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RGD Peptides and Monoclonal Antibodies, Antagonists of α_v-Integrin, Enter the Cells by Independent Endocytic Pathways

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SUMMARY: Cyclic synthetic peptides containing the arginine-glycine-aspartate motif (cRGD) and monoclonal antibodies (mAbs) targeted for individual integrins have been developed as potential therapeutic drugs for the treatment of several diseases. We showed that a cRGD peptide targeted for $\alpha_v\beta_3$ was internalized in α_v-integrin expressing and nonexpressing melanoma cells by an integrin independent fluid-phase endocytosis pathway that does not alter the number of functional integrin receptors at the cell surface. In contrast, a blocking mAb directed to α_v was internalized by an integrin-dependent endocytosis pathway that reduced the number of functional integrin receptors at the cell surface. We prove that melanoma cells pretreated with the mAb do not readhere to the substrate, whereas cells pretreated with cRGD peptide retain their readhesion capacity. Given the growing importance of RGD peptides, knowledge of these cellular mechanisms is required to improve the development of antiangiogenic and anti-inflammatory drugs. (*Lab Invest 2001, 81:1615–1626*).

Integrins are a large family of heterodimeric transmembrane glycoproteins that mediate numerous processes involving cell-cell and cell-matrix adhesion (Hynes, 1992). Integrin-mediated adhesion engages distinct signaling pathways that regulate cell survival, proliferation, and migration (Aplin et al, 1998). Furthermore, α_v-integrins are the main integrin subfamily involved in cell migration (Fujii et al, 1998), cell growth, tumor invasion/metastasis (Mitjans et al, 1995), and angiogenesis (Brooks et al, 1995; Eliceiri and Cheresh, 2000; Yeh et al, 1998). In certain invasive human tumors like metastatic melanoma, the malignant phenotype of the tumor is associated with the expression of $\alpha_v\beta_3$ integrin (Felding-Habermann et al, 1992), which plays a critical role in melanoma tumor survival and malignant progression within human skin (Felding-Habermann et al, 1992; Marshall et al, 1991; Petitclerc et al, 1999).

Malignant melanoma has a profound impact on our population, but its mechanisms of progression in human patients are unknown. Therefore, the achievement of new therapeutic strategies for melanoma treatment is a major issue. Several α_v-integrin antagonists, including blocking anti-integrin monoclonal antibodies and synthetic RGD peptides, have thus been developed (Cheresh and Spiro, 1987; Fujii et al, 1998; Marshall et al, 1991; Mitjans et al, 2000; Petitclerc et al, 1999). The class-specific blocking anti-α_v antibody 17E6 and the cyclo-RGDfV^{Met} peptide (cRGD) (Dechantsreiter et al, 1999) have been shown to be selective $\alpha_v\beta_3$ integrin antagonists with high affinity. They block cell attachment to vitronectin and spreading of several melanoma cell lines (Mitjans et al, 1995), promote detachment from the extracellular matrix (ECM), and alter the cell surface distribution of α_v-integrins and other focal adhesion proteins (Castel et al, 2000), thus subsequently blocking experimental tumor growth in vivo (Mitjans et al, 2000).

Integrin endocytosis has been associated with several phenomena-like cell migration (Lauffenburger and Horwitz, 1996; Pierini et al, 2000; Poumay et al, 1994) and removal of extracellular matrix components (Coopman et al, 1991; Panetti and McKeown-Longo, 1993b). During cell migration, after the detachment of the cell from the ECM, integrins can diffuse across the cell surface (Palecek et al, 1996) or can be endocytosed and recycled to the cell membrane (Bretscher, 1992, 1996; Pierini et al, 2000). However, the mechanism by which integrins are internalized, sorted, and recycled to leading edge remains unclear. $\alpha_v\beta_5$ integrin mediates the internalization of vitronectin from the ECM (Panetti et al, 1995; Panetti and McKeown-Longo, 1993a, 1993b), whereas the $\alpha_v\beta_3$ mediated internalization of altered vitronectin in malignant astrocytomas requires ligation of integrin $\alpha_5\beta_1$ (Pijuan-

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Thompson and Gladson, 1997). Integrin ligation engages distinct signaling pathways that promote viral endocytosis and cell movement (Li et al, 1998). Both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins may mediate the internalization of adenovirus through arginine-glycine-aspartate (RGD) recognition in melanoma cells (Wickham et al, 1993), because many integrins, including $\alpha_v\beta_3$, bind to their ligands by recognizing the RGD sequence.

Recent studies have shown that small peptides containing RGD motif can activate cytosolic caspase-3 by a direct interaction to the RGD recognition site of the caspase (Adderley and Fitzgerald, 2000; Buckley et al, 1999). This leads to the suggestion that the RGD peptides enter into the cell by a passive, nonintegrin dependent pathway that drives the peptides to the cytosol, where it could bind to the RGD-binding motif (Ruoslahti and Reed, 1999). However, this remains to be elucidated.

A study on the internalization of these antagonists by target cells would be very useful, because RGD peptides and blocking antibodies against α_v-integrin are currently tested in clinical trials. Our aim was to determine the role of the $\alpha_v\beta_3$ integrin receptor both in the endocytosis of two α_v-integrin antagonists in melanoma cells and in cell adhesion after antagonists treatment. We show that monoclonal antibody (mAb) 17E6 follows an integrin receptor-mediated internalization and promotes the endocytosis of $\alpha_v\beta_3$ integrin. Therefore, melanoma cells treated with mAb 17E6 do not readhere to the substrate. In contrast, the cRGD peptide endocytosis is independent of α_v-integrin, because it occurs by a fluid-phase endocytic pathway and it does not directly promote the internalization of $\alpha_v\beta_3$ integrin. Furthermore, cells treated with cRGD peptide followed by a washing step readhered to the substrate as control nontreated cells. This suggests that although the cRGD peptide is more efficient in detaching melanoma cells from the ECM, it does not alter the binding capacity of $\alpha_v\beta_3$ integrin to the ECM after the treatment.

Results

Endocytosis of α_v-Antagonists

Antibodies that react with α_v-integrin and block melanoma cell adhesion have been described elsewhere (Cheresh and Spiro, 1987; Mitjans et al, 1995). The detachment effect of the α_v-integrin antagonists mAb 17E6 (and its fragment Fab_2) and the inhibitory cRGD peptide on M21 cells has also been reported (Castel et al, 2000; Mitjans et al, 1995).

To study cell surface binding, M21 cells were grown on glass coverslips for 24 hours and incubated for 30 minutes at 4° C in media supplemented with FITC-17E6, Fab_2-FITC, Fab-FITC, or CF-cRGD. We also studied the distribution pattern of the fluorescein-labeled antagonists when cells were treated for 30 minutes at 37° C. Cell surface binding and internalization of fluorescein-labeled antagonists were determined by confocal microscopy. After binding at 4° C (Fig. 1), mAb FITC-17E6 and its fragments (Fab_2-FITC and Fab-FITC) remained linked to focal contacts of melanoma cells, unlike the CF-cRGD peptide. When cells were treated with the antagonists at 37° C, mAb FITC-17E6, its divalent fragment Fab_2-FITC, and the CF-cRGD peptide induced cell detachment and were internalized by M21 cells. The monovalent fragment Fab-FITC had no effect on detachment and it was not internalized.

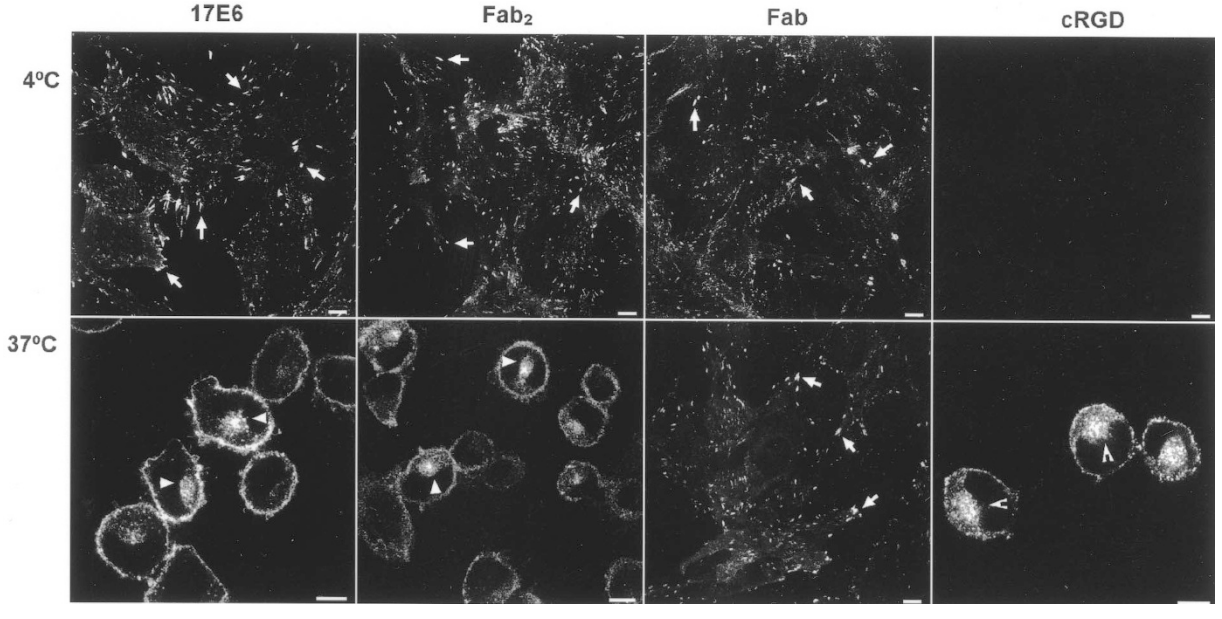

Figure 1.

Binding and endocytosis of α_v-antagonists. After 30 minutes of binding at 4° C, monoclonal antibody (mAb) 17E6 and its fragments (Fab_2 and Fab) remained associated to focal contacts of melanoma cells (*arrows*). Only mAb 17E6, and its divalent fragment Fab_2, induced cell detachment and were internalized when cells were treated for 30 minutes at 37° C (*arrowheads*). Cyclo-RGDfV^{Met} peptide (cRGD) peptide did not remain associated with the melanoma cell surface after binding, but it induced cell detachment and was internalized at 37° C (*barbed arrowheads*). All images are representative confocal microscopy sections. Scale bars, 10 μm.

A pulse-chase experiment was carried out to compare the endocytosis of FITC-17E6 and CF-cRGD. First, cells were incubated for a 15-minute pulse at 37° C with fluorescein-labeled antagonists in complete medium. As we have previously described (Castel et al, 2000), antagonists treatments revealed two main populations: cells detached by the antagonist effect and cells that remained attached to the matrix (Fig. 2a). The latter comprised two types of cells. The attached-spread cell population was resistant to the antagonist-induced detachment and remained adhered and flattened, whereas the attached-rounded cells were rounded and began to detach. After the pulse, all cells were washed with prewarmed medium and monitored separately in a 30, 60, and 120-minute chase in the presence or absence of chloroquine. After the internalization of fluorescein-labeled antagonists, cells were fixed and immunostained with the lysosome marker Lamp-1 to determine whether the endocytosed antagonists reached the lysosomal compartment. In confocal sections (Fig. 2b), attached-spread and attached-rounded cells were differentiated and quantified separately (Fig. 2, c and d). In the first group, after mAb-FITC-17E6 treatments, the antagonist remained associated with the focal contacts and did not internalize. The second showed an α_v-integrin redistribution and they internalized mAb-FITC-17E6. They presented the highest colocalization with Lamp-1 (Fig. 2c) and were sensitive to chloroquine. Detached cells presented some colocalization with the lysosomal marker, but they were not significantly sensitive to chloroquine. Only residual values of colocalization were present in attached-spread cells, as they did not internalize the antibody. However, CF-cRGD was always colocalized with Lamp-1, even in spread-cells resistant to antagonist-induced detachment. When cells were incubated with the CF-cRGD, all populations presented high colocalization with Lamp-1, but they were not sensitive to chloroquine (Fig. 2d).

mAb 17E6 Follows Receptor-Mediated Endocytosis and Promotes $\alpha_v\beta_3$ Integrin Internalization

To determine whether antagonists induce integrin internalization, we used the anti-β_3-AP3 mAb to follow $\alpha_v\beta_3$ integrin during the process. M21 cells were bound to Cy3.5-AP3 at 4° C and incubated for 60 minutes at 37° C. As revealed by confocal microscopy images (Fig. 3a), the anti-β_3 antibody AP3 induced clustering of integrins at the focal contacts (*arrows*), but it did not induce integrin internalization (middle section and 3d reconstruction). In addition, M21 cells that were bound simultaneously with Cy3.5-AP3 and FITC-17E6 at 4° C presented a high colocalization of both antibodies at the focal contacts (data not shown). The same results were observed when cells were bound with one of these antibodies and immunostained with the other one (data not shown), indicating that the anti-β_3-AP3 mAb did not compete with the binding site of the anti-α_v-17E6 mAb. Therefore, after Cy3.5-AP3 binding, M21 cells were treated with FITC-17E6 or CF-cRGD for 30 minutes at 37° C to determine whether the integrin was internalized together with the antagonist (Figs. 3, b and c). After 17E6 treatment, in detached and attached-rounded cells, the AP3 staining was colocalized with 17E6 at the cell surface and in endocytic vesicles (Fig. 3b). In attached-spread cells, both antibodies were localized at the focal contacts. This indicates that 17E6-mAb binds to the $\alpha_v\beta_3$ and that both internalize together. However, when cells were treated with CF-cRGD (Fig. 3c), mAb AP3 staining was only redistributed at the cell surface, but it did not colocalize with CF-cRGD peptide in endocytic vesicles, showing that although cRGD internalizes, the $\alpha_v\beta_3$-integrin remains at the surface of M21 cells. To confirm these results, we detected the α_v-integrin subunit by immunofluorescence using mAb 17E6-TRITC in cells that had endocytosed CF-cRGD (Fig. 3d). The results were similar: endocytic vesicles containing cRGD did not contain the integrin subunit and 17E6 showed a redistribution pattern on the cell surface.

cRGD Follows Fluid-Phase Uptake and Its Entry Is Integrin Independent

The experiments mentioned above indicate that the internalization of mAb17E6 is an integrin-dependent and receptor-mediated pathway. Pulse-chase experiments of the fluorescein labeled antagonists and the fluid phase marker, TRITC-Dextran (TRITC-DX), were performed to further characterize the endocytic pathway of the cRGD peptide (Fig. 4), because the internalization of both receptor-bound and fluid phase ligands may follow various pathways. A high degree of colocalization between CF-cRGD and TRITC-DX was observed in all cell populations. In contrast, in FITC-17E6 treatments only attached-rounded cells and detached cells presented some colocalization, which is supposed to occur at the end of the endocytic pathways.

To confirm the fluid-phase pathway for the cRGD peptide and to test the hypothesis that its entry is integrin independent, we performed a time-course uptake experiment (Fig. 5A) in M21 and M21-L cells, which lack α_v-integrin expression (Cheresh and Spiro, 1987). First, we added a [^{3}H]cRGD peptide to cells at 37° C or 4° C. At designated times after incubations, cells were washed three times with 1 ml of ice-cold PBS to stop the process. M21 and M21-L cells presented similar uptake at 37° C and a small amount of nonspecific signal or residual-bound peptide at 4° C. In another set of similar experiments, M21 and M21-L cells were incubated with CF-cRGD in the same conditions. They presented similar fluorescence intensity and distribution as shown in confocal microscopy middle sections of cells (Fig. 5B). M21-L cells were also incubated for 30 minutes with CF at the same molar concentration as CF-cRGD, as a negative control (Fig. 5B). The entry of CF-cRGD and [^{3}H]cRGD peptides in M21-L cells, which lack the α_v subunit, showed that cRGD uptake is independent of α_v-integrin.

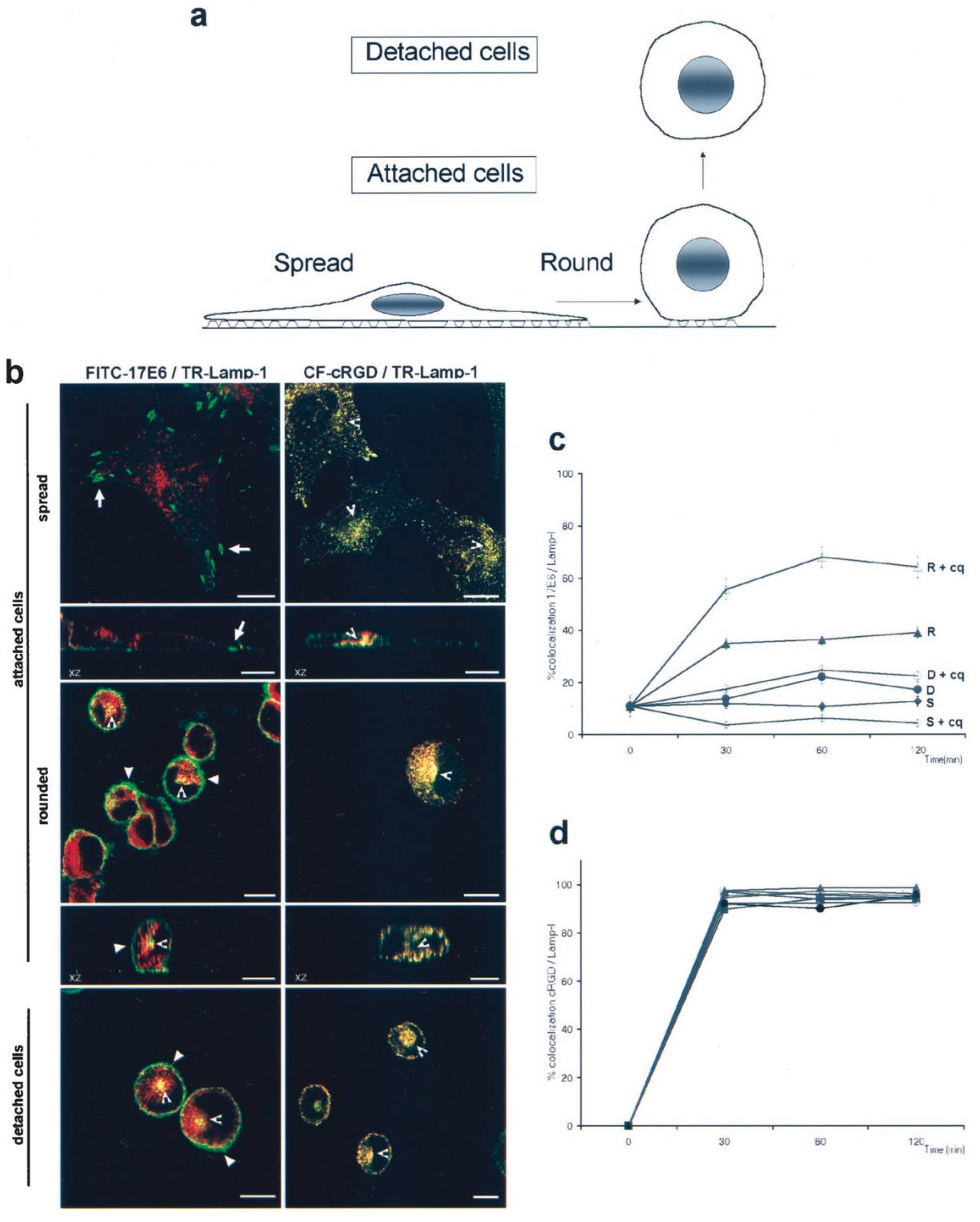

Figure 2.

Internalized 17E6 and cRGD reach lysosomes. a, Treatment of M21 cells with α_v-antagonists for 15 minutes at 37° C (pulse) generated distinct cell populations: attached-spread cells, flattened and resistant to antagonist-induced detachment, attached-rounded cells that appeared rounded and began to detach, and detached cells. b, Double fluorescence confocal images of FITC-17E6 and CF-cRGD (pulse 15 minutes, chase 60 minutes) with the lysosomal marker TR-Lamp-1. FITC-17E6 remains associated to the focal contacts (*arrows*) in attached-spread cells. Attached-rounded cells show an α_v-integrin surface redistribution (*arrowheads*) and internalized FITC-17E6 (*barbed arrowheads*). CF-cRGD is internalized in all populations (right panels, *barbed arrowheads*). c and d, Quantification of colocalization analysis of FITC-17E6 (c) and CF-cRGD (d) with TR-Lamp in the presence (*empty symbols*) or absence of chloroquine (*filled symbols*). Attached-spread cells (◇), attached-rounded cells (▲), and detached cells (●). Results are expressed as a percentage of colocalization of the antagonist with Lamp-1. Scale bars, 10 μm.

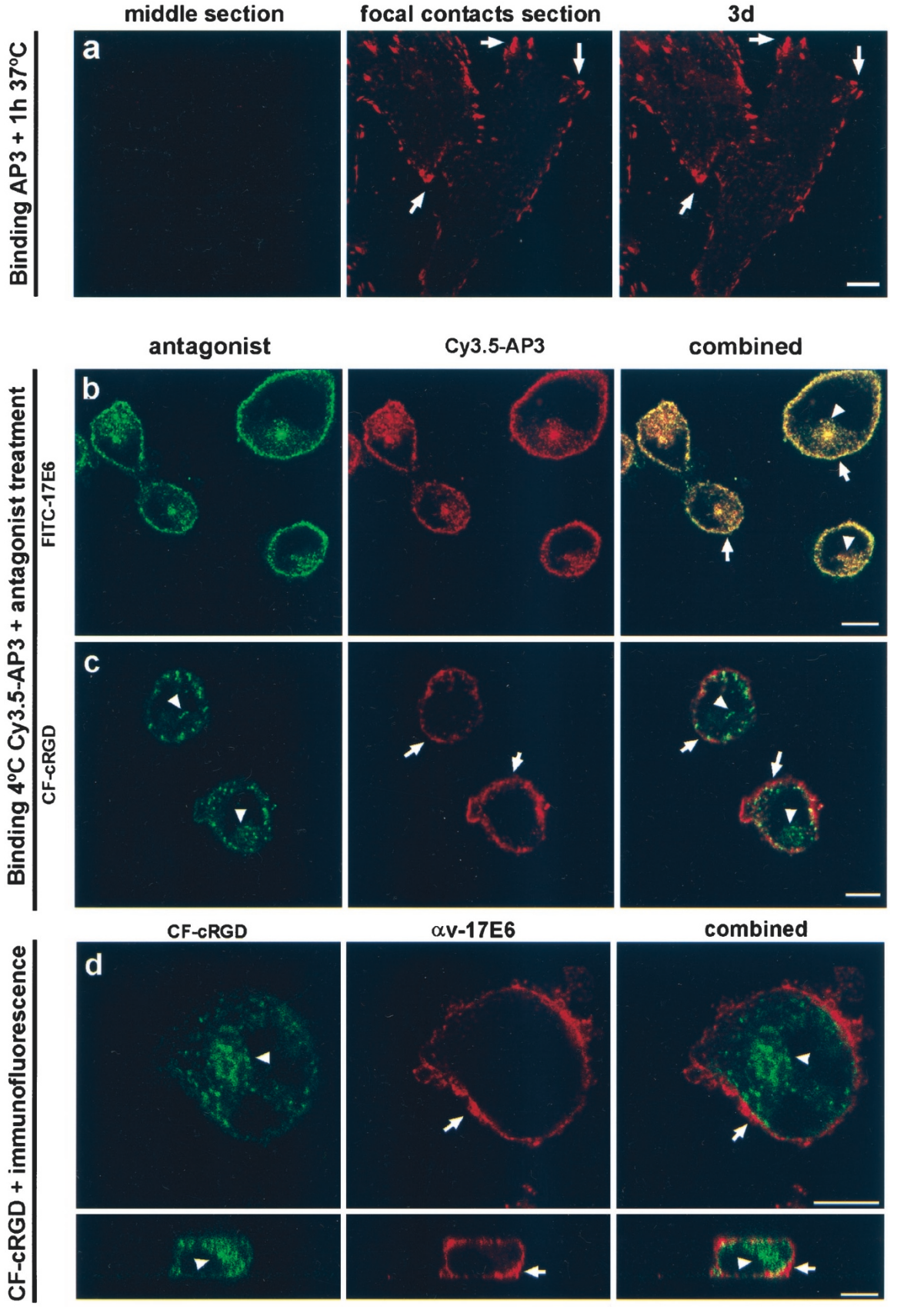

Figure 3.

mAb 17E6 endocytosis promotes $\alpha_v\beta_3$ integrin internalization. a, Integrin aggregation at the focal contact plane (*arrows*) in confocal sections of cells treated with Cy3.5-AP3. b and c, Cells treated with FITC-17E6 (b) or CF-cRGD (c) after AP3 binding. b, Colocalization of α_v and β_3 subunits at the cell surface (*arrows*) and in endocytic vesicles (*arrowheads*). c, Redistribution of the β_3 subunit at the cell surface (*arrows*) and endocytosed CF-cRGD (*arrowheads*). d, Endocytosed CF-cRGD (*arrowheads*) and immunofluorescence-detected α_v-integrin (TRITC-17E6) (*arrows*). All images are representative confocal microscopy sections. Scale bars, 10 μm.

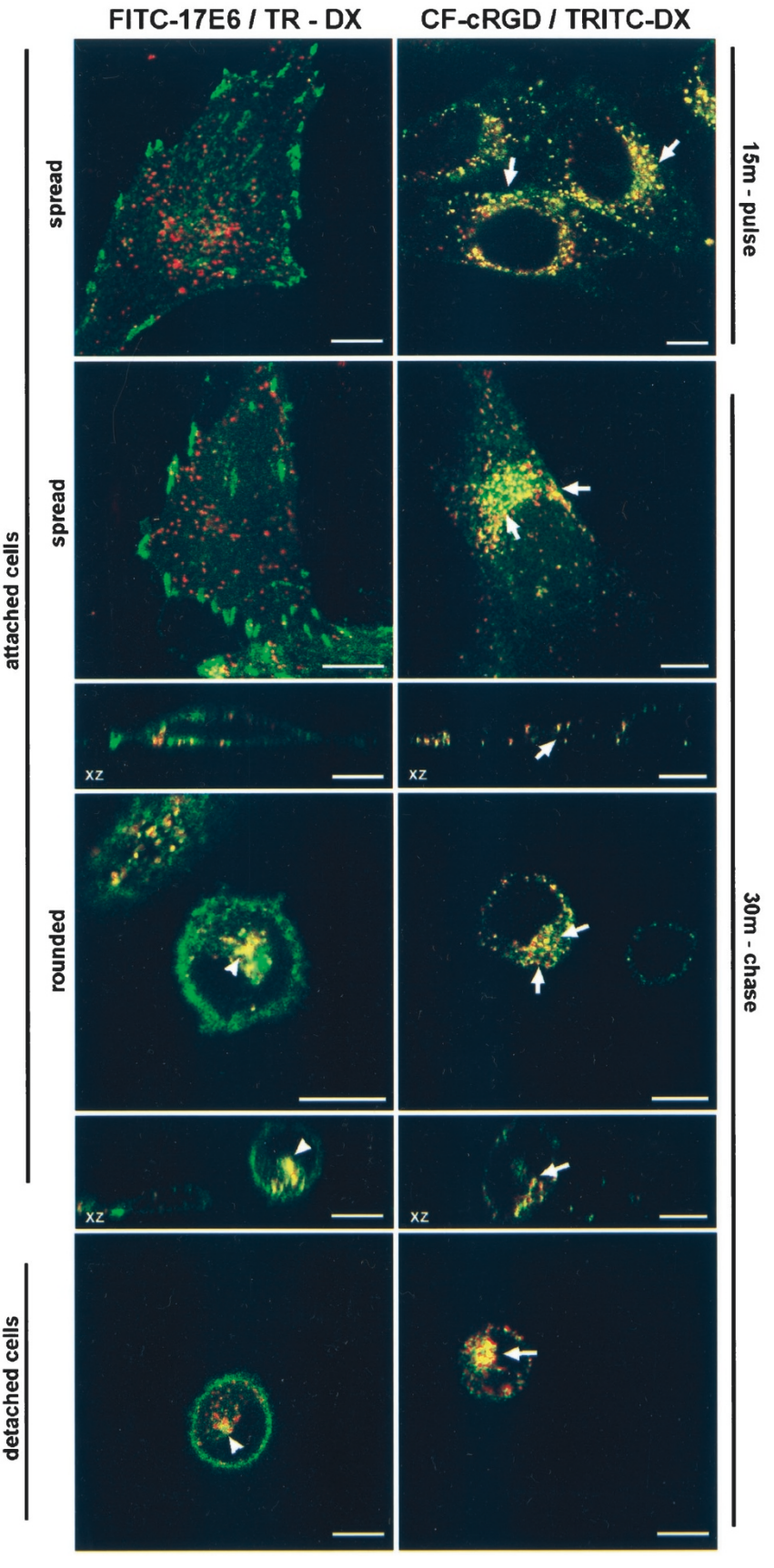

Figure 4.

CF-cRGD fluid-phase uptake. Internalization experiments of fluorescein-antagonists were carried out together with the fluid-phase marker TRITC-Dextran (TRITC-DX). Colocalization of TRITC-DX with CF-cRGD (*arrows*) and FITC-17E6 (*arrowheads*). All images are representative confocal microscopy sections of a 15-minute pulse followed by a 30-minute chase experiment. Scale bars, 10 μm.

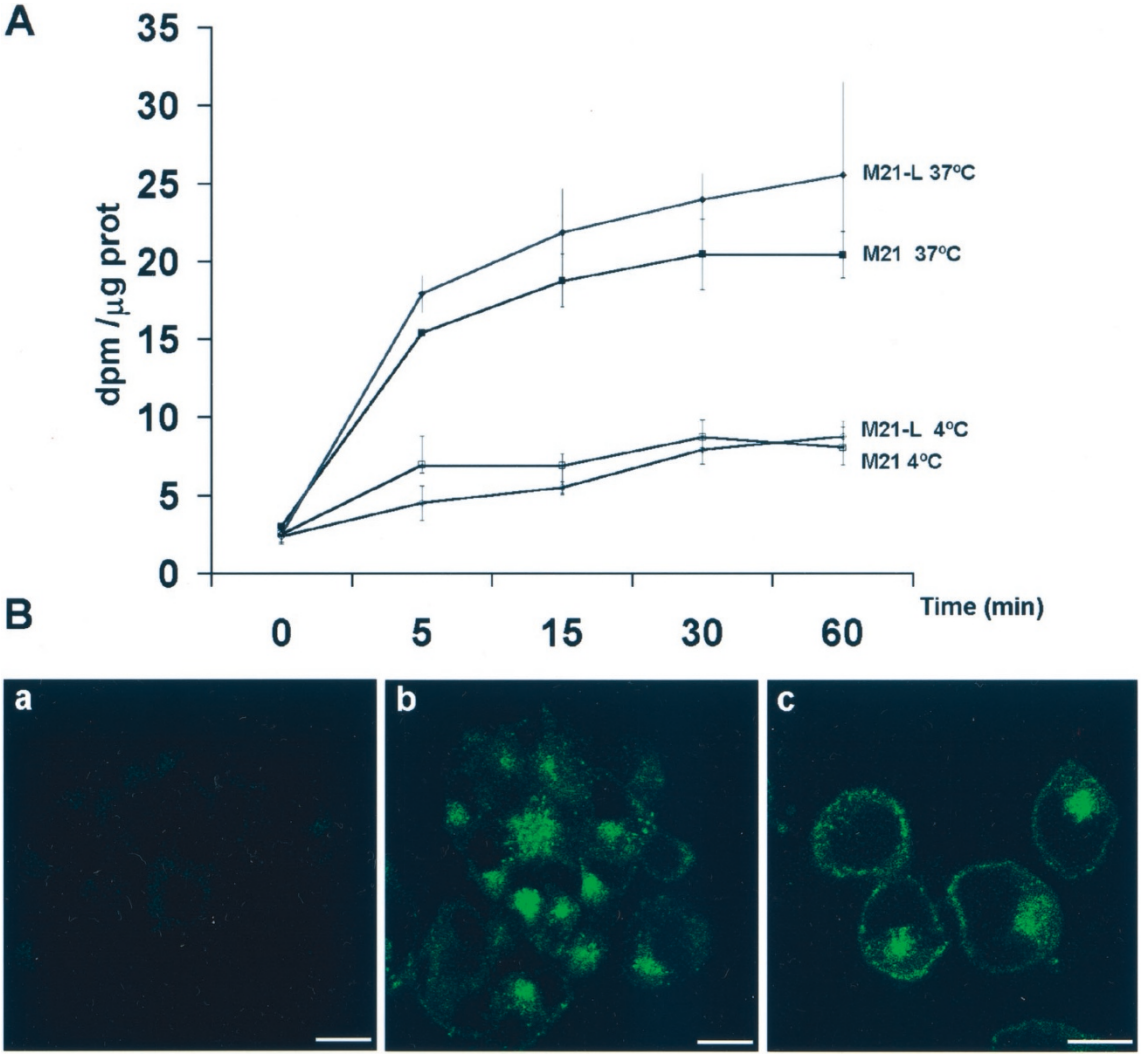

Figure 5.

Integrin-independent uptake of [^{3}H]cRGD in melanoma cells. A, Time-course uptake of [^{3}H]cRGD in M21 (■) and M21-L (◇) cells at 37° C (*filled symbols*) and at 4° C (*empty symbols*). B, Thirty-minute uptake at 37° C of CF (a), CF-cRGD in M21-L cells (b), and CF-cRGD in M21 cells (c). Confocal microscopy middle sections of cells. Scale bars, 10 μm.

mAb α_v-Integrin Antagonist Decrease the Number of Functional $\alpha_v\beta_3$ at the Melanoma Cell Surface

We studied the amount of functional integrin at the melanoma cell surface after antagonists treatments, because α_v-integrins behave as receptors for vitronectin and melanoma cells adhered to the substrate mainly through the vitronectin receptor (Castel et al, 2000; Mitjans et al, 1995). The effect of α_v-integrin antagonists and integrin internalization on the readhesion of M21 cells was quantified by violet crystal dye elution (Fig. 6). After a 15-minute pulse, detached cells were washed in antagonist-free medium and were allowed to adhere for 1 hour at 37° C on vitronectin-coated dishes and on uncoated dishes using FCS-supplemented medium. As control, nonwashed cells were also plated on vitronectin-coated dishes. After 1 hour at 37° C, more than 70% of added cells attach to the vitronectin matrix (Mitjans et al, 1995). In the group of washed cells, only the cells treated with mAb 17E6 presented a significant reduction of cell attachment. The cells treated with cRGD did not present any reduction. No reduction of cell attachment was observed with control mAbs (14E2 and 2E7) or the control peptide cyclic Arginine-Alanine-Aspartate (cRAD). The same results were observed when cells were allowed to adhere onto vitronectin-coated dishes or onto uncoated dishes using FCS-supplemented medium.

Discussion

The purpose of this study was to determine how two α_v-integrin antagonists, which are regarded as potential therapeutic agents in melanoma treatment, are internalized by target cells. We showed that a cRGD peptide and a blocking mAb directed to α_v-integrin follow independent endocytosis pathways after inducing detachment of α_v-expressing melanoma cells, which could have crucial physiological consequences. The cRGD peptide efficiently induced melanoma cell

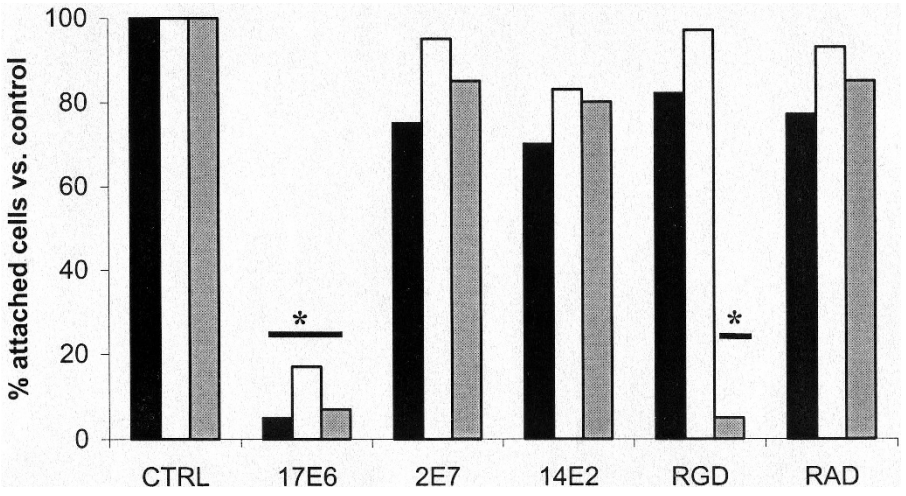

Figure 6.

Readhesion of melanoma cells after treatments. Readhesion on vitronectin-coated dishes (*black bars*) and on uncoated dishes (*empty bars*) of 15-minute, pulse-treated M21 cells followed by a washing step and on vitronectin-coated dishes of same cells without the washing step (*gray bars*). Results are expressed as a percentage of attached cells versus the control cells. * Significant differences.

detachment, but it did not remain bound to the cell surface integrin and it was internalized by an integrin independent fluid-phase endocytosis pathway to lysosomes. In contrast, the blocking mAb 17E6 was internalized by an integrin-dependent endocytosis pathway that internalizes the $\alpha_v\beta_3$/mAb 17E6 complex, drives the mAb to the lysosomes, and blocks the functional integrin at the cell surface. As a result, cells treated with mAb 17E6 could not readhere to the substrate, whereas cells treated with cRGD peptide retained their readhesion capacity.

The human specific α_v-integrin antagonist mAb 17E6 used blocks cell adhesion (Mitjans et al, 1995), induces detachment of previously substrate-attached cells in vitro (Castel et al, 2000), and efficiently blocks the in vivo growth of human melanomas expressing $\alpha_v\beta_3$ (Mitjans et al, 2000). At 4° C, mAb 17E6 has no effect on cell detachment, but the intact antibody, divalent (Fab_2) and monovalent (Fab) mAb fragments were found at focal contacts of adhered cells, indicating that the binding to the integrin receptor is not temperature-dependent. At 37° C, divalent mAb (intact 17E6 and Fab_2 fragment) induced cell detachment and antagonist internalization. Colocalization experiments with a nonblocking anti-β_3 mAb AP3 showed that the $\alpha_v\beta_3$-integrin heterodimer was internalized together with the mAb 17E6, following a nonfluid-phase endocytosis pathway that drives the antibody to the lysosomes where it is probably degraded. Taken together, these results show that the mAb 17E6 behaves as an extracellular ligand, because it triggers the α_v-integrin-mediated endocytosis, as described elsewhere for vitronectin (Memmo and McKeown-Longo, 1998; Panetti et al, 1995; Panetti and McKeown-Longo, 1993a, 1993b) and for adenovirus (Wickham et al, 1993, 1994). The Fab fragment did not induce cell detachment at 37° C (Castel et al, 2000) and was not internalized by α_v-expressing melanoma cells (present study). In addition, the integrin-induced endocytosis seems specific of the epitope recognized by the mAb 17E6 antibody as the nonblocking mAb 2E7, which recognizes a distinct epitope of α_v-integrin and the mAb AP3, which recognizes the β_3-subunit-induced clustering but not integrin internalization. Therefore, only the divalent blocking antibody mAb 17E6 could induce both detachment and internalization of α_v-integrin in melanoma cells, probably by means of the integrin aggregation and clustering induced by mAb 17E6. The ligand-induced clustering of integrins, which induces several outside-in signaling events, is well characterized (Schoenwaelder and Burridge, 1999). However, spread cells (resistant to the detachment induced by mAb 17E6) and cells treated with mAb AP3 showed clustering of the α_v-integrin (Figs. 2 and 3a), suggesting that the clustering induced by the blocking antibodies is necessary, but not sufficient, to induce endocytosis of the integrin-ligand complexes.

Confocal microscopy colocalization analysis of mAb 17E6 and anti-Lamp-1 revealed that the internalization of the blocking antibody mediated by $\alpha_v\beta_3$-integrin is linked to the detachment process. Spread cells presented a residual colocalization between both markers and the effect of chloroquine was not significant. For up to 30 minutes in attached-rounded cells, 35% of 17E6-containing vesicles, or 50–60% in the presence of chloroquine, were lysosomes. Detached cells presented lower costaining with the lysosomal compartment and were not sensitive to chloroquine, suggesting that this process was inhibited when cells were in suspension. Therefore, we suggest that during the 17E6-induced detachment, the antibody is endocytosed, and when cells are in suspension, endocytosis reaches saturation or is inhibited and is not sensitive to chloroquine. As mentioned above, these α_v-antagonists induce redistribution of integrins, reorganize focal adhesion proteins, and alter the actin cytoskeleton (Castel et al, 2000). The link between internalization and focal adhesion is unclear, but it has been shown that the integrity of the actin cytoskeleton

is required for receptor-mediated endocytosis in mammalian cells (Durrbach et al, 1996; Lamaze et al, 1997; Shurety et al, 1998).

α_v-integrin antagonists (antibodies and RGD-based peptides) compete with the endocytosis of α_v-integrin ligands (Nakano et al, 2000; Panetti et al, 1995; Zocchi et al, 1997). However, no study dealing with the endocytosis of cRGD peptides is available. In contrast with the endocytosis of the mAb 17E6, that of the cRGD peptide was not integrin-mediated. The first remarkable difference between the two antagonists was that the cRGD peptide was not associated with the focal contact structures. cRGD may thus act on the α_v-integrin by a "kiss and run" mechanism. This mechanism could consist on an efficient competition between cRGD peptide and extracellular ligands for the binding to the integrin heterodimer, but the peptide does not remain attached to the integrin RGD-binding domain.

Endocytosis of the cRGD was also temperature-dependent but in a different way from that of mAb 17E6. No differences regarding spread, round, or detached cells were detected, which shows that internalization of the cRGD was independent of the detaching process induced by the antagonist. In addition, colocalization of cRGD and the lysosomal marker Lamp-1 was complete, not sensitive to chloroquine, and it reached saturation at 15 minutes (Fig. 5A), suggesting a fast uptake and delivery of the peptide to lysosomes. Confocal microscopy colocalization of cRGD and α_v and β_3 subunits revealed that the process was totally independent of integrin-mediated endocytosis. Colocalization of cRGD and the fluorescent marker TRITC-DX showed that cRGD follows a fluid-phase endocytosis pathway. Experiments with cRGD in M21-L cells that lack α_v-integrin expression (Cheresh and Spiro, 1987), but incorporate [^{3}H]cRGD and CF-cRGD, supported that the endocytosis was integrin independent. Thus, endocytosis of cRGD may follow a nonspecific pathway. Hence, all cell types could potentially take up the cRGD α_v-integrin antagonist.

Peptide mimetics based on the RGD motif have been extensively used to inhibit tumor metastasis (Filardo et al, 1995; Fujii et al, 1998; Humphries et al, 1986), angiogenesis (Brooks et al, 1994, 1995; Sheu et al, 1997), inflammation (Bar-Shavit et al, 1991), and osteoporosis (Engleman et al, 1997). The drugs have been tested in clinical trials or are already under development (Carmeliet and Jain, 2000; Ruoslahti, 1996). In many studies it has been demonstrated that RGD peptides have a potent apoptotic effect (Brooks et al, 1994; Chen et al, 1997; Ferguson et al, 1991; Humphries et al, 1986; Yeh et al, 1998). Linear RGD-peptides induce apoptosis by direct binding and activation of procaspase-3 in lymphocytes (Buckley et al, 1999) and cardiomyocytes (Adderley and Fitzgerald, 2000) by an integrin independent mechanism. This implies that RGD-peptides raise the cell cytoplasm to directly interact with the RGD-binding motif of procaspase, as supported by Adderley and Fitzgerald (Adderley and Fitzgerald, 2000), who detected colocalization of RGD-peptides and caspase-3 within the cells after 48 hours of treatment. In addition, a cRAD peptide was also found inside the cells (Adderley and Fitzgerald, 2000; Buckley et al, 1999). However, no detailed studies on entry mechanisms have been reported by the authors. Our results suggest two possibilities: (a) linear and cyclic RGD peptides enter the cells by various mechanisms and linear RGD peptides cross the cell membrane either by active or passive plasma membrane transport, or (b) because lineal (reported elsewhere) and cyclic RGD peptides (present study) are taken up by an α_v-integrin independent fluid-phase endocytosis pathway, RGD peptides reach the lysosomes and remain or are degraded there. In the absence of permeabilizing agents, a small proportion could leave the lysosomes, reach the cell cytoplasm, and interact with procaspase-3. This could explain why RGD peptides are found in the cell cytoplasm only after a certain period of time (48 to 72 hours) and why high concentrations (above mM) are needed to induce apoptosis (Adderley and Fitzgerald, 2000).

The mAb 17E6 melanoma treatment may be responsible for the decrease in the number of functional α_v-integrin receptors in the cell surface, whereas the cRGD treatment did not alter it, because cRGD treatment of melanoma cells was independent of α_v-integrin endocytosis. Re-adhesion tests (Fig. 6) clearly showed that mAb 17E6-treated melanoma cells lost their adhesion capacity to plastic and to vitronectin-coated substrates. The scarcity of α_v-integrin on the cell surface and the blocking of the remaining α_v-integrin caused by the antibody may account for this loss. As expected, cRGD-treated cells showed the same adhesion capacity as control cells treated either by nonblocking α_v-integrin antibodies or cRAD peptides. Thus, cRGD treatment of a melanoma cell does not alter the number of functional α_v-integrin receptors at the cell surface, allowing them to re-adhere once the concentration of the cRGD peptide is reduced in the culture medium. In conclusion, dimeric and even multimeric cRGD may induce integrin clustering and integrin-dependent endocytosis and, thus, reduce the number of functional receptors at the cell surface of target cells or block them.

Materials and Methods

Antibodies and Peptides

Monoclonal antibodies were 17E6 (anti-α_v-integrin) (Mitjans et al, 1995), 14E2 (anti-200 Kda melanoma surface protein) (Mitjans et al, 1995), and 2E7 (nonblocking anti-α_v-integrin) were from Merck-LBI (Barcelona, Spain). AP3 (nonblocking anti-β_3) was obtained from the American Type Culture Collection (ATCC) (Manassas, Virginia). Cyclic peptides were cRGD (cyclo-RGDfV^{Met}), cRAD (cyclo-RβADfV), and CF-cRGD (cyclo-RGDfK-carboxyfluorescein) and were purified and characterized at Merck KGaA (Darmstad, Gemany). [^{3}H]cRGD (cyclo-RGDfV-[^{3}H] was synthesized at the Institute of Pharmacokinetics

and Metabolism, Merck KGaA (Grafing, Germany). Lamp-1 antibody was kindly provided by Dr. S. Carlsson (Umeå University, Sweden).

Endocytosis of α_v-Antagonists

M21 and M21-L cells (Cheresh and Spiro, 1987) were cultured as described (Castel et al, 2000). Cells were grown on glass coverslips for 24 hours and incubated with FITC-17E6, FITC-Fab_2, FITC-Fab, and CF-cRGD in complete medium at 37° C or 4° C for 30 minutes. For endocytosis experiments, cells were incubated for 15 minutes at 37° C (pulse) in a prewarmed complete medium containing fluorescein-labeled antagonists at 10 μg ml^{-1}, washed, and followed by a 30, 60, 120-minute chase with or without chloroquine (100 μM, Sigma Chemical Co., St. Louis, Missouri). For fluid-phase experiments, endocytosis of fluorescein-labeled antagonists and tetramethylrhodamine-labeled dextran (TRITC-DX, 3 mg ml^{-1}, M_r = 10,000, Molecular Probes, Inc., Eugene, Oregon) was carried out as described in pulse-chase experiments. In all cases, coverslips were washed in ice-cooled 100-mM PBS, fixed in 3% paraformaldehyde/2% sucrose in 0.1 M PB for 15 minutes, and mounted in Mowiol (Calbiochem, San Diego, California).

Internalization of $\alpha_v\beta_3$ Integrin

M21 cells were incubated for 15 minutes in precooled medium at 4° C and for 30 minutes in precooled medium containing anti-β_3 mAb Cy3.5-AP3 (5 μg ml^{-1}) with or without FITC-17E6 (10 μg ml^{-1}). After Cy3.5-AP3 binding, cells were washed 3 times and incubated with FITC-17E6 or CF-cRGD for 30 minutes at 37° C.

Immunocytochemistry

For immunolocalization, cells were grown, treated with fluorescein-labeled antagonists, and fixed as described above. The cells were washed for 10 minutes in 10 mM PBS/20 mM glycine, permeabilized with 10 mM PBS/20 mM glycine containing 0.005% saponin for 7 minutes, and blocked with PBS/20 mM glycine 1% albumin. Cells treated with antagonists were incubated with primary antibody (rabbit anti-Lamp-1; Dr. S. Carlsson, Umeå University, Sweden) for 45 minutes at 37° C, washed 3 times in PBS/20 mM glycine, and incubated with a secondary antibody Texas Red-X (TR) goat anti-rabbit IgG conjugate (2 μg ml^{-1}, Molecular Probes, Inc.). For immunofluorescence detection of the α_v-integrin in CF-cRGD treatment, mAb 17E6 was used as the primary antibody and a TRITC goat-anti mouse IgG conjugate (Dako, Glostrup, Denmark) was used as secondary antibody.

Confocal Microscopy and Colocalization Analysis

Confocal laser scanning microscopy was performed with a Leica TCS 4D (Leica Lasertechnik GmbH, Heidelberg, Germany). Images were taken using a 63X(NA 1.4,oil) Leitz Plan-Apochromatic objective at several zoom factors (Wetzlar, Germany). For fluorescein and TR or TRITC double staining, we used dual channel sequential acquisition exciting with the 488- and 568-nm lines of a krypton-argon laser. Colocalization analysis was performed with a Metamorph Imaging System (version 4.0) (Universal Imaging Corporation, West Chest, Pennsylvania).

Uptake of [^{3}H]cRGD

Uptake of [^{3}H]cRGD (2.5 μCi ml^{-1}) proceeded for 5, 15, 30, and 60 minutes at 37° C or 4° C in M21 and M21-L cells. The cells were washed on ice-cold PBS and the uptake was determined by scintillation in a Packard gamma counter (HP Tri-Carb 2100 TR; Hewlett Packard, Palo Alto, California).

Cell Attachment Assays

After a 15 minute pulse of the antagonists, detached cells were washed in antagonist-free medium and seeded on a 96-well microtiter plate at 37° C for 1 hour. Cells were seeded in FCS-supplemented medium onto uncoated plates or in FCS-free medium onto vitronectin-coated plates (1 μg ml^{-1}) (Collaborative Research, Becton-Dickinson, Bedford, Massachusetts). Cell attachment was measured by the violet crystal dye elution method (Gillies et al, 1986) and then quantified in a spectrophotometer at 630 nm (Bio Tek Instruments, Winooski, Vermont).

The data obtained in triplicate experiments were analyzed by ANOVA and a multiple comparison test (Scheffé post hoc test) applying the SPSS 8.1 statistical program (SPSS, Inc., Chicago, Illinois). The differences were significant ($p < 0.05$).

References

Adderley SR and Fitzgerald DJ (2000). Glycoprotein IIb/IIIa antagonists induce apoptosis in rat cardiomyocytes by caspase-3 activation. J Biol Chem 275:5760–5766.

Aplin AE, Howe A, Alahari SK, and Juliano RL (1998). Signal transduction and signal modulation by cell adhesion receptors: The role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. Pharmacol Rev 50: 197–263.

Bar-Shavit R, Sabbah V, Lampugnani MG, Marchisio PC, Fenton JW II, Vlodavsky I, and Dejana E (1991). An Arg-Gly-Asp sequence within thrombin promotes endothelial cell adhesion. J Cell Biol 112:335–344.

Bretscher MS (1992). Circulating integrins: Alpha 5 beta 1, alpha 6 beta 4 and Mac-1, but not alpha 3 beta 1, alpha 4 beta 1 or LFA-1. EMBO J 11:405–410.

Bretscher MS (1996). Moving membrane up to the front of migrating cells. Cell 85:465–467.

Brooks PC, Montgomery AM, Rosenfeld M, Reisfeld RA, Hu T, Klier G, and Cheresh DA (1994). Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 79:1157–1164.

Brooks PC, Stromblad S, Klemke R, Visscher D, Sarkar FH, and Cheresh DA (1995). Antiintegrin alpha v beta 3 blocks

human breast cancer growth and angiogenesis in human skin. J Clin Invest 96:1815–1822.

Buckley CD, Pilling D, Henriquez NV, Parsonage G, Threlfall K, Scheel-Toellner D, Simmons DL, Akbar AN, Lord JM, and Salmon M (1999). RGD peptides induce apoptosis by direct caspase-3 activation. Nature 397:534–539.

Carmeliet P and Jain RK (2000). Angiogenesis in cancer and other diseases. Nature 407:249–257.

Castel S, Pagan R, García R, Casaroli-Marano RP, Reina M, Mitjans F, Piulats J, and Vilaró S (2000). Alpha v integrin antagonists induce the disassembly of focal contacts in melanoma cells. Eur J Cell Biol 79:502–512.

Chen X, Wang J, Fu B, and Yu L (1997). RGD-containing peptides trigger apoptosis in glomerular mesangial cells of adult human kidneys. Biochem Biophys Res Commun 234: 594–599.

Cheresh DA and Spiro RC (1987). Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen, and von Willebrand factor. J Biol Chem. 262:17703–17711.

Coopman P, Nuydens R, Leunissen J, De Brabander M, Bortier H, Foidart JM, and Mareel M (1991). Laminin binding and internalization by human and murine mammary gland cell lines in vitro. Eur J Cell Biol 56:251–259.

Dechantsreiter MA, Planker E, Matha B, Lohof E, Holzemann G, Jonczyk A, Goodman SL, and Kessler H (1999). N-Methylated cyclic RGD peptides as highly active and selective alpha(V)beta(3) integrin antagonists. J Med Chem 42:3033–3040.

Durrbach A, Louvard D, and Coudrier E (1996). Actin filaments facilitate two steps of endocytosis. J Cell Sci 109:457–465.

Eliceiri BP and Cheresh DA (2000). Role of alpha v integrins during angiogenesis. Cancer J Sci Am 6(Suppl 3):S245–S249.

Engleman VW, Nickols GA, Ross FP, Horton MA, Griggs DW, Settle SL, Ruminski PG, and Teitelbaum SL (1997). A peptidomimetic antagonist of the alpha(v)beta3 integrin inhibits bone resorption in vitro and prevents osteoporosis in vivo. J Clin Invest 99:2284–2292.

Felding-Habermann B, Mueller BM, Romerdahl CA, and Cheresh DA (1992). Involvement of integrin alpha V gene expression in human melanoma tumorigenicity. J Clin Invest 89:2018–2022.

Ferguson TA, Mizutani H, and Kupper TS (1991). Two integrin-binding peptides abrogate T cell-mediated immune responses in vivo. Proc Natl Acad Sci USA 88:8072–8076.

Filardo EJ, Brooks PC, Deming SL, Damsky C, and Cheresh DA (1995). Requirement of the NPXY motif in the integrin beta 3 subunit cytoplasmic tail for melanoma cell migration in vitro and in vivo. J Cell Biol 130:441–450.

Fujii H, Nishikawa N, Komazawa H, Suzuki M, Kojima M, Itoh I, Obata A, Ayukawa K, Azuma I, and Saiki I (1998). A new pseudo-peptide of Arg-Gly-Asp (RGD) with inhibitory effect on tumor metastasis and enzymatic degradation of extracellular matrix. Clin Exp Metastasis 16:94–104.

Gillies RJ, Didier N, and Denton M (1986). Determination of cell number in monolayer cultures. Anal Biochem 159:109–113.

Humphries MJ, Olden K, and Yamada KM (1986). A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. Science 233:467–470.

Hynes RO (1992). Integrins: Versatility, modulation, and signaling in cell adhesion. Cell 69:11–25.

Lamaze C, Fujimoto LM, Yin HL, and Schmid SL (1997). The actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells. J Biol Chem 272:20332–20335.

Lauffenburger DA and Horwitz AF (1996). Cell migration: A physically integrated molecular process. Cell 84:359–369.

Li E, Stupack D, Klemke R, Cheresh DA, and Nemerow GR (1998). Adenovirus endocytosis via alpha(v) integrins requires phosphoinositide-3-OH kinase. J Virol 72:2055–2061.

Marshall JF, Nesbitt SA, Helfrich MH, Horton MA, Polakova K, and Hart IR (1991). Integrin expression in human melanoma cell lines: Heterogeneity of vitronectin receptor composition and function. Int J Cancer 49:924–931.

Memmo LM and McKeown-Longo P (1998). The alphavbeta5 integrin functions as an endocytic receptor for vitronectin. J Cell Sci 111:425–433.

Mitjans F, Meyer T, Fittschen C, Goodman S, Jonczyk A, Marshall JF, Reyes G, and Piulats J (2000). In vivo therapy of malignant melanoma by means of antagonists of alphav integrins. Int J Cancer 87:716–723.

Mitjans F, Sander D, Adan J, Sutter A, Martinez JM, Jaggle CS, Moyano JM, Kreysch HG, Piulats J, and Goodman SL (1995). An anti-alpha v-integrin antibody that blocks integrin function inhibits the development of a human melanoma in nude mice. J Cell Sci 108:2825–2838.

Nakano MY, Boucke K, Suomalainen M, Stidwill RP, and Greber UF (2000). The first step of adenovirus type 2 disassembly occurs at the cell surface, independently of endocytosis and escape to the cytosol. J Virol 74:7085–7095.

Palecek SP, Schmidt CE, Lauffenburger DA, and Horwitz AF (1996). Integrin dynamics on the tail region of migrating fibroblasts. J Cell Sci 109:941–952.

Panetti TS and McKeown-Longo PJ (1993a). Receptor-mediated endocytosis of vitronectin is regulated by its conformational state. J Biol Chem 268:11988–11993.

Panetti TS and McKeown-Longo PJ (1993b). The alpha v beta 5 integrin receptor regulates receptor-mediated endocytosis of vitronectin. J Biol Chem 268:11492–11495.

Panetti TS, Wilcox SA, Horzempa C, and McKeown-Longo PJ (1995). Alpha v beta 5 integrin receptor-mediated endocytosis of vitronectin is protein kinase C-dependent. J Biol Chem 270:18593–18597.

Petitclerc E, Stromblad S, von Schalscha TL, Mitjans F, Piulats J, Montgomery AM, Cheresh DA, and Brooks PC (1999). Integrin alpha(v)beta3 promotes M21 melanoma growth in human skin by regulating tumor cell survival. Cancer Res 59:2724–2730.

Pierini LM, Lawson MA, Eddy RJ, Hendey B, and Maxfield FR (2000). Oriented endocytic recycling of alpha5beta1 in motile neutrophils. Blood 95:2471–2480.

Pijuan-Thompson V and Gladson CL (1997). Ligation of integrin alpha5beta1 is required for internalization of vitronectin by integrin alphavbeta3. J Biol Chem 272:2736–2743.

Poumay Y, Roland IH, Leclercq-Smekens M, and Leloup R (1994). Basal detachment of the epidermis using dispase:

Tissue spatial organization and fate of integrin alpha 6 beta 4 and hemidesmosomes. J Invest Dermatol 102:111–117.

Ruoslahti E (1996). RGD and other recognition sequences for integrins. Annu Rev Cell Dev Biol 12:697–715.

Ruoslahti E and Reed J (1999). New way to activate caspases. Nature 397:479–480.

Schoenwaelder SM and Burridge K (1999). Bidirectional signaling between the cytoskeleton and integrins. Curr Opin Cell Biol 11:274–286.

Sheu JR, Yen MH, Kan YC, Hung WC, Chang PT, and Luk HN (1997). Inhibition of angiogenesis in vitro and in vivo: Comparison of the relative activities of triflavin, an Arg-Gly-Asp-containing peptide and anti-alpha(v)beta3 integrin monoclonal antibody. Biochim Biophys Acta 1336:445–454.

Shurety W, Stewart NL, and Stow JL (1998). Fluid-phase markers in the basolateral endocytic pathway accumulate in response to the actin assembly-promoting drug Jasplakinolide. Mol Biol Cell 9:957–975.

Wickham TJ, Filardo EJ, Cheresh DA, and Nemerow GR (1994). Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization. J Cell Biol 127:257–264.

Wickham TJ, Mathias P, Cheresh DA, and Nemerow GR (1993). Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 73:309–319.

Yeh CH, Peng HC, and Huang TF (1998). Accutin, a new disintegrin, inhibits angiogenesis in vitro and in vivo by acting as integrin alphavbeta3 antagonist and inducing apoptosis. Blood 92:3268–3276.

Zocchi MR, Poggi A, and Rubartelli A (1997). The RGD-containing domain of exogenous HIV-1 Tat inhibits the engulfment of apoptotic bodies by dendritic cells. AIDS 11: 1227–1235.