The $\alpha v\beta 5$ integrin functions as an endocytic receptor for vitronectin

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SUMMARY

Endocytosis and degradation of vitronectin by human skin fibroblasts are regulated by the $\beta 5$ integrin. To determine whether the $\beta 5$ integrin is directly mediating the internalization of vitronectin, both vitronectin and the $\beta 5$ integrin were localized by indirect immunofluorescence during the endocytic process. This analysis showed that both vitronectin and $\beta 5$ were found in intracellular vesicles within 5 minutes of the addition of exogenous vitronectin to fibroblast cell layers. By 15 minutes, approximately 20% of the vitronectin-containing vesicles stained positively for $\beta 5$. In contrast, the $\beta 3$ integrin was not found in any intracellular vesicles. Within 30 minutes, more than 50% of vitronectin-containing vesicles also stained for lamp-1, indicating that internalized vitronectin traveled to lysosomes. Inhibition of clathrin assembly by either potassium depletion or hypertonic buffer inhibited vitronectin internalization, suggesting that vitronectin internalization occurred through coated pits.

Confocal analysis confirmed the colocalization of vitronectin and $\alpha v\beta 5$ in intracellular compartments and further demonstrated that the highest colocalization of the two proteins occurred within 1.8 $\mu$m from the ventral surface of the cell, suggesting endocytosis occurred at the substrate level. Pretreatment of cells with the PI-3 kinase inhibitor, wortmannin, resulted in a marked increase in the coincidence of vitronectin and $\beta 5$ staining within vesicles and prevented the accumulation of vitronectin within lysosomes. This suggests that following internalization, vitronectin and the $\alpha v\beta 5$ integrin are segregated to different cellular compartments. This study provides the first evidence that the $\alpha v\beta 5$ vitronectin receptor directly mediates the internalization of vitronectin.

Key words: Vitronectin, $\alpha v\beta 5$ integrin, Endocytosis

INTRODUCTION

Vitronectin is a glycoprotein with diverse distribution and functions. In the plasma, vitronectin has been found complexed with members of the complement as well as the coagulation cascade (McKeown-Longo and Scalise-Panetti, 1996; Preissner, 1991). In the tissues, vitronectin is found in the extracellular matrix associated with neural crest cell migration (Waltz and Chapman, 1994), angiogenesis (Brooks et al., 1994; Drake et al., 1995) and tumor progression (Gladson et al., 1990, 1995), suggesting that vitronectin may play a role in directed cell migration.

Matrix vitronectin supports integrin-dependent cell adhesion and migration of a number of cell types. A major role of vitronectin in the matrix is the regulation of the activity of both plasminogen activator inhibitor, PAI-1 and urokinase-type plasminogen activator (uPA). Vitronectin is the primary binding site for Type 1 (PAI-1) in the extracellular matrix (Seiffert and Loskutoff, 1991; Seiffert et al., 1994; Deng et al., 1995). In addition, vitronectin directs the uPA/uPAR complex to focal adhesion areas, where it colocalizes with integrin receptors and vinculin (Wilcox et al., 1996; Ciambrone, 1992). The binding of PAI-1 to vitronectin stabilizes PAI-1 in an active conformation where it can inhibit both plasminogen activator and thrombin (Naksi et al., 1993; Ciambrone, 1992; Ciambrone and McKeown-Longo, 1990; Hekman and Loskutoff, 1985), suggesting that matrix vitronectin may be important in the regulation of hemostasis as well as in the control of pericellular proteolysis.

Two conformationally distinct forms of vitronectin, native and conformationally altered, have been distinguished using the 8E6 monoclonal antibody (Tomasini and Mosher, 1988). Native vitronectin is a monomeric plasma protein that binds weakly to heparin (Bittorf et al., 1993; Dahlback and Podack, 1985). Conformationally altered vitronectin results from the treatment of native vitronectin with denaturing agents such as urea. The altered vitronectin has an exposed epitope for the monoclonal antibody 8E6 and is found in a multimeric form with an increased affinity for heparin (Stockmann et al., 1993; Bittorf et al., 1993; Tomasini and Mosher, 1988).

Such multimeric forms of vitronectin have been identified in platelet releasate (Seiffert and Schleef, 1996; Stockmann et al., 1993). Multimerization of native vitronectin also occurs following the binding of PAI-1 or heparin (Seiffert and Loskutoff, 1996; Seiffert, 1997). Our studies have demonstrated that both native and multimeric forms of vitronectin bind directly to sulfated proteoglycans present in the matrix of fibroblast monolayers (Wilkins-Port and
McKeown-Longo, 1996; McKeown-Longo and Panetti, 1993). Although both native and multimeric vitronectin bind to the same site in the cell layer (McKeown-Longo and Panetti, 1993), multimeric vitronectin is readily internalized by fibroblasts and degraded. Degradation of vitronectin by fibroblasts is inhibited by monoclonal antibodies against the \( \alpha_\nu\beta_5 \) integrin receptor (Panetti and McKeown-Longo, 1993b), suggesting that internalization of vitronectin from the matrix is regulated by the \( \alpha_\nu\beta_5 \) integrin. Astrocytoma cells have also been demonstrated to internalize and degrade vitronectin in both an \( \alpha_\nu\beta_5 \)- and \( \alpha_\nu\beta_5 \)-dependent mechanism (Pijuan-Thompson and Gladson, 1997).

Vitronectin integrin receptors have been found to promote the clearance of other molecules such as adenovirus (Wickham et al., 1993, 1994; Goldman and Wilson, 1995) and crocidolite asbestos fibers (Boylan et al., 1995). However, none of these experiments addressed whether the \( \alpha_\nu\beta_5 \) acted directly as an endocytic receptor or regulated the activity of another distinct endocytic receptor. To determine the role of the \( \alpha_\nu\beta_5 \) integrin receptor in the internalization of vitronectin, we localized both the \( \alpha_\nu\beta_5 \) receptor and vitronectin during the process of endocytosis. The data indicate that the \( \alpha_\nu\beta_5 \) receptor localized with vitronectin in early endosomes within 5 minutes of the addition of exogenous vitronectin to fibroblast monolayers. The extent of \( \beta_5 \) and vitronectin colocalization in endosomes was markedly enhanced with wortmannin, indicating that PI-3 kinase activity may regulate intracellular trafficking of both vitronectin and \( \beta_5 \). Internalization of vitronectin was inhibited by potassium depletion, suggesting that endocytosis of vitronectin was dependent on clathrin coated pit formation. After 30 minutes, vitronectin was colocalized with lamp-1 in perinuclear vesicles, indicating that internalized vitronectin was targeted to lysosomes. These results provide the first direct evidence that the \( \alpha_\nu\beta_5 \) integrin functions as an endocytic receptor.

**MATERIALS AND METHODS**

**Cell culture**

Human foreskin fibroblasts (A1-F) were a gift from Dr Lynn Allen-Hoffman (University of Wisconsin, Madison, WI). Cells were cultured with Dulbecco’s Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml Penicillin and 100 U/ml Streptomycin (Gibco Laboratories, Grand Island, NY). Fibroblasts were plated in T75 flasks (Falcon, Lincoln Park, NJ) at 5x10^5 cells/flask and reached confluence in 5-7 days. For experiments, fibroblasts were seeded onto glass coverslips in 6-well plates at a density of 2x10^5 cells/ml. Cells were seeded in serum-containing medium for 3 days, at which time the cells reached between 70% and 90% confluence.

**Purification of vitronectin**

Conformationally altered vitronectin was purified from human plasma by heparin affinity chromatography according to the method of Yatohgo et al. (1988) as previously described (Panetti and McKeown-Longo, 1993a).

**Conjugation of vitronectin with Texas Red**

Conformationally altered vitronectin (0.56 mg) was diluted 1:1 in 0.1 M sodium borate buffer, pH 8.8, and conjugated with Texas Red according to manufacturer’s instructions. 1 mg of Texas Red sulfonyl chloride (Molecular Probes, Inc., Eugene, OR) was reconstituted with 200 \( \mu \)g/ml of Texas Red and added immediately to the protein with constant agitation at 4°C for 1 hour. Conjugated protein was separated from unreacted Texas Red by chromatography on a Sephadex G-25 column. The extent of protein conjugation was measured according to the manufacturer’s instructions by determining the ratio of A_{596}/A_{280}. Proteins with a ratio between 0.8 and 1.2 were used in experiments.

**Binding of vitronectin to fibroblast monolayers**

Conformationally altered vitronectin in serum-free medium at a concentration of 12.5 \( \mu \)g/ml was incubated with cells at 37°C. Medium was supplemented with 0.25% Redu-SER II (Insulin, 25 mg; human transferrin, 25 mg; human selenite, 25 \( \mu \)g; bovine serum albumin (BSA), 2.5 g; oleic acid, 21.4 mg) (Upstate Biotechnology, Inc., Lake Placid, NY). After incubation of the cell layers with vitronectin for various times, cells were fixed, permeabilized and stained as described below. Specific times and fixation protocols are indicated in the figure legends.

In some experiments chloroquine was used at a concentration of 1x10^-4 M. In other experiments, wortmannin was used over a concentration range of 0.01-10 \( \mu \)M. To demonstrate that vitronectin endocytosis required clathrin, potassium depletion and hypertonic buffers were used according to the procedure of Altankov and Grinnell (1993). For potassium depletion experiments, cells were washed twice with potassium-free buffer (140 mM NaCl, 50 mM HEPES, 1 mM CaCl_2, 0.5 mM MgCl_2 and 400 \( \mu \)l of Redu-SER II, pH 6.5). Control cells were washed in the same medium except that it was supplemented with 3 mM KCl. The hypertonic treatment involved incubating cells in the potassium-containing medium supplemented with 0.45 M sucrose. Cells were then preincubated with each of these media for 15 minutes, after which the medium was changed to medium containing vitronectin for 30 minutes. The cells were then fixed, permeabilized and stained for vitronectin.

**Indirect immunofluorescence**

Cells were fixed with 3.7% paraformaldehyde for 10 minutes at 37°C and permeabilized for 5 minutes with 0.5% Triton X-100. Free aldehyde groups were blocked by the addition of glycine at 10 mg/ml. All solutions were made in a physiological buffer that was a modification of Small’s Buffer (Small and Celis, 1978). The buffer consisted of NaCl, 137 mM; KCl, 5 mM; NaHPO_4, 1.1 mM; KH_2PO_4, 0.4 mM; NaHCO_3, 4 mM; glucose, 5.5 mM; EGTA, 2 mM; EDTA, 1 mM; Pipes, 5 mM. Vitronectin was stained using a 1:400 dilution of a polyclonal antibody (AC-7) (McKeown-Longo and Panetti, 1993). The \( \beta_5 \) integrin subunit was visualized using a 1:400 dilution of ascites fluid containing the P1F6 antibody against human integrin \( \alpha_\nu\beta_5 \) (Gibco BRL, Gaithersburg, MD). Hybridoma supernatant containing monoclonal antibody against human lamp-1 (Clone H4A3) was used at a 1:10 dilution (Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, Iowa). The secondary antibodies were used at the following concentrations: Texas Red conjugated-goat anti-mouse (5.0 \( \mu \)g/ml), fluorescein-conjugated goat anti-rabbit (2.0 \( \mu \)g/ml), Texas Red-conjugated goat anti-rabbit (2.0 \( \mu \)g/ml), and fluorescein-conjugated sheep anti-mouse (2.0 \( \mu \)g/ml). Texas Red-conjugated goat anti-mouse was purchased from Calbiochem, LaJolla, CA. All other secondary antibodies mentioned above were purchased from Cappel (ICN), Costa Mesa, CA. All primary and secondary antibodies were diluted in the modified Small’s buffer containing 2 mg/ml BSA. Cells were photographed under epifluorescence microscopy with corresponding phase or interference reflection microscopy (IRM) images using an Olympus BX60 microscope. Fluorescence images were recorded directly from the microscope with T-MAX 400 film (Kodak). IRM and phase contrast images were recorded directly from the microscope using Technical Pan Estar-AH 35 mm negative film (Kodak).

**Confocal microscopy**

The distribution of vitronectin with the \( \beta_5 \) integrin at different sections...
of the cell was analyzed using a Noran Instruments argon krypton laser scanning confocal microscope (Oz) equipped with a Nikon Diaphot 200 inverted microscope (Tokyo, Japan). Representative images corresponding to the ventral surface, dorsal surface and the interior of the cell were selected from a 20-optical-sectioning series of cells. Sections were taken every 0.3 µm starting from the ventral cell surface. The Oz confocal microscope was connected to a Silicon Graphics, Indy, computer equipped with an Intervision imaging hardware and software system (Middleton, WI). Intervision was used to collect, superimpose and store images. Confocal images were then transferred to a Gateway P5-200 PC, IBM-compatible computer (Gateway 2000, North Sioux, SD), prepared for printing through Photoshop (Adobe Systems, Inc., Mountain View, CA), and printed on a Kodak NP1600 dye sublimation printer (Middleburg Heights, OH).

RESULTS

Both the β5 and the β3 integrins remained localized to focal adhesions during vitronectin endocytosis

To evaluate the role of the αvβ3 integrin in the internalization of vitronectin, colocalization studies of vitronectin and β3 were done following the addition of exogenous vitronectin to fibroblast cells. Cells were then fixed, dual-stained for vitronectin and β3 (Fig. 1) or vitronectin and β5 (Fig. 2) and photographed at the substrate level. We found that at time zero, prior to the addition of exogenous vitronectin to cells, human foreskin fibroblasts, growing for 72 hours in complete medium, localized both the β5 and the β3 integrin to focal adhesion sites (Figs 1a-c, 2a-c, matching arrowheads). Furthermore, when the same cells were also stained for vitronectin, vitronectin (presumably serum-derived) was colocalized with the integrin receptors directly at the focal adhesion sites (Figs 1a-c, 2a-c, matching arrowheads). The vitronectin found in the focal adhesions was believed to be serum-derived, since these and most fibroblast cell lines do not synthesize vitronectin (Seiffert et al., 1990; Barnes and Reing, 1985). Focal adhesions were identified by interference reflection microscopy (Figs 1, 2c,f, arrowheads). Virtually all focal adhesions on a particular cell were either fixed and permeabilized prior to the addition of exogenous vitronectin (a-c), or incubated with exogenous purified vitronectin (12.5 µg/ml) in serum-free medium for 15 minutes (d-f), then fixed and permeabilized. Cells were stained by indirect immunofluorescence for vitronectin (a and d) and the β5 subunit of the αvβ5 integrin receptor and endogenous vitronectin in focal adhesions before and after the addition of exogenous vitronectin. Human fibroblasts seeded for 3 days in serum were either fixed and permeabilized prior to the addition of exogenous vitronectin (a-c), or incubated with exogenous purified vitronectin (12.5 µg/ml) in serum-free medium for 15 minutes (d-f), then fixed and permeabilized. Cells were stained by indirect immunofluorescence for vitronectin (a and d) and the β5 subunit of the αvβ5 integrin receptor (b and e). The β5 subunit localized to focal adhesions (b and e, arrowheads), which appeared black by IRM (c and f, matching arrowheads). Serum-derived vitronectin (a, arrowheads) is localized to focal adhesions (c, matching arrowheads) prior to the addition of exogenous multimeric vitronectin. Vitronectin was not detected in focal adhesions after the addition of exogenous vitronectin (d). Scale bar, 10 µm.

The β5 integrin but not the β3 integrin colocalizes with vitronectin in intracellular vesicles

Our earlier studies demonstrated that vitronectin internalization was regulated by the αvβ3 integrin receptor (Panetti et al., 1995; Panetti and McKeown-Longo, 1993b). However, it was not known if the αvβ3 integrin receptor itself participated directly in the cellular entry of vitronectin or if it acted indirectly by regulating another endocytic receptor. Therefore, the intracellular localization of vitronectin with either the β3 or the β5 subunit was examined by manually shifting the plane of focus from the substrate (Figs 1, 2) and evaluating the colocalization of integrin with vitronectin inside the cell where intracellular vesicles were in focus (Figs 3, 4). Vitronectin and β5 or β3 were localized by indirect immunofluorescence at 0, 15 and 30 minutes after the addition of exogenous vitronectin.

At time 0, neither vitronectin nor the β5 subunit were...
localized to any definitive intracellular structure (Fig. 3a,b). However, within 15 minutes after the addition of exogenous vitronectin to the cells, vitronectin could be detected in vesicular-like structures, and some of these vitronectin-staining vesicles also stained positively for $\beta_5$ (data not shown). Within 30 minutes, the vesicular staining of vitronectin became more intense, with vesicles appearing larger in size (Fig. 3c, arrowheads). The $\beta_5$ subunit continued to colocalize with vitronectin in some but not all of the vesicles (Fig. 3d, matching arrowheads). Overlays of photographs indicated that approximately 20% of the vitronectin-containing vesicles also contained $\beta_5$ (data not shown). Colocalization of vitronectin and the $\alpha_v\beta_5$ receptor was observed as early as 5 minutes after the addition of vitronectin (data not shown). These data suggest that the $\alpha_v\beta_5$ receptor and vitronectin may be internalized as a complex, but that vitronectin is also found in vesicles that do not contain $\beta_5$.

In contrast to $\beta_5$, $\beta_3$ was not colocalized to any vitronectin-containing intracellular vesicles at any time before (Fig. 4a,b) or during the internalization of vitronectin (Fig. 4c,d, matching arrowheads). This finding is consistent with earlier data showing that the degradation of vitronectin by human fibroblasts is not dependent on the $\alpha_v\beta_3$ integrin (Panetti and McKeown-Longo, 1993b).

### Colocalization of vitronectin and the $\beta_5$ integrin in intracellular vesicles is enhanced with wortmannin

The results presented above demonstrated that only 20% of the vitronectin-containing vesicles also stained for $\beta_5$. This suggested that the association between $\beta_5$ and vitronectin during endocytosis may be transient, with the $\beta_5$ receptor being rapidly recycled to the plasma membrane. To test for this possibility, the cells were treated with wortmannin, which has been shown to arrest recycling receptors in endosomal compartments (Spiro et al., 1996; Shpetner et al., 1996). Following a 30-minute treatment with wortmannin, localization of vitronectin and the $\beta_5$ integrin was analyzed using confocal microscopy. Fig. 5 represents confocal images of the top (a-c), interior (d-f) and ventral (g-i) sections of the cell. In the presence of wortmannin there was no detectable colocalization on the dorsal cell surface (Fig. 5a-c). However, colocalization increased dramatically in the interior of the cell close to the ventral surface in the presence of wortmannin, as indicated by the large number of yellow vesicles (Fig. 5f). The ventral surface, itself, also contained some vesicles that stained for both vitronectin and $\beta_5$ (Fig. 5i). Therefore, wortmannin increased the accumulation of the $\beta_5$ subunit in intracellular vesicles. Dose-response experiments showed that the wortmannin effects on $\beta_5$ and vitronectin colocalization could...
human fibroblasts seeded for 3 days in serum were either fixed prior to the addition of exogenous vitronectin (a and b), or incubated with exogenous purified vitronectin (12.5 μg/ml) in serum-free medium for 30 minutes (c and d), then fixed and permeabilized. Cells were stained by indirect immunofluorescence for vitronectin (a and c) and the β3 subunit of the αvβ3 integrin receptor (b and d). Arrowheads identify vitronectin-containing vesicles (c), but there is no evidence of β3 in any of these vesicles (d). Scale bar, 10 μm.

Figure 4. The αvβ3 integrin receptor does not colocalize with vitronectin in vesicles after the addition of exogenous vitronectin. Human fibroblasts seeded for 3 days in serum were either fixed prior to the addition of exogenous vitronectin (a and b), or incubated with exogenous vitronectin for 3 minutes in either the potassium-depleted or hypertonic medium. Cells were then incubated with vitronectin for an additional 30 minutes (c and d), then fixed and permeabilized. Cells were stained by indirect immunofluorescence for vitronectin (a and c) and the β3 subunit of the αvβ3 integrin receptor (b and d). Arrowheads identify vitronectin-containing vesicles (c), but there is no evidence of β3 in any of these vesicles (d). Scale bar, 10 μm.

Vitronectin internalization is dependent on clathrin-coated pit formation

The αvβ3 integrin receptor binds to and directly internalizes vitronectin. Human fibroblasts seeded for 3 days in serum were either fixed prior to the addition of exogenous vitronectin (a and b), or incubated with exogenous purified vitronectin (12.5 μg/ml) in serum-free medium for 30 minutes (c and d), then fixed and permeabilized. Cells were stained by indirect immunofluorescence for vitronectin (a and c) and the β3 subunit of the αvβ3 integrin receptor (b and d). Arrowheads identify vitronectin-containing vesicles (c), but there is no evidence of β3 in any of these vesicles (d). Scale bar, 10 μm.

Results presented in Fig. 5 suggested that wortmannin enhanced the colocalization of vitronectin and β3 within endocytic vesicles, suggesting that PI-3 kinase may regulate the segregation of vitronectin and β3 to distinct intracellular compartments. To determine whether wortmannin also blocked the accumulation of vitronectin in lysosomes, wortmannin-treated cells were stained for both vitronectin and lamp-1. As shown in Fig. 8, vitronectin did not localize to lamp-1-containing intracellular vesicles when cells were treated with wortmannin during the 30-minute incubation with exogenous vitronectin. These results indicate that wortmannin inhibits the movement of vitronectin to lysosomes and the recycling of the β3 integrin receptors back to the cell surface, resulting in an accumulation of both vitronectin and β3 integrin within endocytic vesicles.

DISCUSSION

Our earlier studies indicated that vitronectin degradation was inhibited by chloroquine (Panetti and McKeown-Longo, 1993a, 1993b), suggesting that internalized vitronectin is targeted to lysosomes. To determine whether some of the vesicles staining for vitronectin were lysosomes, fibroblasts were fixed after 30 minutes in the presence of vitronectin and stained for vitronectin and lamp-1, a specific marker for lysosomes (Fig. 7). Lamp-1 localized to large intracellular vesicles (Fig. 7a). Vitronectin also localized to intracellular vesicles (Fig. 7b). Many of the lamp-1-labeled vesicles also contained vitronectin and thus appeared yellow after superimposing the two images (Fig. 7d). Quantitation of the vitronectin- and lamp-1-containing vesicles was done using overlays, and demonstrated that at 30 minutes approximately 50% of the vitronectin-containing vesicles were lysosomal (data not shown). Vitronectin colocalization with lamp-1 increased with time and in the presence of chloroquine, such that by 6 hours 90% of the vitronectin-containing vesicles were lysosomal (data not shown).

Earlier biochemical studies have shown that vitronectin degradation was inhibited by chloroquine (Panetti et al., 1995; Panetti and McKeown-Longo, 1993b). However, the exact role of the αvβ3 integrin during the endocytosis of vitronectin was not known. The colocalization of vitronectin with the αvβ3 integrin during the cellular internalization of vitronectin was therefore necessary to determine if the αvβ3 integrin participated directly in the endocytosis of vitronectin or if the αvβ3 integrin acted indirectly by regulating another endocytic receptor. Indirect participation of a receptor in the internalization of its ligand has been demonstrated for the urokinase plasminogen activator receptor (uPAR) in the endocytosis of uPA-PAI-1 complexes. The evidence suggests that the uPAR presents the complex to the low density lipoprotein receptor-related protein (LRP), which in turn binds to and endocytoses the complex (Nykjaer et al., 1994, 1992). The present study, however, demonstrates that the αvβ3 integrin receptor binds to and directly internalizes its ligand, vitronectin.

Vitronectin colocalizes with the αvβ3 integrin receptor prior to the addition of exogenous vitronectin demonstrated that both the β3 and the β5 integrin were localized to focal adhesions. Localization of the αvβ3 integrin to focal adhesions has been previously demonstrated (Wilcox et al., 1996; Bergman et al., 1995;
Pasqualini et al., 1993); however, the localization of β5 to focal adhesions may be cell type specific (Wayner et al., 1991). In the human skin fibroblast cell lines used in the present study, only the β5 appears to function in endocytosis with no contribution of the β3. This result is in contrast to astrocytoma cells where both β3 and β5 participate in the degradation of vitronectin (Pijuan-Thompson and Gladson, 1997). Therefore, it seems that the function of these integrins may also be cell type-specific.

The confocal microscopy analysis confirmed the intracellular colocalization of αvβ3 and vitronectin, with the highest coincidence of colocalization being within the interior of the cell, 1.8 μm above the ventral surface of the cell. The average thickness of the center of cultured A1-F fibroblasts in our studies appears to be 6.0 μm, thus suggesting that endocytosis of vitronectin is occurring from the ventral surface. More specifically, endocytosis could be occurring at or near the focal adhesion sites since this is where the αvβ5 receptor is concentrated. This is not unlikely since clathrin-coated pits have been reported to form at the focal adhesion plaque during initial cell attachment (Woods et al., 1983). These findings are consistent with our previous studies, which suggested that vitronectin binds first to heparan sulfate proteoglycans present in the matrix, before it can be internalized by the cell (Panetti and McKeown-Longo, 1993a; Wilkins-Port and McKeown-Longo, 1996). The αvβ3 integrin receptor colocalized with vitronectin at the focal adhesion but did not colocalize with vitronectin in intracellular vesicles at any time. Therefore, the αvβ3 does not appear to participate in the cellular entry of vitronectin in these cells. Inhibition of clathrin-coated pit formation by either depleting cells of intracellular potassium

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**Fig. 5.** Colocalization of vitronectin and αvβ5 integrin in wortmannin-treated cells using confocal microscopy. Human fibroblasts seeded for 3 days in serum were incubated with exogenous purified vitronectin (12.5 μg/ml) in the presence of 10 μM wortmannin in serum-free medium for 30 minutes. Cells were then fixed, permeabilized and stained by indirect immunofluorescence for the β5 subunit of the αvβ5 integrin receptor (a, d and g) and vitronectin (b, e and h). Confocal images were taken every 0.3 μm starting from the ventral cell surface. Images a-c correspond to the dorsal cell surface (3.6 μm from the ventral cell surface); images d-f correspond to the interior of the cell (1.8 μm from the ventral cell surface); and images g-i correspond to the ventral cell surface. Images c, f, and i correspond to the superimposed images of vitronectin and β5 in a and b, d and f, and g and i, respectively. Scale bar, 10 μm.

**Fig. 6.** Inhibition of vitronectin internalization by treatment of cells with potassium-depleted medium. Human fibroblasts seeded for 3 days in serum were rinsed and incubated with potassium-containing medium for 15 minutes. Vitronectin (12.5 μg/ml) was added to the cells in the same potassium-containing medium (a, b), or in medium depleted of potassium (c, d). After a 30-minute incubation, cells were then fixed, permeabilized and stained for vitronectin (a and c). Vitronectin was detected in intracellular vesicles in the presence of potassium (a, arrows) but not when potassium was removed (c). Scale bar, 10 μm.
or treating cells with hypertonic media also inhibited vitronectin internalization, suggesting that the $\alpha_v\beta_5$ integrin-dependent endocytosis of vitronectin occurred through clathrin-coated pits (Larkin et al., 1983; Altankov and Grinnell, 1993). The accumulation of vitronectin with the lysosomal marker protein, lamp-1, was observed within 30 minutes after the addition of vitronectin to the cell layer, consistent with earlier findings that cellular degradation of vitronectin was sensitive to chloroquine (Panetti and McKeown-Longo, 1993a).

Vitronectin degradation by fibroblasts is inhibited by low levels of soluble heparin, which block internalization of vitronectin from the matrix (Wilkins-Port and McKeown-Longo, 1996; Panetti and McKeown-Longo, 1993a). The inhibition of vitronectin degradation by heparin can be overcome by activators of protein kinase C, demonstrating a role for PKC in the regulation of $\alpha_v\beta_5$ endocytic function (Panetti et al., 1995). PKC activation has been implicated in other $\alpha_v\beta_5$-dependent cellular functions, including motility (Klemke et al., 1994), focal adhesion assembly (Lewis et al., 1996) and gene expression (Ye Bra et al., 1995). These observations suggest that vitronectin’s heparin-binding domain participates in signaling the activation of PKC, which precedes the $\alpha_v\beta_5$-mediated internalization of vitronectin. Similarly, the $\alpha_v\beta_3$ receptor-dependent degradation of vitronectin by astrocytoma cells requires ligation of the $\alpha_v\beta_1$ integrin, suggesting that a necessary signaling event from the matrix precedes the $\alpha_v\beta_3$-mediated internalization of vitronectin (Pijuan-Thompson and Gladson, 1997).

Colocalization of $\beta_5$ and vitronectin in endosomes was increased in the presence of wortmannin. In addition, wortmannin also prevented the movement of internalized vitronectin to lysosomes. Since we were unable to detect $\beta_5$ in lysosomes, even in the presence of chloroquine, the wortmannin effect appears to result from the inability of $\beta_5$ and vitronectin to segregate into distinct intracellular compartments. Previous studies have found that in the presence of wortmannin, recycling receptors such as the transferrin receptor (Spiro et al., 1996) and lysosomally targeted receptors such as the PDGF receptor (Shpetner et al., 1996) became...
arrested in early endosomal compartments, and were not able to return to the cell surface or follow the endocytic pathway to lysosomes for degradation. The fact that wortmannin increases the colocalization of β1 and vitronectin suggests that both receptor and ligand are internalized as a complex into early endosomal compartments. Wortmannin appears to be inhibiting a later step in the trafficking of internalized vitronectin, perhaps at sorting endosomes, similar to what has been described for the effect of wortmannin on the endocytic pathway of the transferrin receptor (Spirito et al., 1996). The wortmannin-dependent accumulation of β1 in the endosomal compartment suggests that this integrin functions as a recycling endocytic receptor, which is normally uncoupled from vitronectin in sorting endosomes and restored to the cell surface. In support of this concept, wortmannin has been shown to decrease the rate of transferrin and transferrin receptor recycling (Shpetner et al., 1996; Spirito et al., 1996).

Enhanced colocalization of β1 and vitronectin was seen with wortmannin levels of 50 nM, which are known to inhibit PI-3 kinase (Okada et al., 1994). However, dose-response experiments indicated that the colocalization of vitronectin with β1 in intracellular vesicles became increasingly more extensive at higher wortmannin levels (1 and 10 μM), where wortmannin may be inhibiting myosin light chain kinase (Yano et al., 1993). These results suggest that both PI-3 kinase and myosin light chain kinase may participate in the intracellular trafficking of endocytosed vitronectin.

Multimeric forms of vitronectin would be expected to be released from platelets (Seiffert and Schleef, 1996) following tissue injury or activation of procoagulant events associated with tumor growth and invasion as well as chronic inflammation (Seiffert and Smith, 1997; Dvorak, 1979). The monomeric forms of plasma vitronectin can also be converted to multimers following the addition of PAI-1 (Seiffert and Loskutoff, 1996). This finding suggests a physiologic mechanism by which excess levels of PAI-1 may trigger conformational changes in vitronectin which ‘signal’ its internalization by the αvβ3 integrin. The binding of PAI-1 to vitronectin may also regulate the migratory activity of the cell by affecting integrin ligation (Stefansson and Lawrence, 1996). These observations suggest that excess levels of PAI-1 may block vitronectin-based motility by preventing αvβ3 binding and promote αvβ3-mediated internalization by triggering the multimerization of vitronectin. Removal of vitronectin from the matrix would be expected to result in an extracellular matrix, which is less conducive to migration. Therefore, activation of this β1-dependent clearance pathway for matrix vitronectin may be associated with the conversion of cells to a less migratory phenotype.

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