Abstract. The mechanisms underlying downregulation of the cadherin/catenin complexes and α-catenin signaling during tumor progression are not fully understood. We have analyzed the effect of oncogenic H-Ras on E-cadherin/catenin complex formation/stabilization and β-catenin distribution in epidermal keratinocytes. Microinjection or stable expression of V12Ras into keratinocytes promotes the loss of E-cadherin and α-catenin and relocalization of β-catenin to the cytoplasm and nucleus. Moreover, these effects are dependent on PI3K (phosphoinositide 3-OH kinase) activity. Interestingly, a strong association of p85α and p110α subunits of PI3K with β-catenin is induced in V12Ras-expressing keratinocytes, and in vitro binding assays show a direct interaction between β-catenin and p85α. Overexpression of either V12Ras or constitutively active p110α induces metabolic stabilization of β-catenin and promotes its accumulation in cytoplasmic and nuclear pools. In addition, the interaction of β-catenin with the adenomatous polyposis coli protein is blocked in V12Ras and p110α transformants though no changes in glycogen synthase kinase 3 β activity could be detected. Nevertheless, in V12Ras as transformants the in vivo phosphorylation of β-catenin in Ser residues is strongly decreased. These results indicate that H-Ras activation induces the relocalization and cytoplasmic stabilization of β-catenin by a mechanism involving its interaction with PI3K.

Key words: H-Ras • E-cadherin • β-catenin • adenomatous polyposis coli • phosphoinositide 3-OH kinase

Cell-cell adhesion mediated by the calcium-dependent cadherin receptors is fundamental for the differentiation and integrity of most adult tissues. Among the different members of the cadherin superfamily, the epithelial E-cadherin plays a pivotal role in the acquisition and maintenance of embryonic and adult epithelia (Takeichi, 1993; Gumbiner, 1996). E-cadherin is organized in adhesion complexes at the lateral membranes of neighboring cells. The receptor associates with cytoplasmic proteins of the catenin family, α-, β-, and γ-catenin (identical to plakoglobin), which in turn mediate the interaction of the complexes with the cortical actin microfilament network (Kemler, 1993; A. Berle et al., 1996). β-catenin and plakoglobin directly bind the cytoplasmic domain of E-cadherin, whereas α-catenin interacts with the other two catenins establishing the link with the actin microfila-

ments (Hinck et al., 1994; Nagafuchi et al., 1994; Jou et al., 1995; Knudsen et al., 1995; Rimm et al., 1995). A novel catenin, p120cas, has been described in some E-cadherin/catenin complexes but apparently with a weak interaction with α-catenin (Reynolds et al., 1994; Daniel and Reynolds, 1995). Recent studies have clearly established that β-catenin has an important signaling activity besides its role in the cadherin-mediated adhesion complexes. β-catenin is homologous to the armadillo protein of Drosophila, and together with other members constitute the arm protein family (Peifer et al., 1992, 1994). An armadillo/β-catenin are downstream effectors of the Wingless/Wnt signaling pathway (for review see Gumbiner, 1995; Miller and Moon, 1996; Willert and Nusse, 1998). Their activity is mediated by the pool of soluble molecules and appears to be independent of their role in adhesive complexes (Orsulic and Peifer, 1996).

In normal resting cells, the cytoplasmic β-catenin levels are very low and are regulated by interaction with the ade-
nomatous polyposis coli (APC) product and by the up-
stream effectors of the Wnt signaling pathway, glyco-
synthetase kinase 3β (GSK 3β) and axin (M unemitsu et al.,
1995; R ubinfield et al., 1996; Y ost et al., 1996; I keda et al.,
1998; Sakanaka et al., 1998). These proteins probably di-
rect the proteolytic degradation of β-catenin by the ubiqui-
tin-proteasome pathway (A berle et al., 1997; O rford et
al., 1997). Wnt signaling inhibits the activity of GSK 3β
(Cook et al., 1996) and leads to the stabilization of cyto-
plasmic hypophosphorylated β-catenin, which is able to in-
teract with lymphocyte enhancer factor 1/T-cell factor
(Lef-1/Tcf) transcription factors and the complex can act
as a transcriptional coactivator in the nucleus (Behrens et
al., 1996; H uber et al., 1996; M olenaar et al., 1996; P apkoff
et al., 1996). Other studies have also reported that β-cate-
nin can be translocated to the nucleus independent of its
interaction with Lef-1/Tcf transcription factors (F agotto et
al., 1998; P rieve and W aterm an, 1999).

It has been recently shown that activation of β-catenin
signaling occurs in intestinal and mammary cells after acti-
vation of the integrin-linked kinase, independent of Wnt
activation but also involving downregulation of GSK 3β
activity (N ovak et al., 1998; D emoccomm enne et al., 1998).
Significantly, the interaction of β-catenin and APC is dis-
turbed in colon carcinoma and melanoma cell lines with
mutations in the interacting regions of either molecule
(K orinek et al., 1997; M orin et al., 1997; R ubinfield et al.,
1997). Mutations in the serine/threonine residues suscepti-
ble of phosphorylation and interaction with APC in the β-
catenin gene have also been found in a variety of human carci-
nomas (M iyoshi et al., 1998; P alacios and G amallo,
1998; V oeller et al., 1998), in some cases associated with
extensive nuclear localization of β-catenin (Palacios and G amallo,
1998). These observations have fostered an in-
creasing interest in the role of β-catenin in tumor progres-
sion regarding the possibility of its functioning as an onco-
gene (P efeler, 1997).

Oncogenic transformation frequently results in alter-
ations of the epithelial properties, including loss of pol-
ardized morphology, less organized cell junctions, and
increased migration of transformed epithelial cells (V lemm
inxk et al., 1991; B ehrens et al., 1993; K inch et al., 1995;
Z hong et al., 1997). In some reports, changes in the E-
cadherin/catenin complexes were associated to a decreased
interaction of the complexes with the cytoskeleton and an
increase in tyrosine phosphorylation of β-catenin and/or
association with p120cas (Behrens et al., 1993; K inch et al.,
1995).

Recently, R as activation has been shown to induce the
destabilization of E-cadherin/catenin complexes in
M DCK cells by mechanisms involving both phospho-
nositide 3-OH kinase (PI3K) and mitogen-activated pro-
tein kinase (M A PK) effector pathways (P ottempa and R id-
ley, 1998). R as activation is a frequent genetic alter-
ation in human and experimental tumors (B arbadic, 1987), but it is
presently unknown whether it is involved in β-catenin sig-
naling during tumor progression. The experimental model
of mouse skin carcinogenesis offers a unique system to in-
vestigate this important issue as H arvey-R as (H -R as) acti-
vation is a frequent genetic event at the initiation stage
(B rown et al., 1986; Q uitanilla et al., 1986). Further alter-
ations in the ras locus, leading to an increase in the dosage
of activated H -R as, accumulate during progression of pap-
illomas to invasive carcinomas (B ianchi et al., 1990; B rem-
ner and B almain, 1990). Downregulation of E-cadherin
expression also occurs at this stage during mouse skin tu-
mor progression (N avarro et al., 1991) and alterations in
the integrity of the E-cadherin–mediated cell–cell adhe-
sion have been found in high risk progressing papillomas
(C ano et al., 1996).

To get further insights into the role of R as activation in
tumor development, we have investigated the effects of ac-
tivated H -R as on the organization of E-cadherin/catenin
complexes and on β-catenin localization in mouse epider-
mal keratinocytes. Here we show that activated H -R as al-
ters the distribution of β-catenin in epidermal kerati-
ocytes in the membrane-bound and cytoplasmic/nuclear
pools in a PI3K-dependent manner. Our results indicate
that R as signaling interferes with the β-catenin–A PC in-
teraction and promotes the interaction of soluble β-cate-
nin with PI3K and the metabolic stabilization of cytoplas-
mic β-catenin pools.

Materials and Methods

Cells and Retroviral Infections

M ouse Pam212 keratinocytes, an immortalized cell line spontaneously de-

erived from a primary keratinocyte culture (Y usa et al., 1980) and GP + E
68 cells producing retroviruses that express either V 12H -R as or the cata-
lytic subunit of PI3K targeted to the plasma membrane (p110CAAX)
were grown in DM E supplemented with 10% FCS (G IBICO B RL), 100
μg/ml ampicillin, 32 μg/ml gentamicin, and 100 μg/ml amphotericin B
(Sigma Chemical Co.). Cells were grown at 37°C in a humidified 5% CO2
atmosphere. Retroviruses encoding V 12H -R as with a neomycin resist-
ance marker or controls with the neomycin resistance marker alone have been
previously described (R odriguez-V iciana et al., 1997). R etroviruses encod-
ing p110CAAX was generated by subcloning the BamHI insert from
pSG5-p110CAAX into pL X SP3. pL X SP3 was a gift from A. Sewing
(Imperial Cancer Research Fund, London, U.K.) and was generated by ligating
the HindIII-XbaI fragment of pBabe puro, containing the puromycin resis-
tance gene, into pL X SN. Infective retroviruses were obtained by grow-
ing GP + E 86 cells to confluence and collecting the culture medium. Su-
pernatants were filtered through a 0.45-mm pores and frozen as aliquots in
the presence of polybrene. R etrovirus-containing supernatants were
added to subconfluent cultures of Pam212 cells and replaced 16 h later
with fresh medium. Pools of infected cells were obtained after selection
with 400 μg/ml G 418 (Calbiochem-N ovabiochem Co.) or 1.5 μg/ml puro-
mycin (Boehringer Mannheim) for 7 d, during which time all control non-
infected cells died. M ore than 200 colonies were pooled from each infec-
tion.

Antibodies

The following antibodies were used: rat monoclonal anti-mouse E-cad-
herin (E CCD-2; a gift from D r. M. T akiechi, K yoto U niversity, K yoto, J a-
pan); mouse monoclonal anti-β-catenin, mouse monoclonal anti-α-cate-
nin, mouse monoclonal anti-GSK 3β, and mouse antiphosphotyrosine
(F Y 20) (Transduction Laboratories); mouse monoclonal anti-c-H-Ras
(A b-1) and mouse monoclonal anti-pan R as recognizing the normal and the activated forms of H-R as (O ncogene Science, Inc.); mouse monoclonal
anti-H-6 His (C L ONTECH Laboratories); rabbit anti-GSK 3β-P Ser9 anti-
silum (Chemicon International Inc.; provided by D r. F. W andosell, Cen-
tro de Biología Molecular, M adrid, Spain); rabbit polyclonal anti-GST
(Sigma Chemical Co.); rabbit antipeptide antisemur anti-α-catenin (V B1)
and rabbit antipeptide antiserum anti-β-catenin (V B2) (Braga et al., 1995); rabbit polyclonal anti-A-PC (N-15) (Santa Cruz Biotechnology, Inc.); rabbit polyclonal anti-Pi3 kinase p85 and anti-Pi3 kinase p110α (Upstate Biotechnology); and mouse monoclonal anti-β-tubulin (A mer- sham). Secondary antibodies included: BODIPY-conjugated goat-anti-rat IgG, anti-mouse IgG and anti-rabbit IgG (Molecular Probes Inc.); A MCA-conjugated rabbit-anti-rat IgG, Cy5-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and peroxidase-conju- gated sheep anti-rat IgG, anti-mouse IgG and anti-rabbit IgG (A mer- sham).

Recombinant Proteins and Microinjection

Recombinant constitutively active Ras (V 12 R as), dominant negative Ras (N 17 R as), C3 transferase and dominant negative Rac (N 17 R ac) were prepared as glutathione-S-transferase (GST) fusion proteins in E scherichia coli, purified using glutathione-Septarose beads, thrombin cleaved, dia- lyzed, and concentrated as described (Ridley et al., 1992). The activity of each batch of recombinant proteins was tested in fibroblasts and keratini- nocytes as described (Braga et al., 1997). Recombinant GST–β-catenin fusi- on protein was generated by isolation of full-length human β-catenin cDNA from plasmid pQE 32 (a gift of D. J. B. Brown) by SmaI-direct ion and ligation into pGEX 5X-2 plasmid (Pharmacia Biotech). Recom- binant His 8 ps 8 wt fusion protein was produced in COS cells as described (Rodriguez-Viciana et al., 1997) and purified with His-Trap columns (Pharmacia Biotech) following the instructions of the manufacturer. Con- fluency-dependent (C) recombinant α-κatenin were essentially as described (Braga et al., 1997) and visualized by mixing the recombinant proteins with dextran conjugated to Texas red (mol wt 10,000; Sigma Chemi- cal Co.).

Immunostaining

A fter microinjection, cells were fixed in either cold methanol or 3.7% buffered formaldehyde for 5 min at 4°C and rinsed in PBS. Staining with the different antibodies was performed as described (Lozano and Cano, 1998). In extraction experiments, cells grown on coverslips were treated with NT buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 5 mM MgCl2, 5 mM CaCl2, 1% NP-40, 1% Triton X-100) for 15 min on ice before fixation and staining. Preparations were viewed either by confocal microscopy or in an A xiphot photomicroscope (Carl Zeiss). Confocal images were obtained using a laser scanner (MRC 1024; BioRad) attached to a Nikon micro- scope (O ptiphoto 2). Pictures were processed using the A dobe Photoshop 4.0 software (A dobe Systems, Inc.).

Preparation of Cell Extracts

Whole cell extracts were obtained from F-25 flasks of 80–90% confluent cells. For total protein extracts, cells were washed twice in cold M F · C a buffer (10 mM Hepes, pH 7.4, 100 mM NaCl, 10 mM CaCl2) and extracted in 1 ml of 5 buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 5 mM MgCl2, 5 mM CaCl2, 2% SDS) containing protease and phosphatase inhibitors (2 mM PM SF2, 20 μg/ml aprotinin, 1 mM N-ethylmaleimide, 1 mM sodium orthovanadate, 2 mM hydrogen peroxide) for 5 min at room temperature under continuous agitation. Soluble and insoluble fractions were obtained as previously described (Lozano and Cano, 1998) using the NT buffer con- taining the inhibitors described above.

Immunoprecipitation and Western Blotting

Immunoprecipitations of soluble fractions were carried out with different antibodies as previously described (Lozano and Cano, 1998) and analyzed in 7.5% or 12% S D · P A G E gels. In P-Tyr immunoprecipitations, con- trols were performed by adding 5 μg P-Tyr or P-Ser (Sigma Chemical Co.) to soluble fractions. Immunoblotting of the different fractions and im- munoprecipitates was performed by transferring the gels to Immobilon P membranes (Millipore Corp.), incubation with the appropriate antibodies, and development with an enhanced chemiluminescent kit (A mershaw).

Pulse-Chase Analysis of β-Catenin

Cells were grown in F-25 flasks to 80% confluence in normal growth me- dium, washed three times in H M F · C a buffer, and pulse-labeled for 1 h in 1 ml of methionine- and glutamine-free minimal essential medium (GIBCO BRL) supplemented with 4 mM glutamine; 10% FCS and 100 μCi [35 S]methionine-cysteine (trans-label; A mershaw; 100 Ci/mmol). La- beled cells were rinsed three times in normal growth medium containing an excess of cold methionine (0.15 mg/ml), chased in this medium for the indicated times, and the soluble extracts subjected to β-catenin immuno- precipitation. The labeled immunoprecipitates were resolved in 7.5% S D · S · P A G E gels, transferred to Immobilon P membranes, and exposed to autoradiography. Bands corresponding to β-catenin were identified by im- munoblotting. 35 S-labeled β-catenin detected at the different experimental points was quantified by scanning and digitalization of the autoradiograms with a A dobe Photoshop 4.0. 4000 NIH Image 1.62f soft- ware. The integrated density obtained at the different times was normal- ly distributed to those obtained at time 0 h in each experiment.

In Vivo Phosphorylation and Phosphoamino Acid Analysis of β-Catenin

M etabolic labeling of keratinocytes with [32 P]orthophosphate was per- formed as previously described (Pérez-M eno et al., 1998) with slight modifications. 20 h after plating the cells, monolayers were labeled in me- dia without phosphate supplemented with 500 μCi/ml of [32 P]orthoph- osphate (acid-free, 5 μCi/ml; D u Font Company; N EN L ife Science Prod- ucts) for 4 h at 37°C in a C02 incubator. A fter labeling, cells were washed twice with PBS and lysed with NT buffer, containing a cocktail of phos- phatase (1 mM sodium orthovanadate, 50 mM sodium fluoride, and 2 mM hydrogen peroxide) and protease inhibitors for 30 min at 4°C with gentle rotation. Extracts were prepared as described, immunoprecipitated with anti-β-catenin antibodies (Transduction Laboratories), and resolved on 7.5% S D · S · P A G E gels. Protease and phosphate inhibitors were main- tained during all the immunoprecipitation procedures. Total [32 P]phospho- phate labeling was detected by autoradiography, and the total β-catenin protein was determined in the same gel by immunoblotting. 32 P-labeled and total β-catenin were quantified by scanning and digitalization of the autoradiograms and blots as described.

Phosphoamino acids were analyzed based on the method of B iyle et al. (1993) with minor modifications. 32 P-labeled phosphorilated β-catenin bands were excised from membranes and hydrolyzed with 6 N HCl at 110°C for 2 h. The hydrolysates were lyophilized using a speed-vac con- centrator and resuspended in 10 μl of pI 8.0 buffer (2.2% formic acid, 7.8% acetic acid, pI 1.9) containing 2 μg each of cold phosphoamino acid standards. The samples were spotted onto thin-layer cellulose plates (5716, M erck) and amino acid sequencing performed on an apparatus (H TLE-7000; C BS Scientific Co.) running at 1,500 V for 45 min in the first dimension with the pI 1.9 buffer. The second dimension thin layer chro- matography was carried out in a mixture of n-buthanol/acetic acid/H2O (100:22:50). The individual amino acids were identified by comparison with the internal standards detected by ninhydrin staining. The position of 32 P-labeled phosphoamino acids were determined by autoradiography.

GSK3β Activity

A citivity of GSK 3β was tested in the absence and presence of 50 mM lith- ium chloride in crude cell extracts prepared following the procedure re- cently described (R yes et al., 1998) and using the specific phosphopep- tide GST SM (R R P A SA V F P P S P S L R H S H O Q R R , where S indicates a phosphoserine introduced during synthesis) (a gift of D r. A. J.) Harwood, M R C Laboratory for Molecular Cell Biology, London, U K. ). The differ- ence between the activity obtained in the absence and presence of lithium chloride was represented as the specific GSK 3β activity.

Pull-down and In Vitro Protein Binding Assays

Pull-down assays were performed using soluble extracts obtained in NT buffer after preclearing with glutathione-Sepharose 4B (Pharmacia Bio- tech). 200 μl of the precleared extracts were incubated with 100 μl of puri- fied GST–β-catenin fusion protein (0.2 μg/ml) for 2 h at 4°C. For in vitro protein binding assays, 200 ng of both GST–β-catenin and His 8 ps 8 wt were mixed in 500 μl of immunoprecipitation buffer and incubated 1 h at 4°C. In all cases, interacting β-catenin–GST complexes were collected by addition of glutathione-Sepharose 4B, washed twice with immunoprecipita- tion buffer, and finally resuspended in L aemmli sample buffer. Complexes were analyzed in 7.5% S D · S · P A G E gels and immunoblotted with the indicated antibodies.

Reporter Gene Assay

Nearly subconfluent cells were transfected in duplicate in P-60 dishes with 4 μg of the pR SV-40lacZ containing a β-galactosidase reporter gene and 4
μg of either pTOPFLASH or pFOPFLASH containing multimerized wild-type or mutated Lef-1/Tcf binding sites, respectively, and a luciferase reporter gene (Korinek et al., 1997) (a gift of Dr. H. Clevers, University Hospital, Utrech, The Netherlands). Transfection was performed with lipofectamine plus (GIBCO BRL). Luciferase and β-galactosidase activities were measured 24 h after transfection.

**Results**

**Microinjection of Keratinocytes with Dominant Active H-Ras Induces Loss of E-Cadherin and α-Catenin and Relocalization of β-Catenin to the Cytoplasm**

To analyze short-term effects of activated H-Ras on the E-cadherin/catenin complexes, we chose the murine epidermal keratinocyte cell line Pam212, obtained after spontaneous immortalization of a primary keratinocyte cell culture (Yuspa et al., 1980). This cell line maintains all the epidermal characteristics of keratinocytes, expresses a normal H-Ras protooncogene, and is nontumorigenic when injected into athymic nude mice (Missero et al., 1991; Sánchez-Prieto et al., 1995). The dominant active form of H-Ras (V12Ras; 0.5 μg/μl) protein was microinjected in confluent Pam212 keratinocytes showing stable cell–cell contacts, and the cells were fixed and stained for different proteins after 1–24 h of incubation. As shown in Fig. 1, 2–4 h after microinjection E-cadherin (Fig. 1 b) and α-catenin

Figure 1. Microinjection of V12Ras in Pam212 keratinocytes induces dismantling of E-cadherin/catenin complexes and the cytoplasmic relocalization of β-catenin. Confluent patches of Pam212 cells were microinjected with V12Ras (0.5 μg/μl) (a–h) or N17Ras (0.5 μg/μl) (i–k), and after 2–4 h fixed in 3.7% formaldehyde. Cells were stained for E-cadherin (b and j), α-catenin (d), and β-catenin (f, h, and k). Microinjected cells were identified by coinjection of dextran–Texas red (a, c, e, g, and i). Cells in c–f were fixed after 2 h of microinjection and the rest of cells after 4 h of microinjection. V12Ras microinjection induces a strong cytoplasmic staining and occasional nuclear localization of β-catenin, whereas E-cadherin and α-catenin disappear from cell–cell contacts on microinjected and adjacent cells. Images in a–h were obtained by confocal laser microscopy and those in i–k in an Axiophot microscope. Bars, 10 μm.
and/or to specific effects of the extracellular matrix (San-
sases because of the cellular context (Braga et al., 1999)
sponse of cadherin receptors to inhibition of small GTP-
1997). These results might reflect a modulation on the re-
fecting cell–cell contacts (data not shown), in contrast to
attachment of the cells from the dish within 2 h without af-
remained absent from the cell–cell contacts and
microinjection in Pam212 cells, E-cadherin and
compared with control Neo cells (Fig. 3 a). E-cadherin and β-catenin
found in both the soluble and insoluble fractions in Neo
cells, whereas in Ras-expressing cells, most of the E-cad-
herin and β-catenin and an increased proportion of α-cate-
nin were detected in the soluble fraction. Quantification of the
data obtained in three independent experiments indicated
that 8% and 11% of E-cadherin and β-catenin, re-
spectively, were detected in the insoluble fractions of Ras
cells. In contrast, in control Neo cells, 39% of E-cadherin
and 29% of β-catenin were associated to the insoluble
fraction. In addition, the distribution in soluble and insol-
ble fractions of a control protein, β-tubulin, was not dis-
turbed upon V12Ras as overexpression (Fig. 3 a). These
results confirm that the components of the E-cadherin
complexes were weakly associated to the actin cytoske-
ton in the Ras-expressing cells.

Immunoblotting analysis of the distribution of the dif-
ferent components of the E-cadherin/catenin complexes
into the detergent-soluble (S) and insoluble (I) fractions of
control neomycin (Neo)- and V12Ras (Ras)-transduced
cells is shown in Fig. 3 a. E-cadherin and β-catenin were
found in both the soluble and insoluble fractions in Neo
cells, whereas in Ras-expressing cells, most of the E-cad-
herin and β-catenin were associated to the soluble fraction.

**Destabilization of E-Cadherin/Catenin Complexes Induced by V12Ras Depends on PI3K and Is Independent of β-Catenin Tyrosine Phosphorylation**

Previous studies have implicated PI3K and MAPK activi-
ties in dismantling E-cadherin/catenin complexes induced by
activated V12Ras in other systems (Potempa and Rid-
ley, 1998; Sander et al., 1998). In murine keratinocytes, the
involvement of PI3K in the destabilization of E-cadherin/
catenin complexes induced by activated H-Ras was con-
firmed by microinjection analysis in the presence of spe-
cific inhibitors. Preincubation of Pam212 cells during 30
min with the PI3K inhibitor wortmannin at 200 nM,
blocked the effect of V12Ras on the adherens junctions
(Fig. 2 a).

To further investigate the effect of activated Ras as and
the involvement of PI3K in the destabilization of the E-cad-
herin/catenin complexes, we generated stable transfec-
tants of Pam212 cells expressing H-V12Ras as by retroviral
transduction. Cells were pooled from three independent
infection assays, which showed similar results. The solubili-
ty of E-cadherin/catenin complexes in control PamNeo
(Neo)- and V12Ras-overexpressing (Ras) cells was first
analyzed by immunostaining after detergent extraction
of the cells. A series of experiments showed that E-cadherin
and β-catenin were preserved at cell–cell contacts of control
Neo cells after treatment with NT buffer (Fig. 2 b, Neo). Both proteins
were removed from cell–cell contacts by the detergent
in H-Ras as cells (Fig. 2 b, Ras), indicating that
H-Ras activation promotes solubilization of the cadherin
complexes. This solubilization was prevented by preincu-
bation of Ras cells with wortmannin during 1 h (Fig. 2 b,
Ras-i-W), further supporting the involvement of PI3K ac-
tivity in H-Ras-induced destabilization of the adhesion
complexes.

Immunoblotting analysis of the distribution of the dif-
gerent components of the E-cadherin/catenin complexes
into the detergent-soluble (S) and insoluble (I) fractions of
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**Interaction of β-Catenin with PI3K Is Strongly Induced in Ras-transformed Keratinocytes**

To get further insights into the H-Ras-induced cytoplas-
mic relocalization of β-catenin, we analyzed the participa-
tion of PI3K. Cell extracts obtained from control (Neo)
and PamV12Ras (Ras)-transduced cells were immuno-
precipitated with antibodies against the regulatory subunit
of PI3K (p85α), and the presence of β-catenin was ana-
yzed by immunoblotting. A series of experiments showed that E-cadherin
and β-catenin were preserved at cell–cell contacts of control
Neo cells, whereas in Ras-expressing cells, most of the E-cad-
herin and β-catenin were associated to the soluble fraction.
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ton in the Ras-expressing cells.
reverse experiment, immunoprecipitation with anti-\(\beta\)-catenin antibodies and immunoblotting with anti-p85\(a\) (Fig. 4 a), also showed an increase in \(\beta\)-catenin/p85\(a\) association in Ras cells. In addition, the strong association of \(\beta\)-catenin with the PI3K complex in Ras cells was also detected when the p110\(a\) catalytic subunit was immunoprecipitated (Fig. 4 a). Interestingly, the interaction of p110\(a\) with its regulatory subunit, p85\(a\), was also significantly increased in Ras cells.

The interaction of \(\beta\)-catenin with PI3K was confirmed in pull-down experiments with recombinant GST-\(\beta\)-catenin. As shown in Fig. 4 b, an excess of recombinant GST-\(\beta\)-catenin interacts similarly with endogenous p85\(a\) derived from protein extracts of Neo and Ras cells. These results suggest that endogenous p85\(a\), from either control or V12R as cells, has the same ability to associate with recombinant \(\beta\)-catenin. The quantitative differences found in the in vivo \(\beta\)-catenin-p85\(a\) interaction might reflect different properties of endogenous \(\beta\)-catenin in control and Ras-expressing cells. GST-\(\beta\)-catenin recombinant protein also showed a strong interaction with E-cadherin and \(\alpha\)-catenin in the pull-down assays (Fig. 4 b, and data not shown), in-

Figure 2. Inhibition of PI3K activity prevents E-cadherin/catenin complexes disassembly induced by V12R as. (a) Confluent patches of Pam212 cells were preincubated with the PI3K inhibitor wortmannin (200 nM) for 30 min, microinjected with V12R as (0.5 \(\mu\)g/\(\mu\)l), and after 3 h, fixed in methanol. Cells were stained for E-cadherin, and microinjected cells were identified by coinjection of dextran-Texas red. (b) Control PamNeo (top, Neo) or PamV12R as (middle and bottom, Ras) cells were extracted in NT buffer for 15 min on ice, fixed in 3.7% formaldehyde, and stained for E-cadherin (left) or \(\beta\)-catenin, using mouse mAb (right). PamV12R as cells showed in the bottom were preincubated with the PI3K inhibitor wortmannin (Ras+W) for 1 h before NT buffer extraction. The nuclear staining detected with \(\beta\)-catenin is background staining because of nonspecific binding of the BODIPY-conjugated anti-mouse IgG observed after detergent extraction. Both E-cadherin and \(\beta\)-catenin are redistributed to the detergent soluble fraction in V12R as-expressing cells and this solubilization is blocked after inhibition of PI3K activity in those cells. Images were obtained in an Axiophot microscope. Bars, 10 \(\mu\)m.
indicating the functionality of the protein in in vitro interactions. A direct interaction of β-catenin with the regulatory subunit of PI3K, p85α, was further demonstrated in in vitro binding assays using recombinant GST-β-catenin and His-p85α wt fusion proteins (Fig. 4 c). Taken together, these results indicate the ability of β-catenin to associate with PI3K in Pam212 keratinocytes. This interaction is significantly increased in V12Ras-overexpressing cells, where

200 ng of recombinant GST-β-catenin fusion protein in immunoprecipitation buffer. Complexes were affinity-purified with glutathione-4B-Sepharose beads (AP:GST) (middle and right lanes) and detection of β-catenin and p85α wt was performed by immunoblotting with anti-GST (GST) (top) and anti-6-His (His) antibodies (bottom), respectively. As an internal control, the presence of His-p85α wt in the precipitation buffer was tested before the addition of GST-β-catenin (left lanes).
both the regulatory and catalytic subunits of PI3K form part of this novel β-catenin complex.

**Activated H-Ras Blocks the Interaction of β-Catenin with APC and Decreases β-Catenin–P-Ser Content**

The above results prompted us to examine the interaction of β-catenin with other known partners in Neo versus Ras cells. Because of the relevance of the β-catenin–APC interaction in the regulation of cytoplasmic β-catenin levels, we investigated whether activated H-Ras could influence such interaction. High levels of soluble β-catenin coprecipitated with APC in control Neo cells (Fig. 5 a, IP: APC, lane Neo), whereas very low levels of β-catenin were detected in the APC immunocomplexes of PamV12Ras as cells (Fig. 5 a, IP: APC, Ras lane), even though the level of APC was similar in both cell types (Fig. 5 a, lysates). On the other hand, similar levels of GSK3β protein were de-

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**Figure 5.** Stable expression of V12Ras in Pam212 keratinocytes inhibits β-catenin–APC interaction, but does not induce stable interaction of β-catenin with GSK3β or affect GSK3β activity. (a) Soluble extracts from PamNeo (Neo) and PamV12Ras (Ras) cells were immunoprecipitated with anti-APC antibodies and subjected to immunoblotting for β-catenin (bottom, IP: APC). As a control of the APC level, the extracts were immunoblotted with anti-APC antibodies (top, lysates). (b) Soluble extracts from PamNeo (Neo) and PamV12Ras (Ras) cells were immunoprecipitated with anti-β-catenin (mouse mAb) (middle, IP: β-catenin) or anti-GSK3β (bottom, IP: GSK3β) and subjected to immunoblotting for β-catenin, GSK3β, and P-SerGSK3β, as indicated. As a control, total cell extracts from Neo and Ras cells (top, lysates) were also included in the immunoblotts. (c) GSK3β activity was assayed in crude cell extracts of control PamNeo (Neo) and PamV12Ras as cells from three independent infections (Ras1, Ras2, and Ras3) in the presence and absence of 50 mM lithium chloride using the specific peptide G5M as substrate. The specific activity is represented as the percentage of the difference between the values obtained in the absence and presence of lithium chloride in each cell line. Samples were analyzed in duplicate, and the results show the average value and SD. (d) Lef-1/Tcf–dependent transcriptional activity was measured in control PamNeo (Neo) and PamV12Ras (Ras) cells using pTOPFLASH (Top) and pFOPFLASH (Fop) reporter vectors containing multimerized wild-type (Top) or mutated (Fop) Lef-1/Tcf binding sites. Assays were performed in duplicate samples, and the results show the average value and SD.
To investigate whether the disturbed interaction of β-catenin with APC induced by activated H-Ras was due to inhibition of GSK3β, we analyzed its activity in crude cell extracts from control PamNeo and PamV12Ras cells. Ras-transformed cells from three independent infection assays (Ras1, 2, and 3) showed a level of GSK3β activity sensitive to lithium chloride similar or slightly higher to that of control cells (Fig. 5c). In addition, endogenous phosphorylation of GSK3β at Ser9 in Ras-expressing cells was slightly higher than in control Neo cells (Fig. 5b).

These results indicate that the overall activity of GSK3β in Pam212 cells was not significantly decreased by overexpression of activated H-Ras. Nevertheless, a slight increase of about twofold in basal Lef-1/Tcf–dependent transcriptional activity was observed in Ras-transformed cells (Fig. 5d), suggesting a weak activation of β-catenin signaling in these cells.

To further analyze the status of β-catenin in control and Ras-transformed keratinocytes, we performed in vivo phosphorylation analysis on both cell types after 4 h of metabolic labeling. The level of β-catenin phosphorylation in PamV12R as cells resulting from two independent infections, Ras1 and Ras2, was significantly lower than that of parental Pam212 cells (Fig. 6a).

Quantitative analysis of the phosphorylated and total immunoprecipitated β-catenin indicated that the ratio of [32P]β-catenin in Ras1 and Ras2 keratinocytes was 6% and 17% relative to that of parental cells. In addition, the phosphoamino acid analysis of immunoprecipitated β-catenin showed that P-Ser was the major phosphorylated amino acid, and, as expected, the relative P-Ser content of β-catenin in Ras-transduced cells was much lower than that of parental cells (Fig. 6b). The P-Tyr content of β-catenin could not be detected in the phosphoamino acid analysis, probably because of the lower stability of P-Tyr residues to acid hydrolysis (Duclos et al., 1991) or to a low level of P-Tyr labeling in the 4-h pulse, in contrast to the steady state levels detected in the immunoprecipitation analysis (Fig. 3c). These results indicate that activated H-Ras induces cytoplasmic accumulation of hypophosphorylated β-catenin and inhibits its interaction with APC.

![Figure 6](image-url)
Figure 7. Expression of constitutively active PI3K inhibits β-catenin–APC interaction and induces cytoplasmic and nuclear accumulation of β-catenin. (a) Soluble extracts from control PamPuromycin (Puro)- and p110CAAX-overexpressing cells from two independent infections (p110S1 and p110S2) were immunoprecipitated with anti-APC antibodies and subjected to immunoblotting for β-catenin (bottom, IP: APC). As control of the APC and β-catenin levels, the extracts were also immunoblotted with anti-APC (top, lysates) and anti-β-catenin (middle, lysates) antibodies. (b) Stable overexpression of V12Ras and p110CAAX induces cytoplasmic and nuclear accumulation of microinjected GST–β-catenin. Confluent patches of PamV12Ras (Ras), Pam110CAAX (p110) and Pam212 (Pam) cells were microinjected with GST–β-catenin protein (1 μg/μl) and after 4 h fixed in cold methanol. Cells were stained for β-catenin (mouse mAb) and E-cadherin, as indicated. Microinjected cells were identified by coinjection of dextran–Texas red. Note the cytoplasmic and nuclear staining of β-catenin in Ras and p110 microinjected cells, whereas the staining of E-cadherin remained at the cell-cell contacts. Images were obtained in an Axiophot microscope. Bars, 10 μm.
with A PC in keratinocytes, through a mechanism apparently independent of stable interactions with or reduced activity of GSK3β.

**Overexpression of Constitutively Active PI3K Is Sufficient to Stabilize Cytoplasmic β-Catenin**

To get additional information on the involvement of PI3K in the stabilization of cytoplasmic β-catenin, Pam212 cells overexpressing the membrane-bound form of p110α subunit of PI3K (p110CAAX) and puromycin-resistant control cells were generated by retroviral transduction. Pam-p110 cells from two independent infections (p110S1 and p110S2) behaved similarly to PamV12Ras cells with respect to β-catenin and A PC content. Thus, no changes in the total level of both proteins (Fig. 7 a, lysates) and a strong reduction in the level of β-catenin–A PC interaction (Fig. 7 a, bottom, IP: A PC) were observed in p110 cells, when compared with control puromycin-resistant (Puro) cells.

The effect of p110α expression on β-catenin localization was further investigated by microinjection of recombinant GST–β-catenin fusion protein. 4 h after microinjection, a strong cytoplasmic and nuclear accumulation of β-catenin was observed in PamV12R as (Ras) and Pamp110α (p110) cells (Fig. 7 b). In both cell types, β-catenin staining was also detected at the cell–cell contacts of the microinjected cells. In contrast, β-catenin staining was exclusively detected at the cell–cell contacts after microinjection of the GST–β-catenin fusion protein in the parental Pam212 (Pam) cells (Fig. 7 b), possibly because of its quick degradation or incorporation into junctions. In Ras and p110 microinjected cells staining with anti-GST antibodies showed localization of the exogenous protein at the membrane, cytoplasm, and nucleus but only at the membrane junctions in Pam cells (data not shown). On the other hand, staining of E-cadherin at the cell–cell contacts was not modified in p110 cells (Fig. 7 b), indicating that PI3K activity alone is not sufficient to disrupt cell–cell adhesion in mouse keratinocytes.

The stabilization of endogenous β-catenin in cells overexpressing either V12R as or p110CAAX was confirmed by pulse–chase experiments. As can be observed in Fig. 8 a, β-catenin was quickly degraded in both control puromycin (Puro) and neomycin (Neo)-transduced cells. In contrast, the metabolic stability of endogenous β-catenin was significantly increased in V12R as (Ras)- and p110CAAX (p110)-overexpressing cells (Fig. 8 a). Quantification of

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**Figure 8.** Increased metabolic stability of β-catenin in Ras- and p110CAAX-overexpressing keratinocytes. (a) Control (Neo and Puro), PamV12R as (Ras), and Pamp110CAAX (p110) cells were pulse-labeled for 1 h with [35S]methionine/cysteine and chased at the indicated times. Labeled β-catenin was detected by immunoprecipitation and processed as described in Materials and Methods. (b) The integrated density of the β-catenin-labeled bands at each chase time was normalized to that obtained at chase time 0 in each cell line. Assays were performed in two independent experiments for each cell type, and the results show the average value and SD. See the increased stability of β-catenin in Ras- and p110-overexpressing cells as compared with control Neo and Puro cells.
the autoradiograms shown in Fig. 8 a indicated a half-life for β-catenin of ~1 h in control Pam cells, and of ~4 h in V 12R as- and p110CA A X-overexpressing cells (Fig. 8 b).

These results demonstrate that the stability of cytoplasmic β-catenin is increased by the expression of V 12R as and constitutively active PI3K in Pam212 keratinocytes.

Discussion

A iterations in the expression or function of the E-cadherin/β-catenin adhesion system occur frequently in a wide variety of human carcinomas (for review see Takei, 1993; Burchmeier and Behrens, 1994). Indeed, a causal role for the loss of E-cadherin has been recently demonstrated during the transition from adenoma to invasive carcinoma (Perl et al., 1998). The molecular mechanisms underlying the loss of expression or functionality of individual components of the cadherin/catenin complexes in tumorigenesis are still poorly understood. The implication of β-catenin in the Wnt signaling pathway has opened new avenues in the study of the modulation of the cadherin–adhesion complexes during tumor progression (Peifer, 1997; Pennisi, 1998). However, it is still largely unknown if other signaling pathways frequently activated in tumor cells and, more specifically, H-Ras activation can influence the signaling activity of β-catenin. Our results indicate that activation of H-Ras induces the dismantling of E-cadherin/β-catenin complexes and the stabilization of hypophosphorylated cytoplasmic β-catenin through signaling pathway(s) involving PI3K that lead to the inhibition of β-catenin–APC interaction and to a stable interaction of β-catenin with PI3K complex. In addition, activation of PI3K is sufficient to promote the stabilization of cytoplasmic β-catenin and its nuclear translocation.

H-Ras expression in mouse epidermal keratinocytes induced a decrease in the total levels of E-cadherin and β-catenin without significantly affecting the levels of β-catenin, as previously described in intestinal and mammary cells (Novak et al., 1998). In addition, in V 12R as-expressing keratinocytes, most of the E-cadherin and associated β-catenin are found in the detergent soluble fraction, indicating a weak interaction of the E-cadherin/β-catenin complexes with the cytoskeleton in those cells. We suggest that activation of H-Ras as can affect the E-cadherin/β-catenin complexes in epidermal keratinocytes via signaling mechanisms different from those leading to increased P-Tyr, in contrast to previous reports using other cell types (Kinch et al., 1995). Despite the biochemical effects on cadherin complexes induced by activated H-Ras in mouse keratinocytes, no significant differences could be observed in the morphological phenotype of the parental Pam212, control PamNeo, and PamV 12R as cells when growing at medium high density. These results differ from those previously reported after Ras transformation of MDCK cells (Behrens et al., 1993; Vlemminckx et al., 1991) or mammary MCF 10A cells (Kinch et al., 1995; Zong et al., 1997), where a more epithelial or fibroblastic phenotype was sometimes observed. These differences may be related to the level of activated H-Ras expression obtained in the different studies or may be specific of the cell system analyzed. In this sense, it is worth mentioning that tyrosine phosphorylation of catenins plays a positive role in the

stratification process in differentiated keratinocytes (Cault et al., 1998). In spite of those differences, our results indicate that even in situations where a full phenotypic transformation is not observed, activated H-Ras as is able to significantly modify the E-cadherin/β-catenin complexes as in the murine Pam212 epidermal keratinocytes. Thus, the modification of the adhesion complexes might be an early event driven by H-Ras as transformation.

Our microinjection studies are in agreement with those showing that V 12R as leads to the loss of E-cadherin and β-catenin from cell–cell contacts in MDCK cells, dependent on both PI3K and MAPK activities (Potempa and Ridley, 1998). However, no cytoplasmic localization of β-catenin was observed and the α-catenin status was not analyzed in this study. These differences can be due to different fixation and permeabilization procedures or to the cell systems analyzed.

In addition to the involvement of PI3K in the Ras-dependent destabilization of cadherin complexes and relocalization of β-catenin, our results provide evidence for a strong association of cytoplasmic β-catenin with PI3K both in vivo and in vitro. In vitro binding studies clearly show a direct interaction between β-catenin and p85α, (Fig. 4 c), suggesting that the in vivo interactions might be mediated by the regulatory subunit of PI3K. In fact, p85α is able to interact with endogenous β-catenin in control Pam212 cells, and this interaction is significantly increased in V 12R as-overexpressing cells. Interestingly, concomitant with the increased association with the regulatory subunit, association of the catalytic subunit of PI3K with β-catenin is strongly induced in PamV 12R as (Fig. 4 a). These observations suggest that the PI3K heterodimer is involved in the stabilization of cytoplasmic β-catenin induced by activated H-Ras as in keratinocytes.

The involvement of PI3K in the Ras-induced β-catenin stabilization is further supported by analysis of Pam212 keratinocytes overexpressing the membrane-bound catalytic subunit of PI3K (Pamp110 cells). In these cells as well as in PamV 12R as cells, microinjected recombinant β-catenin is stabilized in the cytoplasm and translocated to the nucleus after 4 h of microinjection. In contrast, the exogenous β-catenin is apparently degraded or incorporated into junctions in control cells (Fig. 7 b). Furthermore, the pulse-chase analysis demonstrates that expression of V 12R as or p110CA A X significantly reduced the turnover of endogenous β-catenin. These results indicate that constitutive activation of PI3K is sufficient for stabilization of β-catenin and its translocation to the nucleus, although not for disruption of cell–cell junctions. One interesting possibility is that other pathways activated by V 12R as, like MAPK, are required for initial dismantling of adhesion complexes, and that cytoplasmic β-catenin is then stabilized because of its interaction with activated PI3K. This is now being investigated in further detail.

Here, we provide evidence to support that H-Ras-induced stabilization of β-catenin occurs through interference with its APC interaction and decrease in the levels of serine phosphorylation. The ability of β-catenin to associate with APC is significantly reduced in keratinocytes stably expressing V 12R as. However, in contrast to previous reports, our present results indicate that the stabilization of hypophosphorylated cytoplasmic β-catenin induced by
activated H-Ras as might occur by mechanism(s) independent of stable interaction with GSK 3β or significant alterations in the total GSK 3β activity (Delcommenne et al., 1998). Inhibition of additional kinase(s) or activation of phosphatase(s) may be involved in the dephosphorylation of β-catenin upon Ras activation. In this sense, it has been reported that β-catenin can also be phosphorylated by diacylglycerol-independent protein kinase C isoforms (Orford et al., 1997), and that its stability is modulated by PP2A phosphatase (Seelig et al., 1999). In Ras-expressing mouse keratinocytes β-catenin might transiently interact with distinct cytoplasmic partners for its translocation to the nucleus. Interestingly, in our Rα4-expressing keratinocytes only a slight increment in Lef-1/Tcf-dependent transcriptional activity is observed, suggesting that additional factors are needed to fully induce β-catenin transcriptional activation (see Prieve and Waterman, 1999).

Regarding the mechanism(s) leading to β-catenin stabilization, it is not known whether interaction with PI3K can modulate its association to A PC or directly contribute to its stabilization in the cytoplasm. Interestingly, β-catenin– A PC interaction is also blocked in p110CAAX-overexpressing keratinocytes (Fig. 7 a), but similar levels of total GSK 3β activity have been observed in controls, PI3K and V 12R as transformants (Espada, J., and A. Cano, unpublished results). We speculate that p85α (and/or p110α) might compete with other β-catenin partners, such as A PC or E-cadherin, rendering the molecule inaccessible for the ubiquitin-proteasome degradation. On the other hand, PI3K activation by H-Ras can further increase the β-catenin–PI3K interaction. The possibility of a recruitment of β-catenin into vesicles through PI3K interaction cannot be discarded, although it is not supported from the immuno-fluorescent staining (Figs. 1 and 7 b).

In summary, our results show a direct effect of H-Ras activation on the stabilization of β-catenin cytoplasmic pools in epithelial keratinocytes. This effect is mediated by the PI3K effector and involves a novel β-catenin–PI3K complex and the inhibition of β-catenin–A PC interaction. Together with recent data from other groups (Delcommenne et al., 1998; Potempa and Ridley, 1998; Sander et al., 1998), our results highlight the role of PI3K as a main regulator of different signaling pathways impinging on the stabilization of the E-cadherin–mediated adhesion and β-catenin signaling. They also indicate that H-Ras activation can induce β-catenin signaling and, thus, contribute to the present knowledge on the molecular mechanisms of cancer development.

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