BCR-ABL Regulates Phosphatidylinositol 3-Kinase-p110γ Transcription and Activation and Is Required for Proliferation and Drug Resistance*1

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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 is suggested. 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 cyogenetic 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, IA or IB. In general, class IA PI3Ks are heterodimers consisting of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit. PI3Kγ is the only class IA member of the PI3Ks. This enzyme is also composed of a 110-kDa catalytic subunit (p110γ), but in contrast to the class IA members, it associates with a p101 adipotin molecule. In general, class IA 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 βy 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 p110γ (20). BCR-ABL has been shown to interact indirectly with the p85 regulatory subunit of class IA 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 shp110γ reveals that its activity contributes to...
BCR-ABL Regulates p110γ Expression and Activation

the proliferation and apoptosis resistance of these cells. This 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 1-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; 5-trans,5-trans-farnesylthiosalicylic acid (FTS) was obtained from Affiniti Research Products (Exeter, UK); U937 was from Cell Signaling Technology, Hertfordshire, UK; LY294002, pertussis toxin, 8-bromo-cAMP, staurosporine, and protein kinase C inhibitor set from Affiniti Research Products (Exeter, UK); UO126 was from Cell Signaling Technology, Hertfordshire, UK; G418. HeLa cells were transiently transfected using Effectene transfection reagent according to the manufacturer’s instructions (Qiagen Ltd., Crawley, UK).

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), MgCl₂, 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, Tanneal for 1 min, and 72°C for 1 min followed by 72°C for 5 min. Primers were p110γ (forward, 5′-gcaaggagaaatcctcata-3′ reverse, 5′-caacaacctgctcgcttagcttga-3′), p110α (forward, 5′-caacaacctgctcgcttagcttga-3′ reverse, 5′-caacaacctgctcgcttagcttga-3′), and p110β (forward, 5′-caacaacctgctcgcttagcttga-3′ reverse, 5′-caacaacctgctcgcttagcttga-3′). PCR primers were designed to anneal in the junction region between the BCR and ABL genes.

Western Blotting—Western blotting was performed using 1:1000 dilution of polyclonal antibody to Cadherin (Upstate Biotechnology) and counting on a Beckman Coulter LS 6500 scintillation counter. p110γ kinase assay was performed using the P13K enzyme-linked immunosorbent assay kit from Echelon Biosciences Inc (Salt Lake City, UT) according to the manufacturer’s instructions. Macrophage-derived products were electrophoresed on 8% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk and 0.05% Tween-20 in Tris-buffered saline. They were subsequently incubated overnight at 4°C with each antibody, then washed three times with Tris-buffered saline, and then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies. The bands were detected by enhanced chemiluminescence (ECL). Protein expression was calculated using the intensity of the bands compared to an equal loading control.

Kinase Assays—Ras activity was measured using the Rac activation kit from Upstate Biotechnology and following the manufacturer’s instructions. GTP-bound Ras from cell lysates “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. Rac-1 kinase assay was performed using the Raf-1 kinase cascade assay kit from Upstate Biotechnology according to the manufacturer’s instructions. Rac-1 was immunoprecipitated from cell lysates, and kinase activity was measured using MEK, extracellular signal-regulated kinase (ERK), and myelin basic protein (MBP) as partial 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 partial substrates. The Rac-1 kinase activity was measured using MBP 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 dithioretilol, 10 mM MgCl₂, 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-³²P]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 min, and 72°C for 1 min. The PCRs were performed using SYBR Green I on a Light Cycler 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 Na₃VO₄, 1 mM NaF, 1 μg/ml antipain, 1 μg/ml aprotinin, 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 & Schuell). All secondary antibodies were peroxidase-conjugated, and proteins were detected using Enhanced Chemiluminescence (ECL) (Amerham Biosciences). Antibodies to anti-p13K p110γ, Ki-67, and anti-phospho-ERK (Ser-473), and anti-AKT (Cell Signaling Technology), anti-p13K p110α and p110β (Santa Cruz Biotechnology, Heidelberg, Germany), anti-p13K p110β (Calbiochem), anti-phosphotyrosine (PY20) (Transduction Laboratories, San Diego, CA), anti-p101 (Upstate Biotechnology Ltd., Milton Keynes, UK), anti-β-actin (Sigma-Aldrich).

Mutations confirmed by sequencing 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 min, and 72°C for 1 min. The PCRs were performed using SYBR Green I on a Light Cycler instrument (Roche Diagnostics). Primer sequences and PCR conditions were used as above.
min, 72 °C for 1 min followed by 72 °C for 5 min. Primers were 5’-tca-gaggtggcgaaacccgaca-3’/H11032 forward and 5’/H11032-ggcgcgtaatctgctgcttgcaaa-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 g/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 Heps, 2.5 mM CaCl2, 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 catalytic subunits 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 IB 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. 1a 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-
sion of BCR-ABL does not affect the level of expression of either class Iα isoforms p110α or p110β. Expression of the third class Iα isoform p110β 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. 1b). 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 p110β mRNA (Fig. 1a).

The increased p110γ mRNA seen in C4 cells is reflected by an increased level of p110γ protein as seen by Western blots of whole cell lysates from 32D and C4 cells (Fig. 1c). 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 p110β 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. 1d). 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 antibody binding (Fig. 2a). 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 effect on the level of expression of p110β or p110γ (Fig. 2b). 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
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 effect 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. 4a). 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. 4c), 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 G6 6976 (Go), 400 nM Ro 032–0432 (Ro), 100 nM calphostin C (Cal), 1 μM chelerythrine chloride (Chel), 20 μM PKC inhibitor 20–28 (PKC)), and in the case of PI3K, Western blot analysis of PI3K activity (Fig. 4b).

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-regulation...
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-bromocAMP (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. 5a). A Raf-1 activity assay confirmed that Raf-1 activity can be blocked by treatment with 8-bromocAMP (Fig. 5b).

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. 5c 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. 6a 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. 6b 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 p110γ Are Unaffected by BCR-ABL—The class Iα p110 isoforms α, β, and δ form a complex with p85 regulatory subunits, whereas p110γ, as the only class IB 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. 7a) or protein (Fig. 7b) 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 Iα PI3Ks, inactive p110γ is predominantly located in the cytosol, whereas active p110γ is present in the membrane fraction. However, although class Iα 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 βy 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...
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 effects 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. 9a). In addition, a lipid kinase activity assay confirmed that all four selected clones displayed markedly reduced levels of p110γ activity (Fig. 9b). In all cases activity was reduced to less than 30% that of C4 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. 9a). In addition, a lipid kinase activity assay confirmed that all four selected clones displayed markedly reduced levels of p110γ activity (Fig. 9b). In all cases activity was reduced to less than 30% that of C4 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. 9a). In addition, a lipid kinase activity assay confirmed that all four selected clones displayed markedly reduced levels of p110γ activity (Fig. 9b). In all cases activity was reduced to less than 30% that of C4 cells.

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. 10b 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. 10c). Because the increase seen in the level of apoptosis was small (~2-fold), we confirmed this effect using two additional treatments, VP16 (etoposide) (Fig. 10d) and UV irradiation (Fig. 10e). To confirm that the effect 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.
BCR-ABL Regulates p110γ Expression and Activation

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 p110α and p110β 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 Iγ 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 p110α mRNA is also increased in response to BCR-ABL; however, this is not reflected by increased levels of p110γ 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 Iγ 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 activities. 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, whereas p110γ 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 p110. 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 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 finding that p110γ 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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