Altered Growth of Human Colon Cancer Cell Lines Disrupted at Activated Ki-*ras*

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Point mutations that activate the Ki-*ras* proto-oncogene are present in about 50 percent of human colorectal tumors. To study the functional significance of these mutations, the activated Ki-*ras* genes in two human colon carcinoma cell lines, DLD-1 and HCT 116, were disrupted by homologous recombination. Compared with parental cells, cells disrupted at the activated Ki-*ras* gene were morphologically altered, lost the capacity for anchorage-independent growth, grew more slowly both in vitro and in nude mice, and showed reduced expression of c-*myc*. Thus, the activated Ki-*ras* gene plays a key role in colorectal tumorigenesis through altered cell differentiation and cell growth.

Colorectal tumorigenesis in humans is associated with multiple genetic alterations, including activation of the Ki-*ras* proto-oncogene (*1, 2*) and inactivation of the tumor suppressor genes *p53* (*3, 4*), *DCC* (*5*), *MCC* (*6*), and *APC* (*7*). Some colorectal tumors also display a deregulated expression of c-*myc* (*8–10*) that is not attributable to DNA amplification or rearrangement. Progressive accumulation of these genetic alterations reflects the clinical and histopathological progression of colorectal tumors (*11*), but the function and hierarchy of the altered genes have not been elucidated. The product of *ras*, $p21^{ras}$, catalyzes the hydrolysis of guanosine triphosphate to guanosine diphosphate and may control cell proliferation by regulating signal transduction pathways.

To elucidate the role of Ki-*ras* in tumorigenesis, we used gene-targeting methods (*12–14*) to disrupt the activated allele in human colon cancer cell lines (HCCLs). We studied DLD-1 cells [American Type Culture Collection (ATCC) CCL 221] because they contain one normal Ki-*ras* allele and one Ki-*ras* allele with a point mutation at codon 13 that converts Gly^{13} to Asp^{13} in $p21^{ras}$. We detected no mutations in H-*ras* or N-*ras* at codons 12, 13, or 61 but did detect a point mutation in *p53* that converts Ser^{241} to Phe^{241} in the p53 protein (*15*).

To disrupt Ki-*ras* in DLD-1 cells, we constructed an NT cassette (Fig. 1A) composed of the *Escherichia coli* neomycin resistance (*neo*) gene (*16*) and the herpes simplex virus thymidine kinase (*tk*) gene (*17*). The *neo* gene contains no promoter sequences and therefore is inactive, whereas *tk* includes promoter sequences and is active (*18*). The plasmid pBKCalu-1 (Fig. 1C) includes 6.7 kb of the Ki-*ras* gene from the human lung carcinoma cell line Calu-1, which contains a point mutation that converts Gly^{12} to Cys^{12} (*19*). The *ras* sequences in the targeting vector pBKNT-D (Fig. 1D) are interrupted in exon 1 by a 4-kb fragment (*13*) of the NT cassette (*20*). The *neo* gene serves as a positive selection marker for cells that have undergone homologous recombination. The diphtheria toxin fragment-A (*DT-A*) gene (*14*), which lies outside the Ki-*ras* homology region, is a negative selection marker that facilitates enrichment of cells that have undergone homologous recombination. The *tk* gene, which functions only in the presence of the antiviral agent ganciclovir, provides a negative selection marker that facilitates replacement of the disrupted Ki-*ras* by other gene fragments.

The DLD-1 cells were transfected with the targeting vector pBKNT-D by the lipopolyamine-coated DNA method (*21*) and then grown in the presence of G418 (*22*). Of the 1×10^7 cells transfected with pBKNT-D, 24 gave rise to G418-resistant colonies. DNA was extracted from these colonies and screened for integration of the targeting vector by site-specific oligonucleotide (SSO) hybridization analysis (*4, 23*), a polymerase chain reaction (PCR)–based method. One of the primers used for amplification was upstream of the Stu I site where the NT cassette was inserted, and the other was in Ki-*ras* exon 1, starting at codon 31. With this approach, the amplified fragments should be derived only from the nondisrupted allele because the predicted PCR products of the disrupted allele or the targeting vector would be too long (4 kb) to amplify. If homologous recombination occurred at the mutant Ki-*ras* allele, the PCR products should hybridize with the Gly^{13}-SSO probe and not with the Asp^{13}-SSO probe. Conversely, if homologous recombination occurred at the normal Ki-*ras* allele, the reverse hybridization patterns would be expected (*24*). Nonhomologous recombination events would be indicated by hybridization with both probes (Fig. 2A). Seven of 24 G418-resistant colonies showed hybridization patterns that suggest homologous recombination. Three of these cell clones (DKO-3, DKO-4, and DKs-8) were disrupted at the mutant allele (HR-M clones) and four were disrupted at the normal allele (HR-N clones, such as DKO-1). All of the HR-M clones were altered morphologically, whereas the HR-N clones showed no morphological changes.

To confirm that homologous recombination had occurred in these clones, we performed Southern (DNA) blot analyses (*25*) (Fig. 2B). Samples of DNA from the parental cell line and from the derivative clones were digested with Bam HI and hybridized with ^{32}P-labeled probes for Ki-*ras* (Fig. 1B), *neo* (Fig. 1E), and *tk* (Fig. 1E). A clone with properties that suggest nonhomolo-

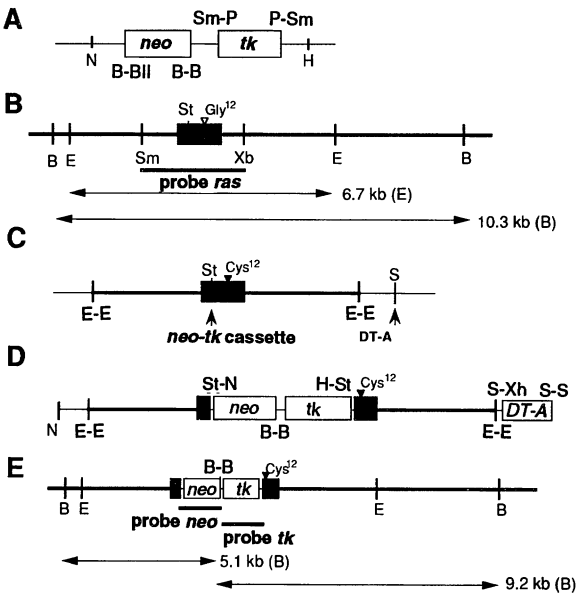

Fig. 1. Constructs used in the targeted disruption of the human Ki-*ras* gene. (**A**) Structure of the NT cassette. (**B**) Partial restriction enzyme map of the Ki-*ras* gene. (**C**) Structure of pBKCalu-1. (**D**) Structure of the disruption vector, pBKNT-D. (**E**) Predicted structure of the disrupted Ki-*ras* locus. Bold lines, Ki-*ras* genomic sequences; thin lines, plasmid sequences; black box, exon 1 of Ki-*ras*; double arrows, predicted fragments from each enzyme digestion (with sizes indicated on the right); B, Bam HI; BII, Bgl II; E, Eco RI; H, Hind III; N, Not I; P, Pvu II; S, Sal I; Sm, Sma I; St, Stu I; Xb, Xba I; Xh, Xho I; *DT-A*, diphtheria toxin fragment A; inverted open triangle, normal codon; inverted solid triangle, mutated codon.

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gous recombination, DKs-5, was also examined as a control. A restriction enzyme map (Fig. 1B) was determined for a Ki-*ras* gene clone that was isolated from a genomic library (JCRB; LI018) (*26*); this map was consistent with a previously reported map (*19*). In the parental line, a 10.3-kb fragment was detected with the Ki-*ras* probe. In all HR-M and HR-N clones, additional fragments of 9.2 and 5.1 kb were detected (Fig. 1E), which hybridized with the *tk* and *neo* probes, respectively (*27*). Probes for *DT-A* did not hybridize with the analyzed cell lines (*27*). Expression of the *tk* gene was confirmed by the observation that these clones did not grow in medium that contained ganciclovir.

We next examined expression of Ki-*ras* by reverse transcriptase (RT) PCR (*4*) and by SSO-probe hybridization. First-strand cDNA was generated from total cellular RNA, and amplification of Ki-*ras* cDNA was performed by PCR (*28*). SSO probes for Gly^{12}, Cys^{12}, Gly^{13}, and Asp^{13} were used (*28*). The HR-M clones hybridized with SSO probes for Gly^{12} and Gly^{13}, whereas the HR-N clones hybridized only with the SSO probe for Asp^{13} (Fig. 2C); this hybridization pattern provides additional evidence for homologous recombination.

Examination of the parental DLD-1 cells and of an HR-M clone (DKs-8) by light microscopy revealed clear morphological differences between the two (Fig. 3). The HR-M cells aggregated into tubule-like structures, a morphology like that seen with the less malignant VACO 235 colon adenoma cell line (*29*). Other HR-M clones had a morphology similar to that of DKs-8 (Table 1). The parental DLD-1 cells, by contrast, were attached to one another in large clusters.

The in vitro doubling time of all three HR-M lines was about twice that of the DLD-1 cells. In contrast, the doubling time of the control DKs-5 cell line (which had undergone nonhomologous recombination; NHR clone) and of the HR-N lines was similar to that of the DLD-1 cells (Fig. 4). We also assayed anchorage-independent growth by seeding cells in semisolid agar (*30*). None of the HR-M lines formed colonies under these conditions (Table 1). These observations, together with the morphology of the cells, indicate that the HR-M lines regained important characteristics of normal growth in vitro.

We then compared the growth of the DLD-1 and HR-M lines in nude mice (*31*). The DLD-1 formed palpable tumors in all mice within 1 week after subcutaneous injection, and the tumors developed rapidly to a volume of 1500 mm^3 at 3 weeks. The HR-M lines produced no palpable tumors over a period of 2 months after the inoculation (Table 1).

To examine whether targeted disruption of the activated Ki-*ras* gene would alter the growth of other HCCLs, we repeated these experiments on HCT 116 cells (ATCC CCL 247). These cells contain a point mutation in Ki-*ras* that converts Gly^{13} to Asp^{13} as well as an insertion mutation in the *DCC* tumor suppressor gene (*5*). We transfected the HCT 116 cells with pBKNT-D and grew them in the presence of G418. Of the 2×10^7 cells transfected

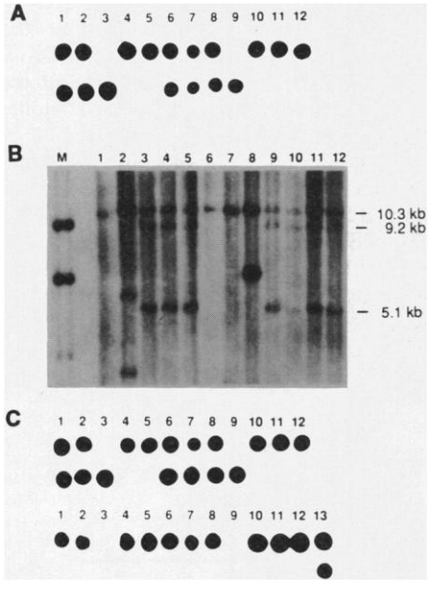

Fig. 2. Screening for disruption of the activated Ki-*ras* gene. (**A**) PCR-SSO analysis with probes for (top row) Gly^{13} and (bottom row) Asp^{13}. (**B**) Southern blot hybridization analysis with probes for Ki-*ras* (Fig. 1B). (**C**) Expression of normal and activated Ki-*ras* genes detected by RT-PCR analysis with probes for (row 1) Gly^{13}, (row 2) Asp^{13}, (row 3) Gly^{12}, and (row 4) Cys^{12}. The numbers above the gel lanes correspond to the cell lines as follows: 1, DLD-1; 2, DKs-5; 3, DKO-1; 4, DKO-3; 5, DKs-8; 6, HCT 116; 7, HCT 116 transfected with NT cassette; 8, HK2-10 (NHR clone); 9, HK2-6; 10, HK2-8; 11, HKe-3; 12, HKh-2; and 13, SW837 (ATCC CCL 235) containing a point mutation that converts Gly^{12} to Cys^{12} (used as a control); M, bacteriophage λ DNA digested with Hind III.

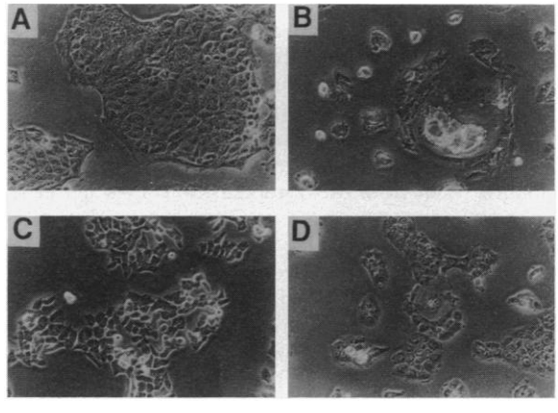

Fig. 3. Comparative morphology of parental cells and HR-M clones. (**A**) DLD-1 cells; (**B**) DKs-8 cells, a derivative of DLD-1 that is disrupted at the activated Ki-*ras* allele; (**C**) HCT 116 cells; and (**D**) HKe-3 cells, a derivative of HCT 116 that is disrupted at the Ki-*ras* allele. Phase contrast; magnification ×50.

Table 1. Properties of parental cells and Ki-*ras* disrupted clones.

Cell line	Ki-*ras* disruption*	Morphological alterations	Tumorigenicity in nude mice†	Soft agar cloning efficiency‡	Expression of c-*myc*§
DLD-1	Parental		5/5	20.0	3.6
DKs-5	NHR	−	5/5	21.8	3.9
DKO-1	HR-N	−	5/5	24.0	3.6
DKO-3	HR-M	+	0/7	0.0	0.3
DKO-4	HR-M	+	0/7	0.0	0.4
DKs-8	HR-M	+	0/7	0.0	0.8
HCT 116	Parental		5/5	18.6	10.6
HK2-10	NHR	−	3/3	19.3	9.2
HK2-8	HR-M	+	0/4	2.1	1.1
HKe-3	HR-M	+	0/7	0.9	0.3
HKh-2	HR-M	+	0/7	0.0	1.1

*NHR, nonhomologous recombination; HR-N, homologous recombination at normal Ki-*ras* allele; HR-M, homologous recombination at mutant Ki-*ras* allele. †The fraction of mice showing tumors after 2 months. ‡Colonies were scored after 3 weeks; each value represents the percent, averaged from duplicate determinations. §Expression was determined by the relative radioactivity measurement with the Bio-Image-Analyzer (BAS2000; FUJIX, Kanagawa, Japan) of c-*myc* and β-tubulin mRNA bands on Northern blots. The ratio of these mRNAs in normal colon mucosae was 0.4 to 0.6.

with pBKNT-D, 93 produced G418-resistant colonies (*22*). These clones were screened for homologous recombination by PCR-SSO hybridization analysis (*24*), Southern blot analysis (*25*), and RT-PCR-SSO (*28*) analysis (Fig. 2). Three of 93 clones were disrupted at the mutant Ki-*ras* allele and five were disrupted at the normal Ki-*ras* allele (HR-N clones such as HK2-6). Like the HR-M derivatives of the DLD-1 cells, all three HR-M clones (HK2-8, HKe-3, and HKh-2) of HCT 116 tended to aggregate into tubule-like structures. The parental HCT 116 cells, by contrast, were not well attached to one another (Fig. 3). The doubling times in vitro of the HKe-3 and HKh-2 lines were extended by a factor of 2 and 3, respectively, compared to that of the HCT 116 cells (Fig. 4). For reasons we do not yet understand, the growth rate of the HK2-8 cells was similar to that of the HCT 116 cells; this clonal variation may be due to the difference in expression of other genes that control cell growth in vitro. The capacity of the HR-M clones for anchorage-independent growth in semisolid agar was 1/10 to 1/500 that of the HCT 116 cells, and none of the HR-M clones formed palpable tumors in nude mice 2 months after inoculation (Table 1). The HK2-8 cells did not form tumors in nude mice, despite their rapid growth in vitro.

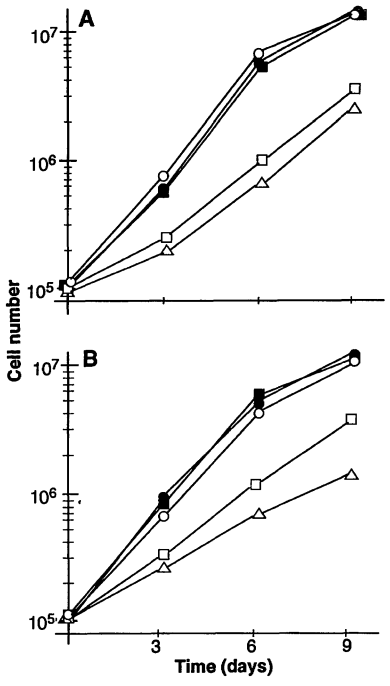

Fig. 4. Growth curves of parental cells, control cells, and HR clones in DMEM containing 10% FCS. Cells (1×10^5) were seeded into nine replicate 60-mm dishes. Cells in three dishes were trypsinized and counted, and the medium was changed every 3 days thereafter. Data points are the average of triplicate determinations. (**A**) Solid circles, DLD-1; solid squares, DKs-5; open circles, DKO-1; open squares, DKO-3; and open triangles, DKs-8. (**B**) Solid circles, HCT 116; solid squares, HK2-10; open circles, HK2-8; open squares, HKe-3; and open triangles, HKh-2.

To explore potential links between Ki-*ras* and other regulated signal transduction pathways, we examined expression of the c-*myc* gene in the DLD-1 and HCT 116 cells and in the derivative clones (*32*). The amount of c-*myc* RNA was less in the HR-M clones than in the parental cells (Table 1). This observation is consistent with the hypothesis that the activated Ki-*ras* product interacts with pathways that are involved in induction of c-*myc* expression.

Our results suggest that the activated Ki-*ras* gene is directly responsible for the malignant phenotype of the DLD-1 and HCT 116 colon cancer cell lines. The coexisting mutations in the *p53* gene (DLD-1 cells) or in the *DCC* gene (HCT 116 cells) are in themselves not sufficient to produce tumors in nude mice. Tumorigenicity in nude mice seems to depend on alteration of Ki-*ras* and perhaps a concomitant deregulation of c-*myc*. Additional genetic alterations may be involved in deregulation of c-*myc* expression (*10*) and tumor progression in colorectal tumors. The only distinction between the parental cell lines and the HR-M lines, except expression of the *neo* and *tk* genes, was expression of the activated Ki-*ras* gene. Thus, the use of these cell lines in studies of oncogene and tumor suppressor gene cooperation may help to elucidate further the molecular mechanisms that underlie colorectal tumorigenesis. The HR-M lines described here, which can be easily grown in vitro but show no tumorigenicity in nude mice, may also be useful for the investigation of other genetic alterations that are associated with the malignant phenotype.

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18. The *neo* gene was derived from pSV2-neo (*16*) by digestion with Bgl II and Bam HI, and the HSV *tk* gene was derived from pAG0 (*17*) by digestion with Pvu II. The 2-kb Pvu II fragment encompassing the *tk* gene was inserted into pBluescript SK+ (pBSK; Stratagene) at the Sma I site, and the 2-kb Bgl II–Bam HI fragment encompassing the *neo* gene was inserted at the Bam HI site. The orientation of transcription was the same for the *neo* and *tk* genes.
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20. The plasmid pLC8 contains a 6.7-kb Eco RI fragment that encompasses Ki-*ras* genomic sequences (including exon 1) from Calu-1 cells; this gene has a mutation at codon 12 that converts Gly^{12} to Cys^{12} (*19*). The Eco RI fragment was inserted into pBSK at the Eco RI site to construct pBKCalu-1. In the targeting vector for disruption (pBKNT-D), the NT cassette (a 4-kb fragment that was generated by digestion with Not I and Hind III) was end-filled with Klenow polymerase and ligated to the Stu I site in exon 1 upstream of the initiation codon ATG of pBKCalu-1. The gene that encodes the diphtheria toxin fragment A [*DT-A*; a 0.9-kb Xho I–Sal I restriction fragment with no poly(A) signal (*14*)] was inserted at the Sal I site downstream of the Ki-*ras* fragment.
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22. The HCT 116 and DLD-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Twelve hours after seeding, approximately 10^6 cells on 100-mm plates were transfected with pBKNT-D DNA (12 μg) that had been linearized by Not I and Sal I digestion and then coated with lipopolyamine (TRANSFECTAM; IBF Biotechnics, Columbia, MD) (*21*). After 2 days, G418 (800 μg ml^{-1}) was added to the medium, which was changed every 2 days for 14 to 18 days. Independent colonies were then trypsinized in cloning cylinders and transferred to 24-well plates. The clones were expanded to stable lines in medium that contained G418 (600 μg ml^{-1}).
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24. To screen recipient cells for homologous recombination events by PCR, we synthesized two primers: PKF1 (5′-TGTGACATGTTCTAATATAGTC-3′) was specific for the first intron (between exon ϕ and exon 1) (*33*) of Ki-*ras*, located about 30 bp upstream of the Stu I site in exon 1; PKR1 (5′-CGTCCACAAAATGATTCTGAAT-3′) was located in exon 1 starting at codon 31. PCR was performed as described (*4*); the reaction mixtures (50 μl) contained 50 pmol each of the PKF1 and PKR1 primers and were subjected to 30 cycles of amplification (96°C for 60 s, 50°C for 80 s, and 72°C for 40 s). The predicted PCR product (149 bp) was visualized by ethidium bromide staining after gel electrophoresis. The SSO probes for normal (Gly) and (Asp) mutated sequences at codon 13 of the Ki-*ras* gene were Gly^{13}-SSO (5′-GGAGCTGGTGGCGTAGGCAAG-3′) and Asp^{13}-SSO (5′-GGAGCTGGTGACGTAGGCAAG-3′), respectively. Amplified DNA fragments (2 μl) were dot-blotted onto nylon membranes, which were then hybridized with ^{32}P-labeled SSO probes and washed as described in (*4*), except that the hybridization and washing temperatures were 59° and 62°C, respectively.
25. Genomic DNAs (10 μg) from parental cells and transfected clones were digested with Bam HI and hybridized with probes for the Ki-*ras* gene

(Sma I–Xba I fragment), the *neo* gene (Bgl II–Bam HI fragment), and the *tk* gene (Pvu II fragment) (Fig. 1). The DNA was subjected to electrophoresis in a 0.6% agarose gel, transferred to nylon filters, and hybridized as described [M. Sasaki *et al.*, *Cancer Res.* **49**, 4402 (1989)]. Because the Sma I–Xba I fragment of the Ki-*ras* gene contains repetitive sequences, the probe DNA was prehybridized with a large excess of human placental DNA to block hybridization to the repetitive region of the probe [P. G. Sealey, P. A. Whittaker, E. M. Southern, *Nucleic Acids Res.* **13**, 1905 (1985)].

26. We performed restriction enzyme mapping on human genomic Ki-*ras* DNA that was isolated from a genomic library (LI018; JCRB) by screening with human Ki-*ras* genomic DNA (2.2-kb Xba I–Eco RI fragment).
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28. First-strand cDNA was generated from total RNA (10 μg) with oligo dT (21-mer). The Ki-*ras* cDNA was amplified by PCR for 30 cycles (96°C for 60 s, 56°C for 60 s, and 72°C for 40 s) with primers PKF2 and PKR2 (*4*). Primer PKF2 (5′-ATGACTGAATATAAACTTTGTGG-3′) was located in exon 1, starting at initiator codon 1 (ATG) 11 bp downstream of the Stu I site. Primer PKR2 (5′-ACAAAGAAAGCCCTCCCCAG-3′) was located in exon 2, which is 12 kb from exon 1 in the Ki-*ras* gene (*19, 33*). The PCR products were shown to be the predicted length (239 bp) by gel electrophoresis and ethidium bromide staining. The SSO probes for normal (Gly) and mutated (Cys) sequences at codon 12 of Ki-*ras* were Gly^{12}-SSO (5′-GTTGGAGCTGGTGGCGTA-3′) and Cys^{12}-SSO (5′-GTTGGAGCTTGTGGCGTA-3′), respectively. The sequences of the Gly^{13}-SSO and Asp^{13}-SSO are shown in (*24*). Dot blot hybridization of the Gly^{13}-SSO and Asp^{13}-SSO probes to the amplified cDNA fragments was performed as described in (*24*). Hybridization with the Gly^{12}-SSO and Cys^{12}-SSO probes was performed as described in (*4*). Hybridization and washing temperatures were 56° and 59°C, respectively.
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31. For tumorigenicity assays, 10^7 cells suspended in DMEM that contained 10% FCS (0.2 ml) were injected subcutaneously into the flanks of 5-week-old nude mice.
32. Expression was determined by Northern (RNA) blot analysis. Total cellular RNA (15 μg) was separated by electrophoresis in a 1% agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized with a probe for c-*myc* (Cla I–Eco RI fragment in exon 3) as described in (*9*). The nitrocellulose filter was subsequently washed to remove the c-*myc* probe and was rehybridized with a probe for human β tubulin (β2) cDNA [S. A. Lewis, M. E. Gilmartin, J. L. Hall, N. J. Cowan, *J. Mol. Biol.* **182**, 11 (1985)] as an internal control.
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14 September 1992; accepted 7 December 1992

Phosphatidylinositol 3-Kinase Encoded by Yeast *VPS34* Gene Essential for Protein Sorting

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The *VPS*34 gene product (Vps34p) is required for protein sorting to the lysosome-like vacuole of the yeast *Saccharomyces cerevisiae*. Vps34p shares significant sequence similarity with the catalytic subunit of bovine phosphatidylinositol (PI) 3-kinase [the 110-kilodalton (p110) subunit of PI 3-kinase], which is known to interact with activated cell surface receptor tyrosine kinases. Yeast strains deleted for the *VPS*34 gene or carrying *vps*34 point mutations lacked detectable PI 3-kinase activity and exhibited severe defects in vacuolar protein sorting. Overexpression of Vps34p resulted in an increase in PI 3-kinase activity, and this activity was specifically precipitated with antisera to Vps34p. *VPS*34 encodes a yeast PI 3-kinase, and this enzyme appears to regulate intracellular protein trafficking decisions.

A variety of peptide growth factors and hormones, such as platelet-derived growth factor (PDGF), nerve growth factor (NGF), and insulin mediate their cellular effects by interaction with cell surface receptor tyrosine kinases. The interaction of these ligands with their cognate receptors induces a series of intracellular signaling events, including stimulation of protein tyrosine kinase activity and the formation and activation of multiprotein complexes at the plasma membrane (*1–3*). These receptor complexes contain enzymes that take part in phosphoinositide metabolism, including phospholipase C (PLC) and PI 3-kinase (*1*). PLC-γ is known to cleave PI 4,5-bisphosphate [$PI(4,5)P_2$] to generate the second messengers diacylglycerol, which activates protein kinase C, and inositol 1,4,5-trisphosphate (IP_3), which can mobilize intracellular Ca (*4*). The association of PI 3-kinase with receptor tyrosine kinases has permitted the identification of a family of phosphoinositides phosphorylated at the 3 position of the inositol ring that appear to act in a signaling pathway distinct from that resulting from turnover of $PI(4,5)P_2$ (*5*). None of the three reaction products of PI 3-kinase—PI 3-monophosphate (PI3P), PI 3,4-bisphosphate [$PI(3,4)P_2$], or PI 3,4,5-trisphosphate [$PI(3,4,5)P_3$]—is a substrate of the known PLC isozymes, suggesting that the lipid compounds themselves function as second messengers in cell signaling (*6, 7*).

Mammalian PI 3-kinase exists as a heterodimer of an 85-kD (p85) and a 110-kD (p110) subunit (*8–10*). The cDNA encoding p110 from bovine brain has been cloned, and the p110 protein has been characterized as the catalytically active subunit of the PI 3-kinase complex (*11*). The p85 subunit is believed to mediate the binding of the p110 subunit to active receptor protein tyrosine kinases through its Src homology 2 (SH2) domains (*12*). This association of p85-p110 with the receptor might lead to the activation of the lipid kinase (*13, 14*). The binding of tyrosine-phosphorylated peptides to the SH2 domains of p85-p110 in vitro stimulates the PI 3-kinase activity fivefold (*2*).

The amino acid sequence deduced from the cDNA sequence of the catalytic subunit of PI 3-kinase from bovine brain is similar to the sequence of the yeast *VPS*34 gene product (*11*), which is essential for the efficient sorting of vacuolar hydrolases in yeast (Fig. 1) (*15*). In eukaryotic cells, vacuolar (lysosomal) enzymes and proteins destined for secretion are transported together through the early stages of the secretory pathway (endoplasmic reticulum and Golgi apparatus) (*16, 17*). Protein transport is mediated by vesicles that bud from one compartment and then dock and fuse with the next compartment in the pathway (*18*). Vacuolar (lysosomal) hydrolases are actively sorted away from proteins destined for the cell surface in a late Golgi compartment (*19, 20*). Mutations in the *VPS*34 gene lead to the missorting and secretion of Golgi apparatus–modified precursor forms of several vacuolar hydrolases, including carboxypeptidase Y (CPY) and proteinase A (PrA) (*15, 17*). Subcellular fractionation experiments have shown that the product of the *VPS*34 gene, Vps34p, is associated through protein-protein interactions with an organelle distinct from the vacuole (*15, 21*).

PI 3-kinase activity is readily detected in yeast cell extracts (*22*). To determine whether *VPS*34 encodes PI 3-kinase, we assayed for PI 3-kinase activity in extracts from wild-type yeast cells, cells with the

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