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Direct Regulation of the *Akt* Proto-Oncogene Product by Phosphatidylinositol-3,4-bisphosphate

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The regulation of the serine-threonine kinase Akt by lipid products of phosphoinositide 3-kinase (PI 3-kinase) was investigated. Akt activity was found to correlate with the amount of phosphatidylinositol-3,4-bisphosphate (PtdIns-3,4-P_2) in vivo, and synthetic PtdIns-3,4-P_2 activated Akt both in vitro and in vivo. Binding of PtdIns-3,4-P_2 occurred within the Akt pleckstrin homology (PH) domain and facilitated dimerization of Akt. Akt mutated in the PH domain was not activated by PI 3-kinase in vivo or by PtdIns-3,4-P_2 in vitro, and it was impaired in binding to PtdIns-3,4-P_2. Examination of the binding to other phosphoinositides revealed that they bound to the Akt PH domain with much lower affinity than did PtdIns-3,4-P_2 and failed to increase Akt activity. Thus, Akt is apparently regulated by the direct interaction of PtdIns-3,4-P_2 with the Akt PH domain.

Stimulation of cells by several growth factors activates PI 3-kinase (*1*). In vivo, the activation of PI 3-kinase increases the intracellular amounts of PtdIns-3,4-P_2 and phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3) (*1*). Various downstream targets of PI 3-kinase have been identified, including the serine-threonine kinase Akt (*1*–*5*). Akt (also referred to as PKBα or Racα) is encoded by the *Akt* proto-oncogene and is defined by an NH_2-terminal regulatory domain of protein-protein interaction [Akt homology (AH) domain)] that contains a PH domain (*2*). Akt participates in the activation of the p70 ribosomal protein S6 kinase ($p70^{S6K}$) (*2*) and inhibits glycogen synthase kinase-3 (*3*), and it has a role in proliferative and anti-apoptotic cell responses (*4*, *6*). Akt activation by growth factors requires PI 3-kinase activity (*2*), but there are other pathways that can also lead to Akt activation (*7*).

To examine whether PI 3-kinase is sufficient to stimulate Akt activity, we coexpressed hemagglutinin epitope–tagged Akt (HA-Akt) and activated PI 3-kinase (*8*). Activated PI 3-kinase (piSH2-p110•MT) induced the activity of HA-Akt in serum-starved COS-1 and NIH 3T3 cells (Fig. 1), and stimulation of HA-Akt by activated PI 3-kinase was blocked by the PI 3-kinase inhibitor wortmannin (*9*). Activation of HA-Akt by piSH2-p110•MT in NIH 3T3 cells was enhanced by platelet-derived growth factor (PDGF) (Fig. 1B). Mutant piSH2-p110(K227E)•MT [in which Lys (K) at position 227 is mutated to Glu (E)] that is deficient in Ras binding (*10*) stimulated the activity of HA-Akt in serum-starved cells, but it was less effective than piSH2-p110•MT at enhancing PDGF stimulation of HA-Akt (*9*). The activity of HA-Akt(R25C), which contains a point mutation of Arg (R) to Cys (C) in the Akt PH domain, was not significantly increased by PDGF treatment or coexpression of piSH2-p110•MT (Fig. 1B). The Akt PH domain was therefore important for Akt activation by PDGF and by PI 3-kinase.

We next examined the relation of Akt activity to phosphoinositide amounts in vivo. After treatment of human platelets with thrombin receptor–activating peptide (TRAP), the amounts of the PI 3-kinase products PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 increase with distinct times (*11*). We measured Akt autophosphorylation (Fig. 2A), Akt phosphorylation of histone H2B (Fig. 2B), and phosphorylation of phosphoinositides (Fig. 2B) as a function of time after the addition of TRAP (*12*). The concentration of PtdIns-3,4,5-P_3 peaked 25 s after TRAP addition, but full Akt activation did not occur until the concentration of PtdIns-3,4-P_2 had peaked

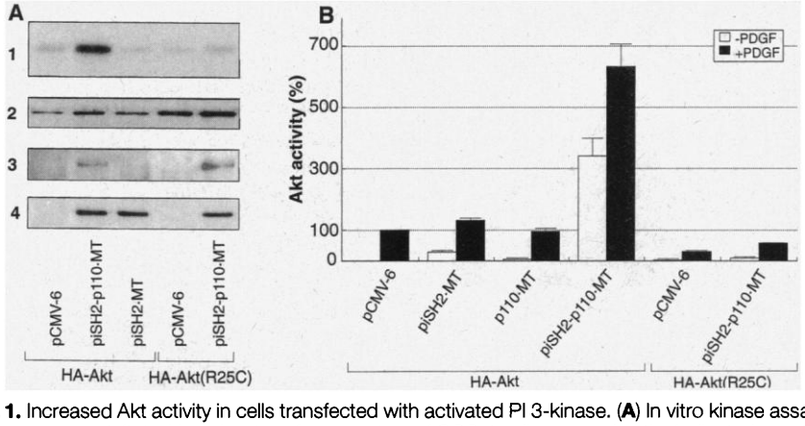

Fig. 1. Increased Akt activity in cells transfected with activated PI 3-kinase. (**A**) In vitro kinase assays of Akt immunoprecipitated from lysates of serum-starved COS-1 cells expressing the indicated constructs (*8*). Immunoprecipitations with monoclonal antibody to HA (anti-HA) (Boehringer) were followed by kinase assays with histone H2B (*3*) (panel 1). Expression of HA-Akt and HA-Akt(R25C) was determined by protein immunoblotting with anti-HA (panel 2). p110•MT (panel 3) and piSH2•MT (panel 4) were immunoprecipitated with monoclonal antibody to MT (*4*) and detected with polyclonal antibody to 9E10 (EQKLISEEDL) (*20*). (**B**) Histone H2B kinase activity in anti-HA immunoprecipitates from lysates of serum-starved or serum-starved and PDGF-stimulated [PDGF (50 ng/ml) for 5 min] NIH 3T3 cells (PDGF-stimulated HA-Akt activity equals 100% in cells transfected only with HA-Akt).

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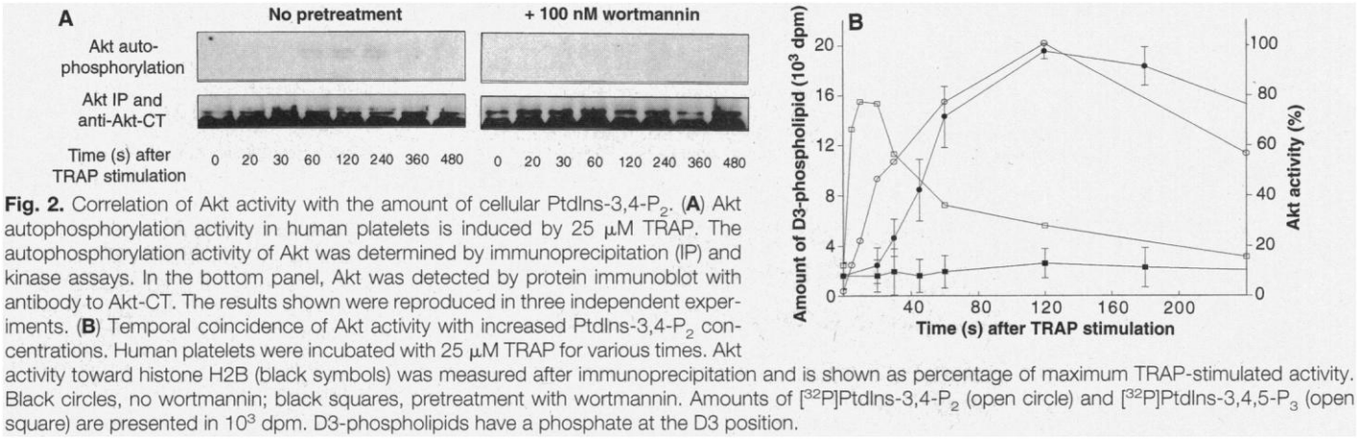

Fig. 2. Correlation of Akt activity with the amount of cellular PtdIns-3,4-P_2. (**A**) Akt autophosphorylation activity in human platelets is induced by 25 μM TRAP. The autophosphorylation activity of Akt was determined by immunoprecipitation (IP) and kinase assays. In the bottom panel, Akt was detected by protein immunoblot with antibody to Akt-CT. The results shown were reproduced in three independent experiments. (**B**) Temporal coincidence of Akt activity with increased PtdIns-3,4-P_2 concentrations. Human platelets were incubated with 25 μM TRAP for various times. Akt activity toward histone H2B (black symbols) was measured after immunoprecipitation and is shown as percentage of maximum TRAP-stimulated activity. Black circles, no wortmannin; black squares, pretreatment with wortmannin. Amounts of [^{32}P]PtdIns-3,4-P_2 (open circle) and [^{32}P]PtdIns-3,4,5-P_3 (open square) are presented in 10^3 dpm. D3-phospholipids have a phosphate at the D3 position.

after 60 s (Fig. 2B). Activation of Akt was blocked by wortmannin at concentrations that blocked TRAP-dependent increases in the concentrations of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 (Fig. 2).

The time course suggested that PtdIns-3,4-P_2 would be critical for in vivo activation of Akt. It has been shown that a product of PI 3-kinase activates immunoprecipitates of Akt (*4*), so we investigated the effects of synthetic phosphoinositides on purified Akt (*13*). We found that DiC_{16}PtdIns-3,4-P_2 stimulated Akt activity in a dose-dependent manner with a more than fourfold increase in activation at 10 μM (9), but neither DiC_{16}PtdIns-3-P nor PtdIns-4,5-P_2 increased Akt activity (Fig. 3), nor did PtdIns (9). DiC_{16}PtdIns-3,4,5-P_3 even inhibited Akt basal activity (Fig. 3). The Akt PH domain mutation R25C abrogated the ability of DiC_{16}PtdIns-3,4-P_2 to induce activity of Akt (Fig. 3).

DiC_{16}PtdIns-3,4,5-P_3 can induce membrane ruffling and chemotaxis when added to whole cells (*14*), suggesting that phospholipid vesicles are able to fuse with the plasma membrane and that they can reach the inner leaflet to trigger biologic responses. When added to intact NIH 3T3 cells at a concentration of 5 μM (*15*), DiC_{16}PtdIns-3,4-P_2 caused more than a threefold increase in the stimulation of Akt autophosphorylation activity (Fig. 4A) that was dose-dependent (9). Other phosphoinositides did not activate Akt, and DiC_{16}PtdIns-3,4,5-P_3 even reduced Akt autophosphorylation activity (Fig. 4A). PI 3-kinase activity was not required because Akt activation by DiC_{16}PtdIns-3,4-P_2 occurred even after treatment of cells with wortmannin (Fig. 4A). PtdIns-4,5-P_2 did not increase Akt activity in vivo (Fig. 4A). Thus, the lipid had similar effects on Akt when added to intact cells compared with those observed with purified Akt.

We also examined the activity of HA-Akt after stimulation with PDGF and PtdIns-3,4-P_2 (Fig. 4B). HA-Akt activity increased when HA-Akt was immunoprecipitated from cells treated with PDGF or DiC_{16}PtdIns-3,4-P_2 (Fig. 4B). The integrity of the Akt PH domain was necessary for Akt activation by DiC_{16}PtdIns-3,4-P_2. Mutant HA-Akt(R25C) (9) or HA-Akt(Δ11–35) (containing a deletion of amino acids 11 through 35) (Fig. 4B) was not activated in cells treated with PDGF or synthetic DiC_{16}PtdIns-3,4-P_2.

To determine whether PtdIns-3,4-P_2 directly interacts with the Akt PH domain, we examined the binding of phosphoinositides to glutathione-S-transferase (GST)–Akt PH domain fusion protein (*16*). ^{32}P-labeled phosphoinositides were incubated with GST–Akt PH protein, and weakly bound lipids were washed away (5). Both PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 bound to the Akt PH domain as determined by high-pressure liquid chromatography analysis of the bound lipids (9). [^{32}P]PtdIns-4,5-P_2 did not significantly bind (9), indicating that binding was stereospecific and required phosphate at the D3 position (Fig. 5A).

To determine relative affinities, we measured the binding of ^{32}P-labeled phosphoinositides to the GST–Akt PH domain (amino acids 1 through 106) fusion protein in the presence of various concentrations of unlabeled phosphoinositides (*17*). DiC_{16}PtdIns-3,4-P_2 displaced binding of [^{32}P]PtdIns-3,4-P_2 to the Akt PH domain with 50% maximal effect at ~5 μM. DiC_{16}PtdIns-3,4,5-P_3 competed for binding at higher concentrations, but PtdIns-4,5-P_2 did not compete at con-

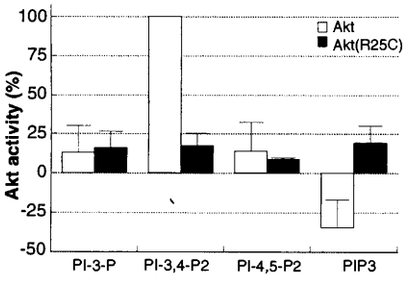

Fig. 3. Activation of Akt by DiC_{16}PtdIns-3,4-P_2 in vitro. Purified Akt and Akt(R25C) were incubated with phosphoinositides (10 μM). Akt and Akt(R25C) activities were determined by phosphorylation of histone H2B and are shown compared with carrier alone (0%) and Akt stimulated by PtdIns-3,4-P_2 (100%). PI-3-P, PtdIns-3-P; PI-3,4-P2, PtdIns-3,4-P_2; PI-4,5-P2, PtdIns-4,5-P_2; PIP3, PtdIns-3,4,5-P_3.

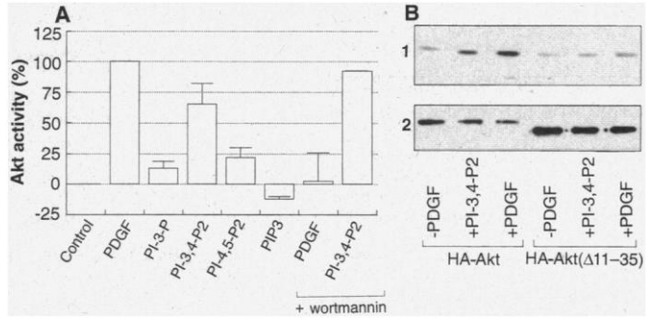

Fig. 4. Activation of Akt by DiC_{16}PtdIns-3,4-P_2 in vivo. (**A**) DiC_{16}PtdIns-3,4-P_2 induces Akt activity in intact cells. Phosphoinositides (5 μM) were added to serum-starved NIH 3T3 cells for 10 min. Cells were pretreated with 100 nM wortmannin where indicated. The activity of immunoprecipitated Akt was determined by autophosphorylation. (**B**) Activation of Akt by DiC_{16}PtdIns-3,4-P_2 depends on the PH domain. Chinese hamster ovary cells were transfected with HA-Akt or HA-Akt(Δ11–35) expression constructs. After serum-starvation, the cells were incubated with DiC_{16}PtdIns-3,4-P_2 (5 μM) or PDGF (50 ng/ml) for 10 min, and Akt activity was determined by phosphorylation of histone H2B (panel 1). Panel 2 shows expression of HA-Akt and HA-Akt(Δ11–35).

centrations up to 100 μM (Fig. 5A). Similar results were observed with displacement of [^{32}P]PtdIns-3,4,5-P_3 (9). Inositol-1,3,4-trisphosphate competed for binding of [^{32}P]PtdIns-3,4-P_2 with a >50% maximal effect at 100 μM (9), whereas inositol-1,4,5-trisphosphate and inositol-1,3,4,5-tetrakisphosphate did not compete at this concentration. Inositol-1,3,4-trisphosphate failed to specifically stimulate Akt activity at these concentrations (9).

Full-length Akt AH domain (amino acids 1 through 147) protein bound somewhat better to [^{32}P]PtdIns-3,4-P_2 than did the PH domain (amino acids 1 through 106) (Fig. 5B); a single point mutation (R25C), however, prevented binding to PtdIns-3,4-P_2 (Fig. 5B). Thus, the Akt AH (PH) domain residue that was critical for Akt activation by PtdIns-3,4-P_2 was important for binding.

We also tested whether binding of PtdIns-3,4-P_2 facilitates Akt dimerization. The GST–Akt AH domain and GST–Akt AH(R25C) domain fusion proteins were incubated with Akt protein from cell lysates in the presence of phosphoinositides. After washing, the bound Akt was revealed by protein immunoblotting (Fig. 5C). Akt dimerization with GST–Akt AH increased more than threefold in the presence of 10 μM DiC_{16}PtdIns-3,4-P_2 (Fig. 5C), but was not observed with GST–Akt AH(R25C) (Fig. 5C). PtdIns-4,5-P_2 and DiC_{16}PtdIns-3,4,5-P_3 failed to cause dimerization.

In vivo, the binding of PtdIns-3,4-P_2, and to a lesser extent of PtdIns-3,4,5-P_3, to Akt may transiently mobilize cytosolic Akt to the plasma membrane where it is activated by PtdIns-3,4-P_2. After stimulation, the presence of PtdIns-3,4-P_2 is no longer required, and immunoprecipitates of Akt remain activated after detergent wash. This persistent activation may be explained by phosphorylation that "locks" Akt into an active conformation (*18*). There are, however, PI 3-kinase–independent pathways that lead to Akt activation, indicating the presence of kinase cascades that bypass the PtdIns-3,4-P_2 dependence (*7*).

PI 3-kinase has been implicated in various cellular processes. The quantities of the three lipid products of this enzyme are regulated by a complex set of kinases and phosphatases (*1*, *19*). Here we show that PtdIns-3,4-P_2 is the only known phosphoinositide that can activate Akt. Although DiC_{16}PtdIns-3,4,5-P_3 at high concentrations bound to the Akt AH (PH) domain, Akt dimerization and Akt activity were not increased by this lipid. Thus, the additional phosphate on PtdIns-3,4,5-P_3 appears to interfere with dimerization. Structural studies are needed to understand the basis for these differences in lipid effects. Our studies further indicate that distinct products of PI 3-kinase have distinct functions and that Akt is likely to be an important mediator of some PI 3-kinase responses.

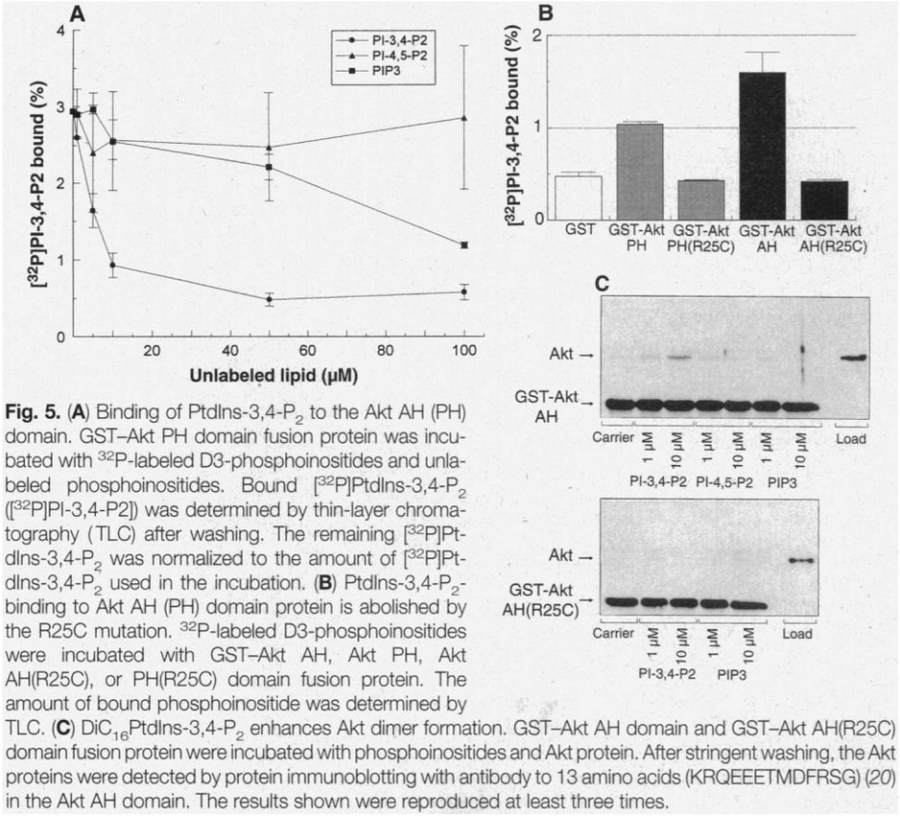

Fig. 5. (**A**) Binding of PtdIns-3,4-P_2 to the Akt AH (PH) domain. GST–Akt PH domain fusion protein was incubated with ^{32}P-labeled D3-phosphoinositides and unlabeled phosphoinositides. Bound [^{32}P]PtdIns-3,4-P_2 ([^{32}P]PI-3,4-P2]) was determined by thin-layer chromatography (TLC) after washing. The remaining [^{32}P]PtdIns-3,4-P_2 was normalized to the amount of [^{32}P]PtdIns-3,4-P_2 used in the incubation. (**B**) PtdIns-3,4-P_2-binding to Akt AH (PH) domain protein is abolished by the R25C mutation. ^{32}P-labeled D3-phosphoinositides were incubated with GST–Akt AH, Akt PH, Akt AH(R25C), or PH(R25C) domain fusion protein. The amount of bound phosphoinositide was determined by TLC. (**C**) DiC_{16}PtdIns-3,4-P_2 enhances Akt dimer formation. GST–Akt AH domain and GST–Akt AH(R25C) domain fusion protein were incubated with phosphoinositides and Akt protein. After stringent washing, the Akt proteins were detected by protein immunoblotting with antibody to 13 amino acids (KRQEEETMDFRSG) (*20*) in the Akt AH domain. The results shown were reproduced at least three times.

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8. HA-Akt and the mutant HA-Akt(R25C) have been described (*4*). The piSH2·MT plasmid is described in [A. Klippel, J. A. Escobedo, Q. Hu, L. T. Williams, *Mol. Cell. Biol.* **13**, 5560 (1993)]. p110·MT was generated by inserting bovine p110α into pCMV-6 with the primers 5′-ATGGTCGACATGCCTCCAAGACCATCA-3′ and 5′-CTTCTGCTCTCCCCCGGGGTTCAATGCATGCTGTTTAATTGTGTG-3′ or 5′-TATGGATCCTCAGTTCAGGTCCTCCTCGGAAATCAGCTTCTGCTCTCCCCCGGG-3′.
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12. Human platelets were stimulated as described (*11*) and lysed in 2× NP-40 lysis buffer [1% NP-40, 10% glycerol, 137 mM NaCl, 20 mM tris-HCl (pH 7.4)] containing inhibitors. Akt kinase activity was determined by immunoprecipitation (*4*).
13. Synthetic DiC_{16}PtdIns-3-P (Matreya, Pleasant Gap, PA), DiC_{16}PtdIns-3,4-P_2 (from C.-S. Chen or from Matreya), DiC_{16}PtdIns-3,4,5-P_3 (*5*), and PtdIns or PtdIns-4,5-P_2 (Sigma) were dried together with phosphatidylserine (PS) and phosphatidylcholine (PC). Lipids were resuspended in 10 mM Hepes (pH 7.0) containing 1 mM EDTA and sonicated. Mixed vesicles containing PS and PC and PtdIns, PtdIns-4,5-P_2, DiC_{16}PtdIns-3-P, DiC_{16}PtdIns-3,4-P_2, or DiC_{16}PtdIns-3,4,5-P_3 were added to purified Akt at 10 μM. Akt and Akt(R25C) baculovirus expression constructs were generated in pVL1392 (Pharmingen) with the primers 5′-TAACCATGGACGACGTAGCCATTGTGAAGG-3′ and 5′-ACCGGATCCTCAGGCTGTGCCACTGGCTGA-3′. Sf9 cells expressing recombinant Akt were homogenized in 40 mM triethanolamine (TEA; pH 7.6) containing inhibitors. Recombinant protein was purified by fast protein liquid chromatography (FPLC) in three steps on HiLoadQ, Heparin, and MonoQ columns (Pharmacia) equilibrated in 40 mM TEA (pH 7.6). Bound proteins were eluted with linear gradients of 0 to 0.5 M, 0 to 0.7 M, and 0 to 0.3 M KCl in TEA (pH 7.6), respectively, and assayed by immunoblotting (*4*). The fractions were assayed for Akt activity, and their purity was determined by staining; a greater than 95% purity was achieved.
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15. Synthetic phosphoinositides were prepared by sonication in the absence of carrier phospholipid and added to serum-starved cells for 10 min. After incubation at 37°C, the cells were harvested and Akt activity was determined.
16. Constructs in pGEX-2T (Pharmacia) were generated from fragments encoding the PH domain of Akt (amino acids 1 through 106) with the primers 5′-TCTGGATCCAACGACGTAGCCATTGTGAA-3′ and 5′-ACCGAATTCCACAGTCTGAATGGCGGT-3′. The primers 5′-TCTGGATCCAACGACGTAGCCATTGTGAA-3′ and 5′-CATGAATTCCATGGTCACACGGTGCTT-3′ were used to generate GST–Akt AH (amino acids 1 through 147).
17. PtdIns-4,5-P_2, DiC_{16}PtdIns-3,4-P_2, and DiC_{16}PtdIns-3,4,5-P_3 were prepared by sonication in the absence of carrier lipids. Inositol-1,3,4-trisphosphate, inositol-

1,4,5-trisphosphate, and inositol-1,3,4,5-tetrakisphosphate were prepared in 10 mM Hepes (pH 7.4). Inositolphosphates and phospholipids were added to bound GST fusion protein on beads together with ^{32}P-labeled phosphoinositides. Bound lipids were resolved by TLC.
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20. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Identification of a Gene That Causes Primary Open Angle Glaucoma

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Glaucoma is a major cause of blindness and is characterized by progressive degeneration of the optic nerve and is usually associated with elevated intraocular pressure. Analyses of sequence tagged site (STS) content and haplotype sharing between families affected with chromosome 1q–linked open angle glaucoma (*GLC1A*) were used to prioritize candidate genes for mutation screening. A gene encoding a trabecular meshwork protein (*TIGR*) mapped to the narrowest disease interval by STS content and radiation hybrid mapping. Thirteen glaucoma patients were found to have one of three mutations in this gene (3.9 percent of the population studied). One of these mutations was also found in a control individual (0.2 percent). Identification of these mutations will aid in early diagnosis, which is essential for optimal application of existing therapies.

In the United States, glaucoma is the second leading cause of legal blindness overall and the leading cause of blindness in African-American individuals (*1*, *2*). Primary open angle glaucoma (POAG) is the most common form of glaucoma, affecting 1 to 2% of the population over age 40 (*3*). Nearly 12,000 people in the United States are blinded annually by this disorder (*2*–*4*). The molecular basis of POAG is unknown, although it is likely to be a genetically heterogeneous disorder that results from the interaction of multiple genes and environmental influences.

One method of identifying genes involved in multifactorial disorders is to study Mendelian diseases with a similar phenotype. Juvenile open angle glaucoma (JOAG) is a term used to refer to a subset of POAG that has an earlier age of onset and a highly penetrant autosomal dominant mode of inheritance (*5*). On clinical examination, patients with juvenile-onset open angle glaucoma are identical to patients with later onset disease in that both groups exhibit elevated intraocular pressure and optic nerve cupping in the presence of a biomicroscopically normal trabecular meshwork. A genetic locus (*GLC1A*) associated with JOAG was identified on chromosome 1q21-q31 by genetic linkage analysis (*6*). A number of other groups subsequently identified additional families in which JOAG mapped to this locus (*7*–*9*). Observed recombinations between the glaucoma phenotype and highly polymorphic genetic markers in two large JOAG kindreds allowed the interval containing the *GLC1A* gene to be narrowed to a 3-centimorgan region of chromosome 1q between markers D1S3665 and D1S3664 (*10*). Further evaluation of marker haplotypes revealed that each of three pairs of glaucoma families shared alleles of the same eight contiguous markers, suggesting that *GLC1A* lies within a narrower interval defined by D1S1619 and D1S3664 (*11*) (Fig. 1).

Several genes mapping to the *GLC1A* region of chromosome 1 were considered as candidates for the disease-causing gene. Three genes [*LAMC1* (*12*), *NPR1* (*13*), and *CNR2* (*14*)] were excluded from the candidate region by genetic linkage analysis with intragenic polymorphic markers (*10*). Five additional candidate genes were determined to lie within the observed recombinant interval by yeast artificial chromosome (YAC) sequence tagged site (STS) content mapping: selectin E (*SELE*) (*15*) (GenBank accession number M24736); selectin L (*SELL*) (*16*) (GenBank accession number

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Table 1. Prevalence of *GLC1A* gene mutations.

Mutation	Familial glaucoma* (n = 227)	Unselected glaucoma† (n = 103)	General population‡ (n = 380)	Normal volunteers§ (n = 91)
Gly357Val	2 (0.9%)	0 (0%)	0 (0%)	0 (0%)
Gln361STOP	6 (2.6%)	3 (2.9%)	1 (0.3%)	0 (0%)
Tyr430His	2 (0.9%)	0 (0%)	0 (0%)	0 (0%)
Totals	10 (4.4%)	3 (2.9%)	1 (0.3%)	0 (0%)

*Unrelated probands having at least one living first-degree relative with a documented history of glaucoma. †Unrelated, consecutive patients with open angle glaucoma seen in the University of Iowa glaucoma clinic. ‡Unrelated patients with retinal disease (n = 333) and unrelated spouses from previous linkage studies (n = 47). These patients were collected without regard to personal or family history of glaucoma and were used as an approximation of the general population. §Unrelated volunteers 40 years of age or older who do not have a personal or family history of glaucoma and whose intraocular pressures were less than 20 mm Hg.