

Cadherin Transfection of *Xenopus* XTC Cells Downregulates Expression of Substrate Adhesion Molecules

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Cadherins are discussed not in terms of their adhesive function but rather as morphoregulatory proteins. Changes in gene expression following cadherin transfection of cells in culture or by overexpression in embryos have, until now, not been reported. We established a protocol for stable transfection of *Xenopus* XTC cells and generated cells bearing high levels of membrane-integrated mouse uvomorulin (E-cadherin) or *Xenopus* XB-cadherin. These cell lines showed drastically impaired substrate adhesion on fibronectin and laminin. In immunoblot and radioimmunoprecipitation experiments, we found that fibronectin and $\alpha 3/\beta 1$ integrin are downregulated. The reduced amounts of proteins result from a decrease of the respective mRNAs as proven by RNase protection assays. Coprecipitations revealed that transfected cadherin molecules are complexed with α -catenin and β -catenin at plasma membranes. However, the α -catenin present in the XB-cadherin complex differs immunologically from that found in the uvomorulin complex. When a truncated form of XB-cadherin lacking 38 of the most C-terminal amino acids was expressed in XTC cells, complex formation with endogenous catenins was abolished. In these transfectants, substrate adhesion was not affected. These results prove that complex formation of transfected cadherins in XTC cells with endogenous β -catenin correlates with altered synthesis of certain substrate adhesion molecules.

Adhesion molecules serve not only in formation and maintenance of tissue structures; their preeminent role as stimulators of cell differentiation has become obvious in recent years. Molecules of the cell-cell, as well as the cell-matrix, adhesion system are involved in these processes. Most of these molecules are spatially and temporally expressed during embryogenesis, directing various cell populations into different cell fates (3, 60).

Cadherins are responsible for Ca^{2+} -dependent cell-cell adhesion via homophilic binding, thereby connecting neighboring cells expressing the same cadherin type. Intracellularly, the cadherin cytoplasmic domain binds to α -catenin, β -catenin, and plakoglobin, which together mediate cadherin linkage to the cytoskeleton (32, 33, 47). α -Catenin shares extensive sequence similarity with vinculin, and β -catenin is similar to plakoglobin (6, 24, 46). Cytoplasmic interactions underlying cadherin-catenin complex formation regulate the cell adhesion and the morphoregulatory function of cadherins. These mechanisms were elucidated by studies of cadherin-transfected cell lines and by analysis of cadherins ectopically expressed in *Xenopus laevis* embryos. In *Xenopus* embryos, interblastomere adhesion up to the late blastula stage is mediated by the two maternal cadherins XB/U-cadherin and EP-cadherin (5, 19, 23, 25, 44). From late blastula and late gastrula stages, E-cadherin and N-cadherin, respectively, are zygotically expressed (5, 12). Overexpression of mouse or *Xenopus* N-cadherin in embryos leads to disruption of ectodermal tissue at the gastrula stage and to partial loss of tissue segregation and neural tube defects observed at the tailbud stage (12, 17). Similar results were obtained when cadherin mutants with the extracellular part deleted were dominantly negatively expressed (14, 34). In addition, mesoderm differentiation was affected, probably because of reduced MyoD expression (30). Truncated cadherins

compete with endogenous ones for catenin binding. The striking sequence similarity between the cytoplasmic domains of maternal and zygotic *Xenopus* cadherins explains why this competition does not depend on the cadherin type. More recently, it was shown by Heasman et al. (22) that dorsal mesoderm induction is impaired when full-length cadherin mRNA is overexpressed in *Xenopus* oocytes before maturation and onset of embryonic development. Taken together, all results emphasize the significance of cadherin-catenin binding. However, they are difficult to interpret; with overexpression of cadherins in embryos, one cannot distinguish between defects due to impaired cell adhesion or cell migration and effects caused by altered gene expression or cytoskeletal rearrangements.

Transfection experiments allow one to study the effects of cadherin expression more precisely. Earlier studies based on mammalian cell lines have shown that uvomorulin induces cell surface polarity after transfection of mouse fibroblasts. Surface polarity was demonstrated by the redistribution of the Na^+ , K^+ -ATPase to basal-lateral membranes of transfected cells. Remarkably, the polarizing activity of uvomorulin was abolished when the cytoplasmic domain was deleted (42). N-cadherin increases the number of adherens junctions at cell-cell contact sites when it is expressed in mouse S180 cells (39). Cytoskeletal reorganizations are considered to be responsible for the morphoregulatory potency of cadherins (33, 60). There is no evidence that changes in cell morphology observed after cadherin transfection are accompanied by an altered gene expression pattern.

By contrast, several reports have indicated that cell-substrate interactions might control cellular gene expression even without alterations in cell shape (7, 15, 58, 63). Enhancer elements have been identified which are activated by cell-matrix binding (13, 38, 56). Cell membrane-integrated receptors, the integrins, form the basis of cell-substrate adhesion by binding different ligands of the extracellular matrix. Integrins are heterodimers consisting of two noncovalently associated subunits (α and β). The substrate specificity of these receptors is attained by combination of different α and β subunits (3, 4,

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31). Striking examples showing that cell-matrix interactions stimulate cell differentiation in terms of altered cell morphology were reported by Adams and Watt (2) for terminal differentiation of keratinocytes, by Klein et al. (35) for kidney tubulus differentiation, and by Grant et al. (21) for endothelial differentiation. In these cases, the crucial role of cell-matrix interactions in changing cell fate was demonstrated by blocking ligand-receptor interaction with peptides or antibodies.

The cell culture systems used to investigate differentiation processes have failed to be established for *Xenopus* cells. Undoubtedly, such experiments would enhance the understanding of cadherin function in *Xenopus* embryogenesis. We generated stably transfected *Xenopus* XTC cell lines expressing either the mouse epithelial cadherin, uvomorulin (XTC-Uvo⁺ [XTC cells transfected with uvomorulin cDNA]), the *Xenopus* XB-cadherin (XTC-XB⁺ [XTC cells transfected with XB-cadherin cDNA]) which mediates interblastomere adhesion, or a cytoplasmic mutant form of deleted XB-cadherin (XTC-XBΔc38 [XTC cells transfected with cDNA encoding a cytoplasmically deleted XB-cadherin]). Surprisingly, both full-length cadherins influenced substrate adhesion by downregulation of α3β1 integrin transcripts. Furthermore, expression of fibronectin, a ligand, was drastically reduced, indicating that the mesenchymal character of the cell had changed. These effects were not observed in transfectants expressing the truncated XB-cadherin.

MATERIALS AND METHODS

Cell culture. *Xenopus* XTC fibroblasts and transfected XTC cells were routinely grown in 70% L15 glutamax medium (Gibco) supplemented with 8% fetal calf serum (Gibco) at 24°C. Transfected cells were grown in the presence of 0.2 mg of G418 (Gibco) per ml after selection.

For metabolic labeling, cells at 75% confluency were grown in 80% methionine-free RPMI (Biochrom) for 2.5 h and subsequently labeled with L-[³⁵S]methionine (specific activity, 1,100 Ci/mmol; Amersham) at 50 μCi/ml for 16 h.

For binding of antibody Fab fragments, cells were depleted of methionine for 30 min and incubated in suspension in methionine-free medium supplemented with 0.1 mg of Fab fragments per ml for 45 min at 4°C on a roller. Cells were seeded and metabolically labeled in the presence of 0.1 mg of antibody Fab fragments per ml for 6 to 12 h as described above.

cDNA constructs and transfection. A full-length cDNA of XB-cadherin was inserted into the polylinker of pRC/CMV (Invitrogen). The truncated XB-cadherin (XBΔc38) was constructed by *EspI-NcoI* cleavage of the full-length XB-cadherin cDNA in pBluescript. The resulting fragment was religated in the presence of a *NheI* nonsense linker which produced a new stop codon downstream from the *EspI* site. Afterwards, the truncated XB-cadherin was transferred into pRC/CMV. The full-length construct of uvomorulin was kindly provided by R. Kemler. For control transfections, plasmid DNA of the vector with no insert was used.

Transfection of XTC cells was carried out by applying a calcium phosphate transfection method (20). Cells (10⁶) were incubated with 5 ml of fresh medium for 4 h. A 1-μg sample of DNA was added as a precipitate, and the mixture was incubated for 4 h. Cells were incubated in fresh medium for a further 48 h before selection was started. Cells were selected in the presence of 2 mg of G418 per ml for 4 weeks. Resistant cells were immunologically selected for membrane-localized cadherin by separation on magnetic beads. Cells (2 × 10⁷) were incubated with 10 μg of affinity-purified gp84 rabbit antibodies (uvomorulin transfectants) or 5 μg of U-cadherin monoclonal antibody (MAB) immunoglobulin G (IgG) (XB-cadherin transfectants) for 45 min at 4°C. Anti-mouse or anti-rabbit IgG antibody-coated beads (Dynal) were added, and the mixture was incubated for 60 min at 4°C. Cells attached to beads were separated on a magnetic stand and cultured further. Positive cells were cloned by limiting dilution. Several cloned cell lines and pooled clones were analyzed.

Antibodies. Fibronectin MAb 6D9 was described elsewhere (9). Previously characterized MAb 6D5 raised against U-cadherin (5) and MAb 8C8 against β1 integrin (18) were gifts from P. Hausen. Affinity-purified antibodies against the 84-kDa tryptic fragment of uvomorulin (62), pan-cadherin antibodies raised against the conserved cytoplasmic domain of uvomorulin (54), and peptide-specific antibodies against α-catenin and β-catenin (6, 24) were gifts from R. Kemler. Peptide-specific antiserum recognizing α3 integrin was a gift from R. Hynes. A MAb against a 19.4-kDa C-terminal fragment of mouse α-catenin was purchased from Transduction Laboratories.

Immunofluorescence. Cells were grown to confluency on glass coverslips for 24 to 36 h. Cells were rinsed in APBS/Ca (amphibian phosphate-buffered saline

[PBS] containing 2.7 mM KCl, 103 mM NaCl, 0.15 mM KH₂PO₄, 0.7 mM NaH₂PO₄, and 2 mM CaCl₂) and then fixed in a fresh solution of 3% paraformaldehyde in APBS/Ca at room temperature for 10 min. After preincubation in 1% bovine serum albumin (BSA) in APBS/Ca for 10 min, cells were incubated with primary antibodies overnight at 4°C. Cells were washed extensively in APBS/Ca and then incubated in a 1:200 dilution of goat anti-rabbit IgG conjugated to fluorescein or a 1:600 dilution of goat anti-mouse IgG conjugated to Cy3 (both from Dianova) for 2 h at room temperature. Cells were washed in APBS/Ca, mounted in Elvanol, and viewed in a Zeiss Axiophot microscope. The micrographs shown are representative fields from repeated experiments.

Preparation of cell extracts, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and immunoblotting. Dishes of confluent cells were rinsed three times with APBS/Ca at room temperature. The cells were scraped from the dishes with a rubber policeman and sedimented by centrifugation for 6 min at 200 × g. For analysis of integrins, cadherins, and associated proteins, harvested cells were lysed on ice in PBS/Ca (PBS containing 103 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 6.5 mM Na₂HPO₄ × 2H₂O [pH 7.2] containing 0.75 mM CaCl₂) containing 1% Triton X-100, 1% Nonidet P-40, and 2 mM CaCl₂ freshly supplemented with 2 μM aprotinin, 2 μM leupeptin, 2 μM pepstatin, 2 mM iodoacetamide, 1 mM *N*-ethylmaleimide, and 2 mM phenylmethylsulfonyl fluoride and incubated on a shaker at 4°C for 20 min. Soluble supernatant lysate was collected after 5 min of centrifugation at 25,500 × g at 4°C. For analysis of extracellular matrix components, cells were harvested as described above and lysed in 0.1 M Tris/Cl (pH 7.5)–2 M urea–10 mM EDTA–10 mM Na₂S₂O₅–1 mM benzamidine and the same cocktail of protease inhibitors. For SDS-PAGE or further precipitation procedures, lysates to be compared were normalized for total protein content on the basis of Bradford protein quantification. SDS-PAGE was done with 7.5% polyacrylamide gels (37). Blotting onto nitrocellulose was performed as described by Towbin et al. (61), in the presence of 15% methanol and, additionally, 2 M urea for transfer of extracellular matrix components. Filters were blocked in 10% low-fat milk powder in PBS and incubated with the respective first antibodies overnight. Horseradish peroxidase-conjugated secondary antibodies (Dianova) were used at a dilution of 1:50,000, and detection was done with the ECL detection system (Amersham).

Concanavalin A (ConA) precipitation of glycoproteins. Lysates were incubated for 1.5 h with ConA-Sepharose (Sigma) at 4°C. The ConA-Sepharose pellet was washed by centrifugation at 800 × g three times in 10 mM Tris/Cl (pH 7.5)–2 mM CaCl₂–0.15 M NaCl–1% Triton X-100–1% sodium deoxycholate–0.1% SDS, once in 0.5 M NaCl–10 mM Tris/Cl (pH 7.5)–2 mM CaCl₂, and once in 10 mM Tris/Cl (pH 7.5)–2 mM CaCl₂. Bound proteins were eluted by boiling in reducing SDS sample buffer for 4 min and subjected to SDS-PAGE and immunoblotting.

Immunoprecipitation. Lysates were incubated with 5 μg of purified mouse antibodies for 1 h. Immune complexes were collected by adding a 10% suspension of protein A-Sepharose (Pharmacia) during 30 min. Beads were washed three times by centrifugation at 800 × g in washing buffer (0.05% Nonidet P-40, 0.5 M NaCl, 50 mM Tris/Cl [pH 8.6], 2 mM CaCl₂, 1 mg of ovalbumin per ml). Bound proteins were eluted with sample buffer at 95°C for 4 min and subjected to SDS-PAGE followed by Western blotting (immunoblotting) or Coomassie blue staining and 30 min of incubation in Amplify solution (Amersham) for fluorography of ³⁵S-labeled proteins.

Biotinylation and streptavidin-agarose precipitation of cell surface proteins. Layers of confluent cells in dishes were rinsed twice with biotinylation buffer (4.5 mM NaHCO₃, 103 mM NaCl, 4 mM KCl, 0.75 mM MgCl₂, 2 mM CaCl₂) and then incubated for 5 min at 24°C in 0.2 mg of NHS-X-biotin (Calbiochem) per ml in biotinylation buffer. Lysates of biotinylated cells were incubated with streptavidin-agarose (Sigma) for 60 min. Bound proteins were washed and eluted as described above for protein A-Sepharose. Eluates were subjected to SDS-PAGE followed by immunoblotting as already described.

Cell-substrate adhesion assay. Ninety-six-well plates were coated with the respective substrates, incubated for 4 h, and blocked by incubation with 1% BSA for another 4 h, both at 4°C. Cells were trypsinized and depleted of serum and incubated at 2.5 × 10⁵/ml in serum-free medium for 30 min. A 100-μl volume of a cell suspension was pipetted into each well. Adhesion was allowed to occur for 20 min at 24°C. Nonadherent cells were washed out, and the remaining cells were fixed with 2.5% glutaraldehyde, stained with 0.1% crystal violet, and lysed in 0.5% Triton X-100 overnight. A₅₉₅ values of different cell types on one plate and substrate were compared.

RNA preparation, in vitro transcription, and RNase protection analysis. The following probes used for RNase protection assays have been described previously: fibronectin (11), α2, α3, α4, α5, and α6 integrins (64); β1 integrin (10); β3 integrin (53); and elongation factor 1α (EF-1α) (36). The latter was used as a control. Protected fragments had the following sizes: fibronectin, 375 nucleotides (nt); α2 integrin, 275 nt; α3 integrin, 460 nt; α4 integrin, 150 nt; α5 integrin, 275 nt; α6 integrin, 305 nt; β1 integrin, 118 nt; β3 integrin, 306 nt; EF-1α, 45 nt. After linearization, in vitro transcription was performed in the presence of 100 μCi of UTP as described by Melton et al. (43), with an appropriate RNA polymerase. After two ethanol precipitations with ammonium acetate, the RNA was dissolved in 100 μl of hybridization buffer [40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 0.4 M NaCl, 1 mM EDTA, 80% formamide]. RNAs from cell lines were prepared with guanidinium isothiocyanate as described by Chirgwin et al. (8). For each RNase protection assay, 5 × 10⁵ cpm of a labeled RNA probe and 30 μg of RNA were dissolved in 30 μl of hybridization buffer,

denatured at 85°C for 5 min, and incubated overnight at 60°C. Afterwards, 350 μ l of RNase digestion buffer (10 mM Tris/Cl [pH 7.5], 300 mM NaCl, 5 mM EDTA, 40 μ g of RNase A per ml, 2 μ g of RNase T₁ per ml) was added, and the mixture was incubated for 1 h at 30°C. To remove RNases, proteinase K digestion was performed by adding 10 μ l of 20% SDS and 5 μ l of proteinase K (10 mg/ml) and heating the mixture to 37°C for 15 min. Afterwards, the solution was extracted with phenol-chloroform and precipitated with ethanol and ammonium acetate in the presence of *Saccharomyces cerevisiae* tRNA as a carrier. The pellet was dissolved in RNA loading buffer (80% formamide, 1 mM EDTA (pH 8.0), 0.1% bromophenol blue) and analyzed on a denaturing 6% polyacrylamide gel. As controls, the labeled probes were hybridized with *S. cerevisiae* tRNA.

RESULTS

Expression of uvomorulin and XB-cadherin in *Xenopus* XTC cells. In an earlier report, it was mentioned that no successful stable transfection of *Xenopus* cells had yet been performed (57). We used pRc/CMV mammalian expression vectors containing the entire coding sequence of uvomorulin (mouse E-cadherin) or XB-cadherin and generated stably transfected *Xenopus* XTC cells. XTC cells are a fibroblast cell line originally established by Pudney et al. (52). An endogenous cadherin of 130 kDa was detected in immunoblots of untransfected XTC cells by using a MAb directed against chicken N-cadherin. Although transmission electron microscopy revealed rare cell-cell contact sites and many extended cell protrusions, XTC cells showed Ca²⁺-dependent cell-cell adhesion probably mediated by the endogenous putative N-cadherin (data not shown).

The lethal concentration of G418, the neomycin derivative geneticin, in the culture medium was determined to be 2 mg/ml, which is twofold higher than that for commonly used mammalian fibroblasts such as mouse L cells. With the respective cadherins, both pRc/CMV plasmids conferred neomycin resistance. Selection was complete 4 weeks after transfection. Cells bearing the respective cadherins were immunologically enriched by magnetic cell separation. After several of these purification steps, FACScan analysis revealed 92% purity in surface expression. Pooled cell clones and several cloned cell lines were analyzed. They all gave the same results. Here, we present data from pooled cell clones.

Uvomorulin and XB-cadherin expressed in transfected cells were found to be of the expected size. Mouse cadherin appeared slightly larger than XB-cadherin (Fig. 1A, gp84 and U-cad). These cadherins are glycosylated, since they could be enriched by binding to ConA-Sepharose. Mouse cadherin was detected with a polyclonal serum raised against the 84-kDa tryptic fragment of the extracellular part of uvomorulin (62). The presence of XB-cadherin was shown by the use of MAb 6D5, raised against *Xenopus* U-cadherin. Both *Xenopus* cadherins, XB-cadherin and U-cadherin, were recently found to be identical (44). In the following text, we use the term XB-cadherin when cDNA or RNA probes were handled and U-cadherin for the use of MAb 6D5 in immunological detections. XTC cells transfected with the pRc/CMV vector with no cDNA insert and untransfected cells served as controls. ConA precipitates from these cells reacted with neither antibodies against uvomorulin nor MAb 6D5 after immunoblotting (Fig. 1A, gp84 and U-cad). The expression of exogenous cadherins dominated but did not alter the amount of the endogenous cadherin, as demonstrated in immunoblots of cell lysates with pan-cadherin antibodies raised against the conserved cytoplasmic domain of uvomorulin (Fig. 1A, pan-cad). Since uvomorulin was of the same size as the endogenous cadherin, both proteins gave a unique band in the immunoblot of XTC-Uvo⁺ lysates which was enhanced compared with the signal of the endogenous cadherin in untransfected XTC cells. In XTC-XB⁺ cell lysates, the smaller 120-kDa protein represented

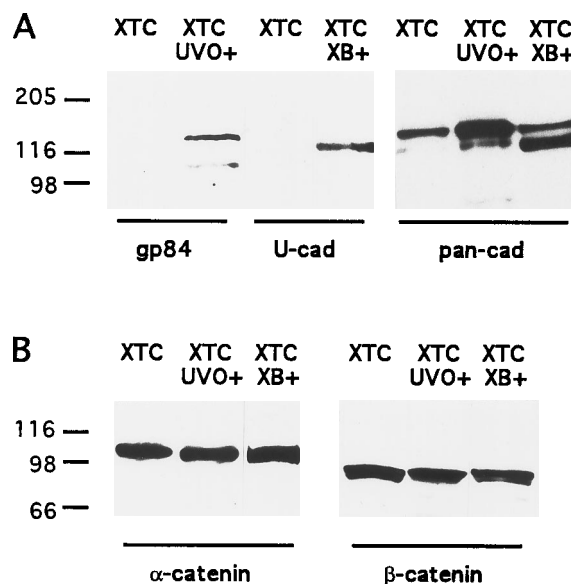


FIG. 1. Immunoblot analysis of cadherins and catenins in untransfected and cadherin-transfected XTC cells. (A) Detection of cadherin. ConA extracts of 100 μ g of total protein were separated per lane on SDS-7.5% PAGE gels, blotted onto nitrocellulose, and probed with gp84 antiserum (lanes XTC and XTC-Uvo⁺; gp84) or U-cadherin MAb 6D5 (lanes XTC and XTC-XB⁺; U-cad). A 50- μ g sample of total protein was separated per lane on SDS-7.5% PAGE gels, blotted, and probed with pan-cadherin antibodies (lanes XTC, XTC-Uvo⁺, and XTC-XB⁺; pan-cad). (B) Detection of catenins. A 20- μ g sample of total protein was separated per lane on SDS-7.5% PAGE gels, blotted, and probed with catenin peptide-specific antibodies. Cell lysates were blotted and probed with α -catenin antibodies (lanes XTC, XTC-Uvo⁺, and XTC-XB⁺; α -catenin) or β -catenin antibodies (lanes XTC, XTC-Uvo⁺, and XTC-XB⁺; β -catenin). The positions of molecular size standards are indicated in kilodaltons.

XB-cadherin, which was overexpressed compared with the level of the endogenous cadherin. The amounts of cadherin-associated proteins α -catenin and β -catenin detected by peptide-specific antibodies did not change upon expression of exogenous cadherins (Fig. 1B).

Immunohistological staining of uvomorulin- and XB-cadherin-transfected XTC cells showed a ubiquitous dot-like distribution of both cadherins on cell surfaces. No accumulation of cadherins was observed at cell-cell contact sites in either XTC-Uvo⁺ or XTC-XB⁺ cells (Fig. 2a and b). This was confirmed by ultrastructural analysis and confocal microscopic stereo imaging (data not shown). Moreover, control and transfected cells showed no obvious differences in morphological structure. Control cells were negative in staining for uvomorulin or XB-cadherin (Fig. 2c).

In cell-cell adhesion assays, we observed no change in the strong Ca²⁺-dependent aggregation of transfected XTC cells compared with wild-type cells. Since adhesion mediated by endogenous cadherin could not be specifically blocked, the contribution of the transfected cadherins to cell-cell adhesion could not be investigated.

Cadherin-catenin complex formation in cadherin-transfected XTC cells. To determine whether the introduced cadherin molecules were bound to catenins, coprecipitation experiments were performed. When uvomorulin was precipitated from XTC-Uvo⁺ cell lysates with gp84 antiserum, α -catenin and β -catenin coprecipitated (Fig. 3A, XTC-Uvo⁺). A complex between uvomorulin and endogenous *Xenopus* catenins was formed in XTC-Uvo⁺ cells. In XB-cadherin precipitates from XTC-XB⁺ cells, only β -catenin was found as a coprecipitate when peptide-specific catenin antibodies were used (Fig.

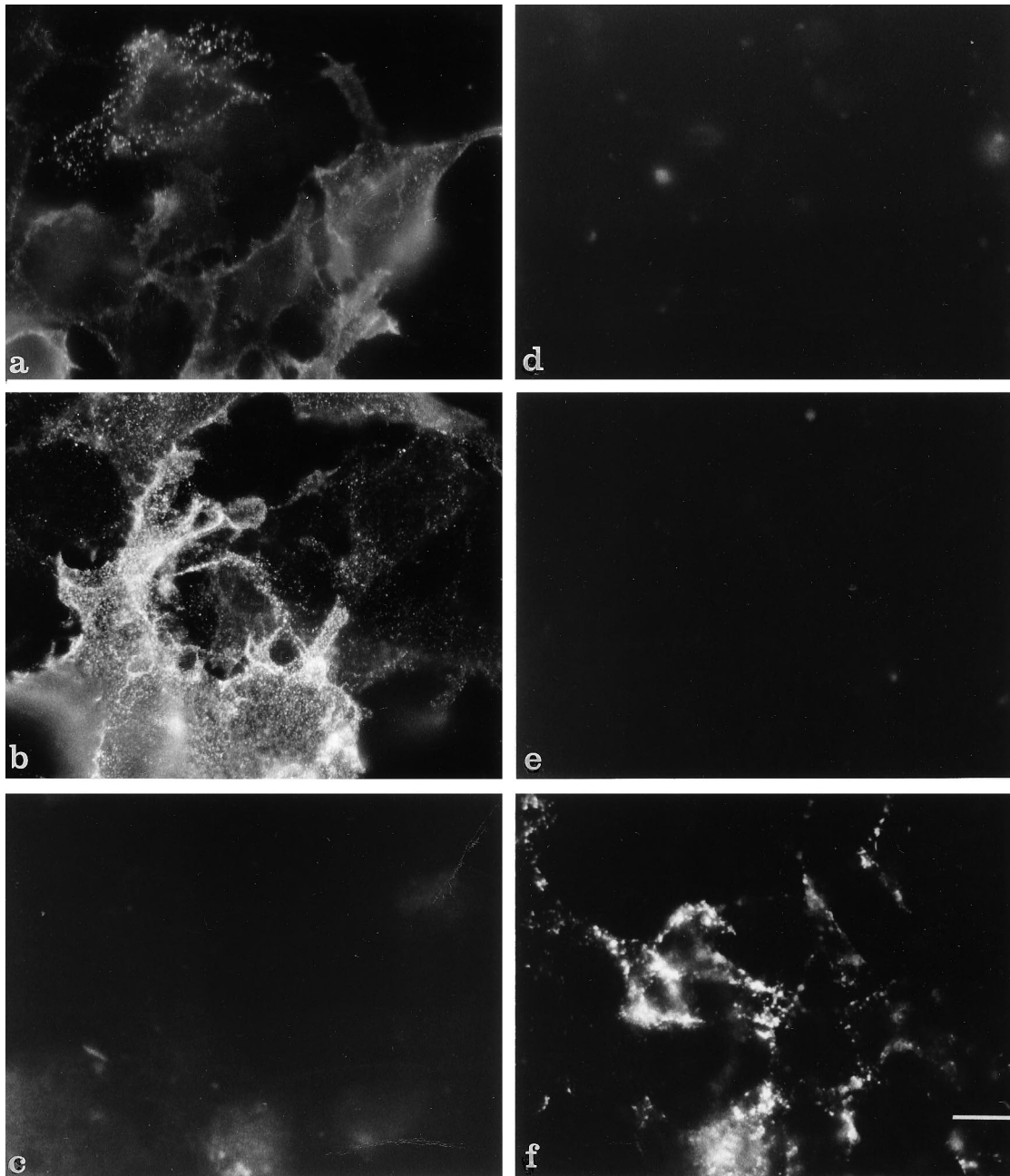


FIG. 2. Immunofluorescence staining for cadherins and extracellular fibronectin in transfected XTC cells. Cells were grown on glass coverslips. Fixed cells were stained with the respective antibodies. Antigens were visualized by incubate with secondary fluorescent antibodies. (a) XTC-Uvo⁺ cells stained for uvomorulin with affinity-purified gp84 antibodies. (b and c) XTC-XB⁺ (b) and vector-transfected XTC (c) cells stained for XB-cadherin with U-cadherin MAb 6D5. (d to f) XTC-Uvo⁺ (d), XTC-XB⁺ (e), and vector-transfected XTC (f) cells stained for fibronectin with *Xenopus* fibronectin MAb 6D9. Bar, 20 μ m.

3A, XTC-XB⁺, probes α -Cat and β -Cat); conversely, in immunoprecipitations performed with α -catenin antibodies, no XB-cadherin was detected (data not shown). When [³⁵S]methionine-labeled XTC-XB⁺ cell lysates were immunoprecipitated with MAb 6D5, in addition to XB-cadherin, two bands corresponding to the expected molecular masses of catenins (102 kDa for α -catenin and 88 kDa for β -catenin) were detected (Fig. 3A, XTC-XB⁺, fluorography). When a MAb raised against a 19.4-kDa fragment of mouse α -catenin was used as a probe, the 102-kDa coprecipitate was identified as a form of α -catenin (Fig. 3A, XTC-XB⁺, probe α -Cat MAb). We

conclude that in XTC cells, two immunologically distinct isoforms of α -catenin are present which differ in binding to uvomorulin- β -catenin and XB-cadherin- β -catenin complexes.

Since a common type of β -catenin was detected in both cadherin-catenin complexes, we determined whether this catenin was shifted from the cytosol to the membrane by binding to membrane-integrated cadherins. Cell surface proteins were labeled via biotinylation and separated from cytosolic proteins by streptavidin-agarose precipitation. In untransfected cells, β -catenin was detected only in the supernatant (Fig. 3B, XTC, SN), whereas in XTC-Uvo⁺ and XTC-XB⁺ cells, β -catenin

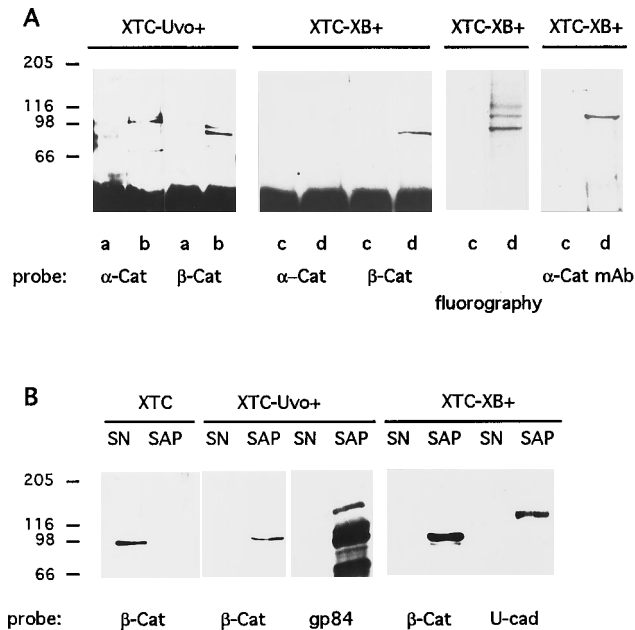


FIG. 3. Cadherin-catenin complex formation in transfectants. (A) α - and β -catenin complex formation with transfected cadherins. Cell lysates were precipitated with either nonspecific antibodies as controls or with respective cadherin antibodies. Immune complexes were boiled in reducing SDS sample buffer and separated on 7.5% polyacrylamide gels. Blotted samples were analyzed for catenins by probing with peptide-specific antibodies for α -catenin (α -Cat) or for β -catenin (β -Cat). XTC-Uvo⁺ cell lysates were incubated with either preimmune rabbit serum (lanes a) as controls or gp84 antibodies to precipitate uvomorulin (lanes b). XTC-XB⁺ cell lysates were incubated with either mouse nonspecific IgG (lanes c) as controls or U-cadherin MAb 6D5 to precipitate XB-cadherin (lanes d). The strong signals at about 45 kDa represent IgGs that were used for immunoprecipitation and detected by the secondary antibodies in the following immunoblot. Radioimmunoprecipitations of XTC-XB⁺ lysates were performed with mouse nonspecific IgG (lane c) as a control or U-cadherin MAb 6D5 (lane d), and precipitated bands were detected by fluorography. For identification of the 102-kDa XB-cadherin coprecipitate, XTC-XB⁺ cell lysates were again incubated with mouse nonspecific IgG (lane c) or U-cadherin MAb 6D5 (lane d). Immune complexes were blotted and probed with a MAb against α -catenin (α -Cat mAb). The positions of molecular size standards are indicated in kilodaltons for all lanes. (B) Localization of β -catenin-cadherin complexes at cell membranes. XTC, XTC-Uvo⁺, and XTC-XB⁺ cells were surface labeled with normal human serum-activated biotin and lysed. Equivalent amounts of total protein were precipitated with streptavidin-agarose. Non-surface-associated proteins in the supernatants after streptavidin-agarose incubation (SN) and surface-bound proteins eluted from streptavidin-agarose after precipitation (SAP) were analyzed on SDS-7.5% polyacrylamide gels under reducing conditions and immunoblotted. β -Cat, lanes probed with β -catenin antibodies. gp84, lanes probed with gp84 antiserum. Lower-molecular-weight signals resulted from proteolytic degradation. U-cad, lanes probed with U-cadherin MAb 6D5. The positions of molecular size standards are indicated in kilodaltons.

was found mainly in streptavidin-agarose fractions, as were both transfected cadherins (Fig. 3B, XTC-Uvo⁺ and XTC-XB⁺, SAP). Although we cannot exclude the possibility that under these experimental conditions small amounts of protein failed to be detected, we conclude that most of the β -catenin molecules were relocated from the cytosolic fraction to the membrane fraction by expression of exogenous cadherins.

Cadherins induce downregulation of fibronectin and β 1 and α 3 integrin synthesis. XTC cells synthesize fibronectin, which is deposited into extracellular fibrils (Fig. 2f). When XTC cells were transfected with uvomorulin or XB-cadherin cDNA, secreted fibronectin was not detected in immunostained cell monolayers (Fig. 2d and e). Immunoblots of urea extracts from cadherin-transfected XTC cells probed with a MAb specific for *Xenopus* fibronectin (6D9) revealed a decrease of the fibronectin

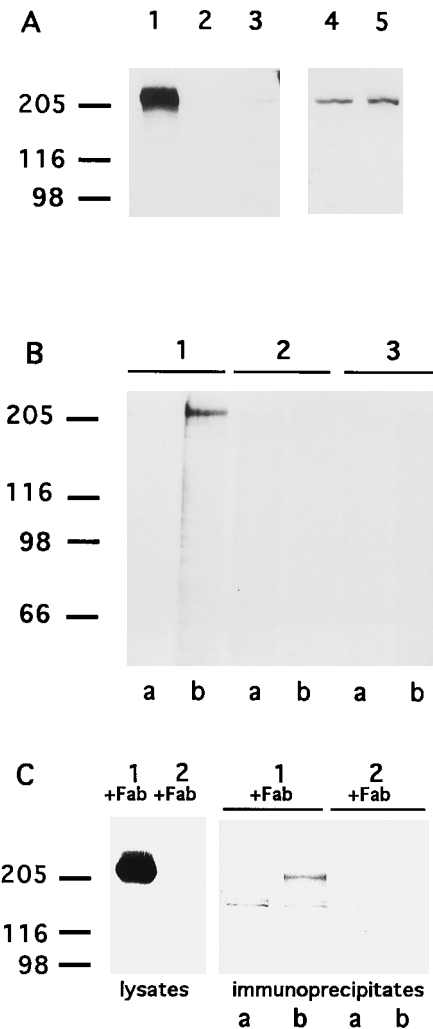


FIG. 4. Expression of fibronectin protein in XTC transfectants. (A) Urea lysates of cells were separated on 7.5% polyacrylamide gels under reducing conditions, blotted onto nitrocellulose, and probed with *Xenopus* fibronectin MAb 6D9. A 20- μ g sample of total protein was loaded per lane of XTC (lane 1), XTC-Uvo⁺ (lane 2), and XTC-XB⁺ (lane 3) cells to show the extent of reduction of fibronectin protein expression. As controls, 5 μ g of total protein was loaded per lane of XTC (lane 4) and vector-transfected XTC (lane 5) cells to rule out nonspecific transfection effects on expression of fibronectin protein. (B) Fibronectin immunoprecipitates of cell lysates metabolically labeled with [³⁵S]methionine for 16 h were separated under equal SDS-PAGE conditions. Radioactive bands were detected by fluorography. Lanes: 1, XTC cells immunoprecipitated with nonspecific mouse IgG (a) and fibronectin MAb 6D9 (b); 2, XTC-Uvo⁺ cells immunoprecipitated with nonspecific mouse IgG (a) and fibronectin MAb 6D9 (b); 3, XTC-XB⁺ cells immunoprecipitated with nonspecific mouse IgG (a) and fibronectin MAb 6D9 (b). (C) Cells were metabolically labeled in the presence of Fab fragments of U-cadherin MAb 6D5 for 6 h. Fab-treated XTC (1+Fab) and XTC-XB⁺ (2+Fab) cells were lysed. A 50- μ g sample of total protein from the lysate was blotted as already described and probed with *Xenopus* fibronectin MAb 6D9 (lysates). Radioactive bands were immunoprecipitated from lysates of Fab-treated cells with nonspecific mouse IgG (a) and fibronectin MAb 6D9 (b) and detected by fluorography (immunoprecipitates). The positions of molecular size standards are indicated in kilodaltons.

band to nearly undetectable amounts (Fig. 4A, lanes 1 to 3). This was also observed when supernatant culture medium of the different cell lines was analyzed by immunodetection with MAb 6D9 (data not shown). Comparison of XTC cells with vector-transfected control cells demonstrated that the decrease in fibronectin was not caused by the vector transfection itself

TABLE 1. Quantification of substrate adhesion molecules^a

Probe	Relative radioactivity (%)		
	XTC	XTC-uvomorulin	XTC-XB-cadherin
Fibronectin mRNA	100	0.1	0.7
Fibronectin protein	100	0.3	4.0
β 1 integrin mRNA	100	59.4	66.7
β 1 integrin protein	100	57.8	83.3
α 3 integrin mRNA	100	70.5	74.0
α 3 integrin protein	ND ^b	ND	ND

^a Radioactive bands of RNase protection analyses for mRNA or radioimmunoprecipitations from [³⁵S]methionine-labeled cells for protein were quantified with a Fuji BAS1000 phosphorimaging system. mRNA values were normalized to equal amounts on the basis of EF-1 α -protected bands. Untransfected XTC cell values served as standards and were taken as 100% expression. Numbers of repeated experiments were $n = 2$ for mRNA data and $n = 4$ for protein data.

^b ND, not done.

(Fig. 4A, lanes 4 and 5). Fibronectin immunoprecipitations of [³⁵S]methionine-labeled cells also gave no signal in XTC-Uvo⁺ and XTC-XB⁺ cells (Fig. 4B, lane groups 2 and 3). In contrast, wild-type cells showed a strong band of 240 kDa (Fig. 4B, lane group 1). When fibronectin signals from several immunoprecipitations were quantified with a phosphorimaging system, residual synthesized fibronectin was detected at levels of 0.3% in XTC-Uvo⁺ cells and 4.0% in XTC-XB⁺ cells in relation to the level in untransfected cells (Table 1).

XTC-XB⁺ cells were cultivated in the presence of Fab fragments of MAb 6D5 (Fig. 4C, lanes 2+Fab) to inhibit XB-cadherin-specific homophilic binding. Earlier, Angres et al. (5) had reported that MAb 6D5 Fab fragments blocked XB/U-cadherin-mediated cell adhesion. Fibronectin synthesis in XB-cadherin transfectants was not reactivated by incubation with MAb 6D5 Fab fragments (Fig. 4C, lanes 2+Fab, lysates and immunoprecipitates). In wild-type XTC cells, cultivation in the presence of Fab fragments did not affect the total amount (Fig. 4C, lane 1+Fab, lysates) or synthesis (Fig. 4C, lanes 1+Fab, immunoprecipitates) of fibronectin. This indicates that fibronectin downregulation in cadherin transfectants occurs independently of the homophilic binding between extracellular domains of the introduced XB-cadherin.

To determine whether downregulation of fibronectin coincides with a reduction of its receptor, β 1 integrin expression was also analyzed. Immunoblots revealed that β 1 integrin expression was diminished in cadherin-transfected XTC cells (Fig. 5A). β 1 integrin synthesis was less downregulated than that of fibronectin. Again, the reduction of β 1 integrin was stronger in XTC-Uvo⁺ cells than in XTC-XB⁺ cells. Three bands of 130, 115, and 100 kDa were identified as radioimmunoprecipitates (Fig. 5B, lane groups 1 to 3). The 115- and 100-kDa bands represent β 1 integrin isoforms due to different posttranslational modifications (18). The majority of β 1 integrin was found to be in the 115-kDa isoform. Both isoforms were diminished to the same extent in XTC transfectants. The observed loss of 42% of β 1 integrin protein in XTC-Uvo⁺ cells explains that the 100-kDa isoform is at the detection limit (Fig. 5A and B, lanes 2, and Table 1). As the reduction in XTC-XB⁺ cells was only about 17% from the wild-type level (Table 1), both isoforms remained visible in immunoblots and immunoprecipitations (Fig. 5A and B, lanes 3, and Table 1). To determine the identity of the 130-kDa signal, β 1 integrin precipitates were transferred to nitrocellulose and probed with α integrin antibodies. The 130-kDa band was identified as α 3 integrin, which coprecipitated with β 1 integrin (Fig. 5C). The decrease of the α 3 integrin coprecipitated in the transfectants

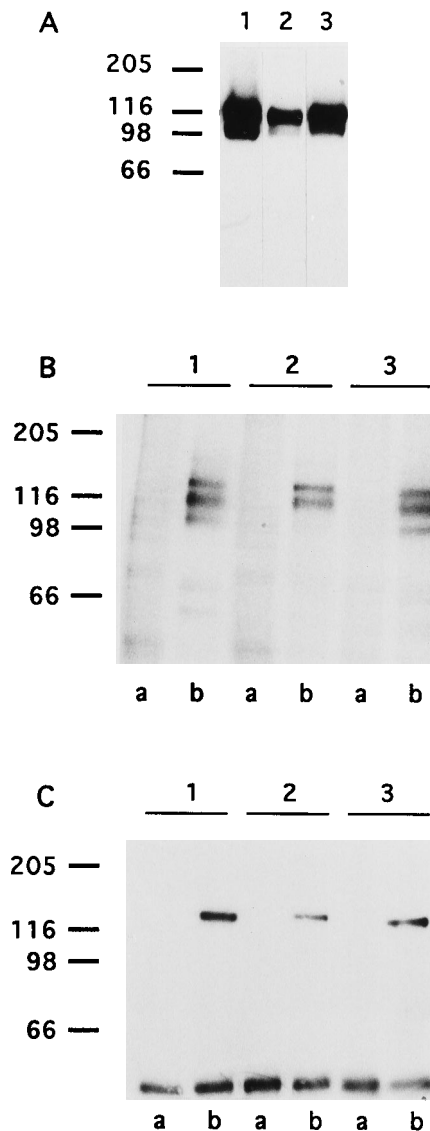


FIG. 5. β 1 integrin protein expression in XTC transfectants. (A) A 2- μ g sample of total protein was separated in each lane under nonreducing conditions and blotted onto nitrocellulose. Lysates of XTC (lane 1), XTC-Uvo⁺ (lane 2), and XTC-XB⁺ (lane 3) cells were probed with β 1 integrin MAb 8C8. (B) XTC (1), XTC-Uvo⁺ (2), and XTC-XB⁺ (3) cells were metabolically labeled with [³⁵S]methionine for 16 h. After lysis, equal amounts of protein were incubated with either nonspecific mouse IgG as controls (lanes a) or with β 1 integrin MAb 8C8 (lanes b). Immune complexes were bound to protein A-Sepharose. Eluted proteins were analyzed on SDS-7.5% PAGE gels under nonreducing conditions. Radioactive precipitates were detected by fluorography. (C) Same immunoprecipitation analyses as in panel B. Eluted proteins were subjected to SDS-PAGE under nonreducing conditions and blotted onto nitrocellulose. The blot was probed with α 3 integrin antibodies and then subjected to immunodetection. The positions of molecular size standards are indicated in kilodaltons.

correlates with the decline of β 1 integrin. The downregulation of fibronectin and β 1 integrin synthesis is a specific effect, since the expression of other proteins, e.g., catenins (Fig. 1C and D) or nucleoplamin (data not shown), was not altered.

To investigate whether reduced quantities of proteins reflected changes in transcription, we performed RNase protection assays. The amount of fibronectin mRNA (Fig. 6) was extremely reduced in cadherin-transfected XTC cells. Amounts of β 1 integrin transcripts decreased to approximately 60% (Fig. 6 and Table 1). For both mRNAs, the decrease was

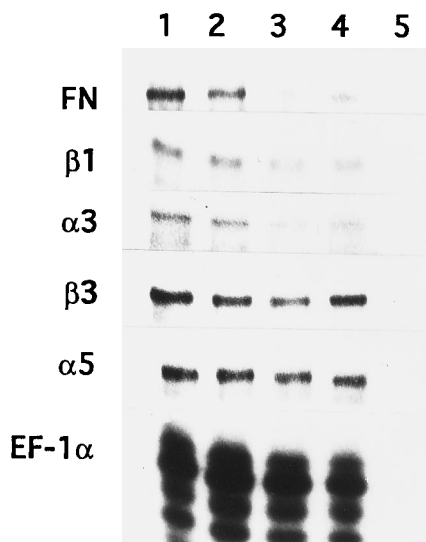


FIG. 6. mRNA expression of fibronectin and integrins in XTC transfectants. Thirty-microgram samples of RNA from XTC (lane 1), vector-transfected XTC (lane 2), XTC-Uvo⁺ (lane 3), and XTC-XB⁺ (lane 4) cells and tRNA (lane 5) were probed with labeled antisense RNA for fibronectin (FN) and different integrins. The EF-1 α probe was used as a control. After RNase digestion and ethanol precipitation, protected fragments were analyzed on denaturing 6% polyacrylamide gels followed by autoradiography. Sizes of protected fragments are 375 nt for fibronectin, 118 nt for $\beta 1$ integrin ($\beta 1$), 460 nt for $\alpha 3$ integrin ($\alpha 3$), 306 nt for $\beta 3$ integrin ($\beta 3$), 275 nt for $\alpha 5$ integrin ($\alpha 5$), and 45 nt for EF-1 α .

stronger in XTC-Uvo⁺ cells than in XTC-XB⁺ cells. When RNase protection was performed with a probe specific for $\alpha 3$ integrin, a decrease of this mRNA was also found to be analogous to that of $\beta 1$ integrin mRNA (Fig. 6 and Table 1). The results shown in Table 1 demonstrate that the amounts of mRNAs correlated with those of the respective proteins.

Thus, reduction of fibronectin and of the $\alpha 3\beta 1$ integrin receptor is induced by transfection of XTC cells with cadherins. Our protein data are in conformity with the results of RNase protection assays. The downregulation of expression is a specific effect, since transcripts of other integrin subunits, such as $\beta 3$, $\alpha 5$ (Fig. 6), $\alpha 2$, $\alpha 4$, and $\alpha 6$ (data not shown), were not affected.

Cadherin-transfected XTC cells lose their substrate adhesive ability. XTC-Uvo⁺, XTC-XB⁺, and vector-transfected and untransfected cells were plated onto fibronectin- or laminin-coated tissue culture wells after the cells had been allowed to recover from trypsinization for 30 min. Adhesion to the substrate was analyzed after 20 min. Untransfected and vector-transfected cells behaved similarly, showing strong adhesion to fibronectin and laminin. Adhesion of XB-cadherin-transfected cells to fibronectin was abolished. Detected binding did not exceed adhesion to BSA, which was used as a nonspecific control (Fig. 7). In contrast, adhesion to laminin showed a reduction to 65% compared with the controls (Fig. 7). A similar result was obtained when XTC-Uvo⁺ cells were analyzed. In contrast to XTC-XB⁺ cells, these cells retained a residual fibronectin adhesion activity of 25%. The adhesion to laminin was in the same range as it was for XTC-XB⁺ cells (Fig. 7). Substrate adhesion of XTC-XB⁺ cells was more severely affected than that of XTC-Uvo⁺ cells.

Substrate adhesion is not affected in XTC cells expressing XB-cadherin with a truncated cytoplasmic domain. A truncated form of XB-cadherin lacking 38 of the most C-terminal amino acids was also expressed in XTC cells via transfection

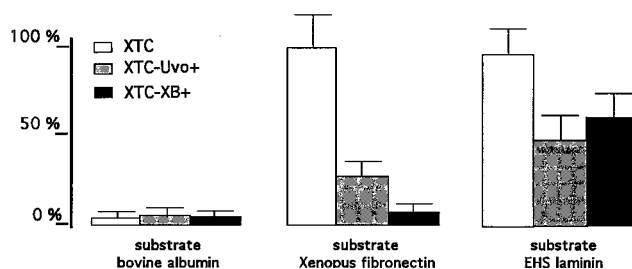


FIG. 7. In vivo adhesion of XTC cells and cadherin transfectants to individual extracellular substrates. Equal numbers of cells were dispersed into wells of 96-well tissue culture plates coated with the respective substrates. Adhesion was allowed to occur for 20 min. Adherent cells were stained with crystal violet, lysed, and quantified photometrically. The data shown are average values of eight separate experiments each comprising 24 wells for every cell-substrate combination. EHS, Engelbreth-Holm-Swarm tumor.

(Fig. 8A). Compared with full-length XB-cadherin, the mutant form showed slightly faster electrophoretic mobility (Fig. 8B, lane 2). The truncated XB-cadherin failed to bind β -catenin in coprecipitation experiments (Fig. 8C, lanes b and c). In contrast to that in XTC-Uvo⁺ and XTC-XB⁺ cells, fibronectin expression was not reduced in XTC-XB Δ 38 transfectants (Fig. 8B, lane 4). RNase protection assays confirmed that the amount of fibronectin mRNA was not altered in XTC cells after transfection with the truncated XB-cadherin (Fig. 9). Expression of $\alpha 3$ integrin and $\beta 1$ integrin was not changed in XTC-XB Δ 38 transfectants either (Fig. 9). In conformity with the integrin data, these transfectants adhered to fibronectin and laminin in the same range as wild-type cells (Fig. 10).

DISCUSSION

Here we report that stable expression of the mouse epithelial cadherin and a *Xenopus* early embryonic cadherin in transfected *Xenopus* XTC cells downregulates certain substrate adhesion molecules, namely, fibronectin and $\alpha 3\beta 1$ integrin. This leads to complete or partial loss of adhesiveness to fibronectin and laminin, respectively. Thus, our results provide strong evidence for an interaction between cell adhesion and substrate adhesion systems in XTC cells. This cross talk is mediated by a change in gene expression or stabilization of specific transcripts, since fibronectin and $\alpha 3\beta 1$ integrin downregulation coincides with the decrease of their mRNAs.

These effects were not dependent on homophilic binding between extracellular domains of transfected cadherins, as blocking of specific cell-cell contacts did not rescue fibronectin synthesis. However, truncation of the cytoplasmic domain by 38 amino acids prevents the cadherins' influence on substrate adhesion.

Despite the high homology in the cytoplasmic domains between uvomorulin and XB-cadherin, cadherin-catenin complexes containing different α -catenin isoforms are formed in XTC cells. The α -catenin isoform in the uvomorulin complex is recognized by antibodies raised against the 12 most C-terminal amino acids of mouse α -catenin (24). The α -catenin isoform in the XB-cadherin complex does not react with these peptide-specific antibodies. However, it is recognized by a MAbs generated against a larger fragment (19.4 kDa, 177 amino acids) of the mouse α -catenin C terminus. Thus, XTC cells express two immunologically distinct α -catenin isoforms. In *X. laevis*, coexpression of closely related protein isoforms is often observed. This might be due to clustered genes arising from tetraploidy of the *X. laevis* genome. Subtypes of catenins, e.g., α N-catenin and α E-catenin (28), were also identified in other species. Both

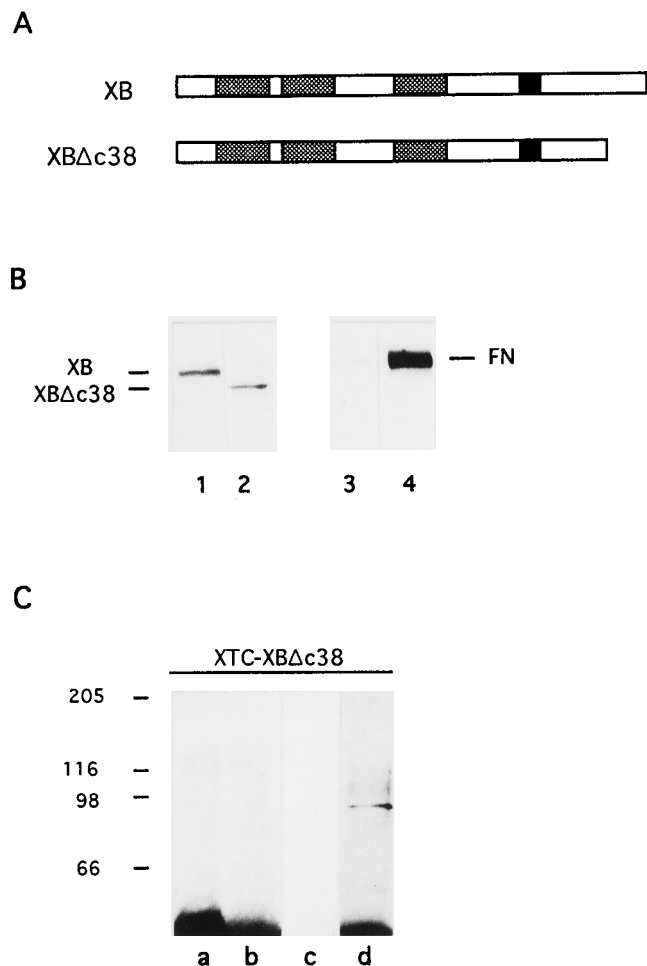


FIG. 8. Truncated XB-cadherin (XBΔc38) neither binds to β -catenin nor downregulates the expression of substrate adhesion molecules. (A) Schematic drawing of the cytoplasmic deletion mutant of XB-cadherin. Dotted boxes represent Ca^{2+} -binding domains, and black boxes represent transmembrane domains. (B) Equal total protein concentrations of XTC-XB⁺ (lanes 1 and 3) and XTC-XBΔc38 (lanes 2 and 4) cell lysates were immunoblotted and probed with MAb 6D5 for detection of XB-cadherin (lanes 1 and 2) and with MAb 6D9 for detection of fibronectin (lanes 3 and 4). (C) Immunoprecipitates of XTC-XBΔc38 lysates with nonspecific IgG (lane a), MAb 6D5 (lane b), and anti- β -catenin antibodies (lanes a, b, and d) or MAb 6D5 (lane c). XB, complete XB-cadherin; XBΔc38, cytoplasmically deleted XB-cadherin; FN, fibronectin. Note that the strong signals at about 45 kDa represent IgGs that were used for immunoprecipitation and detected by the secondary antibodies in the following immunoblot. The positions of molecular size standards are indicated in kilodaltons.

subtypes are able to associate with different cadherins but show *in vivo* preferences for N-cadherin and E-cadherin, respectively.

In transfected mouse fibroblasts (L cells), XB-cadherin integrates the same endogenous α -catenin and β -catenin into its complex as uvomorulin does and mediates Ca^{2+} -dependent cell-cell adhesion (unpublished data). In contrast, in XTC cells, transfected XB-cadherin and uvomorulin recruit different α -catenins into their complexes. Therefore, we conclude that the composition of the complex depends on cellular characteristics.

Although XB-cadherin and uvomorulin are bound to different α -catenins in XTC cells, they interact with the same cellular signaling process in that the same target molecules are downregulated. Complex formation of the transfected cad-

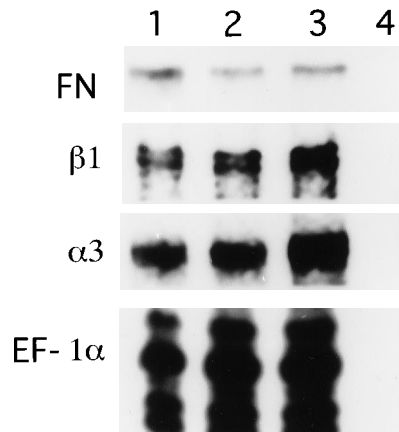


FIG. 9. mRNA expression of fibronectin (FN) and integrins in XTC-XBΔc38 transfectants in comparison with XTC cells and vector-transfected XTC cells. Thirty-microgram samples of RNA from XTC (lane 1), vector-transfected XTC (lane 2), XTC-XBΔc38 (lane 3), and tRNA (lane 4) were probed with labeled antisense RNA for fibronectin and different integrins. The EF-1 α probe was used as a control. After RNase digestion and ethanol precipitation, protected fragments were analyzed on denaturing 6% polyacrylamide gels followed by autoradiography. Sizes of protected fragments are 375 nt for fibronectin, 118 nt for β 1 integrin (β 1), 460 nt for α 3 integrin (α 3), and 45 nt for EF-1 α .

herins with catenins appears to be a prerequisite for altering the synthesis of substrate adhesion proteins. Expression of a truncated XB-cadherin which fails to complex with these proteins does not result in downregulation of fibronectin and α 3 β 1 integrin. β -Catenin is present in uvomorulin and XB-cadherin complexes of XTC cells. The total amount of this catenin is maintained after transfection. Therefore, we assume a competitive situation for the pool of β -catenin between binding to the transfected cadherin and its putative role in an unknown signaling pathway. Recent reports on β -catenin binding and its relationship to other proteins involved in signaling pathways support our idea of this catenin as a possible candidate mediating the cross talk between the different adhesion systems in XTC cells. β -Catenin shows striking homology at the amino acid level to the *armadillo* gene product (41, 49–51). The latter is known to act downstream in the *wingless* signaling pathway in *Drosophila*. In *wnt-1*-transfected murine epithelial cells, cadherin- β -catenin binding was shown to be stabilized (27). Further evidence that β -catenin participates in signaling pathways comes from recent reports by Rubinfeld et al. (55) and Su et al.

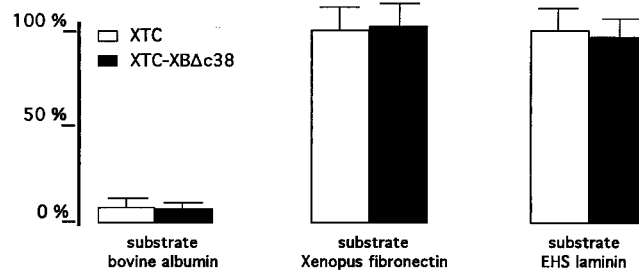


FIG. 10. *In vivo* adhesion of XTC cells and XTC-XBΔc38 transfectants to individual extracellular substrates. Equal numbers of cells were dispersed into wells of 96-well tissue culture plates coated with the respective substrates. Adhesion was allowed to occur for 20 min. Adherent cells were stained with crystal violet, lysed, and quantified photometrically. The data shown are average values of nine separate experiments each comprising 24 wells for every cell-substrate combination. EHS, Engelbreth-Holm-Swarm tumor.

(59). They demonstrated that the *APC* gene product, a cytosolic tumor suppressor protein, binds to catenins. The function of the APC protein is not understood. However, their results imply that within a cell, the APC protein and the cadherins might compete for binding of catenins. Hinck et al. (26) presented a convincing model that takes into account an exchange of catenins between membrane-bound, as well as cytosolic, complexes. For both cadherin-transfected cell lines XTC-Uvo⁺ and XTC-XB⁺, we have shown that β -catenin is shifted from the cytosolic to the membrane fraction upon expression of exogenous cadherins. Thus, our results are in good agreement with the model of Hinck et al. (26).

Conversion of mesenchyme to epithelium is defined by induction of cell polarity, which results in an altered cell shape. In cell culture systems, this morphoregulatory capacity could be assigned to uvomorulin-E-cadherin (42). For kidney development, it was shown that epithelialization in terms of polarization requires the interaction of integrins with the C terminus of the laminin A chain, whereas cadherins play a secondary role in this process (16, 35). Although we did not observe striking alterations in cell shape, we suggest that cadherins are able to initiate a change in the cell type of XTC fibroblasts and in this broader sense possess morphoregulatory abilities. The points of evidence are as follows. (i) Fibronectin synthesis, one of the general characteristics of fibroblasts, becomes downregulated. (ii) The integrin receptor pattern is altered. The loss of function of $\alpha 3\beta 1$ integrin in fibronectin binding provides new possibilities for the cells in terms of capacities to bind to other extracellular components. These interactions could also be mediated by other integrins which gain the advantage of the $\alpha 3\beta 1$ receptor deficiency. Integrin- or extracellular matrix-mediated changes in gene expression do not necessarily coincide with altered cell morphology (1, 63). Terminal differentiation of keratinocytes is accompanied by loss of $\alpha 2\beta 1$, $\alpha 3\beta 1$, and $\alpha 5\beta 1$ receptors (2, 3). More recently, Hodivala and Watt (29) indirectly showed by in situ hybridization that $\alpha 6$ and $\beta 1$ integrin mRNAs were more abundant in some keratinocytes when monolayers were cultured in the presence of E- or P-cadherin antibodies. These results indicate that Ca^{2+} -dependent cell-cell adhesion may inhibit stratification of keratinocytes by preventing the downregulation of integrins. In contrast, downregulation of fibronectin synthesis in XB-cadherin-transfected XTC cells seems to be independent of the extracellular binding domain: when we blocked interaction between XB-cadherin molecules by culturing XTC-XB⁺ cells in the presence of Mab 6D5 Fab fragments, fibronectin synthesis was not reactivated. Taken together, the results from cell culture systems as different as keratinocytes and XTC cells support the idea that the cadherin-catenin system influences cell-substrate adhesion. As a result, the altered integrin and extracellular matrix pattern might initiate a change of cell fate. These findings have important implications for the understanding of cadherin-controlled tissue differentiation in *Xenopus* embryogenesis.

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