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BCR-ABL Regulates Phosphatidylinositol 3-Kinase-p110γ Transcription and Activation and Is Required for Proliferation and Drug Resistance*[S]

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The BCR-ABL oncogene is the hallmark of chronic myeloid leukemia, a clonal hematopoietic stem cell disorder. BCR-ABL displays constitutive tyrosine kinase activity, required for its transformation ability. Although the molecular mechanisms behind this malignancy are not fully understood, a role for phosphatidylinositol (PI) 3-kinase has been repeatedly described. Here we report the specific up-regulation of the class I_B catalytic subunit of PI 3-kinase (p110γ) in response to BCR-ABL expression. We demonstrate that this up-regulation is due to increased transcription and is dependent on both PI 3-kinase and MEK activity. We performed *in vitro* kinase activity assays and show that BCR-ABL also leads to increased p110γ activity and that this activation requires both G protein-coupled receptor and Ras signaling. In addition, by transfection of cells with dominant negative p110γ, we determined that this specific PI 3-kinase isoform is involved in both proliferation and the apoptosis resistance associated with chronic myeloid leukemia. The data presented here define for the first time the ability of BCR-ABL to alter the expression levels of PI 3-kinase isoforms and also demonstrate a previously unreported link between BCR-ABL and p110γ.

Chronic myeloid leukemia (CML)[2] is a clonal disorder of the hematopoietic system that results in the excessive accumulation of immature and mature myeloid cells. The cytogenetic cause of CML is the Philadelphia chromosome (Ph) (1). This results from a translocation between breakpoint cluster region (*BCR*) and *ABL* genes on chromosomes 22 and 9, respectively. Depending on the breakpoint within the *BCR* gene, the fusion protein may be either 210 kDa (p210) or 185 kDa in size (2). The p210 form of the BCR-ABL fusion protein is seen in 95% of all CML cases. *ABL*, which normally encodes a tyrosine kinase, becomes constitutively active when fused to *BCR* (3). This constitutive tyrosine kinase activity is crucial for the transforming ability of the BCR-ABL oncogene (4). CML progresses from an initial chronic phase characterized by the accumulation of apparently normal myeloid precursors and mature cells to an acute phase associated with a poor prognosis. The acute phase is marked by the clonal expansion of differentiation-arrested myeloid precursor cells. The sole curative treatment for CML is allogeneic bone marrow transplantation, which is available only to a very small number of patients. It is hoped that the identification of new genes and signaling pathways associated with BCR-ABL expression will potentially lead to new molecular targets for therapy.

BCR-ABL has been reported to activate numerous signal transduction pathways, normally activated by receptor-tyrosine kinases, including the Ras/mitogen-activated protein kinase pathway (5, 6), Jak/STAT (signal transducers and activators of transcription)(7, 8), and the phosphoinositide 3-kinase (PI3K)/AKT pathways (9). In particular it has been demonstrated that PI3K activity is required not only for BCR-ABL-mediated survival but also for the growth of CML cells (10).

PI3Ks represent a family of cytosolic, intracellular signaling proteins involved in the regulation of several cellular functions including proliferation and differentiation (11), survival (12), and malignant transformation (13). The primary enzymatic activity of these kinases is the phosphorylation of the 3-OH of inositol head groups of phosphoinositide (PI) lipids (14, 15), which have been reported to act as second messengers (16). PI3Ks can be divided into three main classes (I-III). These classes are based on their *in vitro* substrate specificity, structure, and probable mode of regulation. Class I PI3Ks are further categorized into one of two subclasses, I_A or I_B. In general, class I_A PI3Ks are heterodimers consisting of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit. PI3Kγ is the only class I_B member of the PI3Ks. This enzyme is also composed of a 110-kDa catalytic subunit (p110γ), but in contrast to the class I_A members, it associates with a p101 adaptor molecule. In general, class I_A PI3Ks are reported to be activated by receptor and non-receptor tyrosine kinases and small G proteins. Conversely p110γ has been reported to be activated by the α and βγ subunits released upon activation of G protein-coupled receptors (17, 18). However, G protein-coupled receptors have also been shown to activate p85-dependent PI3Ks in human vascular smooth muscle cells (19), and it has been demonstrated that Gβγ can activate p110β (20). BCR-ABL has been shown to interact indirectly with the p85 regulatory subunit of class I_A PI3Ks (21). However, to date PI3Kγ has not been implicated in transformation by BCR-ABL or in the pathogenesis of CML. A potential role for PI3Kγ is substantiated by the fact that cDNA encoding p110γ was first isolated from human myeloid cells (22) and is also preferentially expressed in cells of the hematopoietic lineage (23).

The purpose of this study was to investigate the affect that ectopic expression of BCR-ABL has on the expression levels of different PI3Ks. We demonstrate that transcription of the p110γ gene is selectively up-regulated in response to p210 BCR-ABL in 32D cells transfected with BCR-ABL and also in K562 and Lama-84 CML cell lines. Analysis of this up-regulation shows that it is dependent on both MEK and PI3K activities but occurs independently of Ras, Raf, and PKC. The increased level of p110γ expression in BCR-ABL-positive cells is accompanied by an increase in both the lipid and protein kinase activities of this enzyme. Transfection of cells with dnp110γ reveals that its activity contributes to

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[2] The abbreviations used are: CML, chronic myeloid leukemia; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PI, phosphoinositide; PI3K, PI 3-kinase; PKC, protein kinase C; FTS, *S-trans,trans*-farnesylthiosalicyclic acid; RT, reverse transcription; MBP, myelin basic protein, ERK, extracellular signal-regulated kinase.

the proliferation and apoptosis resistance of these cells. These data not only provide insights into the regulation of p110γ expression and activation but also identify a novel downstream target of BCR-ABL, involved in the drug resistance of CML cells (22).

MATERIALS AND METHODS

Cell Culture and Transfection—The 32D and empty vector cell lines were maintained in RPMI 1640 (Invitrogen) with 10% WEHI-conditioned media as a source of IL-3. The BCR-ABL-expressing C4 clone was maintained in RPMI 1640 containing 0.2 μg/ml puromycin (Sigma-Aldrich). C4 cells treated with STI571 were cultured in the presence of 10% WEHI-conditioned media. C4 cells transfected with dnp110γ (K832R) were maintained in media supplemented with 250 μg/ml G418 sulfate (Geneticin, Sigma-Aldrich). K562, Lama-84, and HeLa cells were cultured in RPMI 1640 containing 200 μM L-glutamine (Sigma-Aldrich). 20 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (Sigma-Aldrich) was added to the media of K562 and Lama-84 cells treated with STI571. All cultures were supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 1 mg/ml streptomycin (Sigma-Aldrich). The pcDNA3 vector encoding dominant negative p110γ (K832R) was kindly provided by Dr. R. Wetzker (University of Jena, Jena, Germany). Transfection of C4 cells was achieved by electroporation using a Gene Pulser apparatus (Bio-Rad). Stably transfected clones were obtained by serial dilution in media containing 700 μg/ml G418. HeLa cells were transiently transfected using Effectene transfection reagent according to the manufacturer's instructions (Qiagen Ltd., Crawley, UK).

Reagents—Radiochemicals were obtained from Amersham Biosciences. STI571 was kindly provided by Novartis, Basel, Switzerland; etoposide (VP-16) and actinomycin D were purchased from Sigma-Aldrich; *S-trans*,*trans*-farnesylthiosalicyclic acid (FTS) was obtained from Affiniti Research Products (Exeter, UK); UO126 was from Cell Signaling Technology, Hertfordshire, UK); LY294002, pertussis toxin, 8-bromo-cAMP, staurosporine, and protein kinase C inhibitor set (bisindolylmaleimide I, calphostin C, chelerythrine chloride, Gö 6976, PKC inhibitor 20–28, Ro 032–0432) were purchased from Calbiochem.

Reverse Transcriptase (RT)-PCR—Total cellular RNA was prepared using Tri Reagent (Biosciences, Dublin, Ireland). Single-stranded cDNA was synthesized according to the Moloney murine leukemia virus reverse transcriptase protocol (Promega, Southampton, UK). Oligo(dT), $MgCl_2$, and RNasin were also purchased from Promega. dNTPs were obtained from Sigma-Aldrich. cDNA was amplified using *Taq* DNA polymerase (Promega) according to the manufacturer's instructions. PCR reactions were carried out as follows: 95 °C for 5 min and 13–30 cycles of 95 °C for 1 min, T_{anneal} for 1 min, and 72 °C for 1 min followed by 72 °C for 5 min. Primers were p110α (forward, 5′-gcaaccgtgaagaaaagatcctcaatcga-3′ reverse, 5′-caacaacatgctccgagtctttttcctga-3′; T_{anneal} 65 °C, 357-bp product), p110β (forward, 5′-atccaaccttcctctcccttttaccaccaa-3′, reverse, 5′-tccagacctcagctgtcctttgaagtca-3′, T_{anneal} 65 °C, 414-bp product), p110δ (forward, 5′-gtcaagctcggaggtgaatgtatgctca-3′, reverse, 5′-gatcttctccagagcggggaagtacaca-3′, T_{anneal} 65 °C, 372-bp product), p110γ (forward, 5′-gctcttggcagaaaaaggtg-3′, reverse, 5′-cctgggcatctcagtggtat-3′, T_{anneal} 55 °C, 169-bp product), p101 (forward, 5′-aactacaccgcaaacttcagtccaa-3′, reverse, 5′-ctggttccagctgtaggtgtagcat-3′, T_{anneal} 60 °C, 352-bp product), 18 S rRNA (forward, 5′-cgaggccctgtaattgga-3′, reverse, 5′-gtttcccgtgttgagtca-3′, T_{anneal} 56 °C, 715-bp product), glyceraldehyde-3-phosphate dehydrogenase (forward, 5′-accacagtccatgccatcac-3′, reverse, 5′-tccaccacccagttgctgta-3′, T_{anneal} 58 °C, 450-bp product). To control for DNA contamination of RNA samples, PCR was also carried out in the absence of reverse transcription (*−RT* in Figs. 1*a* and 7*a*); in this case Moloney murine leukemia virus was omitted from the reverse transcription reaction. Control reactions were performed initially to ensure linearity of amplification over the number of cycles used. Real-time reverse transcriptase-PCR was performed using SYBR Green I on a LightCycler instrument (Roche Diagnostics). Primer sequences and PCR conditions were used as above.

Cell Lysis and Immunoblotting—Cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 1 μg/ml antipain, 1 μg/ml aprotinin, 1 μg/ml chymostatin, 0.1 μg/ml leupeptin, 1 μg/ml pepstatin, and 100 μM phenylmethylsulfonyl fluoride). The lysates were centrifuged at 20,000 × *g* (4 °C) for 15 min to remove insoluble debris. Equivalent amounts of protein, as determined by the Bio-Rad protein assay, were resolved using SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schüll). All secondary antibodies were peroxidase-conjugated, and proteins were detected using Enhanced Chemiluminescence (ECL) (Amersham Biosciences). Antibodies were anti-PI3K p110γ, anti-phospho-AKT (Ser-473), and anti-AKT (Cell Signaling Technology), anti-PI3K p110α and anti-PI3K p110β (Santa Cruz Biotechnology, Heidelberg, Germany), anti-PI3K p110δ (Calbiochem), anti-phosphotyrosine (PY20) (Transduction Laboratories, San Diego, CA), anti-p101 (Upstate Biotechnology Ltd., Milton Keynes, UK), anti-β-actin (Sigma-Aldrich).

Kinase Assays—Ras activity was measured using the Ras activation assay kit from Upstate Biotechnology and following the manufacturer's instructions. GTP-bound Ras from cell lysates was "pulled down" using the GST fusion protein corresponding to the Ras binding domain of Raf-1, bound to agarose. The presence of active Ras was detected by Western blotting using the anti-Ras antibody. Raf-1 kinase assay was performed using the Raf-1 kinase cascade assay kit from Upstate Biotechnology according to the manufacturer's instructions. Raf-1 was immunoprecipitated from cell lysates, and kinase activity was measured using MEK, extracellular signal-regulated kinase (ERK), and myelin basic protein (MBP) as sequential substrates. MEK kinase assays were performed essentially as the Raf-1 kinase assay with some modifications. MEK was immunoprecipitated from cell lysates, and kinase activity was measured using ERK and MBP as sequential substrates. Assay products for Raf-1 and MEK assays were assessed using P81 phosphocellulose paper (Upstate Biotechnology) and counting on a Beckman Coulter LS 6500 scintillation counter. p110γ lipid kinase assays were performed using the PI3K enzyme-linked immunosorbent assay kit from Echelon Biosciences Inc (Salt Lake City, UT) according to the manufacturer's instructions. Protein phosphorylation was assayed essentially according to Stoyanova *et al.* (24) with MBP used as a substrate rather than the measurement of autophosphorylation. Briefly, p110γ was immunoprecipitated from cell lysates and washed twice with kinase buffer without ATP (50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM EDTA, 5 mM dithiothreitol, 10 mM $MgCl_2$, 0.01% Triton X-100) and resuspended in kinase buffer supplemented with ATP to give a final concentration of 20 μM and 10 μCi of [γ-^{32}P]ATP per sample and MBP at a final concentration of 20 μg/sample. The reaction was allowed to proceed for 20 min at 30 °C. Assay products were assessed using P81 phosphocellulose paper (Upstate Biotechnology) and counting on a Beckman Coulter LS 6500 scintillation counter.

PCR—Genomic DNA was extracted from cells using Gen Elute mammalian genomic DNA miniprep kit (Sigma-Aldrich). PCR reactions to confirm the presence of pcDNA3 plasmid were carried out as follows: 95 °C for 5 min and 35 cycles of −95 °C for 1 min, 63 °C for 1

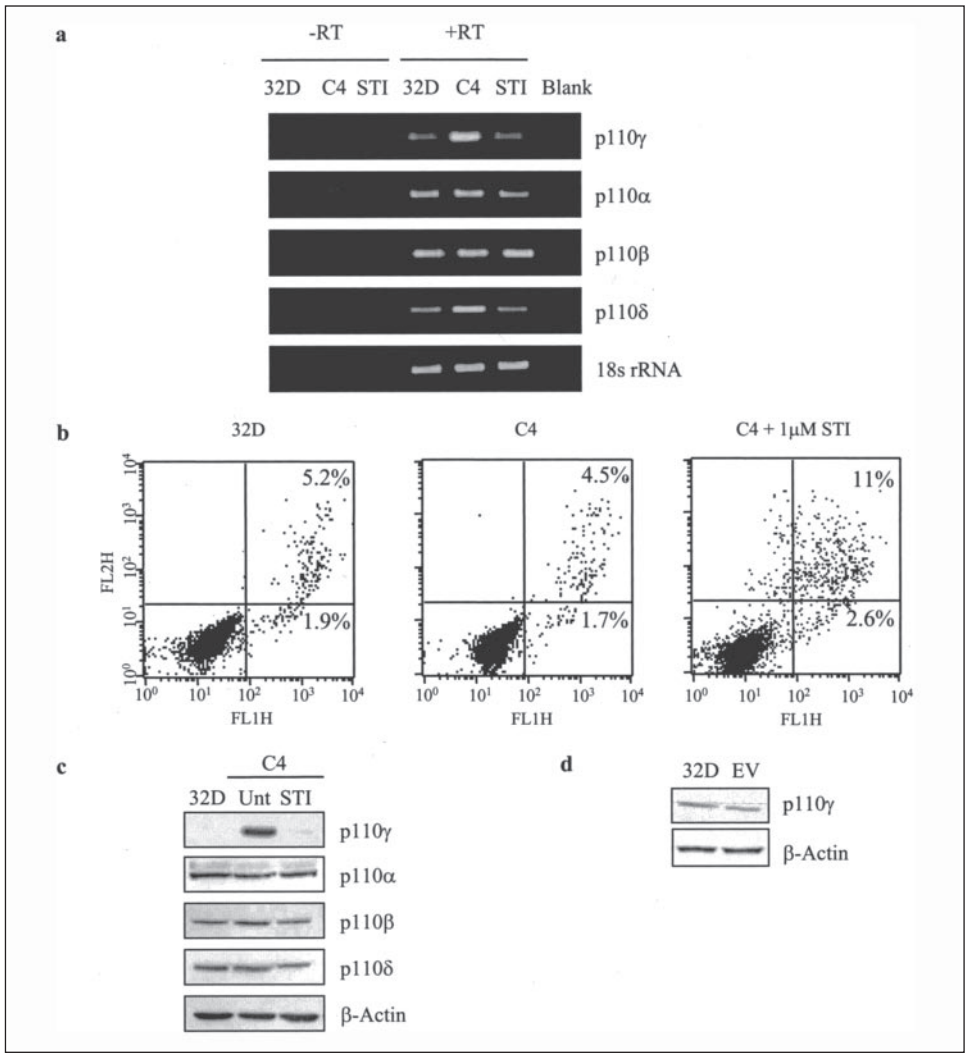

FIGURE 1. **Expression levels of PI3K p110 isoforms in BCR-ABL-expressing cells.** *a*, RT-PCR experiments using cDNA from 32D, untreated C4 cells, and C4 cells treated for 48 h with 1 μM STI571 (*STI*) show expression levels of p110γ, p110α, p110β, and p110δ in the presence (+*RT*) and absence (−*RT*) of reverse transcription. Levels of 18s rRNA PCR product demonstrate equal quantities of cDNA. *b*, untreated 32D and C4 cells and C4 cells treated for 48 h with 1 μM STI571 were assessed for apoptotic cell death by annexin V/propidium iodide staining. Percentages in the *bottom right quadrant* indicate annexin V-positive, propidium iodide-negative cells, and percentages in the *top right quadrant* indicate annexin V-positive, propidium iodide-positive cells. *c*, Western blot analysis of expression of p110γ, p110α, p110β, and p110δ in total cell lysates from 32D, untreated (*Unt*) C4 cells, and C4 cells treated for 48 h with 1 μM STI571. *d*, Western blot analysis of expression of p110γ in total cell lysates from 32D cells and empty vector-transfected 32D cells (*EV*). Actin antibody binding demonstrates equal protein loading.

min, 72 °C for 1 min followed by 72 °C for 5 min. Primers were 5′-tcagaggtggcgaaacccgaca-3′ forward and 5′-gcgcgtaatctgctgcttgcaa-3′ reverse; the product was 498 bp. DNA was amplified using native *Taq* DNA polymerase (Stratagene, Amsterdam, the Netherlands). Electrophoresis was carried out using 1.5% agarose gels containing 0.5 μg/ml ethidium bromide, and DNA was visualized under UV light.

Cell Proliferation Assay—Cells were plated at a density of 0.2×10^6 cells/ml. Cell numbers from aliquots of time course experiments were determined by trypan blue exclusion assay using a Neubauer hemocytometer.

Measurement of Apoptosis (Phosphatidylserine Exposure)—The exposure of phosphatidylserine on the extracellular surface of the plasma membrane was monitored by the binding of annexin V-fluorescein isothiocyanate according to the manufacturer's instructions (IQ Products, Labron Ltd., Dublin, Ireland). Briefly, 5×10^5/ml cells were resuspended in calcium binding buffer (10 mM Hepes, 2.5 mM $CaCl_2$, 140 mM NaCl) and incubated with annexin V-fluorescein isothiocyanate for 5 min at room temperature in the dark. Cells were incubated with 50 μg/ml propidium iodide at room temperature before analysis. Fluorescence resulting from fluorescein isothiocyanate and propidium iodide was measured at 530 nm (FL1) and 590 nm (FL2) respectively and analyzed using Cellquest software on a FACScan flow cytometer (BD Biosciences) using an excitation of 488 nm.

RESULTS

Expression of p110γ Is Selectively Increased in Cells Transfected with p210 BCR-ABL—PI3K has been implicated downstream of BCR-ABL in studies from both mouse (25) and cell line models (9, 26) of CML. It has also been shown recently that PI3K inhibitors can synergize with STI571 to inhibit the growth of human CML cells (27). However, very little is known about the specific PI3K isoforms involved in signaling downstream of BCR-ABL. In particular, the affect of BCR-ABL on expression of PI3K isoforms has not been studied. Microarrays were previously carried out by our group comparing 32D cells (murine IL-3-dependent myeloid progenitor cells) with a transfected clone of this cell line expressing high levels of p210 BCR-ABL (C4). Transfection of these cells with BCR-ABL has been shown to render them growth factor-independent and also to increase their resistance to cytotoxic drug-induced apoptosis (28). Results from microarray comparisons revealed that the class I_B PI3K isoform p110γ is expressed at an increased level in C4 cells compared with the parental 32D cell line.

RT-PCR was used to confirm this increase in p110γ expression and also to investigate the possibility that the expression of other PI3K catalytic subunits is altered by BCR-ABL. Fig. 1*a* demonstrates that p110γ mRNA is in fact expressed at a higher level in C4 cells than 32D cells, as had been shown by microarray results. It was found that ectopic expres-

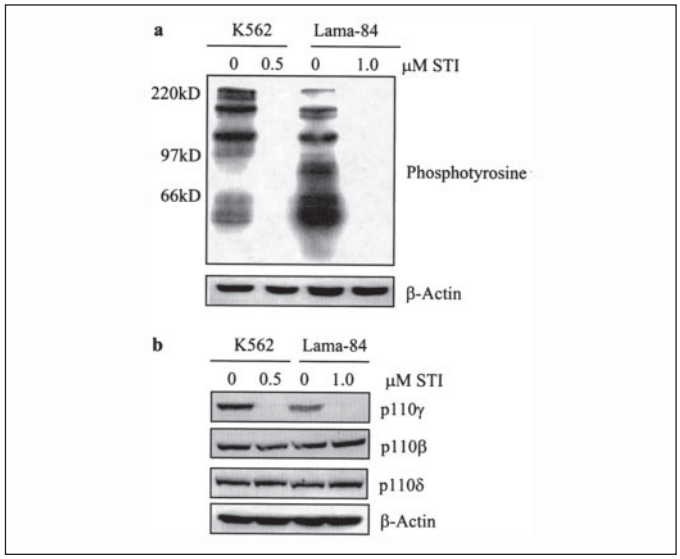

FIGURE 2. **p110γ expression is specifically increased in K562 and Lama-84 cells in a BCR-ABL-dependent manner.** *a*, Western blot analysis of phosphotyrosine levels in total cell lysates from K562 and Lama-84 cells treated with the indicated concentrations of STI571 (*STI*) for 24 h. *b*, Western blot analysis of expression of p110γ, p110β, and p110δ from *a*. Actin antibody binding demonstrates equal protein loading.

sion of BCR-ABL does not affect the level of expression of either class I_A isoforms p110α or p110β. Expression of the third class I_A isoform p110δ was found to be increased in C4 cells, similar to p110γ.

STI571 (Gleevec) is a specific inhibitor of BCR-ABL tyrosine kinase activity (29). When C4 cells are treated with 1 μM STI571 for 48 h, the phenotype reverted to that of 32D cells. That is, levels of phosphotyrosine are reduced, and the cells become dependent on interleukin-3 for survival (data not shown). This treatment does not result in significant cytotoxicity, as determined by annexin V/propidium iodide staining (Fig. 1*b*). As such, STI571 was used to confirm that differences between 32D and C4 cells are in fact due to the tyrosine kinase activity of BCR-ABL. Indeed, treatment of C4 cells with STI571 resulted in a decrease in the levels of expression of both p110γ and p110δ mRNA (Fig. 1*a*).

The increased p110γ mRNA seen in C4 cells is reflected by an increased level of p110γ protein as seen by Western blotting of whole cell lysates from 32D and C4 cells (Fig. 1*c*). It is also shown that treatment with STI571 results in a decrease in levels of p110γ protein in C4 cells. As was observed at the mRNA level, no difference was seen in the level of expression of p110α or p110β protein in C4 cells. In addition, levels of p110δ protein were found to be equivalent between 32D and C4 cells even though an increase in mRNA was seen. No increase in p110γ protein was seen in empty vector-transfected 32D cells, confirming that the up-regulation of p110γ does not occur as a result of transfection alone but is dependent on BCR-ABL expression (Fig. 1*d*). These results demonstrate that expression of the PI3K catalytic isoform p110γ is selectively increased by p210 BCR-ABL.

p110γ Expression Is Also Increased in K562 and Lama-84 Cells in a BCR-ABL-dependent Manner—To confirm that the increase in p110γ expression seen in C4 cells is not unique to this cell line, levels of p110γ were analyzed in two additional CML cell lines, K562 and Lama-84. K562 is an erythroleukemia cell line derived from a chronic myeloid leukemia patient in blast crisis (30). Lama-84 is a chronic myeloid leukemia cell line established from the peripheral blood of a patient 1 month after the onset of blast crisis (31). Both cell lines were treated with STI571 for 24 h to inhibit BCR-ABL tyrosine kinase activity. Concentrations of STI571 were chosen for each cell line that resulted in a complete reduction in phosphotyrosine levels as determined by PY-20

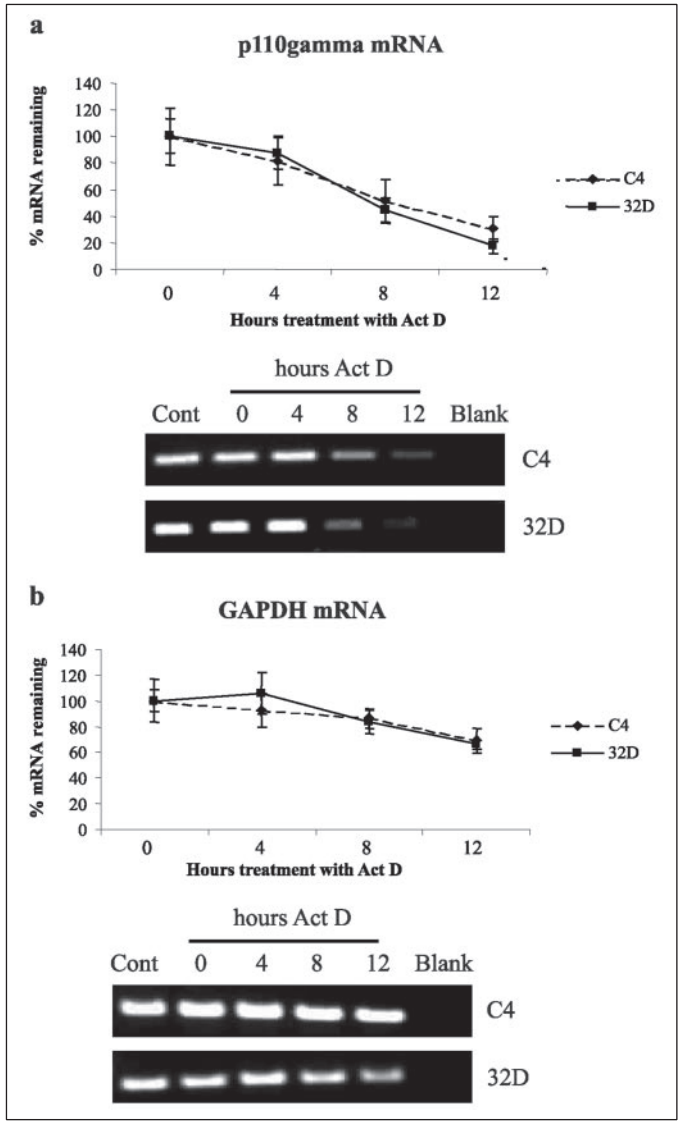

FIGURE 3. **Increased p110γ expression in C4 cells is due to increased transcription.** 32D and C4 cells were treated with 4 μg/ml actinomycin D (*Act D*). Cells were harvested at the indicated times and subsequently analyzed for mRNA levels of p110γ (*a*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (*b*) by real-time RT-PCR. The *upper panel* in *a* and *b* represents the % mRNA remaining compared with samples treated for 0 h. Data are presented as the mean ± S.E. for independent experiments ($n = 4$). The *lower panels* show PCR products run on an agarose gel. The control (*Cont*) in all cases represents untreated cells.

antibody binding (Fig. 2*a*). We also determined by propidium iodide uptake on a FACScan flow cytometer that these concentrations were not significantly cytotoxic to the cells (data not shown).

It was found by Western blotting of whole cell lysates that both cell lines expressed p110γ and that the expression level of this protein was reduced by treatment with STI571. In addition, it is shown that treatment of both cell lines with STI571 had no affect on the level of expression of p110β or p110δ (Fig. 2*b*). We were unable to detect expression of the remaining PI3K catalytic subunit, p110α, in either cell line. These results confirm in two human CML cell lines the results seen in C4 cells, *i.e.* that expression of the p110γ isoform of PI3K is specifically up-regulated by BCR-ABL in a tyrosine kinase-dependent manner.

Increased p110γ Expression in C4 Cells Is Due to Increased Transcription—We have observed in C4 cells that increased p110γ protein is due to increased mRNA. Inhibition of the decay of cytoplasmic mRNA is a common mechanism employed in cells to induce a net

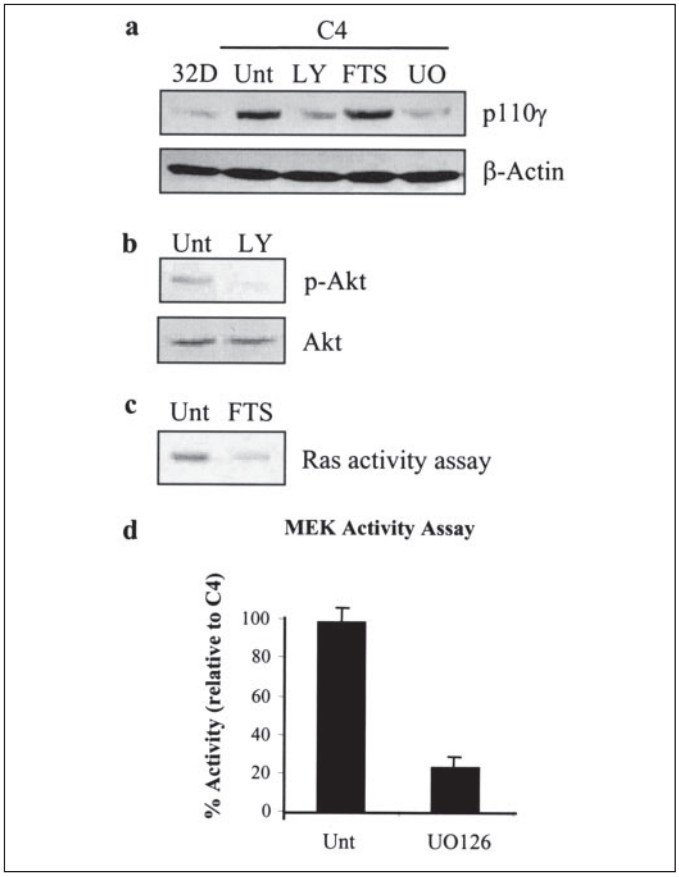

FIGURE 4. **The up-regulation of p110γ in C4 cells is dependent on PI3K and MEK activity but independent of Ras.** *a*, Western blot analysis of p110γ expression in total cell lysates from 32D, untreated (*Unt*) C4 cells, and C4 cells treated for 48 h with 20 μM LY294002 (*LY*) or 10 μM farnesyltransferase inhibitor (*FTS*) or for 24 h with 5 μM UO126 (*UO*) (extracellular signal-regulated kinase 1/2-specific MEK1/2 inhibitor). Actin antibody binding demonstrates equal protein loading. *b*, Western blot analysis of phospho AKT (Ser-473) levels in total cell lysates from untreated C4 cells and C4 cells treated for 48 h with 20 μM LY294002. AKT antibody binding demonstrates equal protein loading. *c*, cell lysates from untreated C4 cells and C4 cells treated for 48 h with10 μM FTS were incubated with Raf-1 Ras binding domain-agarose-conjugated beads. Bound proteins were denatured by boiling in reducing sample buffer and then resolved by SDS-PAGE followed by Western blotting to detect Ras-GTP using a monoclonal antibody to Ras. *d*, cell lysates from untreated C4 cells and C4 cells treated for 24 h with 5 μM UO126 were immunoprecipitated with MEK antibodies, and kinase activity was determined using an *in vitro* kinase assay in which recombinant extracellular signal-regulated kinase (ERK) and MBP were used. MEK activity was determined quantitatively by scintillation counting of the P81 phosphocellulose squares spotted with the assay product. Background counts were subtracted from each sample, and the resultant net counts were expressed as a percentage of the values obtained for C4 cells. Data are presented as the mean and S.D. for independent experiments ($n = 4$).

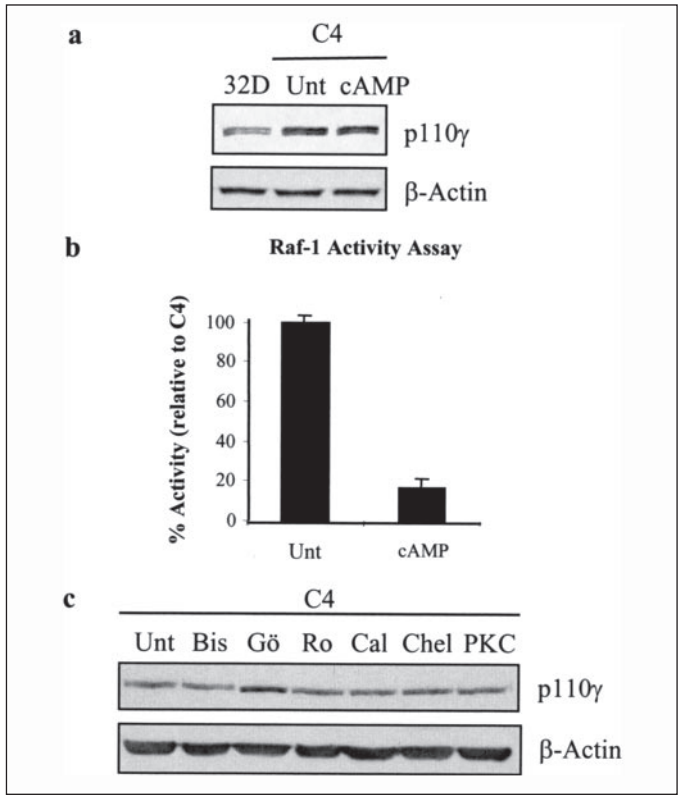

FIGURE 5. **The up-regulation of p110γ in C4 cells is not dependent on Raf-1 or PKC activity.** *a*, Western blot analysis of p110γ expression in total cell lysates from 32D, untreated (*Unt*) C4 cells, and C4 cells treated for 24 h with 400 μM 8-bromo-cAMP (*cAMP*) (Raf-1 inhibitor). Actin antibody binding demonstrates equal protein loading. *b*, cell lysates from untreated C4 cells and C4 cells treated for 24 h with 400 μM 8-bromo-cAMP were immunoprecipitated with Raf-1 antibodies, and kinase activity was determined using an *in vitro* kinase assay in which recombinant MEK, extracellular signal-regulated kinase (ERK), and MBP were used. Raf-1 activity was determined quantitatively by scintillation counting of the P81 phosphocellulose squares spotted with the assay product. Background counts were subtracted from each sample, and the resultant net counts were expressed as a percentage of the values obtained for C4 cells. Data are presented as the mean and S.D. for independent experiments ($n = 4$). *c*, Western blot analysis of p110γ expression in total cell lysates from untreated C4 cells and C4 cells treated for 24 h with a panel of PKC inhibitors (20 nM bisindolylmaleimide I (*Bis*), 40 nM Gö 6976 (*Gö*), 400 nM Ro 032–0432 (*Ro*), 100 nM calphostin C (*Cal*), 1 μM chelerythrine chloride (*Chel*), 20 μM PKC inhibitor 20–28 (*PKC*)). Actin antibody binding demonstrates equal protein loading.

increase in an mRNA species. To investigate the possibility that this mechanism was employed in C4 cells, both 32D and C4 cells were treated with actinomycin D, an inhibitor of *de novo* transcription. RNA was extracted from cells at 0, 4, 8, and 12 h, and the remaining fractions of p110γ mRNA were determined by real-time RT-PCR. Fig. 3*a* demonstrates that there is no difference in the stability of p110γ mRNA between 32D and C4 cells. This excludes the possibility that inhibition of mRNA decay is responsible for the increased level of p110γ mRNA seen in C4 cells. These results favor a direct transcriptional mechanism of BCR-ABL-dependent p110γ expression.

The Up-regulation of p110γ in C4 Cells Is Dependent on PI3K and MEK Activity but Independent of Ras—The ability of p210 BCR-ABL to up-regulate the expression of p110γ represents a novel and potentially important downstream affect of this oncogene. As such, we aimed to determine the pathways and signaling molecules required for this up-regulation. C4 cells were treated for 48 h with 20 μM LY294002 or 10 μM FTS or for 24 h with the extracellular signal-regulated kinase (ERK) 1/2-specific MEK1/2 inhibitor, UO126 (5 μM) to investigate the involvement of the signaling molecules PI3K, Ras, and MEK, respectively. These molecules were chosen due to their reported involvement in transformation by BCR-ABL (26, 32, 33).

It was found that treatment of C4 cells with both LY294002 and UO126 resulted in a down-regulation in the expression of p110γ (Fig. 4*a*). This indicates that downstream of BCR-ABL, both PI3K and MEK activities are required for the increased expression of p110γ. The farnesyltransferase inhibitor (FTS) did not affect p110γ protein levels, suggesting that Ras signaling is not involved in the up-regulation of p110γ expression in response to BCR-ABL. For each of the inhibitors used, it was confirmed that the activity of the target molecule was in fact reduced. This was achieved by the use of a Ras activity assay (Fig. 4*c*) and a MEK activity assay (Fig. 4*d*), and in the case of PI3K, Western blot analysis of levels of AKT phosphorylation (ser473) were used as an indicator of PI3K activity (Fig. 4*b*).

The Up-regulation of p110γ in C4 Cells Is Not Dependent on Raf-1 or PKC Activity—Activation of MEK often occurs in a Ras-dependent manner via Raf-1. Because we found that MEK is involved in the up-reg-

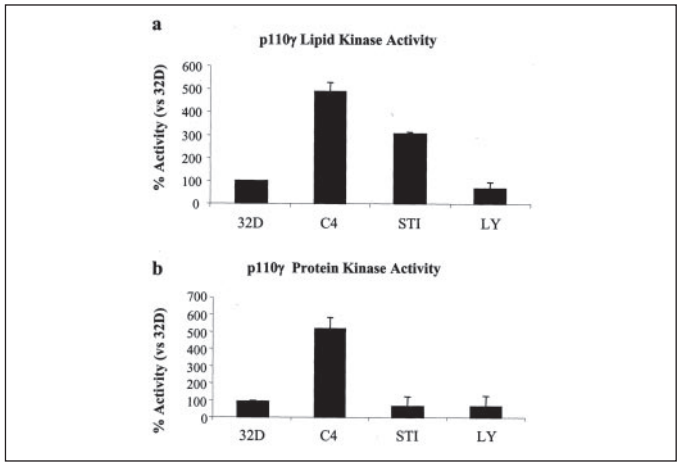

FIGURE 6. **Increased p110γ activity is observed in C4 cells.** *a*, equal amounts of protein from 32D and C4 cells and C4 cells treated for 48 h with 1 μM STI571 (*STI*) or 20 μM LY294002 (*LY*) were immunoprecipitated with anti-p110γ antibodies, and enzymatic activity was determined using a competitive enzyme-linked immunosorbent assay. Results are expressed as a percentage of the values obtained for 32D cells. Data are presented as the mean and S.D. for independent experiments ($n = 4$). *b*, immunoprecipitates from *a* were assayed for protein kinase activity using an *in vitro* kinase assay in which MBP was used as a substrate. p110γ activity was determined quantitatively by scintillation counting of the P81 phosphocellulose squares spotted with the assay product. Background counts were subtracted from each sample, and the resultant net counts were expressed as a percentage of the values obtained for 32D cells. Data are presented as the mean and S.D. for independent experiments ($n = 4$).

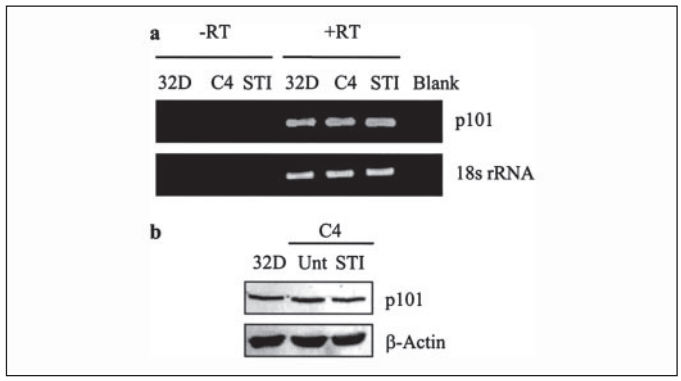

FIGURE 7. **Expression levels of the adaptor molecule p101 are not altered in BCR-ABL expressing cells.** *a*, RT-PCR experiments using cDNA from 32D, untreated C4 cells, and C4 cells treated for 48 h with 1 μM STI571 (*STI*) show expression levels of p101 in the presence (+*RT*) and absence (−*RT*) of reverse transcription. Levels of 18s rRNA PCR product demonstrate equal quantities of cDNA. *b*, Western blot analysis of expression of p101 in total cell lysates from 32D, untreated (*Unt*) C4 cells, and C4 cells treated for 48 h with 1 μM STI571. Actin antibody binding demonstrates equal protein loading.

ulation of p110γ, but Ras is not, we further investigated the possible involvement of Raf-1. C4 cells were treated for 24 h with 400 μM 8-bromo-cAMP (cAMP activates protein kinase A, resulting in the phosphorylation of Raf-1 on Ser-43 and Ser-621, inhibiting Raf-1 activity (34)). It was found that cAMP had no affect on expression levels of p110γ in C4 cells (Fig. 5*a*). A Raf-1 activity assay confirmed that Raf-1 activity can be blocked by treatment with 8-bromo-cAMP (Fig. 5*b*).

These results suggest that in the up-regulation of p110γ in C4 cells MEK is activated in a manner other than the classical Ras/Raf/MEK pathway. Others have shown that MEK can be activated by various PKC isoforms in a Raf-independent manner (35–37). As such, we investigated a possible role for PKC in the up-regulation of p110γ by BCR-ABL. PKC represents a family of at least 11 members that can be divided into three distinct classes: classical PKC, novel PKC, and atypical PKC. These classes differ in their requirements for diacylglycerol, phorbol esters, and calcium for activation *in vitro* and *in vivo* (38–41). To ensure that all PKC isoforms were inhibited, we treated C4 cells with a panel of PKC inhibitors. As shown in Fig. 5*c* none of these inhibitors reduced the expression levels of p110γ protein in C4 cells, suggesting that PKC is not responsible for the activation of MEK or for the increased levels of p110γ expression.

Increased p110γ Activity Is Observed in C4 Cells—Given the increased expression of p110γ in C4 cells, we compared the level of PI3K activity in p110γ immunoprecipitates from 32D and C4 cells. Fig. 6*a* shows that the level of p110γ lipid kinase activity in C4 cells is approximately five times greater than that seen in the parental 32D cell line. It is also demonstrated that this increase in kinase activity can be reduced by treatment of C4 cells with 1 μM STI571, indicating that the tyrosine kinase activity of BCR-ABL is required. C4 cells treated for 48 h with 20 μM LY294002 were also assayed as a control.

p110γ has been described as a pleiotropic signaling protein with both lipid and serine-threonine protein kinase activities. It has been shown to phosphorylate the adaptor protein p101 and MEK-1 along with its capability to autophosphorylate (42). As such we investigated the possibility that p110γ has increased protein kinase activity in C4 cells. Fig. 6*b* shows that this was found to be true, with C4 cells displaying a greater than 5-fold increase in p110γ protein kinase activity when compared with 32D cells. As was seen for the lipid kinase activity of p110γ in C4 cells, its protein kinase activity can be reduced by treatment of cells with the BCR-ABL inhibitor STI571 or with the general PI3K inhibitor LY294002.

Expression Levels of p101 Are Unaffected by BCR-ABL—The class I_A p110 isoforms α, β, and δ form a complex with p85 regulatory subunits, whereas p110γ, as the only class I_B member, is reported to associate with a p101 adaptor subunit (43). This adaptor molecule has been reported to be indispensable for the activation of p110γ in some systems (18). In an attempt to elucidate the mechanism by which p110γ is activated in C4 cells, RT-PCR and Western blotting were employed to determine whether the expression of the p101 regulatory subunit is also increased by BCR-ABL. We found that there is no difference in the level of p101 between 32D and C4 cells at either the mRNA (Fig. 7*a*) or protein (Fig. 7*b*) level. It is also shown that treatment of C4 cells with STI571 has no affect on p101 expression levels, confirming that, although BCR-ABL up-regulates the expression of the PI3Kγ catalytic subunit p110γ, it has no affect on the associated adaptor molecule. The selective induction of the p110γ gene may suggest that the increased kinase activity observed is independent of the p101 adaptor or that p101 is expressed at sufficiently high levels in 32D cells and activation is limited by levels of p110γ.

The Up-regulation of p110γ Lipid Kinase Activity in C4 Cells Is Dependent on Both Ras and G Protein-coupled Receptor Signaling—Similar to class I_A PI3Ks, inactive p110γ is predominantly located in the cytosol, whereas active p110γ is present in the membrane fraction. However, although class I_A PI3Ks are regulated by receptor and non-receptor tyrosine kinases, p110γ has not been reported to be regulated directly by tyrosine phosphorylation. In contrast, p110γ has been reported to be activated by α and βγ subunits of G protein-coupled receptors. In addition, Suire *et al.* (44) have demonstrated that p110γ can be activated by all Ras isoforms. Activation by Ras is thought to occur via allosteric modulation of p110γ in the absence of active recruitment of p110γ to the plasma membrane.

To investigate the mechanism of increased activation of p110γ downstream of BCR-ABL, we treated C4 cells for 24 h with 100 ng/ml pertussis toxin to inhibit the release of Gβγ heterodimers from G protein-coupled receptors or for 48 h with 10 μM FTS (farnesyltransferase

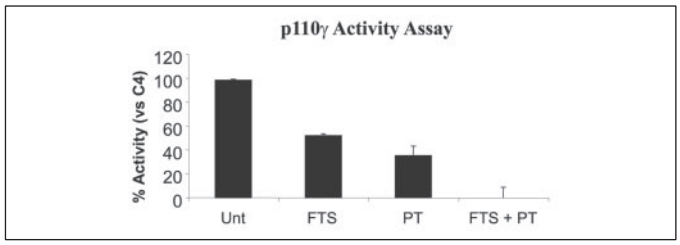

FIGURE 8. **The up-regulation of p110γ lipid kinase activity in C4 cells is dependent on both Ras and G protein-coupled receptor signaling.** Equal amounts of protein from untreated (*Unt*) C4 cells and C4 cells treated for either 24 h with 100 ng/ml pertussis toxin (*PT*) or 48 h with 10 μM FTS (farnesyltransferase inhibitor) or both were immunoprecipitated with anti-p110γ antibodies, and enzymatic activity was determined using a competitive enzyme-linked immunosorbent assay. Results are expressed as a percentage of the values obtained for C4 cells. Data are presented as the mean and S.E. of the mean for independent experiments ($n = 4$).

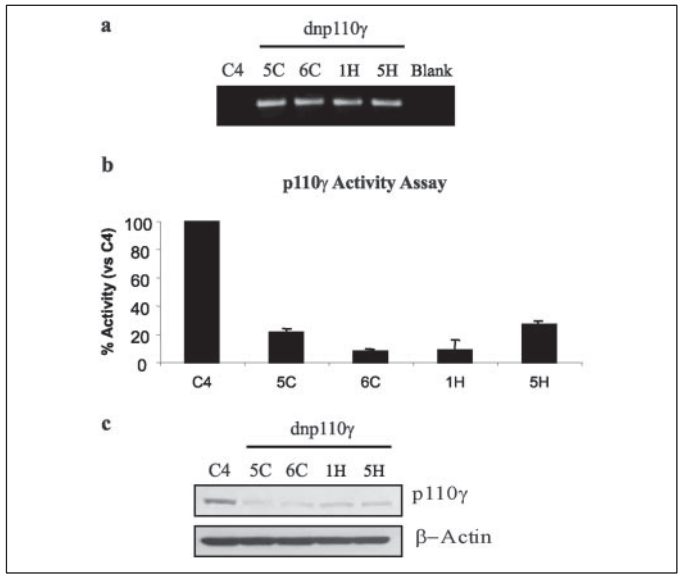

FIGURE 9. **C4 cells transfected with dnp110γ display reduced p110γ lipid kinase activity and express lower levels of p110γ protein.** *a*, PCR experiments using genomic DNA from C4 cells and four clones stably transfected with kinase-dead p110γ (K832R) (5C, 6C, 1H, 5H) show integration of the dominant negative plasmid into genomic DNA. *b*, equal amounts of protein from C4 cells and four clones stably expressing dnp110γ (5C, 6C, 1H, 5H) were immunoprecipitated with anti-p110γ antibodies, and enzymatic activity was determined using a competitive enzyme-linked immunosorbent assay. Results are expressed as a percentage of the values obtained for C4 cells. Data are presented as the mean and S.E for independent experiments ($n = 4$). *c*, Western blot analysis of expression of p110γ in total cell lysates from *b*. Actin antibody binding demonstrates equal protein loading.

inhibitor) to inhibit Ras activity. We found that these treatments led to a 60 and 50% decrease in p110γ activity, respectively (Fig. 8). We also demonstrate that treatment with both inhibitors together led to a complete loss of p110γ activity, suggesting that both pathways are involved. Similar results were seen in 32D cells, *i.e.* G protein-coupled receptor or Ras inhibition alone led to an ~50% decrease in p110γ activity, whereas inhibition of both signals resulted in complete loss of activity (data not shown). This indicates that the same pathways are responsible for the activation of p110γ in both cell lines, suggesting that increased expression of p110γ downstream of BCR-ABL may be critical for the resultant increase in activity seen.

p110γ Activity Is Required for Its Own Expression—To elucidate the downstream affects of increased p110γ activity in C4 cells, we transfected these cells with a dominant negative, kinase-deficient mutant PI3Kγ(K832R) (45, 46). Clones stably expressing this plasmid were selected by serial dilution in media containing G418. Four representative clones were chosen, namely 5C, 6C, 1H, and 5H. Because the vector used did not contain a tag, Western blotting could not be used to verify expression in transfected cells. Therefore, PCR was performed to confirm that the pcDNA3 vector encoding dominant negative p110γ was in fact integrated into the genome of these cells (Fig. 9*a*). In addition, a lipid kinase activity assay confirmed that all four selected clones displayed markedly reduced levels of p110γ activity (Fig. 9*b*). In all cases activity was reduced to less than 30% that of C4 cells, similar to the level of activity seen in 32D cells (Fig. 6). It was also confirmed using isoform-specific activity assays that transfection of C4 cells with dominant negative p110γ did not affect the activity levels of the other class I PI3K isoforms, p110α, p110β, or p110δ (data not shown). It is shown in Fig. 4*a* that treatment of C4 cells with the general PI3K inhibitor LY294002 leads to a down-regulation in the expression of p110γ. We also find that reduction of p110γ activity specifically, results in a decrease in levels of p110γ protein in C4 cells (Fig. 9*c*). This indicates that in our model p110γ activity is required for its own expression. This may suggest a positive feedback loop whereby BCR-ABL leads to increased transcription of p110γ, with subsequent increased activity, which leads to increased p110γ expression.

Expression of dnp110γ Does Not Affect Phospho-AKT but Results in Decreased Proliferation and Increased Sensitivity to Cell Death—Levels of AKT phosphorylation are generally accepted as a measure of PI3K activity in cells. Fig. 10*a* demonstrates that when C4 cells are transfected with dominant negative p110γ, there is no affect on phospho-AKT levels. This does not necessarily indicate that p110γ does not phosphorylate AKT but may imply that in the absence of p110γ, levels of AKT phosphorylation are maintained by the other class I PI3Ks, all of which are expressed in C4 cells (Fig. 1).

PI3K has been extensively shown to be involved in cell proliferation (47–49). In particular, it has been shown that treatment with wortmannin (a specific inhibitor of PI3K) inhibits the growth of both BCR-ABL positive cell lines and also CML primary cells (9). As such we compared the rate of proliferation in C4 cells with that of each of the four dnp110γ-transfected clones to determine whether this specific isoform is involved in proliferation. Fig. 10*b* demonstrates that all four dnp110γ clones displayed a marked reduction in the rate of proliferation based on cell number. This confirms that in C4 cells p110γ is required for cellular proliferation.

We also examined the role of p110γ in the resistance of C4 cells to apoptosis. We found that the percentage of apoptotic cells in response to staurosporine treatment is significantly greater in dnp110γ-transfected cells than in C4 cells (Fig. 10*c*). Because the increase seen in the level of apoptosis was small (<2-fold), we confirmed this affect using two additional treatments, VP16 (etoposide) (Fig. 10*d*) and UV irradiation (Fig. 10*e*). To confirm that the affect seen is due to the absence of p110γ activity rather than the presence of the dnp110γ plasmid, we transfected HeLa cells (which do not normally express p110γ) with dnp110γ. This did not affect the susceptibility of these cells to drug-induced apoptosis (data not shown). Together these results demonstrate that in C4 cells, p110γ activity plays a role both in proliferation and in apoptosis resistance and suggest that drugs targeting PI3Kγ specifically may increase the susceptibility of CML cells to chemotherapeutics.

DISCUSSION

PI3Ks represent a family of intracellular signaling proteins that control a variety of important cellular functions including proliferation (49), apoptosis (12), and migration (50). Recent findings suggest an involvement of PI3Ks in the pathogenesis of numerous diseases including cancer (51), heart failure (52), and autoimmune/inflammatory disorders

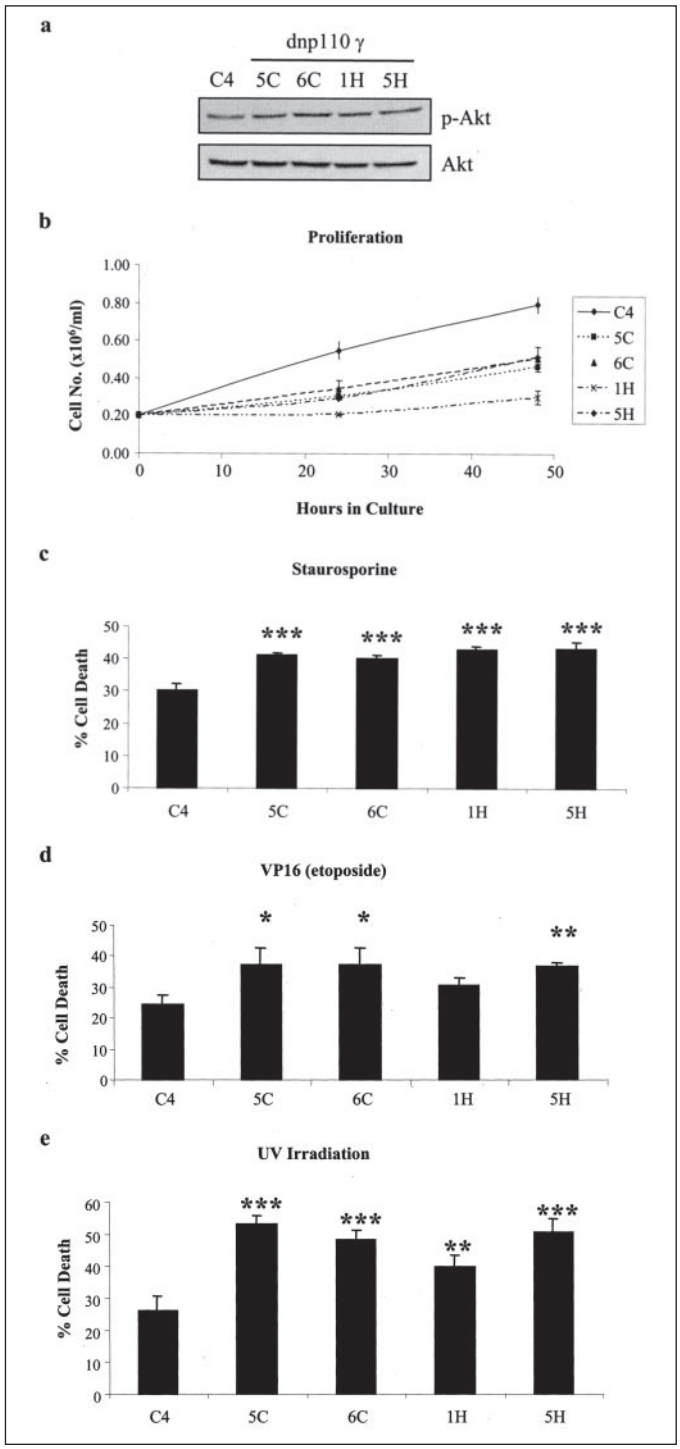

FIGURE 10. **Expression of dnp110γ in C4 cells does not affect phospho (*p*)-AKT levels but results in decreased proliferation and increased sensitivity to cell death.** *a*, Western blot analysis of phospho AKT (Ser-473) levels in total cell lysates from C4 cells and four clones stably expressing dnp110γ (5C, 6C, 1H, 5H). AKT antibody binding demonstrates equal protein loading. *b*, cell number (indicative of proliferation) for C4 cells and four clones stably expressing dnp110γ (5C, 6C, 1H, 5H) was assessed by trypan blue exclusion for 48 h. Data represent the mean ± S.E. for three independent experiments. *c*, cells from *b* were treated for 24 h with 200 nM staurosporine and were assessed for apoptotic cell death by annexin V/propidium iodide staining. Graphical data represent the mean and S.D. for individual experiments ($n = 4$). *d*, cells from *b* were treated for 24 h with 5 μg/ml VP16 (etoposide) and were assessed for apoptotic cell death by annexin V/propidium iodide staining. Graphical data represent the mean and S.D. for individual experiments ($n = 4$). *e*, cells from *b* were exposed to UV irradiation for 10 min and assessed for apoptotic cell death by annexin V/propidium iodide staining 3 h later. Graphical data represent the mean and S.D. for individual experiments ($n = 4$). Statistical analysis was performed using an unpaired Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ compared with levels of apoptosis in C4 cells).

(53). There have also been several reports of PI3K involvement in signaling downstream of the BCR-ABL oncogene (9, 10).

Recently, reports have demonstrated the affects of specific PI3K isoforms on various cellular functions. For example, both p110γ and p110δ have been shown to be involved in lymphocyte chemotaxis (54), whereas p110γ has been reported to regulate glucose-stimulated insulin secretion (55). However, the involvement of specific PI3K isoforms in CML cells is not well described.

The data presented here demonstrate for the first time that expression of the class I_B PI3K isoform, p110γ, is increased (both at the level of mRNA and protein) in a leukemic cell line expressing p210 BCR-ABL (C4). We find that the level of p110δ mRNA is also increased in response to BCR-ABL; however, this is not reflected by increased levels of p110δ protein, suggesting that the increased mRNA detected most likely has little or no function in BCR-ABL signaling. We also demonstrate that BCR-ABL does not affect the expression of the other PI3K p110 isoforms, p110α or p110β. These results indicate that the PI3K-p110γ isoform is specifically up-regulated in C4 cells in a BCR-ABL tyrosine kinase-dependent manner. We also show that the p110γ isoform is specifically up-regulated by BCR-ABL in two additional cell lines developed from CML patients, K562 and Lama-84.

It has been shown that increased levels of mRNA in cells can be due to increased mRNA stability (56, 57). Our data, however, show that the up-regulation of p110γ in C4 cells is due to increased transcription rather than increased mRNA stability, as no difference is seen in the half-life of p110γ mRNA between 32D and C4 cells. This represents a novel finding as, to date, all reports of PI3K involvement in CML have focused on increased activity rather than expression and as such very little is known about the mechanisms of PI3K expression. In an attempt to identify the pathways by which BCR-ABL leads to increased p110γ expression, we demonstrate that both PI3K and MEK activities are required for this increased expression, whereas Ras and Raf-1 activities are not. This suggests that the classical Ras/Raf/MEK pathway is not involved. PKC has also been reported to be involved in the activation of MEK. However, we have not found a role for PKC in the up-regulation of p110γ. Other serine/threonine kinases have also been implicated in MEK activation including Tpl-2 and c-Mos (58, 59). In fact p110γ itself has been shown to phosphorylate MEK-1 (42).

Increased activation of class I_A PI3Ks has been extensively reported in CML cells. Similarly, our study reveals that not only is expression of p110γ increased in C4 cells but that C4 cells also display 5-fold greater lipid and protein kinase activities of p110γ than do the parental 32D cells. We have determined that the activation of p110γ in 32D and C4 cells is dependent on both G protein-coupled receptor signaling and Ras activity. The fact that the mechanism of activation is similar in both cell lines suggests that the increased activity seen in C4 cells may occur as a direct result of increased expression, further demonstrating the importance of differential levels of expression of PI3K isoforms in disease states. We have also noted that inhibition of BCR-ABL tyrosine kinase activity by STI571 has a greater effect on p110γ protein kinase activity than lipid kinase activity. This may suggest that different pathways are involved in these two forms of activation.

The p101 adaptor molecule has been reported by some to be indispensable for the activation of p110γ by Gβγ (18); however, other studies have shown that *in vitro* Gβγ-induced activation of p110γ occurs in the absence of p101 (22). The apparent contradiction between these results may be due to the fact that it has been shown that p101 is required for membrane recruitment of p110γ via the interaction between Gβγ and p101, whereasp110γ already located at the membrane can be activated by Gβγ even in the absence of p101 (60). As such, p101 may not be

required for *in vitro* activation as membrane recruitment is not required. We have found that although BCR-ABL up-regulates expression of the catalytic subunit p110γ, it does not affect either mRNA or protein expression levels of p101. This may suggest that in our model p101 is not necessary for p110γ activation. However, because we have shown that this activation relies on Gβγ, it seems unlikely that p101 is not involved. It is more likely that p101 is expressed at sufficiently high levels in both 32D and C4 cells to allow maximum activation of p110γ and that its activity in 32D cells is limited by the amount of available p110γ.

As stated earlier, PI3Ks have been reported to be involved in many cellular functions. To determine the role of increased p110γ expression and activation in CML cells, we transfected C4 cells with a plasmid encoding a dominant negative version of this kinase. These experiments showed that levels of phospho-AKT in C4 cells are not dependent on p110γ activity. This may suggest that p110γ signaling does not involve the AKT pathway or may reflect redundancy between the different PI3Ks, as has been seen in mice deficient in the various PI3K isoforms (61, 62). Interestingly, we found that p110γ activity is required for its own expression, as levels of p110γ were dramatically reduced in cells expressing the dominant negative. This phenomenon has not been previously reported for any PI3K isoform, although similar affects have been seen for other signaling molecules including PKCβII (63) and αvβ6 integrin (64). In the latter case the authors proposed a self-perpetuating system of colon cancer progression in which the integrin αvβ6 provides a means of sustaining tumor cell proliferation. A similar system may occur in the case of p110γ in our model of CML.

As mentioned above, one of the main cellular functions of PI3K is in the regulation of proliferation. However, the specific p110γ isoform has not to date been reported to play a role. Therefore, our findings that inhibition of p110γ activity results in reduced proliferation represents a novel function of the class I_B PI3K. It has been demonstrated by numerous groups including our own that ectopic expression of BCR-ABL leads to increased resistance of cells to drug-induced apoptosis. Here we show that p110γ activity is involved in this drug resistance in C4 cells using both staurosporine and VP16 (etoposide). Again, this represents the first link between the p110γ isoform of PI3K and drug resistance. This increased susceptibility to apoptosis in dnp110γ-transfected C4 cells was also seen in response to UV treatment. It should be noted that the level of cell death seen in dominant negative p110γ clones treated with either agent, although significantly increased *versus* C4 cells, does not reach the level seen in the parental 32D cell line. This indicates that other factors are also involved in the apoptosis resistance of these cells. These may include other PI3K isoforms or distinct signaling pathways.

It has been shown that expression of p110γ is lost in colon cancer cell lines and also in colorectal adenocarcinomas from patient samples (65). This report also demonstrated that p110γ could be used to block the growth of human colon cancer cells. This study seemingly contradicts our findings that increased expression of p110γ contributes to the growth of a CML cell line. However, p110γ null mice have been generated by several different laboratories who have reported no increase in the development of tumors in these mice (66), suggesting that the antitumor affect of p110γ observed by Sasaki *et al.* (65) may not be of general significance.

Our study is one of the first to demonstrate a direct effect on cellular functions as a result of altered PI3K expression. It is also among the first to examine the mechanism by which the up-regulation of PI3K occurs. We demonstrate that p110γ is involved in the drug resistance of a CML cell line, suggesting that if PI3Kγ could be targeted it would improve the responsiveness of CML cells to chemotherapeutic drugs. Also, our finding that PI3Kγ is involved in transformation by BCR-ABL may not be exclusive to CML but may represent a potentially important target in other drug-resistant leukemias.

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