The \( \alpha_\text{v}\beta_5 \) Integrin Receptor Regulates Receptor-mediated Endocytosis of Vitronectin*

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Vitronectin is an adhesive glycoprotein that binds to the extracellular matrix and interacts with integrin receptors on the surface of adherent cells. Previous studies have demonstrated that the conformationally altered, heparin binding form of vitronectin is removed from the matrix by receptor-mediated endocytosis and degraded through a lysosomal pathway (Panetti, T. S., and McKeown-Longo, P. J. (1993) J. Biol. Chem. 268, 11988–11993). The present studies were undertaken to determine the role of cell surface integrins in the endocytosis and degradation of vitronectin. RGDS peptides, used to disrupt the binding of vitronectin to cell surface integrins, inhibited degradation of vitronectin but had no effect on the binding of vitronectin to the cell layer. Localization of vitronectin in the cell layer by indirect immunofluorescence indicated that the RGDS peptides inhibited degradation by preventing the internalization of vitronectin by the cells. To determine which vitronectin receptor was involved in mediating the endocytosis, vitronectin degradation was measured in the presence of monoclonal antibodies. Antibodies against the \( \alpha_\text{v}\beta_5 \) but not the \( \alpha_\text{v}\beta_6 \) integrin inhibited degradation of vitronectin by 80%. This study demonstrates a new role for integrins in regulating internalization and degradation of molecules from the extracellular matrix.

MATERIALS AND METHODS

Cell Culture—Human foreskin fibroblasts (A1-F) were a gift from Dr. Lynn Allen-Hoffman (University of Wisconsin, Madison, WI). The cells were cultured in Ham’s F-12 nutrient medium (F-12) (BR/A Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), penicillin (100 units/ml), and streptomycin (100 \text{ mg} \text{/ml}). Fibroblasts were plated in T-75 flasks (Falcon, Lincoln Park, NJ) at 5 \times 10^5 cells/flask and reached confluence in 5–7 days. Experiments were done on cultures between passages 4 and 12. For experiments, fibroblasts were grown to confluence in 24-well plates. After the cells achieved confluence, fresh ascorbic acid (50 \text{ mg} \text{/ml}) was added to the medium daily for 3–4 days prior to use in experiments. The addition of ascorbate was found to increase the amount of vitronectin bound to the cell layers.

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1 The abbreviations used are: PAI-1, plasminogen activator inhibitor type 1; F-12, Ham’s F-12 nutrient media; BSA, bovine serum albumin; PBS, Dulbecco’s phosphate-buffered saline.
Integrin Receptors Regulate Vitronectin Degradation

Purification and Iodination of Vitronectin—Conformationally altered vitronectin was purified from human plasma by heparin affinity chromatography according to the method of Yatohgo et al. (26). The human plasma had been previously depleted of fibronectin, fibrinogen, plasminogen, and other gelatin-dependent factors (27). Conformationally altered vitronectin (400 μg) was iodinated with 1.0 mCi of Na125I (Du Pont-New England Nuclear) using 0.4 mM chloramine T in 0.04 M phosphate buffer, pH 7.4. After 60 s the reaction was stopped with an equimolar concentration of sodium metabisulfite (Fisher Scientific, Fair Lawn, NJ) to a final concentration of 1×102 μM (0.8% of iodine content). Alternatively, conformationally altered vitronectin (200 μg/200 μl) was iodinated using 1.0 mCi of Na225I in the presence of lactoperoxidase (20 μg/ml), glucose oxidase (1 unit/ml), and glucose (40 mM) (Sigma). After 10 min, the reaction was stopped by the addition of blue and yellow N-ethylmaleimide (10 mM) (specific activity, ~7.9×104 cpm/μg). Free iodine was removed by chromatography on Sephadex G-25 (Pharmacia, Upsalla, Sweden). Iodinated vitronectin was stabilized with 1% bovine serum albumin (BSA) and 0.1 mM phenylmethylsulfonyl fluoride, dialyzed against phosphate-buffered saline (PBS), and frozen at −80 °C until use. Integrity of the labeled protein was assessed by gel electrophoresis and autoradiography.

Binding of Vitronectin to Fibroblast Monolayers—Monolayers of human fibroblasts were incubated at 37 °C for F-12 containing 0.2% BSA and 620 ng/ml 125I-vitronectin. To measure bound vitronectin, medium containing labeled protein was removed, cultures were rinsed three times with PBS, and the monolayer was solubilized in 1× NaOH. Radioactivity was determined by γ scintillation counting.

Degradation of Vitronectin by Fibroblast Cells—Cells were incubated at 37 °C in F-12 containing 0.2% BSA and 620 ng/ml 125I-vitronectin. Degradation of 125I-vitronectin by the cultures was monitored by the appearance of radioactivity in the medium that was soluble in 10% trichloroacetic acid. To estimate background degradation, blank culture dishes were preincubated with complete medium (F-12; 10% fetal bovine serum) for a minimum of 1 h at 37 °C, rinsed, and subjected to the same procedure as above. Background radioactivity was subtracted from total trichloroacetic acid-soluble radioactivity. Radioactivity was determined in a γ counter. To demonstrate a role for integrins in the regulation of vitronectin degradation, turnover of 125I-vitronectin by the cell layers was measured in the presence of increasing concentrations of RGDS peptides (Sigma), RGES peptides (Sigma), or anti-vitronectin receptor antibodies. Specific amounts of peptides or antibodies are provided in the figure legends.

Purification of IgG—Monoclonal antibodies were purified from ascites fluid according to manufacturer's instructions using the MAPS II kit (Bio-Rad). Briefly, the IgG was purified using Protein A affinity chromatography. The IgG was adsorbed to the column at pH 9 and eluted from the column at pH 6 as recommended for IgG. The LM609 antibody, directed against the α5β1 integrin receptor, was a generous gift from Dr. David Cheresh (Scripps Institute, La Jolla, CA). The P1F6 antibody, directed against the α5β1 integrin receptor, was purchased from Telos/GIBCO (Grand Island, NY). Mouse IgG2a (Sigma) was used as a control.

Indirect Immunofluorescence—Vitronectin was localized within cell monolayers by indirect immunofluorescence. Fibroblasts were grown to confluence on plastic dishes and incubated with exogenous vitronectin (25 μg/ml) for 6 h. The monolayers were rinsed with PBS and fixed with 3% paraformaldehyde. Intracellular vitronectin was visualized by treating fixed cells with −20 °C acetone for 10 min. Cell layers were incubated with hybridoma medium (diluted 1:10 in PBS), which contained a monoclonal antibody (8E6) directed against human vitronectin (generous gift from Dr. Deane F. Mosher, University of Wisconsin, Madison, WI). The secondary antibody was rhodamine isothiocyanate conjugated goat anti-mouse IgG (Cappel, Organon Teknika Corp., Durham, NC). Control studies using no primary antibody were negative. Samples were viewed using a Nikon microphot microscope equipped with epifluorescence.

RESULTS

Vitronectin Degradation Is RGD-dependent—In a previous study, we have shown that exogenous vitronectin binds to the extracellular matrix of cultured fibroblast monolayers. Subsequent to matrix binding, the conformationally altered, heparin binding conformer of vitronectin is removed from the matrix by receptor-mediated endocytosis and degraded intracellularly within a lysosomal compartment (25). The present study was done to determine whether cell surface integrin receptors were involved in the internalization of vitronectin from the extracellular matrix. To determine the role of vitronectin integrin receptor, fibroblast monolayers by fibroblast monolayers by RGDS and RGES peptides, vitronectin binding and degradation were measured in the presence of the RGDS peptide. The RGES peptide does not interact with the vitronectin receptors and was used as a control peptide. RGDS peptides inhibited degradation of vitronectin by 80% at a 0.5 mM concentration (Fig. 1A). The RGES peptide did not affect degradation of vitronectin. Neither RGDS nor RGES peptides inhibited vitronectin binding to the monolayer (Fig. 1B). The peptides did not affect cell attachment or spreading of the postconfluent monolayer during the time course of the experiment as assessed by phase contrast microscopy (data not shown). The inhibition of vitronectin degradation with RGDS peptides suggests that the interaction of vitronectin with cell surface integrins may regulate the internalization of vitronectin.

RGDS Peptides Inhibit Vitronectin Internalization—Indirect immunofluorescence was used to determine if RGDS peptides disrupted the internalization of vitronectin. Exogenous purified human vitronectin was incubated with confluent fibroblasts in the presence of either the RGDS or RGES peptide for 6 h. In the presence of the RGDS peptide, exogenous vitronectin was localized to extracellular fibers (Fig. 2A). In control monolayers incubated with RGES peptide (Fig. 2B), vitronectin was localized to both extracellular fibrils and intracellular vesicles. The localization of vitronectin to extracellular fibrils, but not to intracellular vesicles, is consistent with the results obtained in Fig. 1 and suggests that RGD peptides prevent vitronectin degradation by preventing its internalization from the extracellular matrix.

The α5β1 Integrin Regulates Vitronectin Degradation—To determine which cell surface integrin receptor played a role in vitronectin internalization, monoclonal antibodies known to disrupt the binding of vitronectin to the α5β1 or α5β3 integrin receptors were compared for their ability to inhibit vitronectin degradation. Increasing concentrations of IgG were incubated with confluent fibroblast monolayers in the presence of 125I-vitronectin. After an overnight incubation, the trichloroacetic acid-soluble radioactivity in the culture medium was determined. A monoclonal antibody directed against the α5β3 vitronectin receptor (P1H6) inhibited vitronectin degradation by approximately 80% (Fig. 3A). The antibody had a dose-dependent effect on vitronectin degradation with maximal inhibition at 50 μg/ml. Neither a control IgG nor a monoclonal antibody directed against the α5β1 vitronectin receptor (LM609) inhibited vitronectin degradation. None of the antibodies affected vitronectin binding to the cell layer (Fig. 3B). The inhibition of vitronectin degradation by P1H6 suggests that the α5β3 integrin mediates internalization of vitronectin from the extracellular matrix.
Integrin Receptors Regulate Vitronectin Degradation

**FIG. 2. Immunofluorescent localization of human vitronectin in confluent fibroblasts.** Cells were incubated with human vitronectin (25 μg/ml) in F-12 with 0.2% BSA in the presence of 3 μM RGDS (A) or RGES (B) peptides. After 6 h the cell layers were fixed and permeabilized. Vitronectin was visualized by indirect immunofluorescence. Bar = 10 μm.

**FIG. 3. Degradation and binding of 125I-vitronectin in the presence of antibodies against αβ1 and αβ3 integrin receptors.** Fibroblasts were incubated with increasing concentrations of IgG in F-12 containing 0.2% BSA and 125I-vitronectin. After an overnight incubation, the trichloroacetic acid-soluble radioactivity in the culture medium was measured as an index of degradation, and the cell layer was solubilized in 1 N NaOH to determine bound vitronectin (V_b). Data reflect the mean ± S.E.

**DISCUSSION**

Our previous studies demonstrated that the conformationally altered, heparin binding conformer of vitronectin was cleared from the extracellular matrix by receptor-mediated endocytosis and lysosomal degradation. The degradation of conformationally altered vitronectin was inhibited by exogenous heparin and thrombospondin (25). Since thrombospondin has been shown to be degraded by receptor-mediated endocytosis involving cell surface proteoglycans (28, 29), we proposed that thrombospondin and vitronectin were degraded through similar proteoglycan-dependent pathways. In the present study, we report that RGD peptides also inhibited degradation of 125I-vitronectin to trichloroacetic acid-soluble radioactivity by confluent fibroblast monolayers. Localization of vitronectin by indirect immunofluorescence showed that RGD peptides prevented internalization of vitronectin, suggesting that the integrin receptor mediates internalization of the ligand. Both αβ1 and αβ3 vitronectin receptors recognize vitronectin in an RGD-dependent manner and have been identified on the surface of various adherent cell lines (21, 22). The β subunit of these integrins contains the amino acid sequence, NPXY (30, 31). This sequence has been proposed as a consensus sequence to signal receptor internalization (30). Deletion of this sequence from the low density lipoprotein receptor decreases the efficiency of receptor-mediated low density lipoprotein turnover (30). Previous studies have suggested that integrin receptors can potentially mediate internalization of their ligands. An integrin, α_Vβ_3, is thought to mediate internalization of fibroogen from the plasma into the α granules of platelets (32, 33). Electron microscopy studies have shown that the αβ1 fibronectin receptor can be internalized and recycled (34, 35). The present study is the first to demonstrate that integrin-dependent internalization of matrix molecules leads to lysosomal degradation.

Antibodies directed against the αβ3 integrin receptor inhibited the degradation of vitronectin while antibodies directed against the αβ3 integrin had no effect. Both of these antibodies have been shown to prevent vitronectin-dependent adhesion (21, 36). This suggests that the αβ3 antibody is blocking vitronectin degradation by disrupting the interaction of matrix bound vitronectin with the cell surface receptor. The αβ3 vitronectin receptor recognizes numerous ligands including fibronectin and fibrinogen while the αβ3 receptor recognizes only vitronectin (17, 21, 22). Previously we have shown that other ligands (fibronectin and fibrinogen) for the αβ3 vitronectin receptor did not inhibit vitronectin degradation (25). The inability of known ligands for the αβ3 receptor to inhibit vitronectin degradation is consistent with the finding that αβ3 is regulating vitronectin turnover in fibroblast cells.

The data presented here and in previous studies (8, 25) are consistent with a model where simultaneous binding to both cell surface proteoglycans and the αβ3 integrin may be required for internalization of vitronectin. Coordinate interaction of cell surface proteoglycans and integrins has been proposed to regulate both αβ3-mediated adhesion of melanoma cells to the CS1 fragment of fibronectin (37) and the αβ3-dependent formation of focal contacts on fibronectin (38).

We have demonstrated that receptor-mediated endocytosis of vitronectin is triggered by a conversion of vitronectin to its altered heparin binding conformation and that its internalization from the matrix may depend on the simultaneous binding of altered vitronectin to both proteoglycans (25) and the αβ3 integrin (Fig. 3). Physiologically the conformation of vitronectin can be altered by a variety of thrombin-serpin complexes (5). The thrombin-induced conformational change in vitronectin triggers the receptor-mediated endocytosis of vitronectin and subsequent lysosomal degradation (25). These findings suggest a role for vitronectin in the opsonization of thrombin-serpin complexes, mediating their integrin-dependent clearance from the pericellular environment. These findings suggest that the αβ3 and αβ3 integrins may have distinct functions. The αβ3 may be involved in mediating cell adhesion while the αβ3 regulates matrix turnover and remodeling.
REFERENCES