integrin complexes are clustered primarily to sites of ECM contact of the ventral surfaces (21–23). To determine if the pre-existing β1 integrin complexes with the ECM of adherent cells are resistant to treatment of soluble laminin G peptides or anti-integrin antibodies, LOX cells attached on laminin- and fibronectin-coated gelatin films were treated with

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\text{Adhesion and spreading of LOX cells on immobilized AG-10 and AG-32 peptides and laminin are mediated primarily by α6β1 integrin. A and B: peptide inhibition of LOX cell attachment and spreading, respectively, on 10 μg/ml fibronectin. C and D: peptide inhibition of LOX cell attachment and spreading, respectively, on laminin-1 substratum. Scrambled cell binding-inactive peptides analogous to AG-10-s and AG-32-s were used as controls. Each value represents the mean of three determinations ± S.D. Duplicate experiments gave similar results. E: effects of anti-integrin antibiotics on cell attachment to 50 μg/ml peptides or 10 μg/ml laminin. Inactive peptides AG-10-s and AG-32-s were used as control. Antibodies added were mouse preimmune IgG (IgG, 2 μg/ml), anti-integrin mAb 13 (β1, 2 μg/ml), noninhibitory anti-β1integrin mAb K20 (N1β1, 2 μg/ml), anti-α6 integrin mAb P1B6 (3 μg/ml), anti-α6 integrin mAb P1B5 (3 μg/ml), and anti-α6 integrin mAb GoH3 (2 μg/ml). Each point represents the mean of three determinations ± S.D. F: effect of anti-integrin antibiotics on cell spreading on peptides and laminin. Cell spreading assay was performed using antibodies as shown in E.}
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Soluble laminin G peptides and $\beta_3$ and $\alpha_6$ integrin antibodies promote degradation/invasion by LOX cells in a dose-dependent manner. A, degradation of the film by LOX (five cells in this field) in the presence of AG-10. B, degradation of the film by LOX (five cells in this field) in the presence of AG-10-s. Bar, 25 $\mu$m. C, effect of soluble laminin G peptides. Cell invasiveness was determined by counting the percentage of cells that degraded the film and that exhibit invadopodia. Each value represents the mean $\pm$ S.D. of three independent experiments, in which 200 cell counts were made. D, effect of soluble $\beta_1$ and $\alpha_6$ integrin antibodies. Antibodies added were mouse preimmune IgG (Ig2), anti-$\beta_1$ integrin (mAb13), noninhibitory anti-$\beta_3$ integrin mAb (K20), anti-$\alpha_6$ integrin mAb (P1E6), anti-$\alpha_6$ integrin mAb (P1B5), and anti-$\alpha_6$ integrin mAb (GoH3). Each value was analyzed as shown in C.

peptides and antibodies. Addition of soluble peptides up to concentrations of 100 $\mu$g/ml into the culture of melanoma cells, which had been plated on fibronectin and laminin films, did not affect the number of cells that remained attached (AG-10, 192 $\pm$ 4 cells; AG-10-s, 190 $\pm$ 5 cells; AG-32, 193 $\pm$ 3 cells; AG-32-s, 192 $\pm$ 5 cells. Each value represents the mean $\pm$ S.D., N = 3). Similarly, addition of soluble antibodies up to concentrations of 10 $\mu$g/ml into the culture on fibronectin and laminin films did not affect the number of cells attached (preimmune IgG, 191 $\pm$ 5 cells; anti-$\beta_1$ integrin, 191 $\pm$ 6 cells; noninhibitory anti-$\beta_1$ integrin mAb, 189 $\pm$ 4 cells; anti-$\alpha_6$ integrin, 190 $\pm$ 5 cells; anti-$\alpha_6$ integrin, 189 $\pm$ 5 cells; anti-$\alpha_6$ integrin, 190 $\pm$ 4 cells. Each value represents the mean $\pm$ S.D., N = 3). However, soluble AG-10 and AG-32 peptides as well as anti-$\alpha_6$ and $\beta_1$ integrin antibodies induced melanoma invasion (see below) and a noticeable change in spread morphology, including more round shape and more surface extensions. We suggest that, in this experimental model, AG-10 and AG-32 peptides exert their effects on the $\alpha_6\beta_3$ integrin available on the dorsal surface of melanoma cells instead of the integrin complexed with ECM in the ventral surface.

Ligation of Available $\alpha_6\beta_3$ Integrin Induces Melanoma Invasion.—To examine the effect of laminin peptides on cell invasiveness, we seeded the human melanoma cell lines LOX, RPMI7951, and SKMEL28, which exhibit from high to very low invasiveness (14, 15), on immobilized fibronectin-cross-linked gelatin films in the presence of serum and allow to adhere for 1 h. Soluble peptides or anti-integrin antibodies were then added to cells in the absence of serum for an additional 5 h. AG-10 and AG-32 are more effective on a weight basis than soluble laminin-1, and cause a 2–3-fold increase in invasiveness of invasion-potent cell lines LOX and RPMI7951 but have no effect on a noninvasive line SKMEL28 (not shown). No stimulation was seen with other peptides and including the control peptides AG-10-s and AG-32-s. LOX cells were 2.3- and 1.9-fold more invasive when treated with 10 to 50 $\mu$g/ml AG-10 and AG-32, respectively (Fig. 2C). Consistent with the increase in invasiveness induced by peptides targeted to the available $\alpha_6\beta_3$ integrin were 2.8- and 1.7-fold increases in invasiveness when treated with anti-$\beta_1$ and anti-$\alpha_6$ integrin antibodies, respectively, from 1 to 10 $\mu$g/ml (Fig. 2D). However, this effect was not seen when the cells were treated with control preimmune rat IgG or noninhibitory anti-$\beta_3$ integrin antibody K20 or anti-$\alpha_6$ P1E6 and $\alpha_6$ antibodies P1B5 (Fig. 2D). None of the antibodies and laminin G peptides affected the adhesion of LOX cells that had been incubated on laminin- and fibronectin-coated cross-linked gelatin films (see above). We conclude that soluble laminin G peptides and anti-integrin antibodies exert their inducing effects on melanoma cells through binding to available integrins instead of pre-existing integrin adhesion complexes with ECM.

Laminin G Peptides Promote Re-organization of Seprase on Invadopodia.—We sought to measure potential changes in gelatinase A, seprase, and $\beta_3$ integrins in response to soluble laminin G peptides within the 5-h culture time. Fig. 3 shows that treatment of LOX cells with laminin G peptides resulted in no significant change in total gelatinase A activity, as analyzed by gelatin zymography (Fig. 3A) or in the total amount of seprase (Fig. 3B) and $\beta_3$ integrins (Fig. 3C), by immunoblotting. Similar treatment with anti-integrin antibodies did not increase the level of total expression of gelatinase A, seprase, and $\beta_3$ integrins, a result different from previous studies on matrix metalloprotease expression using long term invasion assays (8, 12, 24, 25). We suggest that, during the short term induction, laminin G peptides and anti-integrin antibodies stimulate invasion via a mechanism other than increasing expression of proteases.

To measure potential changes in cell surface organization of seprase in response to interactions of the cells with soluble laminin G peptides, cell lysates prepared from surface-labeled LOX cells, that were treated with different laminin G peptides, were divided into aliquots containing the same amount of protein and subjected to immunoprecipitation with anti-seprase mAb D8 and ELISA. Immunoprecipitation of surface labeled seprase resulted in intense signals in cells treated with laminin G peptides, while cells treated with control peptides showed a barely detectable signal (Fig. 4A). ELISA analysis showed that cells treated with AG-10 and AG-32 exhibited 5.2–5.0-fold increases in surface seprase than these cells treated with control peptides (Fig. 4B). Immunofluorescence showed that AG-10 induces an intense localization of seprase at invadopodia (Fig. 4C), consistent with a confocal microscopic analysis showing that there was 6-fold more seprase in invadopodia than other surfaces (15). Thus, the increase in peptide-induced surface proteolysis may be due to the increase in expression of seprase at the cell surface and its localization at cell surface invadopodia (Fig. 4C).

DISCUSSION

We have shown that soluble AG-10 and AG-32 laminin G peptides and anti-integrin $\beta_3$ and $\alpha_6$ mAbs can increase LOX
FIG. 4. Soluble laminin peptides induce an increased organization of seprase on surface invadopodia of LOX cells. A, Immunoblot analysis showing surface-expressed seprase in cells treated with different peptides by immunoblotting using mAb D28. B, ELISA of surface seprase in cells treated with peptides using mAb D28 (B). A 5–6-fold increase in surface localization of seprase occurs in LOX cells treated with laminin G peptides. C, localization of seprase on AG-10–induced invadopodia. LOX cells were treated with AG-10 peptide (50 μg/ml). Cell surface seprase was visualized using mAb E34. Sites of local degradation by LOX invadopodia shown in the left panel (arrowheads) correspond to sites of invadopodia-associated seprase shown in the right panel (arrowheads). Bar, 25 μm.

human melanoma cell invasion by stimulating invadopodial activity. The peptides ligated predominantly the α6β1 integrin receptor that induced the subsequent recruitment of seprase to invadopodia found on the leading edge of invading cells, along with a concomitant increase of melanoma cell invasiveness. Our data suggest a mechanism for melanoma cell invasion in which cell invasiveness can be triggered when β1 integrins, particularly α6β1, on ECM-free surfaces of the cell are ligated by laminin G peptides or antibodies.

The biochemical mechanisms by which laminin peptides exert their effect on invasion are less understood. In this report, we showed that binding of laminin peptides to the available integrin triggers a signal necessary for expression of cell invasiveness. Clustering and ligand occupancy of cell surface integrins are both involved in the activation of intracellular signaling (26–30). Furthermore, fragments of laminin and fibronectin and antibodies against integrin stimulate cellular production of collagenases (8,24). Ligation and perturbation of the α6β1 or α6β1 integrins of human melanoma cells by an antibody or soluble adhesion protein promote secretion of gelatinase A and cell migration/invasion regardless of whether the adhesion function of integrins was affected (12,25).

None of the laminin peptides tested had any detectable effect on the adhesion of cells when added in soluble form to LOX melanoma cells that have already been adhered to gelatin substrates. In addition, there were no detectable changes in the expression level of either β1 integrins or membrane proteases, or their localization in focal contact sites, suggesting that the laminin peptides were functioning specifically to signal the recruitment of proteolytic molecules to invadopodia. Thus, the observed effects of the laminin peptides are independent of possible effects on cell adhesion or gene expression.

Tumor cell invasiveness has been linked with either an increased production of ECM-degrading enzymes or surface association and activation of latent proteases. Using long term (72 h) Boyden chamber migration/invasion assays, laminin SIKVAV peptide was shown to stimulate invasive melanoma cells to either produce or activate gelatinase A (4,5,8) and tissue plasminogen activator-catalyzed plasminogen system (6). In addition, ligation of the α6β1 integrin of invasive C8161 human melanoma cells and the α6β1 integrin of A375 human melanoma cells promotes invasion and a concurrent increase in secretion of gelatinase A (12,25). In contrast, we show here the involvement of AG-10 and AG-32 peptides via the α6β1 integrin in shorter term assays (within 5 h) that resulted in re-organization of seprase at invadopodia, increase in local fibronectin degradation, and invasion but not protein expression. We suggest that receptor binding or clustering of available integrins of adherent melanoma cells affected the invasive process via re-organization of cell surface seprase, that leads to enzyme activation (15).

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