

Lack of $\beta 1$ Integrin Gene in Embryonic Stem Cells Affects Morphology, Adhesion, and Migration But Not Integration into the Inner Cell Mass of Blastocysts

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Abstract. A gene trap-type targeting vector was designed to inactivate the $\beta 1$ integrin gene in embryonic stem (ES) cells. Using this vector more than 50% of the ES cell clones acquired a disruption in the $\beta 1$ integrin gene and a single clone was mutated in both alleles. The homozygous mutant did not produce $\beta 1$ integrin mRNA or protein, while $\alpha 3$, $\alpha 5$, and $\alpha 6$ integrin subunits were transcribed but not detectable on the cell surface. Heterozygous mutants showed reduced $\beta 1$ expression and surface localization of $\alpha/\beta 1$ heterodimers. The αV subunit expression was not impaired on any of the mutants. Homozygous ES cell mutants lacked adhesiveness for laminin and fibronectin but not for vitronectin and showed a reduced asso-

ciation with a fibroblast feeder layer. Furthermore, they did not migrate towards chemoattractants in fibroblast medium. None of these functions were impaired in heterozygous mutants. Scanning electron microscopy revealed that homozygous cells showed fewer cell-cell junctions and had many microvilli not usually found on wild type and heterozygous cells. This profound change in cell shape is not associated with gross alterations in the expression and distribution of cytoskeletal components. Unexpectedly, microinjection into blastocysts demonstrated full integration of homozygous and heterozygous mutants into the inner cell mass. This will allow studies of the consequences of $\beta 1$ integrin deficiency in several in vivo situations.

INTERACTIONS of cells with the extracellular matrix and neighboring cells play an important role in a number of biological processes including cell migration, morphogenesis, growth control, and tissue repair (Ekblom et al., 1986). These interactions are mediated largely by the integrin family of cell surface adhesion receptors, which consist of α and β subunits. Each subunit possesses a small cytoplasmic domain, a single transmembrane spanning region, and a large extracellular domain (Hynes, 1992). The $\beta 1$ subunit can be associated with at least 10 different α subunits and thus forms the largest subfamily of integrins. A further level of complexity is added to the $\beta 1$ integrins by the existence of several isoforms with alternatively spliced cytoplasmic domains (Altruda et al., 1990; Languino and Ruoslahti, 1992; Tamura et al., 1991; Hogervorst et al., 1991; Cooper et al., 1991). Antibodies against the $\beta 1$ subunit revealed its presence on virtually all mammalian and avian cell types including unfertilized oocytes (Tarone et al., 1993). This emphasizes a central role for $\beta 1$ integrins during development.

The members of the $\beta 1$ integrin subfamily bind with their

extracellular domain to several matrix proteins such as laminins, collagens, and fibronectin (Hynes, 1992). Such interactions can directly influence cellular events including gene induction, migration, and differentiation. This was demonstrated in many antibody perturbation experiments, which altered adhesion to matrix molecules (Neff et al., 1982) and cell morphology (Greves and Gottlieb, 1982) and blocked cell migration (Bronner-Fraser, 1986), neurite outgrowth (Tomaselli et al., 1986) and cell differentiation (Menko and Boettiger, 1987; Adams and Watt, 1989; Sorokin et al., 1990). The importance of $\beta 1$ integrins in embryonic development has also been documented in a recent knockout of the $\alpha 5$ integrin gene in mice, which caused pronounced mesodermal defects and early death (Yang et al., 1993).

The cytoplasmic domain of the $\beta 1$ subunit can interact with cytoskeletal components such as talin (Horwitz et al., 1986) and α -actinin (Otey et al., 1989) and thereby links the outside of the cell with the inside. Such interactions are accompanied by the reorganization of the actin cytoskeleton and the formation of focal contacts (Burridge et al., 1988). Focal contacts contain structural proteins such as vinculin, talin, and α -actinin, enzymatic regulatory molecules including protein tyrosine kinase pp125^{FAK} (focal adhesion kinase) and protein kinase C and the phosphoprotein paxillin (Sastry

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and Horwitz, 1993). The assembly of these complexes is considered to trigger unknown signal cascades which eventually alter gene expression (Hynes, 1992; Damsky and Werb, 1992).

The high abundance of $\beta 1$ integrins has so far hampered several functional and biological studies on the role of the $\beta 1$ subunit, for example by cell transfection. As could have been predicted from the $\alpha 5$ gene knockout (Yang et al., 1993), a similar knockout of the $\beta 1$ gene caused lethality at an early embryonic stage (Fässler, R., manuscript in preparation). To overcome some of these difficulties, a knockout of the $\beta 1$ gene was recently produced in F9 carcinoma cells and resulted in strongly impaired cell adhesion and migration (Stephens et al., 1993). Since such teratocarcinoma cells are not useful for *in vivo* studies, we have now established a mouse embryonic stem (ES)¹ cell clone with two mutated $\beta 1$ integrin alleles. As a consequence of this mutation, no $\beta 1$ integrin is expressed on these cells. The deficiency profoundly affected cell adhesion and migration and led to an alteration in cell morphology which, however, was not accompanied by significant changes in the distribution of cytoskeletal proteins. Most importantly, the mutant cells still had the potential for integration into the inner cell mass of blastocysts, which opens many interesting prospects for *in vivo* studies.

Materials and Methods

Construction of the Targeting Vector

A 5' mouse $\beta 1$ integrin cDNA probe was provided by R. O. Hynes (MIT, Cambridge, MA) and used to isolate several overlapping cosmid clones from a mouse genomic library from 129/sv strain kindly supplied by J. S. Mudgett (Merck, Rahway, NJ). A genomic 10-kb fragment starting at the first EcoRV site 5' to and ending at the first BamHI 3' to the second exon was produced (see Fig. 1) and subcloned into Bluescript KS- (Stratagene Corp., La Jolla, CA) lacking the HindIII site in the polylinker (construct #1). Subsequently, exon 2 was localized to a 0.8-kb EcoRI/XbaI fragment by Southern analysis, subcloned into Bluescript KS- and sequenced. This exon begins with the ATG start codon and single SspI and BstEII sites were located upstream and one XbaI site downstream of the intron/exon boundary.

PCR was used to insert a promoterless β -galactosidase-neomycin fusion DNA (geo) cassette (provided by P. Soriano, Baylor College, Houston, TX, Friedrich and Soriano, 1991) containing the poly A signal from pMCineopA (Stratagene Corp.) in frame with the $\beta 1$ integrin start codon. Two oligonucleotide primers (5'-ATTTTCTCTATCAATAATATACATTTTCTGTTATAGATGGCGGATCCGTCGAC-3' and 5'-GGAGGTGCCCTCTAGAA-3') were used to amplify a 235 bp fragment of the 0.8-kb EcoRI/XbaI fragment. The first primer begins at the SspI site and contains a BamHI and a Sall site downstream of the ATG. The second primer ends at the XbaI site. The PCR product was digested with XbaI and cloned together with the genomic EcoRI/SspI fragment into a modified Bluescript KS- vector lacking the Sall site in the polylinker (construct #3). After confirming the integrity of the PCR product by sequence analysis, the DNA was linearized with BamHI to insert the BamHI fragment of the β -galactosidase gene (construct #4). The resulting construct was cleaved with ClaI in the inserted β -galactosidase gene and with Sall in order to insert the remaining geo DNA (construct #5). The resulting construct was cleaved with BstEII and NotI to insert the EcoRV/BstEII fragment which was released from construct #1 by BstEII/NotI digestion (construct #6). The final targeting vector pKOgeo21 was constructed in a trimolecular ligation reaction, using construct #1 cleaved with HindIII and NotI (7.4 kb), construct #1 cleaved with HindIII and XbaI (1.8 kb), and construct #6 cleaved with XbaI and NotI (7.2 kb). This ligation reaction was necessary because of the multiple XbaI sites present in construct #1.

1. Abbreviation used in this paper: ES, embryonic stem.

Gene Targeting

Embryonic stem cells D3 (Doetschman et al., 1985) were provided by R. Jaenisch (Whitehead Institute, Cambridge, MA) and grown on a feeder layer of γ -irradiated embryonic fibroblast cells in DME supplemented with 15% heat-inactivated fetal calf serum (GIBCO BRL, Gaithersburg, MD), 0.1 mM β -mercaptoethanol (Sigma Chemical Co., St. Louis, MO) and nonessential amino acids (GIBCO BRL). The embryonic feeder cells were prepared from COL9A1-deficient mice (Fässler et al., 1994) and γ -irradiated (1,500 rad for 30 min) before plating.

About 1×10^8 D3 ES cells were electroporated with 150 μ g of NotI linearized targeting vector using a Bio-Rad Gene Pulser set at 800 V and 3 μ F. After 24 h without selection, G418 (300 μ g/ml; GIBCO BRL) was added to the culture medium. After another 9–11 d, colonies were picked into 24-well dishes (Costar Corp., Danvers, MA) and expanded. Half of each well was frozen and the other half was used to isolate DNA for Southern blot analysis (Laird et al., 1991). The colonies were screened individually by cleaving genomic DNA with BamHI and probing the Southern blots with the external probe A, a 800-bp BamHI/EcoRV fragment which consists of an intron sequence (see Fig. 1).

Establishment of Differentiated $\beta 1$ Integrin-deficient Cell Lines

To establish differentiated, $\beta 1$ integrin-deficient cell lines ES cell clone G-201 was seeded at a density of 10^5 cells/ml on a tissue culture dish in DME containing 10% FCS and 1% (vol/vol) DMSO. After 6 d in culture differentiated cells were infected at 37°C for 2 h with recombinant retroviruses that transduced the SV-40 large T (Jat et al., 1986; a gift from P. A. Sharp, Massachusetts Institute of Technology, Cambridge, MA). After infection cells were further incubated for approximately 14 d in DME supplemented with 10% FCS. Non-infected cells died during this incubation period, infected cells formed clones which were isolated by ring cloning.

Northern Blot Analysis

PolyA + RNA was isolated from wild type, heterozygous, and homozygous ES cells. Cells were grown in two T175 flasks, harvested, and washed once in ice cold PBS (10 mM phosphate buffer, pH 7.3, 0.15 M NaCl, 0.2 mM EDTA). 20 ml of RNA proteinase K buffer (20 mM Tris-HCl, pH 7.4, 0.5% SDS, 0.1 M NaCl, 1 mM EDTA, 200 μ g/ml proteinase K) were added to cell pellets and DNA was sheared by treating cells with a polytron for 1 min. 1 ml of oligo-dT cellulose (Pharmacia Fine Chemicals, Piscataway, NJ) was suspended (vol/vol) and equilibrated for 30 min in high salt buffer (10 mM Tris-HCl, pH 7.4, 0.4 M NaCl, 1 mM EDTA, 0.1% SDS) and then added to the cells and incubated on a rocking platform for 1 h at room temperature. Polyadenylated RNA bound to the oligo-dT cellulose was washed three times in high salt buffer and subsequently put on an Econo column (Pharmacia Fine Chemicals). After three additional washings of the oligo-dT with high salt buffer, polyadenylated RNA was eluted with 1 ml of RNase-free water.

For Northern analysis, 2 μ g of polyadenylated RNA was electrophoretically separated, blotted onto Zeta Probe membrane (Bio-Rad Laboratories, Cambridge, MA), UV cross-linked and probed in Church buffer (Church and Gilbert, 1984) at 65°C. Filters were washed twice in 0.2 \times SCC/1% SDS at 65°C and exposed to an x-ray film for 24 or 72 h at -80°C. The following oligolabeled probes were used: mouse $\beta 1$ integrin cDNA, mouse cDNA for $\alpha 5$ (Holers et al., 1989) and $\alpha 6$ integrins (Hierck et al., 1993), human cDNA for αV (Suzuki et al., 1987) and $\beta 3$ integrins (Fitzgerald et al., 1987), human cDNA for $\alpha 3$ integrin (Takada et al., 1990), and mouse cDNA for β -actin obtained from M. Stürzl (Max-Planck-Institut Biochemie, Martinsried, Germany).

Antibodies

Antibodies against integrins were obtained from the following sources: rabbit anti-rat $\beta 1$ (Bottger et al., 1989) rabbit anti-rat $\beta 3$ from A. Oldberg (Karolinska Institute, Stockholm, Sweden); rabbit anti- $\beta 4$ cytoplasmic domain (P120) and rat monoclonal anti-human $\alpha 6$ (GoH3) from A. Sonnenberg (Netherlands Cancer Institute, Amsterdam, Netherlands), rabbit anti- $\beta 5$ cytoplasmic domain from L. Reichardt (UCSF, San Francisco, CA), rabbit anti- $\alpha 3$ from G. Tarone (University, Torino, Italy), rabbit anti- $\alpha 5$ and αV -cytoplasmic domain from Chemicon Int. (Bad Nauheim, Germany). Antibodies against focal contact components were obtained from the following sources: mouse monoclonal antibodies for staining of talin and vinculin were obtained from Sigma Chemical Co. and used at the dilutions

suggested by the supplier, anti-focal adhesion kinase antibody was purchased from UBI (Lake Placid, NY) and a polyclonal antibody generated against human platelet zyxin was obtained from M. Beckerle (University, Salt Lake City, UT).

Immunoprecipitation of Integrins

Approximately 5×10^7 cells grown to 90% confluency were removed from culture plates by treatment with PBS, 10 mM EDTA, and labeled with NHS-X-biotin (Sigma Chemical Co.) according to von Boxberg et al. (1990). After washing cells once in DME and once in PBS, cells were lysed on ice with 2 ml lysis buffer (50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 3 mM MgCl₂, 1 mM CaCl₂, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin, antipain, aprotinin, each 3 μ g/ml) using a sonifier (Branson, Danbury, CT). Insoluble material was removed by centrifugation at 10,000 g at 4°C for 15 min. Supernatants were stored at -20°C for 24 h. After thawing and recentrifugation, supernatants were diluted with 1 vol of modified lysis buffer containing 5 mM EDTA but no CaCl₂ and MgCl₂ (buffer A) and pretreated with 1/20 vol of protein G-Sepharose 6B (Pharmacia Fine Chemicals) for 2 h at 4°C. Supernatants of this preclearing step were incubated with 1/20 vol of protein G-Sepharose that had been preincubated with anti-integrin antibodies diluted in buffer A. After 15 h at 4°C, protein G-bound components were washed three times in buffer A and subsequently eluted into sample buffer (5 min, 96°C) for SDS-PAGE. Proteins were separated on 5-15% SDS-polyacrylamide gels and transferred onto nitrocellulose using a semidry-blotting apparatus (Bio-metra Biomed. Analytic GmbH, Goettingen, Germany). The efficiency of the transfer and the positions of the marker proteins were verified by Ponceau S staining of the nitrocellulose. After blocking with 5% dry milk powder (Frema Reform) in TBS, (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl), 0.04% Tween 20, biotinylated proteins were identified with Streptavidin-biotinylated peroxidase complex (Amersham Corp., Arlington Heights, IL) diluted 1:1,000 in TBS, 0.04% Tween 20 (2 h) and stained with 0.5 mg/ml 4-chloro-(1)-naphthol, 0.01% H₂O₂ in TBS.

Cell Adhesion Assay

The attachment of ES cells to extracellular matrix ligands followed previously described procedures (Aumailley et al., 1989). Briefly, tissue culture 96-well plastic plates (Costar Corp., Cambridge, MA) were coated with the ligands at 4°C for 15 h. After blocking with 1% BSA, 3.5×10^5 ES cells resuspended in 100 μ l DME were added to each well and incubated for 30 min at 37°C. Adhesion to vitronectin was further evaluated after an incubation period of 60 and 90 min. Wells were then emptied and washed with PBS. The attached cells were fixed with 70% ethanol, stained with crystal violet for 30 min, and washed extensively with H₂O. Cell-bound stain was measured at 550 nm in an ELISA reader after adding 0.2% Triton X-100.

Chemotactic Migration

Chemotactic cell migration was determined as described (Grotendorst, 1984) in a Boyden-type chamber with polycarbonate filters (8- μ m-diam pore; Nucleopore) coated on both sides with gelatine. Cells were released from tissue culture dishes with 0.05% trypsin, 0.02% EDTA in PBS, washed with DME containing 1% fetal calf serum and finally suspended in DME without supplements (2.5×10^5 cells/ml), and introduced into the upper compartment of the chamber (800 μ l). The lower compartment (210 μ l) contained either conditioned medium (serum-free) from human fibroblasts or DME supplemented with 25 ng/ml PDGF or DME without supplements (210 μ l) for assessing background migration. Conditioned medium was harvested from human fibroblasts cultured for 24 h in DME without supplements. Chambers were incubated for 4 h at 37°C and cells that had migrated to the lower surface of the filter were fixed, stained, and counted at 160 \times magnification in 10 microscope fields per filter (1/12 of the total area). Assays were set up in duplicate and values are means \pm SD of 20 fields from two filters.

Scanning Electron Microscopy

Cells were allowed to settle on cover slips for 24 h and were then fixed with 1% glutaraldehyde, 0.02% osmium tetroxide in PBS at room temperature for 30 min. They were flushed with 30% ethanol and further dehydrated through graded alcohol series to 100% ethanol followed by 100% acetone and placed in a Polaron Critical Point Dryer (Polaron, Watford, UK), where they were left for 60 min before being dried. The coverslips were attached to scanning electron microscope stubs with conductive carbon paint and left

overnight. Just prior to examination with a JEOL JSM 35C Scanning Electron Microscope (Tokyo, Japan), they were gold coated to a thickness of 20 nm with a Balzers SCD 020 Coating Unit (Balzers, Liechtenstein).

Immunofluorescence

Fixation was done as described by Small (1981). Briefly, cells grown on coverslips were fixed for 5 min in 3.7% paraformaldehyde and subsequently permeabilized with 0.5% Triton X-100 in PBS for 20 min. They were then treated (10 min) and washed twice with 0.1 M glycine. Immunofluorescence staining for F-actin was done with rhodamine-phalloidin, for all other cytoskeletal proteins with specific antibodies. Incubation was for 2 h at room temperature. Secondary antibodies were Cy3 goat anti-mouse IgG + IgM or Cy3 goat anti-rabbit IgG (Jackson ImmunoResearch Labs., Inc., West Grove, PA) and used for 1 h. After washing three times with PBS, cells were embedded in gelvatol (Langanger et al., 1983) and examined with a Zeiss confocal microscope (LSM 400).

Blastocyst Injection

C57BL/6J blastocysts were isolated at day 3.5 postcoitum and injected with 15 ES cells as described (Bradley, 1987). After incubation of injected blastocysts in M2 medium, they were washed in ice-cold PBS, fixed for 5 min in 4% formaldehyde in PBS, washed three times in PBS and stained overnight at 37°C in a solution of 1 mg/ml X-gal (Sigma Chemical Co.) following established protocols (Friedrich and Soriano, 1991). Stained embryos were incubated for 24 h in PBS at 4°C prior to photography.

Results

Targeted Disruption of β 1 Integrin Alleles in Embryonic Stem Cells

In the 5' region of the mouse β 1 integrin gene (Fig. 1 A) exon 2 begins with the start codon ATG and has a size of 67 nucleotides. To obtain a targeted disruption of the gene, we used a gene trap vector with a geo DNA (Fig. 1 A) inserted in frame to the ATG. The linearized targeting vector was transfected into ES cells by electroporation. After 9-11 d, 104 clones which survived the G418 selection were picked and expanded. Targeted clones were identified by Southern blot analysis of BamHI-digested genomic DNA with probe A (Fig. 1 A) which resulted in a novel 5.2-kb band as illustrated for clones G200 to G204 (Fig. 1 B). The complete data demonstrated a high frequency (56%) of homologous recombination of the β 1 integrin gene with 58 targeted clones per 104 G418-resistant transfectants.

Surprisingly, one out of the 104 clones tested (G201; see Fig. 1 B) showed the 5.2 kb but not the wild type 10-kb band in BamHI-digested genomic DNA. The loss of both wild type alleles was confirmed by Southern blot analysis with two more restriction enzymes, HindIII and XbaI (data not shown). To test whether the loss of the wild type band was due to an aneuploid chromosome complement, metaphase spreads of colcemid-treated G201 cells were used for a chromosome count. The mean number of chromosomes of ten metaphasic spreads was 40.5 consistent with an euploid karyotype. This demonstrated that in clone G201 both β 1 integrin alleles had been inactivated by homologous recombination. All ES cell clones with targeted insertions were tested for additional random insertions by probing genomic DNA with the neo gene. Two clones had one and one clone had two additional hybridizing bands. All other targeted clones as well as clone G201 showed only a single band of the predicted size of 5.2 kb.

A 5' region of the β_1 integrin gene

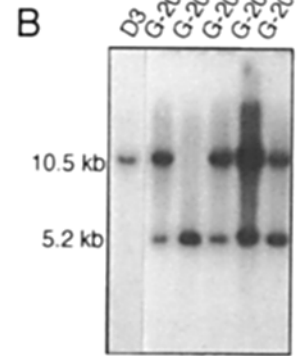
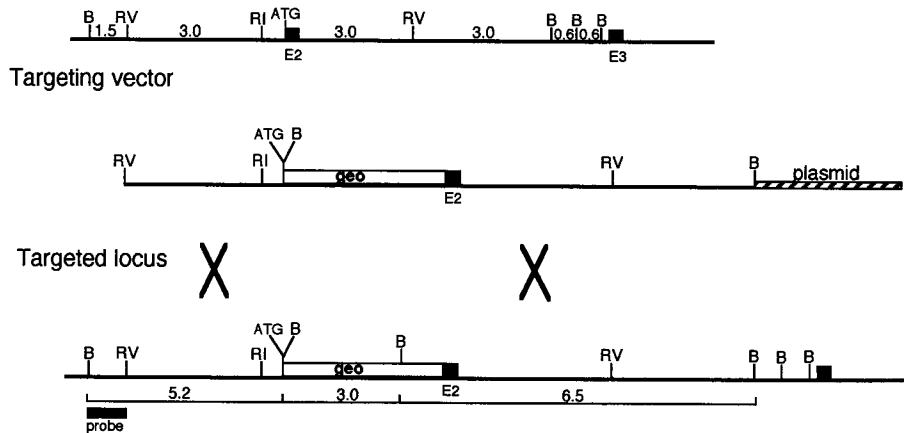


Figure 1. Disruption of the second exon of the β_1 integrin gene in ES cells. (A) Restriction map of the wild type allele, the targeting vector and the mutated allele of the β_1 integrin gene. The *geo*-cassette was inserted in frame with the ATG. The bars under the restriction map of the targeted allele indicate the sizes of restriction fragments hybridizing to the probe used for Southern blot analysis (*heavy line*). Restriction sites: B, BamHI; RI, EcoRI; RV, EcoRV. (B) Southern blot analysis of targeted cells. The 10-kb BamHI fragment was derived from the wild type allele. A novel 5.2-kb BamHI fragment was derived from the targeted allele.

Integrin Subunit Expression in Mutant Embryonic Stem Cells

Northern hybridization was used to analyze the expression of integrin subunit mRNAs in wild type and in heterozygous and homozygous mutant ES cells (Fig. 2). As expected, no signal for β_1 mRNA could be detected in the homozygous mutant and a reduced level in the heterozygotes when compared to normal ES cells. All three cell lines expressed α_3 , α_5 , and α_6 mRNA. Their steady state levels appeared, however, reduced for α_3 and α_5 but not for the α_6 subunit in the homozygous mutant. Because of the surface expression of

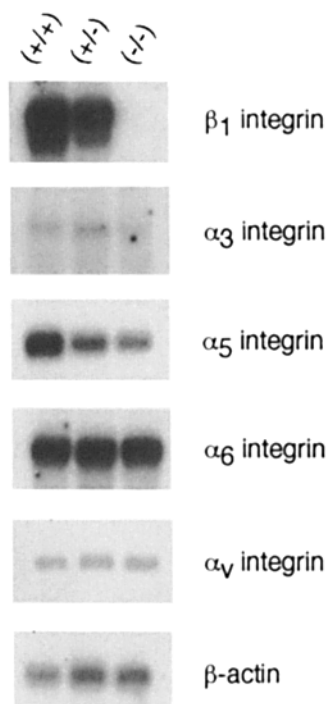


Figure 2. Northern blot analysis of various α and β integrin subunit mRNAs in normal (+/+), heterozygous (+/-), and homozygous (-/-) ES cells. Polyadenylated RNA was isolated from ES cells grown in medium supplemented with leukemia inhibiting factor but in the absence of feeder cells. 2 μ g of RNA was probed with oligolabeled mouse cDNA probes to detect mRNAs of β_1 , α_5 , and α_6 integrin subunits and β -actin. Human cDNA probes were used to detect α_3 , and α_V integrin subunits.

α_V subunits (see below) we also analyzed the level of β_3 mRNA. A weak signal could be detected after long exposure (72 h) in all three cell types (data not shown).

The apparent lack of β_1 mRNA in the homozygous cell line raised the interesting question as to whether there was any surface expression of various α integrin subunits. This was studied by immunoprecipitation of detergent extracts from surface-biotinylated cells (Fig. 3). Specific antibodies against β_1 , α_3 , α_5 , α_6 , and α_V subunits precipitated typical α/β heterodimers from wild type and heterozygous ES cell mutants but, apart from anti- α_V , not from the homozygous mutant. For the identification of the β subunit associated with α_V , further precipitations were carried out with antibodies to β_3 and β_5 but no integrin bands were detected on any of the three cell lines (data not shown). Further studies were performed by Western blot analysis of cell lysates from which integrins were enriched by binding to wheat germ agglutinin (data not shown). The staining patterns matched exactly the data observed by immunoprecipitation.

Reduced Adhesiveness and Migration of β_1 Integrin-deficient Embryonic Stem Cells

Wild type and heterozygous mutant ES cells showed comparable dose-response profiles in their adhesion to fibronectin, laminin (Fig. 4) and gelatine (data not shown) in agreement with the surface expression of the corresponding $\alpha_5\beta_1$ and $\alpha_6\beta_1$ receptors (Fig. 3). Neither of the two cell lines attached to collagens I and IV or tenascin but both showed a low level of binding to vitronectin. In sharp contrast, ES cells deficient for β_1 integrins did not adhere to fibronectin or laminin but still exhibited a low level of vitronectin binding comparable to the other two cell lines. Although several cell lines show slow adhesion to vitronectin this was not the case for normal and β_1 integrin-deficient ES cells: the binding was low after 30 and did not change after 60 as well as 90 min (data not shown).

Since integrins are important for cell migration, we used

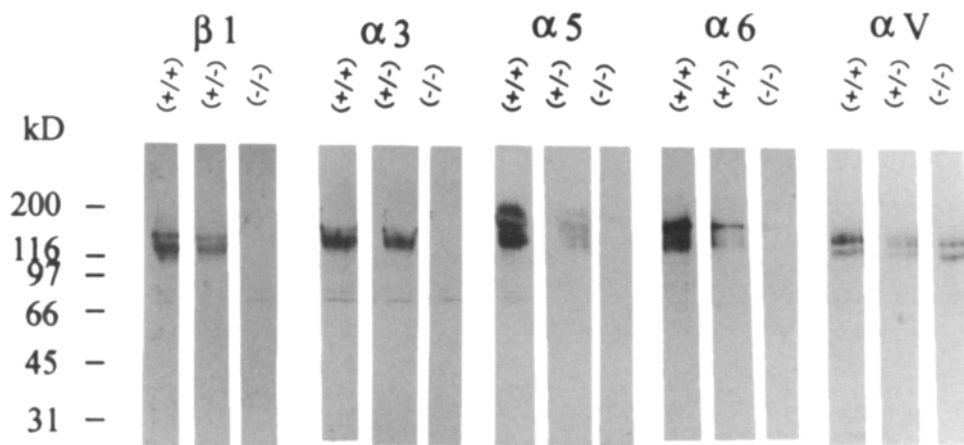


Figure 3. Immunoprecipitation of biotinylated surface proteins from wild type (+/+), heterozygous (+/-), and homozygous (-/-) ES cells with antibodies against $\beta 1$, $\alpha 3$, $\alpha 5$, $\alpha 6$, and αV integrin subunits. Equal amounts of proteins were immunoprecipitated as described in Materials and Methods. Immunoprecipitated proteins were separated on 5–15% gradient gels under non-reducing conditions, except for αV which was run under reducing conditions to allow a better separation of the subunits.

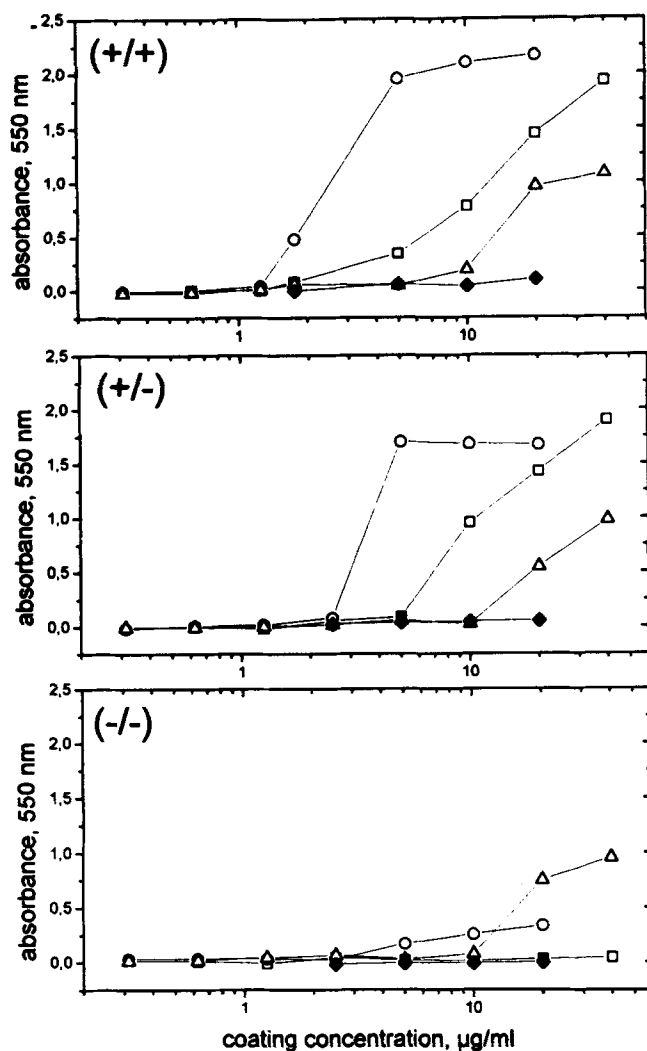


Figure 4. Cell attachment assay. Wild type (+/+), heterozygous (+/-), and homozygous (-/-) ES cells were adjusted to 2.7×10^6 cells/ml and plated on plastic plates coated with human fibronectin (\circ), mouse laminin-1 from the EHS tumor (\square), human vitronectin (\triangle) and mouse collagen type IV (\blacklozenge).

chemotactic stimulation in a Boyden chamber to compare the three ES cell lines (Table I). Human foreskin fibroblasts served as control cells as well as producers of serum-free conditioned medium. Background migration was determined using DME without supplements in the lower compartment. The higher cell number counted for ES cells in the controls reflects the smaller diameter of this cell type compared to fibroblasts. When serum-free conditioned medium from fibroblasts was used as attractant, wild type and heterozygous ES cells exhibited migration rates similar to that of human fibroblasts. In contrast, homozygous mutant ES cells did not migrate above the background level. In contrast to fibroblasts, wild type, and heterozygote ES cells responded very little, if at all, when platelet-derived growth factor was used as attractant. However, even this very small response was absent from the homozygous mutant ES cells.

Morphology and Subcellular Localization of Cytoskeletal Components in $\beta 1$ Integrin-deficient Cells

Normal or heterozygous ES cells adhered well to a feeder layer of fibroblasts and formed small nests of tightly packed cells. However, $\beta 1$ integrin-deficient ES cells showed a weak adhesion and formed colonies of a ball-like structure with a rough surface when plated on fibroblasts. These phenotypic alterations were much less pronounced when $\beta 1$ integrin-

Table I.

Chemoattractant	Cell lines			
	D3 (+/+)	G-119 (+/-)	G-201 (-/-)	Fibroblasts (+/+)
DME	35 ± 17	57 ± 33	28 ± 33	3 ± 3
CM	269 ± 59	287 ± 36	37 ± 24	159 ± 20
PDGF (25 ng/ml)	52 ± 33	66 ± 30	8 ± 6	251 ± 64

Chemotactic migration of normal (+/+), heterozygous (+/-), and homozygous (-/-) ES cells in comparison to fibroblasts. The attractants used were plain medium (DME), conditioned fibroblast medium (CM), and platelet-derived growth factor (PDGF). Analyses were set up in duplicates. Cells on the lower surface of the filters were stained and counted in 20 fields/filter at 160 \times magnification. Presented are mean \pm SD of cells/field from two filters per assay.

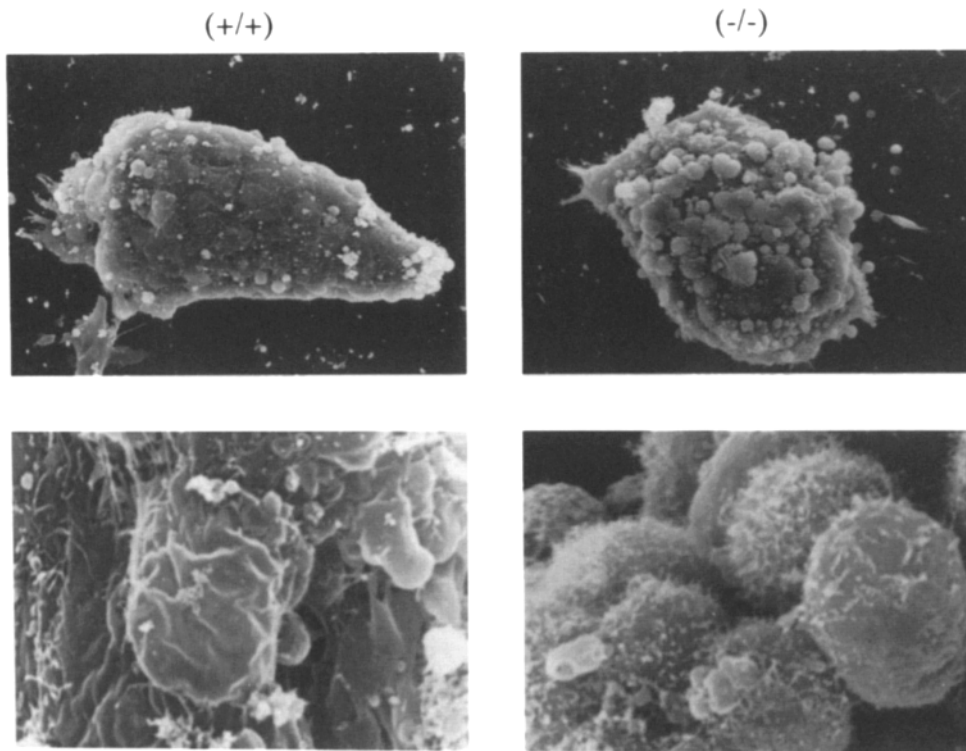


Figure 5. Scanning electron microscopy of wild type (+/+) and homozygous (-/-) mutant ES cells. Cells were grown on cover slips and fixed with glutaraldehyde and osmium tetroxide. The cover slips were then attached to scanning electron microscope stubs and gold coated. The upper parts show a representative ES cell colony, the lower parts show enlargements of single cells. Bar indicates 10 μm for the upper pictures and 1 μm for the lower pictures. Bar: (top) 100 μm ; (bottom) 10 μm .

deficient ES were cultured on gelatinized or untreated plastic dishes in the presence of FCS. Scanning electron microscopy provided a more detailed picture of the changes (Fig. 5). The surface of $\beta 1$ integrin-deficient ES cell colonies was rugged and individual cells which, in wild type colonies are normally tightly sealed to their neighbors, showed fewer cell-cell junctions. Furthermore, the surface of homozygous mutant ES cells was covered by microvilli, whereas they were very scarce on wild type and heterozygous cells.

To test whether this marked alteration of the cell shape is accompanied by pronounced changes in the distribution of cytoskeletal proteins, wild type and homozygous mutant ES cells were immunostained with antibodies for several typical components. Cells were optically sectioned by confocal microscopy in order to evaluate the three-dimensional distribution of the proteins and typical patterns are shown in Fig. 6. These patterns showed primarily a punctate fluorescence in regions close to the plasma membrane in both cell types. No obvious increase in staining intensity at cell-cell or cell-matrix adhesion sites was observed when these contacts were specifically inspected. Some marginal differences between the cell types were, however, noticed in the intensity of staining. Vinculin staining appeared stronger in homozygous mutant ES cells whereas staining for focal adhesion kinase, talin, and zyxin was reduced and more diffuse. F-actin distribution was comparable in both cell lines. Western blot analysis of cell lysates for the same components did not, however, reveal any significant difference in the quantity of these components between wild type and homozygous ES cells (data not shown).

Since it is very difficult to evaluate the cytoskeleton in undifferentiated ES cells we established a total number of 30 permanent cell lines derived from $\beta 1$ integrin-deficient ES

cells after DMSO induced differentiation. One of these cell lines, cell line GD25, had a spindle form and developed focal contacts when plated on untreated plastic dishes as well as on glass coverslips in DME supplemented with 10% FCS. GD25 cells secrete collagen I, fibronectin, and tenascin (data not shown). Immunostaining of these cells (Fig. 7) revealed a fibrillar streak pattern for talin which appears to represent the contact zones with the substratum. In addition a faint overall staining was seen. The vinculin staining patterns were very similar but more pronounced. For α -actinin this staining was less prominent. Rhodamin-phalloidin revealed the presence of stress fibers and also some staining underneath the plasma membrane.

$\beta 1$ Integrin-deficient Embryonic Stem Cells Integrate into the Inner Cell Mass of Blastocysts

A key question was whether $\beta 1$ integrin deficiency has a profound effect even at an early developmental stage. A small fixed number of ES cells was therefore injected into normal mouse blastocysts and their migration followed after 4 and 16 h by β -galactosidase staining. The cells compared were heterozygous and homozygous $\beta 1$ ES cell mutants and, as a control, ES cells carrying a randomly integrated targeting construct. Surprisingly, no difference in distribution was observed (Fig. 8). In the early phase (4 h) cells were associated with the surface of the inner cell mass and after 16 h were completely intermingled with the inner cell mass. In a small fraction of blastocysts ($\sim 20\%$), a few cells also integrated into the trophectoderm cell layer.

Discussion

Gene targeting technology was used to establish ES cell lines

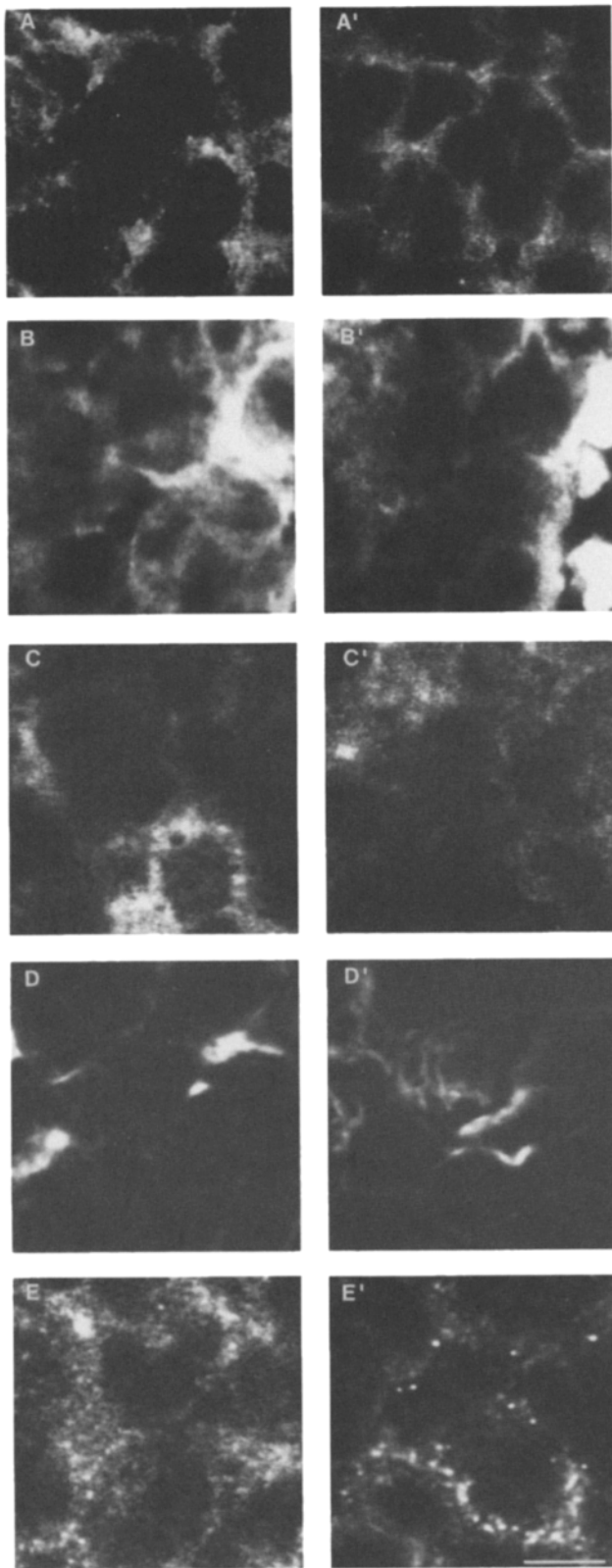


Figure 6. Immunofluorescence localization of cytoskeletal components in wild type (A-E) and homozygous (A'-E') mutant ES cells. ES cells were plated on glass coverslips, fixed with paraformaldehyde, permeabilized with detergent, and then incubated with antibodies reacting with talin (A and A'), vinculin (B and B'), zyxin (C and C') and focal adhesion kinase (E and E') followed by Cy3-labeled secondary antibody. Actin (D, D') was visualized with

carrying a mutation in either one or both genes coding for the $\beta 1$ integrin subunit. Using a gene trap-type vector (Friedrich and Soriano, 1991), we obtained one double and 58 single knockout clones from a total of 104 clones tested. This extremely high recombination frequency probably results from the type of vector used and the high expression rate of $\beta 1$ integrin in normal ES cells. As expected, homozygous mutant ES cells did not produce $\beta 1$ subunit mRNA or protein. In addition, of the α integrin subunits usually associated with $\beta 1$ which were analyzed ($\alpha 3$, $\alpha 5$, $\alpha 6$), none could be detected on homozygous mutant cells. The latter finding was also reported for a triple knockout of the $\beta 1$ integrin gene in F9 cells (Stephens et al., 1993). Similar results have been shown for inherited $\beta 2$ integrin deficiency in man (Springer et al., 1987), where cells from affected patients lack both α and β subunits on the surface. Biosynthetic studies with $\beta 2$ integrin-deficient cells revealed that the α chain precursors are made but not processed and translocated.

Northern hybridization demonstrated that there was also a distinct downregulation of $\alpha 3$ and $\alpha 5$ but not of $\alpha 6$ mRNA in homozygous mutant ES cells. This was not observed in the heterozygotes (Fig. 2). Whether this is a general phenomenon of $\beta 1$ deficiency and for example also found in the corresponding F9 cells (Stephens et al., 1993) remains to be studied. An interpretation of these data can at present only be speculative. One possibility is that a direct feed back loop exists between $\alpha 3\beta 1$ and $\alpha 5\beta 1$ integrins and their corresponding α subunit genes, affecting gene regulation. Clear evidence for integrin-mediated alteration of gene expression is known from several in vitro studies but it is usually dependent on ligand occupancy of the receptors. For example, this was shown for the induction of matrix metalloproteinases, which was apparently mediated by $\alpha 5\beta 1$ or $\alpha V\beta 1$ integrins (Werb et al., 1989; Sefror et al., 1992). A whole series of novel genes in monocytes are induced after binding to fibronectin or collagen (Sporn et al., 1990). Yet other explanations such as effects on mRNA stability or on signal transduction due to cell shape changes are possible and will require further investigations.

The changes in integrin patterns of ES cells correlated well with changes in their functional phenotype. The lack of $\alpha 5\beta 1$ and $\alpha 6\beta 1$ integrins readily explains the failure of homozygous ES cell mutants to adhere to fibronectin and laminin as was also found for F9 cells with a $\beta 1$ knockout (Stephens et al., 1993). The lack of $\alpha 3\beta 1$ integrin should in addition affect their adherence to certain laminin isoforms (Delwel et al., 1994) which were not studied here. The low but distinct adhesion to vitronectin was, however, not changed in the ES cell mutants and probably occurs via an αV integrin since expression of this subunit is not reduced. The associated β subunit of the vitronectin receptor has so far not been identified but, because of their very low expression, may not be $\beta 3$ or $\beta 5$. Interestingly, $\beta 1$ integrin-deficient F9 cells showed a highly increased vitronectin adhesion compared to the parental strain (Stephens et al., 1993). This discrepancy may be due to inherent differences between F9 and ES cells,

rhodamine-phalloidin. Confocal microscopy was used to examine the subcellular distribution of the cytoskeletal components. Bar, 10 μm .

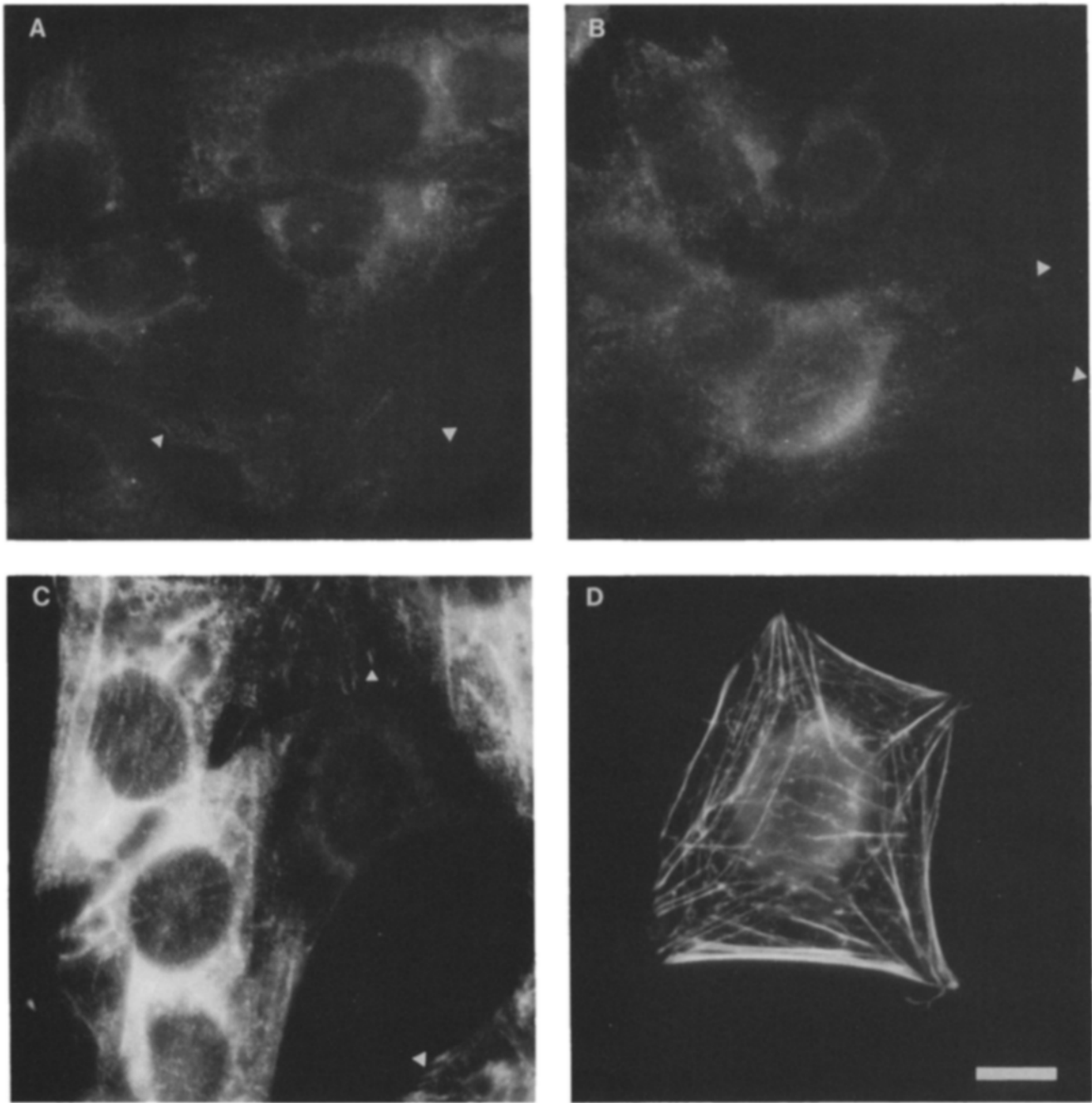


Figure 7. Immunofluorescence localization of cytoskeletal components in a $\beta 1$ integrin-deficient, fibroblast-like cell line. Cells were plated on glass coverslips, fixed with paraformaldehyde, permeabilized with detergent, and then incubated with antibodies reacting with α -actinin (A), talin (B), and vinculin (C) followed by Cy3-labeled secondary antibody. Actin (D) was visualized with rhodamine-phalloidin. Arrowheads indicate focal contact sites. Bar, 10 μm .

including chromosomal duplications which have occurred in F9 cells as indicated from the necessity of a triple knockout for the elimination of the $\beta 1$ gene. The $\beta 1$ deficiency also abolished the chemotactic migration of ES cells towards a fibroblast medium stimulus. Whether this reflects only the general requirement of $\beta 1$ integrins for locomotion or the recognition of a chemoattractant by the same integrins remains an open issue.

$\beta 1$ integrin-deficient ES cells displayed a striking morphological alteration which could be observed by light as well

as by scanning electron microscopy. The cell colonies were spherical and lined on the surface by rounded cells which possessed many atypical microvilli. Furthermore, $\beta 1$ integrin-deficient ES cells adhered badly to a fibroblast feeder layer but much better to an untreated plastic surface in the presence of fetal calf serum. This could indicate that the weak cell adhesion was caused by failure to recognize fibronectin deposited by the feeder cells. An alternative explanation is that lack of $\beta 1$ integrins leads to an alteration of the level of other cell adhesion molecules which are used by

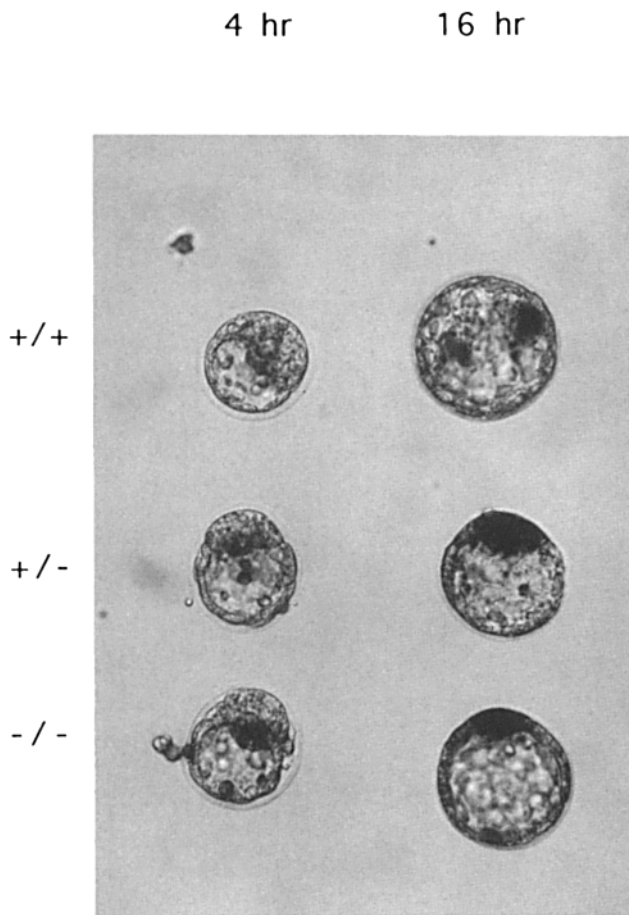


Figure 8. Injection of mock-transfected (+/+), heterozygous (+/-) and homozygous (-/-) ES cells into blastocysts. Blastocysts were isolated at day 3.5 and injected with 15 ES cells. After an incubation period of 4 or 16 h at 37°C, embryos were fixed in formaldehyde and stained for β -galactosidase activity.

these ES cells to interact with the feeder layer. Tight functional associations between integrins and cadherins have been observed during keratinocyte differentiation (Hodivala and Watt, 1994) and in $\beta 1$ integrin-deficient F9 cells (Stephens et al., 1993). The latter express E-cadherin in an abnormal spatial pattern after differentiation into visceral endoderm. Much more experimental evidence indicates that cell morphology is associated with normal integrin function. Antibodies against $\beta 1$ integrin altered the shape of myoblasts in vitro from spindle-shaped cells to rounded-up cells (Greve and Gottlieb, 1982). Furthermore, phosphorylation of the cytoplasmic domain of the $\beta 1$ integrin subunit during mitosis has been suggested to inactivate $\beta 1$ integrin which in turn would enable the rounding and detachment of cells (Hynes, 1992).

The dramatic morphological changes observed for homozygous mutant ES cells could be due to the requirement of interactions between cytoplasmic $\beta 1$ integrin domains and intracellular structural and regulatory proteins for the maintenance of cytoskeletal frameworks. Confocal immunofluorescence microscopy analysis of homozygous ES cells, however, did not reveal any gross alteration in the distribution of several major components implicated in such interactions.

This could indicate that the cytoskeleton is basically independent of $\beta 1$ integrins and that association with the latter is only required for special situations of cell adhesion and migration and to establish certain signal transduction pathways. Yet we cannot exclude subtle but crucial molecular differences which escaped detection in the assay used and would be difficult to verify. A further limitation arises from the fact that wild type ES cells and the mutants do not spread well on various substrates and do not form distinct focal contacts. Since most integrin-cytoskeletal interactions studied so far were performed with cells that form distinct focal contacts in vitro and in vivo, we extended such analysis to a differentiated cell line derived from $\beta 1$ integrin-deficient ES cells which forms focal contacts when cultured in addition with serum. Immunostaining of these cells show no prominent alteration in the subcellular distribution of cytoskeleton proteins. Talin, α -actinin, and vinculin show a diffuse overall staining of the cells and a streak-like staining pattern. Primary mouse fibroblasts which we used as controls showed a similar distribution of these proteins. Focal adhesion-type staining was observed but was much less pronounced than in primary mouse fibroblasts. Stress fibers were well developed in cells derived from $\beta 1$ integrin-deficient ES cells.

Blastocyst injection demonstrated that $\beta 1$ integrin-deficient ES cells integrated themselves into the inner cell mass as perfectly as control ES cells. This indicated dependence on other cell-cell interaction mechanisms than those which are mediated by $\beta 1$ integrins. The transfer of such chimeric blastocysts to foster mothers produced a broad range of individual phenotypes and their full description is beyond the scope of the current study (Fässler, R., manuscript in preparation). The individual phenotypes observed so far range from embryonic death at various stages to apparently healthy chimeric mice. The homozygous ES cell mutants will also be useful for several other studies including differentiation in vitro (Evans and Kaufman, 1981; Martin, 1981) and analysis of their tumorigenic properties (Doetschman et al., 1985). Finally, such cells are ideal vehicles for transfection experiments with $\beta 1$ subunit mutants in order to study structure-function relationships. Work on several of these possibilities is now in progress.

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