Oncogenic Ki-\(\text{ras}\) but Not Oncogenic Ha-\(\text{ras}\) Blocks Integri
\(\beta\)-1-Chain Maturation in Colon Epithelial Cells

(Received for publication, April 3, 1997, and in revised form, September 12, 1997)

Zhongfa Yan, Ming-xing Chen, Manuel Perucchini, and Eileen Friedman

From the State University of New York Health Science Center, Department of Pathology, Syracuse, New York 13210 and the Burnham Institute, La Jolla, California 92037

Human colorectal tumors commonly contain mutations in Ki-\(\text{ras}\) but rarely, if ever, in Ha-\(\text{ras}\). The selectivity for Ki-\(\text{ras}\) mutations in this tumor was explored using the HD6-4 colon epithelial cell line which contains no \(\text{ras}\) mutations. After adhesion to an extracellular matrix, HD6-4 cells polarize into columnar goblet cells with distinct apical and basal regions. Stable HD6-4 transfectants were made with mini-gene constructs of the oncogenic cellular Ki-\(\text{ras}^\text{G12V}\) gene, the oncogenic Ha-\(\text{ras}^\text{G12V}\) gene, or mini-gene constructs of wild-type Ki-\(\text{ras}^\text{4B}\) as a control. Ki-\(\text{ras}\) mutations, but not Ha-\(\text{ras}\) mutations, disrupted colon epithelial cell apicobasal polarity and adhesion to collagen I and laminin. Three Ha-\(\text{ras}\) transfectants and three Ki-\(\text{ras}\) transfectants exhibited Ras proteins expressing the Val-12 mutation by Western blotting with pan-\(\text{ras}\)^\text{G12V} antibody. Only wild-type Ki-\(\text{ras}\) transfectant cells and oncogenic Ha-\(\text{ras}\) transfectant cells synthesized the mature, fully glycosylated forms of \(\beta\)-1 integrin. Instead of the mature integrin \(\beta\)-chain, a faster migrating \(\beta\)-chain intermediate was detected on the cell surface and in the cytoplasm of the oncogenic Ki-\(\text{ras}\) transfectants. Expression of the oncogenic Ki-\(\text{ras}\) gene caused the altered \(\beta\)-1 integrin maturation because phosphorothiolated antisense oligonucleotides to Ki-\(\text{ras}\) reduced expression of both the mutant Ki-\(\text{ras}\) protein and the aberrant integrin \(\beta\)-1-chain and increased expression of the mature integrin \(\beta\)-1-chain. Altered glycosylation generated the new \(\beta\)-1 integrin form since integrin core \(\beta\)-1-chain proteins of the same molecular weight were yielded in Ki-\(\text{ras}\), Ha-\(\text{ras}\), and control transfectants after removal of sugar residues with endoglycosidase \(F\) or following tunicamycin treatment to inhibit glycosylation. The selective effect of oncogenic Ki-\(\text{ras}\) on \(\beta\)-1 integrin glycosylation was not due to selective activation of mitogen-activated protein kinases because both mutated Ki- and Ha-\(\text{ras}\) genes activated this pathway and increased cell proliferation. Since blocking the glycosylation of integrin \(\beta\)-1-chain inhibited the adherence, polarization, and subsequent differentiation of colon epithelial cells, the selective effects of the oncogenic cellular Ki-\(\text{ras}\) gene on integrin \(\beta\)-1-chain glycosylation may account, at least in part, for the selection of Ki-\(\text{ras}\) mutations in human colon tumors.

The mammalian \(\text{ras}\) gene family contains three homologous members, Ki-\(\text{ras}\), Ha-\(\text{ras}\), and N-\(\text{ras}\). Each encodes a 21-kDa protein of either 188 or 189 amino acid residues. The first 85 amino acids of each \(\text{ras}\) isoform are identical, and the next 80 amino acids exhibit an 85% homology. The remaining C-terminal sequence is highly divergent between the isoforms and so is termed the hyper-variable domain. The last four amino acids in the C terminus constitute the CAAX motif, which is required for post-translational modification and subsequent membrane localization after gene-specific lipid modifications (1). The C-terminal region can substitute for the entire gene in localizing the downstream effector \(\text{raf}\) to the plasma membrane (2).

\(\text{Ras}\) proteins switch between an active form that binds GTP and an inactive form that binds GDP (3). Activated \(\text{ras}\) in turn activates the serine-threonine kinase \(\text{raf}-1\) which then activates MAP kinase kinases (MEKs) which in turn activate the p42/44 MAP kinases (Erks). \(\text{Ras}\) also activates \(\text{raf}\)-independent signaling pathways leading to activation of JNK kinases. Although there is evidence linking the \(\text{raf}/\text{MEK/\text{MAP}}\) kinase pathway to cellular transformation, \(\text{ras}\) may mediate some aspects of transformation through \(\text{raf}\)-independent pathways. Non-\(\text{raf}\) candidate \(\text{ras}\) effectors include Rho family proteins, two \(\text{GTPase-activating proteins}\) (p120 and NF1), guanine nucleotide exchange factors for \(\text{Rho}\) proteins, and phosphatidylinositol-3-OH kinase (reviewed in Ref. 4). The possible contributions of \(\text{ras}\) activation to transformation include altered transcription and translation, alterations in the cytoskeleton, and altered cell surface carbohydrates (5, 6). Oncogenic mutations detected in Ha-, Ki-, and N-\(\text{ras}\) genes isolated from different human tumors localize in the N-terminal region controlling GTP binding, suggesting the genes become oncogenic when they remain in the GTP-bound state. Mutations in predominantly one of the three mammalian \(\text{ras}\) genes have been found associated with specific human cancers. For example, Ha-\(\text{ras}\) mutations have been reported in 18% of transitional cell carcinomas of the human urinary bladder, whereas very few mutations in Ki-\(\text{ras}\) were observed (7, 8). In contrast, the vast majority, over 90%, of pancreatic adenocarcinomas contain mutations in the Ki-\(\text{ras}\) gene and not in N-\(\text{ras}\) or Ha-\(\text{ras}\) (9). Specificity of \(\text{ras}\) mutations is also seen in colon cancers, in which roughly 40–50% of cases exhibit activating mutations in the Ki-\(\text{ras}\) gene. Only a few percent of cases exhibit N-\(\text{ras}\) mutations, whereas Ha-\(\text{ras}\) mutations are very uncommon in colon cancers (10, 11). N-\(\text{ras}\) mutations have been found in approximately 20–25% of cases of acute myeloid leukemia, although mutations in Ki-\(\text{ras}\) were infrequent (12). The reason for the selectivity for a specific mutated \(\text{ras}\) isoform in a tumor type is not known but may be due to the importance of that...


isoform in controlling proliferation within that cell type. Recently it was shown using gene-specific antisense oligonucleotides that Ki-ras, but not Ha-ras, contributes to the proliferation of normal human lung fibroblasts (MCR-5 cells), whereas oncogenic Ha-ras drives proliferation of T24 human bladder carcinoma cells (13).

Both Ha-Ras and Ki-Ras proteins are expressed in colon epithelial cells (14) but may have different functions. We wished to determine whether mutations in the Ki-ras gene which are found in colon carcinoma cells induce another function in addition to cell growth through the MAP kinase cascade. Viral Ki-ras prevents the polarization of MDCK kidney epithelial cells (15, 16) suggesting that the mutated cellular Ki-ras gene may have a similar function in colon epithelial cells. Studies in tissue culture have shown that cellular polarization may require both E-cadherin-mediated cell to cell contact and integrin-mediated cell-substratum interactions (17). E-cadherin is critical to the formation of the basolateral domain, whereas the orientation of the apicobasal axis depends on integrin-mediated associations with the substratum.

We now compare the effects of two ras genes, the Ki-ras which is often mutated in colon cancer and the Ha-ras which is rarely, if ever, mutated in colon cancer, on colon epithelial cell polarity, integrin-mediated adhesion, and integrin expression and function. The expression plasmids utilized are a mini-gene construct of the large cellular Ki-ras4B gene (18) and an oncogenic cellular Ha-ras gene, both mutated at codon 12 from Gly to Val. The colon epithelial cell used is the HD6-4 line, which binds strongly to collagen I through the integrin heterodimer α2β1 (19). After binding to collagen I, HD6-4 cells then polarize into columnar cells with distinctive apical and basal compartments. These columnar cells differentiate into a specialized epithelial cell type, the colon goblet cell, with a basal nucleus and an apical thickening mucin granules (20). HD6-4 cells contain inactivating mutations in the tumor suppressor genes p53 and APC but have wild-type ras genes, making them suitable recipients for oncogenic ras genes (21). We now find that constitutive expression of oncogenic Ki-ras, but not oncogenic Ha-ras, prevents the establishment of columnar cell polarity in HD6 cells by blocking glycosylation and maturation of integrin β1-chain, thus reducing the capacity of this integrin to mediate binding to extracellular matrix components.

EXPERIMENTAL PROCEDURES

Materials—[α-32P]GTP, [32P]ATP, [32P]P3HTP, and [32P]methionine were obtained from NEN Life Science Products, protein A-Sepharose from Pharmacon Biotech Inc., PVDF transfer paper Immobilon-P from Millipore, and PEI-cellulose-F TLC plates from EM Separations. Pan-ras rat monoclonal antiserum Y13-259 which reacts with the p21 translation products of the Ha-, Ki- and N-ras human oncogenes; monoclonal pan-ras (31, 32) antibody which reacts with only the forms of the ras oncogenes mutated at codon 12 to valine; c-Ki-ras Ab1 (clone 234-4.2) a mouse monoclonal antibody specific for c- and v-Ki-ras p21 and not recognizing c-Ha-ras or c-Ha-ras p21; c-Ha-ras (Ab-1) clone 235-1.7.1 a mouse monoclonal antibody specific for Ha-ras and not Ki- or N-ras p21 proteins, and purified p21 recombinant protein Ki-ras30-121 or -122 were purchased from Oncogene Science. Phosphorothioate oligodeoxynucleotides were a gift of Dr. Brett Monia, Isis Pharmaceuticals, Carlsbad, CA. Isis-2503 is a 20-mer targeted to the initiation codon (AUG) of c-Ha-ras mRNA (TCC-GTC-ATC-GCT-CCT-CAG-GG) and Isis-13177 is a 20-mer of random sequence; Isis-6957 is a 20-mer targeted to the 5′-UTR of Ki-ras (CAG-TGC-CTG-CGC-GGC-GCT-CAG). This sequence is found within the promoter region of c-Ki-ras4B gene cloned into pMIKVal-12 (see below), about 60 base pairs upstream of the translational start. Anti-integrin α1, a polyclonal antibody with clone 18 raised to a protein fragment corresponding to amino acids 76–256 of human integrin β1-chain, a region of the extracellular domain, was purchased from Transduction Laboratories and was used for immunoblotting. Monoclonal antibody clone P4C10, an IgG1 isotype directed to integrin β1-chain was purchased from Life Technologies, Inc. and was used for immunoprecipitation and cell binding studies. Phospho-specific MAP kinase antibody and p44/42 MAP kinase antibody were rabbit polyclonal IgGs from New England BioLabs, and endoglycosidase F (N-glycosidase F-free), also known as endo-β-N-acetylglucosidase F, was purchased from Boehringer Mannheim. Collagen I was purchased from the Collagen Corp., Palo Alto, CA. Fibronectin and tunicamycin were purchased from Calbiochem, La Jolla, CA. Anti-integrin α1, a polyclonal mouse IgG-clone 18 raised to integrin α1, a protein fragment corresponding to amino acids 76–256 of human integrin β1-chain, or 5 µg/ml of the Ki-ras-specific, Ha-ras-specific, or pan-ras antibodies and detected by enhanced chemiluminescence. After SDS-PAGE and blocking as above, tyrosine-phosphorylated erk1 and erk2 and total erk1 and erk2 were detected, respectively, by 1 µg/ml phospho-MAP kinase and 1 µg/ml total MAP kinase antibodies and then detected using the Western blotting detection system provided by the

β1 Integrin Maturation Blocked by Oncogenic Ki-ras, Not Ha-ras

kinase antibody and p44/42 MAP kinase antibody were rabbit polyclonal IgGs from New England BioLabs, and endoglycosidase F (N-glycosidase F-free), also known as endo-β-N-acetylglucosidase F, was purchased from Boehringer Mannheim. Collagen I was purchased from the Collagen Corp., Palo Alto, CA. Fibronectin and tunicamycin were purchased from Calbiochem, La Jolla, CA. Anti-integrin α1, a polyclonal mouse IgG-clone 18 raised to integrin α1, a protein fragment corresponding to amino acids 76–256 of human integrin β1-chain, or 5 µg/ml of the Ki-ras-specific, Ha-ras-specific, or pan-ras antibodies and detected by enhanced chemiluminescence. After SDS-PAGE and blocking as above, tyrosine-phosphorylated erk1 and erk2 and total erk1 and erk2 were detected, respectively, by 1 µg/ml phospho-MAP kinase and 1 µg/ml total MAP kinase antibodies and then detected using the Western blotting detection system provided by the
manufacturer: a 1-h incubation at room temperature with 1:1000 dilution of alkaline phosphatase-conjugated anti-rabbit secondary antibody and 1:1000 dilution of alkaline phosphatase-conjugated anti-biotin antibody in blocking buffer, followed by a wash, and detection using a 1:5,000 dilution of CDP-Star for 5 min, followed by autoradiography.

**Antibody Oligonucleotide Treatment—**Treatment of Ki-ras and Ha-ras transfectants was essentially as described (13). Cells were seeded at 2 × 10^5 per well in 6-well plates. 48 h later the cells were washed with pre-warmed serum-free ITS-DME medium and then incubated in this medium with a fixed ratio of oligonucleotide to Lipofectin (2.4 μl of Lipofectin per 40 pmol of oligonucleotides) for 4 h. The oligonucleotide-containing medium was then replaced with normal growth medium, and growth was continued for 48 h to allow ras turnover plus reduced ras mRNA levels to result in reduced Ras protein level (13).

**Differentiation Assay—**Cells were cultured on Costar transwells with 2 ml of medium under the layer and 1.5 ml above the layer for 2 weeks post-confluence with 3 times weekly media changes, fixed, and stained for mucin detection by Aclian blue dye with a nuclear fast red counterstain, exactly as described (20).

**Kinase Assays in Polyacrylamide Gels Containing MBP—**The method is adapted from one used previously (22). Cell lysates in high salt EBC buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 10 mM KCl, 1% Nonidet P-40, 200 μM sodium orthovanadate, 10 μM each aprotinin and leupeptin, and 1 μM phenylmethylsulfonyl fluoride) were boiled in Laemmli sample buffer for 5 min and then electrophoresed in a 7.5% SDS-PAGE gel (0.5 mm thick and 5 cm long) containing 0.5 mg/ml MBP (Sigma). After fixing the gel with four changes of 20% 2-propanol in 50 mM Tris-HCl buffer, pH 8.0, for 2 h, SDS was removed by washing the gel twice for 2 h each in several gel volumes of 50 mM Tris-HCl, pH 8.0, containing 5 mM 2-mercaptoethanol. The MBP kines were then re-denatured with 6 M guanidine HCl for 2 h and then renatured by 10 washes of 20 min each in several gel volumes of 50 mM Tris-HCl, pH 8.0, containing 0.04% Tween 20 and 5 mM 2-mercaptoethanol. After preincubation for 1 h with 5 ml of 40 mM HEPES, pH 8.0, containing 2 mM 2-mercaptoethanol and 10 mM MgCl2, phosphorylation of MBP within the gel was carried out by incubating the gel at room temperature for 1 h in 5 ml of 40 mM HEPES, pH 8.0, containing 25 μCi of [γ^32P]ATP, 40 μM ATP, 0.5 mM EGTA, and 10 mM MgCl2, and then washing the gel in 5% (w/v) trichloroacetic acid containing 1% sodium pyrophosphate several times until the radioactivity reached background levels.

**Digestion with Endoglycosidase F and Tunicamycin Treatment—**Cells were treated with 3 μg/ml tunicamycin for 24 h before lysis. Cell lysates were denatured by boiling in 2% SDS-Laemmli sample loading buffer, boiled for 5 min. For digestion with endoglycosidase F, 0.5 units of endoglycosidase F were added after the lysates were made 1% in Nonidet P-40 and 0.1% in SDS and boiled for 5 min. Denaturation by heating at 100 °C in the presence of SDS, but not Nonidet P-40, increases the deglycosylation rate considerably according to the vendor. The lysates were incubated for 36 h before SDS-PAGE and Western blot analysis.

**Preparation of Membrane Fraction—**After washing with PBS, the cells were swollen for 5 min on ice in hypotonic buffer A (20 mM Tris-HCl, pH 7.5, containing 1 mM NaF, 100 μM sodium orthovanadate, 2 mM EDTA, 1 mM EGTA) containing protease inhibitors exactly as described (25). After 20–25 strokes with a Dounce homogenizer on ice, the post-nuclear supernatant was layered on top of a 15% sucrose cushion in buffer A and centrifuged at 100,000 × g for 1 h. The sedimentable membrane pellet was suspended in 1% Nonidet P-40 in buffer A, and any Nonidet P-40-insoluble material was removed at 10,000 × g for 60 min.

**Cell Surface Iodination and Immunoprecipitation of β1 Integrin—**Cells were plated in 6-well dishes so that the majority of cells would be doublings 2 days post-plating, after doubling once. IODO-BEADS (Pierce) were washed twice in freshly prepared PBS, pH 6.5, and dried on filter paper just before using. Cells were rinsed with PBS, pH 6.5, twice, and then 1 ml of PBS, pH 6.5, two washed IODO-BEADS, and 10 μl (1 μCi) of 125I were added per dish. The cells were incubated for 20 min with rotation on an orbital shaker at room temperature. Cells were then washed 3 times with cold PBS and lysates prepared as above. Immunoprecipitations were performed using 5 μg of rabbit antbody clone P4C10 to 400 μg of cell lysate exactly as described (22).

**Tumorigenicity in Athymic Mice—**Cells were collected by trypsinization and adjusted to 10^5/ml, pelleted, resuspended in PBS at this volume, and 0.1 ml (10^5 cells) was injected in each of 5 male BALB/c nu/nu athymic mice between the shoulder blades. Cells were >99% viable by trypan blue exclusion. Tumor size was measured in two dimensions every 2–3 days using calipers, and volume was calculated as (width)^2 × length/2. Sections were fixed in formalin, then processed for routine histology, and stained with either hematoxylin and eosin or Aclian blue with a nuclear fast red counterstain.

**RESULTS**

Transfectant Lines Express Mutated Ras Proteins—Transfectants of HD6-4 cells were performed with a mini-gene construct of the cellular Ki-ras4B gene mutated at codon 12 to valine (Ki-ras^G12V), an expression plasmid encoding a mini-gene construct of the wild-type cellular Ki-ras4B gene, and a cellular Ha-ras^G12V expression plasmid. We isolated three independent transfectant clones expressing the Ki-ras^G12V oncogene: 4V1, 4V2, and 4V3; three independent transfectant clones expressing the Ha-ras^G12V oncogene: H15, H18, and H25; and several independent clones expressing the transfected wild-type Ki-ras4B gene. One of the latter, 4G1, was arbitrarily selected for a control for expression of excess copies of Ki-Ras protein. The transfected oncogenes were expressed as active proteins. Proteins in lysates from the transfected lines were size-fractionated by SDS-PAGE and then transferred to PVDF membrane and analyzed for the presence of Ki-Ras proteins by immunoblotting with a Ki-ras-specific antibody (Fig. 1A, top panel), analyzed for the presence of Ha-Ras proteins by immunoblotting with an Ha-ras-specific antibody (Fig. 1A, second panel), analyzed for the presence of total Ras proteins by immunoblotting with a pan-ras antibody (Fig. 1A, bottom panel), and analyzed for Ras proteins mutated at Val-12 by immunoblotting with a pan-ras^Val-12 antibody (Fig. 1A, bottom panel). Cells transfected with mutant Ki-ras (4V1, 4V2, and 4V3) expressed elevated levels of Ki-Ras protein, presumably mutant, compared with the other transfectant and parental lines (Fig. 1A, top panel). Cells transfected with mutant Ha-ras (H15, H18, and H25) expressed elevated levels of Ha-Ras protein, presumably mutant, whereas other transfectant lines and parental cells displayed lower levels of Ha-Ras protein (Fig. 1A, second panel). All of the transfectant lines displayed similar levels of total Ras proteins (Fig. 1A, third panel) suggesting endogenous Ras proteins were down-regulated in the transfectant cells (Fig. 1A, third panel). Similar levels of total Ras proteins
were also observed by immunoprecipitation experiments (data not shown).

Each of the three lines transfected with the Ki-ras4B^{G12V} oncogene and each of the three lines transfected with the Ha-ras^{G12V} oncogene expressed mutant Ras proteins, which migrated at the expected mobility for proteins of 21 kDa and at the same position as recombinant mutant Ki-ras^{Val-12} (Fig. 1A). No Ras proteins mutated at valine 12 were detected in the parental line or in the Ki-ras4B wild-type control transfectant (Fig. 1A, bottom panel).

To confirm that the 21-kDa protein bearing the Val-12 mutation detected by immunoblotting was indeed ras, Ras proteins were immunoprecipitated from 35S-prelabeled HD6-4 parental cells, wild-type c-Ki-ras 4G1 transfectant cells, and 4V transfectant cells expressing the mutated c-Ki-ras gene (Fig. 1B). Similar amounts of total Ras proteins were immunoprecipitated from each cell line (data not shown). Immunoprecipitated wild-type and mutant Ki-Ras proteins were transferred to PVDF membrane and analyzed for the presence of mutated Ras proteins by immunoblotting with the pan-ras^{Val-12} antibody. Only the 4V line expressed Ras proteins mutated at valine 12 (Fig. 1B), confirming the results of the Western blot analysis (Fig. 1A).

The mutant Ras proteins bound elevated levels of GTP showing they were functional. The ratio of GTP/GDP bound to immunoprecipitated Ras proteins was determined by thin layer chromatography (22). In duplicate experiments, the 4V line had a ras-bound GTP/GDP ratio of 15.5%, parental cells a ratio of 8.5%, and wild-type c-Ki-ras transfectants a ratio of 7.0%. The presence of an activated ras was confirmed by a second method. Ras proteins exchange guanine nucleotides at a very low rate that can be measured by addition of [α-32P]GTP to permeabilized cells, followed by analysis of nucleotides bound to Ras proteins (23). In 4V cells over three times as much labeled GTP specifically bound to Ras proteins than in control 4G1 transfectant cells or in the parental line (data not shown).

Thus mutated Ras proteins were expressed in both oncogenic Ki-ras and Ha-ras transfectant lines, and the immunoprecipitated oncogenic Ki-ras and Ha-Ras proteins exhibited increased GTP binding.

Oncogenic Ki-ras, Not Oncogenic Ha-ras, Prevents Colon Epithelial Cell Polarization into a Columnar Cell Shape—Parental cells, oncogenic Ha-ras transfectants H25, H18, and H15, and oncogenic Ki-ras transfectants 4V, 4V1, and 4V2 were each injected into five athymic mice. The relatively small inoculum of 10^5 cells was used to maximize any differences in tumor cell growth between the three lines. Tumor take was identical in each line, with 4 of 5 of each set of mice showing tumor growth. However, each ras transfectant line grew more rapidly in vivo than the parental line. The Ha-ras transfectants grew 2–4 times as quickly as the parental line, whereas the Ki-ras transfectants grew 4–7-fold as rapidly (Fig. 2). Thus the presence of oncogenic Ras proteins in each transfectant line was correlated with increased cell growth in athymic mice.

The HD6-4 cell line organized into glands similar to normal colonic crypts seen in cross-section when cells were injected subcutaneously into athymic mice (21). Sections from tumors induced by the transfectant lines were examined after routine histology. Disorganized glands were seen in each tumor induced by each of three oncogenic Ki-ras transfectant cell lines (Fig. 3D and data not shown), whereas the Ha-ras transfectants and wild-type Ki-ras transfectants displayed normal appearing glands (Fig. 3B and C). These data suggested that the oncogenic Ki-ras gene disrupted either the tight cell to substratum adherence characteristic of HD6-4 cells or that cell to cell lateral adherence was disrupted.
tyrosine-phosphorylated forms of erk1 and erk2. Both forms were detected in each oncogenic Ha-ras transfectant (H15, H18, and H25) and each oncogenic Ki-ras transfectant (4V1, 4V2, and 4V) but were not detected in the parental HD6-4 line. erk2, purified unphosphorylated erk2 run as a negative control; erk2-P, purified tyrosine phosphorylated erk2 run as a positive control. Middle panel, Western blot with antibody specific for protein abundance of erk1 and erk2. The positions of erk1 at 44 kDa and erk2 at 42 kDa are indicated at right. Bottom panel, myelin basic protein kinases seen in in gel kinase assay (see “Experimental Procedures”) showing increased MBP kinase activities at the 42/44-kDa position in each Ki-ras and each Ha-ras transfectant line, compared with the parental line.

at various locations in the cytoplasm. 4V cells grown in vitro secreted mucins into intercellular spaces (Fig. 3H) instead of secreting mucins by degranulation from apical surfaces as the parental cells. Thus expression of an oncogenic c-Ki-ras gene, but not an oncogenic c-Ha-ras gene, blocked HD6-4 colon epithelial cell polarization.

The Differential Effect of Oncogenic Ki-Ras and Ha-Ras Proteins on Colon Epithelial Cell Polarity Is Not Due to Differences in Activation of MAP Kinases—Introduction of either mutated Ha-ras or Ki-ras into PC12 cells or NIH3T3 cells leads to activation of the MAP kinases erk1 and erk2 through sequential activation of raf and MEK (26). The MAP kinases erk1 and erk2 are activated by phosphorylation on tyrosine and threonine residues in the motif T*EY*. Antibody specific for the tyrosine-phosphorylated forms of erk1 and erk2 was utilized in Western blot analysis of cell lysates to demonstrate the presence of constitutively activated erk1 and erk2 in each of the three oncogenic Ha-ras and each of the three oncogenic Ki-ras transfectant lines, whereas no activated MAP kinases were detected in the parental HD6-4 line (Fig. 4 top). To confirm that the tyrosine-phosphorylated MAP kinases in the Ki-ras and Ha-ras transfectant cells were active kinases, the proteins in cell lysates were size-fractionated by SDS-PAGE in a gel containing immobilized myelin basic protein, a MAP kinase substrate. After removal of the SDS and renaturation of the lysate proteins, radiolabeled ATP was added and an in gel kinase assay was performed. Increased myelin basic kinase activity was detected in each of the Ki-ras and Ha-ras cell lysates at the 42/44-kDa position, the molecular masses of erk1 and erk2 (Fig. 4 bottom). Western blot analysis of the lysates with an antibody that detects both phosphorylated and unphosphorylated forms of erk1 and erk2 showed that the transfectant and parental cell lysates contained equal levels of these MAP kinases (Fig. 4, middle). Thus the presence of tyrosine-phosphorylated MAP kinases and active MBP kinases of 42/44 kDa confirm that the both oncogenic Ki-ras and Ha-ras transfectants expressed activated MAP kinases, with greater activity than those in the parental line (Fig. 4, bottom).

In earlier studies we had determined that colon carcinoma cell lysates that exhibited 42/44-kDa proteins with kinase activity on myelin basic protein also contained tyrosine-phosphorylated erk1 and erk2, as shown by immunoprecipitation of these kinases (22). Thus the increased cell growth rate detected in each transfectant line in vivo (Fig. 2) and in vitro (data not shown) was consistent with an activated ras-MAP kinase pathway. However, differences in Ki-ras and Ha-ras effects on cell polarization were not caused by differences in MAP kinase activation as MAP kinase activation was seen in each type of transfectant.

Oncogenic Ki-Ras, Not Oncogenic Ha-Ras, Interrupts β1 Integrin Maturation—The first step in HD6-4 cell polarization and differentiation is adherence to collagen I (20). HD6-4 cells bind much tighter to collagen I than undifferentiated colon carcinoma cells (19). In these cells collagen binding is mediated by the α2β1 integrin heterodimer (19). Both undifferentiated colon carcinoma cells with poor collagen I binding (19) and the oncogenic Ki-ras transfectants displayed a disorganized monolayer of unpolarized cells when cultured on transwells. These data suggested that the Ki-ras transfectants might have abnormal α2β1 integrin collagen receptors. The establishment of cell polarity is controlled by both cell to cell and cell to substrate interactions (17). Orientation of the apical-basal axis in MDCK cells is known to depend on integrin-mediated interactions with the growth surface. When grown in suspension MDCK cells aggregate to form cysts with the apical domain facing outwards. These cysts reverse their polarity when collagen is added to the culture medium causing apical markers to disappear from the external domain and reappear on the luminal face (27). Collagen interaction with integrins on the basal surface of cells is believed to orient the apical-basal axis, since addition of blocking antibody to β1 integrin prevents this polarity reversal (28). Because of these data, our studies concentrated on β1 integrin.

Integrin β1-chain is present in cells in several forms as follows: partially glycosylated precursors of 105–115 kDa and a more fully glycosylated mature form of lower electrophoretic mobility, about 130 kDa in fibroblasts, both of which do not migrate as sharp bands because of variations in their glycosylation (29). N-Glycosylation of both the α and β subunits of the integrin receptor in the Golgi is essential for the association of the heterodimer and for its optimal function (30). Under reducing conditions the 105–115-kDa integrin β1-chain precursors in the HD6-4 colon epithelial cells formed a large, tight band of 115 kDa, and the mature integrin β1-chain unexpectedly migrated as a doublet of roughly 140–145 kDa (Fig. 5A, arrow). HD6-4 cells can differentiate into a colon goblet-like cell (Fig. 3E) that secretes copious amounts of mucins. Possibly post-translational modifications other than those typical of mesenchymal cells occur in this differentiated epithelial cell, and these modifications result in a mature integrin β1-chain with lower electrophoretic mobility.

Each of the three c-Ha-ras transfectant lines (Fig. 5A) and the wild-type Ki-ras transfectant 4G1 line (Fig. 5B, arrow) also exhibited the mature integrin β1-chain doublet and the more abundant precursor. In sharp distinction, the mature integrin β1-chain doublet was absent in each of the three c-Ki-rasG12V transfectants (Fig. 5, A and B). In its place was an integrin β1-chain species with migration intermediate between the 115-kDa precursor and the 145-kDa mature doublet. We postulated that integrin β1-chain maturation was impaired in the oncogenic c-Ki-ras transfectants.

Under standard growth conditions immature integrin β1-chain molecules are not transported to the cell surface but remain in the Golgi. Prolonged treatment of fibroblasts with 1-deoxymannojirimycin, a mannose analogue that specifically inhibits Golgi α-mannosidase I, resulted in the appearance of immature integrin β1-chains on the cell surface (31). Similar observations have also been made with keratinocytes (32). Membrane fractions of parental HD6-4 cells and Ha-ras transfectant H18 cells exhibited an enrichment of the mature forms of integrin β1-chains, as expected since the mature forms are
transferred to the cell surface (Fig. 5C). Membrane fractions of the oncogenic Ki-ras transfectant contained the abnormal integrin β1-chain form (Fig. 6C), suggesting that the abnormal β1-chain was transported to the cell surface where it formed a heterodimer with an α-chain and functioned as a matrix receptor.

To confirm that the aberrant integrin β1-chain was transported to the cell surface, HD6 and HD6-4V cells were surface-labeled with 125I, and integrin β1-chain species were immunoprecipitated and then analyzed by SDS-PAGE and autoradiography. The β1-chain intermediate form was iodinated, proving its location on the cell surface (Fig. 6, left). A protein that might be an integrin α2-chain was co-immunoprecipitated with integrin β1-chain in both parental and oncogenic Ki-ras transfectant 4V cells but appeared in slightly less abundance in the 4V cells. To confirm the two large bands immunoprecipitated by β1-chain integrin antibody in HD6-4 parental cells and 4V cells, respectively, were the β1 mature form and the aberrant form, the same blot was probed by Western analysis for total integrin β1-chain abundance, indicated by arrows at right. + lanes, immunoprecipitations with anti-β1 integrin monoclonal antibody P4C10; − lanes, control immunoprecipitations with isotype control monoclonal MOPC-21.

N-linked oligosaccharides of glycoproteins, cleaving high mannose structures, hybrid structures, and biantennary complex structures. In less complete digestions, the mature β1 integrin forms in the 4G1 control transfectant, the H18 Ha-ras transfectant, and the parental line were digested to a form roughly the same size as the unusual β1 integrin found in 4V2 cells (data not shown) suggesting, but not proving, that the aberrant β1 integrin was an immature form. Further studies are in progress to determine the biochemical structure of the aberrant β1 integrin form. Abolition of N-glycosylation by tunicamycin pretreatment of cells before lysis and Western analysis also demonstrated that the β1 integrin core protein was identical in both parental cells and oncogenic Ki-ras transfectant 4V cells (Fig. 7B). Thus the aberrant β1 integrin found in oncogenic Ki-ras transfectant cells was the result of impaired N-glycosylation and thus impaired maturation.

**Impaired β1 Integrin N-Glycosylation Shown to Be Caused by Oncogenic Ki-ras Gene by Use of Gene-specific Antisense Oligonucleotides—**Blocking transcription of the transfected oncogenic Ki-ras gene in 4V2 cells partially reversed accumulation of the β1 integrin intermediate. Chen and colleagues (13) used phosphorothioated antisense oligonucleotides directed to the 5'-untranslated region of the Ki-ras gene to demonstrate the dominant role for c-Ki-Ras proteins in controlling the proliferation of diploid human fibroblasts. A 4-h treatment with oligonucleotides (see “Experimental Procedures”) was followed by a 48-h chase to allow turn-over of the endogenous Ki-ras mRNA and protein (13). We used the same conditions to treat 4V2 cells. 4V2 cells treated with antisense oligonucleotides
directed to the Ki-ras gene (A) exhibited almost no mutated Ki-Ras protein (Fig. 8A) compared with 4V2 cells treated with random sequence oligonucleotides (R) or untreated 4V2 (C) or parental cells. In each of two experiments performed in duplicate a slower migrating integrin β1-chain form was seen in 4V2 cells treated with antisense oligonucleotides directed to the Ki-ras gene (A) compared with 4V2 cells treated with random sequence oligonucleotides (R) (Fig. 8B). The abnormally glycosylated integrin β1-chain seen in untreated cells (C lane) was not completely replaced by the mature form, perhaps because the long half-life of the abnormally glycosylated integrin β1-chain. However, we have shown that specifically inhibiting c-Ki-ras gene transcription by antisense techniques increases the degree of maturation of integrin β1-chain. Thus the impairment in β1 integrin maturation seen in three of independently cloned transfectant lines is not due to some extraneous event in the establishment of these lines but is directly due to the functioning of the oncogenic c-Ki-ras gene.

Aberrantly N-Glycosylated Integrin β1-Chain Mediates Cell Adherence Poorly—HD-6-4 cells tightly adhere to collagen I-coated transwells as a first step in establishing their apicobasal and lateral polarity. HD-6-4 cells use integrin β1-chain to mediate binding to both collagen I and laminin as inclusion of 1 μg/ml anti-β1-chain monoclonal antibody to a cell adherence assay decreased binding to these substrates 42 and 39%, respectively (Fig. 9, left). Binding to collagen I films was decreased by one-third in the oncogenic Ki-Ras transfectant line 4V2. HD-6-4 parental line contains mature β1 integrin at 130 kDa and β1 integrin precursor at 115 kDa. Phosphorothiolated random sequence antisense oligonucleotides (R) do not alter the β1 integrin migration pattern of 4V2 cells; C, control untreated 4V2 cells.

to other cells (33). Nine different β-chains have been identified. The integrin β1-chains form 10 different αβ heterodimers that mediate binding to collagen, fibronectin, laminin, vitronectin, and VCAM-1, a cell adhesion molecule. Coordinate control between maturation of α and β1 integrin chains has been indicated by several studies. In mesenchymal cells iodination of surface proteins followed by immunoprecipitation of either a specific α integrin or β1 integrin has demonstrated that the mature forms of α2, α3, α5, or α6 integrin chains co-immunoprecipitate with mature β1 integrin chains (29, 34). Furthermore, an increase in conversion of β1 integrin precursor to the mature form led to an increase in the assembly of β1 with mature α, showing coordinate regulation of maturation. The immature integrin chains β1 and α6 each transiently associate with the chaperone calnexin prior to assembly of the mature integrin chains to heterodimers in human kidney epithelial 293 cells (35) or human epidermal keratinocytes (32).

In the current study we observed blocked maturation of β1 integrin in each of three independently cloned oncogenic Ki-ras transfectants and in none of three independently cloned oncogenic Ha-ras transfectants. Blocking expression of the Ki-ras gene by a transient 4-h transfection of an antisense oligonucleotide partially restored the normal maturation pattern, demonstrating that expression of the oncogenic Ki-ras gene inhibited β1 integrin glycosylation. The faster-migrating β1 integrin forms seen in oncogenic Ki-ras transfectants and the mature β1 integrin species found in control cells yielded products of the same size when digested with endoglycosidase F or when cells were pretreated with the N-glycosylation inhibitor tunicamycin. These results suggested that oncogenic Ki-ras blocked post-translation N-glycosylation of β1 integrin at some intermediate point in maturation.

Most of the immature β1 integrin molecules remain in the endoplasmic reticulum. After translocation to the cis-Golgi complex, immature forms of integrin β1-chains may be processed by multiple glycosyltransferases including branching enzymes, such as GlcNAc-T III, IV, and V, and elongating enzymes, such as β1,4-galactosyltransferases and β1,3-GlcNAc-T. Twelve potential N-glycosylation sites (Asn-X-(Ser/Thr) sequences) occur in β1 integrin. N-Glycosylation of both the α and β subunits of the integrin receptor in the Golgi is essential for the association of the heterodimer and for its optimal function (30). Removal of GlcNAc residues from purified integrin
receptor or from K562 cells bearing such receptors dissociated the heterodimer, preventing co-immunoprecipitation of α and β1 integrin chains. The N-glycosylation of precursor β1-integrin chains, in particular the addition of GlcNAc residues, is thus an essential component of their maturation. Partial elimination of N-linked structures located at the surface domain of integrin β1 abolishes adhesive function, mainly by dissociating the heterodimer, suggesting that only a few carbohydrate chains are essential (30). During suspension-induced terminal differentiation of keratinocytes, the maturation of the β1 subunit and the associated α subunits was prevented by blocking the N-linked glycosylation of both integrin chains (36). We do not know the biochemical nature of the glycosylation step altered by expression of oncogenic Ki-ras in HD6-4 cells. However, N-acetylgalactosaminyltransferase III (GlcNAc-T III) catalyzes the addition of a bisecting GlcNAc structure that inhibits further processing of oligosaccharides by other glycosyltransferases (37). Thus activation of GlcNAc-T III or a similar transferase could block β1 integrin processing. Analysis of the biochemical nature of the β1 integrin form found in Ki-ras transfectants should allow us to define the pathways altered by expression of this oncogene.

 Oncogenic forms of ras genes have been implicated in modulating various Golgi N-acetylgalactosaminyltransferases in different cell types. β1–6 N-acetylgalactosaminyltransferase V (GlcNAc-T V) is increased severalfold in activity in rodent fibroblast lines transfected with the oncogenic T24 Ha-ras gene (38). Expression of transfected GlcNAc-T V in MvLu lung epithelial cells led to an acquisition of tumorigenicity and an increase in the apparent molecular weights of α5, αv, and β1 integrins, indicating that the oligosaccharides on these integrins are substrates for GlcNAc-T V (39). NIH3T3 cells expressing the N-ras proto-oncogene exhibited larger cell surface asparagine-linked glycans. Possibly this increase was caused by 5–7-fold increases in the activities of the levels of elongating β1-galactosyltransferase and β3-N-acetylgalactosaminyltransferase, both of which synthesize polyacetylasaminoglycan chains (40). R-ras is a GTP-binding protein highly homologous to Ha-ras but, unlike Ha-ras, induces neither cell proliferation nor differentiation. Transfection of a constitutively active R-ras into myeloid cells grown in suspension activated integrins by a yet unknown mechanism, increasing cell adhesiveness (41). Thus H, N, and R ras genes either modify the activity of various acetylgalactosaminyltransferases which could alter β1 integrin maturation in various cell types or, in the case of R ras in myeloid cells, alter integrin β1-chain function directly. Cell type specificity in the action of different ras isoforms is also deduced from the following studies. Viral Ki-ras prevents the apical polarization of MDCK kidney epithelial cells (15) by decreasing expression of β1 integrin precursor, whereas no decrease in the abundance of mature integrin β1-chain was seen (16). No effects on glycosylation of integrin β1-chain were observed in contrast to the current study with colon epithelial cells, and an aberrant β1 integrin intermediate was not observed in the v-Ki-ras-transformed MDCK cells (16). Transfection of a different ras gene, oncogenic Ha-ras, into another epithelial cell type, CACO2 colon cancer cells, did not lead to loss of growth control or to loss of cell polarization but led instead to induction of multiple markers of normal intestinal brush border differentiation and the capacity for terminal differentiation (42). These studies support our observations in this study that expression of a mutated cellular Ha-ras gene in differentiated colon epithelial cells did not alter cell polarity.

 Studies by other investigators have implicated loss of the α2 integrin chain in colon cancer progression. Low expression of α2β1 integrin collagen receptors has been found in the more aggressive histological subtypes of colon cancers (43). A second group also reported that reduced expression of the α2 integrin subunit was commonly found in both locally invasive and in metastatic colon cancers (44). The decrease in α2 integrin observed in these colon cancers may be caused by impaired maturation of β1 integrin, since maturation of α integrin is regulated in part by the size of the intracellular β1 integrin precursor pool (45). Expression of β1-integrin antisense RNA decreased synthesis of the β1-integrin, decreasing the size of the precursor pool. The β1 integrin precursors that were synthesized exhibited accelerated maturation, whereas the α integrin precursors remained in the Golgi, yielding fewer mature α integrin molecules. Therefore, the impaired β1 integrin maturation we observed in this study could contribute to the decreased α2 integrin expression seen by others in colon cancers.

 Additional clinical evidence has linked aberrant β1 integrin processing to colon cancer progression. Alteration of the ratio of the β1 integrin precursor to the mature form of β1 integrin is found in invasive and metastatic colorectal cancers (46). Fifteen of 19 colorectal cases (79%) with alteration of β1 integrin processing showed lymph node metastases compared with 12 of 32 (38%) without detectable alterations in β1 integrin processing (p < 0.01). Cancer invasion beyond the muscularis propria was observed in all 19 cases with alterations in the ratio of β1 integrin precursor to mature form. The mechanism for the alteration in β1 integrin protein processing in colorectal cancer is not known. We suggest, from the results of the current study, that the mutation in the cellular Ki-ras gene found in a large subset of colon cancers may prevent the normal processing of β1 integrin precursor and lead to characteristic areas of cell multilayering or dysplasia. Expression of the oncogenic Ki-ras gene in benign colon tumors is also seen within areas of dysplasia where cells form multilayers (47). In the current study, the oncogenic Ki-ras HD6-4 cell transfectants also formed multilayers when grown in transwells (Fig. 3). Mutations of both Ki-ras and Ha-ras genes may occur randomly within the colon. However, the specificity of the effects of oncogenic Ki-ras on colon epithelial cell polarization through impairment of β1 integrin maturation may lead to selection of cells bearing Ki-ras mutations. Selection of colon adenoma cells with oncogenic Ki-ras mutations is seen during the growth of benign tumors (47), and this selection is maintained in the transition to malignancy and during further progression of the cancer cells to highly invasive and metastatic states (48, 49). Tumor cells freed from tight cell to cell and cell to substratum adherence because of the synthesis of aberrant β1 integrin could have a selective advantage in both growth and invasion and become the dominant cell type within a tumor. It has been observed that cells with oncogenic Ki-ras mutations form as foci within benign tumors but rapidly become the sole tumor cell type when benign tumors progress to malignancy (47).

REFERENCES

1. Lowy, D., and Willumsen, B. (1993) Annu. Rev. Biochem. 62, 851–891
2. Levers, S., Paterson, H., and Marshall, C. (1994) Nature 369, 411–414
3. Boguski, M., and McCormick, F. (1993) Nature 366, 643–654
4. Cahill, M. A., Janknecht, R., and Nordheim, A. (1996) Curr. Biol. 6, 16–19
5. Davis, R. J. (1995) J. Biol. Chem. 268, 14503–14506
6. Dennis, J. W., Kosh, K., Bryce, D. M., and Breitman, M. L. (1989) Oncogene 4, 855–860
7. Burchill, S., Neal, D., and Lance, J. (1994) J. Urol. 153, 516–521
8. Sandberg, A., and Berger, C. (1994) J. Urol. 154, 545–560
9. Smit, V., Boot, A., Smits, A., Flueren, G., Cornelisse, C., and Bos, J. (1988) Nature 337, 1237–1241
10. Bos, J., Fearon, E., Hamilton, S., Verlaan-de Vries, M., van Boom, J., van der Eb, A., and Vogelstein, B. (1987) Nature 327, 293–297
11. Forrester, K., Almoquera, C., Han, K., Grizzle, W. E., and Perucchi, M. (1987) Nature 327, 298–303
12. Bos, J., Verlaan-de Vries, M., van der Eb, A. J., Janssen, J., Delwel, R., Lowenberg, B., and Colly, L. (1987) Blood 69, 1237–1241
13. Chen, G., Oh, S., Mania, B. P., and Stacey, D. W. (1996) J. Biol. Chem. 271, 30935

β1 Integrin Maturation Blocked by Oncogenic Ki-ras, Not Ha-ras
β1 Integrin Maturation Blocked by Oncogenic Ki-ras, Not Ha-ras

28259–28265

14. Augenlicht, L., Augeron, C., Yander, G., and LaBoisse, C. (1987) Cancer Res. 47, 3763–3765
15. Schoenberger, C.-A., Zuk, A., Kendall, D., and Matlin, K. (1991) J. Cell Biol. 112, 873–889
16. Schoenberger, C.-A., Zuk, A., Zinkl, G., Kendall, D., and Matlin, K. (1994) J. Cell Sci. 107, 527–541
17. Eaton, S., and Simons, K. (1995) Cell 82, 5–8
18. Kahn, S., Yamamoto, F., Almoguera, C., Winter, E., Forrester, K., Jordano, J., and Perou, C. (1997) Anticancer Res. 17, 639–652
19. Hafez, M., Hsu, S., Yan, Z., Winawer, S., and Friedman, E. (1992) Cell Growth Differ. 3, 753–762
20. Hafez, M., Inflante, D., Winawer, S., Friedman, E., Yan, Z., Huang, F., and Friedman, E. (1990) Cell Growth Differ. 1, 617–626
21. Huang, F., Hsu, S., Winawer, S., and Friedman, E. (1994) Oncogene 9, 3701–3706
22. Yan, Z., Winawer, S., and Friedman, E. (1994) J. Biol. Chem. 269, 13231–13237
23. Buday, L., and Downward, J. (1995) Mol. Cell. Biol. 15, 1903–1910
24. Yan, Z., Hsu, S., Winawer, S., and Friedman, E. (1992) Oncogene 7, 801–805
25. Lee, H., Ghose-Dastidar, J., Winawer, S., and Friedman, E. (1995) J. Biol. Chem. 268, 5255–5263
26. Robbins, D., Zhen, E., Cheng, M., Xu, S., Ebert, D., and Cobb, M. (1994) Adv. Cancer Res. 63, 93–116
27. Wang, A., Ojakian, G., and Nelson, W. (1990) J. Cell Sci. 95, 153–156
28. Ojakian, G., and Schwimmer, R. (1991) J. Cell Sci. 107, 561–576
29. Heino, J., Ignotz, R., Hemler, M., Crouse, C., and Massague, J. (1993) J. Biol. Chem. 264, 380–388
30. Zheng, M., Fang, H., and Hakamori, S. (1994) J. Biol. Chem. 269, 12325–12331
31. Akita, K., Yamada, S., and Yamada, K. M. (1989) J. Biol. Chem. 264, 18011–18018
32. Hotchin, N., Gandarillas, A., and Watt, F. (1995) J. Cell Biol. 128, 1209–1219
33. Hynes, R. (1999) Cell 100, 11–25
34. Wayner, E., and Carter, W. (1997) J. Cell Biol. 105, 1873–1884
35. Lenter, M., and Vestweber, D. (1994) J. Biol. Chem. 269, 12263–12268
36. Hotchin, N., and Watt, F. M. (1992) J. Biol. Chem. 267, 14852–14858
37. Miyoshi, E., Ibara, H., Hayashi, N., Fusamoto, H., Kamada, T., and Taniguchi, N. (1995) J. Biol. Chem. 270, 28311–28315
38. Dennis, J., Laferte, S., Waghorne, C., Breitman, M., and Kerbel, R. (1987) Science 236, 582–585
39. Demetriou, M., Nabi, I., Coppolino, M., Dedhar, S., and Dennis, J. (1995) J. Cell Biol. 130, 383–392
40. Easton, E. W., Bolscher, J. G., and van den Eijnden, D. H. (1991) J. Biol. Chem. 266, 21874–21880
41. Zhang, Z., Vuori, K., Wang, H.-G., Reed, J., and Roulaht, E. (1996) Cell 85, 61–69
42. Celano, P., Berchtold, C. M., Mabry, M., Sidransky, D., Casero, R. A., Jr., and Lopu, R. (1995) Cell Growth Differ. 4, 341–347
43. Pigattelli, M., Smith, M. E. F., and Bodmer, W. F. (1990) Br. J. Cancer 61, 636–638
44. Korpetz, K., Schlag, P., Boumsell, L., and Muller, P. (1991) Am. J. Pathol. 138, 741–750
45. Koivisto, L., Heino, J., Hakkila, L., and Larjava, H. (1994) Biochem. J. 300, 771–779
46. Fujita, S., Watanabe, M., Kubota, T., Teramoto, T., and Kitajima, M. (1995) Cancer Lett. 91, 145–149
47. Shibata, D., Schaeffer, J., Li, Z.-H., Capella, G., and Perou, M. (1993) J. Natl. Cancer Inst. 85, 1058–1063
48. Finkelstein, S., Sayegh, R., Bakker, A., and Swalsky, P. (1993) Arch. Surg. 128, 526–532
49. Suchy, B., Zietz, C., and Rabes, H. (1992) Int. J. Cancer 52, 30–33