Rac1 mutations produce aberrant epithelial differentiation in the developing and adult mouse small intestine

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SUMMARY

The mouse small intestinal epithelium undergoes continuous renewal throughout life. Previous studies suggest that differentiation of this epithelium is regulated by instructions that are received as cells migrate along crypt-villus units. The nature of the instructions and their intracellular processing remain largely undefined. In this report, we have used genetic mosaic analysis to examine the role of Rac1 GTPase-mediated signaling in controlling differentiation. A constitutively active mutation (Rac1Leu61) or a dominant negative mutation (Rac1Asn17) was expressed in the 129/Sv embryonic stem cell-derived component of the small intestine of C57Bl/6-ROSA26→129/Sv mice. Rac1Leu61 induces precocious differentiation of members of the Paneth cell and enterocytic lineages in the proliferative compartment of the fetal gut, without suppressing cell division. Forced expression of the dominant negative mutation inhibits epithelial differentiation, without affecting cell division, and slows enterocytic migration along crypt-villus units. The effects produced by Rac1Leu61 or Rac1Asn17 in the 129/Sv epithelium do not spread to adjacent normal C57Bl/6 epithelial cells. These results provide in vivo evidence that Rac1 is involved in the import and intracellular processing of signals that control differentiation of a mammalian epithelium.

Key words: Rac1, Epithelial differentiation, Cell migration, Intestine, Genetic mosaic analysis, Mouse

INTRODUCTION

The adult mouse intestinal epithelium undergoes rapid and continuous renewal throughout life. Proliferation, differentiation and death are coordinated to maintain a proper cellular census. These programs are executed in anatomically well-defined units consisting of invaginated flask-shaped crypts of Lieberkühn and evaginated finger-like villi. Crypts represent the proliferative units of the intestine. Each crypt contains a population of 4-5 long-lived multipotent stem cells anchored at or near its base (Cheng and Leblond, 1974c; Bjerknes and Cheng, 1981a,b, 1999). The daughters of these stem cells undergo 4-6 rounds of division in the middle and upper portions of crypts and give rise to four distinct epithelial lineages. Three of these, enterocytes, goblet and enteroendocrine cells complete their differentiation as they migrate upwards, out of the crypt, onto adjacent villi (Cheng, 1974a; Cheng and Leblond, 1974a-c). These cells move up the villus in coherent well-ordered columns (Schmidt et al., 1985) over a 3-5 day period. Upon reaching the villus tip, they undergo apoptosis and/or extrusion (Hall et al., 1994). The fourth lineage (Paneth cell) executes its terminal differentiation program during a downward migration to the crypt base (Cheng, 1974b). Each crypt contains 30-50 mature Paneth cells with an average lifespan of 18-23 days (Cheng et al., 1969; Troughton and Trier, 1969; Cheng, 1974b). Paneth cells secrete a variety of factors involved in regulation of cell proliferation (e.g. epidermal growth factor), modification of the extracellular matrix (e.g. matrixisin; Wilson et al., 1997) and host defense against microbial pathogens (e.g. cryptdins; Ouellette, 1997; Wilson et al., 1999).

Villi first appear at embryonic day (E) 15, as a proximal-to-distal wave of cytodifferentiation converts the pseudostratified intestinal epithelium to a simple monolayer. Crypt morphogenesis is not completed until the end of the second postnatal week (Calvert and Pothier, 1990). Studies of chimeric mice, composed of cells from two distinct genetic backgrounds, indicate that as crypts form from the intervillus epithelium, their stem cell population undergoes ‘purification’ from polyclonality (i.e. a mixture of both genotypes) to monoclonality so that all active stem cells in a fully formed crypt are ultimately descended from a progenitor of a single genotype (Schmidt et al., 1988). Absorptive enterocytes, mucus-producing goblet cells, and enteroendocrine cells are apparent in late fetal life, while mature Paneth cells do not appear until postnatal day (P) 7 to P14 (Bry et al., 1994).

A central question concerning this renewing epithelium is how decisions about proliferation, differentiation and death are coordinated. Recent in vivo studies suggest that these decisions are not exclusively cell autonomous. Forced expression of E-cadherin in epithelial cells distributed throughout the length of crypt-villus units slows their rate of migration (Hermiston et
al., 1996). If regulation of intestinal epithelial differentiation were entirely cell autonomous, expression of terminal differentiation markers should be affected by the time that has elapsed since migrating cells exit the cell cycle, and not by the position they occupy along the crypt-villus axis. However, the location of expression of terminal differentiation markers in the intestinal epithelium of these E-cadherin overproducing mice is unchanged, even though migration is slowed appreciably (Hermiston et al., 1996). This finding suggests that as epithelial cells arrive at specific locations along crypt-villus units, they receive instructions that allow them to proceed through their differentiation programs. The results invite an obvious question: what is the nature of these instructions and what molecules are involved in their transmission and interpretation? In the present study, we have examined the role of Rac1, a member of the Rho family of GTP-binding proteins.

The Rho GTPase family includes at least eight distinct groups of proteins. Thus far, the best characterized members are Rho (A, B and C subtypes), Cdc42 (Cdc42Hs and G25K), and Rac (1, 2 and 3; Mackay and Hall, 1998; Aspenström, 1999). Studies in cultured cells have demonstrated that members of this family integrate information from a variety of signaling pathways and act as molecular switches to mediate effects on proliferation, differentiation, or migration (Mackay and Hall, 1998). For example, Rac1 stimulates quiescent 3T3 cells to progress through G1/S (Olson et al., 1995). It also receives instructions that allow them to proceed through their differentiation state (i.e. free of Hepatitis, Minute, Lymphocytic Choriomeningitis, C. Inc.) ad libitum. Mice were maintained in a specified pathogen-free environment.

Constitutively activated form of the protein, in transgenic mice using thymus-specific transcriptional regulatory elements from the lck gene, produces apoptosis in developing thymocytes (Lores et al., 1997). A constitutively active Rac1 expressed in the cerebellar Purkinje neurons of transgenic mice has differential effects on axons, dendritic trunks and spines (Luo et al., 1996).

There are no reports of the results of in vivo tests of Rac function in mammalian epithelia. In the present study, we have examined the effects of forced expression of constitutively active and dominant negative Rac1 mutations in the developing and adult small intestinal epithelium of chimeric mice. Our results indicate that this protein plays an important role in regulating cellular differentiation.

**MATERIALS AND METHODS**

**Generation of chimeric mice**

pl596GHpNeoAB2 (Hermiston et al., 1996) contains nucleotides −596 to +21 of the rat fatty acid binding protein gene (Fabpl) linked to nucleotides +3 to +2150 of the human growth hormone gene (hGH). The neomycin resistance gene, under the control of pgk regulatory elements, is positioned downstream of hGH. Plasmids containing cDNAs encoding previously characterized, constitutively active and dominant negative human Rac1 mutations, each with an N-terminal c-myc epitope tag, were generously provided by Alan Hall (University College, London). The constitutively active mutation contains a Gln61→Leu substitution (abbreviated Rac1Leu61) (Lamarche et al., 1996). The dominant negative mutation contains a Thr17→Asn substitution (Rac1Asn17). 600 bp ClaI EcoRI fragments, containing Rac1Leu61 or Rac1Asn17, were blunt-end ligated to BamHI-digested (and Klenow treated) pl596GHpNeoAB2 so that the Rac1 sequences were placed between the Fabpl and hGH sequences. The 5.2 kb Fabpl-Rac1Leu61-hGH-pgkneo and Fabpl-Rac1Asn17-hGH-pgkneo inserts in the resulting plasmids (pLFRAc1Leu61 and pLFRac1Asn17, respectively) were excised with XbaI, separated from vector sequences by electrophoresis in low melting point agarose, extracted from the gel, and electroporated into D3 129/Sv ES cells (Hermiston and Gordon, 1995a). Stably transfected ES cells were selected using G418, cloned, and the presence of Fabpl-Rac1Leu61-hGH-pgkneo or Fabpl-Rac1Asn17-hGH-pgkneo in various clones was verified by PCR using primers that recognize Rac1 sequences and sequences in exon 2 of hGH (5′-AAATTACCTGGAGTGCTCGGC-3′; 5′-GGCAGAGCAGGCCAAAAGGCC-3′). Cloned ES cell lines, or nontransfected D3 ES cells (controls) were injected into C57Bl/6-Rosa2A6 blastocysts (Wong et al., 1998) to produce B6-Rosa2A6→129/Sv chimeric mice. Chimeras were produced from 6 different ES cell clones containing Fabpl-Rac1Leu61, and from 6 different ES cell clones containing Fabpl-Rac1Asn17. A ‘line of chimeric-transgenic mice refers to animals generated using a given ES cell clone. ‘Normal chimeras’ refer to mice produced from non-transfected D3 ES cells. The 129/Sv contribution in adult chimeras was determined by coat color, and in the embryos and neonates by an electrophoretic glucose 6-phosphate isomerase (GPI) isoenzyme assay of limb tissue (Nagy and Rossant, 1992).

**Maintenance of mice**

All mice used in this study were housed in microisolator cages, in a barrier facility, under a strict 12 hour light cycle. Mice were fed a standard irradiated chow diet (PicoLab Rodent Chow 20, Purina Mills Inc.) ad libitum. Mice were maintained in a specified pathogen-free state (i.e. free of Hepatitis, Minute, Lymphocytic Choriomeningitis,
**Assays for transgene expression**

**Reverse transcriptase-PCR**

Total cellular RNA was isolated from the entire small intestine and brain of E18.5 B6-ROSA26e+129Sv chimeric-transgenic and normal chimeric mice using the RNeasy kit (Qiagen, Santa Clara, CA). RNA was also purified from 2 cm segments harvested from the middle third of the small intestine, and from the intact brain of P42 animals. Oligo dT-primed cDNA was generated and mutant Rac1 mRNAs were identified by PCR using two different sets of primers, both of which recognize Rac1 sequences and sequences from exon 2 of hGH (5'-AAATACCTGTGGTCTGGC-3' and 5'-GACAGTTGTTGACGAAAGCA-3'; 5'-GGAGACAGACGCAAGAAGC-3' and 5'-GAA-TGGTTGGAAAAGCAGT-3'). Thermocycling conditions were as follows: (i) 95°C for 1 minute (denaturation); (ii) 60°C for 1 minute (annealing), and (iii) 72°C for 2 minutes (extension) for 30 cycles.

**Immunoblotting**

Proteins from the small intestine and brain of E18.5 Rac1Leu61 chimeric-transgenic mice and age matched normal chimeras were extracted by placing these tissues in a solution containing 8 M urea, 0.19 M Tris-HCl pH 6.8, 1% SDS, and 1% β-mercaptoethanol. Tissues were disrupted by passage through an 18-gauge needle, and insoluble material was removed by centrifugation at 12,000 g for 5 minutes. Protein concentrations in the resulting supernatants were determined according to Bradford (1976).

Protein was also isolated from the small intestinal epithelium of normal adult (P42) chimeras. To do so, the epithelium was separated from the underlying mesenchyme using a protocol adapted from Weiser (1973). Epithelial and mesenchymal proteins (50 µg/sample) were fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were pre-treated for 1 hour at 24°C with blocking buffer (PBS containing 5% powdered nonfat milk), and then probed for 1 hour, at 24°C with rabbit anti-human/mouse Rac1 (raised against the C terminus of the protein; final dilution in blocking buffer = 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Duplicate blots were also probed with rabbit anti-human RhoA (1:1000, Santa Cruz Biotechnology), or with rabbit antibodies raised against the C-terminal 11 residues of all actins (1:2000, Sigma, St. Louis, MO). Antibodies-antibody complexes were visualized with horseradish peroxidase-conjugated donkey anti-rabbit Ig using protocols and reagents provided in the Super Signal West Femto Detection kit (Pierce Chemical, Rockford, IL).

**Histochemistry**

Small intestine whole mounts (Wong et al., 1998) were prepared from P42 normal chimeras and B6-ROSA26e+129Sv-Rac1Asn17 chimeric-transgenic mice as follows. The small intestine was removed, flushed gently with ice-cold PBS, opened along its mesenteric side, pinned villus-side up on wax sheets, and fixed in periodate-lysine-paraformaldehyde (PLP) for 1 hour at 24°C. The whole mount was then washed three times in PBS, incubated in a solution containing 20 mM DTT, 20% ethanol, and 150 mM Tris-HCl (pH 8.0) for 45 minutes at 24°C to disrupt any adherent mucus, and washed once again in PBS. The epithelium was then genotyped by incubating the preparation overnight at 4°C in PBS containing 2 mM X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside), 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, and 2 mM MgCl2 (final pH, 7.6). After the epithelium was photographed, the whole mount was embedded in 2% agar, cut in half along its cephalocaudal axis, and placed in tissue cassettes so that histologic sections could be prepared parallel to the cephalocaudal axis and perpendicular to the crypt-villus axis.

For E18.5 chimeras, intestines were removed en bloc, submerged in ice-cold PBS, and the mesentery was removed using a stereoscope and a pair of fine forceps. The tissue was then fixed in PLP for 2 hours at 24°C, washed in PBS, and stained as above, except that the staining solution was supplemented with 0.1% deoxycholate and 0.02% IGEPAL CA-630 (Sigma) to improve X-gal penetration. Fixed and stained intestines were then embedded in 2% agar and divided into 2 mm wide ‘donuts’ by cutting perpendicular to the cephalocaudal axis. Donuts from each intestine were placed in tissue cassettes so that their original cephalocaudal relationships could be preserved.

**Single and multi-label immunohistochemistry**

PLP-fixed tissue sections were de-waxed with xylene, rehydrated, and pre-treated for 15 minutes at room temperature with blocking buffer (1% bovine serum albumin, 0.3% Triton X-100, and 1mM CaCl2 in PBS). Sections of E18.5 or P42 small intestine were stained with the following antisera: (i) rabbit anti-human/mouse Rac1 (Santa Cruz Biotechnology; final dilution in blocking buffer = 1:100); (ii) mouse anti-c-myc epitope (clone 9E10.2; Calbiochem; see Evan et al., 1985; 1:100); (iii) rabbit anti-rat liver fatty acid binding protein (L-FABP; Sweetser et al., 1988; 1:1000); (iv) rabbit anti-rat intestinal fatty acid binding protein (I-FABP; Cohn et al., 1992; 1:1000-1:10,000); (v) rabbit antiserum against residues 4-35 of c-myc (clone H-129; Santa Cruz Biotechnology; final dilution in blocking buffer = 1:100); (vi) mouse anti-c-myc (clone MAB102; Oncogene; 1:200); (vii) rabbit anti-sheep alkaline phosphatase (ASAP; Sigma, 1:100); and (viii) rabbit anti-sheep alkaline phosphatase (ASAP; Sigma, 1:100). Prior to incubation with antibodies to Rac1, c-myc, or β-catenin, sections were microwaved at 50% power in a 1.5 kW machine for 15 minutes in 100 mM sodium citrate, pH 6.0 (for antigen retrieval). Prior to incubation with antibodies to β-actin, antigen was retrieved by treating sections with 0.1% chymotrypsin (prepared in 0.1% CaCl2) for 10 minutes at 37°C.

Cycling intestinal epithelial cells in E16.5 or E18.5 chimeras were labeled in S-phase by injecting their Swiss-Webster foster mothers intraperitoneally with an aqueous solution of 5-bromo-2'-deoxyuridine (BrdU, 120 mg/kg) and 5'-fluoro-2'-deoxyuridine (12 mg/kg; Sigma). Chimeric mice labeled at E18.5 were killed 1.5 hours later for analysis of cell proliferation. Mice labeled at E16.5 were killed 2 days later for analysis of cell migration. Intestines from both groups of chimeric animals were fixed in PLP, stained with X-gal as above, and then post-fixed in Bouin’s solution for 6 hours. Sections were prepared and incubated with goat anti-BrdU (Cohn et al., 1992; 1:2000). Antigen-antibody complexes were detected with indocarbocyanine (Cy3)- or FITC-conjugated donkey anti-rabbit, anti-mouse, or anti-goat Ig (1:500, Jackson ImmunoResearch Laboratories). Nuclei were stained with bis-benzimide (20 ng/ml PBS).

Sections of PLP-fixed and X-gal stained chimeric intestine were also incubated with a series of FITC- or biotin-tagged lectins (all at a final concentration of 5 µg/ml blocking buffer; Falk et al., 1994). The lectin panel consisted of the following members: (i) Dolichos biflorus agglutinin (Sigma; carbohydrate specificity = GalNAc-containing glycans); (ii) Helix pomatia agglutinin (Sigma; α-GalNAc/GalNAcβ4Gal-glycans); (iii) Arctium lappa integirifolia agglutinin (Jalacini-1; E.Y. Laboratories, Inc., San Mateo, CA); (iv) Ulex europaeus I agglutinin (Sigma), GalNAcβ4Neu5Acα2,3Gal; (v) Maackia amurensis (E. Y. Laboratories; Neu5Acα2,3Galβ4Glc/GlcNAc); and (vi) Ulex

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europeaus agglutinin-1 (Sigma; Fucα1,2Galβ). Bound biotinylated lectins were detected using horseradish peroxidase-conjugated avidin, VIP substrate, and protocols provided by their manufacturer (Vector Labs, Burlingame, CA). The lineage-specific, differentiation-dependent, and cephalocaudal patterns of reactivity of these lectins with the mouse intestinal epithelium have been described previously (Falk et al., 1994; Hermiston and Gordon, 1995a,b; Hermiston et al., 1996).

**RESULTS**

**Rationale for using chimeric mice to test the function of Rac1 in the intestinal epithelium**

The mouse intestine establishes and maintains complex cephalocaudal differences in the differentiation programs of its component lineages, the organization of its mucosal immune system, and the composition of its microflora (Falk et al., 1998). This spatial complexity makes it imperative that audits of gene function pay attention to cellular location and environment. One way to control for this complexity is to use chimeric mice produced by injection of genetically manipulated 129/Sv embryonic stem cells into C57Bl/6-ROSA26 (B6-ROSA26) blastocysts (Wong et al., 1998). Genotyping of the intestines of these mice is straightforward. The B6-ROSA26-derived intestinal epithelium produces E. coli β-galactosidase (lacZ) throughout the lifespan of the mouse and can be visualized by staining whole-mount preparations of gut, or histologic sections, with X-gal. The 129/Sv ES cell-derived epithelium is lacZ-negative (Fig. 1A, B). Crypts are monoclonal in the small intestine of adult chimeras: i.e. they are populated with either B6-ROSA26 or 129/Sv epithelial cells, but not a mixture of both (Fig. 1B). Each villus is supplied by several crypts. Therefore, a chimeric mouse offers the opportunity to compare epithelial cell phenotypes in adjacent villi that are supplied exclusively by 129/Sv ES cell-derived crypts, or exclusively by B6-ROSA26-derived crypts. Some villi will be supplied by both types of crypts. These ‘polyclonal’ villi contain juxtaposed columns of normal B6-ROSA26 cells and genetically manipulated 129/Sv cells (Fig. 1A, B). A single polyclonal villus in a chimeric mouse represents a well-controlled experiment where the effects of a genetic manipulation on proliferation, migration, differentiation, or death can be ascertained simply by comparing cells of each genotype at a given location along the length of the crypt-villus axis.

In late fetal life, before crypts have formed, the intervillus epithelium of chimeric mice contains a polyclonal population of stem cells. Therefore, the fetal intervillus epithelium and its associated nascent villi can contain intermingled populations of B6 or 129/Sv cells that can be compared (Fig. 1C).

**Studies of normal chimeras indicate that Rac1 is induced as 129/Sv and B6-ROSA26 intestinal epithelial cells undergo terminal differentiation**

Previous northern blot analyses indicated that Rac1 mRNA is expressed in most organs of the adult mouse (Moll et al., 1991). The cellular patterns of Rac1 expression in the developing and adult mouse small intestinal epithelium have not been reported. Therefore, we began our study by addressing this point in normal adult chimeras generated by introducing non-manipulated (‘wild type’) 129/Sv ES cells into B6-ROSA26 blastocysts. Epithelium was recovered from the middle third of the small intestine (jejunum) of P42 chimeras. When western blots of total epithelial cell proteins were probed with antibodies specific for Rac1, a single immunoreactive protein of the expected mass (25 kDa) was detected (Fig. 2A). Rac1 was present in the intact small intestine of E18.5 chimeras but at a considerably lower level than in adults (Fig. 2A) (n=2 mice surveyed/developmental time point).

The same antibody preparation was used to define the cellular patterns of Rac1 accumulation. Immunohistochemical surveys disclosed that in normal P42 chimeras, Rac1 is not detectable in epithelial cells located in the middle and upper thirds of crypts where relatively undifferentiated cycling
epithelial cells reside. The finding applied to crypts positioned in the proximal, middle and distal thirds (duodenum, jejunum, ileum) of the small intestine (n=15 mice surveyed). Rac1 levels rise abruptly as epithelial cells migrate from the upper crypt to the base of their associated villi. Levels do not change appreciably as cells complete their migration to the villus tip. Rac1 is not only prominent throughout the villus epithelium but also at the crypt base where mature Paneth cells are located (Fig. 2B). In each region of the intestine, there was no appreciable difference between the cellular pattern of Rac1 expression in adjacent 129/Sv and B6-ROSA26 crypt-villus units, or between the 129/Sv and B6 components of polyclonal villi. Control experiments revealed that in each region of the small intestine, villus epithelial and Paneth cell staining could be blocked by pre-treating Rac1 antibodies with the peptide used for their generation (Fig. 2C). Together, these results indicate that Rac1 accumulation correlates with terminal differentiation of small intestinal epithelial cells in adult chimeric mice.

Although the very sensitive detection methods employed for western blot analysis revealed Rac1 in the late fetal small intestine, we were unable to reliably detect the protein in E16.5-18.5 intervillus or villus epithelium (or mesenchyme) using the immunohistochemical techniques employed for adult intestine (n=10 normal fetal chimeras/time point; e.g. Fig. 2D,E). However, Rac1 levels rose appreciably as gut morphogenesis proceeded. The protein was readily detectable in the villus epithelium by P3. In nascent P3 crypts, only a small subset of cells belonging to the goblet lineage were Rac1-positive (Fig. 2F). This cohort of goblet cells was lost, beginning at P7, as crypt morphogenesis proceeded to completion by the third postnatal week (data not shown).

**Generation of B6-ROSA26→129/Sv chimeras that express Rac1Leu61 in their 129/Sv intestinal epithelium**

We reasoned that if Rac1 functioned as an effector of intestinal epithelial differentiation, rather than a marker of differentiation, then expression of a constitutively active Rac1 in the epithelium of nascent or fully formed crypts could result in ‘precocious’ differentiation within this proliferative compartment. Of course, this would require that the cells contain other components of a Rac1-dependent signaling pathway that mediates differentiation.

We selected a previously characterized (Lamarche et al., 1996) constitutively active human Rac1 mutation (Gln61→Leu) with an N-terminal c-myc tag for this gain-of-function experiment. (Note, the amino acid sequences of the orthologous human and mouse Rac1 proteins are identical; Moll et al., 1991). 129/Sv ES cells were stably transfected with
a recombinant DNA consisting of nucleotides −596 to +21 of a fatty acid binding protein gene (Fabpl) linked to an open reading frame encoding c-myc-Rac1Leu61. These Fabpl elements were chosen because previous studies had shown that other Fabpl-reporter transgenes are expressed throughout the intervillus and villus epithelium. Highest levels of expression occur in the jejunum. Expression commences by E15, is sustained after crypt-villus units have formed, and occurs in all four epithelial lineages throughout the course of their differentiation (e.g. Hermiston and Gordon, 1995b; Hermiston et al., 1996; Wong et al., 1998).

Six cloned, stably transfected Fabpl-Rac1Leu61 ES cell lines were injected (individually) into B6-ROSA26 blastocysts (10-15 ES cells/blast). Three of the clones produced high percentage adult chimeras (30-50% 129/Sv by coat color). However, these chimeras did not show expression of the Fabpl-Rac1Leu61 transgene, as determined by RT-PCR of P42 small intestinal RNA (data not shown). The three remaining Fabpl-Rac1Leu61 ES cell lines produced high percentage chimeras (defined by glucose 6-phosphate isomerase assay) that died within a day after birth (n=2-4 litters of mice examined/ES cell line). None of these mice had any obvious histopathologic changes in their organs, and the cause of their death remains unclear.

We examined B6-ROSA26→129/Sv-Rac1Leu61 chimeras, generated from each of the three Rac1Leu61 lines that produced neonatal mortality, at E18.5. Sixty-nine of 71 chimeric-transgenic mice (97%) surveyed at this stage of development were alive and free of any gross deformities. This level of viability was identical to that of normal chimeras (33 of 34 mice). RT-PCR analysis of small intestinal RNA showed the expected size product of Fabpl-Rac1Leu61 in each of these chimeric-transgenic ‘lines’ (n=2 mice surveyed/line) (Fig. 3A). The RT-PCR product was not present in RNA prepared from their brain, or in RNA isolated from the small intestine and brain of E18.5 normal chimeras (Fig. 3A).

Production of Rac1Leu61 was verified by immunoblot analysis. A single 25 kDa reactive species was present in the small intestines of E18.5 chimeric-transgenic mice. The level of immunoreactive protein was several-fold higher than the level of endogenous Rac1 in age-matched normal chimeric mouse intestine (Fig. 3B). This difference is consistent with our engineered forced expression of the Rac1Leu61 mutation.

X-gal plus antibodies to either Rac1 or its N-terminal c-myc epitope tag were used to stain adjacent serial sections of the mid-portions of the small intestine of E18.5 chimeric-transgenic mice. The same results were obtained with both antibodies: Rac1 was present in the cytoplasm of 129/Sv intervillus and villus epithelial cells and absent from adjacent B6-ROSA26 epithelial cells (see Fig. 3C,D and compare with Fig. 2D,E).

Expression of Rac1Leu61 causes precocious differentiation of Paneth cells and enterocytes within the proliferative compartment of the E18.5 small intestinal epithelium

E18.5 chimeric-transgenic mice generated from two of the three Fabpl-Rac1Leu61 ES cell lines were analyzed in detail. Animals produced from both lines had the same phenotype.

During late fetal life, the Paneth cell and enterocytic lineages can be used as sensitive reporters of precocious differentiation in the intervillus epithelium. As noted in the Introduction, mature Paneth cells do not normally appear until P7-P14 (Bry et al., 1994). Surveys of serial sections of E18.5 B6-ROSA26→129/Sv-Fabpl-Rac1Leu61 intestine, stained with hematoxylin and eosin and X-gal, revealed 129/Sv cells with large apical eosiophilic granules scattered throughout the intervillus epithelium (Fig. 4A). These granules reacted with antibodies raised against residues 4-35 of one of the most abundant intestinal defensins, cryptd-1 (Fig. 4B). These granules also reacted with antibodies specific for a secerted phospholipase A2 encoded by the Pla2g2a gene (Mulkerhar et al., 1994). Surveys of serial sections of E18.5 B6-ROSA26→129/Sv-Fabpl-Rac1Leu61 intestine, stained with hematoxylin and eosin and X-gal, revealed 129/Sv cells with large apical eosinophilic granules scattered throughout the intervillus epithelium (Fig. 4A). These granules reacted with antibodies raised against residues 4-35 of one of the most abundant intestinal defensins, cryptd-1 (Fig. 4B). These granules also reacted with antibodies specific for a secerted phospholipase A2 encoded by the Pla2g2a gene (Mulkerhar et al., 1994). Surveys of serial sections of E18.5 B6-ROSA26→129/Sv-Fabpl-Rac1Leu61 intestine, stained with hematoxylin and eosin and X-gal, revealed 129/Sv cells with large apical eosinophilic granules scattered throughout the intervillus epithelium (Fig. 4A). These granules reacted with antibodies raised against residues 4-35 of one of the most abundant intestinal defensins, cryptd-1 (Fig. 4B). These granules also reacted with antibodies specific for a secerted phospholipase A2 encoded by the Pla2g2a gene (Mulkerhar et al., 1994). Surveys of serial sections of E18.5 B6-ROSA26→129/Sv-Fabpl-Rac1Leu61 intestine, stained with hematoxylin and eosin and X-gal, revealed 129/Sv cells with large apical eosinophilic granules scattered throughout the intervillus epithelium (Fig. 4A). These granules reacted with antibodies raised against residues 4-35 of one of the most abundant intestinal defensins, cryptd-1 (Fig. 4B). These granules also reacted with antibodies specific for a secerted phospholipase A2 encoded by the Pla2g2a gene (Mulkerhar et al., 1994). Surveys of serial sections of E18.5 B6-ROSA26→129/Sv-Fabpl-Rac1Leu61 intestine, stained with hematoxylin and eosin and X-gal, revealed 129/Sv cells with large apical eosinophilic granules scattered throughout the intervillus epithelium (Fig. 4A). These granules reacted with antibodies raised against residues 4-35 of one of the most abundant intestinal defensins, cryptd-1 (Fig. 4B). These granules also reacted with antibodies specific for a secerted phospholipase A2 encoded by the Pla2g2a gene (Mulkerhar et al., 1994). Surveys of serial sections of E18.5 B6-ROSA26→129/Sv-Fabpl-Rac1Leu61 intestine, stained with hematoxylin and eosin and X-gal, revealed 129/Sv cells with large apical eosinophilic granules scattered throughout the intervillus epithelium (Fig. 4A). These granules reacted with antibodies raised against residues 4-35 of one of the most abundant intestinal defensins, cryptd-1 (Fig. 4B). These granules also reacted with antibodies specific for a secerted phospholipase A2 encoded by the Pla2g2a gene (Mulkerhar et al., 1994). Surveys of serial sections of E18.5 B6-ROSA26→129/Sv-Fabpl-Rac1Leu61 intestine, stained with hematoxylin and eosin and X-gal, revealed 129/Sv cells with large apical eosinophilic granules scattered throughout the intervillus epithelium (Fig. 4A).
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al., 1993; Harwig et al., 1995) (data not shown). Large apical granules containing these two protein products are characteristic features of mature Paneth cells (Cheng, 1974a; Bry et al., 1994; Garabedian et al., 1997). Cells with these features were not detectable in the B6-ROSA26 intervillus or villus epithelium of E18.5 chimeric-transgenic mice (n=30). In addition, they were absent from the 129/Sv and B6-ROSA26 intestinal epithelium of age-matched normal chimeras (n=20).

The enterocytic lineage can also be readily scored for precocious differentiation in the E18.5 intervillus epithelium. In normal chimeras, 129/Sv and B6-ROSA26 intervillus epithelial cells do not contain detectable levels of two homologous cytoplasmic fatty acid binding proteins, I-FABP and L-FABP. These proteins only appear as differentiating enterocytes move to the base of nascent villi (Fig. 5A,C). In contrast, there was a large population of L- and I-FABP-positive 129/Sv intervillus epithelial cells in the intestines of all E18.5 chimeric-transgenic mice surveyed (n=5-8 animals/line) (Fig. 5B,D). Adjacent B6-ROSA26 intervillus epithelial cells did not contain detectable levels of the FABPs (Fig. 5B,D). The effect of forced expression of Rac1Leu61 on these proteins was limited to the intervillus epithelium: there were no appreciable changes in the steady state levels of either FABP in 129/Sv villus enterocytes (reference controls = adjacent B6-ROSA26 cells and 129/Sv jejunal villus enterocytes in normal chimeras).

Goblet and enteroendocrine cells are present in the intervillus and villus epithelium of normal E18.5 chimeras and, thus, are not sensitive reporters of precocious differentiation at this stage of development. Nonetheless, after staining X-gal-marked sections with (i) antibodies to Rac1, c-myc, and chromogranin A, (ii) Alcian blue and periodic acid Schiff, or (iii) lectins that recognize glycans produced by members of these two lineages, we concluded that Rac1Leu61 does not impede goblet or enteroendocrine cell differentiation (data not shown).

Fig. 4. Expression of a constitutively active Rac1 causes precocious differentiation of Paneth cells. (A) Hematoxylin and eosin-stained section of jejunum from an E18.5 Rac1Leu61 chimeric-transgenic mouse showing a Paneth cell with large eosinophilic granules (arrows) in a region of wholly of 129/Sv intervillus epithelium. The inset provides a higher power view of these granules. (B) Adjacent section stained with rabbit anti-cryptdin-1, Cy3-donkey anti-rabbit Ig and bis-benzimide, reveals that these large apical Paneth cell granules contain cryptdins. Bars, 25 μm.

Fig. 5. Forced expression of Rac1Leu61 causes premature expression of enterocyte-specific proteins. Sections of X-gal-stained E18.5 jejunum from a normal chimeric mouse (A,C), and an age-matched chimeric-transgenic animal (B,D). For each animal, a given section was genotyped with X-gal and then stained with either rabbit anti-L-FABP (A,B) or rabbit anti-I-FABP (C,D), Cy3-conjugated donkey anti-rabbit Ig, and bis-benzimide. In the normal chimeric mouse, L-FABP and I-FABP are only detectable in differentiating villus enterocytes, regardless of genotype. The intervillus epithelium (below the dashed line) is negative for each protein, irrespective of genotype. In the Rac1Leu61 chimeric-transgenic mouse, both L-FABP and I-FABP show staining within the villus epithelium. However, both of these proteins are also expressed in 129/Sv (but not B6-ROSA-26) intervillus epithelial cells. Bars, 25 μm.
**Rac1Leu61 does not block proliferation or migration in the E18.5 small intestinal epithelium**

We considered two possible mechanisms that may contribute to the effect of Rac1Leu61 on enterocytic and Paneth cell differentiation. First, the mutation inhibits proliferation in the intervillus epithelium, allowing cells to differentiate. Second, the mutation does not affect proliferation but impedes migration, thereby giving cells additional time to execute their terminal differentiation before they arrive on nascent villi. If cell non-autonomous mechanisms act to normally prohibit differentiation in the intervillus compartment, then either of the two postulated mechanisms would require that Rac1Leu61 allows cells to override, or ignore, differentiation-restrictive signals produced in the compartment.

To address the issue of proliferative status, E18.5 Rac1Leu61 chimeras were exposed in utero to BrdU for 1.5 hours to mark cells in S-phase. Subsequently, sections of their jejunum were stained with antibodies to BrdU (Cohn et al., 1992). No discernible differences were found in the number of cycling BrdU-positive cells in the 129/Sv- and B6-ROSA26 components of the intervillus epithelium (Fig. 6A) \((n=3-4\) chimeric-transgenic mice surveyed/ES cell line plus 5 normal chimeric mice). Because cycling 129/Sv and B6-ROSA26 epithelial cells are intermingled in various proportions within the polyclonal intervillus epithelium, a more detailed quantitative assessment of the ratios of BrdU-positive to -negative cells of each genotype was not feasible.

To assess the effects of Rac1Leu61 on cell migration, we took advantage of two observations. RT-PCR assays established that at E16.5 *Fabpl*-Rac1Leu61 is expressed in the small intestine of chimeric-transgenic mice. In addition, 1.5 hours after intraperitoneal administration of BrdU to pregnant mothers, S-phase cells are only rarely encountered in the villus epithelium of E16.5 and E18.5 chimeric-transgenic or normal chimeric mice (data not shown). Therefore, we were able to examine the migratory behavior of 129/Sv Rac1Leu61-producing intervillus cells by giving a single injection of BrdU to the pregnant mothers of E16.5 chimeric-transgenic fetuses, and then analysing the fetuses 2 days later. Migration was defined by noting the distribution of BrdU-positive cells along the intervillus-villus axis. Cells were genotyped by staining adjacent sections with X-gal. The results revealed that Rac1Leu61 does not block the ability of 129/Sv cells to move up the villus \((n=2\) mice/ES cell line) (Fig. 6B).

**The effects of Rac1Leu61 on the intracellular distribution of β-actin in intervillus and villus epithelial cells**

Rac1Leu61 can have varied effects on the shape, mobility, and actin cytoskeleton of cultured epithelial cells, depending upon the cell line studied, and the culture conditions employed (e.g., Braga et al., 1997; Takaishi et al., 1997; Jou and Nelson, 1998; Sander et al., 1998). We characterized the effects of Rac1Leu61 on the actin cytoskeleton of intestinal epithelial cells by staining adjacent sections of jejunum, harvested from E18.5 normal chimeras and chimeric-transgenic mice, with X-gal and antibodies to β-actin. There were no appreciable differences in the intracellular distribution of actin between *intervillus*129/Sv-Rac1Leu61 and B6-ROSA26 epithelial cells. Differences in the actin cytoskeleton were apparent by the time cells migrated to the villus epithelium. In normal E18.5 chimeras, the bulk of the actin cytoskeleton is localized at the apex of 129/Sv and B6-ROSA26 jejunal villus enterocytes, which constitute the majority of the epithelial population. Much smaller amounts are present at cell-cell and cell-substratum junctions (Fig. 7A). In chimeric-transgenic mice, Rac1Leu61 expression was associated with a modest decrease in apical actin (Fig. 7B) \((n=5\) mice surveyed/ES cell line).

Other markers of enterocytic polarization were not affected in 129/Sv-Rac1Leu61 villus epithelial cells. For example, there were no detectable differences in the intensity of staining of adherens junction proteins (E-cadherin, β-catenin) between adjacent 129/Sv and B6-ROSA26 enterocytes (data not shown; \(n=5\) chimeric-transgenic mice/ES cell line; 5 normal chimeras).

**A loss-of-function experiment supports conclusions made from the gain-of-function experiment**

Thr17→Asn substitution in Rac1 (Rac1Asn17) yields a dominant negative mutation (Ridley et al., 1992). B6-ROSA26→129/Sv-*Fabpl*-Rac1Asn17 chimeric-transgenic mice were created by injecting each of six cloned, stably transfected ES cell lines into B6-ROSA26 blastocysts. Unlike the situation with the constitutively active mutation, three *Fabpl*-Rac1Asn17 ES cell clones gave rise to adult chimeric-
transgenic mice that expressed the transgene (e.g., inset to Fig. 8A). All three ‘lines’ of mice produced from these ES cell clones had a similar abnormal phenotype, although in two lines the changes were more pronounced. This phenotype was characterized by alterations in the morphology of jejunal crypt-villus units, an inhibition of differentiation without a change in proliferation, and a slowing of epithelial migration. These abnormalities were not associated with increased lethality, or with any statistically significant alterations in adult body weight compared to age-matched normal chimeras.

Crypt-villus units have abnormal morphology

Surveys of jejunal whole mounts prepared from members of two of the three lines of P42 Rac1Asn17 mice \( (n \geq 3/\text{line}) \) showed that 129/Sv villi were 1.5-2 times wider than adjacent normal B6-ROSA26 villi (Fig. 8A). This effect was most prominent in the distal half of the jejunum where >75% of 129/Sv villi were widened. All of these widened villi were supplied by at least one elongated 129/Sv crypt, although not all 129/Sv crypts that were associated with a widened villus had this abnormal morphology. The elongated crypts extended into the core of the villus, emerging at various locations along its length (Fig. 8B,C). Widened villi and elongated crypts were not present in the B6-ROSA26 component of the proximal or distal jejunum, nor were they evident in either genotypic component of the normal chimeric gut. A quantitative comparison of proliferation in juxtaposed elongated 129/Sv Rac1Asn17 crypts and normal B6 crypts revealed no significant differences in the number of M-phase cells.

As noted above, Rac1 is normally induced at the crypt-villus junction. Therefore, we hypothesized that the ‘elongated’ phenotype was due to Rac1Asn17-mediated inhibition of epithelial differentiation and hence an expansion (extension) of the upper crypt into what would normally be the base of a villus. Cells at the crypt-villus junction should be particularly susceptible to the effects of the dominant negative mutation since levels of endogenous Rac1 are lower in this epithelial population compared to more differentiated cells positioned on the villus. Our hypothesis was supported by the following observations. As enterocytes move from the upper portions of normal 129/Sv and B6-ROSA26 crypts onto villi, three prominent changes occur: I- and L-FABP are induced, levels of apical actin increase, and the concentration of Fucα1,2Galβ-glycans in apical membranes rises (Fig. 8E,G,I). I- and L-FABP-positive cells were absent in the upper halves of elongated Rac1Asn17 crypts (e.g., Fig. 8D). In addition, these crypt cells had low levels of apical actin and Fucα1,2Galβ-glycans (Fig. 8F,H).

**Fig. 7.** Forced expression of Rac1Leu61 decreases the apical actin cytoskeleton in 129/Sv villus epithelial cells. Sections of jejunum from an E18.5 normal chimera (A) and a chimeric-transgenic mouse (B). Each section was genotyped with X-gal and then stained with FITC-conjugated mouse antibodies to β-actin, and bis-benzimide. (A), villi sectioned perpendicular to the intervillus-villus axis. There is equivalent staining of apical actin in 129/Sv and B6-ROSA26 epithelial cells. (B), 129/Sv villus epithelial cells in the Rac1Leu61 chimeric-transgenic mouse have decreased apical actin staining compared to juxtaposed B6-ROSA26 cells. This is most clearly seen in the enlarged views of the boxed area. Bars, 25 μm.
the crypt to villus (n=4-5 mice/line (Fig. 9A,B). Like intermediate cells in normal chimeras, members of this expanded population of Fabpl-Rac1Asn17 intermediate cells contained cryptdins and the product of Pla2g2a (Fig. 9C, plus data not shown).

The expansion of 129/Sv intermediate cells was accompanied by a marked decrease in the number of mature 129/Sv goblet cells, and a less prominent reduction in Paneth cells. The decrease in mature goblet cells was defined using members of our lectin panel, and by staining with Alcian blue (e.g. Fig. 9D; compare with the normal chimera in Fig. 9E). These results suggest that intermediate cell differentiation is blocked by Rac1Asn17. However, since intermediate cells may not be the only precursor of goblet cells, it is possible that the pronounced loss of members of this lineage reflects Rac1Asn17-mediated inhibition of differentiation of other progenitors.

In contrast to its effect on the goblet and Paneth cell lineages, Rac1Asn17 did not produce a detectable alteration in the number, or crypt-villus distribution, of chromogranin A-positive enteroendocrine cells (n=6 mice from both lines).

The effect of Rac1Asn17 on epithelial differentiation was not associated with changes in proliferation. Proliferation was measured by examining X-gal- and hematoxylin and eosin-stained sections containing normal appearing 129/Sv and B6-Rosa26 crypts affiliated with 50 proximal jejunal polyclonal villi (n=two P42 chimeric-transgenic mice from 2 lines). The number of M-phase cells was similar irrespective of genotype.

Fig. 8. Expression of a dominant negative Rac1Asn17 in adult chimeric-transgenic mice affects the morphology of crypt-villus units. (A) Whole-mount preparation of X-gal stained distal jejunum from a P42 Rac1Asn17 chimeric-transgenic mouse. 129/Sv villi are wider than adjacent B6-Rosa26 villi. The inset presents an RT-PCR analysis of RNAs isolated from the jejunums of P42 chimeric-transgenic and normal chimeric mice. A 250 bp PCR product, generated from the mRNA transcript of Fabpl-Rac1Asn17, is present in RNA recovered from the chimeric-transgenic animal. (B,C) Hematoxylin and eosin-stained sections from a Rac1Asn17 mouse showing widened 129/Sv distal jejunal villi with elongated crypts (examples indicated by arrows). (D) I-FABP-stained section of a widened villus with a central elongated crypt extending throughout its core (outlined with the dashed lines). I-FABP (red) is limited to villus enterocytes (arrows). Epithelial cells lining the elongated crypt do not contain detectable levels of this cytoplasmic protein (e.g. arrowheads). Normal appearing crypts flank the widened villus. (E) Distal jejunal crypt-villus unit from a normal chimera showing induction of I-FABP expression as enterocytes emerge from crypts. (F) Section from a single widened 129/Sv-Rac1Asn17 villus stained with FITC-conjugated antibodies to β-actin (green) and bis-benzimide. Two elongated crypts, each indicated by an asterisk, penetrate the core of the villus. A normal appearing crypt is located to the left. Apical actin only becomes prominent as epithelial cells migrate to the upper portions of the elongated crypts. (G) Distal jejunal crypt-villus unit from a normal chimera processed as in (F). (H) Higher power view of a crypt located in a widened 129/Sv villus. The section has been stained with Ulex europeaus agglutinin 1 (UEA-1). Fucτ1,2Galβ glycans recognized by the lectin become apparent in the apical membranes of enterocytes (e.g. arrow), as they emerge from the elongated crypt. (I) Distal jejunal crypt-villus unit from a normal chimeric mouse, stained as in H. The arrow points to Fucτ1,2Galβ glycans present in the apical membrane of a villus enterocyte. Bar in A 235 μm; in all other panels, 25 μm.
Rac1 affects intestinal epithelial differentiation

(73-85 cells/100 sectioned 129/Sv Rac1Asn17 crypts; 78-80 cells/100 sectioned B6-ROSA26 crypts). The number was also similar to values obtained from 129/Sv and B6-ROSA26 proximal jejunal crypts present in age-matched normal chimeras (82-84 M-phase cells/100 sectioned crypts/genotype). No M-phase cells were encountered in the 129/Sv-Rac1Asn17 or B6 villus epithelial compartments of polyclonal villi.

Fig. 9. The dominant negative Rac1Asn17 mutation inhibits epithelial differentiation and slows enterocytic migration. (A) Whole-mount preparation of X-gal stained proximal jejunum from a chimeric-transgenic mouse showing a polyclonal villus with coherent columns of juxtaposed 129/Sv and B6-ROSA26 cells. (B) Phloxine- and tartrazine-stained section of the proximal jejunum from a P42 Rac1Asn17 chimeric-transgenic animal. The arrow points to an intermediate cell located at the base of a 129/Sv villus. These cells contain apical tartrazine-positive granules (arrow) that are noticeably smaller than the granules of mature Paneth cells located at the base of crypts (arrowhead). (C) Section of jejunum from a Rac1Asn17 chimeric-transgenic mouse, incubated with rabbit anti-cryptdin-1, Cy3-conjugated donkey anti-rabbit Ig, and bis-benzimide. The entire area shown in this photograph was genotyped as 129/Sv, with X-gal. Intermediate cells express cryptdins (arrow) as do Paneth cells (arrowhead). (D) Staining with Helix pomatia agglutinin (HPA) discloses a marked loss of goblet cells in the 129/Sv component of this polyclonal villus (arrowheads indicate goblet cells). (E) HPA-stained section prepared from the jejunum of a normal chimera (age matched with the mouse in D). There are no appreciable differences in the number of goblet cells in the juxtaposed 129/Sv and B6-ROSA26 epithelium of this polyclonal villus. (F,G) Forced expression Rac1Asn17 in 129/Sv epithelial cells slows cell migration. A P42 Rac1Asn17 chimeric-transgenic mouse (F), and an age-matched normal chimera (G), were pulse labeled with BrdU and killed 48 hours later. Sections of their X-gal stained proximal jejenum were incubated with goat anti-BrdU, Cy3-donkey anti-goat Ig and bis-benzimide. In the normal chimeric mouse, B6-ROSA26 and 129/Sv epithelial cells, tagged with BrdU (red) in the crypt during S-phase, have migrated equivalent distances up the polyclonal villus during the 48 hour period (see arrowheads). In contrast, 129/Sv-Fabpl-Rac1Asn17 enterocytes have not moved as far up the polyclonal villus as enterocytes in the adjacent band of B6-ROSA26 epithelium. Bar in A, 235 μm; in all other panels, 25 μm.

Rac1Asn17 produces a slowing of cell migration

Recent studies of cultured rat embryo fibroblasts have shown that Rac1Asn17 inhibits protrusive activity/migration in a monolayer wound closure assay. The effect is correlated with the ability of Rac1Asn17 to reduce lamellipodia formation (Nobes and Hall, 1999). We were able to examine the effects of the dominant negative mutation on epithelial migration in vivo by surveying adjacent sections prepared from the same proximal jejunal polyclonal crypt-villus units that had been used to quantitate proliferation. We chose these proximal jejunal units because of their normal morphology. The lack of architectural complexity simplified our task of accurately assessing whether Rac1Asn17 altered cell movement.

Mice harboring these polyclonal villi had received a single intraperitoneal injection of BrdU 48 hours prior to being killed. Sections containing polyclonal villi were incubated with BrdU antibodies plus bis-benzimide (to visualize all nuclei). The positions of 129/Sv epithelial cells that had migrated the greatest distance up a given villus were scored relative to the positions of adjacent B6-ROSA26 cells that had migrated the greatest distance up the same villus. For example, if the leading 129/Sv-Rac1Asn17 cells had moved to a position that averaged 6.4 cell diameters below the leading B6-ROSA26 cells (mean values for each mouse = −6.5 and −6.3) (e.g. Fig. 9F). Intestinal epithelial cells move at a rate of 5-10 μm/hour (Heath, 1996). Therefore, a 6 cell-diameter difference should correspond to an approx. 6 hour time difference. Of course, we can not
formally rule out the possibility that Rac1Asn17 produces its effect on migration by delaying the initiation of upward movement, rather than by reducing the speed of movement.

Surveys of X-gal-stained jejunal whole mounts disclosed that the alteration in distance migrated per unit time was not accompanied by a perturbation in the orderliness of cell migration. Polyclonal villi were composed of coherent columns of wholly 129/Sv-Rac1Asn17 cells and wholly B6-ROSA26 cells. The borders between these columns were sharply demarcated and there was no intrusion of cells of one genotype into cellular columns of the opposite genotype (Fig. 9A).

Two control experiments were performed to help interpret the results of the migration assay. First, analysis of a comparable number of polyclonal villi from normal chimeras revealed no significant difference in the relative positions of the leading, BrdU-positive 129/Sv and B6-ROSA cells (mean values = −0.4 and −0.2, for the two mice analyzed; e.g. Fig. 9G). When migration is defined in this way, we found that the difference between the movement of normal 129/Sv and Rac1Asn17 129/Sv villus enterocytes was statistically significant (P<0.001). Second, although our quantitative assessment of proliferation in crypts supplying the polyclonal villi ruled out reduced cell production in 129/Sv-Rac1Asn17 crypts as a cause of reduced cell migration, we still had to rule out increased cell loss. Therefore, hematoxylin and eosin-stained sections that had been used to quantitate the number of M-phase cells in crypts were reviewed so that apoptosis could be scored using well defined morphologic criteria (Hall et al., 1994; Wong et al., 1998). The results established that Rac1Asn17 did not produce a significant change in basal apoptosis: there were 9-22 apoptotic cells per 100 sectioned Rac1Asn17 crypts versus 7-19 apoptotic cells per 100 sectioned (adjacent) B6-ROSA26 crypts.

Epithelial movement along the crypt-villus axis is viewed by some as a ‘passive’ process driven solely by mitotic pressure generated in the crypts (reviewed by Heath, 1996). Data obtained from these chimeric-transgenic mice indicate that intestinal epithelial cell movement may be an active process orchestrated, at least in part, by the epithelial cells themselves through Rac1-mediated pathways. Since Rac1Asn17 does not affect the orderliness of migration, it appears that the level of inhibition of Rac1 activity achieved in these chimeras results in a partial blockage of epithelial traffic flow without an associated disruption in the functional organization of the underlying ‘roadbed’.

DISCUSSION

Three observations described in this report provide in vivo evidence that Rac1 participates in a pathway that affects epithelial differentiation in the small intestine. First, in the normal developing and adult mouse, Rac1 is induced as post-mitotic cells execute their terminal differentiation program. Second, forced expression of a constitutively active Rac1 mutation induces precocious differentiation of members of the Paneth cell and enterocytic lineages. Third, forced expression of a dominant negative Rac1 results in an inhibition of epithelial differentiation. These effects on differentiation occur without an alteration in proliferation, and do not generalize to juxtaposed B6-ROSA26 epithelial cells.

Studies using cultured cells have assigned a variety of functions to the Rac subgroup of Rho GTPases. The challenge has been to determine which of these functions are expressed in specified cell populations in vivo and how function is affected by various physiologic, or pathophysiologic, states. The stem cell hierarchy of the self-renewing mouse small intestinal epithelium makes it particularly well suited for using genetic mosaic analysis to assess the in vivo role of Rac GTPases in regulating proliferation, differentiation and migration. Chimeric mice can be generated that contain easily identifiable and juxtaposed cohorts of genetically manipulated 129/Sv ES cell-derived and normal B6-ROSA26 blastocyst-derived cells. These cells can be compared and contrasted at various locations along the small intestine’s cephalocaudal and crypt-villus axes. By transfecting ES cells with gain-of-function or loss-of-function mutations under the control of regulatory elements that function at specified locations along these axes, it is possible to perform an internally controlled experiment which is restricted to a subset of the 129/Sv gut epithelium.

Activation of Rac in the intervillus epithelium of the fetal mouse gut represents the first reported example that we are aware of where precocious differentiation has been engineered in the proliferative compartment of the developing intestine without an accompanying general inhibition of the cell cycle. The one other report of precocious differentiation in the fetal gut involved mice homozygous for a null allele of the T-cell factor-4 gene (Korinek et al., 1998a). Tcf-4 encodes a HMG box transcription factor that mediates Wnt signaling in the small intestinal epithelium (Korinek et al., 1998b). Loss of Tcf-4 causes a complete block in proliferation in the intervillus epithelium. The intervillus epithelium of these knockout mice is populated with cells that have the same morphologic appearance as ‘differentiated’ post-mitotic villus epithelial cells (Korinek et al., 1998a).

Interestingly, Ramalho-Santos et al. (2000) have shown that Indian hedgehog (Ihh) is expressed in the intervillus region in E18.5 mice. Their analysis of E18.5 Ihh−/− fetuses indicate that loss of Indian hedgehog leads to a reduction in proliferation (manifested by reduced villus size) and a loss of differentiation (reduced numbers of enteroendocrine cells). These changes are not accompanied by detectable alterations in Tcf4.

In adult chimeras, the junction between the upper crypt and lower villus served as a very sensitive reporter of the effects of the dominant negative Rac1Asn17 on differentiation. As noted in Results, levels of Rac1 rise abruptly as epithelial cells pass through this junction, making them vulnerable to the impact of a dominant negative mutation. Inhibition of Rac1 appears to delay the differentiation of post-mitotic cells in the upper crypt, resulting in a marked elongation of crypts, and a distortion of villus architecture. Normally, as cells move from the upper crypt to the lower villus, their actin cytoskeleton undergoes reorganization and a variety of genes involved in terminal differentiation are induced. The ability of Rac1Asn17 to impede actin re-organization and to modulate gene transcription at the crypt-villus junction is consistent with the known effects of this molecule in cultured cells. The inability of Rac1Asn17 to produce sustained alterations in cell polarity as enterocytes complete their migration up the villus is likely due to the fact that levels of endogenous Rac1 normally increase as these cells exit the crypt.
Results obtained from in vitro or in vivo experiments using dominant negative mutations are subject to questions about whether the level of expression was sufficient to fully interfere with the function of the endogenous protein, and whether the effect of the mutation was restricted to the target protein of interest. Despite these caveats, the results of expressing the dominant negative Rac1Asn17 complement the results obtained with the constitutively active Rac1Leu61: they both enforce the idea that Rac1 signaling is critical for programming epithelial differentiation.

Our findings support the notion that expression of Rac1 mutations perturbs the proper entry and/or processing of (unspecified) extracellular cues that normally help define the state of epithelial differentiation in vivo. This idea is consistent with the formulation that differentiation of intestinal epithelial cells is regulated in large part by cell non-autonomous mechanisms, and that position-dependent cues are received during the course of cellular migration along crypt-villus units (see Introduction). We do not know whether the constitutively active or dominant negative Rac1 perturbs receipt or processing of signals imported from the mesenchyme underlying the small intestinal epithelium, or from adjacent epithelial cells. We do know from the chimeric system that the effect of the constitutive active mutation on differentiation is not 'exported' to juxtaposed normal B6-ROSA26 cells. This suggest that the protein acts directly within expressing cells, and not by re-sculpting the local extracellular environment so as to generate instructions sufficient to enforce differentiation of non-expressing cells.

We believe that the real challenge in understanding the molecular mechanisms involved in Rac1-mediated regulation of intestinal epithelium is to first identify upstream components of its signaling pathway(s). Numerous in vitro studies of cultured cell lines have emphasized how extracellular context (milieu) can influence cellular responses to Rac GTPase-mediated pathways (e.g. Sander et al., 1998). The intestine establishes and maintains a remarkable degree of spatial diversity in its structure and function. In such a system, it is critical that Rac1-mediated signaling be considered in the context of where epithelial cells are in space and time. Therefore, it will be important to define how potential upstream signaling molecules, derived from mesenchymal (Kaestner et al., 1997), and/or epithelial populations (Lefevre et al., 1999), or the microflora (Hooper et al., 1999), are distributed along crypt-villus units.

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REFERENCES


