The Catalytic Subunit of Phosphoinositide 3-Kinase: Requirements for Oncogenicity

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The retroviral oncogene p3k (v-p3k) of avian sarcoma virus 16 (ASV16) codes for the catalytic subunit of phosphoinositide (PI) 3-kinase, p110α. The v-P3k protein is oncogenic in vivo and in vitro; its cellular counterpart, c-P3k, lacks oncogenicity. Fusion of viral Gag sequences to the amino terminus of c-P3k activates the transforming potential. Activation can also be achieved by the addition of a myristylation signal to the amino terminus or of a farnesylation signal to the carboxyl terminus of c-P3k. A mutated myristylation signal was equally effective; it also caused a strong increase in the kinase activity of P3k. Mutations that inactivate lipid kinase activity abolish oncogenicity. The transforming activity of P3k is correlated with the ability to induce activating phosphorylation in Akt. Point mutations and amino-terminal deletions recorded in v-P3k were shown to be irrelevant to the activation of oncogenic potential. Interactions of P3k with the regulatory subunit of PI 3-kinase, p85, or with Ras are not required for transformation. These results support the conclusion that the oncogenicity of P3k depends on constitutive lipid kinase activity. Akt is an important and probably essential downstream component of the oncogenic signal from P3k.

PI 3-kinases of class IA consist of a catalytic subunit p110 and a regulatory subunit p85. P110 can bind to p85 and is controlled by p85. These lipid kinases affect many biological functions such as cell proliferation, differentiation, apoptosis, and glucose transport (1–8). A possible role of PI 3-kinases in oncogenic transformation was first suggested by the observation that PI 3-kinase activity was associated, via the regulatory subunit, with oncogene products such as polyoma middle T antigen (9, 10) or v-Src (11, 12). The catalytic subunit p110α can also be activated by a direct interaction with GTP-loaded Ras, another oncogene (13, 14). The isolation of a retroviral oncogene, v-p3k, coding for a homologue of p110α established an active role of PI 3-kinase in oncogenic transformation (15). v-p3k was cloned from the genome of avian sarcoma virus 16 (ASV16), an agent causing hemangiosarcomas in chickens (15). The v-P3k protein differs from its cellular counterpart, c-P3k, in two major points. 1) The first 13 amino acids of c-P3k are deleted in v-P3k and replaced by retroviral Gag sequences, and 2) v-P3k carries several amino acid substitutions; they are located outside the kinase domain. Expression of v-P3k induces oncogenic transformation in cultures of chicken embryo fibroblasts (CEF) and hemangiosarcomas in young chickens, suggesting that a constitutively active PI 3-kinase is sufficient for the transformation of the chicken cells (15).

Recent findings further support the involvement of PI 3-kinase in development of cancer. These include amplification of PIK3CA, the human counterpart of c-p3k, in human ovarian cancer cell lines (16), isolation of an oncogenic mutant of p85 which can transform mammalian fibroblasts in collaboration with the v-raf oncogene (17), and demonstration of a partially transformed phenotype in mammalian fibroblasts transfected with constitutively active forms of p110α (18). Downstream targets of PI 3-kinase, such as the Akt protein kinase (also called protein kinase B α or PKBα) and the related Akt2 (or PKBβ) are also amplified and overexpressed in some cancer cells (19–21). Up-regulation of Akt3 (or PKBγ) was found in breast cancers (22). The tumor suppressor protein PTEN was recently shown to dephosphorylate the D3-lipid product of PI 3-kinase, phosphatidylinositol 3,4,5-trisphosphate, thus interfering with potentially oncogenic signals emanating from PI 3-kinase (23, 24).

The v-P3k protein is much more potent in inducing oncogenic transformation than the wild type cellular c-P3k. Here we report that this difference in activity is caused by the fusion of v-P3k to viral Gag sequences. We show that membrane localization guided by a myristylation or a farnesylation signal can substitute for Gag in activating the transforming potential. We also describe the isolation of a variant v-p3k gene from a new avian sarcoma virus, ASV9895. An analysis of this variant and of deletion mutants of p110α suggests that oncogenic transformation does not require binding of p110α to the regulatory subunit p85, but is dependent on amino-terminal domains and on lipid kinase activity.

EXPERIMENTAL PROCEDURES

Culture of CEF for Transfection and Transformation Assays—CEF cultures were prepared from White Leghorn embryos obtained from SPAFAS (Preston, CT). For focus assays, DNA was transfected into CEF by using the dimethyl sulfoxide/Polybrene method (25). Focus assays with virus stocks were performed as described previously (26). Foci were counted on day 20 after infection or transfection. After counting foci of transformed cells, the assay plates were stained with crystal violet and photographed. Transfection experiments were performed at least three times. The results shown are from a representative experi-
Oncogenic PI 3-Kinase

RESULTS

Fusion to Activates the Oncogenicity of c-P3k—v-P3k expressed by the retrovector vector RCAS induced at least 10 times more transformed cell foci per microgram of transfected DNA than did c-P3k expressed by the same vector in CEF

Immunofluorescence—Cells grown on glass coverslips were washed with PBS and fixed with 3% paraformaldehyde in PBS for 30 min. The coverslips were washed with PBS, cells were permeabilized with PBS containing 0.1% Triton X-100, and then the organic phase was dried down. Samples were dissolved in chloroform, spotted onto Silica Gel 60 TLC plates (Merck), and then developed in a borate buffer system (27). The plates were exposed to Kodak X-Omat films for autoradiography.

Cellular Fractionation—Subcellular fractionation of CEF was performed as described (28). In short, CEF were washed with phosphate-buffered saline and then resuspended in low salt buffer (10 mM Hepes pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 0.3 mM EDTA, 1 mM dithiothreitol, 100 KIU/ml aprotinin, 10 mg/ml leupeptin, 1 mg/ml pepstatin A. Anti-FLAG antibody (M2, KODAK) was added to the labeled cell lysates. The immune complexes adsorbed to protein G-Sepharose were washed extensively with lysis buffer and then analyzed by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorography.

Western Blots and In Vitro Lipid Kinase Assay—Cells were lysed in lysis buffer. For Western blots, lysates consisting of 400 μg of proteins were separated by SDS-PAGE (7.5%) and transferred to Immobilon P membranes (Millipore). The membranes were then probed with the indicated antibodies. Polyclonal anti-FLAG was obtained from Zymed Laboratories Inc.; polyclonal anti-phospho-Akt (Ser473), polyclonal anti-phospho-P44/42 mitogen-activated protein kinase were obtained from New England Biolabs; polyclonal anti-p85 was obtained from Santa Cruz Biotechnology. After incubation with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech), bound proteins were detected by incubation with a chemiluminescent substrate (Renaissance plus, NEN Life Science Products Inc.) according to the manufacturer's protocol. For immunoprecipitation/ Western blotting, lysates consisting of 400 μg of proteins were incubated with 2 μl of polyclonal anti-FLAG antibody for 1 h at 4 °C and immunoprecipitates were collected with protein G-Sepharose. The beads were washed five times with lysis buffer and then extracted by boiling with SDS-PAGE sample buffer. The extracts were dissolved in 10% SDS-PAGE sample buffer, and then analyzed by 7.5% SDS-PAGE, transferred to the membrane, and probed with polyclonal anti-FLAG antibody as described above.

For in vitro lipid kinase assays, the immune complexes prepared as described above were incubated with 50 μl of kinase reaction buffer containing 20 mM Hepes pH 7.5, 10 mM MgCl2, 200 μg/ml phosphatidylserine (sonicated), 60 μM ATP, 200 μCi/ml [γ-32P]ATP for 5 min at 25 °C. The reaction was terminated by adding 80 μl of 1 N HCl. The phosphorylated lipids were extracted with 160 μl of chloroform/methanol and then the supernatant was centrifuged for 30 min at 120,000 × g. The suspension was incubated for 30 min. The coverslips were washed first with PBS, and then mounted on glass coverslips with 3% paraformaldehyde in PBS for 30 min. The coverslips were washed with PBS, cells were permeabilized with PBS containing 0.1% Triton X-100, and then the organic phase was dried down. Samples were dissolved in chloroform, spotted onto Silica Gel 60 TLC plates (Merck), and then developed in a borate buffer system (27). The plates were exposed to Kodak X-Omat films for autoradiography.

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Immunofluorescence—Cells grown on glass coverslips were washed with PBS and fixed with 3% paraformaldehyde in PBS for 30 min. After a wash with PBS, cells were permeabilized with PBS containing 0.1% Triton X-100 for 30 min, the coverslips were washed with PBS, and then incubated with rabbit anti-FLAG antibody (Zymed Laboratories Inc.) at a dilution of 1:5000 for 30 min at room temperature in a humidifying chamber. After three washes with PBS, the coverslips were incubated with fluorescein isothiocyanate-conjugated goat anti rabbit IgG (Sigma) for 30 min. The coverslips were washed with PBS, and then mounted on glass slides with SlowFade medium (Molecular Probes).

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cultures. c-P3k foci also took 3–4 weeks to develop as compared with v-P3k-induced foci which came up after 1 week. However, progeny virus released by the c-P3k-transformed CEF was as effective in inducing oncogenic transformation as v-P3k. Hence, c-P3k must have undergone a genetic change during its passage as an insert in the replication-competent retroviral vector. In order to define this genetic change of c-P3k, we isolated and cultured single foci of CEF transformed by RCAS-c-P3k. The c-P3k in this vector was marked at the carboxy terminus with a FLAG epitope, so it could be distinguished immunologically from the endogenous c-P3k protein. Six cultures, each derived from a different c-P3k-induced focus and therefore representing six independent, discrete genetic events, were characterized by immune precipitation (Fig. 1). All expressed different sized c-P3k proteins, and in each the c-P3k protein was substantially larger than 110 kDa, the expected size for c-P3k. Immunoprecipitation of the same cell lysates with antibody directed against the p19 Gag protein of the RCAS retroviral vector brought down the same proteins and showed that the c-P3k in all six clones was fused to Gag sequences that vary in length from clone to clone. Such fusions would have taken place during replication of the RCAS-c-P3k retrovirus and suggest that an amino-terminal Gag tail can activate the oncogenic potential of c-P3k. The role of Gag in oncogenic transformation by c-P3k was further defined by replacing the p110a-derived coding region in v-P3k by that of c-P3k thus producing a Gag-c-P3k fusion. This construct was as strongly transforming as v-P3k. Addition of Gag to c-P3k sequences is, therefore, sufficient to elicit full oncogenic activity, and the point mutations seen in v-P3k are not essential for transformation (Table I).

c-P3k-transformed CEF that were not selected for the transformed phenotype expressed much lower levels of the c-P3k protein than did cultures derived from transformed cell foci (Fig. 1). This inefficient expression could be the cause for the low degree of transforming activity seen with c-P3k protein. The cDNA used to express c-P3k in these experiments contained in its 5’ region a poor match of the Kozak consensus sequence for the initiation of translation plus a long 5’-untranslated region. In a modified construct (Koz-c-P3k), the initiation sequence was changed to CCACCATGCCC with good homology to the consensus except C at position +3 instead of G, and the 5’-untranslated region was deleted. In addition, a construct termed c-P3k-N was made in which c-P3k was amino terminally tagged with a FLAG epitope with a good initiator sequence (Fig. 2). Western blots of CEF transfected with these constructs showed levels of P3k expression comparable to those seen in v-P3k-transfected cells (Fig. 3). Yet the efficiency of transformation for these modified c-P3k constructs remained low (Fig. 4 and Table I). The possibility that the FLAG epitope at the amino terminus of P3k could itself interfere with transforming activity was ruled out because addition of a farnesylation signal to the carboxy terminus of FLAG-tagged c-P3k resulted in efficient transformation (c-P3k-CAAX, Fig. 2 and Table II, see also below). These results suggest that high levels of expressed c-P3k are not sufficient for oncogenic transformation.

### Table I

| Infiltrating Tumor | Focus forming units/ml |
|-------------------|-----------------------|
| **Vector (RCAS)** | 0                     |
| c-P3k from nontransformed culture | 1.2 × 10^4 |
| c-P3k from transformed focus clone 1 | 1.1 × 10^6 |
| c-P3k from transformed focus clone 2 | 1.4 × 10^6 |
| c-P3k from transformed focus clone 3 | 2.4 × 10^6 |
| c-P3k from transformed focus clone 4 | 1.8 × 10^6 |
| c-P3k from transformed focus clone 5 | 1.6 × 10^6 |
| c-P3k from transformed focus clone 6 | 1.4 × 10^6 |
| v-P3k^1^ | 1.0 × 10^6 |
| gag-c-P3k | 2.5 × 10^6 |
| v-P3k^2^ | 3.5 × 10^6 |
| Myr-c-P3k | 4.0 × 10^6 |
| G2A-Myr-c-P3k | 1.6 × 10^6 |
| a Derived from avian sarcoma virus ASV16. |
| a Derived from avian sarcoma virus ASV8905. |
| * Myr, myristylation signal of the Src protein. |
| b G2A, mutated myristic acid acceptor. |

### Membrane Localizing Signals Activate the Oncogenicity of c-P3k—Gag sequences can pilot fusion proteins to cell membranes (29), and membrane localization by myristylation or farnesylation has previously been shown to make PI 3-kinase constitutively active (28, 30, 31). In order to test for a possible role of membrane localization in c-P3k-induced oncogenic transformation, constructs were produced with either a membrane binding sequence of c-Src that contains a myristylation signal and basic residues at the amino terminus or a farnesylation signal at the carboxy terminus of c-P3k (Myr-c-P3k and c-P3k-CAAX, Fig. 2). Both constructs were expressed as inserts of the RCAS vector, and both were highly transforming in CEF cultures (Tables I and II). (Differences in focus forming titers between Tables I and II are due to the fact that one represents infection with RCAS viruses, the other transfection with DNA constructs.) In the myristylation signal, MGSSKSKP, the Gly residue functions as the acceptor for myristic acid, and a mutation of this residue to Ala has been shown to prevent myristylation (32). Surprisingly, such a G to A mutation in the myristylation signal of c-P3k did not reduce transforming activity (G2A-Myr-c-P3k, Fig. 2; see also Table I and Fig. 4). However, in vitro lipid kinase assays of these constructs showed that both Myr-c-P3k and G2A-Myr-c-P3k have much stronger PI 3-kinase activity than c-P3k or even v-P3k of ASV16 (Fig. 5). Immunoprecipitation and Western blots had shown these proteins to be expressed at similar levels (Fig. 3)
except c-P3k-CAAX. These results demonstrate that c-P3k proteins with a Src amino terminus contain higher specific kinase activity even if the myristic acid acceptor is mutated. The reason for the low expression of c-P3k-CAAX is unclear (Fig. 3). Higher levels may be toxic to the cells. However, the high specific lipid kinase activity may compensate for the low expression level of c-P3k-CAAX (Fig. 5).

Immunofluorescence revealed distinct staining patterns in cells stably transfected with c-P3k and v-P3k constructs (Fig. 6A). KOZ-c-P3k and c-P3k-N showed multivesicular cytoplasmic staining. The Gag-linked v-P3k transfected cells were characterized by vesicular, possibly endosomal staining at the periphery of the cell. Myr-c-P3k or c-P3k-CAAX induced a cytoplasmic staining pattern that was localized in elongated vesicles. In subcellular fractionation, c-P3k was found exclusively in the cytosol (Fig. 6B). Small but significant fractions of v-P3k, Myr-c-P3k, and G2A-Myr-c-P3k were membrane-bound. The higher than expected cytosolic distributions of these constructs may reflect separation from membranes during the fractionation procedure.

Interaction with p85 Is Not Required, but Most Other Regions Are Essential for the Oncogenicity of P3k—In a search for new oncogenes, a second retrovirus, avian sarcoma virus 8905 (ASV8905), with an independent insert of P3k was discovered. A map of the single protein product of ASV8905 is shown in Fig. 2 (v-P3k<sup>8905</sup>). The amino terminus of the P3k insert in this genome is fused to Gag sequences as in ASV16, but the Gag portion is short, consisting only of p19 sequences. The fusion also results in a deletion of 72 amino acids from the amino terminus of p110α. In ASV16 only 13 amino acids of p110α are deleted at the amino-terminal fusion point. The remainder of p110α in ASV8905 contained only one amino acid substitution, providing additional evidence for the conclusion that other amino acid substitutions in v-P3k of ASV16 are not relevant for transformation. The v-P3k of ASV8905 was cloned into RCAS and was found to induce oncogenic transformation of CEF and hemangiosarcomas in young chickens (Table II and data not shown).

In order to test for a possible activating role of the amino-terminal deletions in transformation, a c-P3k construct with a 72-amino acid amino-terminal deletion was made and expressed in the RCAS vector (Δ72-c- P3k, Fig. 2). Its transforming activity was low and comparable to that of KOZ-c-P3k and c-P3k-N (Table II and Fig. 4). Again, addition of a wild type or G2A-mutated myristylation signal resulted in strong transforming activity (Myr-Δ72-c-P3k and G2A-Myr-Δ72-c-P3k in Fig. 2, see also Table II and Fig. 4). These observations together with the results on Gag-c-P3k, Myr-c-P3k, and c-P3k-CAAX show that the amino-terminal deletions in v-P3k of ASV16 or ASV8905 are neither necessary nor sufficient for oncogenic transformation.

Since the deletion of 72 amino acids from the v-P3k of ASV8905 means the loss of more than half of the p85-binding domain, the interaction of the ASV16 and the ASV8905-derived forms of v-P3k with the regulatory subunit p85 was examined. p85 was co-immunoprecipitated with P3k from ASV16 but not with the P3k protein of ASV8905 (Fig. 7). These data show that oncogenic transformation by P3k does not require interaction with the regulatory subunit p85.

Additional deletions in non-kinase domains of P3k (Fig. 8A) were tested for oncogenic activity. All of these constructs contained a myristylation signal. Amino-terminal deletions of 132...
amino acids and larger failed to transform CEF suggesting that the region adjacent to the p85-binding domain is required for oncogenicity. The precise function of this region remains to be determined. All of the internal deletions and a small carboxyl-terminal deletion also failed to induce transformation of CEF suggesting a functional or structural role of domains between amino acids 132 and 768 and of the carboxyl terminus in the oncogenic activity of P3k. These amino-terminal, internal and carboxyl-terminal deletion mutants were efficiently expressed but lacked lipid kinase activity (Fig. 9 and 10). We also tested the role of Ras binding in P3k transformation. The construct Myr-Δ72-c-P3k/K227E carries a mutation in the Ras-binding domain and fails to interact with Ras (13) yet it showed strong transforming activity in CEF (Fig. 8A). This result suggests that binding to Ras is not required for transformation by constitutively active P3k.

Kinase Activity Is a Prerequisite for Oncogenic Transformation by P3k—P3k codes for a lipid kinase that phosphorylates inositol phospholipids at the 3 position of the inositol residue. In order to test whether this kinase activity is necessary for P3k-induced oncogenic transformation, two different mutations were introduced into Myr-Δ72-P3k. One is the deletion of the entire kinase domain, the other is a point mutation substituting Lys at position 802 with Arg (33) (Fig. 8B). These mutations abolish the kinase activity of p110α (Fig. 9). The mutations were expressed in CEF with the RCAS vector, and expression was verified by Western blots (Fig. 10). Both kinase negative constructs failed to induce oncogenic transformation in CEF cultures. This result and the observation made with the amino-terminal and internal deletions suggest that kinase activity is essential for the oncogenicity of P3k. A downstream target of P3k is the serine-threonine kinase Akt (34–36). P3k indirectly activates Akt via the production of D3-phosphorylated phosphoinositides which in turn activate PDK1 and PDK2, the two kinases that phosphorylate Akt at threonine 308 and serine 473, respectively, and thus activate its enzymatic function (37–39). Since transformation by P3k requires kinase activity, the transformed cells should show elevated levels of phosphorylated Akt. Fig. 11 shows that in serum-starved, RCAS-transfected control CEF, Akt is not significantly
FLAG antibody from CEF lysates transfected with RCAS-v-p3k or from ASV8905. v-P3k proteins were immunoprecipitated with anti-p85 or anti-FLAG antibody. SDS-polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membranes. The blot was probed with anti-p85 or anti-FLAG antibody.

phosphorylated at serine 473, but addition of PDGF induces phosphorylation at this site. In serum-starved CEF transformed by v-P3k, Akt is constitutively phosphorylated to a level significantly higher than in the PDGF-treated control CEF. The level of Akt phosphorylation in P3k-transformed cells is not increased further by PDGF, a result in keeping with the independence of oncogenic P3k from upstream p85-mediated input. Similar results were obtained with an antibody that recognizes Akt phosphorylation at threonine 308, the phosphorylation site of PDK1 (data not shown). In contrast to Akt, another signaling molecule, ERK, was not constitutively activated in P3k-transformed cells (Fig. 11). The basal levels of Erk phosphorylation in RCAS transfected CEF were high (Fig. 10), but they were further increased by PDGF. A comparison of various oncogenic and non-oncogenic P3k constructs shows a correlation between transformation and Akt phosphorylation (Fig. 12). All oncogenic versions of P3k induce phosphorylation of Akt; the non-oncogenic P3k proteins fail to effect phosphorylation of Akt. These data suggest that the oncogenic signal of P3k travels through Akt, a conclusion that is also supported by the ability of dominant-negative mutants of Akt to interfere with P3k-induced oncogenic transformation (40).

**DISCUSSION**

The c-P3k protein does not induce oncogenic transformation, even if it is overexpressed with the help of improved initiator sequences. The low numbers of transformed cell foci observed in c-P3k-transfected cell cultures in our previous work (15) and in the present work represent oncogenic mutants. In all of these mutants, the amino terminus of the c-P3k is fused to Gag sequences of the retroviral vector RCAS. The fusion to Gag sequences during retroviral replication activates the oncogenic potential of c-P3k. If the Gag sequences of v-P3k are fused to c-P3k, the protein also becomes oncogenic. The amino acid substitutions found in the coding domain of v-P3k are therefore irrelevant for oncogenicity. The two forms of v-P3k derived from ASV16 and ASV8905 also carry deletions of 13 and 72 amino acids, respectively, at the amino terminus of the P3k sequences. Constructs that lack these deletions but are linked to Gag, a myristylation signal, or a fatty acylation signal are oncogenic. Yet introducing the 72-amino acid amino-terminal deletion into c-P3k fails to activate the transforming potential of this protein. The amino-terminal deletions in the two forms of v-P3k are therefore neither necessary nor sufficient for oncogenicity.

The Gag sequences acquired by the mutant c-P3ks are derived from an avian retrovirus and, unlike Gag sequences of murine retroviruses (41), are not myristylated (42). However, recent studies have shown that the amino-terminal half of the avian retroviral Gag protein p19 functions as a membrane-binding domain (29, 43). The suggestion that membrane localization is critical for the oncogenicity of P3k is supported by the effects of a myristylation or a farnesylation signal; both activate oncogenicity. Both have also been shown to make the lipid kinase constitutively active. We have confirmed that the G to A mutation in the myristylation signal of P3k does indeed prevent myristylation (data not shown). Yet some of the mutant protein remains localized in the cellular membrane fraction (Fig. 6B). The extent of membrane localization for the mutant is comparable to that of v-P3k and could explain the undiminished oncogenic activity of the G to A mutation in the myristylation signal. The data are in agreement with the conclusion that membrane localization is critical for oncogenic activity of P3k. However, they do not rule out the possibility that added Gag, myristylation, or farnesylation sequences but not FLAG sequences induce a conformational change that results in enhanced lipid kinase activity as suggested by others (44), and that high enzymatic activity is sufficient for oncogenic transformation. This alternative appears less likely, but the two explanations for the oncogenic activation of P3k are not mutually exclusive. The analysis of terminal and internal deletion mutants identified kinase activity as a prerequisite for cellular transformation. Amino-terminal deletions of 132 amino acids and more, internal deletions, and a small carboxyl-terminal deletion all where devoid of transforming and kinase activity. The deleted sequences may represent distinct and indispensable functional domains of P3k or they may be necessary structural elements that sustain the active conformation of the molecule. These results are in agreement with a previous study on the effects of amino-terminal and internal deletions on the kinase activity of constitutively active p110a except that P3k tolerates a larger amino-terminal deletion, probably because its activity is independent of interaction with p85 (45). Targeted inactivation of the kinase function by single amino acid substitution in the kinase domain or by the deletion of the kinase domain both result in a non-oncogenic protein, even if the protein is directed to cellular membranes by a myristylation signal. These observations suggest that constitutive kinase activity of P3k is necessary and perhaps sufficient for transformation, while membrane localization may or may not be needed but in the absence of kinase activity is not sufficient to activate the oncogenicity of P3k.

Genetic analysis also ruled out two known interactors of P3k from the transformation process: p85 and Ras. The v-P3k protein encoded by ASV8905 has a 72-amino acid amino-terminal

**Table II**

| Transfecting construct | Focus forming units/μg of DNA |
|------------------------|------------------------------|
| Vector (RCAS)                  | 0                            |
| KOZ-c-p3k                  | 60                           |
| Myr-c-p3k<sup>a</sup>               | 2 × 10<sup>3</sup>       |
| c-p3k-N                     | 1 × 10<sup>2</sup>         |
| c-p3k-CAAX<sup>b</sup>         | 1.5 × 10<sup>3</sup>   |
| Δ72-c-p3k                  | 1 × 10<sup>2</sup>         |
| Myr-Δ72-c-p3k               | 2 × 10<sup>3</sup>         |
| v-p3k<sup>8905</sup><sup>c</sup> | 5 × 10<sup>2</sup>       |

<sup>a</sup> Myr, myristylation signal of the Src protein.
<sup>b</sup> CAAX, farnesylation signal.
<sup>c</sup> P3k derived from avian sarcoma virus ASV8905.
deletion and therefore cannot bind p85, yet it is strongly transforming (Fig. 7 and Table II). Its kinase activity is even higher than that of v-P3k from ASV16 which retains p85 binding capability (Fig. 5). It has been shown previously that p85 can inhibit the lipid kinase activity of GST-p110α (44). GTP-activated Ras can bind to the region of amino acids 190 to 288 in P3k and can activate the kinase function. A Lys to Glu mutation at position 227 of P3k has been shown to interfere with Ras binding (13) yet this mutant is still transforming. Therefore, binding to GTP Ras does not play a role in P3k-induced transformation.

A downstream target of PI 3-kinase, Akt, can also induce transformation in CEF cultures and hemangiosarcomas in chickens (40). Membrane localization and kinase activity are
requirements for these transforming events. Dominant negative mutants of Akt interfere with P3k-induced transformation suggesting that Akt is a necessary component of the oncogenic signal issued by P3k (40). The current study provides additional evidence for this relationship between P3k and Akt by establishing a correlation between oncogenicity of a P3k construct and activating phosphorylation of Akt and by showing that Akt is constitutively active in P3k-transformed cells, independent of growth factor signaling (Figs. 11 and 12). In contrast to Akt, another putative downstream target of PI 3-kinase (28), Erk, is not constitutively activated in P3k-transformed CEF (Fig. 11), consistent with a recent report (46). Transformation by P3k therefore does not require a global up-regulation of P3k-dependent activities and targets. It will now be interesting to follow the signal from Akt downstream and identify further components that are critical to oncogenicity.

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