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Fibronectin Receptor Functions in Embryonic Cells Deficient in $\alpha_5\beta_1$ Integrin Can Be Replaced by α_V Integrins

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$\alpha_5\beta_1$ integrin mediates cell adhesion to extracellular matrix by interacting with fibronectin (FN). Mouse lines carrying null mutations in genes encoding either the α_5 integrin subunit or FN have been generated previously. Both mutations are embryonic lethal with overlapping defects, but the defects of α_5-null embryos are less severe. Primary embryonic cells lacking $\alpha_5\beta_1$ are able to adhere to FN, form focal contacts, migrate on FN, and assemble FN matrix. These results suggest the involvement of (an)other FN receptor(s). In this study, we examined functions of $\alpha_4\beta_1$ and α_V integrins in embryonic cells lacking $\alpha_5\beta_1$. Our analysis of cells lacking both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ showed that $\alpha_4\beta_1$ is also not required for these FN-dependent functions. Using α_V-specific blocking reagents, we showed that α_V integrins are required for α_5-null cells, but not wild-type cells, to adhere and spread on FN. Our data also showed that, although the expression levels of α_V integrins on the wild-type and α_5-null cells are similar, there is an increase in recruitment of α_V integrins into focal contacts in α_5-null cells plated on FN, indicating that α_V integrins can compensate *functionally* for the loss of $\alpha_5\beta_1$ in focal contacts of α_5-null cells. Finally, our data suggested possible roles for α_V integrins in replacing the role of $\alpha_5\beta_1$ in FN matrix assembly in vitro and in FN-dependent embryonic functions in vivo.

INTRODUCTION

Integrins are a family of cell adhesion receptors that mediate cell adhesion to extracellular matrix proteins such as fibronectin (FN), laminin, vitronectin, and collagen (reviewed by Hynes, 1987, 1992). The interactions between integrins and their ligands play important roles in a number of cellular processes, including cell proliferation (Rosales *et al.*, 1995), cell differentiation (Adams and Watt, 1993), cell survival (Meredith *et al.*, 1993; Frisch and Francis, 1994), and cell migration (Hynes and Lander, 1992; Lauffenburger and Horwitz, 1996). Structural analyses have revealed that integrins are heterodimeric transmembrane glycoproteins; each has an α and a β subunit, and each subunit has a large extracellular domain, a single transmembrane domain, and a small cytoplasmic domain. The extracellular domains of the two subunits are noncovalently associated, forming a ligand-binding site, and the cytoplasmic domains interact with cytoskeletal proteins and other cytoplasmic components (Hynes, 1992). It has been shown that integrins are involved in signal transduction pathways (Clark and Brugge, 1995; Schwartz *et al.*, 1995).

The integrin family comprises >20 heterodimers with 16 α subunits and 8 β subunits (Hynes, 1992; Rosales *et al.*, 1995). Many integrins share the same ligands, and one integrin may have multiple ligands. We are particularly interested in $\alpha_5\beta_1$, which belongs to a subclass of integrins that interact with FN. There are at least eight members of this subclass, including $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_6$, and $\alpha_{IIb}\beta_3$. $\alpha_5\beta_1$ binds to the RGD region of FN and is one of the major FN receptors (Pytela *et al.*, 1985). To study the in vivo functions of $\alpha_5\beta_1$, we had previously generated a null mutation in the gene encoding the α_5 integrin subunit and shown that the mutation is a recessive embryonic lethal (Yang *et al.*, 1993). The α_5 integrin-

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deficient (α_5-null) embryos display embryonic and extraembryonic mesodermal defects similar to those resulting from a null mutation of the FN gene (George *et al.*, 1993). However, the defects of the α_5-null embryos are less severe than those in FN-deficient (FN-null) embryos. FN-null embryos die at day 8.5 of gestation with shortened anterior–posterior axis and a failure to form heart, somites, and notochord. In contrast, α_5-null embryos die at days 9.5–10 of gestation. The α_5-null embryos develop the anterior 10–12 somites and are able to form heart and notochord. We have also shown that primary α_5-null embryonic cells in culture are able to adhere to FN and form focal contacts, migrate on FN, and assemble a FN matrix (Yang *et al.*, 1993). These observations led us to propose that there must be (an)other FN receptor(s) involved in mediating FN-dependent functions in α_5-null embryos and in cultured α_5-null embryonic cells.

We have also knocked out the gene encoding the α_4 integrin subunit and shown that α_4-null embryos develop heart, notochord, and many somites (Yang *et al.*, 1995); others have reported that α_3-null and α_8-null mutants both develop to birth (Kreidberg *et al.*, 1996; Mueller and Reichardt, unpublished observations). Therefore, elimination of any one of several β_1 integrin FN receptors fails to reproduce the severe phenotype of FN-null mutants. These results raise the possibility either of overlapping functions or compensation between or among different FN receptor integrins.

In this paper, we extend the analysis of FN receptor functions in vitro with cells from singly or doubly mutant mice in combination with inhibition of the functions of specific integrins. We have focused particularly on $\alpha_5\beta_1$, $\alpha_4\beta_1$, and α_V integrins, all of which have been reported to be FN receptors. The α_V subunit is structurally the most closely related to α_5 (Hynes, 1992), and, like $\alpha_5\beta_1$, α_V integrins also bind to the RGD sequence of FN. Therefore, α_V integrins are good candidates as functional FN receptors in α_5-null embryos and may be able to replace the functions of $\alpha_5\beta_1$ in the mutant embryos. On the other hand, α_4 integrins bind to a different V25/CS1 region (Wayner *et al.*, 1989; Guan and Hynes, 1990; Ruegg *et al.*, 1992) and are thought to have different biological functions.

In this study, we examined the functions of $\alpha_4\beta_1$ and α_V integrins in primary cells cultured from mutant embryos. By analyzing embryonic cells lacking both $\alpha_4\beta_1$ and $\alpha_5\beta_1$, we showed that $\alpha_4\beta_1$ is not required for these cells to perform FN-dependent functions. Using α_V-specific blocking reagents, we showed that α_V integrins can replace the in vitro functions of $\alpha_5\beta_1$ in embryonic cells lacking $\alpha_5\beta_1$. In addition, we show that $\alpha_5\beta_1$ and α_V integrins are colocalized in the somites and the heart of embryonic day (E) 9.5 embryos, suggesting that α_V integrins may also be able to replace certain functions of $\alpha_5\beta_1$ in α_5-null embryos.

MATERIALS AND METHODS

Embryonic Cells

Embryonic cells were cultured as described previously with some modifications (Yang *et al.*, 1993). E9.25 embryos were dissected out, and the yolk sac of each embryo was saved for genotyping. The head and the heart of each embryo were removed and the remainder washed twice in phosphate-buffered saline (PBS) and twice in embryonic cell culture medium containing Dulbecco's modified Eagle's medium (DMEM, high glucose; JRH Biochemical, Lenexa, KS) with 26 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.5, 14 mM sodium bicarbonate, 15% fetal bovine serum (Intergen, Purchase, NY), 1× nonessential amino acids (Life Technologies, Grand Island, NY), and 0.1 mM β-mercaptoethanol (Fluka, Ronkonkoma, NY). The embryo was then dissociated with sterile tweezers and plated in 1 ml of embryonic cell culture medium in a well of a 24-well tissue culture plate (Falcon, Oxnard, CA) precoated with 0.1% gelatin (swine skin type II; Sigma, St. Louis, MO). After 4–5 d in culture, cells were trypsinized and plated in two wells of a 24-well plate. Cells were subcultured in the same manner through two or three more passages. The cells showed a fibroblastic morphology.

Genotyping Mice and Embryos

Heterozygous mice carrying either α_4 or α_5 knockout mutations were genotyped by polymerase chain reaction (PCR) as described previously (Yang *et al.*, 1993, 1995). These mice were crossed to obtain α_4 and α_5 double heterozygous mice, which were genotyped by Southern blot analysis on DNA extracted from tail biopsy as described previously (Yang *et al.*, 1993, 1995). Genotypes of embryos were determined by Southern blot analysis on yolk sac DNA (Yang *et al.*, 1993, 1995).

Antibodies and Peptides

Hamster monoclonal antibodies 8B3 and 82D, recognizing α_V and β_3 integrin subunits, respectively, were generously provided by Drs. Gerber and Tonegawa (Massachusetts Institute of Technology, Cambridge, MA; Gerber *et al.*, unpublished observations). Polyclonal antibodies against various integrin subunits for immunoprecipitation and immunofluorescence experiments were α_5 (160; Hynes *et al.*, 1989), β_1 (363; Marcantonio and Hynes, 1988), α_V (Chemicon, Temecula, CA), α_3 (8-4; DiPersio *et al.*, 1995), β_3 (8275; a gift of Dr. Mark Ginsberg, Scripps Research Institute, La Jolla, CA), β_5 (a gift of Dr. Martin Hemler, Dana Farber Cancer Institute, Boston, MA; Ramaswamy and Hemler, 1990), and β_6 (206; a gift of Dr. Robert Pytela, Gladstone Research Foundation, San Francisco, CA). The anti-vinculin antibody was a monoclonal antibody (V4505) from Sigma. For immunohistochemistry, 8B3 and a monoclonal antibody against α_5 (MFR5; PharMingen, San Diego, CA) were used. The sequences for the RGD and RGE peptides are GRGDSP and GRGESP, respectively. The α_V-specific peptide (27R, Telios, San Diego, CA) was a generous gift of Dr. Erkki Ruoslahti (Burnham Institute, La Jolla, CA).

Adhesion Assay

Wells of 24-well plates were coated with 20 μg/ml mouse plasma FN (Life Technologies) in PBS by incubating at 37°C for 2 h. The wells were then washed one time with PBS and blocked overnight at 4°C with 2 mg/ml bovine serum albumin (BSA; heat-inactivated by incubating at 70°C for 1 h) in PBS. Embryonic cells were trypsinized and washed one time in serum-free medium (Hybridoma-SFM; Life Technologies). For the qualitative assay, 5×10^4 cells were preincubated for 1 h at room temperature with 0.5 ml of Hybridoma-SFM or supernatant of 8B3 or 82D hybridoma cells grown in Hybridoma-SFM. The cells in the same SFM or antibodies were then plated on the FN-coated wells and incubated in a tissue

culture incubator. After 1 h, the nonadherent cells were gently washed off the plate by submerging the plate in PBS and shaking off the cells. The plate was washed three times in this manner. The remaining adherent cells in the wells were fixed in methanol and stained with Giemsa (Sigma). The adherent cells were photographed (Ektachrome 160T film) with an Axiophot microscope (Carl Zeiss, Thornwood, NY). The quantitative assay was the same as described above except that the cells (1×10^4/well) were plated in wells of a 96-well plate, and, after the cells were plated for 1 h with or without the blocking reagents (1 mM of each peptide in the Hybridoma-SFM), the wells were washed only once, in the same manner as described above. The total numbers of adherent cells in each well were counted. Effects of the blocking reagents were quantitated as a percentage of cells that failed to be blocked for adhesion in the presence of the blocking reagents. The number of adherent cells without any blocking antibodies was used as the denominator. Two independent experiments were performed. The γδ T cell line (T3.13.1), a generous gift of Drs. Gerber and Tonegawa, was a hybridoma line derived by fusing primary murine γδ T cells with BW5147 cells (Pereira *et al.*, 1995).

Cell Migration Assay

The cell migration assay has been described previously (Yang *et al.*, 1993). Briefly, rat plasma FN was diluted in DMEM (10 μg/ml), and 35 μl were dispensed into blind-well portions of a micro chemotaxis chambers (NeuroProbe, Cabin John, MD). Membranes (8-μm pore size; NeuroProbe) were overlaid onto the wells, the chambers were assembled, and 50 μl of cell suspension (3×10^5 cells/ml in serum-free DME) was added to each top well of the assembly. Chambers were incubated for 4 h at 37°C, and the membranes were removed and fixed in methanol for 5–7 min, stained with Giemsa (Sigma), rinsed with water, and air dried. Cells that had migrated through the membrane were counted; 15 nonoverlapping high-power fields (200×) per well were counted, and the data were analyzed by the *t* test in Statworks (Cricket Software, Malvern, PA).

Surface Iodination and Immunoprecipitation

Cell surface labeling with ^{125}I was performed by the lactoperoxidase–glucose oxidase method (Hynes, 1973). Embryonic cells cultured in 6-cm plates were labeled with 500 μCi of Na [^{125}I] (DuPont NEN, Boston, MA) per plate, washed, and lysed with 2 ml of cold lysis buffer (50 mM tris(hydroxymethyl)aminomethane [Tris], pH 8.0, 150 mM NaCl, 0.5 mM $CaCl_2$, 0.5% NP-40, 2 mM phenylmethylsulfonyl fluoride [PMSF], 0.02 mg/ml aprotinin, and 0.0125 mg/ml leupeptin). Lysates were preabsorbed with 200 μl of protein A-Sepharose (Pharmacia LKB, Piscataway, NJ) for 1 h, the Sepharose beads were removed by centrifugation, and the resulting supernatants were used for immunoprecipitation.

For immunoprecipitation, 10 μl of 5% NP-40 and 90 μl of 10 mg/ml BSA (Boehringer Mannheim, Indianapolis, IN) in lysis buffer were mixed with 200 μl of cell lysate and 5 μl of antiserum. After 1 h at 4°C, 30 μl of protein A-Sepharose beads (a 1:1 slurry preabsorbed with 10 mg/ml BSA in lysis buffer) were added to each tube. After 1 h at 4°C, the beads were sedimented and washed four times with cold lysis buffer, followed by boiling in 40 μl of nonreducing SDS sample buffer. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis on 6.5% resolving gels with 3% stacking gels (Laemmli, 1970).

Immunofluorescence

For examining focal contacts, cells were cultured overnight in 1% fetal bovine serum or Hybridoma-SFM on coverslips coated with 10 μg/ml mouse plasma FN. The cells were then washed with PBS and fixed for 15 min in 4% paraformaldehyde (Fluka) in PBS, permeabilized for 15 min in 0.5% NP-40 (Sigma) in PBS, and incubated with a mixture of antibodies against vinculin (diluted 1:50; Sigma) and α_V integrin (diluted 1:100; Chemicon) at 37°C. After 30 min, the coverslips were washed three times in PBS, incubated at 37°C for 30 min with a mixture of fluorescein isothiocyanate (FITC) and rhodamine-conjugated secondary antibodies (diluted 1:500; Biosource International, Camarillo, CA), and washed three times in PBS. Both the primary and the secondary antibodies were diluted in 10% normal goat serum (Vector, Burlingame, CA) in PBS.

For examining FN matrix assembly in embryonic cells lacking both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins, the cells were cultured in embryonic cell culture medium overnight on coverslips coated with 0.1% gelatin (Swine skin type II; Sigma). The cells were processed and stained as described above with the use of an antiserum against rat plasma FN (61.1, prepared as described by Mautner and Hynes, 1977). For the antibody blocking experiment, the cells were cultured in Hybridoma-SFM with or without blocking antibodies, and 20 μg/ml mouse laminin (Collaborative, Bedford, MA/Becton Dickinson, San Jose, CA) was used to coat the coverslips.

Immunohistochemical Analysis of Embryos

Frozen sections were warmed to room temperature, and all of the following steps were done at room temperature. The sections were washed twice, 5 min each, in PBS containing 0.1% BSA, blocked with blocking buffer (0.1% BSA and 10% normal goat serum in PBS) for 30 min, and incubated for 1 h with 8B3 (biotinylated, diluted 1:100) or a monoclonal antibody against α_5 integrin (purified, diluted 1:500; PharMingen). For staining with the anti-α_5 integrin antibody, the sections were washed three times in PBS and incubated with biotinylated goat anti-rat immunoglobulin (Ig) G secondary antibody (Vector) diluted 1:200 in blocking buffer for 30 min. After three washes in PBS, the sections were incubated with 0.3% H_2O_2 in methanol for 30 min and washed again three times in PBS. The sections were then incubated with ABC reagent mix (Vector) for 30 min and DAB/H_2O_2 mix (DAB substrate kit, Vector) for 30 sec to 2 min, counterstained with 0.05% methylene blue, dehydrated in ethanol and xylene, and mounted with Permount (Fisher Scientific, Pittsburgh, PA).

RESULTS

$\alpha_4\beta_1$ Is Not Required for Mediating FN-dependent Functions in α_5-Null Cells

We have shown previously that in vitro cultured cells derived from α_5-null embryos were able to adhere to FN, form focal contacts and migrate on FN, and assemble FN matrix (Yang *et al.*, 1993). To examine whether $\alpha_4\beta_1$ is involved in mediating these functions in the α_5-null cells, we cultured cells lacking both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins from embryos carrying both the α_4 and α_5 knockout mutations. The embryonic cells derived from wild-type, $\alpha_4^{-/-}$, $\alpha_5^{-/-}$, or $\alpha_4^{-/-}$; $\alpha_5^{-/-}$ embryos were all able to adhere to FN. To examine focal contact formation, the cells were stained with antibodies against vinculin after plating on FN. The singly and doubly mutant cells formed focal contacts indistinguishable from those of wild-type cells (Figure 1).

Next, we examined the ability of the embryonic cells to migrate on FN by using a modified Boyden chamber assay (Yang *et al.*, 1993). The results are shown in Figure 2, which shows that the singly and doubly mutant cells migrate in a FN-dependent manner at least as well as the wild-type cells.

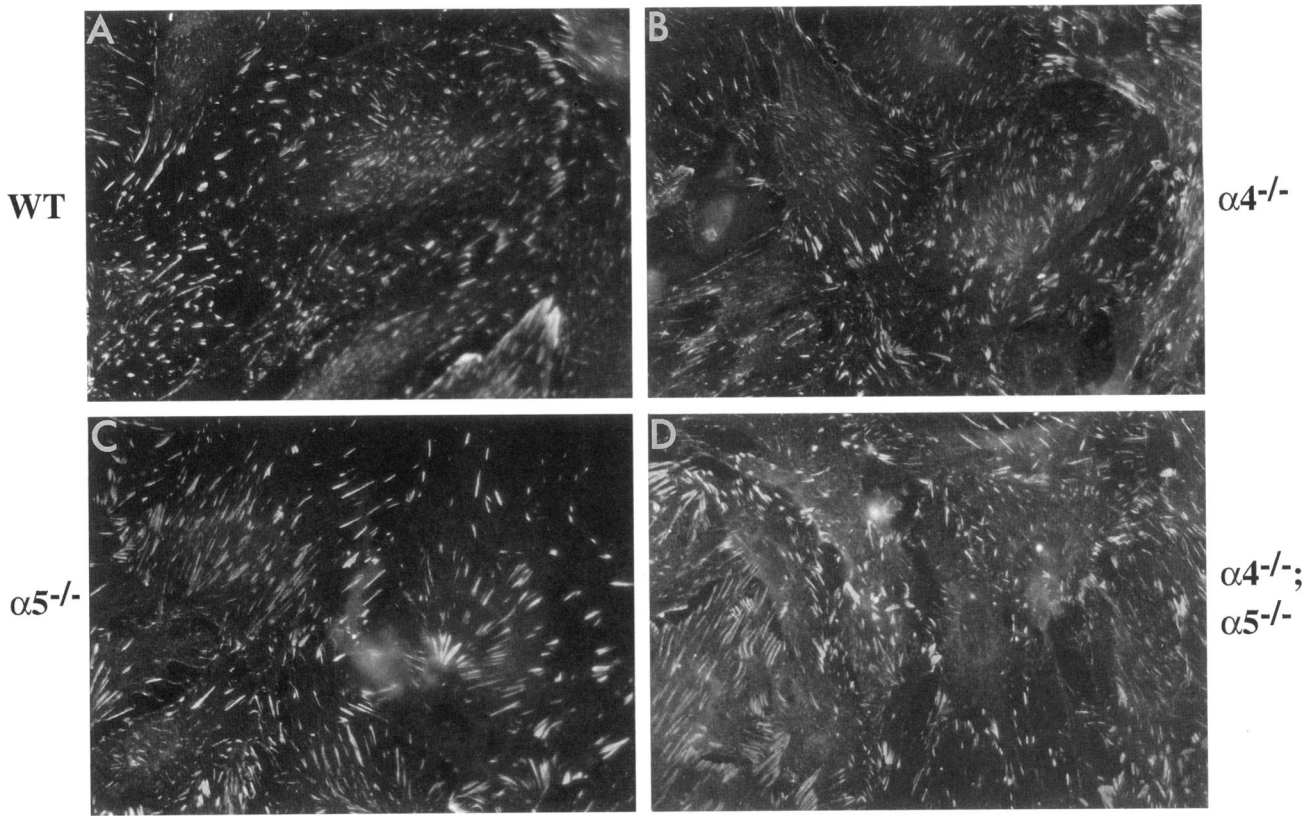

Figure 1. Immunofluorescence showing formation of focal contacts by cells derived from wild-type, $\alpha_4^{-/-}$, $\alpha_5^{-/-}$, or $\alpha_4^{-/-}$; $\alpha_5^{-/-}$ embryos. Cells derived from (A) wild-type, (B) $\alpha_4^{-/-}$, (C) $\alpha_5^{-/-}$, or (D) $\alpha_4^{-/-}$; $\alpha_5^{-/-}$ embryos were cultured on coverslips coated with FN and stained with an antibody against vinculin. Notice that the mutant cells formed focal contacts similar to those of the wild-type cells.

To examine whether cells lacking both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins are able to assemble FN matrix, we plated the embryonic cells derived from wild-type, $\alpha_4^{-/-}$, $\alpha_5^{-/-}$, or $\alpha_4^{-/-}$; $\alpha_5^{-/-}$ embryos on gelatin-coated coverslips and stained them with an antiserum against FN after 2 d of incubation. The matrices formed by the singly and doubly mutant cells were indistinguishable from those produced by wild-type cells (Figure 3).

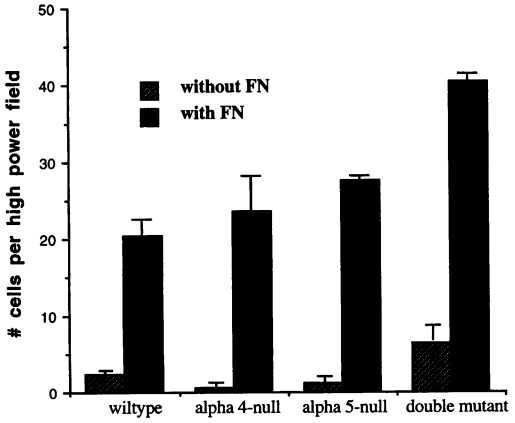

Figure 2. Migration of cells derived from wild-type, $\alpha_4^{-/-}$, $\alpha_5^{-/-}$, or $\alpha_4^{-/-}$; $\alpha_5^{-/-}$ embryos. The abilities of wild-type, $\alpha_4^{-/-}$, $\alpha_5^{-/-}$, or $\alpha_4^{-/-}$; $\alpha_5^{-/-}$ embryonic cells to migrate on FN were examined by the use of a modified Boyden chamber assay. Cells were tested in duplicate wells with 10 μg/ml of FN. The number of migrated cells per high power field (HPF, 200×) was counted. Fifteen nonoverlapping HPF were counted per well; the means and SD are shown.

These results show that various FN-dependent functions proceed normally in cells lacking either one or both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins. Therefore, overlapping functions between $\alpha_4\beta_1$ and $\alpha_5\beta_1$ or compensation for loss of $\alpha_5\beta_1$ by $\alpha_4\beta_1$ (or vice versa) do not explain the continued ability of these cells to respond to FN.

Involvement of $\alpha_5\beta_1$ and α_V Integrins in Adhesion of Embryonic Cells to FN

To examine whether α_V integrins are functional FN receptors in the α_5-null embryonic cells, we tested the effects of an α_V integrin-specific blocking antibody, 8B3, on adhesion and spreading of the α_5-null cells on FN. The results are shown in Figure 4. Without the blocking antibody, both the wild-type and α_5-null cells adhered and spread on FN equally well (Figure 4, A and B). When the anti-α_V blocking antibody was

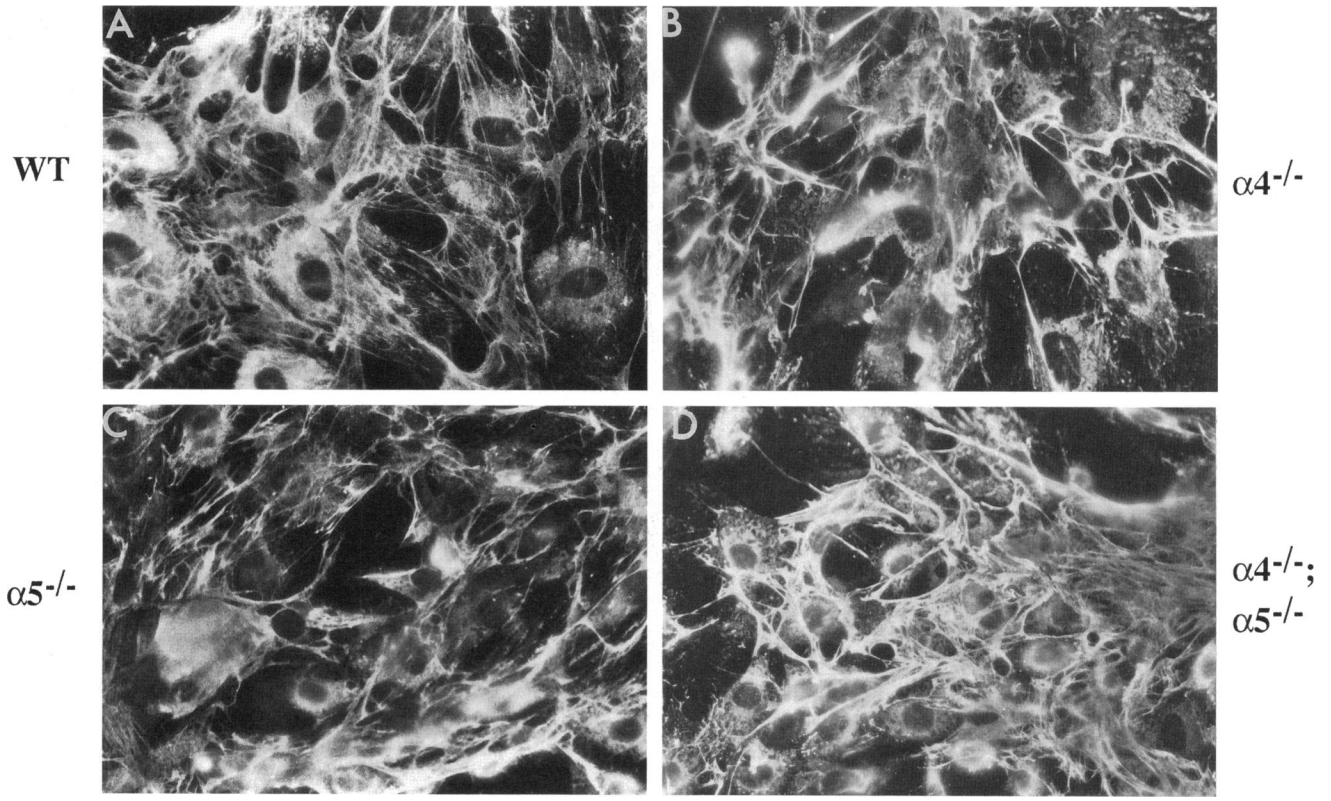

Figure 3. Immunofluorescence showing assembly of FN matrix by cells derived from wild-type, $\alpha_4^{-/-}$, $\alpha_5^{-/-}$, or $\alpha_4^{-/-}$; $\alpha_5^{-/-}$ embryos. Cells derived from (A) wild-type, (B) $\alpha_4^{-/-}$, (C) $\alpha_5^{-/-}$, or (D) $\alpha_4^{-/-}$; α_5 embryos were plated on gelatin-coated coverslips and stained with an antiserum against FN after 48 h of incubation. Notice that the FN matrix assembled by the mutant cells is similar to that formed by the wild-type cells.

added, adhesion and spreading of wild-type cells on FN were hardly affected (Figure 4C); on the other hand, the majority of the α_5-null cells failed to adhere to FN, and those α_5-null cells that did adhere failed to spread (Figure 4D). We also tested the effect of a β_3-specific blocking antibody, 82D, on adhesion and spreading of wild-type and α_5-null cell on FN. 82D was of the same isotype as 8B3 and, as a control, was able to block adhesion of cultured $\gamma\delta$ T cells to vitronectin (Figure 4, G and H). However, 82D did not block adhesion and spreading of either wild-type or α_5-null cells on FN (Figure 4, E and F).

We also examined the effects of 8B3 and 82D and several peptides, including RGD and an α_V-specific blocking peptide (27R), on adhesion of wild-type and α_5-null cells to FN in a quantitative adhesion assay (Figure 5). 27R peptide almost completely abolished adhesion of α_5-null cells to FN, although it had a much weaker effect on the wild-type cells. 8B3 (anti-α_V) blocked 80% of the adhesion of α_5-null cells to FN, although it had little effect on the wild-type cells. On the other hand, the antibody isotype control 82D (anti-β_3) did not have any blocking effects. Finally, RGD peptide blocked adhesion of both wild-type and α_5-null cells to FN, although the control RGE peptide did not (Figure 5). These results showed that disruption of function of both α_5 and α_V was required to block the ability of wild-type embryonic cells to adhere to and spread on FN.

Involvement of $\alpha_5\beta_1$ and α_V Integrins in FN Matrix Assembly

We also tested the effects of 8B3 and 82D on FN matrix assembly in wild-type and α_5-null cells. Wild-type and α_5-null cells were plated on laminin-coated coverslips in the presence of the blocking antibodies and stained with an antiserum against fibronectin after overnight incubation. Without blocking antibodies, the wild-type and α_5-null cells were able to assemble FN matrix equally well (Figure 6, A and B). In the presence of 8B3, matrices formed by α_5-null cells were significantly reduced (Figure 6D); matrices formed by wild-type cells were also reduced, but to a lesser extent (Figure 6C). 82D (anti-β_3) did not have any effects on the ability

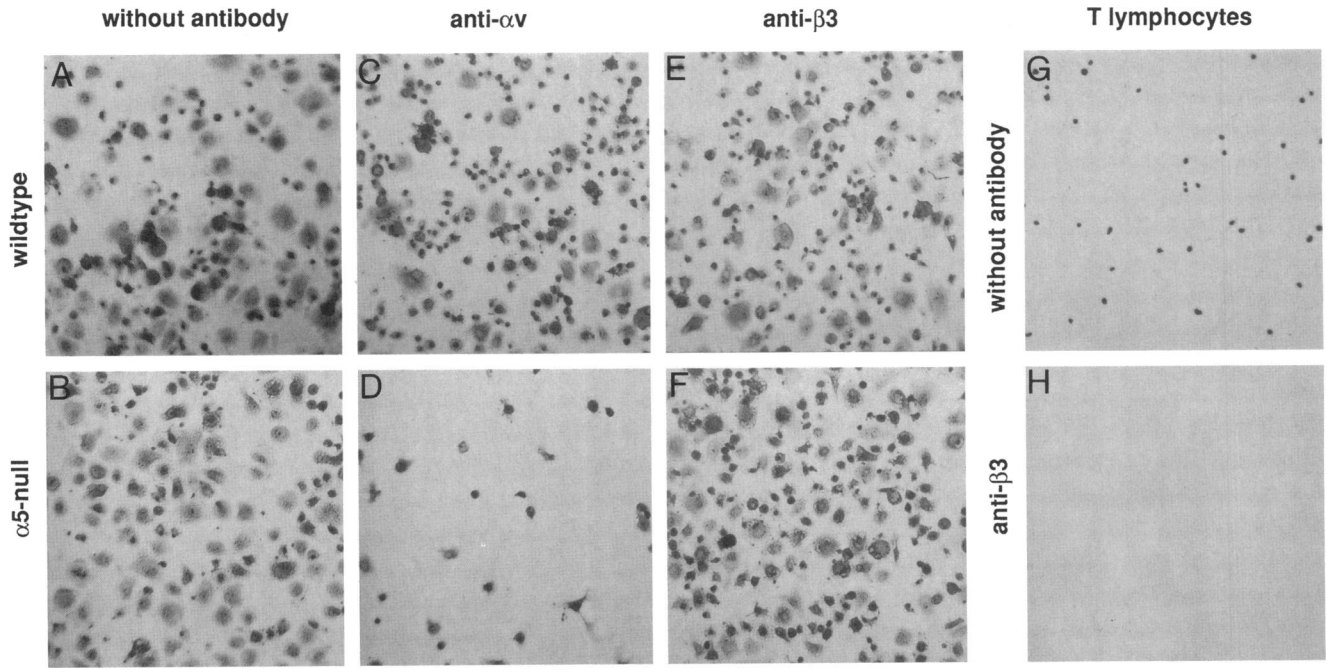

Figure 4. Effects of an anti-α_V integrin antibody on adhesion and spreading of embryonic cells on FN. Cells derived from wild-type (A, C, and E) or α_5-null (B, D, and F) embryos were plated on FN with serum-free medium (A and B), an anti-α_V integrin antibody 8B3 (C and D), or an anti-β_3 integrin antibody 82D, which has the same isotype as 8B3 (E and F). Notice that 8B3 blocked adhesion and spreading of the α_5-null cells but not the wild-type cells on FN, whereas the serum-free medium and 82D had no effect. Cultured $\gamma\delta$ T cells were plated on vitronectin without (G) or with (H) 82D to confirm the ability of this antibody to block β_3 integrin functions.

of either wild-type or α_5-null cells to form FN matrix (Figure 6, E and F).

The Expression of α_V Integrins on the Surfaces of α_5-Null Cells Is Not Up-regulated

We compared the expression of α_V integrins, including $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$ and $\alpha_V\beta_6$, on the surfaces of wild-type and α_5-null cells. Wild-type and α_5-null cells were surface iodinated and lysed; equal amounts of lysates were immunoprecipitated with antibodies against α_5, α_V, β_1, β_3, β_5, and β_6 (Figure 7). The results showed that, whereas α_5 was expressed on the surfaces of wild-type, but not α_5-null cells (Figure 7A), the expression levels of α_V and the associated β_1, β_3, and β_5 subunits on the surfaces of wild-type and α_5-null cells were not significantly different (Figure 7, A–C), and β_6 was not expressed on the surface of either cell type (Figure 7C). Immunoprecipitation with the anti-α_V antibody also showed that the expression of $\alpha_V\beta_1$ appeared much higher than that of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ (Figure 7, B and C).

Recruitment of α_V Integrins into Focal Contacts Is Increased in α_5-Null Cells Plated on FN

We examined the distribution of α_V integrins on the surfaces of embryonic cells. Wild-type and α_5-null cells were plated on FN, and the expression of α_3, α_5, and α_V in focal contacts was examined by immunofluorescence staining with the use of antibod-

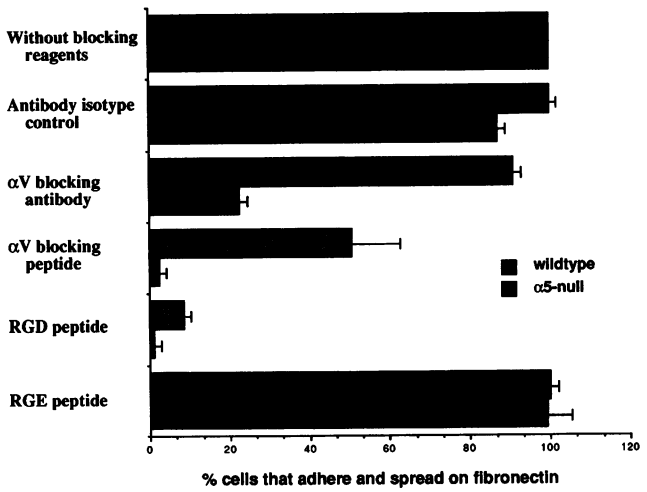

Figure 5. Involvement of $\alpha_5\beta_1$ and α_V integrins in FN-mediated adhesion and spreading. Histogram showing the effects of blocking reagents on adhesion of embryonic cells to FN. The percentages of cells that adhered and spread are mean values of two independent experiments. Notice that an anti-α_V integrin antibody and an α_V-specific blocking peptide both inhibited adhesion of α_5-null cells, but not the wild-type cells, to FN. An RGD peptide that blocks both $\alpha_5\beta_1$ and α_V integrins blocks adhesion of both cell types.

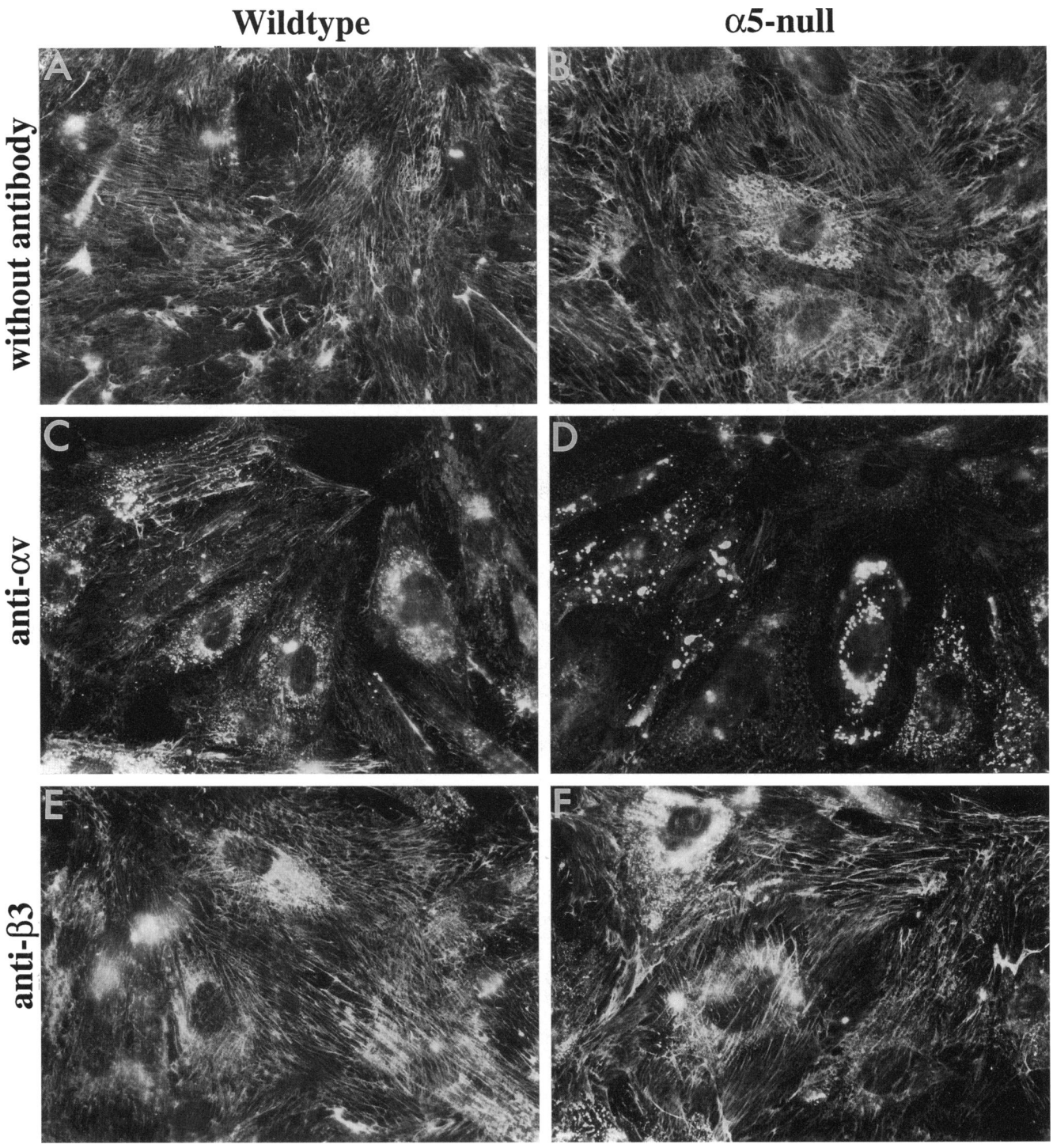

Figure 6. Effects of the anti-α_V integrin antibody on FN matrix assembly. Cells derived from wild-type (A, C, and E) or α_5-null (B, D, and F) E9.25 embryos were plated on laminin-coated coverslips with serum-free medium (A and B), an anti-α_V integrin antibody 8B3 (C and D), or an anti-β_3 integrin antibody 82D (E and F). After overnight incubation, the cells were stained with an antiserum against rat plasma FN. Notice that 8B3 inhibited FN matrix assembly in both wild-type and α_5-null cells, but the inhibitory effect on α_5-null cells was stronger, whereas the serum-free medium and 82D had no effect.

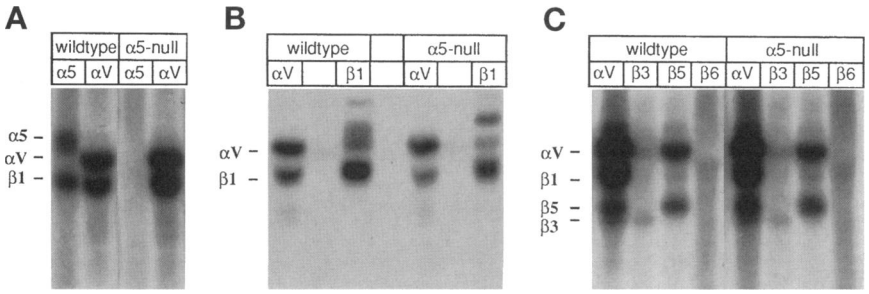

Figure 7. Expression of α_V integrins on cultured embryonic cells. Immunoprecipitation from lysates of wild-type and α_5-null cells with antibodies against (A) α_5 and α_V, (B) α_V and β_1, and (C) α_V, β_3, β_5, and β_6. Panel C was overexposed to reveal the lower levels of β_3 and β_5. Notice that the expression levels of all α_V integrins are similar on wild-type and α_5-null cells.

ies against these integrins and vinculin, a cytoskeletal protein localized in focal contacts (Figure 8). Interestingly, although the antibody against α_3 stained focal contacts of wild-type and α_5-null cells at a similar intensity (Figure 8, C and D), the staining patterns of the antibody against α_V in the wild-type and α_5-null cells were dramatically different (Figure 8, E and F). In the wild-type cells, the staining by anti-α_V antibody was diffuse on the cell surface and was very weak in the focal contacts. In contrast, in the α_5-null cells, the staining by the anti-α_V antibody in focal contacts was much stronger, indicating that recruitment of α_V integrins into focal contacts is increased in α_5-null cells plated on FN. On the other hand, the antibody against vinculin, in a double-label immunofluorescence staining with anti-α_V (Figure 8, E and F), stained focal contacts equally well in the wild-type and α_5-null cells (Figure 8, G and H). Finally, the antibody against α_5 stained the focal contacts in the wild-type cells but not those in the α_5-null cells (Figure 8, A and B).

$\alpha_5\beta_1$ and α_V Integrins Are Partially Colocalized in E9.5 Embryos

To test whether the involvement of α_V integrins in mediating the interactions of embryonic cells with FN might be relevant in vivo, we examined the expression of α_5 and α_V in E9.5 embryos by immunohistochemical studies. The results showed that $\alpha_5\beta_1$ and α_V integrins were both expressed in the heart (Figure 9, A and B) and somites (Figure 9, C and D) of E9.5 embryos. Our unpublished observations have suggested that the expression patterns of $\alpha_5\beta_1$ and α_V integrins in the anterior and posterior somites are similar.

DISCUSSION

α_V Integrins, but not $\alpha_4\beta_1$, Are Required for Adhesion and Spreading of α_5-Null Embryonic Cells on FN

We have previously shown that cells derived from α_5-null embryos are able to adhere to FN, form focal contacts, and migrate on FN, suggesting that there must be (an)other FN receptor(s) involved in these processes (Yang *et al.*, 1993). In this study, we show that cells lacking *both* $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins are also able to perform these FN-dependent functions, indicating that $\alpha_4\beta_1$ is not required for performing these functions in embryonic cells lacking $\alpha_5\beta_1$. We also show that an α_V-specific antibody and an α_V-specific peptide are able to block cell adhesion and spreading of α_5-null cells, but not wild-type cells, on FN. Because two different α_V-specific blocking reagents (a monoclonal antibody and an oligopeptide) give similar results, the blocking effects seem specific. These results indicate that, in the absence of $\alpha_5\beta_1$, α_V integrins are required to mediate adhesion and spreading of the embryonic cells on FN. These results are consistent with an observation by Marshall *et al.* (1995) that antibodies against α_5 and α_V were both required to abrogate binding of an $\alpha_V\beta_1$-expressing human melanoma line to FN.

It is not yet absolutely clear which α_V integrins function as FN receptors in the α_5-null embryonic cells. We showed that a β_3-specific blocking antibody failed to block adhesion of α_5-null cells to FN. Unfortunately, because we have not able to obtain an effective function-blocking antibody against mouse β_1, we cannot conclude that $\alpha_V\beta_3$ is not involved, because our data do not rule out the possibility that $\alpha_V\beta_1$ and $\alpha_V\beta_3$ are both functional FN receptors in the α_5-null cells. Wennerberg *et al.* (1996) have reported that $\alpha_V\beta_3$ can mediate FN-dependent adhesion in β_1-null cells. In addition, it is not clear whether $\alpha_V\beta_5$, which is also expressed in the embryonic cells, is involved in mediating any embryonic functions of FN. Nevertheless, we show that $\alpha_V\beta_1$ is the predominant α_V integrin expressed in the embryonic cells (Figure 7), suggesting that $\alpha_V\beta_1$ is likely to play the major role in cell adhesion and spreading in the α_5-null cells.

Functions of $\alpha_5\beta_1$ Can Be Compensated by α_V Integrins in α_5-Null Cells

Functional replacement of $\alpha_5\beta_1$ in α_5-null cells could be a result of a *functional overlap* between $\alpha_5\beta_1$ and the α_V integrins or *compensation* by up-regulation (of level

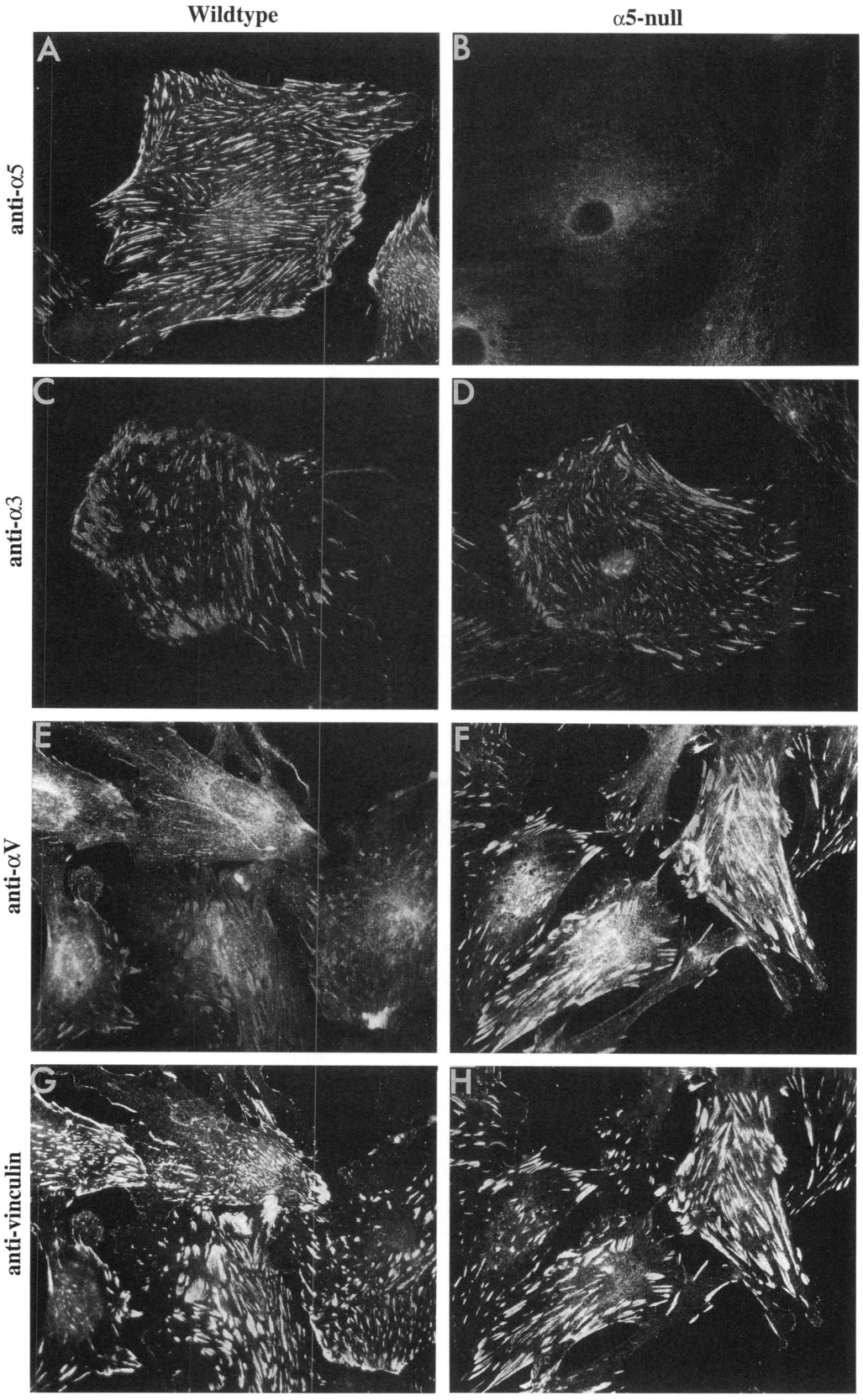

Figure 8. α_V integrins in focal contacts. Shown is immunofluorescence staining of wild-type (A, C, E, and G) and α_5-null cells (B, D, F, and H) plated on FN with antibodies against α_5 (A and B), α_3 (C and D), α_V (E and F), and vinculin (G and H). In E–H, cells were double stained with anti-α_V and anti-vinculin. Notice that the staining of anti-α_V in the focal contacts of α_5-null cells is much stronger than that in the focal contacts of wild-type cells.

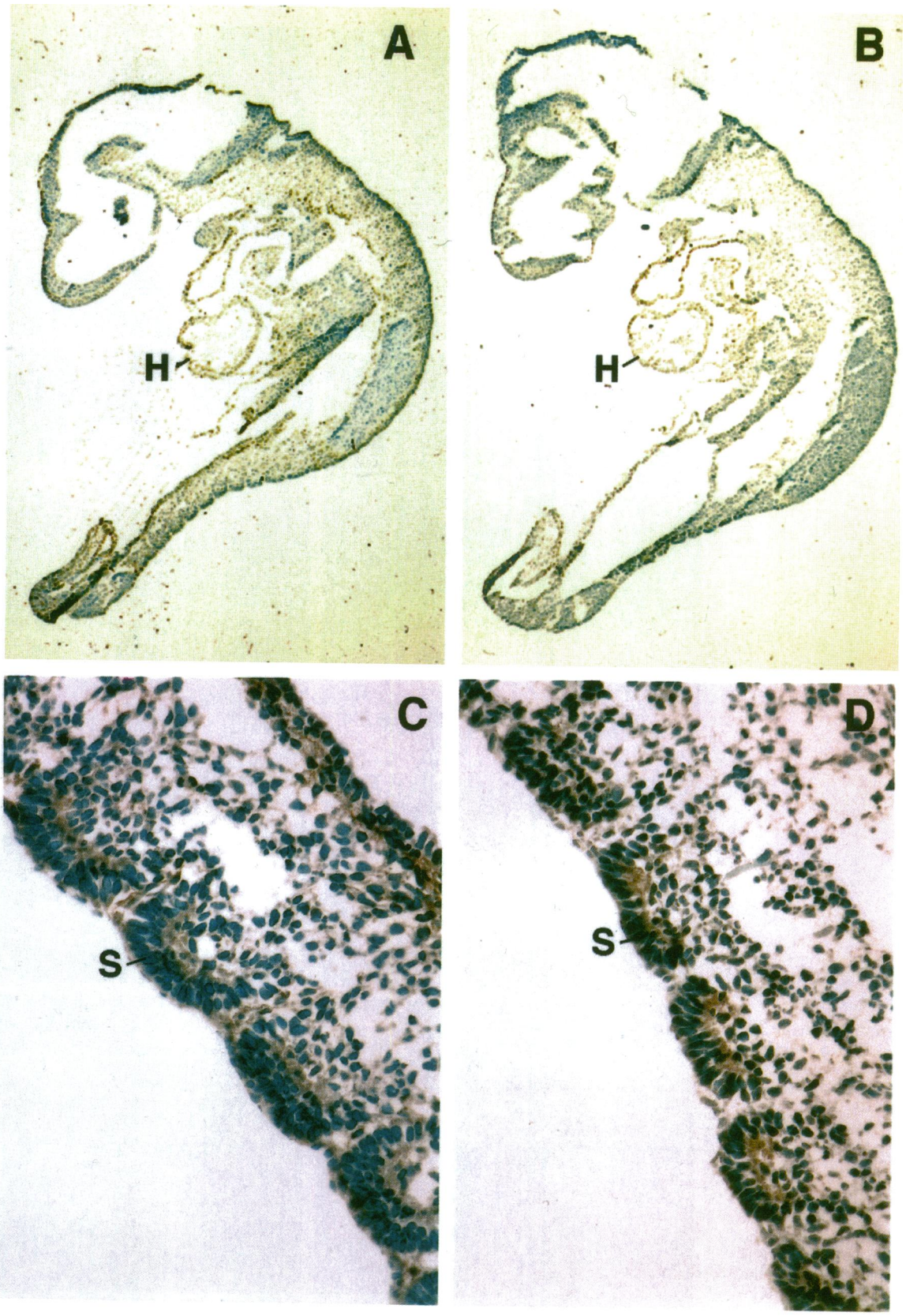

Figure 9.

or function) of α_V integrins in α_5-null cells. Surface iodination and immunoprecipitation experiments on wild-type and α_5-null cells show that α_V integrins are *not* up-regulated at the level of expression on the cell surface (Figure 7). However, we have shown by immunofluorescence that, when the cells are plated on FN, the distributions of α_V integrins on the surfaces of wild-type and α_5-null cells are dramatically different. In the wild-type cells, α_V is diffuse on the surface with very weak localization in the focal contacts, whereas in α_5-null cells the recruitment of α_V into the focal contacts is increased (Figure 8). We have previously reported that, when embryonic cells are plated on FN, α_5 and β_1 are both in the focal contacts of wild-type cells, and β_1 is in focal contacts of α_5-null cells (Yang et at., 1993). One interpretation of the results is that, in wild-type cells, $\alpha_5\beta_1$ plays the major role in mediating FN-dependent functions of these cells, and α_V integrins are largely excluded, perhaps by competition. When $\alpha_5\beta_1$ is absent, α_V integrins, most likely $\alpha_V\beta_1$, are engaged with FN, recruited into the focal contacts, and take over the role of $\alpha_5\beta_1$. A role for α_V β_1 as a FN receptor is somewhat unexpected. It has been reported by Zhang *et al.* (1993) that when $\alpha_V\beta_1$ is expressed in CHO-B2 cells that lack $\alpha_5\beta_1$, $\alpha_V\beta_1$ is distributed diffusely over the cell surface. In contrast with our results, Zhang *et al.* (1993) did not observe concentration of $\alpha_V\beta_1$ in focal contacts in cells lacking $\alpha_5\beta_1$. Because Zhang *et al.* (1993) have reported that the $\alpha_V\beta_1$-expressing cells had lower affinity for FN than those expressing $\alpha_5\beta_1$, it is possible that in our wild-type cells α_V integrins are in a low-affinity state and fail to engage with FN and move into focal contacts; however, in the α_5-null cells, α_V integrins are activated to a higher affinity state and are able to bind to FN and move into focal contacts to compensate the functions of $\alpha_5\beta_1$. On the other hand, $\alpha_V\beta_1$ in CHO-B2 cells may fail to be activated. In conclusion, our results indicate that α_V integrins ($\alpha_V\beta_1$ and perhaps $\alpha_V\beta_3$ and/or $\alpha_V\beta_5$) can compensate functionally for the absence of $\alpha_5\beta_1$ in the focal contacts and that, when $\alpha_5\beta_1$ is present, there is little or no overlap in function between $\alpha_5\beta_1$ and α_V integrins in the focal contacts.

α_V Integrins Are Required for FN Matrix Assembly in Embryonic Cells

We show that neither $\alpha_4\beta_1$ nor $\alpha_5\beta_1$ is required for FN matrix assembly in embryonic cells; on the other hand, α_V integrins are required in this process. Unlike cell adhesion, FN matrix assembly was inhibited by the α_V-specific antibody in both wild-type and α_5-null cells, although the inhibitory effects on the mutant cells were much stronger than those on the wild-type cells. Perhaps α_V integrins have multiple roles for FN matrix assembly in the embryonic cells: a role of their own and a role in replacing the functions of $\alpha_5\beta_1$ when $\alpha_5\beta_1$ is absent from the cells. The replacement of $\alpha_5\beta_1$ could be a result of a functional overlap between $\alpha_5\beta_1$ and α_V integrins or functional compensation by α_V integrins when $\alpha_5\beta_1$ is absent. Our results on FN matrix assembly are consistent with two reports showing that integrins other than $\alpha_5\beta_1$ can mediate FN matrix assembly. Wu *et al.* (1995) showed that $\alpha_{IIb}\beta_3$ expressed in CHO cells can mediate FN matrix assembly, and Wennerberg *et al.* (1996) showed that $\alpha_V\beta_3$ expressed in β_1-null cells can also do so. However, Wennerberg *et al.* (1996) observed that $\alpha_V\beta_3$ seemed to play little role in FN matrix interactions when β_1 integrins were present but became involved when they were absent, whereas we have observed that disruption of α_V functions had inhibitory effects when $\alpha_5\beta_1$ was present in the embryonic cells, suggesting that α_V integrins do play a role in cells expressing β_1 integrins. The discrepancy may be explained by the fact that different cells were used in these studies.

$\alpha_5\beta_1$ and α_V Integrins as Potential FN Receptors during Mouse Embryogenesis

Our observation that α_V integrins can replace $\alpha_5\beta_1$ functions in α_5-null embryonic cells in culture suggests that α_V integrins might also be functional FN receptors in α_5-null embryos. Such a function could account for the less severe embryonic defects of α_5-null embryos, as compared with those of FN-null embryos. There are three possible mechanisms by which $\alpha_5\beta_1$ and α_V integrins could be involved in embryogenesis. First, $\alpha_5\beta_1$ and α_V integrins may each have *independent* embryonic functions and mediate, respectively, subsets of FN functions; second, $\alpha_5\beta_1$ and α_V integrins may have partially *overlapping* embryonic functions; and third, $\alpha_5\beta_1$ may play a major role in embryonic development, and α_V integrins may be up-regulated (in level or function) in the absence of $\alpha_5\beta_1$ to *compensate* for the loss of $\alpha_5\beta_1$ in α_5-null embryos. Because our studies on embryonic cells have suggested that the functions of $\alpha_5\beta_1$ in the focal contacts of α_5-null cells can be replaced by α_V integrins mainly through a compensation mechanism, the same mechanism may also apply in vivo. However, our data do not exclude functional overlap between $\alpha_5\beta_1$ and α_V integrins in embryonic development. Because $\alpha_5\beta_1$ and α_V integrins colocalize in the heart and somites of E9.5 embryos, where the defects resulting from the FN knockout are more severe than in the α_5 integrin knockout, α_V integrins may replace the functions of

Figure 9 (on facing page). $\alpha_5\beta_1$ and α_V integrins in embryonic tissues. Shown is immunohistochemical staining of sections of E9.5 wild-type embryos with antibodies against α_5 (A and C) and α_V integrins (B and D). (C and D) The trunk region of the embryos. Notice that α_5 and α_V colocalize in the heart (A and B) and somites (C and D).

$\alpha_5\beta_1$ in cardiac development and somitogenesis in α_5-null embryos through either functional overlap or compensation or both. One unresolved problem is why the functions of $\alpha_5\beta_1$ during embryogenesis were not completely rescued by other FN receptors. It seems that the other FN receptors, perhaps α_V integrins, can replace the functions of $\alpha_5\beta_1$ in the anterior, but not in the posterior, region of the α_5-null embryos. Finally, to test whether α_V integrins are indeed able to replace the functions of $\alpha_5\beta_1$ in vivo, it will be necessary to examine the consequence of knocking out both α_5 and α_V integrin genes during mouse embryogenesis.

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