Development 126, 2285-2298 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV3981

β-catenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion

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Accepted 25 February; published on WWW 19 April 1999

SUMMARY

We found that cultured human keratinocytes with high proliferative potential, the putative epidermal stem cells, expressed a higher level of noncadherin-associated β-catenin than populations enriched for keratinocytes of lower proliferative potential. To investigate the physiological significance of this, a series of β-catenin constructs was introduced into keratinocytes via retroviral infection. Full-length β-catenin and a mutant containing only nine armadillo repeats had little effect on proliferative potential in culture, the full-length protein being rapidly degraded. However, expression of stabilised, N-terminally truncated β-catenin increased the proportion of putative stem cells to almost 90% of the proliferative population in vitro without inducing malignant transformation, and relieved the differentiation stimulatory effect of overexpressing the E-cadherin cytoplasmic domain. Conversely, β-catenin lacking armadillo repeats acted as a dominant negative mutant and stimulated exit from the stem cell compartment in culture. The positive and negative effects of the β-catenin mutants on proliferative potential were independent of effects on cell-cycle kinetics, overt terminal differentiation or intercellular adhesion, and correlated with stimulation or inhibition of transactivation of a TCF/LEF reporter in basal keratinocytes. We conclude that the elevated level of cytoplasmic β-catenin in those keratinocytes with characteristics of epidermal stem cells contributes to their high proliferative potential.

Key words: β-catenin, Keratinocyte, Stem cell, TCF/LEF

INTRODUCTION

β-catenin is a vertebrate homologue of the *Drosophila* Armadillo protein, which was first identified as a segment polarity gene product that determines cell fate (Wieschaus and Riggleman, 1987). β-catenin is a major structural component of adherens junctions, linking the classical cadherins to the actin cytoskeleton through its ability to bind the cadherin cytoplasmic domain and α-catenin (McCrea and Gumbiner, 1991; Ozawa and Kemler, 1992). β-catenin is also a downstream effector of the Wingless (*Drosophila*)/Wnt (vertebrates) signalling pathway that controls morphogenetic events during embryonic development. In the absence of Wnt, β-catenin that is not complexed to cadherins is rapidly degraded by a mechanism that involves binding of β-catenin to the adenomatous polyposis coli gene product (APC) and phosphorylation of β-catenin by glycogen synthase kinase-3β (GSK-3β). When the Wnt pathway is activated GSK-3β is negatively regulated, and as a result high levels of β-catenin accumulate in the cytoplasm. Thereafter β-catenin translocates to the nucleus, forms a complex with transcription factors of the TCF/LEF family and acts as a transcriptional activator (reviewed by Miller and Moon, 1996; Clevers and van de Wetering, 1997; Cox and Peifer, 1998).

One of the organs in which β-catenin signalling is known to regulate growth and morphogenesis is the skin. Mammalian keratinocytes express several members of the TCF/LEF family of transcription factors, including LEF-1 (Zhou et al., 1995; Byrne, 1997), TCF-3 (N. Barker and H. Clevers, personal communication) and TCF-4 (C. Niemann and F. M. Watt, unpublished observation). LEF-1 is expressed throughout the mouse embryonic ectoderm and at high levels in the hair germ placodes; when the expression pattern is perturbed abnormalities in hair follicle patterning and orientation are observed (Zhou et al., 1995). Mice that are homozygous null for LEF-1 show defects in epithelial-mesenchymal interactions, including a failure to develop body hairs and whiskers (van Genderen et al., 1994; Kratchowil et al., 1996). Specific Wnts are expressed in the skin and Wnts have been implicated in feather and hair development (Chuong et al., 1996; St-Jacques et al., 1998). Most recently, expression of N-terminally truncated β-catenin, which is stabilised through loss of the GSK-3β phosphorylation sites, in the basal layer of mouse epidermis has been shown to cause keratinocytes to revert to a pluripotent state in which they can differentiate into hair follicles or interfollicular epidermis (Gat et al., 1998).

While the importance of β-catenin in hair follicle formation is thus well established, less is known about its function in post-embryonic interfollicular epidermis. Self-renewal of the epidermis occurs throughout adult life and is believed to depend on proliferation of a subpopulation of keratinocytes in the basal layer known as stem cells. In human epidermis, the stem cells

can be distinguished from their daughters that are destined to differentiate within a few rounds of division, the transit amplifying cells, because they express two- to threefold higher surface levels of β1 integrins (Jones and Watt, 1993; Jones et al., 1995). Primary diploid human epidermal keratinocytes can be grown in culture under conditions in which cells with characteristics of stem, transit amplifying, and suprabasal, terminally differentiating, cells are all maintained (reviewed by Watt, 1989, 1998). Populations enriched for stem or transit amplifying cells can be selected by FACS after labelling with anti-β1 integrin antibodies or by differential extracellular matrix adhesiveness, and the proportion of stem and transit amplifying cells in a mixed population can be estimated by scoring the proportion of cells that form actively growing or abortive colonies (Jones and Watt, 1993; Jones et al., 1995; Gandarillas and Watt, 1997). Such cultures therefore provide an attractive experimental model for studies of β-catenin function in the absence of specific mesenchymal influences.

MATERIALS AND METHODS

Retroviral vectors

The full-length *Xenopus* β-catenin construct, MT1, and three deletion mutants, T2, T3 and MT10, were kind gifts from B. M. Gumbiner (Funayama et al., 1995; Fagotto et al., 1996). The cDNAs were adapted with *Bam*HI linkers and subcloned into the *Bam*HI site of the retroviral vector, pBabe puro (Zhu and Watt, 1996; Gandarillas and Watt, 1997). Both ecotropic (GP+E) and amphotropic (AM12) packaging cells producing each retroviral vector were generated. Clones of amphotropic packaging cells were isolated as described previously (Zhu and Watt, 1996). The titre of the MT1 producer cells was 7.7×10^5 cfu/ml, that of the T2 producer cells was 9.7×10^5 cfu/ml, that of the T3 producer cells was 1.4×10^6 cfu/ml and that of the MT10 producer cells was 8.0×10^5 cfu/ml.

As a control keratinocytes were infected with polyclonal AM12 cells expressing the empty retroviral vector encoding only the puromycin resistance gene, puro (3.2×10^6 cfu/ml) (Zhu and Watt, 1996; Gandarillas and Watt, 1997). In some experiments keratinocytes were infected with pBabe puro containing the dominant negative E-cadherin mutant, H-2K^d-E-cad, or a control construct in which the catenin binding site was destroyed, H-2K^d-E-cadΔC25 (Zhu and Watt, 1996).

Keratinocyte culture and retroviral infection

Normal human epidermal keratinocytes from neonatal foreskins (strains z, km, kq; passages 2-6) were cultured on a J2-3T3 feeder layer as described previously (Jones and Watt, 1993; Zhu and Watt, 1996; Gandarillas and Watt, 1997) and were retrovirally infected by using producer cells as feeders for the first 3 days (Zhu and Watt, 1996; Gandarillas and Watt, 1997). Cells expressing the retroviral vectors were selected in the presence of 1.0 μg/ml puromycin and close to 100% transduction efficiency was achieved. Double infections were performed in the same way, with keratinocytes expressing H-2K^d-E-cad or H-2K^d-E-cadΔC25 (Zhu and Watt, 1996) being cocultured with T2-, T3- or puro-expressing producer cells. Subconfluent cultures were used in all experiments unless stated otherwise. The effects of the retroviral vectors on keratinocyte proliferative potential were observed with cells from three different individuals (i.e. the three strains referred to above) and in both primary infections and subsequent passages.

Antibodies

The following monoclonal antibodies were used: 9E10 (mouse anti-human c-Myc; a gift from G. Evan; Gandarillas and Watt, 1997), 12CA5 (mouse anti-influenza HA, a gift from J. M. White; Funayama et al., 1995), ALI-12-28 (mouse anti-APC; a gift from W. F. Bodmer; Efstathiou et al., 1998), CD29 conjugated to FITC (mouse anti-human β1 integrins, DAKO A/S, Denmark; Jones and Watt, 1993), clones 5, 14 and 15 (mouse anti-α-, β-catenin and plakoglobin, Transduction Laboratories, NY, USA), HECD-1 (mouse anti-E-cadherin, a gift from M. Takeichi; Zhu and Watt, 1996), mAb13 (rat anti-human β1 integrins; a gift from K. Yamada; Hodivala and Watt, 1994), MP4F10 (mouse anti-human α6β4 integrin, a gift from M. Pignatelli; Anbazhagan et al., 1995), P5D2 (mouse anti-human β1 integrins, Develpmental Studies Hybridoma Bank; Zhu and Watt, 1996), SY5 (mouse anti-human involucrin; Zhu and Watt, 1996), TAG-1A2 (mouse anti-tubulin; Sigma, Dorset, UK) and UCHT4 conjugated to FITC (mouse anti-CD8, Sigma; Gandarillas and Watt, 1997). The following rabbit antisera were also used: A14 (rabbit anti-Myc, Santa Cruz Biotech. Inc., Santa Cruz, CA, USA), FWCAD (rabbit anti-pan-cadherin; Braga et al., 1995), VB1, VB2 and VB3 (rabbit anti-α-, β-catenin and plakoglobin; Braga et al., 1995) and Y11 (rabbit anti-HA, Santa Cruz Biotech., CA, USA). Fluorescein (FITC)-conjugated goat anti-mouse and anti-rabbit IgG and Texas Red (TRSC)-conjugated goat anti-mouse and anti-rabbit IgG were purchased from Jackson ImmunoResearch Lab., West Grove, PA, USA.

Integrin FACS and flow cytometry

Preconfluent, uninfected keratinocyte cultures were harvested with trypsin/EDTA, labelled with an anti-β1 integrin antibody, CD29-FITC, and sorted with a Becton-Dickinson FACStar Plus (Becton-Dickinson, Lincoln Park, NJ, USA). Suprabasal cells were gated out on the basis of forward and side scatter profiles (Jones and Watt, 1993). Stem cells (i.e. the 20% of basal cells with the highest β1 integrin levels) and transit amplifying cells (i.e. the 20% of basal cells with the lowest β1 integrin levels) were isolated from the basal cell fraction (Jones and Watt, 1993). UCHT4-FITC was used as the negative control antibody.

To compare surface integrin levels on total basal keratinocytes expressing different retroviral vectors, labelling was carried out as described above, except that P5D2 was used to label β1 integrins and MP4F10 was used to label the α6β4 integrin. The primary antibodies were detected with FITC-anti mouse IgG.

Cell lysis, immunoprecipitation, SDS-PAGE and western blotting

For immunoprecipitation, cells were washed twice in ice-cold PBS containing 1 mM Mg^{2+} and 1 mM Ca^{2+} ions, then extracted in Triton X-100 extraction buffer (Zhu and Watt, 1996) for 20 minutes on ice. The cells were scraped into tubes and centrifuged at 16,000 ***g*** for 10 minutes at 4°C. Protein concentrations of the supernatants were determined by the Bio-Rad Dc protein assay (Bio-Rad, Hercules, CA, USA). The pellets were discarded and the supernatants were divided into equal portions and frozen at –80°C.

Prior to immunoprecipitation, samples were pre-cleared by incubation with Protein A-Sepharose CL-4B beads (Pharmacia Biotech., Uppsala, Sweden) or Protein G-agarose (Sigma) and then incubated with antibodies for 4 hours at 4°C. For some experiments antibodies were chemically crosslinked to Protein A-Sepharose or Protein G-agarose beads as described by Zhu and Watt (1996). Immunoprecipitates were washed twice in the HSB buffer, once in the HSB buffer supplemented with 0.5 M NaCl, once in the HSB buffer, then once in the LSB buffer (Zhu and Watt, 1996). Immunoprecipitates were then separated by PAGE on 7.5% or 10% SDS-gels (Laemmli, 1970). In some experiments keratinocytes were extracted in SDS-PAGE sample buffer and directly subjected to electrophoresis. Rainbow markers (Amersham International, Buckinghamshire, UK) were used in each gel.

For western blotting, protein lysates or immunoprecipitates that had been separated by SDS-PAGE under reducing (or non-reducing for β1 integrin blotting) conditions were transferred to Immobilon PVDF

membranes (Millipore, Harrow, UK) at 200 mA for 1 hour using a semi-dry MilliBlot graphite electroblotter I system (Hoffer Scientific Instruments, CA, USA). Membranes were blocked with 5% skimmed milk (Marvel, Cadbury, UK) in PBS/0.05% Tween-20 (PBS/Tween) overnight at 4°C and incubated with the antibodies diluted in 2.5% milk in PBS/Tween for 1 hour. After five washes in PBS/Tween, the membranes were incubated for 1 hour with horseradish peroxidase-conjugated sheep anti-rabbit (or anti-mouse, or anti-rat) IgG (Amersham) diluted in milk in PBS/Tween. The membranes were washed five times in PBS/Tween. All incubation and washing steps were performed at room temperature. Finally immunoreactive proteins were visualised by chemiluminescence (ECL, Amersham) on Hyperfilm-MP (Amersham). In some experiments, blots were reprobed with a tubulin antibody to check for equal loading. Quantitation of the intensity of bands detected by immunoprecipitation or western blotting was performed using NIH IMAGE software.

For pulse-chase labelling, preconfluent cells were starved by incubation in labelling medium that lacked methionine and cysteine for 1 hour at 37°C. Cells were then incubated with fresh labelling medium containing 150 μCi/ml ^{35}S-cysteine and -methionine (Trans ^{35}S-label, specific activity >1180 Ci/mmol, ICN, High Wycombe, UK) for 30 minutes at 37°C. The labelling medium was removed and the cells were washed three times with ice-cold PBSABC. For the 0 hour time point, cells were lysed immediately. For the chase period, normal growth medium supplemented with 5 mM cysteine and methionine (Sigma) was added to the cells. Equal volumes of protein lysates were precipitated as described above and separated by 10% SDS-PAGE under reducing conditions. The gels were then incubated for 30 minutes with Amplify (Amersham), dried and exposed to Kodak X-OMAT X-ray film (Sigma) at −80°C.

For immunodepletion of cadherins cells were radioactively labelled overnight using the conditions described for pulse-chase labelling. After labelling, stem cells were selected by adhesion to 100 μg/ml type IV collagen for 20 minutes and transit amplifying cells were selected as those that adhered between 1-3 hours, as described by Jones and Watt (1993). The different populations were extracted and immunoprecipitated with FWCAD, as described above, except that the antigen bound to protein G-agarose was discarded and the supernatant immunoprecipitated again with FWCAD. The FWCAD immunoprecipitations were carried out a total of six times; after the sixth round of immunoprecipitation no cadherin was detected in the lysates. The lysates were then immunoprecipitated with antibodies to E- and P-cadherin, α-, β-catenin or plakoglobin, and visualised as described for the pulse-chase experiment.

Indirect immunofluorescence staining

Keratinocytes grown on coverslips were fixed in 3% paraformaldehyde for 20 minutes at room temperature and quenched in freshly made 50 mM NH_4Cl for 10 minutes. Subsequently, cells were permeabilised in 0.1% Triton X-100 in PBS for 4 minutes at room temperature. Prior to immunostaining, cells were incubated for 1 hour in 0.2% fish skin gelatin in PBS (FSG/PBS) in order to inhibit non-specific antibody binding. Antibody incubations were carried out at room temperature for 1 hour; specimens were washed thoroughly in FSG/PBS after each incubation. DAPI (Molecular Probes Inc., PoortGebouw, The Netherlands) counterstaining was used to identify nuclei in some experiments. Stained coverslips were mounted in Gelvatol (Monsanto Chemicals, St Louis, MO, USA) and examined with a Nikon Diaphot 200 inverted microscope (Nikon UK Ltd., Telford, UK), equipped with an MRC-1000 laser scanning confocal microscope attachment (Bio-Rad Microscience, Hemel Hempstead, UK). Confocal images were captured and processed with Adobe Photoshop (Adobe Systems Inc., Mountain View, CA, USA).

Growth, motility, terminal differentiation and clonogenicity assays

To generate growth curves, the total number of keratinocytes per dish was determined using a Coulter Counter (Coulter Electronics Ltd., Herts, UK) (Zhu and Watt, 1996; Gandarillas and Watt, 1997). Terminal differentiation was evaluated by flow cytometry as the percentage of involucrin-positive cells in preconfluent adherent cultures or after suspension for 24 hours in methyl cellulose-supplemented medium (Gandarillas and Watt, 1997). Cell motility assays were carried out essentially as described by Zhu and Watt (1996).

For clonogenicity assays the cultures were fixed in formaldehyde 14 days after plating and stained for 30 minutes at room temperature with Rhodanile Blue (Jones and Watt, 1993; Jones et al., 1995; Gandarillas and Watt, 1997). All colonies (i.e. >1 cell) were scored on each dish and colony forming efficiency was calculated as percentage of all plated cells that formed colonies; this differs from Jones and Watt (1993), who calculated colony forming efficiency as percentage of basal cells that formed colonies. Abortive colonies were defined as colonies that contained fewer than 40 cells, the majority of the cells being large and terminally differentiated (Jones and Watt, 1993; Jones et al., 1995; Gandarillas and Watt, 1997).

To determine whether colony size differed between puro-, T2- and T3-expressing cells Rhodanile Blue-stained dishes from clonogenicity assays were scanned into a Power Macintosh computer using Adobe Photoshop software. The perimeter of stem cell colonies was traced using IPLab Scientific Imaging software (Signal Analytics Corp., Vienna, VA, USA), which calculated the area of each colony and assigned a unitary value, such that 100 units corresponded to 2.4 mm^2. More than 100 colonies were scored per construct. Abortive, transit amplifying colonies had an area of less than 50 units (i.e. <1.2 mm^2) and they were excluded from the analysis.

As described by Gandarillas and Watt (1997), the proportion of cells in different phases of the cell cycle was measured by flow cytometry after resuspending cells in 10 μg/ml propidium iodide, and the proportion of S-phase cells was measured after incubating cells for 3 hours with 10 μM BrdU.

Karyotyping

Metaphase chromosome spreads were prepared from 5th passage keratinocytes expressing T2 or puro in log-phase growth. J2 feeder cells were first removed by EDTA treatment. Fresh medium was added to the cultures and the cells were incubated with 0.5 μg/ml colcemid for 1-2 hours, harvested and subjected to hypotonic shock. Chromosome spreads were prepared and karyotyping was carried out by P. Gorman of the ICRF Human Cytogenetics Laboratory.

Luciferase assays

The following luciferase reporters were used: pTOPtkLuciferase (TOPFLASH, containing three copies of TCF/LEF consensus sequence), pFOPtkLuciferase (FOPFLASH, containing three copies of mutated consensus sequence) (gifts from H. Clevers; van de Wetering et al., 1997), and p4xAP1Luc (containing four copies of AP-1 binding site) (a gift from A. Corbi; Rincón and Flavell, 1994). The *Xenopus* β-catenin cDNAs were subcloned into the *Bam*HI site of pCMV5 (Olah et al., 1992) for the transient transfection experiments.

For transient transfections, primary epidermal keratinocytes (strains kc, km, kq) were grown in keratinocyte serum-free medium (Maciag et al., 1981) supplemented with EGF and bovine pituitary extract (Gibco, Paisley, UK). 2×10^5 keratinocytes were plated per 35 mm dish and 6 hours later the SuperFect transfection reagent (Qiagen, Crawley, UK) was used to transfect each dish with a total of 1.8 μg of the various combinations of plasmids: 1.5 μg of β-catenin constructs in pCMV5 or empty pCMV5; 0.3 μg TOPFLASH, FOPFLASH or p4xAP1Luc. Cells were harvested after 24 hours. Transfection efficiency was examined by western blotting and indirect immunofluorescent staining. Cells were lysed in situ in 1× reporter lysis buffer (Promega, Southampton, UK) and luciferase activity was determined using reconstituted luciferase assay substrate (Promega) on a BioOrbit 1251 luminometer. The Bio-Rad Dc protein assay was

used to confirm that equal amounts of protein were present in each sample used for the luciferase assay.

The same procedure was used to measure promoter activity in retrovirally infected cells and in uninfected cells that had been enriched for stem and transit amplifying cells by adhesion selection on type IV collagen (as described above). In these experiments the luciferase reporter constructs (1.0 μg) were cotransfected with a constitutively active LacZ expression vector, pJ7LacZ (0.25 μg), kindly provided by P. Parker, ICRF.

RESULTS

β-catenin levels in subpopulations of cultured keratinocytes enriched for stem and transit amplifying cells

In order to compare β-catenin levels in stem and transit amplifying cell-enriched populations, cultured keratinocytes were labelled with a fluorescein conjugated β1 integrin antibody, CD29-FITC. Based on the forward (FSC) and side (SSC) scatter profiles suprabasal cells were gated out (Jones and Watt, 1993) (Fig. 1A). Within the basal cell compartment, enrichment for stem cells (S) (i.e. the 20% of basal cells with the highest β1 integrin levels) and transit amplifying cells (TA) (i.e. the 20% of basal cells with the lowest β1 integrin levels) was obtained by fractionation using a fluorescence activated cell sorter (FACS) (Jones and Watt, 1993) (Fig. 1A).

Total and Triton X-100-soluble protein lysates were prepared from FACS-sorted cells. The gels shown in Fig. 1 are representative of data obtained from four separate FACS sorts. Equal amounts of protein lysate were separated by SDS-PAGE, transferred to PVDF membranes and sequentially blotted with antibodies to E-cadherin (E-cad; HECD-1), α-catenin (α-cat; VB1), β-catenin (β-cat; VB2) and plakoglobin (pk; VB3) (Fig. 1B). Plakoglobin, a protein with sequence homology to β-catenin, binds to cadherin cytoplasmic domains and to desmosomal proteins (Cowin and Burke, 1996). As a control, equal amounts of Triton-soluble lysates were also blotted with a β1 integrin antibody, mAb13: stem cells expressed approximately twofold higher levels of β1 integrin than transit amplifying cells (band intensity ratio, S:TA=2.0 in the blot shown), showing that the difference between the populations was in total as well as in surface integrin levels (Fig. 1B). A further loading control was to reprobe the blots of Triton-soluble lysates with an anti-tubulin antibody (Fig. 1B); quantitation confirmed that the intensity of the tubulin bands was equal in stem and transit amplifying lysates (S:TA=1.1 for the integrin blot, 1.0 for the cadherin/catenin blot). There was no significant difference between the levels of E-cadherin or plakoglobin in either the Triton-soluble pool (S:TA=1.0 for E-cadherin, 1.1 for plakoglobin) or the total protein pool of stem and transit amplifying cells (S:TA=1.0 for E-cadherin, 1.0 for plakoglobin). The total amount of α- and β-catenin was similar in stem and transit amplifying cells (S:TA=1.1 for α-catenin, 1.2 for β-catenin). However, there was 2- to 4-fold more α- and β-catenin in the Triton-soluble fraction of stem cells compared with transit amplifying cells (S:TA=2.7 for α-catenin, 4.2 for β-catenin; Fig. 1B). The amount of Triton-

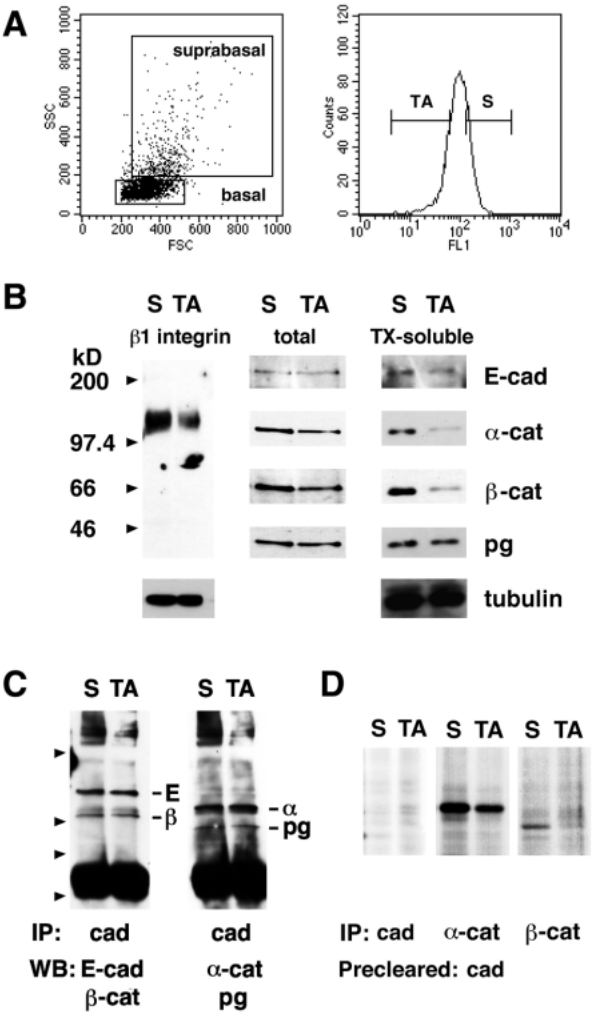

Fig. 1. β-catenin levels in stem and transit amplifying cells. (A) Isolation of populations of keratinocytes enriched for stem and transit amplifying cells. Keratinocytes were immunolabelled with an anti-β1 integrin antibody. Basal and suprabasal cells were distinguished on the basis of forward (FSC) and side (SSC) scatter (left panel). Within the basal cell compartment, the stem cell-enriched population (S) (i.e. the 20% of cells with the highest β1 integrin levels) and transit amplifying cell-enriched population (TA) (i.e. the 20% of cells with the lowest β1 integrin levels) were selected (right panel). (B) Equal amounts of total and Triton-soluble (TX-soluble) protein lysates of stem (S) and transit amplifying (TA) cells were separated by SDS-PAGE under reducing conditions, transferred onto PVDF membranes and blotted sequentially with antibodies specific for the proteins shown. Cell lysates were also western-blotted with a β1 integrin antibody under non-reducing conditions. As a control the blots of TX-soluble lysates were re-probed with an anti-tubulin antibody. Positions of molecular mass standards are shown in B and C. (C) Equal amounts of TX-soluble lysates were immunoprecipitated (IP) with a pan-cadherin antibody. The immunoprecipitates were separated by SDS-PAGE and blotted (WB) sequentially with a mixture of antibodies to E-cadherin (E) and β-catenin (β) and to α-catenin (α) and plakoglobin (pg) (reducing conditions). The band migrating between the 46 and 66 kDa markers is rabbit IgG. (D) ^{35}S-cysteine and -methionine-labelled stem and transit amplifying cells were selected by differential adhesion to type IV collagen. Equal amounts of TX-soluble lysates were sequentially immunoprecipitated six times with FWCAD to remove all of the E- and P-cadherin (cad) and then immunoprecipitated with antibodies to E- and P-cadherin (cad), α- or β-catenin.

soluble α- and β-catenin in the 60% of basal keratinocytes with modal β1 integrin levels (i.e. excluded from selection in Fig. 1A) was intermediate between the amount in the stem and transit amplifying cell-enriched populations (data not shown).

The cytoplasmic domains of the classical cadherins form a complex with α-catenin and either β-catenin or plakoglobin. In order to see whether there was any difference in the level of the β-catenin associated with the cadherins of stem and transit amplifying cells we immunoprecipitated equal amounts of Triton-soluble protein extracts of each cell fraction with a pan-cadherin antiserum, FWCAD, that recognises P- and E-cadherin. The immunoprecipitates were sequentially blotted with a mixture of antibodies to E-cadherin and β-catenin (HECD-1 and VB2) or to α-catenin and plakoglobin (clone 5 and VB3) (Fig. 1C). There was no difference in the level of E-cadherin in stem and transit amplifying cells or in the levels of α-, β-catenin or plakoglobin in the cadherin-associated complexes of each population, suggesting that the excess α- and β-catenin in the stem cell population (Fig. 1B) was not bound to cadherins.

To confirm that the excess β-catenin in stem cells was not cadherin-associated, keratinocytes were metabolically labelled with ^{35}S-cysteine and -methionine and then enriched for stem or transit amplifying cells by differential adhesion to type IV collagen, using the method of Jones and Watt (1993). All of the cadherin in the lysates was removed by six rounds of immunoprecipitation with FWCAD (Fig. 1D). The cadherin-immunodepleted lystates were then immunoprecipitated with antibodies to α- or β-catenin or plakoglobin (Fig. 1D and data not shown). There was considerably more β-catenin in the stem than in the transit amplifying cell lysates (S:TA=2.9); in the transit amplifying cells very little β-catenin remained. There was also more α-catenin in the stem cell lysates (S:TA=1.3), but the difference was less pronounced than in the case of β-catenin. There was no difference in the level of non-cadherin associated plakoglobin (data not shown).

Expression of β-catenin constructs in human epidermal keratinocytes

Having shown that putative epidermal stem cells had a higher level of noncadherin-associated β-catenin than transit amplifying cells, we investigated whether increased β-catenin expression had any effect on keratinocyte proliferation. We obtained cDNAs of full-length *Xenopus* β-catenin (MT1) and three deletion mutants (T2, T3 and MT10) (Funayama et al., 1995; Fagotto et al., 1996) from B. M. Gumbiner of Memorial Sloan-Kettering Cancer Center, New York, USA (Fig. 2A). Full-length *Xenopus* β-catenin consists of 13 imperfect armadillo (arm) repeats flanked by N- and C-terminal domains, and shows 97% sequence identity with human β-catenin (Peifer et al., 1992). The MT10 construct contains only nine arm repeats. In the T2 mutant the N terminus (N-terminal 147 amino acids) of β-catenin has been deleted. All the arm repeats except for half of the first repeat and the last one and half repeats are absent in the T3 mutant. MT1 and MT10 are Myc-tagged at the N terminus and the T2 and T3 mutants are tagged with influenza haemagglutinin (HA) at the C terminus. The β-catenin cDNAs were subcloned into a retroviral vector, pBabe puro, and introduced into cultured primary human

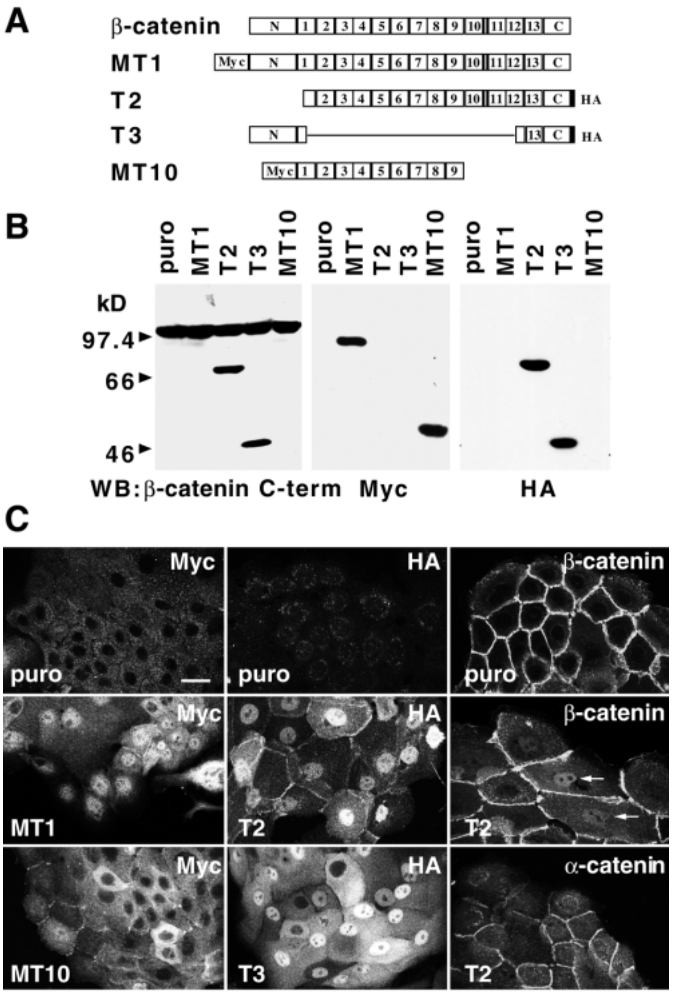

Fig. 2. Expression of β-catenin mutants in keratinocytes. (A) Schematic illustration of wild-type β-catenin and the mutants that were introduced into keratinocytes. (B) Equal amounts of total protein lysates of keratinocytes expressing puro, MT1, T2, T3 and MT10 were separated and immunoblotted (WB) with antibodies to the proteins shown. Positions of molecular mass standards are indicated. (C) Colonies of keratinocytes expressing puro, MT1, T2, T3 and MT10 were immunolabelled with anti-Myc, anti-HA or antibodies specific for endogenous α- or β-catenin. Nuclear staining of endogenous β-catenin in T2 cells is indicated with arrows. Bar, 20 μm.

epidermal keratinocytes as described previously (Zhu and Watt 1996; Gandarillas and Watt, 1997).

In order to examine expression levels of the different constructs, total protein lysates were prepared from puro- (empty vector), MT1-, T2-, T3- and MT10-expressing keratinocytes and immunoblotted with an antiserum specific for the β-catenin C terminus, VB2, or with Myc- or HA-specific antibodies, A14 or Y11, respectively (Fig. 2B). In addition to detecting endogenous β-catenin, VB2 could detect T2 and T3. VB2 could not be used to distinguish MT1 from endogenous β-catenin because the two proteins comigrated, nor to detect MT10, since the C terminus had been deleted. The expression levels of T2 and T3 relative to endogenous β-catenin were 60% (T2) and 51% (T3). Since the Myc-tagged constructs were not detected with VB2, direct comparison of

their abundance relative to the endogenous proteins was not possible; however, MT1 and MT10 had the same relative abundance (MT10:MT1=1.2 in Fig. 2B).

We next examined the cellular localisation of the β-catenin constructs in keratinocytes. Cells were immunolabelled with 9E10, to detect MT1 and MT10, or 12CA5, to detect T2 and T3 (Fig. 2C). Full-length *Xenopus* β-catenin, MT1, and the N terminus deletion mutant, T2, localised at cell-cell contacts in the cytoplasm and in the nucleus. The T3 mutant accumulated in the cytoplasm and in the nucleus, but was absent from cell-cell borders; its nuclear localisation confirms that β-catenin can translocate to the nucleus independently of interaction with TCF/LEF transcription factors (Fagotto et al., 1998). MT10 had a patchy and punctate distribution at cell-cell contacts with some cytoplasmic accumulation and very weak nuclear localisation. Keratinocytes expressing puro served as negative controls for the Myc and HA staining.

Immunofluorescent labelling was used to detect endogenous cadherins and catenins in the infected cells. Endogenous human β-catenin could be distinguished using a monoclonal antibody (clone 14), which does not cross-react with *Xenopus* β-catenin (confirmed by western blotting; data not shown). The localisation patterns of E-cadherin and catenins were unaffected in keratinocytes expressing MT1, T2, T3 and MT10, with the exception that endogenous β-catenin was weakly detected in the nucleus of T2-expressing cells (Fig. 2C and data not shown). We failed to detect β-catenin in the nucleus of uninfected keratinocytes, although we used a range of fixation protocols designed to optimise staining of nuclear proteins (Schmidt et al., 1997). Expression of the β-catenin constructs did not cause any obvious changes in keratinocyte morphology or intercellular adhesion (Fig. 2C and data not shown) and had no effect on the total level of cadherins, α-, β-catenin or plakoglobin, as evaluated by western blotting of total or Triton-soluble proteins (Fig. 2B and data not shown).

The motility of keratinocytes expressing the T2 or T3 mutants was compared with that of cells expressing the empty retroviral vector, puro. The cells were plated under standard growth conditions in the presence of a J2-3T3 feeder layer and monitored with a time-lapse video recording unit for 48 hours at a rate of one frame every 2 minutes. The speed of 12 individual cells from each population was determined. The average motility of T2-expressing cells (23 μm/hour, range 17-26 μm/hour) was similar to that of puro-expressing cells (25 μm/hour, range 19-38 μm/hour) while that of T3-expressing cells was slightly increased (36 μm/hour, range 20-43 μm/hour).

β-catenin forms complexes with E-cadherin and α-catenin through its central arm repeats and N-terminal region, respectively (Orsulic and Peifer, 1996). Having determined the expression levels and cellular distribution of the β-catenin constructs we examined the ability of each protein to associate with endogenous cadherins and α-catenin (Fig. 3A). Equal amounts of Triton-soluble protein lysates were immunoprecipitated with rabbit antisera specific for the Myc or HA tags as positive controls (A14 or Y11, respectively), with a pan-cadherin antibody (FWCAD) or anti-α-catenin (VB1). The immunoprecipitates were then western-blotted with Myc-specific (9E10) or HA-specific (12CA5) monoclonal antibodies. Full-length β-catenin, MT1, co-immunoprecipitated with cadherins and α-catenin. The T2 mutant formed a complex with cadherins but not with α-catenin. The T3 mutant associated with α-catenin but not cadherins. The MT10 mutant did not form a complex with either cadherins or α-catenin. These biochemical data are consistent with predictions based on the protein sequences of the β-catenin mutants (Funayama et al., 1995; Fagotto et al., 1996).

It has been suggested that E-cadherin and APC compete for β-catenin binding (Su et al., 1993; Hülsken et al., 1994): the binding site for E-cadherin overlaps with that for APC and both sites lie in the central arm repeats of β-catenin. In order to examine the association of APC with the β-catenin mutants and compare it with that of E-cadherin, equal amounts of Triton-soluble protein lysates from puro-, MT1-, T2-, T3- and MT10-expressing keratinocytes were immunoprecipitated with antibodies specific for cadherins

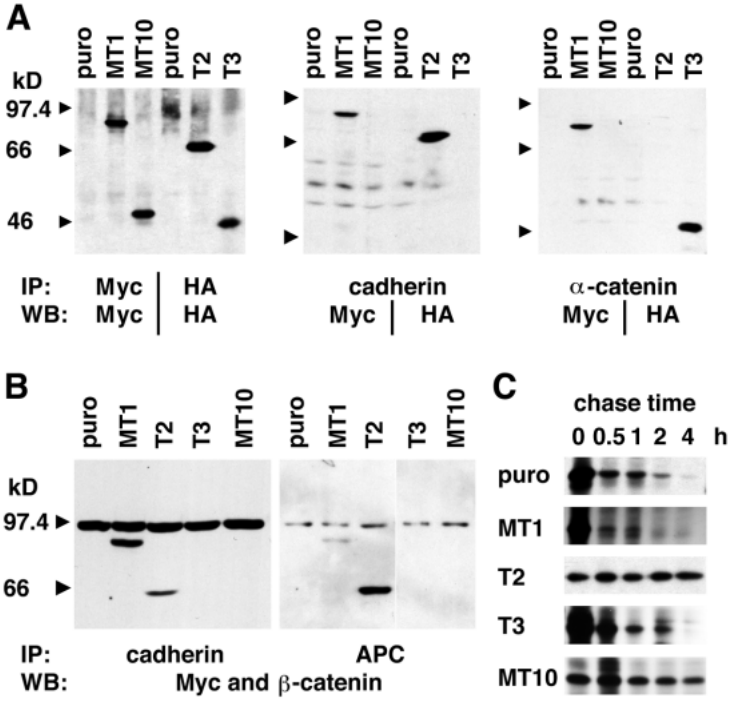

Fig. 3. β-catenin half-life and association with cadherin, α-catenin and APC. (A) Equal amounts of Triton-soluble protein lysates of infected keratinocytes were precleared with pre-immune serum and immunoprecipitated (IP) with antibodies to the proteins shown. Immunoprecipitates were separated by SDS-PAGE under reducing conditions, transferred onto PVDF membranes and immunoblotted (WB) with antibodies specific for the Myc and HA tags. (B) Equal amounts of Triton-soluble protein lysates of infected keratinocytes were precleared with pre-immune serum and immunoprecipitated (IP) with antibodies to the proteins shown. Immunoprecipitates were separated by SDS-PAGE under reducing conditions, transferred onto PVDF membranes and immunoblotted (WB) with a combination of antibodies specific for the Myc tag and the β-catenin C terminus. (C) Infected keratinocytes were starved, pulse-labelled with ^{35}S-cysteine and -methionine for 30 minutes, chased in non-radioactive medium for the number of hours shown and immunoprecipitated with a β-catenin antiserum, VB2 (puro; endogenous β-catenin), a Myc antibody, A14 (MT1, MT10) or an HA antibody, Y11 (T2, T3). The positions of molecular mass standards are shown in A and B.

(FWCAD) or APC (ALI-12-28), separated by SDS-PAGE and immunoblotted with a combination of a monoclonal antibody (9E10) to Myc and a rabbit antiserum (VB2) to β-catenin. As shown in Fig. 3B, both full-length MT1 and the N terminus-deleted T2 mutant were able to associate with E-cadherin and APC; there was more MT1 associated with cadherin than with APC and, conversely, there was more T2 associated with APC. As expected MT10 and T3 did not associate with APC.

The N terminus of β-catenin contains several GSK-3β phosphorylation sites, which facilitate the rapid degradation of β-catenin (Rubinfeld et al., 1996; Yost et al., 1996). Wild-type β-catenin has a very short half-life and deletion or mutation of the GSK-3β phosphorylation sites at the N terminus results in accumulation of stable β-catenin in the cytoplasm. To examine the half-life of the β-catenin mutants infected cells were starved for 1 hour, pulse-labelled with ^{35}S-cysteine and -methionine for 30 minutes and chased in radioactive-free medium for up to 4 hours (Fig. 3C). As predicted, endogenous human β-catenin and full-length *Xenopus* β-catenin, MT1, had a half-life of less than 30 minutes, whilst T2 and MT10, both lacking the N terminus, were stable throughout the time period examined. The T3 mutant, which has most of the arm repeats deleted but the N terminus intact, had a half-life of around 45 minutes.

Effects of β-catenin mutants on keratinocyte growth and terminal differentiation

Having examined the expression, stability, subcellular localisation and complex formation of the β-catenin mutants, we examined their effects on growth and terminal differentiation. To prepare growth curves of infected keratinocytes 500 cells were plated per 35 mm dish. Cell numbers were measured in triplicate dishes at intervals for up to 26 days (Fig. 4A). There was little difference in the growth rates of puro-, MT1- and MT10-expressing keratinocytes. However, cells expressing the T2 mutant had a higher growth rate than the control cells from day 10 onwards. The difference in cell numbers between T2-expressing cells and control keratinocytes was 2- to 2.5-fold at day 26. Conversely, the T3 mutant strongly suppressed keratinocyte growth. Growth curves were prepared for three different strains of keratinocytes (i.e. isolated from three different individuals) and all showed the same effects of the β-catenin mutants.

The proportion of keratinocytes that had left the basal layer and were undergoing terminal differentiation was estimated using flow cytometry to score the percentage of cells expressing involucrin, which in culture is expressed by all suprabasal keratinocytes but is absent from the basal layer (Gandarillas and Watt, 1997). Keratinocyte terminal differentiation can be induced by placing cells in methyl cellulose-supplemented medium (Green, 1977; Zhu and Watt, 1996). There was no significant difference in the percentage of involucrin-positive cells between control keratinocytes and cells expressing the different β-catenin mutants, either in adherent cultures or after suspension in methyl cellulose for 24 hours (Fig. 4B).

Having shown that the T2 mutant was very stable and increased keratinocyte proliferation dramatically, we examined whether T2-expressing cells were transformed by two different

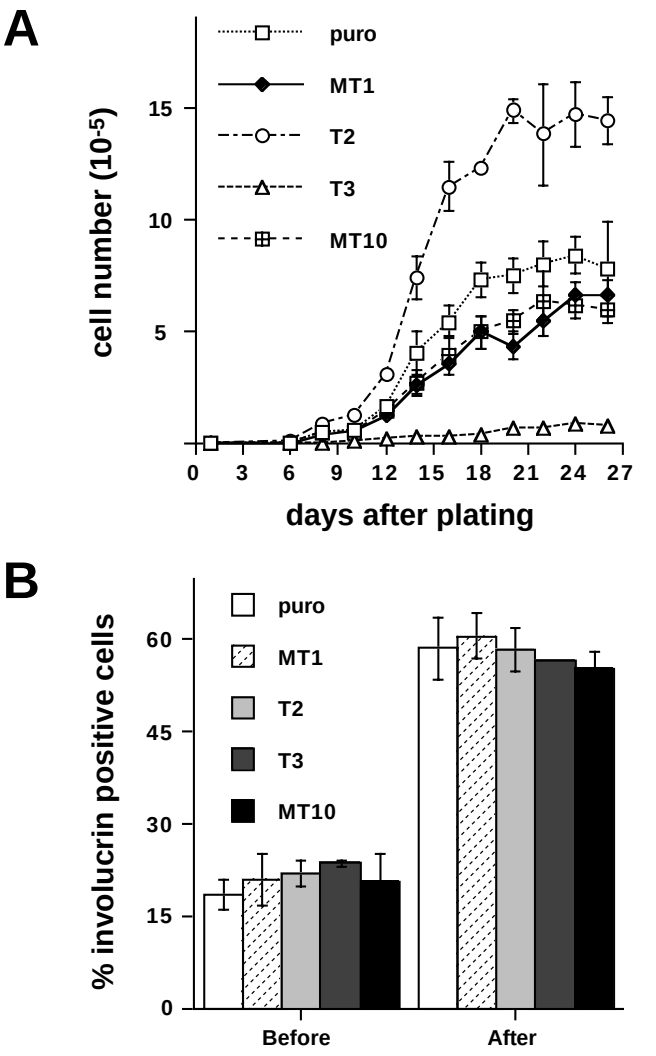

Fig. 4. Effects of the β-catenin mutants on keratinocyte proliferation and terminal differentiation. (A) Equal numbers of keratinocytes expressing puro, MT1, T2, T3 and MT10 were plated per 35 mm dish. Triplicate dishes were harvested on the days shown and cell numbers were determined. Error bars represent standard deviation of the mean of triplicate samples within one experiment. (B) The proportion of involucrin-positive keratinocytes was determined by flow cytometry in preconfluent adherent cultures (Before) and after suspension for 24 hours in methyl cellulose-supplemented medium (After). Error bars represent standard deviation of the mean of duplicate samples within one experiment.

criteria. Both puro- (control) and T2-expressing keratinocytes failed to divide in suspension and were thus incapable of anchorage independent growth (data not shown). In addition, when metaphase spreads of puro- and T2-expressing keratinocytes were prepared, both populations had a diploid karyotype with no obvious chromosomal abnormalities (data not shown).

Effect of β-catenin mutants on the proportion of stem and transit amplifying cells in culture

Having established that the T2 and T3 β-catenin mutants were able to modulate keratinocyte growth independently of

inducing terminal differentiation, we went on to investigate whether they had any effect on the proportion of putative stem cells and transit amplifying cells in the proliferative compartment, using the two markers currently available to distinguish them, namely surface β1 integrin levels and clonogenicity (Jones and Watt, 1993; Watt, 1998). T3-expressing basal keratinocytes showed a reduced level of surface β1 integrins when compared to control keratinocytes, while T2-expressing cells showed an increase (Fig. 5A and data not shown). In contrast, the level of the α6β4 integrin, which is not a stem cell marker (Jones and Watt, 1993) was not significantly different between T2- and T3-expressing cells (Fig. 5A).

For the clonogenicity assays equal numbers of cells expressing puro, MT1, T2, T3 and MT10 were plated on tissue culture plastic in the presence of J2-3T3 feeder cells and cultured for 14 days (Table 1). Two parameters were measured: colony forming efficiency and percentage of abortive colonies. Colony forming efficiency was calculated as the percentage of all plated cells that divided at least once. A colony was classified as abortive if it contained fewer than 40 cells, the majority of which were large and terminally differentiated, by 14 days after plating; such colonies were founded by transit amplifying cells (Jones and Watt, 1993). At 14 days nonabortive colonies (i.e. founded by putative stem cells) contained more than 40 cells and consisted of a mixture of stem, transit amplifying and terminally differentiating cells (Jones and Watt, 1993). There was no difference in overall colony forming efficiency between puro-, MT1-, T2- and MT10-expressing cells; however, percentage colony forming efficiency was reduced twofold in T3-expressing cells. Percentage of abortive colonies was unaffected by expression of MT1 and MT10, but was increased in the presence of T3 and decreased in the presence of T2. Thus T3 stimulated keratinocytes to become transit amplifying cells or stop dividing altogether while T2 expanded the stem cell compartment to almost 90% of clonogenic cells. The same results were obtained with keratinocytes isolated from three different individuals.

β-catenin has been shown to mediate the interaction of cadherin/catenin complexes with the EGF receptor (Hoschuetzky et al., 1994) and EGF is a component of the keratinocyte culture medium used in our experiments. In order to test whether the growth stimulatory effect of T2 was dependent on EGF receptor signalling, the clonogenicity assays were repeated in the absence of EGF. There were fewer keratinocyte colonies formed in each population (i.e. overall reduction in colony forming efficiency), but the proportion of abortive colonies was still reduced in T2 keratinocytes relative to the puro controls (data not shown).

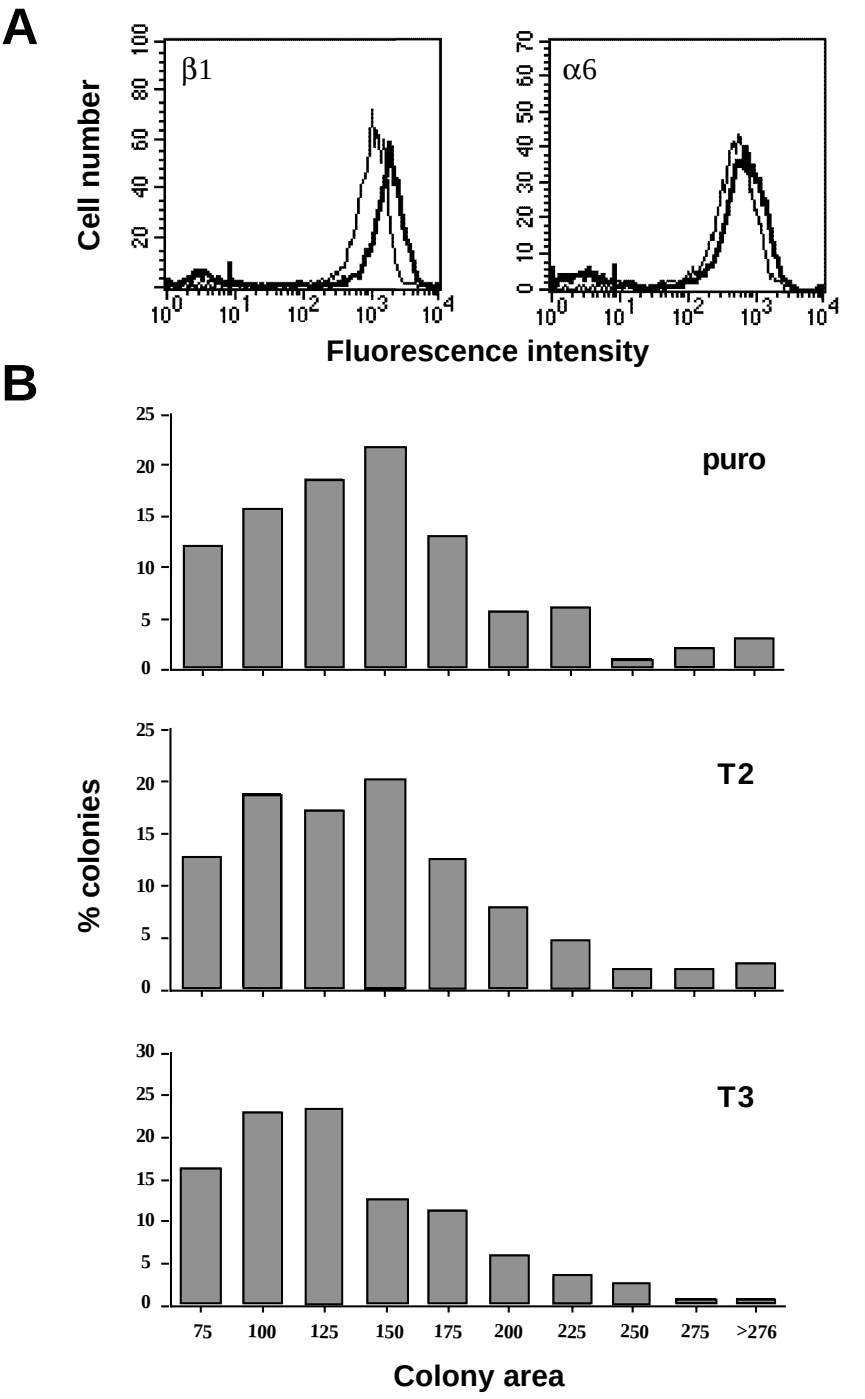

Fig. 5. Integrin levels and colony size of keratinocytes expressing β-catenin mutants. (A) Surface levels of total β1 integrins and α6β4 integrin in basal keratinocytes expressing T3 (thin lines) or T2 (thick lines). Marks on the *x* axes indicate the maximum fluorescence of cells labelled with secondary antibody alone. (B) Size of individual stem cell colonies founded by keratinocytes expressing puro, T2 or T3. Colonies were grouped according to area, such that the column labelled 75 contains colonies with an area of 51-75 units, 100 contains colonies with an area of 76-100 units and so on. 100 units corresponds to a colony with an area of 2.4 mm^2. The total number of colonies scored for each construct was as follows: 198 (puro), 204 (T2), 136 (T3).

Table 1. Effect of the β-catenin mutants on colony formation

Mutant	% colony forming efficiency	% abortive colonies
puro	22.6±3.2	32.6±3.6
MT1	19.6±3.6	35.1±4.0
T2	24.5±2.7	11.0±3.3
T3	9.0±0.9	53.0±4.4
MT10	20.4±4.0	36.5±5.2

Colony forming efficiency was calculated as the percentage of plated cells that formed colonies (i.e. divided at least once). Data are means of triplicate dishes from one experiment.

Effects of β-catenin on cell cycle kinetics and the size of stem cell colonies

The integrin flow cytometry (Fig. 5A) and the clonogenicity experiments (Table 1) showed that T2 and T3 affected the proportion of putative stem and transit amplifying cells in the cultures. We next examined whether there was any effect on the rate of growth of cells within each compartment by testing whether T2 and T3 changed the cell cycle distribution of keratinocytes and the number of cells per colony in colonies founded by stem cells. The proportion of cells in each phase of the cell cycle was determined by flow cytometry of propidium iodide labelled cells and is shown in Table 2. There was no difference in the proportion of cells in G_1, S or G_2+M in keratinocytes expressing puro, T2 or T3. When cells in S-phase were labelled by a 3 hour incubation with BrdU the percentage of labelled cells was the same in T2- and puro-expressing populations, but was reduced in the T3-expressing population (Table 2).

Keratinocyte colonies expand as the number of cells per colony increases and there is a linear relationship between log colony area and log cell number for the first 14 days of culture (Barrandon and Green, 1985). We measured the area of over 120 actively growing (stem cell) colonies founded by T2-, T3- and puro-expressing keratinocytes and plotted the data, using a linear scale in which 100 units corresponds to a surface area of 2.4 mm^2 (Fig. 5B). There was no significant difference between the range of clone sizes in each population. The modal stem cell colony size (approximately 3.6 mm^2, corresponding to 10^4 cells; Barrandon and Green, 1985) was also the same in T2-, T3- and puro-expressing populations (T2 versus puro, P=0.51; T3 versus puro, P=0.36; student's t-test). Taken together with the analysis of cell cycle parameters we conclude that T2 and T3 did not affect the proliferation rate of cycling keratinocytes.

Table 2. Cell cycle analysis of keratinocytes expressing β-catenin mutants

Mutant	Propidium iodide DNA profile (% of cells)			% BrdU labelled cells
	G_1	S	G_2+M	S
puro	60.7	15.6	22.6	31.0
T2	61.7	13.5	23.7	33.3
T3	62.6	13.5	22.9	23.7

Data are means of duplicate dishes from one experiment.

β-catenin rescues cells from the growth inhibitory effect of a dominant negative cadherin mutant

Expression of a dominant negative cadherin mutant, H-2K^d-E-cad, consisting of the extracellular domain of H-2K^d and the transmembrane and cytoplasmic domains of E-cadherin, has previously been shown to inhibit keratinocyte growth in culture (Zhu and Watt, 1996). One hypothesis is that the cadherin mutant acts by binding cytoplasmic β-catenin and depleting the size of the free β-catenin pool (Heasman et al., 1994; Fagotto et al., 1996; Sanson et al., 1996). Since the N terminus-deleted β-catenin, T2, had a potent stimulatory effect on keratinocyte growth, we investigated whether overexpression of the T2 mutant could overcome the inhibitory effect of H-2K^d-E-cad.

Keratinocytes expressing the dominant negative cadherin mutant, H-2K^d-E-cad (E), or a control mutant, H-2K^d-E-cadΔC25 (Δ), in which the β-catenin binding site in E-cadherin has been destroyed (Zhu and Watt, 1996), were doubly infected by co-cultivation with the empty retroviral vector, puro, or with

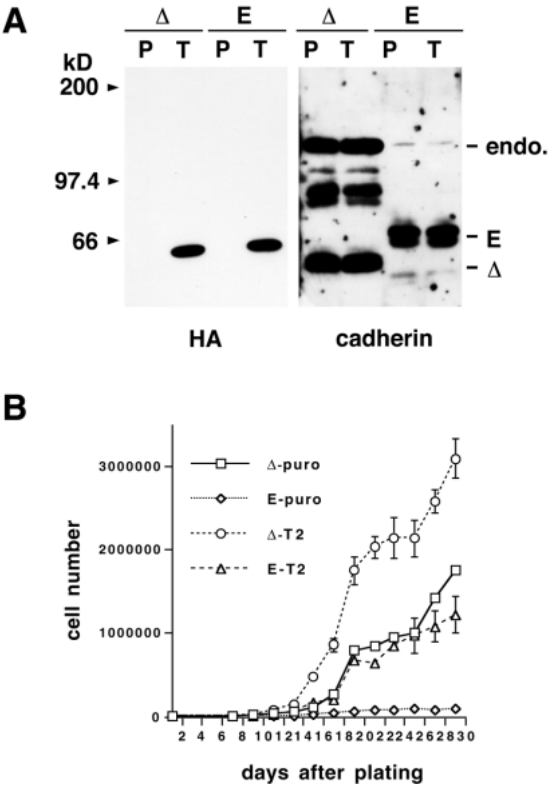

Fig. 6. Rescue of keratinocytes expressing the dominant negative E-cadherin mutant with the T2 β-catenin construct. Keratinocytes expressing the dominant negative cadherin mutant, H-2K^d-E-cad (E), or the control mutant, H-2K^d-E-cadΔC25 (Δ), were doubly infected with retroviral vectors producing puro or T2. (A) Equal amounts of total protein from doubly infected cells were separated by SDS-PAGE under reducing conditions and immunoblotted with antibodies specific for HA and total cadherins. endo., endogenous E- and P-cadherin band. Additional bands in the cadherin blot may be degradation products. Positions of molecular mass standards are indicated. (B) Equal numbers of doubly infected keratinocytes were plated per 35 mm dish. Triplicate dishes were harvested on the days shown and cell numbers were determined. Error bars represent standard deviation of the mean of triplicate samples within one experiment.

the retroviral vector containing the T2 mutant. These cells were designated Δ-puro, Δ-T2, E-puro and E-T2. Total protein lysates were examined by western blotting (Fig. 6A): the level of the T2 mutant was the same in Δ- and E-expressing cells doubly infected with the T2 mutant and there was no significant difference in the levels of endogenous cadherins or the Δ or E constructs in cells expressing puro or T2. Expression of puro or T2 did not affect cell morphology: Δ cells remained as compact keratinocyte colonies and E cells showed the defects in intercellular adhesion reported previously (Zhu and Watt, 1996) (data not shown).

Equal numbers of doubly infected Δ-puro, Δ-T2, E-puro and E-T2 cells were plated on tissue culture plastic in the presence of feeder cells and cell number was determined at intervals for up to 29 days (Fig. 6B). The T2 mutant increased the growth rate of Δ cells to the same extent as its effect on puro cells (cf. Fig. 4A). In the presence of T2 the growth of E cells was increased to that of Δ cells doubly infected with puro. Thus introduction of the T2 mutant largely rescued the growth inhibitory effect of the dominant negative cadherin mutant. Clonogenicity assays could not be performed, because E cells did not form cohesive colonies in the presence or absence of T2.

Effect of β-catenin mutants on TCF/LEF transactivation

The TCF/LEF transcription factors are downstream targets of the Wnt signalling pathway (Behrens et al., 1996; Huber et al., 1996; van de Wetering et al., 1997). It has been demonstrated that the N-terminal region of TCF/LEFs associates with the central arm repeats of β-catenin. In addition, the C terminus of β-catenin has been implicated in the transactivation of TCF/LEFs, thereby affecting gene transcription. We therefore investigated whether the effects of the β-catenin constructs on proliferation correlated with their ability to modulate TCF/LEF transcriptional activity.

We examined whether keratinocytes that had not been transduced with β-catenin constructs had detectable TCF/LEF activity and, if so, whether there was any difference between the putative stem and transit amplifying cells. Stem and transit amplifying cell-enriched populations were isolated on the basis of differential adhesiveness to type IV collagen (Jones and Watt, 1993) and compared with unfractionated basal cells. The cells were transfected with two luciferase reporter constructs: TOPFLASH, which contains three copies of TCF/LEF consensus sequence, and FOPFLASH, which has mutated TCF/LEF sites (van de Wetering et al., 1997). As shown in Fig. 7A the FOPFLASH reporter had no activity in keratinocytes. The activity of TOPFLASH in the stem cell population (14 units) was three times greater than that of the transit amplifying population (4 units) and twofold higher than in unselected basal cells (6 units) (Fig. 7A). A constitutively active LacZ expression vector was co-transfected with the luciferase constructs and used to normalise transfection efficiency (data not shown).

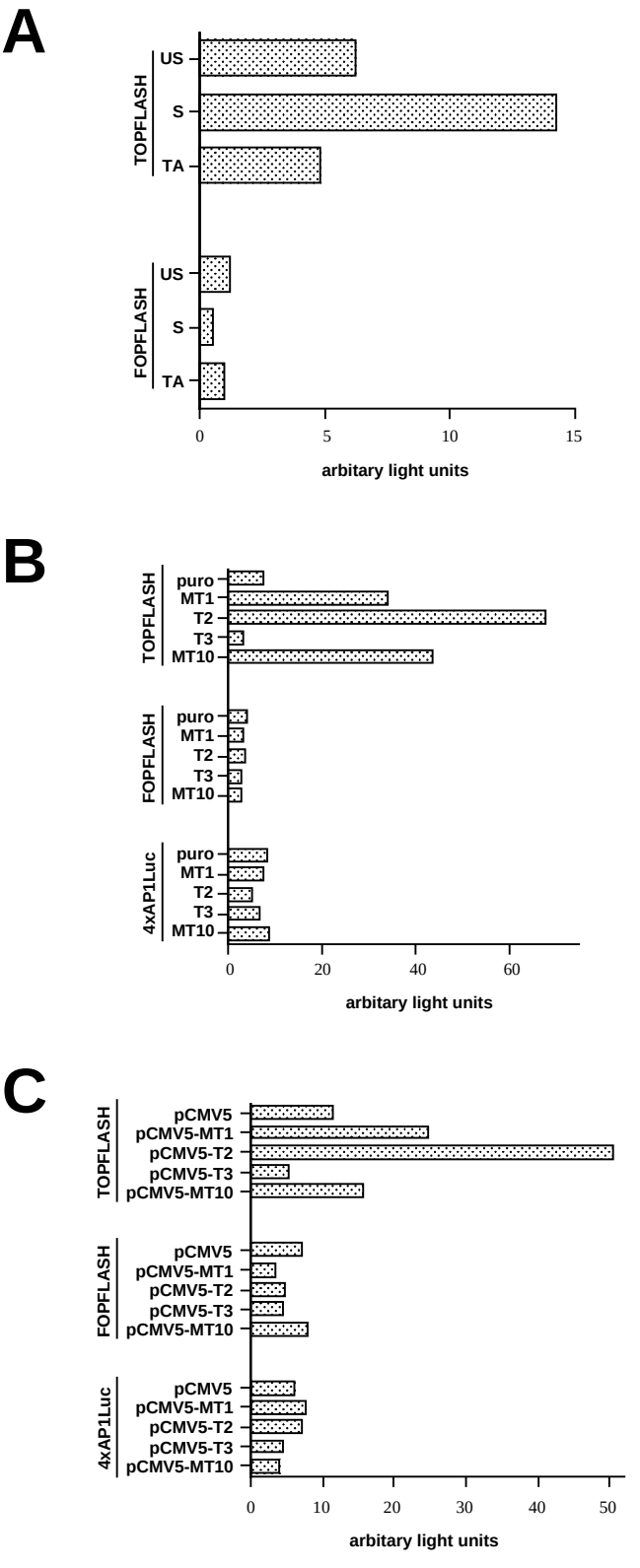

Fig. 7. TCF/LEF activity in human keratinocytes. (A) Stem (S) and transit amplifying (TA) cells were selected by differential adhesion to type IV collagen and compared with unselected (US) basal cells. Cells were transiently transfected with TOPFLASH or FOPFLASH and the luciferase activity of each reporter construct was measured. (B) Keratinocytes stably transduced with the β-catenin constructs or puro were transiently transfected with TOPFLASH or FOPFLASH or the 4 x AP1Luc reporter. (C) β-Catenin mutants in pCMV5 vector or the vector alone were transiently co-transfected with TOPFLASH, FOPFLASH or the 4 x AP1Luc reporter. The luciferase activity of each reporter construct was measured. A-C are data from a typical experiment.

We also investigated whether introduction of the various β-catenin constructs by stable retroviral infection had any effect on TCF/LEF transcriptional activity (Fig. 7B). Keratinocytes expressing puro, MT1, T2, T3 or MT10 were transiently transfected with TOPFLASH, FOPFLASH or an irrelevant reporter, 4xAP1Luc, with 4 AP1 sites (Rincón and Flavell, 1994) and the data normalised as described above. Cells transduced with MT1 (34 units), MT10 (44 units) and T2 (68 units) all activated transcription of TOPFLASH to a greater extent than puro (7 units), the T2 construct being the most active. In contrast T3 reduced activity (3 units) relative to the puro control. The background activity of FOPFLASH was the same in all cells. The AP1 reporter showed some activity above background but this was similar in all cell populations.

The differences in LEF/TCF activity in cells transduced with the retroviral vectors (Fig. 7B) could either be a direct effect on β-catenin signalling or a secondary consequence of altering cell fate, given the differences in endogenous transcriptional activity between stem and transit amplifying cells (Fig. 7A). We therefore examined whether the β-catenin mutants were able to affect transactivation of TCF/LEFs in transient transfection assays (Fig. 7C). The β-catenin mutants were subcloned into a plasmid expression vector, pCMV5, in which the β-catenin constructs are expressed under the CMV promoter, and co-transfected with TOPFLASH, FOPFLASH and 4xAP1Luc. 24 hours after transfection, total proteins from each population were prepared and the expression levels were examined by western blotting: all β-catenin mutants had comparable levels of protein expression (data not shown).

For the luciferase assays, lysates of cells co-transfected with different combinations of reporters and β-catenin mutants were prepared. Equal volumes of cell lysates were used for measuring luciferase activity (Fig. 7C) and determination of protein contents confirmed that equal amounts of protein were present (data not shown). Endogenous β-catenin (pCMV5) was able to activate TOPFLASH luciferase activity (i.e. 12 units compared with 5-6 units for FOPFLASH and 4xAP1Luc). The MT10 mutant showed a slightly higher basal luciferase activity (16 units compared with 12 units). TOPFLASH activity was stimulated a further twofold when MT1 was introduced (25 units) and fourfold relative to basal levels by the introduction of the T2 mutant (51 units). Conversely, the T3 mutant caused a 50% decrease in basal luciferase activity (5 units). None of the β-catenin mutants was able to activate FOPFLASH or 4xAP1Luc.

DISCUSSION

We have shown that the level of noncadherin-associated β-catenin differs between subpopulations of primary human epidermal keratinocytes enriched for stem and transit amplifying cells and that the elevated level of β-catenin in the putative stem cells is likely to contribute to maintenance of high proliferative potential. When the stabilised, N-terminally truncated, form of β-catenin (T2) was introduced the proportion of colonies attributable to stem cells was increased. Conversely, a β-catenin mutant lacking the arm repeats (T3) acted as a dominant negative and keratinocytes were stimulated to form colonies characteristic of transit amplifying cells. Stem cells had a higher basal level of TCF/LEF transcriptional activity than transit amplifying cells and the effects of the mutants correlated with modulation of TCF/LEF activity. Gat et al. (1998) have recently reported that when N-terminally truncated β-catenin is overexpressed in the basal epidermal layer of transgenic mice there is conversion of adult basal epidermal cells to pluripotent embryonic ectoderm. It would therefore be interesting to know whether the T2 construct can cause a similar conversion of human epidermal cells when recombined with dermis.

While stabilising mutants of β-catenin have been found in a variety of tumour cells (reviewed by Ben-Ze'ev and Geiger, 1998) expression of N-terminally truncated β-catenin was not sufficient to cause keratinocytes to become anchorage-independent or aneuploid, two markers of malignant transformation. Nevertheless such a mutation arising in a keratinocyte that had already acquired a mutation in, for example, Ras (Brown et al., 1998), could contribute to tumour development by increasing the proliferative potential of the target cells. Consistent with our findings, the transgenic mice of Gat et al. (1998) developed tumours of the hair follicle but not epidermal basal cell carcinoma or squamous cell carcinoma.

While the proportion of keratinocytes in culture with characteristics of stem and transit amplifying cells was controlled by the β-catenin constructs, the number of rounds of division that each type of cell underwent was not, since the number of cells per stem cell clone and the proportion of suprabasal, involucrin-positive cells were unaltered. The proportion of cells in each phase of the cell cycle was the same in cells expressing the constitutively active or dominant negative β-catenin mutants, consistent with previous observations that the cell cycle characteristics of stem and transit amplifying cells in culture are similar and that cells can initiate involucrin synthesis at any phase of the cell cycle (Gandarillas and Watt, 1997, and references cited therein). The only difference we observed in this respect between T2- and T3-expressing cells was a reduction in percentage of S-phase cells in the T3 population, as would be expected when cells initiate terminal differentiation and lose the ability to divide.

When we compared keratinocytes enriched for stem or transit amplifying cells on the basis of surface integrin levels there was little difference in the total amount of β-catenin, but the amount in the Triton-soluble pool that was not cadherin-associated was higher in the putative stem cell population. It seems likely that this 'free' β-catenin pool is in the nucleus, since otherwise it would be rapidly degraded; our failure to detect it by immunostaining of epidermis and cultured keratinocytes (Braga et al., 1995; Molès and Watt, 1997; Fig. 2C) may be due to epitope masking or to its low abundance relative to the pool of cadherin-associated β-catenin. Confocal microscopy of sections of human epidermis has previously shown reduced levels of membrane-associated E-cadherin and β-catenin in the stem cell compartment (Molès and Watt, 1997); since the tissue sections had been postfixed and detergent-extracted those observations cannot be compared directly with the biochemical analysis, but they are not incompatible with the conclusion that the stem cells have more noncadherin-associated β-catenin than the transit amplifying cells. Plakoglobin, which has some Wnt signalling activity (Karnovsky and Klymkowsky, 1995; Simcha et al., 1998; White et al., 1998), was equally abundant in stem and transit

amplifying cells. Within the Triton-soluble protein pool there was more α-catenin in stem than transit amplifying cells; while this could potentially influence both E-cadherin-mediated adhesion (Kuroda et al., 1998) and β-catenin signalling (Sehgal et al., 1997), the abundance and cellular distribution of α-catenin was not affected by any of the β-catenin constructs we tested.

Expression of the β-catenin constructs had no obvious effects on intercellular adhesion. Cell morphology was unaffected, and the T2 mutant did not restore cell-cell adhesion to keratinocytes transduced with the dominant negative cadherin mutant. The endogenous cadherins, α-, β-catenin and plakoglobin were expressed at normal levels in the presence of the mutants and it is therefore unlikely that β-catenin downregulates transcription of the E-cadherin gene in keratinocytes (cf. Huber et al., 1996). The subcellular distribution of the endogenous cadherins and catenins was also unaffected by the β-catenin constructs, with the exception that endogenous β-catenin was detected in the nucleus in some cells expressing T2: it probably escaped degradation in the cytoplasm because the T2 mutant was specifically enriched in APC complexes (cf. Papkoff et al., 1996; Barth et al., 1997). This is consistent with the proposal that overexpression of membrane-tethered β-catenin can affect the balance of membrane-bound and -free endogenous β-catenin and that endogenous β-catenin can subsequently translocate to the nucleus (Miller and Moon, 1997; Hsu et al., 1998). The small increase in motility of T3-expressing cells relative to T2- and puro-expressing cells may reflect the reduction in surface integrins characteristic of the transit amplifying compartment (Jensen et al., 1999).

Of the constructs examined, full-length β-catenin, MT1, and the MT10 mutant, consisting of the first nine arm repeats, had little effect on keratinocyte proliferation even though both constructs have been shown to induce axis duplication in *Xenopus* embryos (Funayama et al., 1995; Fagotto et al., 1996). In the case of MT1 this is probably because, like the endogenous β-catenin, it was rapidly degraded and so caused a relatively small increase in the pool of β-catenin available for signalling, an interpretation supported by the modest stimulation of TCF/LEF transcriptional activity relative to T2 we observed. The half life of MT10 was increased, as would be expected because it lacks the N-terminal GSK-3β phosphorylation sites; however it also lacks the C terminus of β-catenin that is required for TCF/LEF transactivation (Orsulic and Peifer, 1996; Sanson et al., 1996; Pai et al., 1997; van de Wetering et al., 1997; White et al., 1998). MT10 did not accumulate to any great extent in the nucleus, in contrast to its behaviour in *Xenopus* embryos (Fagotto et al., 1996). Given these observations it is surprising that the transcriptional activity of the TCF/LEF reporter was higher in keratinocytes expressing MT10 than in cells expressing puro (Fig. 7B), raising the possibility of interference with co-repressors of TCF/LEF (Cavallo et al., 1998; Roose et al., 1998; Waltzer and Bienz, 1998) rather than direct activation of TCF/LEF transcription factors.

On the basis of its effects on MDCK cells (Barth et al., 1997; Pollack et al., 1997) and intestinal epithelium (Wong et al., 1998), its potency in causing *Xenopus* axis duplication (Funayama et al., 1995) and the types of β-catenin mutation found in cancer cells (Ben-Ze'ev and Geiger, 1998), N-terminally truncated T2 is the form of β-catenin that would be predicted to have the most potent positive effects in β-catenin signalling. T2 stimulated keratinocyte proliferation by increasing the proportion of proliferative keratinocytes that formed large, actively growing colonies characteristic of stem cells to almost 90%. Furthermore, T2 was able to rescue cells from the growth inhibitory effect of a dominant negative E-cadherin mutant (Zhu and Watt, 1996), probably by increasing the size of the free β-catenin pool available for signalling. An association between elevated β-catenin and increased transcriptional activity has already been observed in several cancer cell lines (see, for example, Morin et al., 1997; Rubinfeld et al., 1997) and in fibroblasts and PC12 cells overexpressing Wnt-1 (Porfiri et al., 1997; Young et al., 1998). Since T2 activated TCF/LEF to a greater extent than MT1 and MT10 and was the only construct with a positive effect on proliferative potential, we believe that the strength and duration of β-catenin signalling are important for maintenance of the stem cell compartment. Gat et al. (1998) have shown that co-transfection of N-terminally truncated β-catenin and LEF-1 in mouse keratinocytes results in much greater transcriptional activity of a TCF/LEF reporter gene than transfection of either alone, raising the possibility that proliferative potential of keratinocytes could be further enhanced by the combination of T2 and TCF/LEF.

The T3 mutant, lacking most of the arm repeats, does not transduce Wnt signals or cause axis duplication in *Xenopus* embryos (Funayama et al., 1995) and in keratinocytes it localised to the nucleus (see also Orsulic and Peifer, 1996) and acted as a dominant negative inhibitor of endogenous β-catenin signalling, strongly inhibiting proliferation and downregulating TCF/LEF transcriptional activity. In the presence of T3, keratinocytes transduced with the dominant negative cadherin mutant were unable to proliferate at all (data not shown). Deletion of arm repeats in *Drosophila* Armadillo has also been reported to result in dominant negative mutants (Orsulic and Peifer, 1996). It has been suggested that, in addition to the central arm repeats, the last arm repeat and C-terminal region of β-catenin are important in the transactivation of the TCF/LEF transcription factors (Behrens et al., 1996; Orsulic and Peifer, 1996; Sanson et al., 1996; Pai et al., 1997; van de Wetering et al., 1997; Hsu et al., 1998; White et al., 1998). The T3 mutant may act as a dominant negative by titrating away one or more components that bind to the C terminus of β-catenin and are essential for formation of a transcriptionally active TCF/LEF complex (Pai et al., 1997; van de Wetering et al., 1997; Cavallo et al., 1998; Hsu et al., 1998; White et al., 1998). It will be interesting to compare the effects of T3 with those of dominant negative TCF/LEF constructs in which the β-catenin binding domain has been deleted (Hsu et al., 1998; Roose et al., 1998).

Our experiments suggest an important role for transcription factors of the TCF/LEF family in regulating epidermal stem cell fate: the basal level of TCF/LEF activity was higher in the stem than the transit amplifying cell-enriched population (Fig. 7A) and the positive (T2) or negative (T3) effects of the β-catenin mutants on proliferation correlated with their effects on transcriptional activity (see also Gat et al., 1998). That these factors may be of general importance in regulating stem cell fate is highlighted by the finding that in mice that are homozygous null for TCF-4 the stem cell compartment of the

small intestine is lacking (Korinek et al., 1998). Furthermore, targeting N-terminally truncated β-catenin to the proliferative compartment of the intestine in transgenic mice results in increased proliferation, although this is accompanied by increased apoptosis and augmentation of E-cadherin at adherens junctions (Wong et al., 1998).

In order to find out more about how β-catenin regulates epidermal stem cell fate the challenge now is to investigate upstream events and downstream targets. Since β-catenin signalling does not appear to be controlled by differences in the level of cadherins in stem and transit amplifying cells (Fig. 1B,C) it is important to examine the role of Wnts, whether secreted by keratinocytes themselves or by cells in the underlying dermis (Chuong et al., 1996; St-Jacques et al., 1998). It has previously been suggested that there is cross-talk between β1 integrins and cadherins (Burdsal et al., 1993; Hodivala and Watt, 1994; Finnemann et al., 1995; Huttenlocher et al., 1998) and expression of the T3 construct or the dominant negative cadherin mutant that we now know to decrease β-catenin signalling (Fig. 6B) decreases β1 levels in keratinocytes (Zhu and Watt, 1996; Fig. 5A). Thus it will be interesting to discover whether β-catenin plays any direct role in maintaining the elevated level of β1 integrins characteristic of the stem cell compartment and, if so, whether this is mediated transcriptionally, via TCF/LEFs, or post-translationally, for example, via the integrin-linked kinase (Novak et al., 1998). Finally, whereas β-catenin maintains keratinocytes in the stem cell compartment, c-Myc stimulates entry into the transit amplifying compartment (Gandarillas and Watt, 1997); the observation that c-Myc transcription can be activated by β-catenin (He et al., 1998) raises the intriguing possibility of a feed-back loop controlling the proportion of stem and transit amplifying cells in the epidermis.

We are very grateful to everyone who provided us with advice and reagents, in particular Barry Gumbiner, Hans Clevers and Angel Corbi. A. J. Z. is an Imperial Cancer Research Fund graduate student and this work was supported by the Imperial Cancer Research Fund.

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