Protein Kinase C Activation Upregulates Intercellular Adhesion of α-Catenin–negative Human Colon Cancer Cell Variants via Induction of Desmosomes

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Abstract. The α-catenin molecule links E-cadherin/β-catenin or E-cadherin/plakoglobin complexes to the actin cytoskeleton. We studied several invasive human colon carcinoma cell lines lacking α-catenin. They showed a solitary and rounded morphotype that correlated with increased invasiveness. These round cell variants acquired a more normal epithelial phenotype upon transfection with an α-catenin expression plasmid, but also upon treatment with the protein kinase C (PKC) activator 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). Video registrations showed that the cells started to establish elaborated intercellular junctions within 30 min after addition of TPA. Interestingly, this normalizing TPA effect was not associated with α-catenin induction. Classical and confocal immunofluorescence showed only minor TPA-induced changes in E-cadherin staining. In contrast, desmosomal and tight junctional proteins were dramatically rearranged, with a conversion from cytoplasmic clusters to obvious concentration at cell–cell contacts and exposition at the exterior cell surface. Electron microscopical observations revealed the TPA-induced appearance of typical desmosomal plaques. TPA-restored cell–cell adhesion was E-cadherin dependent as demonstrated by a blocking antibody in a cell aggregation assay. Addition of an antibody against the extracellular part of desmoglein-2 blocked the TPA effect, too. Remarkably, the combination of anti–E-cadherin and anti-desmoglein antibodies synergistically inhibited the TPA effect.

Our studies show that it is possible to bypass the need for normal α-catenin expression to establish tight intercellular adhesion by epithelial cells. Apparently, the underlying mechanism comprises upregulation of desmosomes and tight junctions by activation of the PKC signaling pathway, whereas E-cadherin remains essential for basic cell–cell adhesion, even in the absence of α-catenin.

Cell adhesion is essential for a wide variety of regulatory and developmental processes. The cadherins comprise a family of transmembrane, cell surface glycoproteins that mediate Ca^{2+}-dependent cell–cell adhesion (Takeichi, 1995). In cells with well developed intercellular junctions, E-cadherin is concentrated in the adherens junctions but appears to influence other intercellular junctions such as gap junctions (Jongen et al., 1991; Musil et al., 1990), tight junctions (Gumbiner et al., 1988; Watabe et al., 1994), and desmosomes (Gumbiner et al., 1988; Watabe et al., 1994; Amagai et al., 1995). E-cadherin also plays a crucial role in development and maintenance of cell polarity (Nelson, 1992; Eaton and Simons, 1995), and its dysfunction has been strongly implicated in the invasiveness and carcinogenesis of tumor cells and human tumors (Vleminckx et al., 1991; Schipper et al., 1991; Berx et al., 1995, 1996; Bracke et al., 1996).

Extracellularly, the individual cadherin molecules are proposed to associate in a homophilic way to promote specific cell–cell interactions (Shapiro et al., 1995; Nagar et al., 1996). Intracellularly, they interact with a group of proteins, collectively termed catenins. α-catenin has been postulated to link either cadherin/β-catenin or cadherin/plakoglobin complexes to F-actin, either directly or indirectly via α-actinin (Knudsen et al., 1995; Rimm et al., 1995). The $p120^{cas}$ protein associates also directly with the cytoplasmic domain of E-cadherin, but is not binding to α-catenin (Daniel and Reynolds, 1995). α-catenin is divided into two subtypes: αE-catenin, which is expressed in many kinds of tissues (Nagafuchi et al., 1991; Herrenknecht et al., 1991; Nagafuchi and Tsukita, 1994), and αN-catenin, expressed

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mainly in the nervous system (Hirano et al., 1992; Uchida et al., 1994). For both subtypes two isoforms are known to be expressed (Oda et al., 1993; Rimm et al., 1994).

In malignant cancers, inhibition of cell–cell adhesion is indispensable as first step in the metastatic process. It has been suggested that this detachment is related to reduced expression of E-cadherin (van Roy and Mareel, 1992). Moreover, it has been reported that in certain malignancies, disruption of cell–cell adhesion was caused by reduced functionality of E-cadherin even though its expression level was normal (Bracke et al., 1996). Shimoyama et al. (1992) reported an impairment of intercellular adhesion in the lung cancer cell line PC-9, resulting from the loss of expression of αE-catenin. Using this cell line, recovery of E-cadherin function by transfection of αN-catenin or αE-catenin cDNA was demonstrated (Hirano et al., 1992; Watabe et al., 1994). A similar observation of impaired adhesion was made by Morton et al. (1993) for the prostate cancer cell line PC-3 and ascribed to deletion of α-catenin genes. Vermeulen et al. (1995) showed that transition from the noninvasive to the invasive phenotype might occur during culture of the human colon cell lines, HCT-8 and DLD-1. This transition was associated with loss of αE-catenin expression. Furthermore, in particular human cancer tissues immunohistological studies confirmed the loss of α-catenin expression in vivo (Kadowaki et al.,1994; Shiozaki et al., 1994). In the human gastric cancer cell line HSC-39, loss of E-cadherin–mediated intercellular adhesion was explained by an in frame deletion of the β-catenin gene (Oyama et al., 1994; Kawanishi et al., 1995). Also, tyrosine phosphorylation of catenins and cadherins down-modulates the function of the cadherin/catenin system (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993; Empereur et al., 1997).

In addition, phosphorylation by protein kinase C (PKC)[1] represents a regulatory mechanism of intercellular adhesion. The PKC protein family comprises several isoforms of related serine/threonine-specific, phospholipid-dependent kinases (Dekker et al., 1995). The phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) is a potent activator of PKC. TPA has been shown to induce premature compaction in the mouse embryo and a coincidental shift in the localization of E-cadherin to the cell–cell contacts (Winkel et al., 1990). A monoclonal antibody to E-cadherin as well as sphingosine, a potent PKC inhibitor, could completely block this premature compaction.

In the present work, we analyzed the effect of the PKC activator TPA on invasive α-catenin–negative human colon cell lines. The cell lines used are the HCT-8 derivatives described before by Vermeulen et al. (1995). Our findings reveal that it is possible to bypass by PKC activation the need for α-catenin to establish tight intercellular adhesion. The underlying mechanism implicates a striking induction of desmosomal junctions.

Materials and Methods

Cell Culture and Transfection

Human colon carcinoma cell lines were obtained and characterized as described previously (Vermeulen et al., 1995). In brief, epithelioid variants HCT-8/E8, HCT-8/E10, and HCT-8/E11 were obtained by subcloning the HCT-8 cell line (ATCC CCL24; American Type Culture Collection, Rockville, MD). In this and the following nomenclature, E stands for epithelioid and substitutes for S, used before to indicate the smooth edges of epithelial cell colonies. HCT-8/E11R1 (R, round cell variants) were obtained by subcloning HCT-8/E11 cultures and selecting for a round cell morphotype. HCT-8/R1 and DLD-1/R1 cell lines were obtained by subcloning and selecting G418-resistant round cell variants from, respectively, HCT-8 and DLD-1 (ATCC CCL221) cultures that were electroporated with the pPNT vector, encoding a neo^r gene (Tybulewicz et al., 1991). All cells were cultured in RPMI 1640 (GIBCO BRL, Ghent, Belgium), supplemented with 10% FCS, 100 μg/ml streptomycin, and 250 IU/ml penicillin. HCT-8 derivatives received 1 mM sodium pyruvate, whereas 2 mM L-glutamine was added to DLD-1 derivatives. Cells were used between passage 5 and 30 after subcloning.

The expression vector of chicken αN-catenin cDNA, pMiw-αN (Hirano et al., 1992), was a generous gift from Dr. S. Hirano and Dr. M. Takeichi (Kyoto University, Kyoto, Japan). HCT-8/E11R1 cells (4×10^6 cells/75 cm^2) were cotransfected by calcium phosphate coprecipitation with 20 μg of linearized pMiw-αN plus 1 μg of linearized pSV2neo. The cells were replated and cultured in the presence of 600 μg/ml of G418 to select stable transfectants. Colonies of G418-resistant cells were isolated, tested by immunofluorescence for expression of αN-catenin, and recloned if necessary. Several stable clones were isolated.

Antibodies and Reagents

The following mouse monoclonal antibodies were used: antibodies HECD-1 (Takara Biochemicals, Otsu, Japan) and MB2 (Bracke et al., 1993) both functionally blocking human E-cadherin, 1G5 against α-catenin (Becton Dickinson, San Jose, CA), anti–β-catenin against β-catenin (Transduction Laboratories, Lexington, KY), anti-pp120 against $p120^{cas}$ (Transduction Laboratories), PG 5.1 against plakoglobin and DG 3.10 against desmoglein (both from Cymbus Bioscience Ltd., Southampton, UK), 10G11 against desmoglein-2 (Progen, Heidelberg, Germany), DP 2.15 against desmoplakin I and II (Boehringer Mannheim, Mannheim, Germany), hVIN-1 against vinculin (Sigma Immunochemicals, St. Louis, MO), and htr-5 against the human TNF-receptor p55 (Brockhaus et al., 1990). The rabbit polyclonal antibody pan-cadherin was obtained from Sigma. The rat monoclonal MOC37 against human occludin and the mouse monoclonal antibody against ZO-1 were kindly provided by, respectively, Dr. M. Furuse (Furuse et al., 1994) and Dr. M. Itoh (Itoh et al., 1991). The rat monoclonal antibody NCAT-2 against αN-catenin and the rat monoclonal antibody α18 against α-catenin were generous gifts of Dr. S. Hirano and Dr. M. Takeichi (Hirano et al., 1992) and Dr. A. Nagafuchi (Nagafuchi and Tsukita, 1994), respectively. Secondary and tertiary antibodies coupled to biotin, FITC, or Texas red were obtained from Amersham Life Science (Buckinghamshire, UK).

The concentration of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA; Sigma) was 250 ng/ml, unless otherwise stated. A stock solution of TPA was made at 250 μg/ml in DMSO. The final concentration of DMSO in the culture medium was adjusted to 0.1%, and at this concentration the solvent alone had minor or no effects on the cells. 1,2-dioctanoyl-*sn*-glycerol (Sigma) was made up at a concentration of 50 mm in DMSO and used at a final concentration of 0.5 mM.

Video Registration

To evaluate the effect of TPA on living cultures, time-lapse phase contrast video registrations were made. Cells were placed on a Zeiss IM inverted phase contrast microscope that was set up in a thermostabilized chamber at 37°C, with continuous gassing with humidified air containing 5% CO_2. An F-LD 20/0.25 objective was used in combination with a long working distance condensor (Zeiss No. 465224). Image capture was done via a high resolution WV-1850C camera (National Panasonic, Tokyo, Japan) equipped with an animation control unit AC-580 (EOS, Barry, UK), a time date generator, and an impulse generator. For time-lapse films, two video images were recorded every 15 s. The video films were viewed at the normal play-back speed of 25 images per second.

Ca^{2+}-dependent Fast Aggregation

Cell–cell adhesion was numerically evaluated in an aggregation assay as described before (Bracke et al., 1993). In brief, cultures were dissociated

1. *Abbreviations used in this paper*: PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

into single cell suspensions under E-cadherin–saving conditions. They were incubated under Gyrotory shaking at 80 rpm for 30 min in an isotonic buffer containing either 1 mM EGTA or 1.25 mM Ca^{2+}. Treatment of cells at 4°C with various antibodies was started 30 min before the aggregation period and continued throughout incubation at 37°C. The aggregation index was expressed as $1-(N_{30}/N_0)$, where N_0 indicates the initial number of particles and N_{30} the number of particles after 30 min of aggregation as measured by a Coulter counter ZM (Coulter Electronics Ltd., Luton, UK). All N_0 and N_{30} measurements were done in duplicate and the experiments were repeated at least twice. Cell aggregation was also measured by use of an LS particle size analyzer (Coulter). Not the decreasing number of particles but the increasing size of particles was then used as an indicative parameter for aggregation.

Metabolic Labeling and Immunoprecipitation

Cells were incubated for 3 h with [^{35}S]methionine/cysteine (125 μCi/ml, Promix, SJQ 0079; Amersham Life Science) in methionine- and cysteine-free MEM containing 5% dialyzed FCS. The cells were rinsed and extracted in a lysis buffer A, containing 0.5% NP-40, 6 mM $CaCl_2$, 5 mM $MgCl_2$, 8 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 0.3 μM aprotinin in PBS. Samples were diluted to contain equivalent trichloroacetic acid-precipitable radioactivity, followed by preabsorption with protein-G Sepharose 4 Fast Flow beads (Pharmacia, Uppsala, Sweden) for 1 h. Then, the supernatant was incubated with 1 μg mAb for 3 h at 4°C. Subsequently, protein-G Sepharose 4 was added for one h. Adsorbed immunoprecipitates were washed four times with lysis buffer and eluted with sample buffer (Laemmli, 1970). Protein separation was by 8% SDS-PAGE. Radioactive signals were amplified by En^{3}hance (Du Pont, Boston, MA) before fluorography. Precipitated proteins were quantified with the aid of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In some experiments, cells were metabolically labeled for 3 h with [^{32}P]orthophosphate (PBS 13; Amersham Life Science) using 0.5 mCi/ml in phosphate-free MEM. Cells were extracted in lysis buffer A, which contained also the phosphatase inhibitors NaF at 10 mM and Na_3VO_4 at 1 mM.

Biotinylation of Cell Surface Proteins

Proteins at the exterior cell surface were specifically labeled by biotinylation with the membrane-impermeable reagent Sulfo-NHS-biotin (Pierce Chemical Co., Rockford, IL) in PBS for 30 min at 4°C. Untreated cells were compared with TPA-treated cells. After labeling, the monolayers were washed four times with PBS and extracted with 0.5% NP-40, 0.5% deoxycholate, 0.1% SDS, 8 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 0.3 μM aprotinin in PBS. Proteins (400 μg) were incubated with 50 μl of 50% avidin-agarose (Pierce) for 18 h at 4°C to recover the biotinylated proteins. The complexes were washed four times with lysis buffer and proteins were eluted with sample buffer and resolved by 8% SDS-PAGE. Proteins were detected by immunoblotting as described below.

Immunoblotting

Cells were dissolved in sample buffer (Laemmli, 1970) and boiled in the presence of 5% 2-mercaptoethanol. Proteins were separated by 8% SDS-PAGE, and transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA). After being blocked with 5% nonfat dry milk in PBS, 0.01% Tween-20, the membranes were incubated with the primary antibody. After extensive washing, the membranes were incubated with alkaline phosphatase-conjugated antibodies using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as substrate.

Cell Fractionation

Cells in 80-cm^2 culture dishes were washed three times with PBS, collected into a 1.5-ml microtube by scraping, and pelleted by mild centrifugation. After removing the supernatant, 100 μl of 2.5% NP-40, 8 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 0.3 μM aprotinin in PBS was added to the cell pellet. Samples were incubated for 5 min with mild pipetting, and then centrifuged at 22,000 *g* for 15 min. Three-times concentrated sample buffer (Laemmli, 1970) was added to the supernatant, to make a total volume of 150 μl, and used as the detergent soluble fraction. On the other hand, the pellet fraction was dissolved in 100 μl of sample

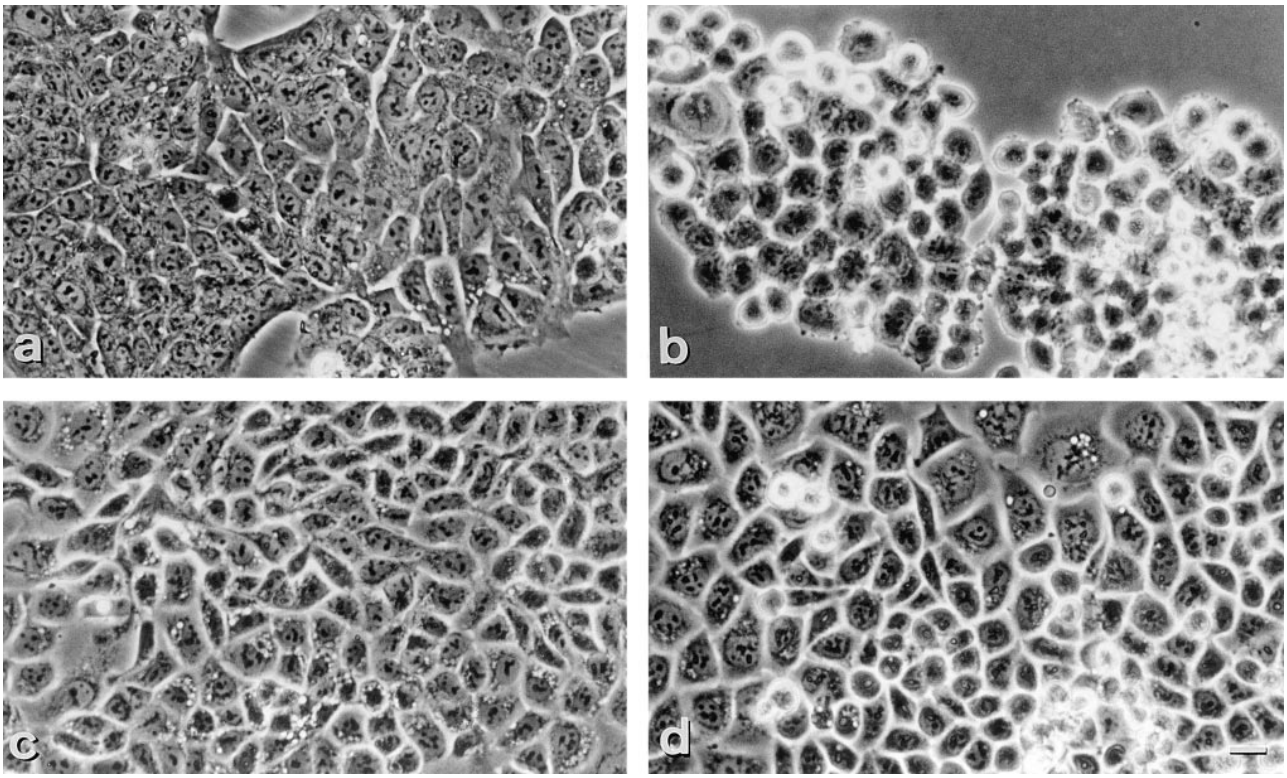

Figure 1. TPA induces an epithelioid morphotype in round cell variants of colon cancer cells HCT-8. Epithelioid (HCT-8/E8, *a* and *c*) and round cell variants (HCT-8/R1, *b* and *d*) were either untreated (*a* and *b*) or treated with 250 ng/ml TPA for 3 h (*c* and *d*). Similar observations were done for other round cell variants of HCT-8 and DLD-1. Bar, 20 μm.

buffer and used as the detergent insoluble fraction. Both fractions were subjected to immunoblotting.

Immunofluorescence Microscopy

Monolayers prepared for fluorescent staining were grown on glass coverslips or on tissue culture-treated polycarbonate filters with a pore size of 0.4 μm (Transwell; Costar Corp., Cambridge, MA). Cell cultures were treated with the appropriate agents, rinsed briefly with PBS, and fixed with either ice-cold 100% methanol for 15 min at −20°C or with 3% paraformaldehyde in PBS for 20 min at room temperature. Fixation by paraformaldehyde was followed by quenching in 50 mM NH_4Cl solution in PBS and by permeabilization in 0.2% (wt/vol) Triton X-100 in PBS for 5 min at room temperature. Then, cells were incubated for 1 h at 37°C with primary antibody diluted in PBS, 0.04% gelatin. A washing step was followed by the biotinylated secondary antibody under the same conditions and then by Texas red–conjugated streptavidin for 30 min at 37°C. Finally, cells were incubated in a 4′-6-diamidino-2-phenylindole-solution and mounted with Glycergel (Dako Corporation, Carpinteria, CA) or Vectashield (Vector Laboratories, Burlingame, CA) to prevent photobleaching. Samples were examined with a Zeiss Axiophot photomicroscope or with a Zeiss LSM 410 confocal laser-scanning immunofluorescence microscope (Carl Zeiss, Jena, Germany).

Electron Microscopy

For the morphological analysis of ultrathin sections via EM, cells were grown on Permanox plastic (Nunc, Roskilde, Denmark) and fixed with 2% glutaraldehyde in 100 mM cacodylate buffer (pH= 7.4) for 2 h. After washing with plain buffer, samples were postfixed in 1% (wt/vol) OsO_4 in 100 mM cacodylate buffer for 1 h. Samples were then dehydrated with a graded series of ethanol and embedded in Epoxy Resin (ERL 4206; Merck, Darmstadt, Germany). Ultrathin sections (50 nm) were cut with a diamond knife, double stained with uranyl acetate and lead citrate, and examined under an electron microscope (1200 EX II; Jeol, Tokyo, Japan) at an accelerating voltage of 80 kV.

Results

Characterization of Round Cell Variants of Human Cancer Cells

Round cell (R) variants, e.g., HCT-8/R1, HCT-8/E11R1, DLD-1/R1, seemed to emerge in a spontaneous way from the human colon cancer cell lines HCT-8 and DLD-1 (Vermeulen et al., 1995). In contrast to the epithelioid subclones (Fig. 1 *a*), R-variants grew as loosely adherent clusters of cells, including spherical halo-forming cells on top of confluent parts of the monolayer (Fig. 1 *b*). At the protein level, the R-variants were found to be negative for α-catenin as revealed by Western blotting and coimmunoprecipitation experiments (Fig. 2, *a* and *b*; Vermeulen et al., 1995). Nevertheless, they contained both E-cadherin and β-catenin in a macromolecular complex containing additional proteins too (Fig. 2 *b*). E-cadherin–mediated cell–cell adhesion of R-variants was strongly reduced, as demonstrated in the Ca^{2+}-dependent fast aggregation assay (Fig. 3; Vermeulen et al., 1995). Dysfunction of the E-cadherin/catenin complex was also demonstrated by formation of loose colonies inside collagen and by invasion into organ cultures, whereas the epithelioid E-variants formed tight colonies inside collagen and were noninvasive (Vermeulen et al., 1995). In the following experiments, we used the HCT-8/E8 and HCT-8/R1 cell lines as prototypes of variants with, respectively, the epithelioid (E) and round (R) morphotype. Several other isolated S- and R-clones (see Materials and Methods) behaved essentially in the same way.

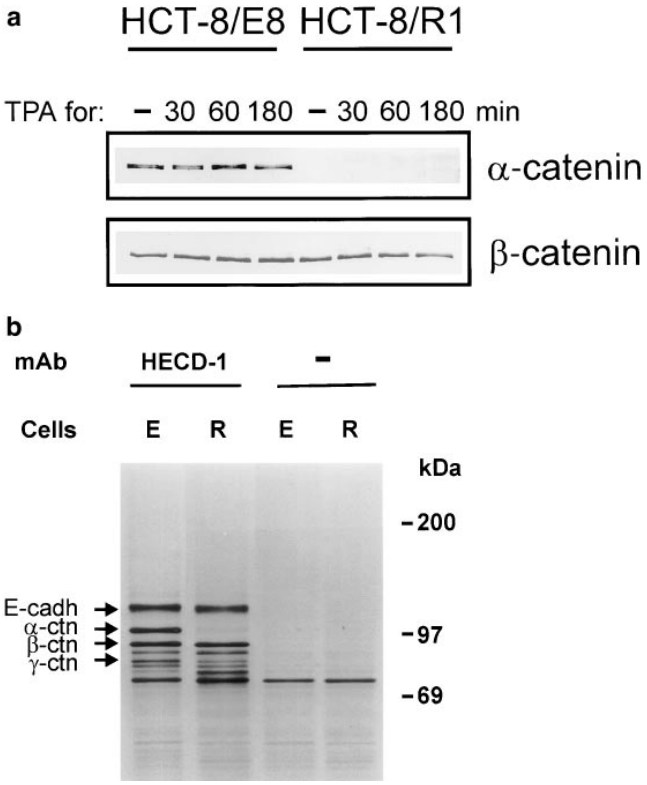

Figure 2. Round cell variants of HCT-8 lack α-catenin in the E-cadherin/catenin complex. (*a*) Western blotting for α- and β-catenin in total lysates of the epithelioid variant HCT-8/E8 and the round cell variant HCT-8/R1. Cells were treated with TPA for the time periods indicated. (*b*) Metabolically labeled cell extracts of the epithelioid variant HCT-8/E10 (*E*) and of the round cell variant HCT-8/R1 (*R*) were subjected to immunoprecipitation with (*left two lanes*) or without (*right two lanes*) E-cadherin–specific mAb HECD-1. Positions of E-cadherin (*E-cadh*) and catenins (*ctn*) are indicated by arrows. Molecular mass markers are indicated on the right. In cell line HCT-8/E10, E-cadherin was associated with α-, β-, and γ-catenin (*left lane*). The round cell variant HCT-8/R1 lacked α-catenin in the complex (*second lane*).

TPA Treatment Restores Cell–Cell Adhesion of R-variants

Interestingly, addition of TPA (250 ng/ml) to adherent colonies of R-variants of HCT-8 or DLD-1 caused striking alterations in cell morphology. By time-lapse video registration, nonconfluent monolayers of cells were followed before and during 4 h of TPA treatment. The first changes were apparent within 30 min. The treated cells adopted progressively a typical polygonal epithelioid morphology (illustrated for HCT-8/R1 in Fig. 1 *d*). Besides this remarkable change in R-variants, TPA showed additional effects that were similar for both epithelial and round cell variants. Cell ruffling activity increased very much as well as cell spreading on the surface in the case of subconfluent cultures (Fig. 1, *c* and *d*; video registration data not shown).

In line with the TPA-induced morphological changes in R-variants, we noticed in a cell–cell aggregation assay that, upon treatment with increasing doses of the phorbol ester TPA, the deficient aggregation of R-variant cell suspen-

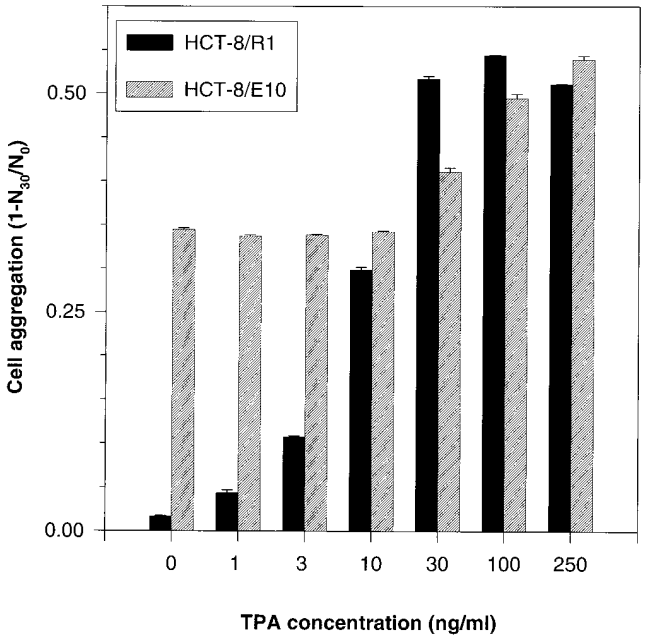

Figure 3. TPA induces cell aggregation in α-catenin–negative variants of HCT-8. Monolayer cultures of the epithelioid HCT-8/E10 cell line and of the α-catenin–negative variant HCT-8/R1 were pretreated for 3 h with the indicated amounts of TPA. Then, cells were dissociated under E-cadherin saving conditions and allowed to reaggregate from single cell suspensions for 30 min at 37°C in the presence of the same TPA concentrations. The degree of cell aggregation (decreased particle number) was calculated using the formula $1\text{-}N_{30}/N_0$. Similar observations were done for other round cell variants of HCT-8 and DLD-1.

sions could be progressively normalized to the aggregation level of the epithelioid E-variants (Fig. 3). Three independently obtained round cell populations, HCT-8/R1, HCT8/E11R1, and DLD-1/R1, responded in the same way. This restoration was evident as soon as 30 min after application of 100 ng/ml TPA (data not shown). In most subsequent experiments TPA was used at 250 ng/ml. The restored intercellular adhesion was Ca^{2+}- and E-cadherin–dependent since it could be prevented by adding, together with TPA, either EGTA (data not shown), or the E-cadherin blocking monoclonal antibody MB2 (open circles in Fig. 4 *a*). These features resemble the Ca^{2+}- and E-cadherin–dependent adhesion observed in the E-variants in the absence of TPA (as shown for HCT-8/E8 in Fig. 4 *b*). TPA (≥30 ng/ml) increased also to some extent the cell–cell aggregation of the E-variants HCT-8/E8 (not shown) and HCT-8/E10 (Fig. 3). TPA-induced intercellular adhesion was transient for both cell types, as might be expected for a PKC activation process (Hecht et al., 1994). By 6 h after starting the TPA treatment, cell–cell adhesion was abolished again (data not shown). Finally, 1,2-dioctanoyl-*sn*-glycerol, a synthetic analogue of diacyl glycerol, showed at 0.5 mM similar effects to those of 250 ng/ml TPA in HCT-8/R1 cells (not shown).

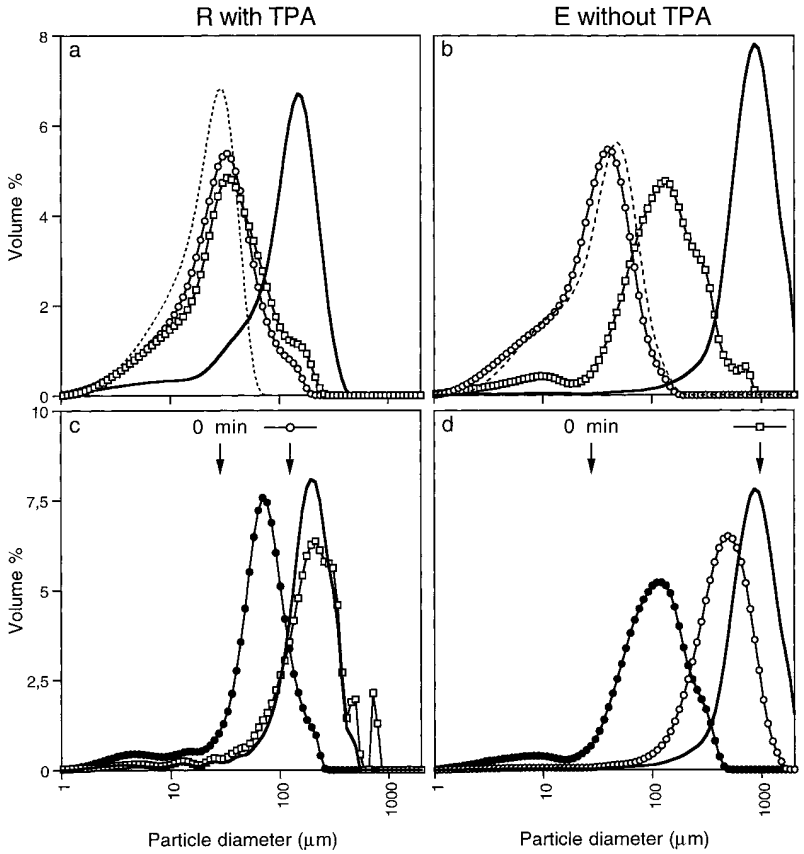

Figure 4. Cell–cell adhesion via E-cadherin and desmoglein-2 occurs by a cooperative mechanism in both E-variants and TPA-treated R-variants. Monolayers of HCT-8/R1 cells (*R*) were treated with 250 ng/ml TPA as indicated in Materials and Methods. Monolayers of HCT-8/E8 cells (*E*) were not treated with TPA here (*b* and *d*), although analysis of TPA-treated E-variants yielded essentially identical data (not shown). Both R- and E-variants were analyzed for cell aggregation (increasing particle volume) either after 0 min (broken lines in *a* and *b*; peak positions indicated by 0-min arrows in *c* and *d*) or after 30 min of incubation at 37°C (full lines in all panels). Aggregation for 30 min by R-variants, which were not treated with TPA, yielded curves coinciding with the 0-min curves (not shown). Other curves depicted correspond with the following antibody treatments (see also Materials and Methods). The mAb MB-2 against human E-cadherin (O) was used at an effective concentration (dilution 1:20; *a* and *b*), or at a more ineffective concentration (1:200; *c* and *d*, with in *c* only peak position indicated by an arrow). The mAb 10G11 against desmoglein-2 (□) was also used at an effective concentration (1:2; *a* and *b*), or at an ineffective concentration (1:8; *c* and *d*, with in *d* only peak position indicated by an arrow). These two antibodies, both at the lower concentrations, were also combined as indicated by filled circles (●) in *c* and *d*. Upon such combination, a clear-cut synergistic inhibition of cell aggregation was observed for both cell types.

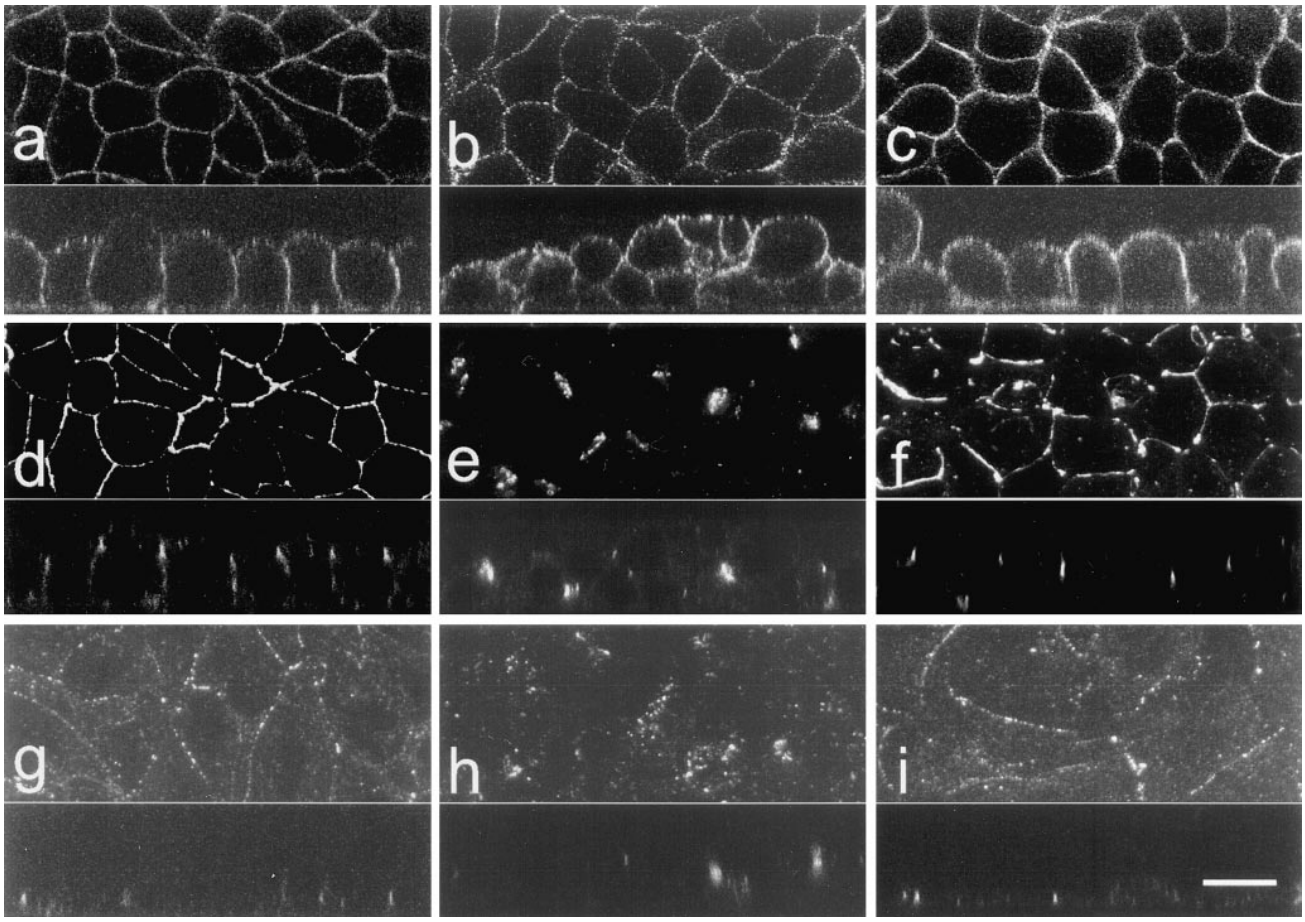

Figure 5. No major effect on E-cadherin, but formation of typical tight junctions and spot desmosomes upon TPA treatment of α-catenin–negative variants of HCT-8. Cells were double stained for E-cadherin (*a–c*) and occludin (*d–f*), or stained for desmoglein-2 (*g–i*). Each panel comprises an extended depth view by overlaying several confocal *X-Y* sections (*top*) and confocal *X-Z* sections of the same field (*bottom*). Cells analyzed were: epithelioid HCT-8/E8 cells (*a*, *d*, *g*), untreated HCT-8/R1 cells (*b*, *e*, *h*), and TPA-treated (250 ng/ml for 3 h) HCT-8/R1 cells (*c*, *f*, *i*). Bar, 14 μm.

TPA Effects on the E-Cadherin/Catenin Complex of R-variants

TPA induction of cell–cell adhesion by α-catenin–negative tumor cells may occur via upregulation of the E-cadherin function. Therefore, the molecular elements of the E-cadherin/catenin complex were studied by immunoblotting, immunohistochemistry, and coimmunoprecipitation. HCT-8 cells have an epithelial origin and express E-cadherin as expected. Another classical cadherin was not present as demonstrated by immunoblotting with a pan-cadherin antibody (results not shown). The simplest explanation for the observed phenomenon is that TPA induces α-catenin expression. This was assessed by Western blotting with two different mAb recognizing, respectively, the NH_2-terminal and middle part of αE-catenin. TPA did not at all induce α-catenin expression in HCT-8/R1 cells and also the level of β-catenin expression was not influenced by TPA treatment (Fig. 2 *a*; plus data not shown).

For immunohistochemistry, cells were treated by TPA for increasing time periods, fixed, and stained with antibodies against E-cadherin, β-catenin, and $p120^{cas}$ proteins. Both classical and confocal immunofluorescence was applied. In untreated E- and R-variants, these three proteins were distributed predominantly at plasma membrane sites involved in lateral cell–cell contacts (illustrated for E-cadherin in Fig. 5, *a* and *b*). The spherical cells on top of monolayers of R-variants especially expressed E-cadherin on their whole surface (Fig. 5 *b*). Treatment with TPA for 3 h emphasized the honeycomb pattern of E-cadherin staining, particularly in R-variants, but there was no evidence for redistribution of E-cadherin, β-catenin, or $p120^{cas}$ into restricted positions (adherens junctions) at the lateral cell–cell contacts (Fig. 5 *c*, and data not shown).

We next addressed the question whether vinculin, which shows sequence similarities to α-catenin (Herrenknecht et al., 1991), would replace α-catenin in the E-cadherin/catenin complex of TPA-treated R-variants. In the untreated cells, vinculin is found at the level of the substratum contacts. TPA treatment partly redistributed the vinculin to a diffuse cytoplasmic staining but not at all to cell–cell contacts (data not shown). Moreover, coimmunoprecipitation experiments did not show any change in the E-cadherin/catenin complex upon TPA treatment. The same pattern was seen as depicted in Fig. 2 *b*, and neither α-catenin nor another protein like vinculin seemed to be induced by TPA in the complex (Fig. 2 *a*; and data not shown). This was also obvious

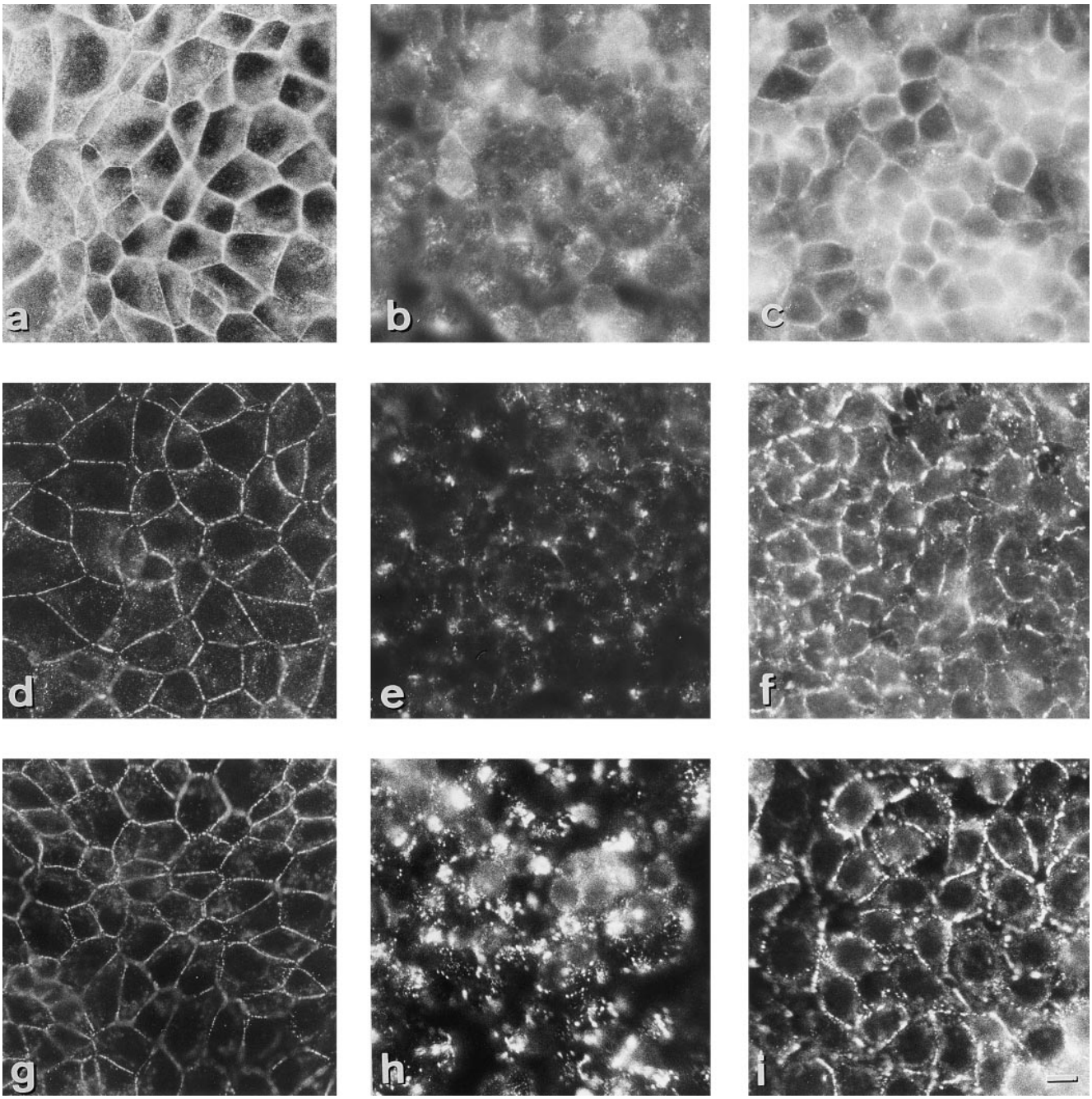

Figure 6. Relocalization of desmosomal proteins to cell–cell contacts upon TPA treatment of α-catenin–negative variants of HCT-8. Cells were stained for plakoglobin (*a*, *b*, and *c*), desmoglein-2 (*d*, *e*, and *f*), or desmoplakin (*g*, *h*, and *i*). In the untreated epithelioid variant HCT-8/E8, desmosomal proteins were localized at the cell–cell contacts (*a*, *d*, and *g*). HCT-8/R1 cultures were either untreated (*b*, *e*, and *h*) or treated for 3 h with 250 ng/ml TPA (*c*, *f*, and *i*). Similar observations were done for other R-variants of HCT-8. Bar, 10 μm.

from analysis of phosphorylated proteins in the E-cadherin/catenin complex after TPA treatment (data not shown).

Immunostaining for plakoglobin (γ-catenin) revealed a honeycomb pattern in the epithelioid HCT-8/E8 cells (Fig. 6 *a*). In contrast, plakoglobin was located in small granular dots in the cytoplasm of R-variants and was rarely detectable at sites of cell–cell contact (Fig. 6 *b*). TPA treatment for 30 min induced formation of bigger dots and after a 3-h treatment a honeycomb pattern appeared with continuously stained lines at cell–cell contacts (Fig. 6 *c*). In conclusion, several components of the cadherin/catenin complex were originally present at the cell surface of R-variants, except for plakoglobin and α-catenin. Plakoglobin was induced to relocalize to the cell–cell contacts upon TPA treatment, whereas α-catenin expression remained totally defective.

TPA Reorganizes Desmosomal and Tight Junctional Proteins in R-variants

Plakoglobin is the only protein within the E-cadherin/cate-

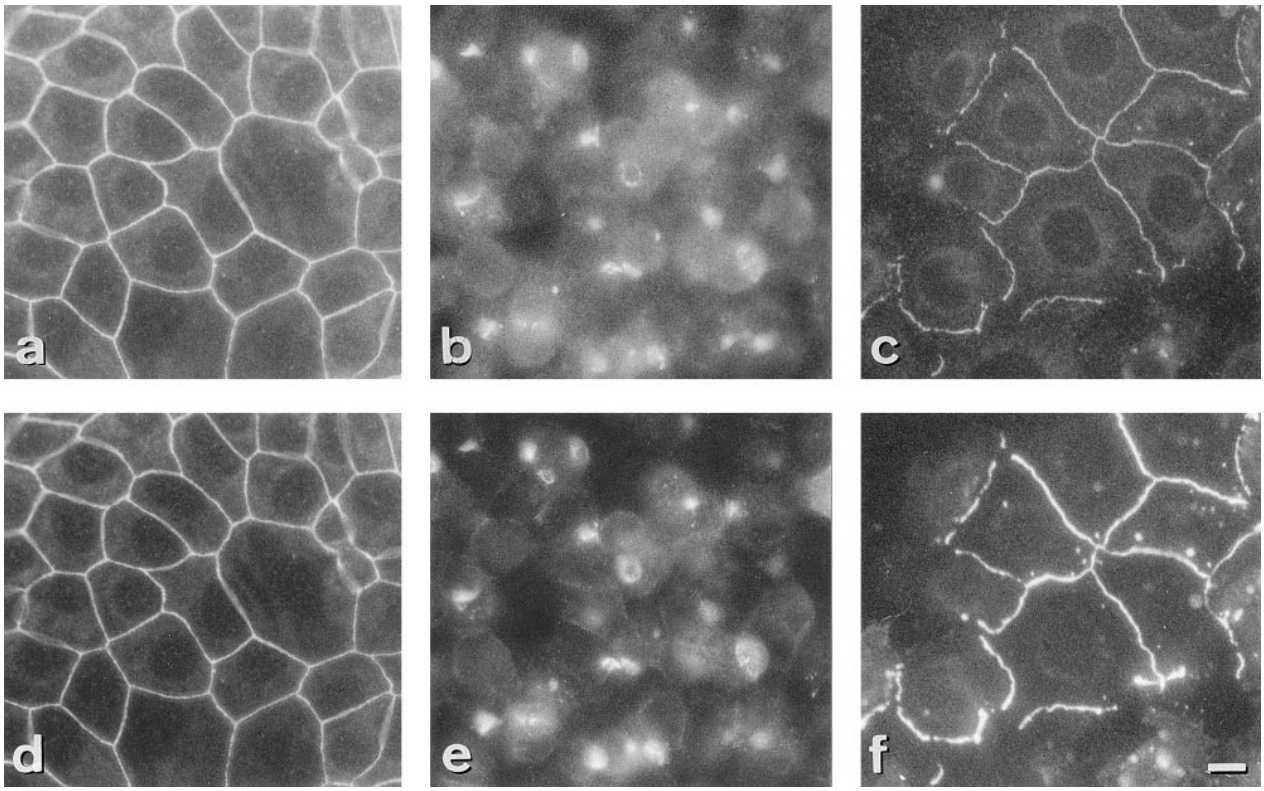

Figure 7. Relocalization of tight junctional proteins to cell–cell contacts upon TPA treatment of α-catenin–negative variants of HCT-8. Double immunofluorescence was performed for ZO-1 (*a–c*) and occludin (*d–f*). In the untreated epithelioid variant HCT-8/E8, the ZO-1 and occludin proteins were localized at the apical side of lateral cell–cell contacts (*a* and *d*). ZO-1 and occludin colocalized in a granular, dot-like pattern in untreated HCT-8/R1 cells (*b* and *e*), but after treatment for 3 h with 250 ng/ml TPA, they became largely concentrated at sites of cell–cell contact (*c* and *f*). Bar, 10 μm.

nin complexes that is known to localize also in spot desmosomes. Colon carcinoma cell lines express the desmoglein-2 isoforms (Schmidt et al., 1994). This was confirmed also for HCT-8 and DLD-1 cells by a desmoglein-2 specific antibody. In the epithelioid variants, both desmoglein-2 and desmoplakin proteins were detectable as punctuated lines along the cell outlines (Figs. 5 *g*; 6, *d* and *g*). In the R-variants, desmoglein-2 and desmoplakin proteins were present as granular dots in the cytoplasm nearby the plasma membrane and at rare points of cell–cell contacts (Figs. 5 *h*; 6, *e* and *h*). An increase in the intensity and number of desmoglein-2 and desmoplakin-containing fluorescent spots throughout the cytoplasm was induced by 30 min of TPA treatment (not shown). This may be the result of antigen clustering. Longer treatment resulted in clearing of the intracellular staining with concomitant increase in staining at the plasma membrane. By 4 h of TPA treatment, the punctuated peripheral staining characteristic of desmosomes was apparent and about no intracellular staining remained (Figs. 5 *i*; 6, *f* and *i*).

Besides desmosomal proteins, also the tight junctional proteins ZO-1 and occludin relocalized to the membrane upon TPA treatment of R-variants. In the epithelioid HCT-8/E8 cells, ZO-1, and occludin-specific immunoreactivities were situated at the expected location of tight junctions, i.e., at the apical ends of lateral cell–cell contacts and extending over the whole cell perimeter (Figs. 5 *d*; 7, *a* and *d*). ZO-1 and occludin were apparently clumped together nearby or at cell–cell contacts of untreated R-variants (Figs. 5 *e*; 7, *b* and *e*), but they reorganized dramatically to the typical tight junction line pattern upon TPA treatment (Figs. 5 *f*; 7, *c* and *f*). Nevertheless, some discontinuities remained even after 4 h of TPA treatment, and transepithelial electrical resistance was not built up (the latter data not shown).

We performed an ultrastructural analysis to determine whether our immunofluorescence data represented the TPA-induced assembly of genuine cell junctions in R-variants. E-variants exhibited many well developed intercellular junctions and showed interdigitation of juxtaposed plasma membranes (Fig. 8 *a*). Moreover, these cells possessed short microvilli (not shown). In contrast, untreated R-variants revealed no detectable adhesion specializations along the whole length of their non-interdigitated cell–cell contact surfaces (Fig. 8, *b* and *d*). The TPA-treated R-variants, however, demonstrated an evident array of junctions, including tight junction–like structures and classical desmosomes with attached intermediate filaments (Fig. 8, *c* and *e*).

TPA-induced Cell Aggregation Is Desmosome Dependent

Next, we wondered whether desmosomal cadherins were causally involved in restoration of TPA-induced cell–cell adhesion of the α-catenin–negative R-variants. Addition

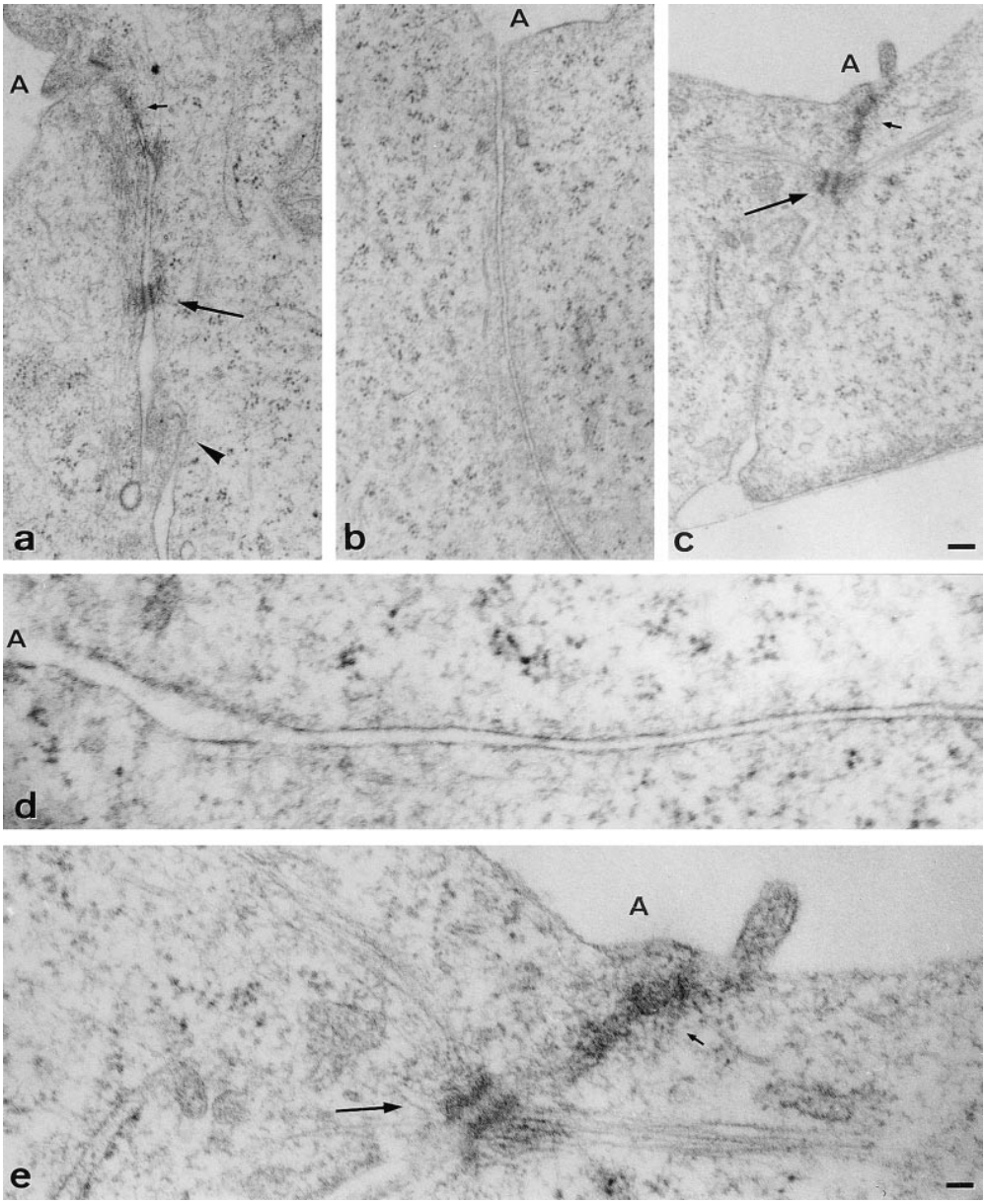

Figure 8. Upon TPA treatment, α-catenin–negative cells start to exhibit various cell junctions as demonstrated by electron microscopy. Untreated HCT-8/E8 cells (*a*) show tight-junction-like structures (*small arrows*), desmosomes (*large arrows*), and membrane interdigitations (*arrowhead*) at lateral cell–cell contacts. All these features are consistently lacking in untreated HCT-8/R1 cells (*b* and *d*). In contrast, TPA-treated HCT-8/R1 cells (*c* and *e*) form morphologically typical tight junctions (*small arrows*) and desmosomes (*large arrows*). The latter are clearly linked to 10-nm intermediate filaments (*e*). *A*, apical side of the cells. Bars: (*a–c*) 280 nm; (*d* and *e*) 120 nm.

of antibody 10G11, directed against the extracellular part of desmoglein-2, blocked indeed the TPA-induced cell aggregation (open squares in Fig. 4 *a*). This inhibition was antibody concentration dependent (not shown). The mAb htr5 against TNF-receptor p55 is of the same IgG isotype as 10G11, but showed no effect on TPA-induced aggregation (not shown). This result was clearly reminiscent of the above mentioned anti–E-cadherin antibody effect (open circles in Fig. 4 *a*). This double E-cadherin–plus desmoglein-2 dependence was also noticed for E-variants, although antibodies against desmoglein-2 were less inhibitory than anti–E-cadherin antibodies (Fig. 4 *b*). In E-cells, the E-cadherin–mediated cell–cell adhesion is apparently more important, or the desmoglein-2 molecules are less accessible to blocking antibodies. In agreement with this, the anti–desmoglein-2 antibody used here was originally described to be nonblocking in epithelial cell monolayers (Demlehner et al., 1995), and this was essentially confirmed by us.

To investigate in more detail the observed antibody effects, a noneffective anti–E-cadherin antibody concentration was combined with a noneffective anti–desmoglein-2 antibody concentration. It is interesting that this resulted in a synergistically enhanced inhibition of cell–cell adhesion, both in TPA-treated R-variants (filled circles in Fig. 4 *c*) and in E-variants with or without TPA (partly shown in Fig. 4 *d*).

Antigen Exposition at the Cell Surface Antigens and Detergent Solubility

To further verify the presence of E-cadherin and desmoglein-2 at the outer side of the plasma membrane, we selectively biotinylated the cell surface of TPA-treated E- and

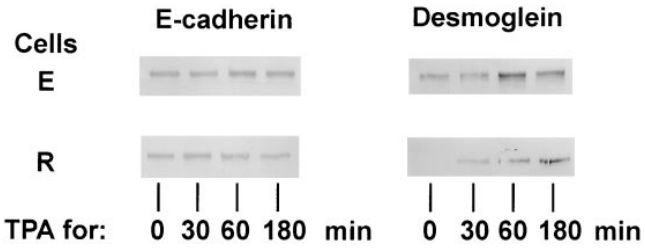

Figure 9. TPA induces exposition of desmoglein-2 molecules at the cell surface in α-catenin–negative variants of HCT-8. Monolayers of the epithelioid HCT-8/E8 (*E*) and of the round cell variant HCT-8/R1 (*R*) were treated for the indicated times with 250 ng/ml TPA, and then labeled with NHS-biotin for 30 min and extracted with lysis buffer. Biotinylated proteins were purified by adsorbing to avidin-agarose beads and analyzed by Western blotting using antibodies to E-cadherin (HECD-1) or desmoglein (DG 3.10).

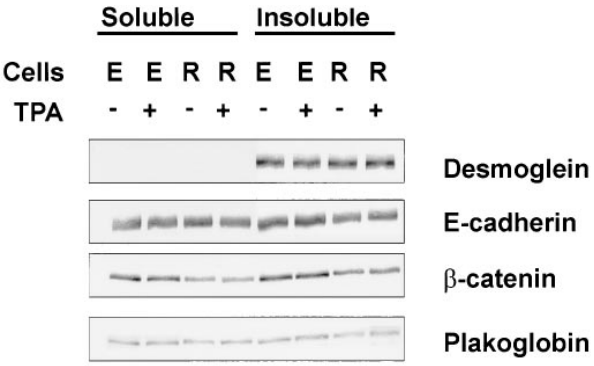

Figure 10. TPA does not modify the detergent solubility of E-cadherin, β-catenin, plakoglobin, and desmoglein-2. Monolayers of HCT-8/E8 (*E*) and HCT-8/R1 (*R*) were either untreated or treated with 250 ng/ml TPA for 3 h as indicated. Detergent (2.5% NP-40) soluble and insoluble fractions were prepared and analyzed by Western blotting using antibodies to the antigens indicated at the right.

R-variants with a membrane-impermeable reagent, sulfo-NHS-biotin. Biotinylated proteins were recovered with immobilized avidin and subjected to immunoblotting with antibodies against E-cadherin or desmoglein-2 (Fig. 9). E-cadherin was expressed at the plasma membrane of both cell types, HCT-8/E8 and HCT-8/R1, and TPA treatment had no obvious influence on the extent of biotinylation of E-cadherin. Upon prolonged exposure to TPA, the intensity decreased slightly, probably due to downregulation of PKC (Winkel et al., 1990; Hecht et al., 1994). Desmoglein-2 was detectable at the plasma membrane of the epithelioid HCT-8/E8 cells and a 1-h treatment with TPA increased to some extent the biotinylation of desmoglein-2. In clear contrast to E-variants, no desmoglein-2 could be biotinylated in the plasma membrane of the untreated R-variants. However, TPA treatment of the R-cells increased desmoglein-2 biotinylation progressively (Fig. 9). These results show that E-cadherin is exposed at the plasma membrane of the α-catenin–negative R-variants and that desmoglein-2 appears at the cell surface solely upon TPA treatment. These findings consolidate the results obtained by immunohistochemistry and EM.

Nevertheless, in cell fractionation experiments the bulk of desmoglein-2 molecules was recovered in the detergent insoluble fraction and this was found for both E- and R-variants, irrespective of TPA treatment (Fig. 10). This points at a cytoskeleton linkage of desmoglein-2 molecules, even if not exposed at the cell surface of untreated R-cells. E-cadherin, β-catenin, and plakoglobin molecules were detected in both insoluble and soluble fractions under our fractionating conditions and TPA treatment did not induce any obvious changes (Fig. 10).

Transfection of the Round Cell Variants with αN-Catenin cDNA

Finally, we examined whether α-catenin cDNA expression could restore the deficient cell adhesion and induce desmosome formation in the R-variants. HCT-8/E11R1 cells were transfected with a G418-resistance plasmid in combination with pMiw-αN, encoding chicken αN-catenin (Hirano et al., 1992). Colonies with stable expression of this ectopic αN-catenin were selected by Western blotting using mAb NCAT-2, specific for αN-catenin (Fig. 11; Hirano et al., 1992). From immunohistochemistry, it turned out that it was difficult to obtain a stable homogeneous αN-catenin positive cell population. The cloned transfectants HRpCαN1 and HRpCαN2 were further studied because of more consistent expression of αN-catenin in an epithelial honeycomb pattern at the cell–cell contacts (Figs. 11 and 12 *a*; Table I). No α-catenin expression was detected in transfectant HRpCαN3 (Fig. 11 and Table I), and this served as a negative control in our experiments. Immunohistochemistry of transfectants HRpCαN1 and HRpCαN2 revealed a honeycomb staining for αN-catenin, E-cadherin, β-catenin, $p120^{cas}$, and also the proteins of spot desmosomes and tight junctions (illustrated for desmoglein-2 in Fig. 12 *b*). Restoration of the intact E-cadherin/catenin complex in the HRpCαN1 and HRpCαN2 transfectants was confirmed also by immunoprecipitation (data not shown), and the functionality of this complex was demonstrated in a fast aggregation assay (Table I).

Discussion

It is generally accepted that in assembling epithelia, surface interactions between adhesion molecules of the cadherin superfamily are the starting point of the molecular cascade that leads to the formation of other junctions like desmosomes and tight junctions (Lewis et al., 1994; Amagai et al., 1995; Gumbiner, 1996). The possibility of cadherin-mediated cell–cell adhesion in the absence of α-catenin, as obtained in this study upon TPA treatment of R-variants, seems to contradict this. Many studies (Ozawa et al., 1990; Hirano et al., 1992; Shimoyama et al., 1992; Breen et al., 1993; Morton et al., 1993; Watabe et al., 1994; Ewing et al., 1995; Rimm et al., 1995) have led to the generally accepted principle of cadherin function: cadherins must bind catenins inside the cell for them to function properly outside the cell. This molecular complex is thought to confer adhesive strength because catenins link the cadherins to the actin cytoskeleton and because clustering of the cadherin molecules should increase the avidity of their

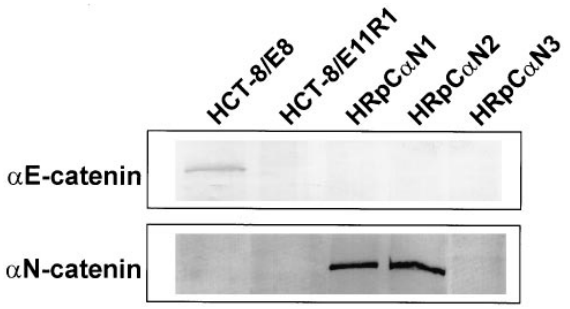

Figure 11. Expression of αE- and αN-catenin in HCT-8 variants and transfected derivatives. Expression levels were assessed by Western blotting using anti-αE-catenin mAb 1G5 and anti-αN-catenin mAb NCAT-2. Total cell lysates of epithelioid HCT-8/E8 cells contained αE-catenin, whereas the round cell variant HCT-8/E11R1 expressed neither αE- nor αN-catenin. The transfected clones HRpCαN1 and HRpCαN2 expressed the exogenous αN-catenin cDNA. Transfectant HRpCαN3 expressed no detectable α-catenins and served as a negative control in further experiments.

Table I. Effects of Exogenous α-Catenin Expression by Round Cell Variants on Cell Aggregation

			Fast aggregation assay
Cells*	Western blot‡	Immunostaining‡	$1-(N_{30}/N_0)$
HCT-8/E8	+	+	0.6
HCT-8/E11R1	−	−	0.0
HRpCαN1	+	+	0.5
HRpCαN2	+	+	0.5
HRpCαN3	−	−	0.1

*The epithelial HCT-8/E8 cells express endogenous αE-catenin and serve as a positive control.
‡+, positive for αE-catenin in the case of HCT-8/E11R1, and positive for αN-catenin in the case of HRpCαN clones (see Figs. 11 and 12); −, negative for both αE- and αN-catenin.

interactions. However, in spite of this model, evidence exists that in special situations the extracellular part of cadherins might retain some biological activity in the absence of catenin linkage. A chimeric molecule consisting of the extracellular part of E-cadherin and the transmembrane and intracellular part of N-CAM is able to function in adhesion (Jaffe et al., 1990). Likewise, the intracellular domain of desmoglein-3 confers adhesive functionality on the extracellular domain of E-cadherin (Roh and Stanley, 1995). Both types of chimeric molecules do not use α-catenin to mediate their adhesive function. Also, T-cadherin, which is anchored to the membrane by a glycosyl phosphatidylinositol group instead of a transmembrane domain, does not interact with catenins, but it can still mediate cell–cell adhesion to some extent (Vestal and Ranscht, 1992). On the other hand, an E-cadherin–α-catenin fusion molecule confers a strong and inflexible adhesive phenotype upon cells (Nagafuchi et al., 1994). The latter finding emphasizes, besides a putative modulating role of β-catenin, the importance of the α-catenin–cytoskeleton connection for tight cell adhesiveness.

Our antibody inhibition experiments show that E-cadherin plays an essential role in the TPA-induced cell aggregation by R-variants, despite the sustained absence of any α-catenin expression. In line with this, E-cadherin–defective cells such as Colo320DM did not show this TPA-induced cell aggregation (our data not shown). From coimmunoprecipitation, immunofluorescence, and cell surface biotinylation experiments, we could conclude that E-cadherin molecules are expressed at the cell surface of α-catenin–negative R-variants. They form a complex with β-catenin and with part of the intracellular plakoglobin and $p120^{cas}$ molecules. R-variants expressed E-cadherin, β-catenin, and $p120^{cas}$ also at free cell borders besides cell–cell contacts, whereas plakoglobin was largely stained as cytoplasmic clumps. Upon TPA treatment of R-variants, cadherin–catenin complexes did not translocate completely to cell–cell contacts, contrary to what might be expected for a functional cadherin–catenin complex. We tried to clarify this phenomenon by determining the detergent soluble and insoluble fractions of the cells. In accordance with a

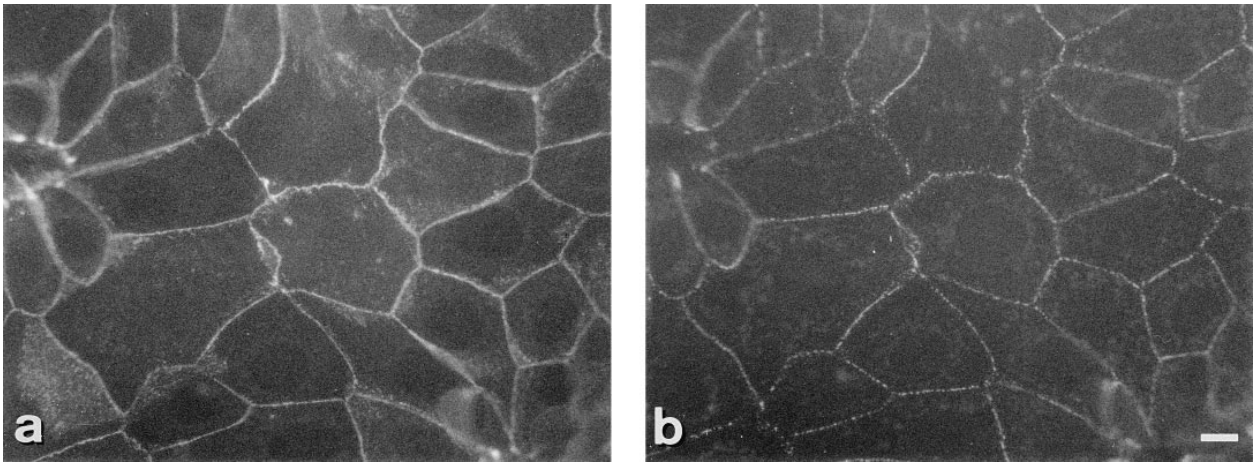

Figure 12. Transfection of αN-catenin cDNA into the round cell variant HCT-8/E11R1 induces desmosome formation. Transfected cell clone HRpCαN2 was fixed and double stained with anti-αN-catenin mAb (*a*) and anti-desmoglein mAb (*b*). Note the different location of the two antigens, with the immunoreactivity of desmoglein being punctate as might be expected for typical desmosomes. Bar, 5 μm.

report on α-catenin–negative PC-9 cells (Shimoyama et al., 1992), our data suggest that α-catenin–lacking E-cadherin complexes are still associated in some way with detergent insoluble material. Moreover, the striking effect of TPA on cell adhesion by R-variants was apparently not due to strengthened association of E-cadherin with, e.g., the cytoskeleton. Another possible level of cell adhesion regulation is via modulation of β-catenin. Cook et al. (1996) described that the Wingless protein of *Drosophila* could inactivate the GSK-3 kinase in murine fibroblasts and suggested the involvement of an upstream TPA-sensitive PKC isoform in this process. Inactivation of GSK-3 should lead to stabilization of β-catenin and maybe increased cell adhesion besides signaling to the nucleus (Miller and Moon, 1996). However, TPA-treated R-variants did not show increased levels of β-catenin, as shown by direct Western blotting and coimmunoprecipitation with E-cadherin.

The suboptimal E-cadherin comprising cell–cell adhesion events of R-variants might act as indispensable organization centers for the TPA-induced establishment of extra strengthening cell junctions, i.e., desmosomes and tight junctions, which are clearly implicated in the observed phenomena. Indeed, the most dramatic effects of TPA were not seen on E-cadherin–containing junctions but on desmosome and tight junction organization (Figs. 5–7). This was further consolidated by the antibody experiments. Efficient blocking of either E-cadherin or desmoglein-2 was fully abrogating the TPA-induced cell aggregation. This finding excludes also that R-variants comprise two subpopulations, which are dependent on either E-cadherin or desmoglein-2 for TPA-induced aggregation. It is interesting that combination of anti–E-cadherin and anti–desmoglein-2 antibodies at noneffective concentrations produced a clear-cut cooperative inhibition of TPA-induced aggregation of R-variants. A similar phenomenon was seen for antibody-treated E-variants in the absence of TPA. As this effect was synergistic instead of additive, it cannot be simply explained by a threshold of adhesive bridges, composed of either E-cadherin or desmoglein-2, that should be reached before cell aggregation is achieved. Except for particular conditions such as lymphatic endothelial cells (Schmelz and Franke, 1993) and plakoglobin-negative heart cells (Ruiz et al., 1996), classic studies on cell junctions show no evidence for mixed structures, composed of both E-cadherin and desmosomal proteins (except for plakoglobin). Such peculiar junctions could have explained the observed cooperative action of the antibodies. In contrast, both confocal microscopy and EM suggested that no such hybrid structures were present or induced in our cell lines. It can be suggested, therefore, that both α-catenin–lacking E-cadherin complexes and desmosomes are involved in mutually intercommunicating signaling pathways.

The main role of α-catenin–lacking E-cadherin complexes in TPA-induced cell aggregation might be the establishment of a basic though essential level of intercellular adhesion. A slightly similar situation was reported for HeLa cells, in which exogenous adhesion protein zero (P_0) was expressed (Doyle et al., 1995). Such cells showed obvious development of desmosomes and redistribution of tight junction proteins to apical cell contacts, as a function of P_0 expression. However, intracellular levels of desmoplakin, N-cadherin, and even α-catenin were increased, too, and this is in clear variance with our findings on R-variants, where α-catenin was never found to be induced. On the other hand, αN-catenin-expressing transfectants of R-variants showed normal intercellular cell adhesion and also an epithelioid pattern of desmosomes, even in the absence of TPA treatment. The latter data emphasize the apparently complex interplay between E-cadherin containing junctions and desmosomes.

Nevertheless, it is likely from our various observations that the molecular target of PKC with respect to cell–cell adhesion in the present system belongs to or influences the desmosomal junctions, rather than the tight junctions or E-cadherin–comprising junctions. Although tight junctional proteins occludin and ZO-1 were strikingly redistributed upon TPA treatment, no fully sealed tight junctions were built up as evaluated by immunofluorescence and transepithelial electrical resistance measurements. Probably, formation of desmosomes is the driving but too transient event to induce functional tight junctions. Regarding the E-cadherin complex, no obvious change in phosphorylation of E-cadherin plus associating proteins could be observed in TPA-treated R-variants (data not shown). Therefore, the phosphorylation status of the many desmosomal components, recently reviewed or newly reported (Cowin and Burke, 1996; Mertens et al., 1996; Pin and Sugrue, 1996), deserves further investigation. Specific phosphorylation events may mediate the maturation of the complicated desmosomal junction. Additional immuno-EM studies could reveal whether the various desmosomal components in untreated R-variants are colocalized in so-called half-desmosomal structures (Demlehner et al., 1995). This possibility is supported by our finding in untreated R-variants that the bulk of desmoglein-2 molecules is in a detergent-insoluble fraction, like in E-variants. Assembly and disassembly of desmosomes in MDCK cells was proposed to be regulated via reversible protein phosphorylation involving both protein kinases such as PKC and protein phophatases (Pasdar et al., 1995). However, in that cell system phosphatases seemed to be involved in assembly rather than disassembly.

It is widely assumed that desmosomal cadherins are involved in cell–cell adhesion, but little direct evidence to support this belief is available so far. Expression of full-length desmocollin-1 is not sufficient to confer adhesiveness to transfected L929 fibroblasts (Chidgey et al., 1996). Similarly, a chimeric protein consisting of the desmocollin-1 extracellular domain and the E-cadherin transmembrane and cytoplasmic domains was not adhesive in the same system, even though the chimeric protein interacted with endogenous α- and β-catenins (Chidgey et al., 1996). A similar chimeric molecule consisting of the desmoglein-3 extracellular domain linked to the membrane-spanning and intracellular domains of E-cadherin is adhesive in L929 cells, but only weakly (Amagai et al., 1994). On the contrary, as mentioned above, the cytoplasmatic domain of desmoglein-3 is able to confer strong adhesiveness on the extracellular domain of E-cadherin (Roh and Stanley, 1995). Our antibody studies show that the extracellular domain of desmoglein-2 is indeed essential for intercellular adhesion, at least under the conditions tested. The same

anti–desmoglein-2 antibody was previously reported to be nonblocking, but those studies were performed on adhering instead of suspended cells (Demlehner et al., 1995). It is interesting that defective expression of desmoglein and desmoplakin molecules is correlated with loss of differentiation and lymph node involvement in various human carcinomas (Nei et al., 1996; Hiraki et al., 1996). The detailed analysis of our data is complicated by the transient nature of PKC activation. Downregulation of PKC by sustained TPA treatment is well established and should be considered when comparing our results with the often contradictory literature on PKC activation in epithelial cells. Disruptions (Ben-Ze'ev, 1986; Citi, 1992; Dong et al., 1993; Fabre and de Herreros, 1993; Lampe, 1994; Nilsson and Ericson, 1995) as well as assembly of various junctional complexes in epithelial cells (Sheu et al., 1989; Winkel et al., 1990; Balda et al., 1993; Williams et al., 1993; Denisenko et al., 1994; Lewis et al., 1995; Stuart and Nigam, 1995) have been described to result from PKC activation. Downregulation of PKC has been forwarded as a fair explanation for transient effects on cell junction functionality (Sheu et al., 1989; Winkel et al., 1990; Lewis et al., 1995), and also we observed reversibility upon prolonged TPA treatment. Despite this complexity, it might be clear from our findings that remarkably genuine cell junctions can be formed in the absence of the important bridge molecule α-catenin. The key role of PKC in either the basic assembly or the strengthening of various epithelial cell junctions is emphasized and so is the functional interplay between E-cadherin and desmosomal cadherins. Further studies on R-variants may allow identification of the relevant PKC substrate. Such a protein may play a key role in cell–cell adhesion.

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