

Expression of a dominant negative cadherin mutant inhibits proliferation and stimulates terminal differentiation of human epidermal keratinocytes

Alan Jian Zhu and Fiona M. Watt*

Keratinocyte Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

*Author for correspondence

SUMMARY

Cell adhesion molecules are not only required for maintenance of tissue integrity, but also regulate many aspects of cell behaviour, including growth and differentiation. While the regulatory functions of integrin extracellular matrix receptors in keratinocytes are well established, such functions have not been investigated for the primary receptors that mediate keratinocyte intercellular adhesion, the cadherins. To examine cadherin function in normal human epidermal keratinocytes we used a retroviral vector to introduce a dominant negative E-cadherin mutant, consisting of the extracellular domain of H-2K^d and the transmembrane and cytoplasmic domains of E-cadherin. As a control a vector containing the same construct, but with the catenin binding site destroyed, was prepared. High levels of expression of the constructs were achieved; the dominant negative mutant, but not the control, formed complexes with α -, β - and γ -catenin. In cells expressing the dominant negative mutant there was a 5-fold decrease in the level of endogenous cadherins and a

3-fold increase in the level of β -catenin. Cell-cell adhesion and stratification were inhibited by the dominant negative mutant and desmosome formation was reduced. Expression of the mutant resulted in reduced levels of the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins and increased cell motility, providing further evidence for cross-talk between cadherins and the β_1 integrins. In view of the widely documented loss of E-cadherin in keratinocyte tumours it was surprising that the dominant negative mutant had an inhibitory effect on keratinocyte proliferation and stimulated terminal differentiation even under conditions in which intercellular adhesion was prevented. These results establish a role for cadherins in regulating keratinocyte growth and differentiation and raise interesting questions as to the relative importance of cell adhesion-dependent and -independent mechanisms.

Key words: Cadherin, Catenin, Differentiation, Epidermis, Keratinocyte

INTRODUCTION

The classical cadherins are a family of cell surface glycoproteins that mediate calcium-dependent intercellular adhesion (Kemler, 1993; Ranscht, 1994; Takeichi, 1995). Cadherins are single chain polypeptides that span the plasma membrane once; recent crystallographic data suggest that cadherins may form dimers within the plane of the plasma membrane and may interact with cadherins on neighbouring cells via the N-terminal domains, in an analogous fashion to a zipper (Overduin et al., 1995; Shapiro et al., 1995; Nagar et al., 1996).

The adhesive function of cadherins is dependent on their association with regulatory proteins, the best characterised being α -, β - and γ -catenin (plakoglobin) (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989; McCrea and Gumbiner, 1991). α -Catenin shares sequence similarity with vinculin (Herrenknecht et al., 1991), while β - and γ -catenins are homologous to the product of the *Drosophila* segment polarity gene *armadillo* (Peifer and Wieschaus, 1990; McCrea et al., 1991; Gumbiner, 1995). Catenins link cadherins with the actin cytoskeleton and can also form complexes with other proteins (Kemler, 1993; Su et al., 1993; Hoschuetzky et al., 1994; Peifer, 1996). A short core region within the last 70 amino

acids of the cadherin cytoplasmic domain is responsible for binding catenins (Ozawa et al., 1990; Stappert and Kemler, 1994; Jou et al., 1995). Mutations and deletions in this region result in abolition of cadherin/catenin complex formation and a loss of adhesive properties of the cells (Stappert and Kemler, 1994; Hertig et al., 1996).

Dominant negative cadherin mutants provide a powerful approach to the study of cadherin and catenin function, both in cultured cells and in intact tissues and organisms (Takeichi, 1995). Two types of construct have been used, one in which the cadherin cytoplasmic domain is deleted and one in which the extracellular domain is deleted; the cytoplasmic domain is often fused with the extracellular domain of a different (marker) protein. The cytoplasmic domain deletion mutants specifically inhibit the cadherin from which they are derived and may act by competing with the endogenous cadherin for homophilic binding (Levine et al., 1994). The extracellular domain deletion mutants appear to have a more powerful dominant negative effect than the cytoplasmic domain deletion mutants (Fujimori and Takeichi, 1993) and they inhibit all endogenous cadherins, probably because of the high degree of sequence conservation of the cytoplasmic domain; their mode of action is via binding of endogenous catenins (Kintner, 1992;

Fujimori and Takeichi, 1993; Amagai et al., 1995; Hermiston and Gordon, 1995; Hertig et al., 1996).

Two cadherins are expressed in human epidermis: E-cadherin is found in all the living cell layers, whilst P-cadherin is only expressed in the basal layer (Nicholson et al., 1991, and references cited therein). Cadherin function is required for epidermal stratification, since stratification is inhibited by growing keratinocytes in low calcium medium (Watt and Green, 1982), by addition of anti-cadherin antibodies (Wheelock and Jensen, 1992; Hodivala and Watt, 1994; Lewis et al., 1994) or by expression of a dominant negative mutant, corresponding to a deletion of the extracellular domain of N-cadherin (Amagai et al., 1995). Extracellular matrix receptors of the integrin family are normally downregulated when keratinocytes undergo terminal differentiation, but in low calcium cultures differentiating, involucrin-positive, keratinocytes continue to express functional integrins, suggesting that there may be an element of cross-talk between the two classes of receptor (Hodivala and Watt, 1994).

The keratinocyte integrins not only mediate extracellular matrix adhesion but also regulate the initiation of terminal differentiation (Adams and Watt, 1989; Watt et al., 1993) and are markers of epidermal stem cells (Jones and Watt, 1993). In view of the possible interaction between cadherins and integrins we decided to examine whether cadherins also regulate keratinocyte growth and differentiation. We have therefore used retroviral infection to express a dominant negative cadherin mutant, consisting of the extracellular domain of the class I major histocompatibility antigen H-2K^d and the transmembrane and cytoplasmic domains of E-cadherin (Ozawa et al., 1990), in cultured human epidermal keratinocytes.

MATERIALS AND METHODS

Construction of retroviral vectors and producer cell lines

The dominant negative E-cadherin mutant cDNA construct (pKSH-2UMTC) was a kind gift from R. Kemler (Ozawa et al., 1990). It encodes a 66 kDa chimeric protein consisting of the extracellular domain of H-2K^d (297 amino acids) linked to the C terminal 191 amino acids of mouse E-cadherin, which comprise 16 amino acids of the extracellular domain and the entire transmembrane and cytoplasmic domains (see Fig. 1). As a control, a construct was prepared in which the catenin binding site was destroyed (pKSH-2UMTCΔC25). The control construct was constructed by PCR, using pKSH-2UMTC as the template, synthetic oligo 5'-ATGAAATTGGAACTTCATC-GATAGTGGTTCTGAAGCCGC-3' as the 5'-primer and oligo 5'-ATTAACCCTCACTAAAG-3' as the 3'-primer. To facilitate recloning of the amplified fragment, the 5'-primer contained a *Clal* restriction site (sequence underlined). After cleavage, the fragment was subcloned into *Clal/XhoI* cut pKSH-2UMTC. The sequence of the amplified fragment was confirmed by restriction analysis and DNA nucleotide sequencing using T7 Sequenase (USB). The cDNAs were then subcloned into the *Sall* site of the retroviral vector pBabe puro (see Fig. 1; Morgenstern and Land, 1990).

Retroviral DNAs (25 µg) were transfected into the ecotropic cell line GP+E via calcium phosphate mediated transfection (Sambrook et al., 1989). After 48 hours of growth, supernatants from the transfected ecotropic cells were used to infect the amphotropic packaging cell line AM12 using a standard infection protocol (Morgenstern and Land, 1991). Infected cells were selected and cloned in the presence of 2.5 µg/ml puromycin; 24 clones from each cell line were expanded and

subjected to indirect immunofluorescence staining for the H-2K^d tag to confirm expression of the retroviral vectors. The viral titre was determined by infection of HeLa cells. The positive clones exhibiting the highest titre for each retroviral construct (AM12-H-2UMTC clone 17, titre 5.5×10^6 cfu/ml and AM12-H-2UMTCΔC25 clone 10, titre 5.1×10^5 cfu/ml) were used for further studies. A producer cell line expressing the empty vector pBabe puro was also generated as a control. No helper virus contamination was detected in the clones.

Keratinocyte culture

Normal human epidermal keratinocytes from neonatal foreskins (strains z, kb, km; passages 2-6) were cultured according to the method of Rheinwald (1989) on a feeder layer of J2-3T3 mouse embryo cells pre-treated with 4 µg/ml mitomycin C (Sigma). The culture medium (FAD+FCS+HICE) consisted of one part Ham's F12 medium and three parts Dulbecco's modification of Eagle's medium, supplemented with 1.8×10^{-4} M adenine (FAD), 10% foetal calf serum (FCS), 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, 10^{-10} M cholera toxin and 10 ng/ml epidermal growth factor (HICE).

Retroviral infection was carried out by plating keratinocytes onto producer lines that had been pre-treated with 4 µg/ml mitomycin C (10^5 keratinocytes per 100 mm dish). After 2 days 1 µg/ml puromycin was added to the medium. The producer cells were usually removed after 3-4 days and replaced with puromycin-resistant J2-3T3 cells (prepared by transfection of J2-3T3 with pBabe puro and kindly provided by L. Goodman). Infected keratinocytes were used for experiments immediately or following passage on puromycin-resistant J2-3T3 cells in medium supplemented with 1 µg/ml puromycin. Cells were grown at 37°C in a humid atmosphere containing 5% CO₂. The culture medium was changed every 2 days. Cells were harvested by first removing the feeders with EDTA and then treating the keratinocytes with trypsin/EDTA. Pre-confluent or newly confluent cultures were used for all experiments.

Growth curves were obtained by plating 500 cells per 35 mm dish in the presence of mitomycin C-treated feeder cells and harvesting triplicate dishes at 2-day intervals. The total number of keratinocytes per dish was determined using a Coulter Counter (Coulter Electronics Ltd, UK).

Antibodies

The following monoclonal antibodies were used: DPI/II (mouse anti-desmoplakin, ICN, Thame, UK), GoH3 (rat anti- α_6 -integrin subunit, SeroTec; Sonnenberg et al., 1986), HAS4 (mouse anti- α_2 -integrin subunit; Tenchini et al., 1993), HECD-1 (mouse anti-E-cadherin, a gift from M. Takeichi; Shimoyama et al., 1989), K9-18 (mouse anti-H-2K^d, a gift from B. Arnold; Arnold et al., 1985), mAb16 (rat anti- α_5 -integrin subunit, a gift from K. Yamada; Akiyama et al., 1989), NCC-CAD-299 (mouse anti-P-cadherin, gift from S. Hirohashi; Shimoyama et al., 1989), P5D2 (mouse anti- β_1 -integrin, Developmental Studies Hybridoma Bank; Dittel et al., 1993), SY5 (mouse anti-involucrin; Hudson et al., 1992), VM-2 (mouse anti- α_3 -integrin subunit, ATCC; Kaufmann et al., 1989), 3E1 (mouse anti- β_4 -integrin subunit, Gibco BRL; Rynänen et al., 1991). The following rabbit antisera were also used: DH1 (rabbit anti-involucrin; Dover and Watt, 1987); FWCAD (rabbit anti-pan cadherin; Braga et al., 1995); VB1, VB2 and VB3 (rabbit anti- α -, β - and γ -catenin, respectively; Braga et al., 1995).

Fluorescein (FITC)-conjugated anti-rabbit, anti-mouse and anti-rat IgG and Texas red (TRSC)-conjugated anti-rabbit and anti-mouse IgG were purchased from Jackson ImmunoResearch Lab. Inc., West Grove, PA. A FITC-conjugated rat anti-mouse H-2K monoclonal antibody, E12.265 (Seikagaku Co., Tokyo), and FITC-conjugated HECD-1 (kindly prepared by C. Bagutti) were also used.

Indirect immunofluorescence staining

For staining of desmoplakin, cells were permeabilised with 0.1% Triton X-100 at room temperature for 5 minutes. For double staining of H-2K^d and cadherin or catenins, cells were treated with absolute

methanol for 20 minutes at -20°C . For involucrin staining, cells were fixed in 3.7% formaldehyde for 10 minutes at room temperature, then permeabilised in absolute methanol on ice for 5 minutes. For double staining of β_1 integrins and involucrin, cells were incubated with 3.7% formaldehyde and 0.4% Triton X-100 in PBS for 10 minutes at room temperature. Prior to immunostaining, cells that had been grown on coverslips were incubated for 1 hour in 0.2% fish skin gelatin in PBS at room temperature in order to inhibit non-specific antibody binding. Antibody incubations were carried out at room temperature for 30-40 minutes; cells were washed thoroughly in PBS after each incubation.

Stained cells were mounted in Gelvatol (Monsanto, St Louis, MO) and either examined with a Zeiss Axiophot microscope (Carl Zeiss Ltd, Herts, UK) or with a Nikon Diaphot 200 inverted microscope (Nikon UK Ltd, Telford, UK), equipped with a MRC-1000 laser scanning confocal microscope attachment (Bio-Rad Microscience, Hemel Hempstead, UK). Photographs were taken using plus-X Pan film (Kodak). Confocal images were directly taken from the video screen.

Flow cytometry

Keratinocytes to be labelled with anti-integrin or H-2K^d antibodies were harvested using trypsin/EDTA. Keratinocytes to be labelled with anti-cadherin antibodies were incubated with 0.02% EDTA for 5 minutes and then in thermolysin solution for 3-5 minutes (Germain et al., 1993), conditions that minimised proteolysis of the extracellular domains of P- and E-cadherin.

Single cell suspensions were resuspended in medium at 4°C and incubated with a saturating concentration of first antibody for 25-30 minutes. The cells were then washed in cold medium and incubated with an appropriate FITC-conjugated secondary antibody for 30 minutes on ice. After washing, cells were filtered through a $75\ \mu\text{m}$ nylon mesh (Becton-Dickinson and Company, NJ). Immediately before analysis, propidium iodide ($5\ \mu\text{g}/\text{ml}$) was added for viability gating. Cells were analysed in a FACScan (Becton-Dickinson Immunocytometry Systems, CA). In some experiments the differentiating cells were gated out as described by Jones and Watt (1993).

Cell lysis, immunoprecipitation, SDS-PAGE and western blotting

For immunoprecipitation, subconfluent keratinocytes were washed twice in ice-cold PBS containing 1 mM magnesium and 1 mM calcium ions, then extracted in lysis buffer containing 0.5% Triton X-100, 120 mM sodium chloride, 2 mM calcium chloride, 25 mM potassium chloride, 15 mM Tris-HCl, pH 7.5, 1 mM PMSF, 10 μM leupeptin and 0.1 mM DTT (Ozawa et al., 1990) for 20 minutes on ice on a rocking platform. The cells were scraped from the flasks and cell residues were pelleted at 10,000 rpm for 10 minutes at 4°C . Protein concentrations were determined by the Bio-Rad Dc protein assay (Bio-Rad, Hercules, CA). The supernatants were aliquoted into clean tubes and frozen at -70°C .

Prior to immunoprecipitation, samples (60 μg protein) were pre-cleared by incubation with Protein A-Sepharose CL-4B beads (Pharmacia Biotechnology, Uppsala, Sweden) for 1 hour on a rotating wheel at 4°C . The beads were discarded and the supernatant was incubated with K9-18 that had been chemically cross-linked to Protein A-Sepharose beads (Harlow and Lane, 1988). The samples were incubated overnight on a rotating wheel at 4°C . Immunoprecipitates were washed 3 times in HSB buffer (5 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 120 mM sodium chloride, 25 mM potassium chloride, 15 mM Tris-HCl, pH 7.5, 10 μM leupeptin and 0.1 mM DTT), then washed once in LSB buffer (2 mM EDTA, 10 mM Tris-HCl, pH 7.5 and 0.5 mM DTT) (modified from Shore and Nelson, 1991). Immunoprecipitates were separated on 7.5% SDS-PAGE gels (Laemmli, 1970) under reducing conditions.

Transfer to Immobilon PVDF membrane (Millipore) was performed at 200 mA for 1 hour using a semi-dry MilliBlot graphite

electroblotter I system (Hofer Scientific Instruments, CA). Membranes were blocked with 5% skimmed milk powder (Marvel, Cadbury) in PBS/0.05% Tween-20 (PBS/Tween) overnight at 4°C and incubated with the antibodies diluted in 2.5% skimmed milk in PBS/Tween for 1 hour. After 5 washes in PBS/Tween, the membranes were incubated for 1 hour with horseradish peroxidase-conjugated anti-rabbit (or anti-mouse) IgG (DAKO) diluted in PBS/Tween. The membranes were washed 5 times in PBS/Tween. Finally immunoreactive proteins were visualised by chemiluminescence (ECL, Amersham). All incubation and washing steps were performed at room temperature.

In some experiments keratinocytes were extracted in PAGE sample buffer directly. Equal amounts of protein (20 μg) were loaded and separated on 7.5% SDS-PAGE gels under reducing conditions. Western blotting was performed as described above.

Suspension induced terminal differentiation

Terminal differentiation was induced by suspending disaggregated keratinocytes (10^5 cells/ml final concentration) in culture medium (FAD+FCS+HICE) supplemented with 1.65% methyl cellulose (Aldrich Chemical Co. Ltd, Gillingham, UK). The cell suspension was transferred to bacterial culture grade plastic dishes coated with 0.4% polyHEMA (Hydron Laboratories, New Brunswick, NJ) for 24 hours and recovered from suspension by diluting the methyl cellulose with PBS as described previously (Adams and Watt, 1989; Watt et al., 1993). The single cell suspension was then air-dried onto coverslips, fixed in 3.7% formaldehyde in PBS and permeabilised in methanol (Read and Watt, 1988).

The degree of terminal differentiation after 24 hours in suspension was assessed by indirect immunofluorescence staining of cells with SY5 for the presence of involucrin. The percentage of involucrin-positive cells was calculated from photographs. A minimum of 1,000 cells was scored per sample. Results were expressed as the percentage increase in the number of cells expressing involucrin compared to the starting population (Hotchin et al., 1993).

Time lapse video recordings

Confluent keratinocytes were harvested with trypsin/EDTA and washed twice in complete medium to inactivate the trypsin. Keratinocytes (2×10^4) were plated onto feeder cells in a 35 mm dish and grown for 2 days. Cells were then enclosed in a 10% CO_2 , 37°C environmental chamber and cell motility was monitored for 24 hours. Migration of the cells was recorded every 2 minutes. Olympus IMT1 or IMT2 inverted microscopes fitted with monochrome CCD cameras, video recorders (Sony SCC M370CE and PVW-2800P, respectively) and driven by Broadcast Animation Controllers (BAC 900) were used. Films were viewed on a SVHS video recorder linked to a 386 PC and cell motility analysed using the program 'Cell Motility' (EOS Electronics, South Glamorgan, Wales).

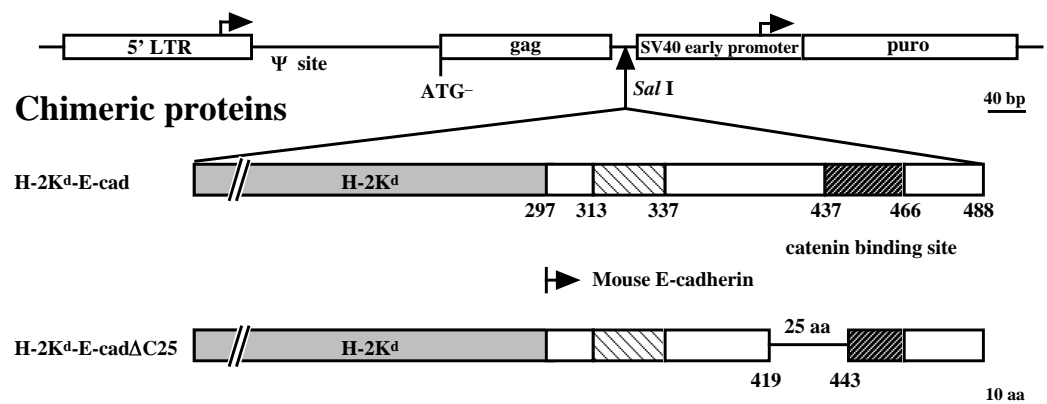
RESULTS

Expression of a dominant negative E-cadherin mutant in keratinocytes

Normal human epidermal keratinocytes are difficult to transfect and so we used retroviral infection to express the cadherin mutant constructs. The retroviral vector, pBabe puro, is a high titre, direct orientation vector which expresses the gene of interest under the control of the Mo MuLV LTR and the puromycin resistance gene from an internal SV40 early region promoter. As illustrated in Fig. 1 we introduced two inserts into pBabe puro. The first (H-2K^d-E-cad) consists of the transmembrane and cytoplasmic domains of mouse E-cadherin and the extracellular domain of H-2K^d, which acts as a con-

Fig. 1. Schematic illustration of construction of the dominant negative E-cadherin mutant and the mutant with the catenin binding site destroyed. Arrows in the pBabe puro vector indicate the start sites of transcription. The dominant negative E-cadherin protein H-2K^d-E-cad consisted of the extracellular part of mouse MHC class I antigen H-2K^d (shaded) linked to a small part of the extracellular region (297-313 amino acids), the transmembrane domain (light hatching) and the cytoplasmic domain of mouse E-cadherin. The H-2K^d-E-cad Δ C25 construct had a 25 amino acid deletion that destroys the catenin binding site. The catenin binding site is indicated by dark hatching.

Retroviral vector pBabe puro



venient marker of infected cells (Ozawa et al., 1990). The second (H-2K^d-E-cad Δ C25) is a control in which the catenin binding site has been destroyed by a 25 amino acid deletion in the cytoplasmic domain. In the experiments to be described, four populations of keratinocytes were compared and are designated K, P, Δ and E: K were normal, uninfected keratinocytes; P were infected with the empty vector, pBabe puro; Δ were infected with the H-2K^d-E-cad Δ C25 construct; and E were infected with the H-2K^d-E-cad construct.

Keratinocytes were labelled with an antibody specific for H-2K^d (Fig. 2). As expected, H-2K^d was not detected on the surface of K and P cells, but gave positive staining of Δ and E cells. The majority of Δ and E cells were positively labelled with H-2K^d (i.e. fluorescence greater than with second antibody alone) and the modal fluorescence of each population

was similar. However, the proportion of cells with higher fluorescence (>10 units in Fig. 2) was consistently greater in the E than in the Δ population.

Effects of the dominant negative mutant on endogenous cadherins and catenins

To examine expression levels and catenin binding of the H-2K^d-E-cad and H-2K^d-E-cad Δ C25 constructs infected keratinocytes were extracted and immunoprecipitated with anti-H-2K^d. The immunoprecipitates were transferred to Immobilon membrane and either blotted with an antiserum that recognises the cytoplasmic domain of E- and P-cadherin (Braga et al., 1995) or with catenin-specific antisera. As shown in Fig. 3A, anti-H-2K^d immunoprecipitated a band of 66 kDa in E cells (see Ozawa et al., 1990) and of 62 kDa in Δ cells

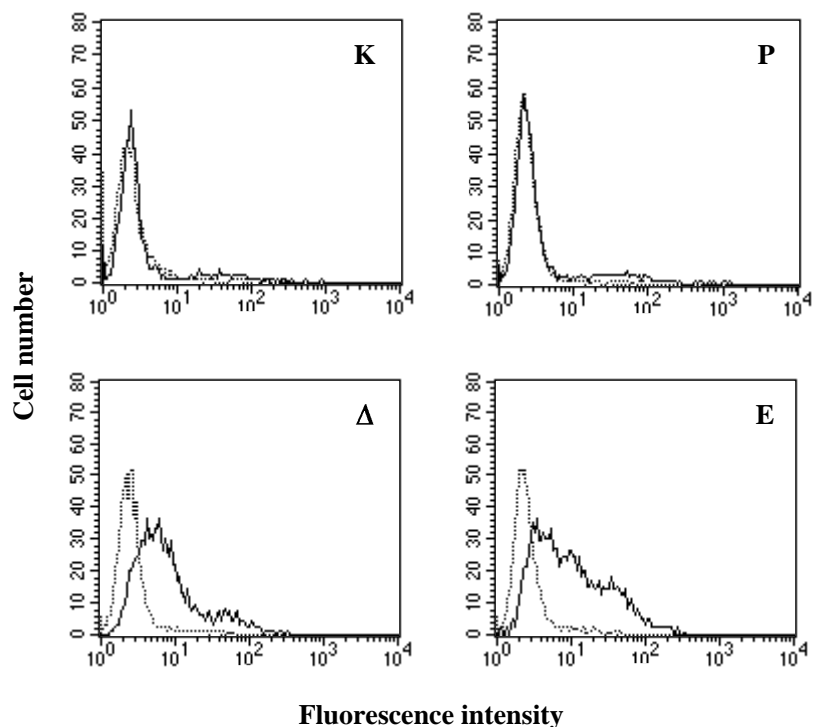


Fig. 2. Flow cytometry of cells labelled with antibody K9-18, which recognises H-2K^d (continuous lines) or with secondary antibody alone (dotted lines). Profiles show total population (i.e. involucrin-positive and -negative cells).

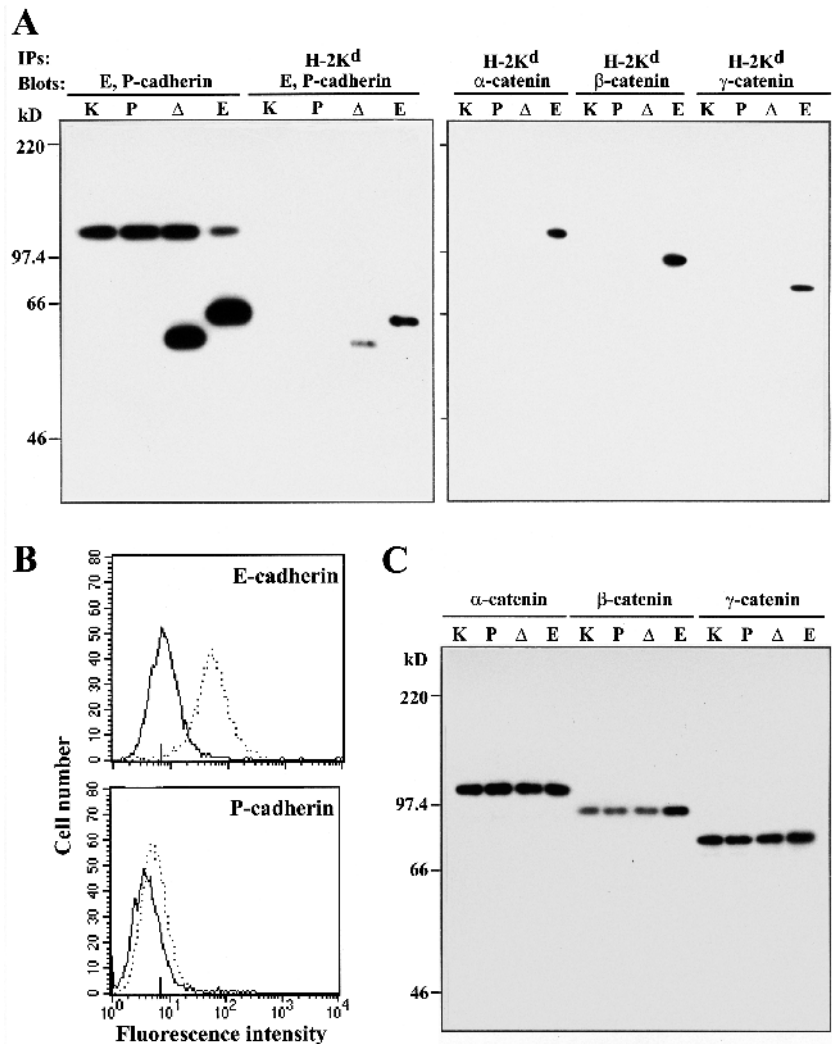


Fig. 3. Functional analysis of the chimeric proteins H-2K^d-E-cad and H-2K^d-E-cadΔC25. (A,C) Unlabelled cell extracts were immunoblotted with the antibodies shown; where indicated the extracts were immunoprecipitated (IPs) with anti-H-2K^d prior to blotting. Positions of molecular mass standards are shown. Samples were run on 7.5% SDS-PAGE gels under reducing conditions. Equal amounts of protein from the different cell populations were compared. Immunoprecipitations were carried out on Triton-soluble proteins; proteins to be blotted without prior immunoprecipitation were extracted in PAGE sample buffer. (B) Flow cytometry of E cells (continuous lines) and Δ cells (dotted lines) with monoclonal antibodies specific for the extracellular domains of E-cadherin and P-cadherin. Marks on x axes show maximum fluorescence of cells labelled with second antibody alone.

that were recognised by the anti-cadherin antiserum. The 62 kDa band tended to be slightly less abundant than the 66 kDa band, consistent with the flow cytometry results (Fig. 2); note that the left hand panel of Fig. 3A has been overexposed in order to reveal the endogenous cadherin band in E cells. The anti-H-2K^d immunoprecipitates of E cells contained α-, β- and γ-catenin, while those of Δ cells did not, confirming that the catenin binding site had been destroyed in the Δ construct (Fig. 3A). K and P cells served as negative controls.

The antiserum to the cadherin cytoplasmic domain not only recognised the H-2K^d/cadherin chimeric proteins but also endogenous E- and P-cadherin (which co-migrate on SDS-PAGE; see Braga et al., 1995). As shown in Fig. 3A the levels of total endogenous cadherins were similar in K, P and Δ cells, but were decreased by approximately 5-fold in E cells (data in Fig. 3A are representative of 4 experiments). Flow cytometric analysis showed that there was a marked reduction in the level of E-cadherin on the surface of E cells but not Δ cells (Fig. 3B and data not shown); the same was true of P-cadherin although the level of surface P-cadherin in control cells (P, K, Δ) was considerably lower than that of E-cadherin (Fig. 3B, and data not shown). Expression of H-2K^d-E-cad had no effect on the levels of α- and γ-catenin in infected keratinocytes, but the level of both total and Triton-soluble β-

catenin was increased approximately 3-fold (Fig. 3C and data not shown).

Double label immunofluorescence staining was used to compare the distribution of the H-2K^d tag and endogenous E-cadherin and catenins in E cells and Δ cells (Fig. 4, and data not shown). All cells stained positive for H-2K^d. In cells expressing either construct the majority of the H-2K^d label was cytoplasmic, the perinuclear distribution suggesting localisation predominantly to the endoplasmic reticulum (Fig. 4; Hotchin et al., 1995; A. J. Zhu and F. M. Watt, unpublished observations). In E cells there was extensive co-localisation of E-cadherin and the catenins with H-2K^d in the cytoplasmic compartment and no concentration of E-cadherin and catenins at cell-cell borders (Fig. 4B,D). In contrast, the majority of E-cadherin and catenin labelling was at cell-cell borders in Δ cells (Fig. 4A,C).

Effects of the dominant negative mutant on intercellular adhesion and motility

Keratinocytes expressing the H-2K^d-E-cadΔC25 construct grew as compact colonies that were indistinguishable from control keratinocytes (cf. Δ, K, P; Fig. 5A). In contrast, E cells failed to form compact colonies and stratification, as determined by conventional and confocal microscopy, was

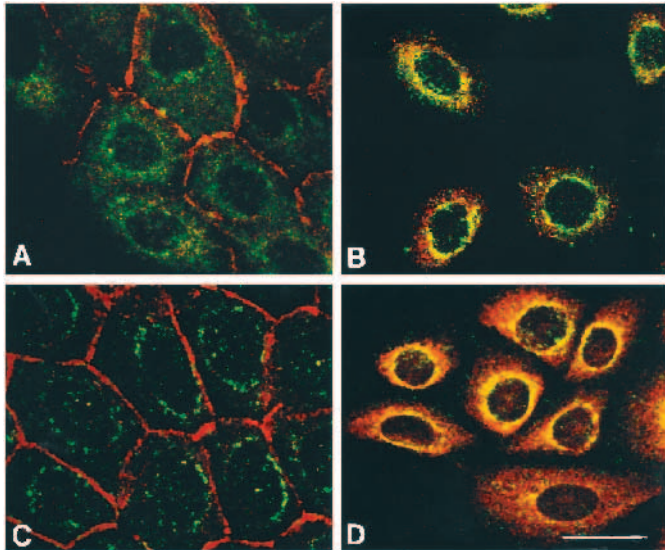


Fig. 4. Double label immunofluorescence staining of Δ cells (A,C) and E cells (B,D) with anti-H-2K^d (green) and anti-E-cadherin (red, A,B) or anti- α -catenin (red, C,D). Bar, 15 μ m.

greatly inhibited. E cell morphology was dramatically different from Δ , P or K cells: E cells tended to be elongated with prominent lamellipodia and formed very few intercellular contacts.

The motility of P, Δ and E cells was monitored using an Olympus time-lapse photography unit. Cultures were filmed for 24 hours at a rate of one frame every 2 minutes. The speed and travel distance of 24 individual cells from each population was determined from traces (Fig. 5B). There were no significant differences in the speed or travel distances of P and Δ cells, but E cells showed a twofold increase in speed and travel distance ($P < 0.0001$; Fig. 5C). No P, Δ or E cells detached from the culture dish during the recording period.

It has already been reported that classical cadherins are required to maintain and stabilise desmosomal junctions (Amagai et al., 1995; Marrs et al., 1995). As expected, desmosome formation was impaired in E cells. Fig. 6 shows immunofluorescence staining of keratinocytes for desmoplakin, one of the protein components of desmosomal junctions. Intense punctate staining for desmoplakin was observed at cell-cell borders of K, P and Δ cells, consistent with extensive desmosome formation (cf. Watt et al., 1984). In E cells desmoplakin staining was observed at sites of cell-cell contact, but was greatly decreased compared to control cells, reflecting the reduction in intercellular adhesion. In the absence of cell-cell contact, punctate desmoplakin staining was seen throughout the cytoplasm of E cells, possibly representing endocytosis of unstable desmosomal complexes (Demlehner et al., 1995).

Effects of the dominant negative mutant on integrin expression

In view of the effects of H-2K^d-E-cad on keratinocyte morphology and motility, we examined the levels and cellular distribution of integrins. Surface expression of integrin subunits in basal (involucrin-negative) keratinocytes was examined by flow cytometry (Fig. 7A). There was a twofold reduction in the

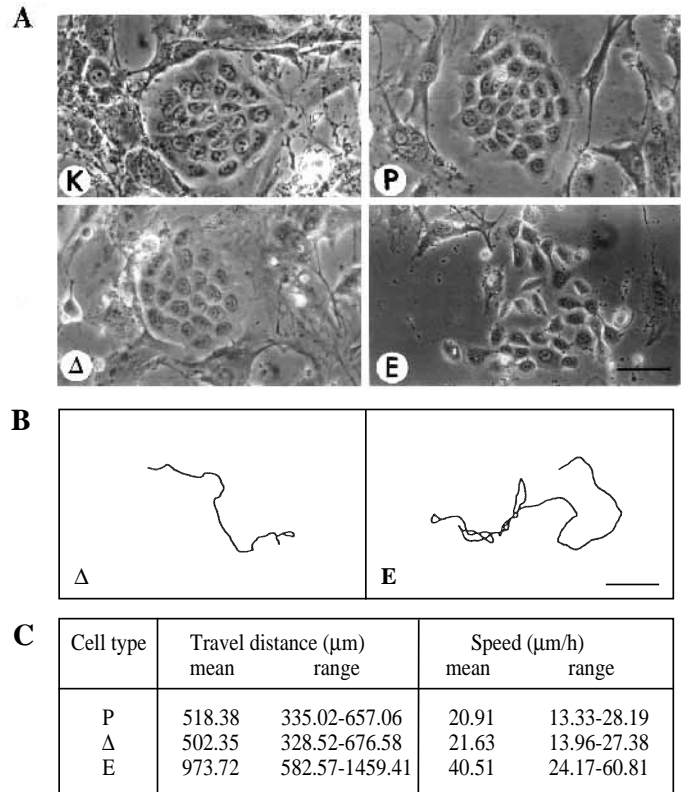


Fig. 5. Effects of H-2K^d-E-cad on cell morphology and motility. (A) Phase contrast images of individual keratinocyte colonies surrounded by feeder cells. Bar, 30 μ m. (B) Traces showing the movement of an individual Δ and an individual E cell. These cells were chosen because they migrate with the mean travel distance of each population. Bar, 100 μ m. (C) Comparison of the travel distance and speed of 24 cells from each cell population (P, Δ , E).

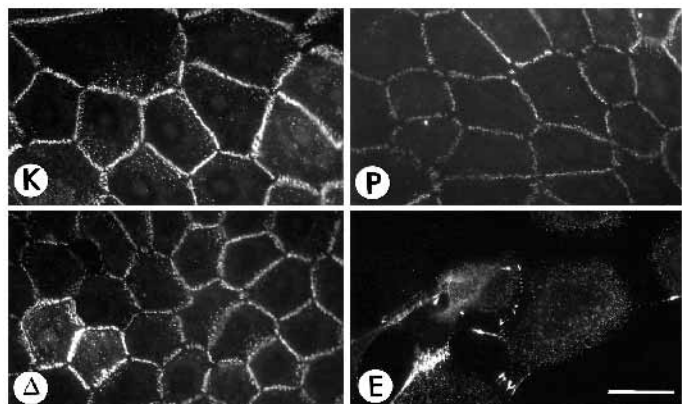


Fig. 6. Desmosome assembly. Subconfluent sheets of keratinocytes were permeabilised and stained by immunofluorescence with an antibody to desmoplakin. Bar, 20 μ m.

modal fluorescence of E cells labelled with antibodies to the α_2 , α_3 and β_1 integrin subunits when compared to Δ cells; there was no difference in the levels of the α_5 , α_6 or β_4 integrin subunits. The levels of surface integrins were similar in K, P and Δ cells (data not shown).

In epidermis and in stratified cultures of keratinocytes

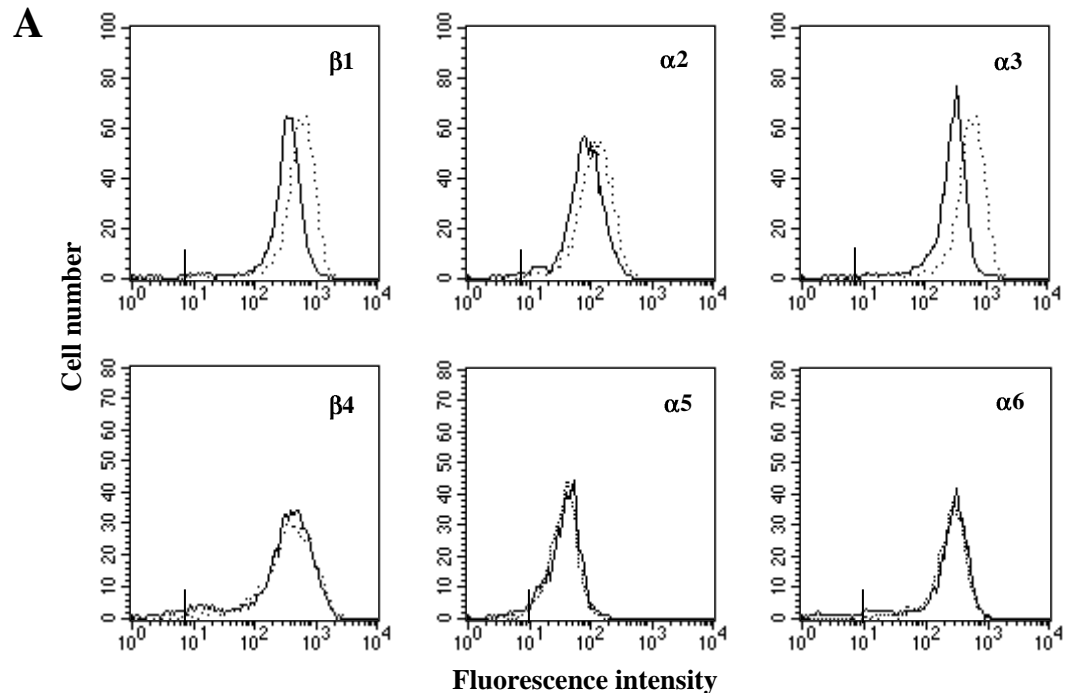
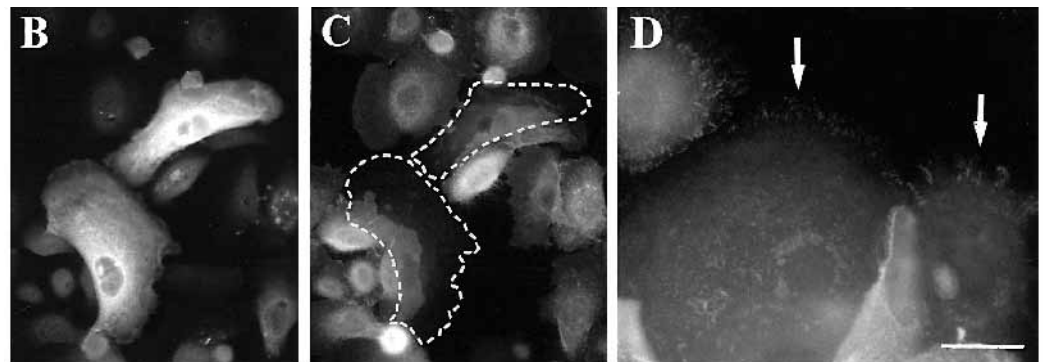


Fig. 7. Integrin expression. (A) Flow cytometry of basal keratinocytes with antibodies to the integrin subunits indicated. Continuous lines, E cells; dotted lines, Δ cells. Marks on x axes show maximum fluorescence of cells labelled with second antibody alone. (B,C) Double label immunofluorescence of E cells stained with antibodies to involucrin (B) and β_1 integrins (C). Broken lines in C show the position of the two involucrin-positive cells in B. (D) β_1 integrin staining of focal adhesions. The two cells marked with arrows are involucrin-positive (as determined by double labelling with anti-involucrin; not shown). Bar, 40 μm (B,C); 16 μm (D).



integrin expression is largely confined to the basal cell layer, but when stratification of keratinocytes is inhibited by culture in low calcium medium, terminally differentiating, involucrin-positive cells continue to express integrins on the apical and lateral membrane domains and in focal adhesions (Hodivala and Watt, 1994; F. M. Watt, unpublished data). As described above, E cells failed to stratify when grown in standard medium (1.8 mM calcium ions). Involucrin-positive E cells expressed β_1 integrins in focal adhesions and remained attached to the culture substratum; however, integrins were absent from the apical and lateral domains of the cells (Fig. 7B,C,D). Involucrin-negative E cells expressed β_1 integrins in focal adhesions and over the entire cell surface (Fig. 7B,C, and data not shown).

Effects of the dominant negative mutant on keratinocyte growth and differentiation

In order to determine whether H-2K^d-E-cad had any effect on keratinocyte proliferation, we prepared growth curves of K, P, Δ and E cells. Five hundred cells of each population were plated per 35 mm dish on mitomycin C treated feeder cells. Cell numbers were measured in triplicate dishes at intervals for

up to 25 days (Fig. 8A). Cells could not be counted with accuracy prior to day 7 as keratinocytes in these early cultures tended to detach during the EDTA treatment that was used to release the feeder cells, but in older cultures keratinocytes could only be detached with trypsin/EDTA. There were no differences in the growth rates of K, P and Δ cells, even though P and Δ cells were grown in the presence of puromycin. E cells appeared to grow at the same rate as the other cells during the first week, but grew more slowly from day 9 onwards. Confluence was reached by day 19 in K, P and Δ cells, but not until day 25 in E cells.

The proportion of cells undergoing terminal differentiation was measured in pre- and post-confluent adherent cultures and in single cell suspensions of pre-confluent cultures maintained in methyl cellulose for 24 hours (Fig. 8B). In pre-confluent cultures there was no significant difference between the proportion of involucrin-positive cells in the four cell populations. However, in confluent cultures and following suspension-induced differentiation the proportion of involucrin-positive cells was significantly higher in the E cell populations than in the P, Δ and K cell populations ($P < 0.005$; Fig. 8B).

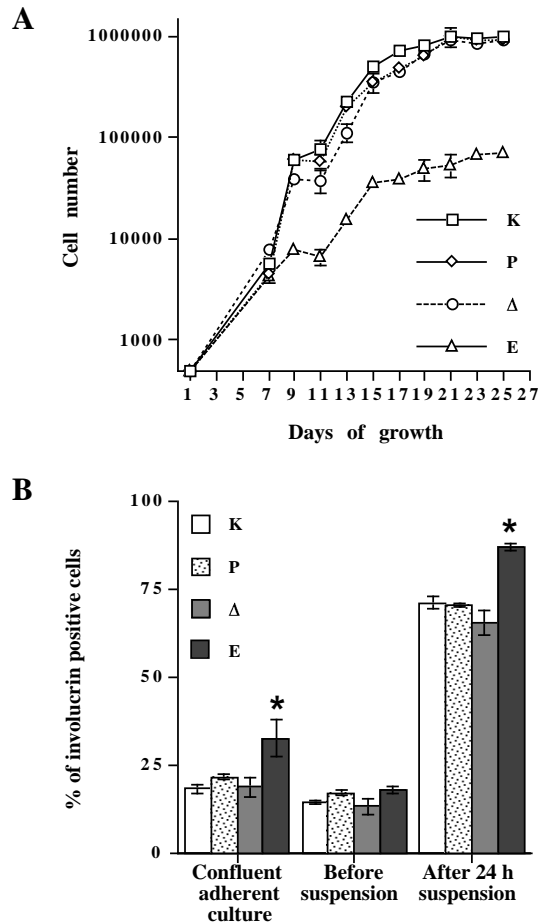


Fig. 8. Keratinocyte growth and differentiation. (A) 500 cells were plated per 35 mm dish and triplicate dishes were harvested on the days shown. Error bars are s.e.m. (B) The proportion of involucrin-positive keratinocytes was determined in pre-confluent cultures (Before suspension) and following 24 hours suspension in methyl cellulose (After suspension). In a separate experiment, the proportion of involucrin-positive cells was determined in a confluent adherent culture. Asterisks indicate that the proportion of involucrin-positive E cells was significantly different from that of Δ cells ($P < 0.005$). There was no significant difference between the proportion of involucrin-positive K, P and Δ cells ($P > 0.05$). Error bars represent standard deviation of the mean of replicate samples within a single experiment.

DISCUSSION

We have achieved high level expression of a dominant negative cadherin mutant by retroviral infection of cultured human keratinocytes. The H-2K^d/cadherin chimeric protein was found on the cell surface and in the cytoplasm. The mutant formed complexes with α -, β - and γ -catenin. The total and cell surface levels of endogenous E- and P-cadherin were reduced in cells expressing the dominant negative mutant and the total level of β -catenin was increased. Destruction of the catenin binding site abolished the activity of the mutant, although it should be noted that larger deletions of the cytoplasmic domain of N-cadherin still have dominant negative activity in *Xenopus* embryos (Kintner, 1992).

Two mechanisms have been proposed for the dominant

negative effect of cadherins that lack the extracellular domain: competition with endogenous cadherins for binding catenins (Kintner, 1992; Hertig et al., 1996) and substitution of non-functional cadherin for endogenous cadherins at cell-cell junctions (Fujimori and Takeichi, 1993). In keratinocytes expressing H-2K^d-E-cad there was evidence for both the competition and substitution mechanisms: the chimeric protein bound endogenous catenins and was expressed at the cell surface. It has previously been shown that E-cadherin forms complexes with β - and γ -catenins in the endoplasmic reticulum and that α -catenin becomes associated during traffic to the plasma membrane (Ozawa et al., 1989, 1990; Hinck et al., 1994a). Further experiments will be required to determine whether in keratinocytes expressing H-2K^d-E-cad there is some inhibition of transport of endogenous cadherins to the cell surface or whether when they reach the surface they are unstable and undergo endocytosis and degradation.

The changes in the levels of cadherins and β -catenin that we observed have not been reported in other experimental models in which dominant negative cadherin mutants have been expressed (Fujimori and Takeichi, 1993). There is some evidence that homophilic binding of E-cadherin prolongs the half-life of the protein (Shore and Nelson, 1991) and that association of E-cadherin with α -catenin may stabilise cell surface expression (Nagafuchi et al., 1994). Indeed the latter observation fits with our finding that higher expression of H-2K^d-E-cad than of H-2K^d-E-cad Δ C25 could be achieved in keratinocytes (Figs 2, 3). We therefore suggest that the reduction in endogenous E- and P-cadherin in keratinocytes expressing H-2K^d-E-cad is the result of decreased protein stability, either due to competition for catenin binding or failure to reach the cell surface. This would be consistent with our preliminary northern blot analysis which shows that E- and P-cadherin mRNA levels are the same in E and Δ cells (A. J. Zhu and F. M. Watt, unpublished observations). β -Catenin stability is affected by Wnt signalling, APC binding and glycogen synthase kinase phosphorylation (van Leeuwen et al., 1994; Peifer et al., 1994; Papkoff et al., 1996), potential regulatory mechanisms that remain to be explored in keratinocytes.

The decrease in levels of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ on the surface of basal keratinocytes expressing H-2K^d-E-cad is similar to the decrease in β_1 integrin levels seen in uninfected involucrin-negative keratinocytes transferred to low calcium medium. It is also striking that in both low calcium cultures of normal keratinocytes and in standard cultures of E cells involucrin-positive cells continued to express integrins, although in E cells the integrins were largely confined to focal adhesions (Hodivala and Watt, 1994). Our observations and those of other workers lend support to the concept of cross-talk between cadherins and integrins (Hodivala and Watt, 1994). Hermiston and Gordon (1995) found a reduction in adhesion of epithelial cells to the basement membrane when a dominant negative cadherin was expressed in the intestine of transgenic mice. In contrast, expression of E-cadherin in a *Xenopus* cell line leads to reduced extracellular matrix adhesion (Finnemann et al., 1995). It is interesting that the effect of H-2K^d-E-cad in basal keratinocytes was selective for $\alpha_2\beta_1$ and $\alpha_3\beta_1$, since these integrins are predominantly localised to cell-cell borders, whereas $\alpha_5\beta_1$ and $\alpha_6\beta_4$ are not (see Adams and Watt, 1991; Hertle et al., 1991). The nature of the interaction between integrins and cadherins is obscure at present, but direct binding

of the two types of receptor is one possibility (Cepek et al., 1994).

H-2K^d-E-cad expression inhibited stratification, reduced desmosome formation and stimulated motility in the total E cell population. The inhibition of stratification and suppression of desmosome formation were to be expected, because of earlier findings of the effects of growing cells in low calcium medium (Watt and Green, 1982), applying anti-cadherin antibodies (Wheelock and Jensen, 1992; Lewis et al., 1994; Hodivala and Watt, 1994) or expressing a dominant negative cadherin mutant (Amagai et al., 1995). H-2K^d-E-cad reduced surface expression of E- and P-cadherin and the effects on stratification and desmosome formation are consistent with the finding that antibodies to both E- and P-cadherin are required for maximum inhibition of those processes in uninfected cells (Hodivala and Watt, 1994; Lewis et al., 1994). The stimulation of motility could be due to the small decrease in $\alpha_3\beta_1$ expression (see Tenchini et al., 1993), since cell migration is believed to be maximal at intermediate cell surface integrin levels (Huttenlocher et al., 1995), or to release of physical constraints imposed by stable intercellular adhesion. The motility of E cells was very similar to that of normal keratinocytes in low calcium medium (Hodivala et al., 1994).

H-2K^d-E-cad expression inhibited keratinocyte proliferation and increased terminal differentiation. These results are surprising in view of the observations that loss or decrease of E-cadherin expression is a feature of squamous cell carcinomas (see, for example, Schipper et al., 1991), that activation of E-cadherin in lung carcinoma cells leads to inhibition of growth (Watabe et al., 1994) and that perturbation of desmosome assembly with a mutant desmosomal cadherin results in enhanced proliferation in transgenic mouse epidermis (Allen et al., 1996). Several observations suggest that the effects of H-2K^d-E-cad are not simply a consequence of the inhibition of intercellular adhesion. The effects were seen with a lag time of one week or more, whereas the inhibition of intercellular adhesion was observed within one day of plating. Growth in low calcium medium, which also inhibits cadherin-dependent intercellular adhesion, does not affect the proportion of cells that undergo terminal differentiation (Watt and Green, 1982; Drozdoff and Pledger, 1993; F. M. Watt, unpublished) and H-2K^d-E-cad cells were stimulated to differentiate in suspension in the absence of any cell-cell contact.

There are two potential mechanisms by which H-2K^d-E-cad could regulate proliferation and differentiation independently of its effect on intercellular adhesion. There is now good evidence for a signalling function of β -catenin that is independent of its role in adhesion; indeed it has been suggested that cadherins act as negative regulators of β -catenin signalling (Hinck et al., 1994b; Funayama et al., 1995; Gumbiner, 1995; Fagotto et al., 1996). It is thus possible that the effects of H-2K^d-E-cad are due to the combination of the increased level of β -catenin and decreased levels of E- and P-cadherin within the cells. Alternatively, the effects could be a consequence of the decrease in $\alpha_2\beta_1$ and $\alpha_3\beta_1$ levels: a twofold reduction in β_1 integrin levels occurs when keratinocytes leave the stem cell compartment (high proliferative potential) and enter the transit amplifying cell compartment (low proliferative potential, increased probability of terminal differentiation) (Jones and Watt, 1993); thus reduced integrin expression in E cells might shift the balance between proliferation and differentiation in

favour of differentiation. It is of considerable interest to test these ideas as the results will provide new insights into the role of cadherins in regulating cell behaviour.

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