Functional Analysis of PIK3CA Gene Mutations in Human Colorectal Cancer

Tsuneo Ikenoue,[1,3] Fumihiko Kanai,[1,2] Yohko Hikiba,[3] Toshiyuki Obata,[4] Yasuo Tanaka,[1] Jun Imamura,[1] Miki Ohta,[1] Amarsanaa Jazag,[1] Bayasi Guleng,[1] Keisuke Tateishi,[1] Yoshinari Asaoka,[1] Masayuki Matsumura,[2] Takao Kawabe,[1] and Masao Omata[1]

[1]Department of Gastroenterology, Graduate School of Medicine and [2]Clinical Research Center, University of Tokyo Hospital, Bunkyo-ku; [3]Division of Gastroenterology, Institute for Adult Diseases, Asahi Life Foundation, Nishi-Shinjuku, Shinjuku-ku, Tokyo Japan; and [4]Division of Molecular Genetics, Institute for Enzyme Research, University of Tokushima, Tokushima City, Tokushima Japan

Abstract

Mutations in the *PIK3CA* gene, which encodes the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K), have been reported in human cancers, including colorectal cancer. Most of the mutations cluster at hotspots within the helical and kinase domains. Whereas H1047R, one of the hotspot mutants, is reported to have elevated lipid kinase activity, the functional consequences of other mutations have not been examined. In this study, we examined the effects of colon cancer–associated *PIK3CA* mutations on the lipid kinase activity *in vitro*, activation of the downstream targets Akt and p70S6K *in vivo* and NIH 3T3-transforming ability. Of eight mutations examined, all showed increased lipid kinase activity compared with wild-type p110α. All the mutants strongly activated Akt and p70S6K compared with wild-type p110α as determined by immunoblotting using phospho-specific antibodies. These mutants also induced morphologic changes, loss of contact inhibition, and anchorage-independent growth of NIH 3T3 cells. The hotspot mutations examined in this study, E542K, E545K, and H1047R, all had high enzymatic and transforming activities. These results show that almost all the colon cancer–associated PIK3CA mutations are functionally active so that they are likely to be involved in carcinogenesis. (Cancer Res 2005; 65(11): 4562-7)

Introduction

Phosphatidylinositol 3-kinases (PI3K) are a family of lipid kinases that regulate the signaling pathways involved in cell proliferation and survival, cell motility and adhesion, differentiation, cytoskeletal rearrangement, and intracellular trafficking (1).

Mammalian cells contain multiple isoforms of PI3Ks, which are subdivided into three classes (2). The class I PI3Ks comprise a p110 catalytic subunit and a regulatory adaptor subunit. The catalytic subunits are composed of several modular domains, and most PI3K members share four homologous regions: the catalytic lipid kinase domain, the helical domain, the C2 domain, and the Ras-binding domain (RBD), as well as the NH_2-terminal domain that interacts with the regulatory subunit (3, 4). The catalytic lipid kinase domain exhibits weak homology to protein kinases. The available structural data suggest that the helical domain serves as scaffolding for the other domains. The C2 domain is involved in phospholipid membrane binding.

Class I PI3Ks can be further subdivided into two groups based on their structure and mode of activation: class Ia and class Ib (2). Class Ia PI3Ks are primarily regulated downstream of receptors with intrinsic tyrosine kinase activity, or are associated with nonreceptor tyrosine kinases. The binding of p85 SH2 domains to specific phosphotyrosine residues within the receptors or other signaling proteins activates the PI3K and recruits the cytosolic complex to the plasma membrane. At the membrane, PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) and converts it to phosphatidylinositol-3,4,5-triphosphate (PIP3), which recruits the serine-threonine protein kinases Akt and PDK1 to the membrane. PDK1 consequently phosphorylates and activates Akt, which in turn regulates a number of downstream targets (1). Synergistic with this activation, class Ia PI3Ks are stimulated directly by GTP-bound Ras (5). The ability of activated Ras to stimulate PI3K is important in Ras transformation of mammalian cells and is essential in Ras-induced cytoskeletal reorganization (6).

The isolation of a retroviral oncogene, v-p3k, encoding a homologue of p110α established that PI3K has active role in oncogenic transformation (7). The overexpression of constitutively active p110α has been reported to transform murine and chicken cells (7–9). The oncogenic potential of PI3K is correlated with the activation of the downsteram target Akt, which can also induce transformation (10, 11). Oncogenic transformation by PI3K and Akt depends on downstream targets that control translation, such as p70S6K and eIF4E (12).

Recent findings support the involvement of PI3K in tumorigenesis. Aberrant activation of the PI3K/Akt pathway, including the PTEN mutation and Akt overexpression and gene amplification has been implicated in several human cancers (13, 14). Somatic mutations in the gene for the p85α regulatory subunit of PI3K (*PIK3R1*) have been reported in human colon and ovarian tumors (15). The copy number of the gene for the p110α catalytic subunit of PI3K (*PIK3CA*) is frequently increased in ovarian cancers, resulting in increased PIK3CA transcription, p110α protein expression, and PI3K activity (16).

Recently, a large-scale mutational analysis of the entire coding regions of all the major classes of PI3K revealed somatic mutations in the PIK3CA in a significant fraction (25-30%) of colorectal cancers, gastric cancers, and glioblastomas and in a smaller fraction (<10%) of breast and lung cancers (17). Another report revealed that the PIK3CA mutations were found in 40% of breast cancers and 7% of ovarian cancers (18). The mutations clustered in hotspots within the helical (exon 9) and kinase (exon 20) domains. In that study, H1047R, a hotspot mutation in the kinase domain,

Requests for reprints: Fumihiko Kanai, Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Phone: 81-3-3815-5411; Fax: 81-3-3814-0021; E-mail: kanaif-int@h.u-tokyo.ac.jp.

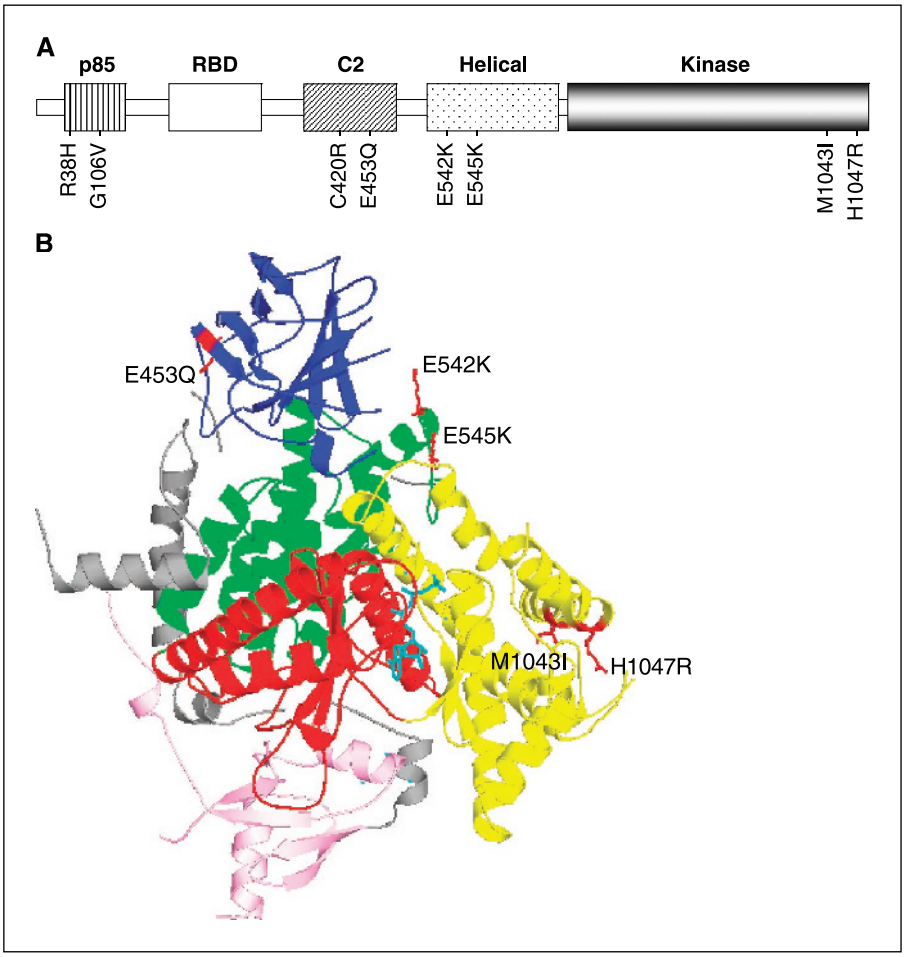

Figure 1. *A,* PIK3CA mutations analyzed in this study. E542K, E545K, and H1047R are reportedly hotspot mutants (11). No cancer-associated RBD mutations have been reported. *B,* three-dimensional locations of mutations in the catalytic subunit of PI3K. The mutations E453Q, E542K, E545K, M1043I, and H1047R are drawn as red sticks in the ribbon diagram of p110γ (3) using the open-source molecular modeling package PyMOL (http://www.pymol.org/). The domains of the catalytic subunit are colored as follows: Ras-binding domain (*pink*), C2 domain (*blue*), helical domain (*green*), N-lobe of catalytic domain (*red*), C-lobe of catalytic domain (*yellow*), ATP (*cyan*), stick and other regions including linker regions (*gray*).

was shown to have an elevated kinase activity compared with the wild type. However, the functional consequences of the other mutations are poorly understood. In this study, we did the functional analyses of PIK3CA mutations within different domains.

Materials and Methods

Constructs. The NH_2-terminally Myc-tagged p110α expression vector pCMV6-Myc-p110α and constitutively active p110α expression vector pCMV6-iSH2p110α were kindly provided by Lewis C. Cantley (Harvard Medical School, Boston, MA). To construct the p110α expression vector with a neomycin-resistant gene, a *Kpn*I/*Sac*I-digested fragment containing Myc-p110α cDNA from pCMV6-Myc-p110α was cloned into *Kpn*I/*Sac*I-digested pCMV3 vector, which was kindly provided by Phillip T. Hawkins (Babraham Institute, Cambridge, United Kingdom). To construct expression vectors for cancer-associated p110α mutants (R38H, G106V, C420R, E453Q, E542K, E545K, M1043I, and H1047R), the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used according to the manufacturer's instructions. Mutations were verified by sequencing both strands of DNA in the region of interest.

Cell lines and transfection. NIH 3T3 cells were cultured in DMEM/10% FCS. Transfections were done using FuGENE6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. To generate NIH 3T3 cells stably expressing the p110α wild type or mutants, the cells were maintained in growth medium containing 400 μg/mL geneticin (Invitrogen, Carlsbad, CA). Multiple geneticin-resistant colonies were then pooled (>50 colonies) for use in soft agar assays, as described previously (19).

Antibodies and reagents. Anti-p70 S6K monoclonal antibody (H-9) and protein A/G agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Myc monoclonal antibody (9B11) and anti-phospho-Akt, anti-Akt, and anti-phospho-p70 S6K polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-p85α monoclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY).

Immunoprecipitation and immunoblotting. HEK293T cells were grown in 3.5-cm dishes and transfected with 2 μg of Myc-tagged wild type or each mutant p110α. Twenty-four hours after transfection, the cells were washed twice with ice-cold PBS and lysed in lysis buffer A [50 mmol/L Tris-HCl (pH 7.4); 1% NP40, 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L phenylmethylsulfonyl fluoride; 1 mmol/L NaF; and 1 mmol/L Na_3VO_4, containing 1 μg/mL each of aprotinin, leupeptin, and pepstatin]. Myc-p110α was immunoprecipitated by anti-Myc coupled to protein A/G-agarose overnight at 4°C, subjected to SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) as previously described (20). The membranes were blotted with anti-p85 antibody overnight at 4°C and then with peroxidase-conjugated secondary antibody for 1 hour at room temperature. The immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England) according to the manufacturer's instructions.

Lipid kinase assay. HEK293T cells in 10-cm dishes were transfected with 10 μg of p110α constructs. The cells were serum-starved for 24 hours and lysed with lysis buffer B [20 mmol/L Tris-HCl (pH 7.4), 1% NP40, 137 mmol/L NaCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 0.1 mmol/L Na_3VO_4, and 1 mmol/L phenylmethylsulfonyl fluoride]. The cell lysates were immunoprecipitated with anti-Myc coupled to protein A/G-agarose for 3 hours at room temperature. The immune complex was washed thrice

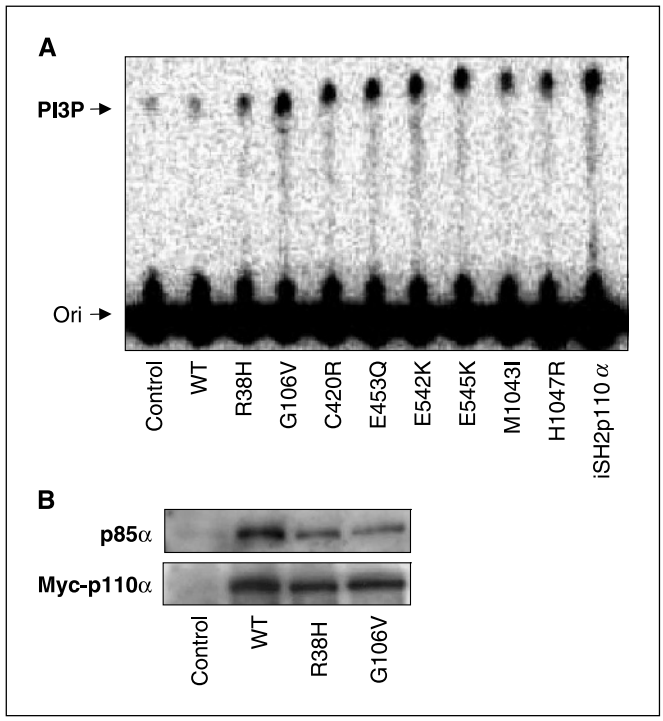

Figure 2. *A, in vitro* lipid kinase activity of p110α mutants. HEK293T cells were transfected with Myc-tagged p110α constructs. After 24 hours, the kinase activities of the immunoprecipitated Myc-p110α proteins were assayed as described in Materials and Methods. The reaction products were analyzed using TLC. Representative experiment. *B,* association of p110α mutants with p85α. HEK293T cells were transiently transfected with empty vector, Myc-p110α wild type, or p85 binding domain mutants R38H and G106V. The cells were lysed, and Myc-tagged p110α was immunoprecipitated with anti-Myc antibody. The immunoprecipitated proteins were analyzed on immunoblots with anti-Myc or anti-p85α antibodies. Representative experiment.

with wash I (PBS containing 1% NP40), thrice with wash II [100 mmol/L Tris-HCl (pH 7.5), 500 mmol/L LiCl, and 0.1 mmol/L Na_3VO_4], and twice with wash III [10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, and 0.1 mmol/L Na_3VO_4]. The beads were resuspended in 70 μL of kinase buffer [8 mmol/L Tris-HCl (pH 7.5), 80 mmol/L NaCl, 0.8 mmol/L EDTA, 15 mmol/L $MgCl_2$, 180 μmol/L ATP, and 5 μCi [γ-^{32}P]ATP] and 10 μL of sonicated PI mixture [20 μg L-α-PI (Sigma, St. Louis, MO), 10 mmol/L Tris-HCl (pH 7.5), and 1 mmol/L EGTA] for 15 minutes at room temperature. The reaction was stopped by the adding of 20 μL of 8 mol/L HCl, mixed with 160 μL of $CHCl_3/CH_3OH$ (1:1), and centrifuged. The lower organic phase was spotted on an oxalate-treated silica gel 60 TLC plate (Merck, Darmstadt, Germany) and developed in $CHCl_3/CH_3OH/H_2O/NH_4OH$ (60:47:11.6:2). The plate was dried and visualized by autoradiography using an FLA 3000 image analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Akt and p70 S6K activation assay. NIH 3T3 cells stably expressing p110α constructs were maintained in DMEM containing 0.1% serum for 12 hours and lysed in lysis buffer A. The cell lysates were analyzed by SDS-PAGE and immunoblotting using anti-phospho-Akt, anti-Akt, anti-phospho-p70 S6K, and anti-p70 S6K antibodies.

Focus formation assay. Focus formation assays were done as previously described (19, 20). Briefly, NIH 3T3 cells in 10-cm dishes were transfected with 0.5 μg of empty vector or p110α constructs, cultured in DMEM containing 5% FCS, and stained with crystal violet 14 days later; the number of transformed foci was counted.

Soft agar assay. Soft agar assays were done as previously described (19). Briefly, NIH 3T3 cells stably expressing p110α wild type or mutants were trypsinized, suspended in medium containing 0.3% agar and 10% FCS, and plated onto a bottom layer containing 0.6% agar. The cells were plated at a density of 3×10^4 cells per 6-cm dish, and the number of colonies >0.5 mm in diameter was counted 14 days later.

Results

***In vitro* lipid kinase activity of p110α mutants.** To clarify the functional consequences of cancer-related PIK3CA mutations, we first examined the lipid kinase activity of the eight different mutants found in colorectal cancers (Fig. 1*A*). As the structure of p110α has not been determined, we showed the putative three-dimensional location of the mutations examined here based on the reported structure of p110γ (ref. 3; Fig. 1*B*). We could not show the location of the R38H, G106V, or C420R mutations because the structure of the region where they might be located has not yet been determined (3).

HEK293T cells were transfected with wild-type or mutant p110α expression vectors and then immunoprecipitated p110α protein was assayed using an *in vitro* lipid kinase assay with PI as the substrate. As shown in Fig. 2*A*, constitutively active p110α, iSH2p110α, showed much greater lipid kinase activity than the wild type. Although the lipid kinase activities of all the cancer-associated p110α mutants examined in this study were not as high as that of iSH2p110α, they all had greater lipid kinase activities than wild-type p110α (Fig. 2*A*). These results suggested that all the cancer-associated PIK3CA mutations examined in this study act as gain-of-function mutations.

To examine whether the increased lipid kinase activity induced by the p110α mutants within p85 binding domain is due to the strength of interaction with p85α, we did coimmunoprecipitation analysis of overexpressed p110α with endogenous p85α. As shown in Fig. 2*B*, the p85 binding domain mutants R38H and G106V did not interact with the p85 as strongly as the wild-type p110α did.

Akt and p70S6K activation induced by p110α mutants *in vivo*. To examine whether the cancer-associated p110α mutants activate downstream components of PI3K signaling *in vivo*, we tested p110α mutation-induced activation of Akt and p70S6K, two well-defined PI3K downstream molecules. The protein extracts from NIH 3T3 cells stably expressing p110α mutants were probed with antibodies against phospho-Akt and phospho-p70S6K. As shown in Fig. 3, the phosphorylation of both Akt and p70S6K was elevated in all the p110α mutant-expressing cells compared with wild-type expressing cells. The elevated levels of phospho-Akt and phospho-p70S6K with the C2 domain mutant C420R, the helical

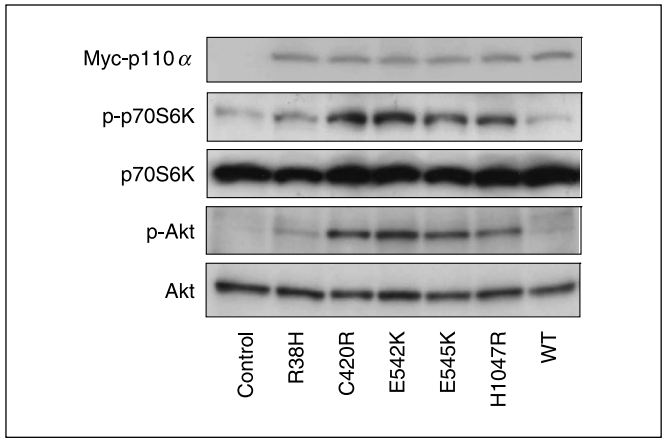

Figure 3. Activation of Akt and p70S6K *in vivo* by p110α mutants. NIH 3T3 cells stably expressing p110α mutants were grown in DMEM containing 0.1% serum. After 12 hours, cell lysates were analyzed using SDS-PAGE and immunoblotting with anti-phospho-Akt, anti-Akt, anti-phospho-p70 S6K, anti-p70 S6K, and anti-Myc antibodies.

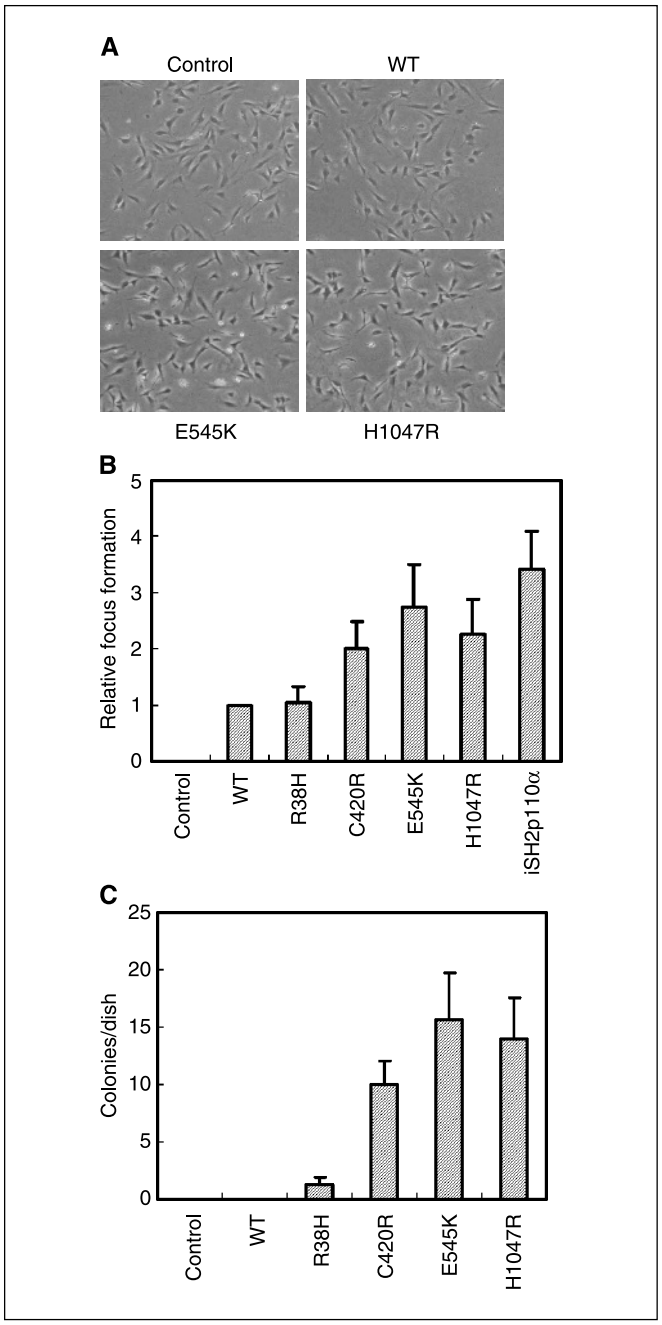

Figure 4. *A,* morphology of NIH 3T3 cells stably expressing p110α mutants. The cells stably expressing empty vector, wild type, E545K, and H1047R p110α were cultured in medium containing 10% FCS. *B,* focus-forming ability of p110α mutants. NIH 3T3 cells were transiently transfected with the p110α constructs. The number of transformed foci was counted 14 days later. *Columns,* mean of three independent experiments; *bars,* ±SD. *C,* focus-forming ability of p110α mutants. NIH 3T3 cells were transiently transfected with the p110α constructs. The number of transformed foci was counted 14 days later. *Columns,* mean from three independent experiments; *bars,* ±SD.

domain mutants E542K and E545K, and the kinase domain mutant H1047R were much greater than the levels with the p85α binding domain mutant R38H.

Morphologic change induced by p110α mutants. The morphology of the NIH 3T3 cells stably expressing p110α mutants was examined. Cells expressing oncogenic H-Ras G12V and B-Raf V599E were used as positive controls of NIH 3T3 transformation. In our previous study, oncogenic H-Ras– and B-Raf–expressing NIH 3T3 cells were shown more refractile and less flattened than parental cells (19). In this study, the NIH 3T3 cells expressing p110α C420R, E545K, and H1047R were slightly more retractile and less flattened than parental cells, whereas cells expressing wild-type p110α showed no significant morphologic changes compared with the parental cells (Fig. 4*A*). The cells expressing the C420R mutant also showed slight morphologic changes, but those expressing the R38H mutant did not (data not shown).

Focus formation activity induced by p110α mutants. Next, we examined whether the cancer-associated p110α mutants have transforming abilities. The overexpression of constitutively active p110α has been reported to transform murine fibroblast NIH 3T3 cells or chicken fibroblasts but not rat intestinal epithelial cells (7–9, 21). Furthermore, the overexpression of constitutively active Akt or wild-type Akt2 has also been found to transform NIH 3T3 cells (11, 22). Therefore, we did transformation assays using NIH 3T3 cells. The cells were transiently transfected with each p110α construct, and the number of transformed foci was counted 14 days later. As shown in Fig. 4*B*, the p110α C420R, E545K, and H1047R induced more foci than did the wild-type p110α (2.0- to 3.4-fold compared with the wild type). The focus-forming abilities of these mutants were comparable to that of the constitutively active p110α construct iSH2p110α. The R38H mutant did not have elevated focus-forming ability compared with the wild-type p110α (Fig. 4*B*).

Anchorage-independent growth induced by p110α mutants. We examined anchorage-independent growth induced by cancer-associated p110α mutants. NIH 3T3 cells stably expressing each mutant were seeded into soft agar, and their colony-forming abilities were determined 14 days later. The p110α mutant-expressing cells formed colonies in soft agar, whereas wild-type p110α-expressing cells did not (Fig. 4*C*). However, the p110α mutants induced far fewer colonies than did oncogenic H-Ras or B-Raf (data not shown).

Discussion

Most of the reported PIK3CA mutations in human cancers are in the helical or kinase domains, although some mutations have been reported in the p85 binding or C2 domains (17). A point mutation within the RBD, K227E, was shown to elevate the basal lipid kinase activity of PI3K, suggesting a direct influence of the RBD on the catalytic activity of PI3K; however, no RBD mutation has been reported in human cancers (17, 23). In this study, we analyzed the functional consequences of PIK3CA mutations found in colorectal cancers. We selected eight of the 36 reported mutations: the p85 binding domain mutations R38H and G106V, the C2 domain mutations C420R and E453Q, the helical domain mutations E542K and E545K, and the kinase domain mutations M1043I and H1047R (Fig. 1*A*). The H1047R mutation elevates basal lipid kinase activity compared with that of the wild type *in vitro* (17). In this study, all the other mutants examined also had elevated lipid kinase activity, suggesting that they act as gain-of-function mutations. The strength of the activity resulting from the mutations seemed unrelated to the domains in which they were located. However, hotspot mutations, such as E542K, E545K, and H1047R, have been shown to produce high activity.

In the helical domain, another hotspot mutation, Q546K, has been reported in addition to the E542K and E545K mutations (17). These residues are located between the hA1 and hB1 helices of the

domain, based on the structure of p110γ (ref. 3; Fig. 1*B*). Although the functions of the helical domain other than its scaffold function are unclear, our results suggest that amino acid substitutions that insert a positive charge into the region recruit the molecule to the membrane where it functions.

Besides the hotspot mutation H1047R, some mutations surrounding His^{1047} have been reported in colorectal cancers (17). We showed that the M1043I mutation also elevated lipid kinase activity. Based on the structure of the kinase domain of PI3Kγ, these mutations are located in the kα11 helix in the kinase domain (Fig. 1*B*; ref. 3). As the kα11 helix is positioned near the kinase activation loop on the surface of the enzyme between the kα12 helix on one side and kα10 on the other, amino acid substitutions in the kα11 helix may affect the conformation of the kinase activation loop, leading to elevated activity. Other kinase domain mutations that affect Thr^{1025} in the kα10 helix have been reported (17). Although we did not examine their activities, it seems reasonable to speculate that these mutants have elevated lipid kinase activities owing to changes in the conformation of the activation loop.

Reported mutations within the p85 binding and C2 domains account for only a small fraction of the colon cancer associated PIK3CA mutations (<15%). The C2 domain is often involved in phospholipid membrane binding using three loops known as CBR1, CBR2, and CBR3. According to the amino acid sequences of p110β and p110δ, CBR3 (residues 395-417 of p110δ) is particularly rich in basic residues that may be important for membrane binding. The C420R mutation, one of two C2 domain mutations examined in this study, has been reported in breast and colon cancers (17, 18). As the C420R mutation is located in the region of p110α that corresponds to CBR3 (residues 407-429 of p110α), it may lead to increased membrane binding by shifting the polarity of the CBR3 to more basic. Perhaps, the E453Q mutation produces elevated lipid kinase activity by changing an acidic residue to a neutral one, leading to increased membrane binding.

In mammalian cells, the activity of the p110α monomer is inhibited by p85α at 30°C, a temperature at which p110α is stable. By contrast, p85α is considered an activator of p110α at 37°C, a temperature at which p110α is unstable, not by increasing basal lipid kinase activity but by stabilizing p110α (24). The p85 binding domain mutants that we examined had increased lipid kinase activity. Under our experimental conditions, at a temperature of 37°C, binding to p85α is likely to increase the activity of p110α. However, these mutations did not increase the binding ability to p85α in our coimmunoprecipitation assays (Fig. 2*B*). Therefore, these mutations are unlikely to increase the lipid kinase activity by stabilizing the p110α monomer by increasing the binding affinity to p85α. These mutations might increase the basal activity or stability of p110α regardless of the p85 binding ability.

The mutant proteins with higher lipid kinase activity induced Akt and p70S6K activation *in vivo* and NIH 3T3 transformation as determined using focus formation or colony formation assays. Although we cannot exclude the possibility that some of the reported mutations are chance passenger mutations, our results suggest that most of the cancer-associated PIK3CA mutations contribute to tumorigenesis via the up-regulation of lipid kinase activity. Whereas this article was under revision, Kang et al. made a similar observation that supports the oncogenic potential of the PIK3CA mutations found in human cancers (25).

K-Ras mutations are commonly observed in human colorectal cancers (26). As oncogenic Ras interacts with and activates PI3K (5), the activation of the PI3K signaling pathway via K-Ras or PIK3CA mutations was considered one of the most common mechanisms involved in colorectal carcinogenesis. In fact, the PI3K pathway is reportedly activated in colon cancer cells frequently (27). Therefore, p110α, which is encoded by the *PIK3CA* gene, is a potential target for developing drugs to treat colon cancer.

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