A Novel Protease-docking Function of Integrin at Invadopodia*

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Invasion is a common feature of invasive tumor cells that results in the activation of membrane-bound proteases. In this study, we report new and previously unreported functions of integrin in docking proteases to sites of invasion, termed invadopodia. In the absence of collagen, α3β1 integrin and the gelatinolytic enzyme, seprase, exist as nonassociating membrane proteins. Type I collagen substratum induces the association of α3β1 integrin with seprase as a complex on invadopodia. The results show that α3β1 integrin is a docking protein for seprase to form functional invadopodia. In addition, α3β1 integrin may participate in the adhesion process necessary for invadopodial formation. Thus, α3β1 and αβ3 integrins play major organizational roles in the adhesion and formation of invadopodia, promoting invasive cell behavior.

The integrin family of transmembrane adhesion proteins has been shown to exhibit multiple functions, including adhesion to extracellular matrix (ECM), degradation of ECM, and signal transduction. Because integrins can be regulated by the interaction between integrins and extracellular matrix (ECM) and signal transduction, we hypothesized that integrins may also be involved in recruiting proteases to sites of cell invasion. In support of this hypothesis, the αβ3 integrin has been shown to modulate ECM proteolytic activities by recruiting a major soluble protease, matrix metalloproteinase-2, to the cell surface. Moreover, both adhesive and signaling activities of integrins can be regulated by the interaction between integrins and extracellular matrix (ECM). The results show that α3β1 integrin is a docking protein for seprase to form functional invadopodia. In addition, α3β1 integrin may participate in the adhesion process necessary for invadopodial formation. Thus, α3β1 and αβ3 integrins play major organizational roles in the adhesion and formation of invadopodia, promoting invasive cell behavior.

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†† The abbreviations used are: ECM, extracellular matrix; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; BLOT, immunoblotting; IP, immunoprecipitation.

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collagen I films (rat tail type I collagen at 1 mg/ml according to manufacturers instructions, Collaborative Biomedical Products, Becton and Dickinson Labware, Bedford, MA) and cultured overnight until 80–90% confluence. To harvest lysates, each 175-cm² plate was washed once with 25 ml of PBS, pH 7.4, at 25 °C and then extracted with 25 ml of PBS containing 0.1% Triton X-100 and 0.02% NaN₃ by incubating for 2 h at 25 °C on a rotary shaker (25 rpm, Belco Orbital Shaker, Vine-
land, NJ). The cell layer and buffer (or gelatin plus cell layer and buffer) were transferred to a 50-ml conical tube and incubated a further 3 h at 4 °C with end-over-end agitation. The extract was clarified by centrifuga-
tion at 10,000 × g for 20 min at 4 °C and the supernatants were used for immunoprecipitation reactions. Cell body (cb) and invadopodia membranes (in) were rapidly harvested by shearing the cell bodies in 25 ml PBS after a brief PBS wash. The invadopodia and cell bodies were extracted in 25 ml of extraction buffer as described above for lysates. Purified rat and mouse Mabs against membrane proteins (2.5 mg) were coupled to 1 ml of Sepharose 4 MB (50% slurry) and 0.25 ml used to immunoprecipitate complexes from 25 ml of cell extract overnight at 4 °C with end-over-end agitation. After 3 × washes in 25 ml of extraction buffer, the beads with coupled antibody-antigen complexes were resuspended in extraction buffer (equal to the bead volume) and the sample subjected to 3 cycles of sonication on ice (setting 20, 10 s each using a KOMET Micro Ultrasonic Cell Disrupter). Immediately, the sample was transferred to an Amicon filter insert (0.45 µm, 400-µl capacity) and centrifuged 20 min at 10,000 rpm in an Eppendorf mi-
crofuge at 4 °C. The bead filtrate was used either for Western blotting of integrin and seprase or for zymography to detect seprase gelatinase activity. To test for complete extraction of the seprase and integrin complexes from the beads, Laemmli sample buffer (equal to the bead volume) was added, and the samples were heated by microwaves (2 cycles on low setting, 30 s each, followed by 1 cycle on medium for 30 s). Then, the samples were immediately centrifuged at 25 °C. The filtrates were subjected to immunoblotting and gelatin zymography as described (14, 15). Antigens were essentially absent from post-sonication beads. We concluded that sonication detached antigens from antibodies, but the antibodies were only removed from the Sepharose beads after extraction with Laemmli sample buffer.

Biotinylation and Chemical Cross-linking—LOX cells were cultured on plastic or collagen overnight. After washing cells with PBS, pH 7.4, 1 mM Ca²⁺, 1 mM MgCl₂ at 4 °C, cell surface proteins were cross-linked at 4 °C for 30 min in the same buffer using BS-3 as described by the manufacturer (Pierce). Following additional washing in the above buffer, cells were surface biotinylated at 4 °C for 120 min using SH-biotin (Pierce) as described by the manufacturer. Cross-linking and biotinylation of cell surface proteins were carried out at 4 °C to prevent internalization of cell surface proteins. In addition, these reactions were carried out in the absence of detergent to ensure that intracellular pools of integrins and seprase would not be labeled or cross-linked. Cells were then solubilized in RIPA buffer and immunoprecipitated with C27 or D28. After multiple washes, immunoprecipitated complexes were solubilized in Laemmli sample buffer by boiling without reduction, and the cross-linked proteins were separated on a 7.5% SDS-polyacrylamide gel electrophoresis gel. Ferritin (440 kDa, Amersham Pharmacia Biotech) was used as the high molecular weight standard in addition to the routinely used 205-, 116-, 97.4-, 66-, 45-kDa standards (Sigma).

RESULTS

To determine invadopodial proteins that associate with seprase and participate in matrix degradation and invasion, the mAb C27 was generated using detergent soluble proteins derived from LOX melanoma cells that exhibited gelatinolytic activities. Immunoaffinity chromatography using mAb C27 identified two major bands in the LOX cell extract (Fig. 1A). The first band at 120 kDa, co-migrated with β₁ integrin, and the second migrated at 150 kDa. To determine whether the 120-kDa C27 antigen was β₁ integrin, the C27 antigen was isolated from LOX RIPA extracts by affinity chromatography using either mAb C27 or mAb 13 that recognizes β₁ integrin (17). The eluates were immunoblotted with each of the mAbs and with polyclonal antibodies against β₁ (3847) or β₃ (anti-vitronectin receptor) integrin. The results in Fig. 1B indicate that the C27 antigen band at 120 kDa is β₁ integrin. The C27 antigen was not β₁ integrin, because β₃ integrin was expressed at high levels in platelets but was not detected in LOX antigen preparations (Fig. 1B). The C27–120 and 150-kDa antigens were also affinity-purified from MDA-MB-231 breast carcinoma cells, and the 120-kDa band in C27 antigen preparations was identified as β₁ integrin in these cells (Fig. 1C).

We sought to identify the 150-kDa band by subjecting it to N-terminal peptide sequencing. The following sequence was obtained: F N L D T R F L. The data base search program “FindPatterns” was used to search for the identical protein sequence allowing zero mismatches in the protein data banks (PIR-Protein and SwissProt). This sequence is 100% identical to the N-terminal residues 1–8 of the human integrin α₃ chain/galactoprotein α₃/very late antigen-α₃ chain; residues 38–45 of human integrin VLA-3 α₃ chain precursor; and residues 38–45 of golden hamster cell surface glycoprotein α₃ precursor (20–22). Also, the MDA-MB-231 150-kDa band was sequenced and found to be α₃ integrin. We conclude that mAb C27 recognizes β₁ integrin on Western blots, and appears to preferentially immuno-isolate α₃β₁ heterodimers from these two cell lines.

To probe further the possible interaction between α₃β₁ integrin and seprase, immunoprecipitation was performed on lysates of LOX cells cultured on plastic (Fig. 2A) or collagen or gelatin (Fig. 2, B and C) with mAbs D8 or D28 to detect seprase (14, 15, 18) and C27 to detect β₁ integrin. In three independent experiments, a stable association of integrin and seprase was not detected when cells were cultured on plastic (Fig. 2A) but was reproducibly detected in lysates prepared from cells that were cultured on collagen or gelatin (Fig. 2B). This association was specific because anti-α₃ integrin or rat mAb E19 (control) did not co-immunoprecipitate either β₁ integrin or seprase (Fig. 2B).
To determine the localization of the seprase-α5β1 complex at invadopodia, we fractionated LOX cells into an invadopodia-enriched fraction (in) and the cell body fraction (cb) as described previously (8, 18). Association of seprase and integrin occurred specifically in the invadopodia fraction rather than in the cell body fraction (Fig. 2B, IP: seprase and β1, cb versus in) despite the predominant localization of β1 integrin in the cell body fraction (Fig. 2B, IP and BLOT: β1, cb versus in). As previously demonstrated (18), we found that seprase was concentrated in the invadopodia-enriched membrane fraction (in) with very little detected in the remaining cell body (Fig. 2B, IP and BLOT: seprase, cb versus in). These data suggest the existence of a stable invadopodial complex consisting of seprase and β1 integrin. Furthermore, gelatin zymography detected a 170-kDa gelatinase activity in immunoprecipitates of anti-seprase mAb D28 (Fig. 2C, IP: seprase) or anti-β1 integrin (Fig. 2C, IP: β1). Lysates (ly) from cells cultured on cross-linked gelatin films or on collagen I layers contained equal amounts of seprase gelatinase activity co-immunoprecipitating with β1 integrin. This demonstrated that both native or denatured collagen matrices were equally effective in eliciting co-immunoprecipitation of seprase and β1 integrin (Fig. 2C, IP: β1). Similar to what was observed by immunoblot detection of seprase, the association of seprase gelatinase activity with integrin occurred predominantly in the invadopodia fraction (Fig. 2C). Control immunoprecipitations using mAb E19 (control) and anti-α, mAb did not result in immunoprecipitation of any detectable seprase gelatinase activity (Fig. 2C).

Immunoprecipitations of individual α subunits of integrin were used to determine the specificity of seprase interactions with integrins. Lysates from LOX cells cultured on plastic or collagen were immunoprecipitated using anti-α2, α3, or α6 integrin mAbs or anti-seprase mAb D28 (Fig. 3). Western blotting of immunoprecipitates revealed that anti-α3 mAbs, but not mAbs against α2 or α6, were able to precipitate seprase from cells cultured on collagen (Fig. 3A, BLOT seprase, IP α3 versus IP α2 or α6 obtained from lysates of LOX cells cultured on collagen, lane 7 versus lanes 6 and 8). In the complementary immunoprecipitation, anti-seprase mAb D28 only co-precipitated β1 integrin from cells cultured on collagen (Fig. 3A, BLOT β1, IP seprase obtained from lysates of LOX cells cultured on collagen, lane 5). Western blotting using secondary antibody only (control) revealed background bands that were present particularly in the α6 lane (see Fig. 3A, brackets, rat IgG). These bands, however, were not related to seprase as demonstrated by zymography (Fig. 3B). The increased background bands might be due to impurities that were present in the α6 antibody preparation or the fact that this particular antibody results in higher background binding to lysate proteins. Zymography was used to detect seprase gelatinolytic activity. Activity was only immunoprecipitated from cells cultured on collagen using anti-α3 or seprase mAbs (Fig. 3B). Thus, we conclude that collagenous matrix can induce the seprase-α5β1 association in invadopodia, which results in the localization of the 170-kDa gelatinase activity at sites of matrix degradation.

In vitro experiments to determine the interaction between α5β1 and seprase are not very feasible, because the association of these dimeric membrane molecules requires cell attachment to matrix and occurs only in membranes isolated from the invadopodia-enriched fraction. Therefore, cross-linking and immunoprecipitation analyses were used to explore the cell surface association of seprase and integrin. Chemical cross-linking experiments were used to determine whether seprase interacts directly with β1 integrin complexes on the cell surface. Because immunoprecipitation experiments demonstrated that this interaction occurred only in cells cultured on collagen, but not on plastic, we expected that cells cultured on plastic would not form oligomers of seprase and α5β1 integrin. And, conversely, cells cultured on collagen would be expected to contain complexes of a molecular weight corresponding to seprase dimer (170 kDa) plus α5β1 dimer (−270 kDa), thus a complex of about
Cultured on collagen 1; and 3) this high molecular weight complex at 430 kDa is formed only when cells are
lanes
C27 lanes (Fig. 4, a).

Immunofluorescence studies on cells cultured in the absence of either of these proteins on either substratum (data not shown). 10,000 suspended cells for each antibody, relative to secondary
sorter and immunofluorescence microscopy of cells in suspension or cultured on collagenous substrata. Comparison of the

Chemical cross-linking of seprase and α6β1 integrin on the surface of LOX cells. Cross-linked and biotinylated cell surface proteins were immunoprecipitated using anti-seprase (D28) or anti-β1 integrin (C27) antibodies. When LOX cells were cultured on plastic (plastic), D28 precipitated predominantly the seprase dimer that migrates at 170 kDa (seprase dimer) from cell lysates, whereas C27 immunoprecipitated primarily β1 integrin monomer (120 kDa, β1) and α6β1 dimer (~270 kDa, α6β1). In contrast, following cell culture on collagen (coll1), D28 immunoprecipitated a ~430-kDa complex of α6β1 plus seprase (α6β1 (~270 kDa) + seprase (170 kDa)), seprase dimer (170 kDa, seprase dimer), β1 monomer (120 kDa, β1), and seprase monomer (95 kDa, seprase monomer). C27 immunoprecipitated a high molecular weight complex of the same size as that immunoprecipitated by D28 (α6β1 + seprase).

440 kDa. Comparison of D28 and C27 immunoprecipitates from lysates of cells cultured on plastic versus collagen, confirmed our prediction that seprase and α6β1 integrin were associating in a direct manner at the cell surface (Fig. 4, a, β1 + seprase). Specifically, a complex of ~430 kDa was precipitated both by C27 and D28 demonstrating that the complex contains both α6β1 integrin and seprase dimers (Fig. 4, coll1 lanes). This high molecular weight band was only observed in the lanes derived from cells cultured on collagen (Fig. 4, α6β1 + seprase, IP D28 and C27, compare plastic versus coll1).

In cells cultured on plastic, D28 seprase dimer was the major species observed, whereas C27 precipitated α6β1 dimer as well as a prominent band at the position of β1 monomer (Fig. 4, α6β1 and β1). In cells cultured on collagen, seprase dimers were detected in D28 immunoprecipitates (Fig. 4, seprase dimer, IP D28, coll1 lane). Bands co-migrating with the expected molecular weight of seprase monomer also appear in the D28 and C27 lanes (Fig. 4, seprase monomer, IP D28 and C27, coll1 lanes). We conclude that 1) the immunoprecipitates observed in Fig. 4 are derived from the cell surface; 2) the high molecular weight complex at 430 kDa is formed only when cells are cultured on collagen 1; and 3) this high molecular weight complex corresponds to seprase dimer plus α6β1 dimer, because it is immunoprecipitated by anti-seprase or anti-integrin antibodies.

In addition, genistein, a tyrosine kinase inhibitor, inhibits tyrosine phosphorylation of proteins at invadopodia as well as the degradative and motile activities of invadopodia (8, 9). Immunoprecipitation and immunofluorescence studies demonstrated the association between α6β1 integrin and seprase that occurs only when cells are cultured on matrix. This scenario is also consistent with the state of tyrosine phosphorylation of these cytoskeletal/signaling molecules and their localization at invadopodia (8, 9). In addition, genistein, a tyrosine kinase inhibitor, inhibits tyrosine phosphorylation of proteins at invadopodia as well as the degradative and motile activities of invadopodia (8, 9). Immunoprecipitation and immunofluorescence data shown in this paper demonstrate that α6β1 may participate in the formation of invadopodia by docking seprase, and α6β1 integrin appears to function in adhesion structures throughout the cell, particularly at the base of invadopodia. α6β1 is primarily a receptor for the basement membrane-associated molecules epiligrin and laminin/merosin, but also for fibronectin and collagen types I and IV (25–27). A related laminin binding integrin, αvβ6, does not associate with seprase, even though we found that it was localized at the membrane in fibopodia and invadopodia in a pattern very similar to α6β1 (data not shown). However, α6β1 integrin previously was demonstrated to play a role in signal transduction that promotes invadopodial activities (28) suggesting that these integrins may coordinateantly regulate the activities of invadopodia. Taken...
together, these results suggest that proteolytic activity at the tip of the invadopodia degrades the matrix to weaken resistance to invasion and that collagen-induced $\alpha_5\beta_3$ association with seprase participates in this process. We speculate that $\alpha_5\beta_3$ supports the localized membrane attachment for extension of invadopodia into the matrix.

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