Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ Contribute to Cell Attachment to Vitronectin but Differentially Distribute on the Cell Surface

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Abstract. We investigated the role of the integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ in mediating vitronectin adhesion of three phenotypically distinct cell types. M21 human melanoma cells and H2981 lung carcinoma cells use both αv-containing integrins in adhering to vitronectin while UCLA-P3 lung carcinoma cells adhere exclusively with $\alpha v\beta 5$. Specifically, monoclonal antibodies directed to functional epitopes on both receptors were required to block adhesion of M21 or H2981 cells while adhesion of UCLA-P3 cells to vitronectin could be blocked with a monoclonal antibody to $\alpha v\beta 5$. Although both receptors are involved in M21 and H2981 cell adhesion to vitronectin, only $\alpha v\beta 3$ can be detected in focal contacts, colocalizing with vinculin, talin, and the ends of actin filaments, while $\alpha v\beta 5$ shows a distinct, nonfocal contact, distribution on the cell surface. These results provide the first evidence that two homologous integrins that recognize the same ligand distribute differentially on the cell surface.

INTEGRINS are a family of heterodimeric proteins responsible for a wide variety of cellular adhesive functions involving both cell–cell and cell–matrix interactions. The term integrin was derived from the ability of these proteins to link the extracellular environment with the cytoskeleton (Hynes, 1987). Numerous reports have documented that cells attach and spread on ligands such as vitronectin, fibronectin, or collagen, and organize their respective integrin receptors into focal contacts that form at the end of actin filaments (Chen et al., 1985; Damsky et al., 1985; Burridge et al., 1988; Singer et al., 1988; Dejana et al., 1988; Carter et al., 1990). These focal contacts contain not only the appropriate integrin but also cytoplasmic proteins such as vinculin, talin, and α-actinin (Geiger, 1979; Horwitz et al., 1986; Tapley et al., 1989; Nuckolls et al., 1990; Otey et al., 1990), which are thought to mediate the interaction between the integrin/ligand structure on the outside of the cell with the actin-containing microfilaments on the inside of the cell.

The functional diversity of integrins is dictated by the particular α/β subunit composition. However, it is now clear that several integrins with distinct subunit composition recognize the same ligand. For example, $\alpha 2$, $\alpha 3$, and $\alpha 6$ when coupled to the $\beta 1$ subunit have demonstrated laminin recognition capability (Elices and Hemler, 1989; Languino et al., 1989; Gehlsen et al., 1989; Sonnenberg et al., 1988). Variation of the integrin α subunit can also give rise to multiple receptors for collagen (Wayner and Carter, 1987; Elices and Hemler, 1989), fibronectin (Pytela et al., 1985; Wayner et al., 1989), and fibrinogen (Cheresh et al., 1989*a*, *b*; Smith et al., 1990*a*, *b*).

Recently, it became evident that the αv subunit was capable of associating with multiple integrin β subunits (Cheresh et al., 1989*a*; Freed et al., 1989; Smith et al., 1990*b*; Krissansen et al., 1990; Vogel et al., 1990; Bodary and McLean, 1990). At present there are at least three distinct β subunits that associate with αv, namely, $\beta 1$, $\beta 3$, and $\beta 5$. Some of these receptors have been shown to bind vitronectin in an Arg-Gly-Asp-dependent manner (Cheresh et al., 1989*a*; Bodary and McLean, 1990). However, $\alpha v\beta 3$ is capable of recognizing multiple Arg-Gly-Asp-containing ligands (Cheresh and Spiro, 1987, Smith et al., 1990*a*; Charo et al., 1990) whereas $\alpha v\beta 5$ (Cheresh et al., 1989*a*: Smith et al., 1990*a*) and $\alpha v\beta 1$ (Vogel et al., 1990; Bodary and McLean, 1990) are clearly more restricted in their adhesive functions.

At present it is not clear what, if any, biological significance can be attributed to the presence of different integrins with similar or identical ligand specificities. It is conceivable that distinct integrins that recognize the same ligand may convey differential signals to the cell. Alternatively, one receptor could be used for adhesion while a second receptor may potentiate cell migration.

In this report, we investigated the role of the two vitronectin receptors, $\alpha v\beta 3$ and $\alpha v\beta 5$, in mediating cellular adhesion to vitronectin. Our results demonstrate that while both receptors are involved in the adhesion of a given cell to vitronectin, postligand binding events cause these receptors to differentially distribute on the cell surface. Once cells attach and spread on a vitronectin substrate, $\alpha v\beta 3$ exclusively associates with focal contacts and therefore colocalizes with vinculin and the ends of actin filaments, but $\alpha v\beta 5$ on the same

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cell does not. This is the first evidence that two integrins on a given cell recognize the same ligand yet differentially segregate on the cell surface. These results clearly demonstrate that distinct integrins bound to the same ligand can promote differential postligand binding events.

Materials and Methods

Cells and Cell Culture

UCLA-P3 human lung carcinoma cells and M21 human melanoma cells were obtained from Dr. Donald Morton (Department of Surgery, University of California, Los Angeles). A549 human lung carcinoma cells were obtained from the ATCC. H2981 human lung carcinoma cells were obtained from Dr. Diane Horn (Oncogen, Seattle, WA). Cells were grown in RPMI-1640 containing 10% fetal bovine serum and were free of mycoplasma during the course of these studies.

Antibodies

mAbs P5H9 and P3G2 (IgG1) directed to the integrin receptor $\alpha v\beta 5$ were produced as described by Wayner and Carter (1987) and Wayner et al. (1988, 1989). Spleens from RBF/DnJ mice immunized with A549 lung carcinoma cells were removed and fused with NS-1/FOX-NY myeloma cells. Hybridomas producing antibody directed to carcinoma cell vitronectin receptors were screened by the specific inhibition of UCLA-P3 adhesion to vitronectin-coated surfaces and cloned by limiting dilution on thymocyte feeder layers. The resulting monoclonal antibodies, P3G2 ($\alpha v\beta 5$), P5H9 ($\alpha v\beta 5$), and P3G8 (αv) were used throughout the present studies.

Other anti-integrin mAbs used included: LM609 ($\alpha v\beta 3$), LM142 (αv), LM534 ($\beta 1$ nonfunctional), P4C10 ($\beta 1$ functional), have been described (Cheresh and Harper, 1987; Cheresh, 1987; Carter et al., 1990). mAB AP3 (Newman et al., 1985) to the integrin $\beta 3$ subunit was a generous gift from Dr. Peter Newman (Blood Center of Southeastern Wisconsin, Milwaukee, WI). Monoclonal anti-vitronectin, 8E6 (Hayman et al., 1983), was a generous gift from Dr. Deane Mosher (University of Wisconsin, Madison, WI). Polyclonal anti-vitronectin was prepared by immunizing rabbits with purified human vitronectin. This reagent did not cross-react with any known adhesive ligand by immunofluorescence or Western blotting. A rabbit polyclonal antibody raised against a 20 residue peptide (CTHVDFTFNKFNKSYNGTVD) derived from the COOH terminus of $\beta 5$ (Ramaswamy and Hemler, 1990) was a gift from Dr. Martin Hemler (Dana Farber, Boston, MA). mAb to the cytoskeletal protein vinculin was obtained from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-talin was generously provided by Dr. Keith Burridge (University of North Carolina, Chapel Hill, NC). All antibodies were purified on protein A-Sepharose.

Adhesive Ligands

Human plasma fibronectin was prepared as described (Wayner and Carter, 1987). Collagen type I was obtained from Collaborative Research (New Bedford, MA). Vitronectin was prepared as described (Yatohgo et al., 1988).

Cell Adhesion Assay and Inhibition with Monoclonal Antibodies

The cell adhesion assays were performed as previously described (Wayner and Carter, 1987; Wayner et al., 1988; Carter et al., 1990) except that the following buffer was used for the adhesion experiments: RPMI 1640 buffered with Hepes (Gibco Laboratories, Grand Island, NY) supplemented with 1% BSA and 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 100 μM $MnCl_2$. Briefly, virgin styrene 48-well plates (Costar, Cambridge, MA) were coated with adhesive ligands (200 μl per well containing 5 μg/ml ligand) and were blocked with PBS supplemented with 5% BSA. Melanoma and carcinoma cells in logarithmic growth were suspended by a 10-min treatment with versene, washed, resuspended in adhesion buffer and labeled with $Na_2{}^{51}CrO_4$ (50 μCi/ml for 30 min). The chromium-labeled cells were allowed to adhere to the protein-coated surfaces for 15–30 min at 37°C in the presence or absence of purified mAbs or ascites fluid to specific adhesion receptors diluted 1:200. At the end of the incubation, the nonadherent cells were removed and the adherent cells were dissolved in SDS/NaOH and bound ^{51}Cr cpm were quantitated in a gamma counter.

Fluorescence Analysis of Receptor Expression and Localization of Receptors in Focal Adhesions

Adherent cells were suspended with versene (as above), washed, and allowed to adhere to glass coverslips coated with 10 μg/ml fibronectin, collagen type I, or vitronectin in the absence of serum for 30 min at 37°C. After adhesion, the nonadherent cells were removed and the adherent cells were fixed with 3% paraformaldehyde in HBSS for 30 min. They were permeabilized with 0.5% Triton X-100 for 1 min, washed, and blocked with 5% BSA in PBS. The permeabilized cells were stained with antibodies to specific receptors or adhesive ligands (60 min at room temperature), washed, incubated with either FITC-conjugated goat anti-mouse (Tago, Inc., Burlingame, CA) or rhodamine-conjugated goat anti-rabbit IgG (Tago, Inc.) (60 min at room temperature), and washed again. The coverslips were inverted onto glass slides for fluorescence microscopy as described (Wayner et al., 1989; Carter et al., 1990). The actin-containing cytoskeleton was visualized by staining with FITC or rhodamine-conjugated phalloidin diluted 1:50 in HBSS containing 1% BSA (Sigma Chemical Co.). Focal adhesions formed during the attachment of cells to vitronectin-coated surfaces were visualized by the exclusion of a ligand specific antibody from the close contacts (Burridge et al., 1988; Carter et al., 1990) and by vinculin colocalization (Geiger, 1979; Burridge et al., 1988).

Immunoprecipitation Analysis

Cells were either surface labeled with ^{125}I or metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine as previously described (Cheresh et al., 1989*a*). Radiolabeled cells were lysed with LCL (10 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 0.02% NaN_3, pH 8.5) containing 2% Renex 30 detergent. The lysates were then subjected to immunoprecipitation as previously described (Cheresh et al., 1989*a*). Immunoprecipitated proteins were analyzed by SDS-PAGE under nonreducing conditions on 6% polyacrylamide gels. Radiolabeled proteins were visualized by autoradiography as previously described (Cheresh, 1987).

Results

Monoclonal Antibody P5H9 Blocks Adhesion of UCLA-P3 to Vitronectin

UCLA-P3 lung carcinoma cells express the vitronectin receptor $\alpha v\beta 5$, originally designated as $\alpha v\beta x$ (Cheresh et al., 1989*a*). mAbs were selected for their ability to block the attachment of these cells to vitronectin. As shown in Fig. 1, mAbs P5H9 and P3G2 (not shown) were selected based on their ability to inhibit the adhesion of UCLA-P3 cells to vitronectin while not affecting cell attachment to collagen, or fibronectin. mAb LM142 or P3G8 (not shown) directed to the αv subunit of this receptor (Cheresh et al., 1989*a*) or mAb LM609 directed to the $\alpha v\beta 3$ complex (Cheresh and Spiro, 1987) failed to inhibit UCLA-P3 cell attachment to any of the adhesive ligands (Fig. 1). In addition, mAb P4C10, directed to a functional epitope on the integrin $\beta 1$ subunit, inhibited attachment of UCLA-P3 cells to collagen but failed to affect cell attachment to vitronectin or fibronectin. These results suggest that mAbs P5H9 and P3G2 recognize the primary vitronectin receptor on UCLA-P3 cells.

In a second experiment, we tested various concentrations of purified mAb P5H9 for their ability to block UCLA-P3 adhesion to vitronectin. As shown in Fig. 2, this mAb inhibited cell attachment to vitronectin in a dose-dependent manner with half-maximal inhibition at ~0.5 μg/ml and complete inhibition was obtained at 10 μg/ml. In contrast, mAb LM609 directed to the vitronectin receptor $\alpha v\beta 3$ (Cheresh and Spiro, 1987) failed to inhibit UCLA-P3 cell attachment at a concentration of 100 μg/ml. These results demonstrate that the mAb P5H9 recognizes the sole vitronectin receptor on UCLA-P3 lung carcinoma cells.

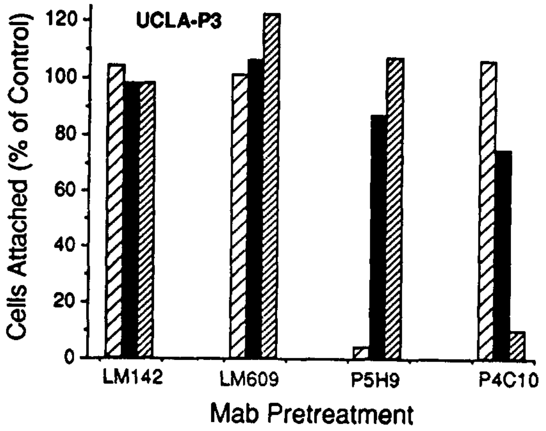


Figure 1. UCLA-P3 cell adhesion to vitronectin, fibronectin, and collagen: effect of mAb P5H9. UCLA-P3 cells were radiolabeled with ^{51}Cr and allowed to attach to microtiter wells coated with vitronectin, fibronectin, or collagen in the presence of various mAbs (ascites fluid diluted 1:200) as described in Materials and Methods. After adhesion, nonadherent cells were washed away and the remaining cells were harvested and the cpm were determined in a gamma counter. Data are expressed as percent of control adhesion, i.e., cells incubated in the absence of antibody. In control wells, >50% of the cells added were attached. The mAbs used are as follows: LM142 (αv), LM609 (αvβ3), P5H9 (αvβ5), and P4C10 (β1). Each bar represents the mean of triplicates the standard error of which is routinely <10%. (▨) VN; (■) FN; (▩) COL.

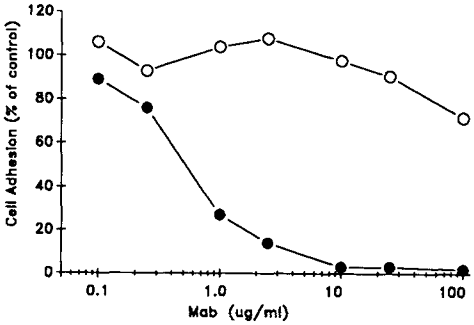


Figure 2. The effect of varying concentrations of mAb P5H9 on the attachment of UCLA-P3 cells to vitronectin. UCLA-P3 cells were allowed to adhere to wells coated with vitronectin as in Fig. 1 in the presence of varying concentrations of mAb P5H9 (*solid circles*) or mAb LM609 (*open circles*). Data are expressed as the percent of control adhesion (i.e., cells incubated in the absence of antibody). In control wells, ~50% of the cells of the cell population was adherent. Each point represents the mean level of adhesion in duplicate wells.

mAb P5H9 Immunoprecipitates the Vitronectin Receptor αvβ5 from UCLA-P3 and H2981 Lung Carcinoma Cells

To characterize the molecule on the surface of UCLA-P3 carcinoma cells recognized by mAb P5H9, cells were surface labeled with ^{125}I, lysed in detergent, and the lysates were subjected to immunoprecipitation. As shown in Fig. 3 (*left*, lane *C*), mAb P5H9 immunoprecipitates a heterodimer consisting of an α chain (160 kD) and a β chain (95 kD) under nonreducing conditions. This identical pattern was obtained with mAb LM142 directed to the αv subunit (Fig. 3, lane *A*). In contrast, mAb LM609, directed to the αvβ3 complex or mAb AP3, directed to the β3 subunit, failed to immunoprecipitate any proteins from these cells (Fig. 3 *left*, lanes *B* and *D*, respectively) consistent with previous results demonstrating that UCLA-P3 cells lack αvβ3 (Cheresh et al., 1989*a*).

To examine the expression of these receptors on another lung carcinoma cell, H2981 cells were surface labeled and subjected to immunoprecipitation as above. As shown in Fig.

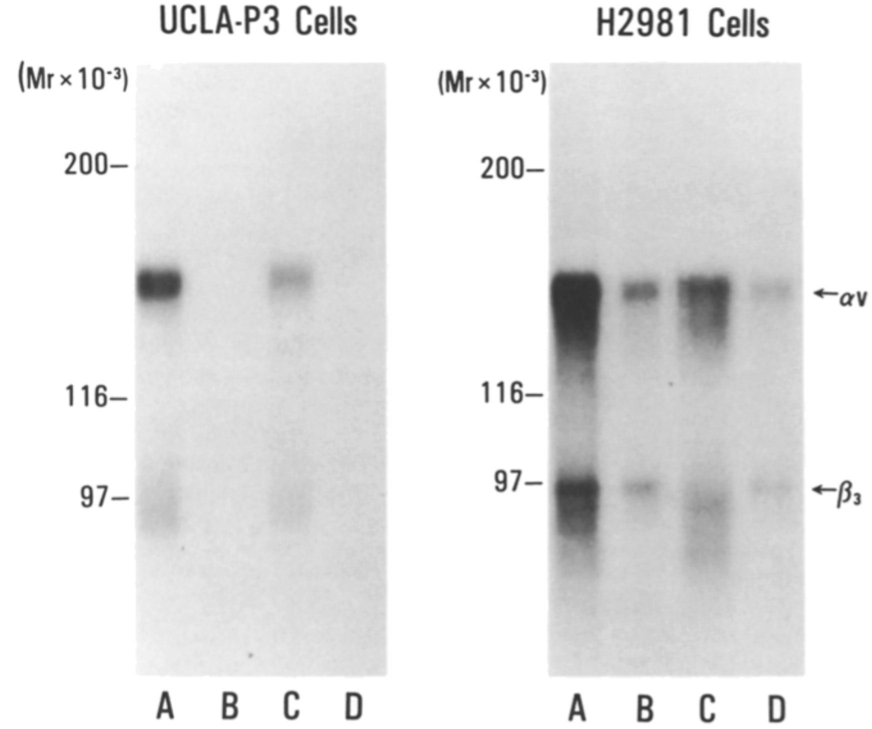


Figure 3. Molecular profile of integrins αvβ3 and αvβ5 on UCLA-P3 and H2981 cells. UCLA-P3 cells (*left*) or H2981 cells (*right*) were surface-labeled with ^{125}I, extracted in detergent, and the lysates were subjected to immunoprecipitation with various mAbs as described in Materials and Methods. Samples were resolved by SDS-PAGE on 6% gels under nonreducing conditions and radiolabeled bands were visualized by autoradiography as described in Materials and Methods. Molecular mass markers are indicated to the left of each panel. mAbs used include LM142, directed to αv (lane *A*); LM609, directed to αvβ3 (lane *B*); P5H9, directed to αvβ5 (lane *C*) and AP3, directed to β3 (lane *D*). The migration of αv and β3 on these gels is depicted by arrows.

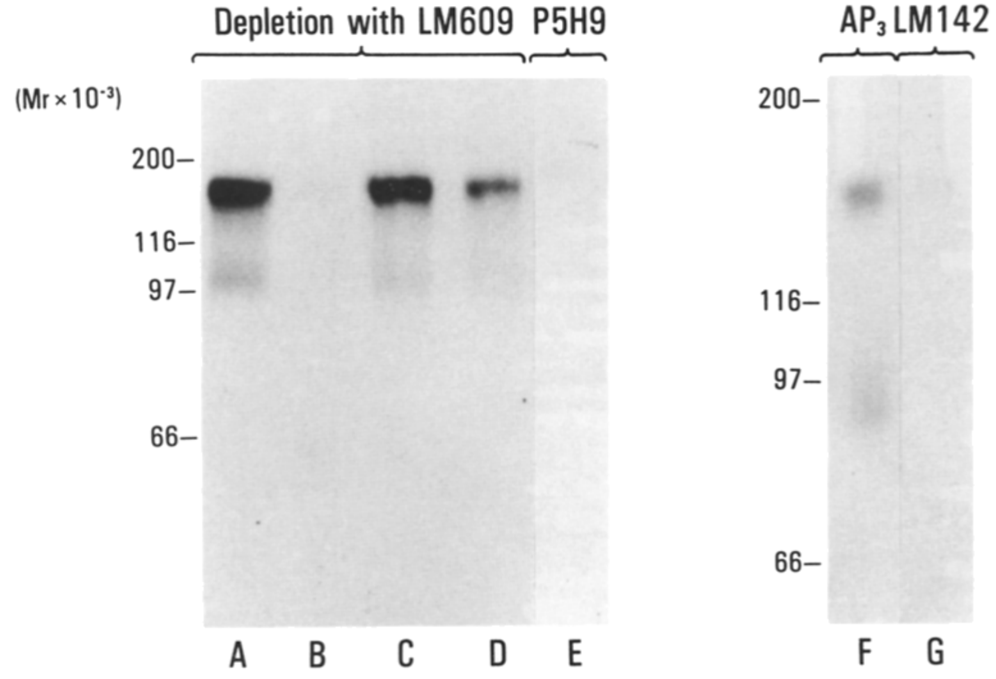


Figure 4. mAb P5H9 recognizes the integrin αvβ5. UCLA-P3 cells were surface labeled, extracted with detergent, the lysate of which was subjected to immunoprecipitation as described in Fig. 3. However, before immunoprecipitation, lysates were precleared three times with mAb LM609 (anti-αvβ3, lanes *A-D*), mAb P5H9 (putative anti-αvβ5, lane *E*), mAb AP3 (anti-β3, lane *F*) or mAb LM142 (anti-αv, lane *G*) as previously described (Cheresh et al., 1989). Immunoprecipitation of these precleared lysates was as follows: lane *A*, mAb 142 (anti-αv); lane *B*, mAb LM609 (anti-αvβ3); lanes *C*, *F*, and *G*, mAb P5H9 (putative anti-αvβ5); and lanes *D* and *E*, polyclonal anti-β5. Samples were analyzed by SDS-PAGE on 7.5 (*left*) or 6% (*right*) gels run under nonreducing conditions and radiolabeled proteins were visualized by autoradiography as described in Fig. 2.

3 *C* (*right*), mAb P5H9 immunoprecipitates a heterodimer identical to that observed from UCLA-P3 cells. However, H2981 cells also express the integrin αvβ3 (Fig. 3 *right*, *arrows*) since mAbs LM609 (αvβ3 complex) and AP3 (β3) immunoprecipitate both αv and β3 from these cells (Fig. 3, lanes *B* and *D*, respectively). Therefore, mAb LM142 directed to the αv subunit immunoprecipitates αv together with β3 (*arrow*) as well as the β subunit identified by mAb P5H9.

To establish the identity of the heterodimer immunoprecipitated with mAb P5H9, UCLA-P3 cell lysates were first subjected to immunodepletion with subunit specific anti-integrin antibodies. This was followed by a second round of immunoprecipitation. As shown in Fig. 4 (*left*), the radiolabeled lysate was depleted with mAb P5H9 or LM609, and then subjected to immunoprecipitation with mAb LM142, LM609, P5H9, or a β5-specific antibody (Ramaswamy and Hemler, 1990). Immunodepletion with mAb P5H9 (Fig. 4, lane *E*) completely depleted the β5 immunoreactive material, whereas depletion with mAb LM609, directed to the αvβ3 complex, did not (Fig. 4, lane *D*). As expected, depletion with mAb P5H9 eliminated immunoreactivity with itself and with mAb LM142 but failed to deplete immunoreactivity with a β1-specific mAb (data not shown). Moreover, cross-depletion studies using mAb LM609 and P5H9 on H2981 cell lysates, show absolutely no cross-reactivity between these antibodies (data not shown). Taken together, these results indicate that the β subunit immunoprecipitated by mAb P5H9 is β5.

To identify the α subunit associated with β5 on these cells, lysates were first depleted with mAb AP3 (β3) or mAb LM142 (αv) and then subjected to immunoprecipitation with mAb P5H9. As shown in Fig. 4 (*right*), depletion with mAb LM142 specifically precleared the lysate of P5H9 reactivity whereas depletion with mAb AP3 failed to do so. These results indicate that the receptor recognized by mAb P5H9 is the integrin αvβ5. This is consistent with our previous results where we demonstrated that mAb LM142 immunoprecipitated αvβx (αvβ5) from UCLA-P3 cells (Cheresh et al., 1989*a*). That mAb P5H9 completely blocks UCLA-P3 cell adhesion to vitronectin (Figs. 1 and 2) suggests that αvβ5 is the sole vitronectin receptor expressed on these cells.

In an attempt to characterize the relative abundance of vitronectin receptors αvβ5 and αvβ3 on various cell types, lung carcinoma cell lines UCLA-P3 or H2981 or M21 human melanoma cells were metabolically labeled and subjected to immunoprecipitation with mAb P5H9 directed to αvβ5 or mAb LM609 directed to αvβ3. As shown in Fig. 5, mAb P5H9 immunoprecipitates a heterodimer from all three cell lines. In contrast, mAb LM609 directed to αvβ3 immunoprecipitates a corresponding heterodimer from M21 and H2981 cells yet fails to identify any protein in UCLA-P3 cells. Therefore these cell lines represent three distinct phenotypes since M21 cells express mostly αvβ3 and minimal levels of αvβ5, H2981 cells express similar levels of both integrins while UCLA-P3 cells express αvβ5 exclusively.

Integrins αvβ5 and αvβ3 Are Both Involved in Adhesion of M21 Melanoma and H2981 Carcinoma Cells to Vitronectin

To determine whether αvβ5 and αvβ3 are both involved in cell adhesion to vitronectin M21 melanoma and H2981 car-

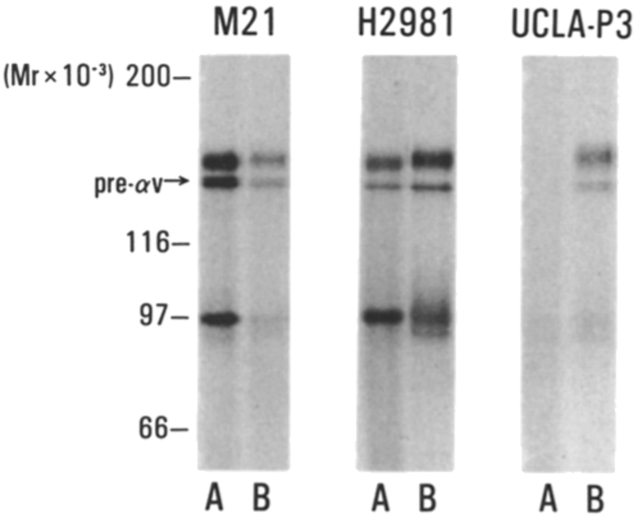


Figure 5. Immunoprecipitation of $\alpha v\beta 3$ and $\alpha v\beta 5$ from metabolically labeled M21, H2981, and UCLA-P3 cells. M21, H2981, or UCLA-P3 cells were metabolically labeled under steady-state conditions (18 h) with [^{35}S]cysteine or [^{35}S]methionine, lysed in detergent, and the lysates were subjected to immunoprecipitation with mAb LM609 ($\alpha v\beta 3$, lane *A*) or mAb P5H9 ($\alpha v\beta 5$, lane *B*) as described in Materials and Methods. Samples were analyzed on 6% polyacrylamide gels under nonreducing conditions and visualized by autoradiography as described in Fig. 2. Arrow refers to the biosynthetic precursor to αv as previously described (Cheresh and Harper, 1987; Cheresh and Spiro, 1987).

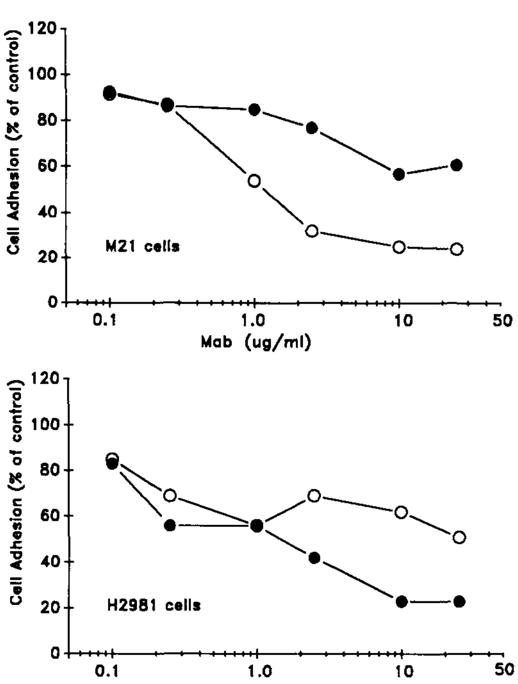


Figure 6. The effects of mAb P5H9 ($\alpha v\beta 5$) and mAb LM609 ($\alpha v\beta 3$) on H2981 and M21 cell adhesion to vitronectin. M21 cells (*top*) or H2981 cells (*bottom*) were radiolabeled with ^{51}Cr, and allowed to attach to microtiter wells coated with vitronectin in the presence of varying concentrations of mAb P5H9 (*solid circles*) or mAb LM609 (*open circles*) as described in Materials and Methods. After adhesion, nonadherent cells were washed away and the remaining cells were harvested and the cpm were determined in a gamma counter. Data are expressed as percent of control adhesion, i.e., cells incubated in the absence of antibody. In control wells, >50% of the cells added were attached. Each point represents mean of duplicate samples.

cinoma cells were allowed to attach to vitronectin in either the presence or absence of mAb P5H9 and LM609. As shown in Fig. 6, neither mAb alone (0.1-25 μg/ml) completely blocks M21 or H2981 cell adhesion to vitronectin. It is apparent that mAb LM609 significantly reduced the adhesion of M21 cells while mAb P5H9 significantly reduced the adhesion of H2981 cells. These results are consistent with the relative levels of $\alpha v\beta 3$ and $\alpha v\beta 5$ expressed by these cells (Fig. 5). However, when these cells were allowed to react with a mixture of mAbs P5H9 and LM609 virtually all M21 cell adhesion to vitronectin was abolished (Fig. 7). The combined effects of both of these mAbs reduced H2981 cell adhesion by 80% (Fig. 7). As a control, mAb W6/32 directed to HLA class I antigens present on these cells failed to inhibit cell adhesion alone or when combined with either vitronectin receptor mAb (Fig. 7). In addition, mAbs P5H9 or LM609 either separately or together had negligible effects on the adhesion of these cells to fibronectin, collagen or laminin (data not shown). These results indicate that M21 and H2981 cells use both $\alpha v\beta 3$ and $\alpha v\beta 5$ in adhesion to vitronectin.

Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ Exhibit a Distinct Distribution on the Surface of M21 or H2981 Cells Attached to Vitronectin

To determine the distribution of $\alpha v\beta 3$ and $\alpha v\beta 5$ on cells attached to vitronectin, immunofluorescence experiments were performed. M21, H2981, or UCLA-P3 cells were allowed to attach and spread on coverslips coated with vitronectin or various other ligands. As shown in Fig. 8, once M21 or H2981 cells attach to vitronectin a focal distribution is detected upon staining with mAb AP3 directed to the $\beta 3$ subunit (Fig. 8, *B* and *D*). This staining pattern completely codistributes with focal contacts as detected by these same cells stained with anti-vitronectin (Fig. 8 *A*). In this case, anti-vitronectin is specifically excluded from the regions of focal contact (Fig. 8, *A*, *C*, and *E*, *arrows*) and these regions codistribute with the staining pattern of $\beta 3$ on M21 cells or H2981 cells (Fig. 8, *B* and *D*, respectively, *arrows*). In contrast, when H2981 cells are stained with anti-$\beta 5$ (Fig. 8 *F*), a punctate distribution is observed over the entire ventral surface (Fig. 8 *F*) which shows little or minimal colocalization with focal contacts as depicted by anti-vitronectin exclusion (Fig. 8 *E*). A composite staining pattern of $\beta 3$ and $\beta 5$ is observed (Fig. 8 *H*) when H2981 are reacted with mAb LM142 directed to the αv subunit on these cells. Thus, it can be clearly seen that the immunolocalization of the αv subunit on these cells reveals both focal contact staining (identified in Fig. 8 *G*, *arrows*) as well as the punctate nonfocal contact staining pattern.

When M21 (Fig. 9, *A* and *B*) or H2981 cells (Fig. 9, *E* and *F*) are attached to vitronectin and cells are stained with both anti-$\beta 3$ (Fig. 9, *A* and *E*) and anti-$\beta 5$ (Fig. 9, *B* and *F*) completely distinct patterns are observed. Thus, $\beta 3$ is localized in a focal contact distribution (Fig. 9, *arrows*) while $\beta 5$ on

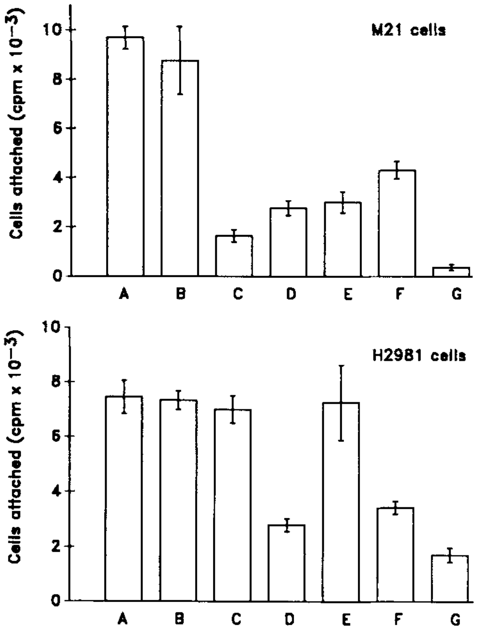


Figure 7. Integrins $\alpha v\beta 5$ and $\alpha v\beta 3$ are involved in M21 and H2981 cell adhesion to vitronectin. Radiolabeled M21 cells (*top*) or H2981 cells (*bottom*) were allowed to adhere to wells coated with vitronectin as described in Materials and Methods. Cell adhesion was performed either in the absence of antibody (*A*) or in the presence 25 μg/ml of the following: mAb W6/32 (*B*; anti-HLA, class I); mAb LM 609, (*C*; anti-$\alpha v\beta 3$); mAb P5H9, (*D*; anti-$\alpha v\beta 5$); mAb W6/32 + mAb LM609, (*E*); mAb W6/32 + mAb P5H9 (*F*); or mAb LM609 + mAb P5H9 (*G*). Data are expressed as the cells attached (cpm) to the wells, which in the absence of antibody treatment, represents approximately 50% of the cells added to each well. Each bar represents the mean ± SD of triplicates.

the same cell is not. As expected, when M21 cells are stained with a mAb directed to vinculin (Fig. 9, *C*) the focal contacts are detected. This staining pattern shows no colocalization with these same cells stained with the anti-$\beta 5$ antibody (Fig. 9 *D*). As shown in Fig. 9, *G* and *H*, H2981 cells stained with anti-vinculin show the expected focal contact distribution (*arrows*). When these cells are stained with antibody to talin (Fig. 9 *I*) there is significant colocalization with vinculin (Fig. 9 *H*) but not $\beta 5$ (Fig. 9, *D* and *F*). Thus, when cells are attached to vitronectin integrin $\alpha v\beta 3$ is found in focal contacts colocalizing with vinculin and talin while integrin $\alpha v\beta 5$ does not. As a control, we examined the $\beta 3$ distribution on H2981 and M21 cells attached to collagen. In this case, the cells did not organize $\beta 3$ into focal contacts, but as expected mAb LM534, directed to a nonfunctional epitope on $\beta 1$, located this subunit in focal contacts on collagen (data not shown). These results are consistent with cell adhesion experiments in which mAb P4C10, directed to a functional epitope on the $\beta 1$ subunit, blocked collagen adhesion of all three cell lines (data not shown). Taken together, these results demonstrate that when cells are attached to vitronectin, $\alpha v\beta 3$ and $\alpha v\beta 5$ demonstrate distinct distributions on the cell surface even though both receptors are clearly involved in the attachment to this ligand.

UCLA-P3 cells readily attach to vitronectin but fail to spread and form focal contacts. As shown in Fig. 10 *A*, the $\beta 5$ distribution on these cells shows a similar punctate staining pattern as observed on M21 cells (Fig. 8) and H2981 cells (Figs. 8 and 9). mAb LM142, directed to αv or a polyclonal antibody directed to $\alpha v\beta 3$ that react with both receptors (Cheresh et al., 1989*a*) on UCLA-P3 cells demonstrate this identical staining pattern confirming the $\beta 5$ staining pattern on these cells (data not shown). This lack of focal contact distribution is correlated with the inability of UCLA-P3 cells to organize actin during their adhesion to vitronectin (Fig. 10 *B*). In contrast, M21 cells (Fig. 10 *D*), or H2981 (Fig. 10 *F*) attached to vitronectin demonstrate well organized actin filaments as depicted on cells stained with rhodamine-phalloidin. As expected the $\beta 3$ distribution on M21 cells localizes at the ends of actin filaments (Fig. 10 *C*, *arrows*) whereas $\beta 5$ on M21 cells (not shown) or H2981 cells does not (Fig. 10 *E*). It is of interest to note that on vitronectin-attached H2981 cells the actin filaments appear to organize around the circumference of the ventral cell surface (Fig. 10 *F*) whereas on M21 cells the actin filaments appear to span the diameter of the cell (Fig. 10 *D*).

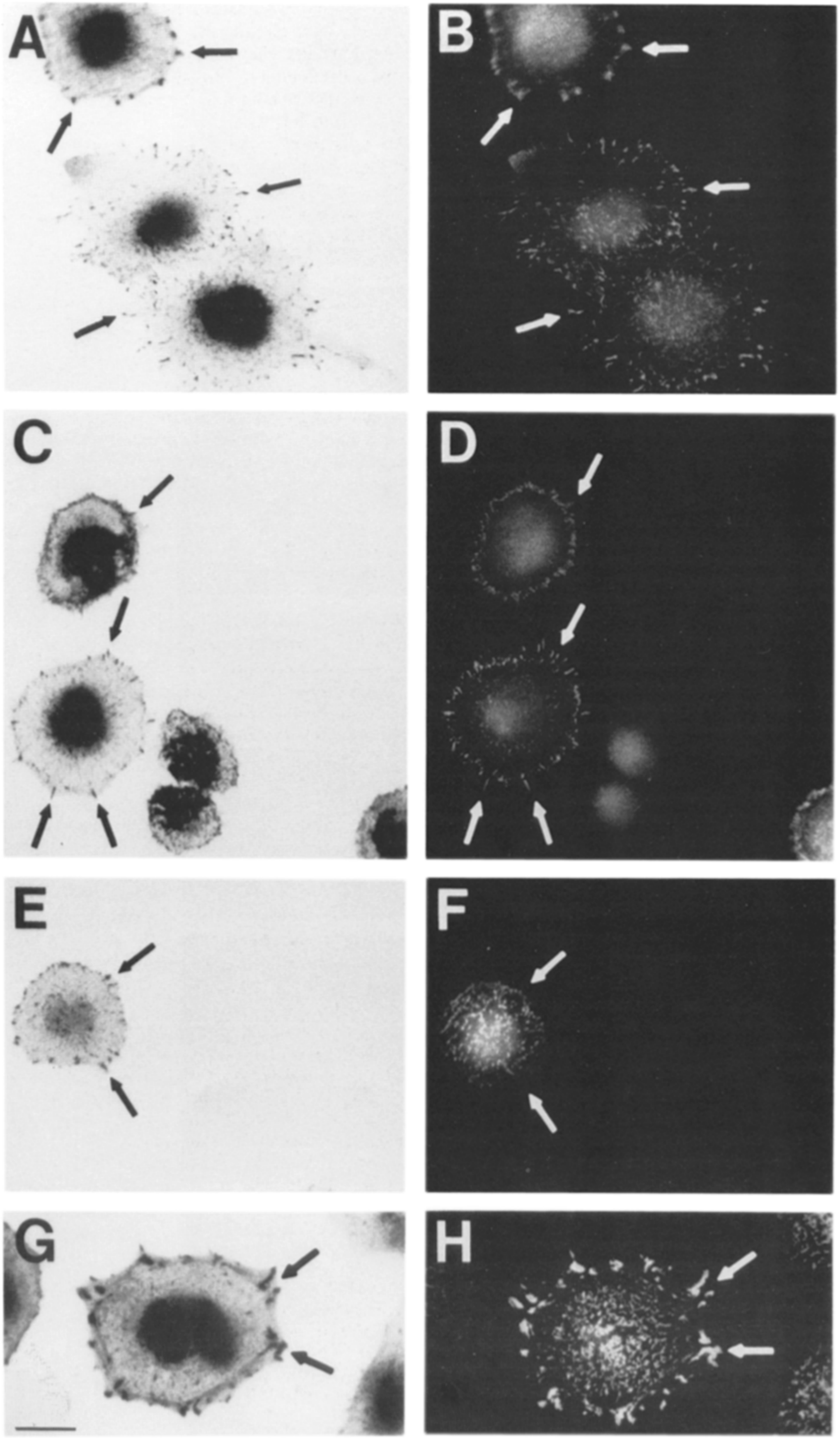
The distinct localization of $\beta 3$ and $\beta 5$ on M21 and H2981 cells attached to vitronectin (as shown above) were based on experiments in which cells were allowed to attach and spread on vitronectin for 30 min. However, when M21 or H2981 cells were allowed to attach and spread for 120 min we were still unable to observe focal contact distribution of $\alpha v\beta 5$ (data not shown). These results indicate that integrin $\alpha v\beta 5$, involved in cell attachment to vitronectin, is incapable of associating with the actin-cytoskeleton and therefore does not localize in focal contacts.

Discussion

In this report we investigated the mechanism of vitronectin adhesion of three phenotypically distinct cell types: (*a*) M21 melanoma cells which express primarily $\alpha v\beta 3$ and a smaller amount of $\alpha v\beta 5$; (*b*) H2981 carcinoma cells which express similar levels of $\alpha v\beta 5$ and $\alpha v\beta 3$; and (*c*) UCLA-P3 carcinoma cells which exclusively express $\alpha v\beta 5$ (originally defined as integrin $\alpha v\beta x$ [Cheresh et al., 1989*a*; Smith et al., 1990*a*]). The M21 and H2981 cells use $\alpha v\beta 3$ and $\alpha v\beta 5$ to adhere and spread on vitronectin whereas UCLA-P3 cells adhere, but fail to spread on this substrate.

Although $\alpha v\beta 3$ and $\alpha v\beta 5$ are required for optimal adhesion of M21 and H2981 cells, these receptors distribute to

Figure 8. Immunofluorescence detection of $\alpha v\beta 3$ and $\alpha v\beta 5$ on the surface of M21 and H2981 cells. M21 (*A* and *B*) of H2981 (*C–H*) cells were allowed to attach and spread on vitronectin-coated coverslips for 30–45 min at 37°C, after which cells were fixed, permeabilized, and reacted with primary antibodies to vitronectin, either mAb 8E6 (*A* and *C*) or rabbit anti-vitronectin antibody (*E* and *G*). Localization of $\beta 3$ (*B* and *D*) or $\beta 5$ (*F*) on these cells was achieved using the primary antibodies mAb AP3 and rabbit anti-$\beta 5$ peptide, respectively. The composite staining of both receptors was identified by mAb LM142 directed to αv (*H*). These antigens were detected with FITC-conjugated anti-mouse or rhodamine-conjugated anti-rabbit IgG. Arrows indicate focal contact staining as defined by anti-vitronectin exclusion in *A*, *C*, *E*, and *G*. Representative fields were photographed using a Zeiss microscope fitted with epifluorescence. Bar, 10 μm.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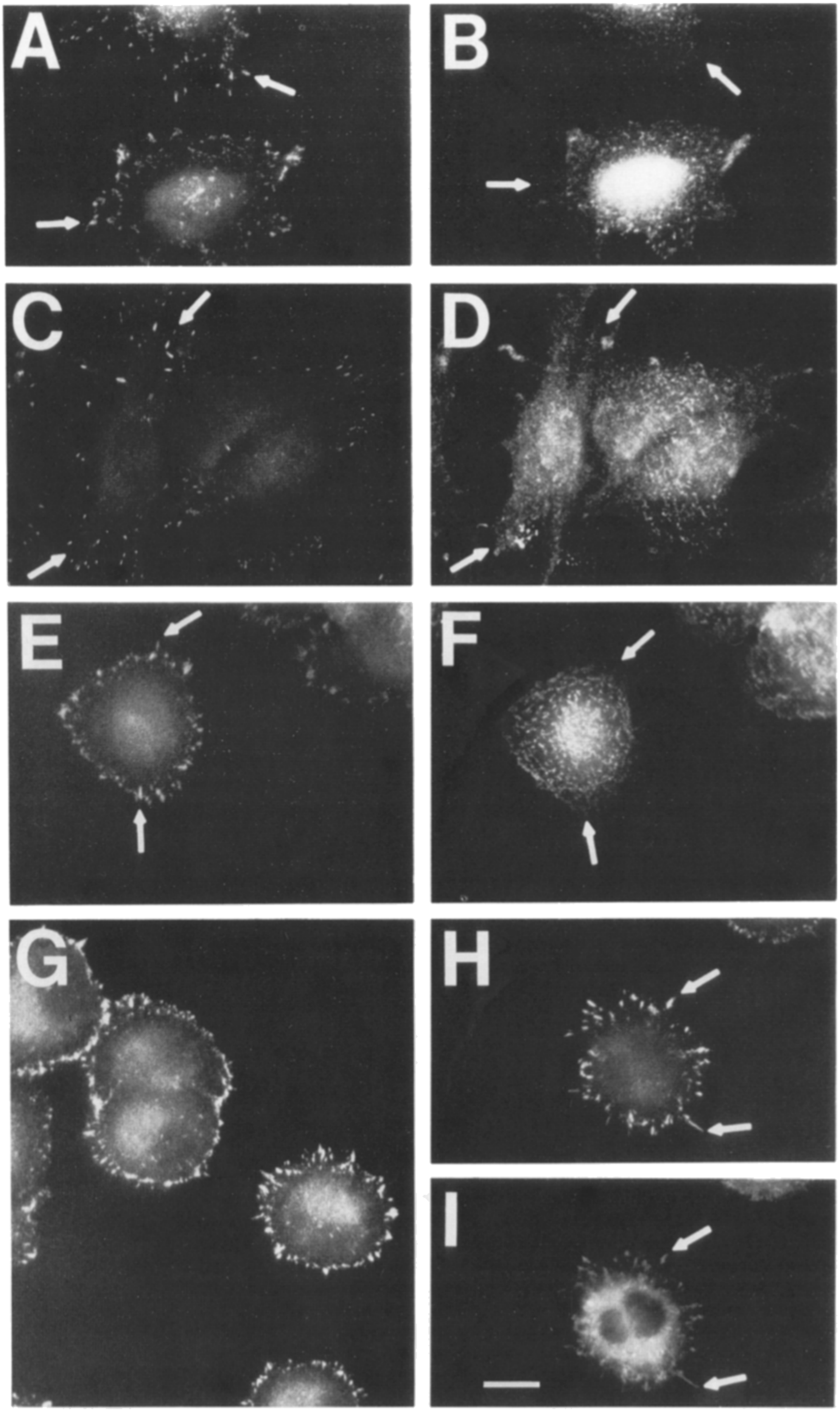
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distinct regions of the cell surface after ligand binding. Thus, after adhesion of M21 or H2981 cells, all detectable surface-associated $\alpha v\beta 3$ is localized to focal contacts whereas $\alpha v\beta 5$ is not. Instead, $\alpha v\beta 5$ appears in a punctate distribution over much of the ventral cell surface. UCLA-P3 cells, which exclusively use $\alpha v\beta 5$ to attach to vitronectin, also show a similar nonfocal distribution. In fact, in none of the three cell lines examined did $\alpha v\beta 5$ localize to the ends of actin filaments or associate with vinculin. These results suggest one of two possibilities. Either the $\beta 5$ subunit is unable to associate with these cytoskeletal proteins or is unable to mobilize them into focal contacts. This is of interest since previous studies demonstrated that both integrins recognize vitronectin in an Arg-Gly-Asp-dependent manner (Cheresh et al., 1989*a*). These results suggest that distinct localization of these two receptors is not a consequence of a major difference in their ligand binding capability.

Of the three cell lines we examined, M21 exhibited the greatest degree of spreading on vitronectin, while H2981 cells had a moderate capacity to spread and UCLA-P3 cells failed to do so. Consistent with these morphological observations, immunoprecipitation analysis demonstrated that M21 cells expressed a high $\alpha v\beta 3/\beta 5$ ratio whereas H2981 cells revealed a relatively low $\alpha v\beta 3/\beta 5$ ratio and UCLA-P3 cells express only $\alpha v\beta 5$. By comparison, M21 cells spread considerably less on collagen indicating that these cells are not simply more capable of spreading in general. However, it should be pointed out that like M21 cells, UCLA-P3 cells use a $\beta 1$-containing integrin to attach to collagen (Fig. 1), yet UCLA-P3 cells were unable to significantly spread on collagen or vitronectin suggesting the failure of these cells to spread on vitronectin is not entirely due to their exclusive expression of $\alpha v\beta 5$ as a vitronectin receptor. Although cell spreading is a complex molecular event, it is conceivable that the failure of $\alpha v\beta 5$ to associate with the actin-cytoskeleton may render this receptor incapable of inducing cell spreading on a vitronectin substrate. Thus, in order to spread on vitronectin, cells may require surface expression of $\alpha v\beta 3$, which is capable of promoting the organization of the actin-cytoskeleton in order to spread.

Our inability to localize $\alpha v\beta 5$ to focal contacts is somewhat surprising in light of previous published reports which have demonstrated both $\beta 1$ and $\beta 3$ integrin association with focal contacts. When cells are attached to fibronectin, collagen, and laminin, integrins containing the $\beta 1$ subunit have been localized to focal contacts (Singer et al., 1988; Wayner et al., 1989; Carter et al., 1990), whereas on vitronectin $\beta 3$ was localized to focal contacts (Singer et al., 1988). These results suggest that integrins direct the organization of cytoskeletal components in response to specific and relevant ligands. This is biologically important since redistribution of integrins into focal contacts causes cells to change shape in response to cytoskeletal rearrangements. These shape changes may affect cellular signaling and the migratory or proliferative capacity of the cell. Thus, the relative expression of $\alpha v\beta 5$ and/or $\alpha v\beta 3$ on a cell surface may have profound effects on the biological behavior of that cell in the presence of vitronectin.

In this study, the immunolocalization of $\alpha v\beta 5$ on vitronectin-attached cells was performed using a polyclonal antibody directed to a 20 residue synthetic peptide derived from the COOH terminus of $\beta 5$ (Ramaswamy and Hemler, 1990). It is conceivable that such an antibody might be sterically inhibited when the $\beta 5$ subunit is associated with the actin-cytoskeleton. In such a case one would expect selective exclusion of staining at the sites of focal contact formation. However, this is not the case, rather, the staining with the $\beta 5$ antibody is punctate over most, if not all, of the ventral surface of all three cell lines. It is also unlikely that all epitopes represented by a polyclonal antibody directed to a peptide of 20 residues would be sterically unable to recognize $\beta 5$ in association with the actin-cytoskeleton, when a polyclonal antibody prepared to a cytoplasmic peptide from $\beta 1$ does localize within focal contacts (Marcantonio and Hynes, 1988). Moreover, staining these cells with a polyclonal antibody to $\alpha v\beta 3$ or a mAb to αv, either of which will recognize both receptors (Cheresh et al., 1989*a*; Smith et al., 1990*a*), demonstrates the composite $\beta 3$ and $\beta 5$ staining pattern of M21 and H2981 and the exclusive $\beta 5$ staining pattern on UCLA-P3 cells.

It is likely that the distinct distribution of $\alpha v\beta 3$ and $\alpha v\beta 5$ is attributable to a structural difference between the β subunits since both receptors share a common α subunit. The primary amino acid sequence of $\beta 3$ (Fitzgerald et al., 1987) and $\beta 5$ (Ramaswamy and Hemler, 1990; Suzuki et al., 1990; McLean et al., 1990) show 56% identity. However, $\beta 5$ contains a cytoplasmic domain that is distinct from that of $\beta 3$. In fact, the cytoplasmic domain of $\beta 5$ is only 10–20% homologous to $\beta 3$ or the other integrin β subunits (Ramaswamy and Hemler, 1990). Moreover, $\beta 5$ contains a 10 residue carboxy-terminal extension not found on $\beta 3$ or any of the known integrin β subunits. Since the cytoplasmic tail of the integrin $\beta 1$ subunit has been implicated in focal contact formation (Solowska et al., 1989; Hayashi et al. 1990) it is conceivable that the unique cytoplasmic portion of $\beta 5$ prevents its association with the actin-cytoskeleton. Alternatively, $\alpha v\beta 5$ might preferentially associate with cytoplasmic proteins other than the actin-cytoskeleton such as intermediate filaments. In either event, this would likely result in the failure of this receptor to localize in focal contacts even though it maintains contact with its ligand. A similar model has been proposed for the integrin $\alpha 6\beta 4$ (Suzuki and Naitoh, 1990; Tamura et al., 1990). This integrin has an unusually long cytoplasmic tail not found on any other integrin β subunits and also fails to enter focal contacts probably due to its association with intermediate filaments (Quaranta, V., personal communication). In epithelial tissues $\alpha 6\beta 4$ exclusively localizes to the basolateral surface whereas the $\beta 1$ subunit is uniformly expressed on the entire cell surface (Carter et al., 1990).

The results presented in our study provide the first evi-

Figure 9. $\alpha v\beta 5$ and $\alpha v\beta 3$ expressed on M21 and H2981 cells demonstrate distinct distributions. M21 (*A–D*) or H2981 (*E–I*) cells attached to vitronectin coated coverslips were stained as in Fig. 8, except the primary antibodies used were mAb AP3 directed to $\beta 3$ (*A* and *E*), rabbit anti-$\beta 5$ (*B*, *D*, and *F*), mAb directed to vinculin (*C*, *G*, and *H*), or rabbit anti-talin (*I*). The secondary antibodies used were identical to those in Fig. 8. Arrows refer to focal contacts as detected by $\beta 3$ localization (*A* and *E*) or vinculin localization (*C*, *G*, and *H*). Representative fields were photographed using a Zeiss microscope fitted with epifluorescence. Bar, 10 μm.

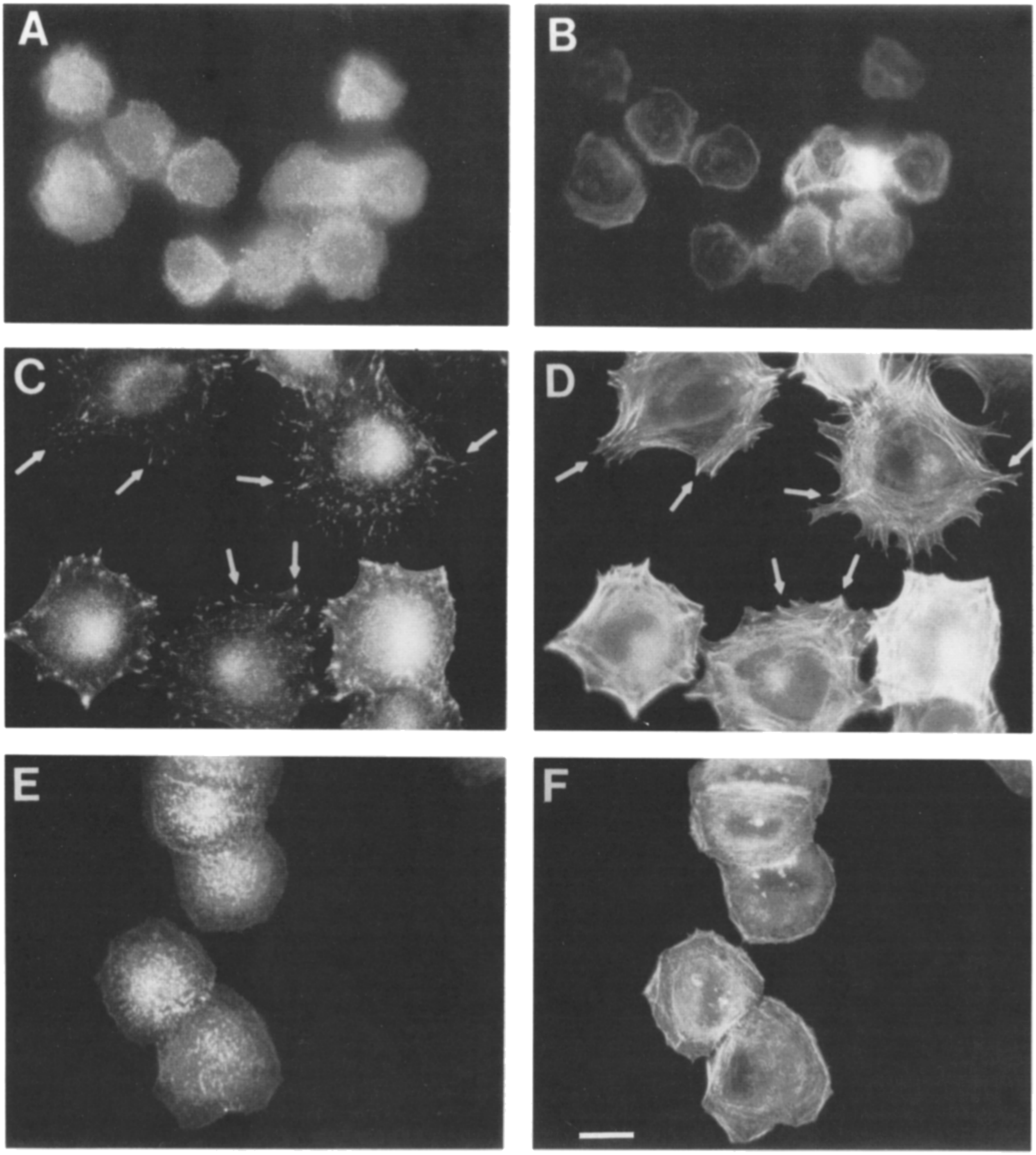

Figure 10. Integrin $\alpha v\beta 3$ colocalizes with the ends of actin filaments whereas $\alpha v\beta 5$ does not. UCLA-P3 (*A* and *B*), M21 cells (*C* and *D*) or H2981 cells (*E* and *F*) were allowed to attach to vitronectin-coated coverslips for 30 min and were stained with anti-$\beta 5$ (*A* and *E*) or the anti-$\beta 3$ mAb AP3 (*C*) as described above. Actin was visualized with FITC (*B* and *F*) or rhodamine-conjugated phalloidin (*D*). Photos of representative fields were taken with a Zeiss microscope fitted with epifluorescence. Magnification is 1,000 (*A* and *B*) or 630 (*C–F*). When M21 or H2981 cells were allowed to spread for longer period (1–2 h) increased actin filament organization was observed whereas UCLA-P3 cells remained unspread with minimal actin organization. The 30-min time was chosen to correspond to the localization of $\beta 3$ and $\beta 5$. Bar, 10 μm.

dence that two homologous integrins that recognize the same ligand differentially segregate on the cell surface. This distinct distribution can be accounted for by differential association with the actin-cytoskeleton. Therefore, two integrins that bind the same ligand can provide distinct cellular signals. Future experiments are aimed at elucidating these signals at the molecular level and determining their consequence in terms of cellular differentiation and proliferation.

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