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Membrane Localization of all Class I PI 3-Kinase Isoforms Suppresses c-Myc-Induced Apoptosis in Rat1 Fibroblasts via Akt

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Abstract Phosphoinositide 3′-kinases (PI3Ks) constitute a family of lipid kinases implicated in signal transduction through tyrosine kinase receptors and heterotrimeric G protein-linked receptors. PI3Ks are heterodimers made up of four different 110-kDa catalytic subunits (p110α, p110β, p110γ, and p110δ) and a smaller regulatory subunit. Despite a clear implication of PI3Ks in survival signaling, the contribution of the individual PI3K isoforms has not been elucidated. To address this issue, we generated Rat1 fibroblasts that co-express c-Myc and membrane targeted derivates of the different p110 isoforms. Here we present data for the first time showing that activation of PI3-kinase signaling through membrane localization of p110β, p110γ, and p110δ protects c-Myc overexpressing Rat1 fibroblasts from apoptosis caused by serum deprivation like it has been described for p110α. Expression of each p110 isoform reduces significantly caspase-3 like activity in this apoptosis model. Decreased caspase-3 activity correlates with the increase in Akt phosphorylation in cells that contain one of the myristoylated p110 isoforms. p110 isoform-mediated protection from cell death was abrogated upon expression of a kinase-negative version of Akt. J. Cell. Biochem. 95: 979–989, 2005.

Key words: PI3K isoforms; Akt; c-Myc; Rat1 fibroblasts; apoptosis

Phosphoinositide 3′-kinases are lipid kinases that phosphorylate phosphatidyl-inositide lipids at the D3 position of the inositol ring producing lipid second messengers. The products of PI3-kinase activity, PI(3,4) P_2 and PI(3,4,5)-P_3 are present in very low level in quiescent cells but are rapidly produced during cell stimulation and are involved in the regulation of multiple biological responses including mitogenesis, apoptosis, vesicular trafficking, and cytoskeleton rearrangement [Fruman et al., 1998; Backer, 2000; Vanhaesebroeck et al., 2001; Cantley, 2002]. The class I PI3Ks comprise a p110 catalytic subunit and a regulatory adapter subunit. Four isoforms of the p110 subunit have been described (p110α, p110β, p110γ, and p110δ). The type IA PI3Ks, p110α, p110β, and p110δ associate with the p85 family of regulatory subunits that bind to tyrosine-phosphorylated tyrosine kinase receptors. This interaction results in the translocation of the cytosolic enzyme to the inner leaflet of the plasma membrane, giving p110 access to its lipid substrates [Gillham et al., 1999]. Similar to class IA PI3Ks, unstimulated p110γ, the only class IB member, is predominantly localized in the cytosol, however membrane recruitment and activation involves an interaction with Gα or Gβγ subunits, which are released upon agonist binding to receptor linked G proteins [Stephens et al., 1994; Lopez-Ilasaca et al., 1997]. p110γ associates with the regulatory subunit p101, that shows no homology with any known protein. All p110 isoforms display, at least in vitro the same lipid substrate specificity [Vanhaesebroeck et al., 2001]. The main product of PI3-kinase activity, PI(3,4,5)-P_3 acts as a binding site for many intracellular proteins that contain pleckstrin homology (PH) domains with selectivity for this lipid. The best studied PH domain containing protein, and probably the most

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important downstream effector of PI3Ks, is the serine/threonine kinase Akt. Akt has been implicated in the suppression of apoptosis through inactivation of several components of the cell-death machinery including Bad [Datta et al., 1997], Caspase 9 [Cardone et al., 1998], the Forkhead family of transcription factors [Brunet et al., 1999] and the NF-κB regulator kinase [Romashkova and Makarov, 1999]. Since evasion of apoptosis appears to play a critical role in tumor formation, it is not surprising that genetic alterations in PI3K/Akt signaling pathway occur frequently in human cancers. A number of additional considerations make the PI3K/Akt pathway an attractive target for therapeutic anti-cancer intervention. The pathway is relatively inactive in resting cells and involves several kinases, one of the most druggable classes of intracellular targets, ideal for the development of small molecule inhibitors.

However, PI3K/Akt signaling plays an important role for a variety of physiological activities, including glucose metabolism and survival of lymphocytes. Because only a subset of the cellular processes regulated by the PI3K-Akt pathway are involved in tumorigenesis, the choice of drug targets must take into account the adverse effects resulting from the inhibition of other PI3K-Akt-dependent cellular processes. It would be desirable, therefore, to target components that are more exclusively involved in cell growth, survival, and proliferation. This might be achieved by targeting branches further downstream in the PI3K/Akt pathway or developing isoform-specific inhibitors that will spare other PI3K isoforms. Although much effort in anticancer drug development is directed toward generating more specific PI3K inhibitors, surprisingly little information is currently available on the contribution of the different p110 isoforms to survival signaling. We investigated the antiapoptotic properties of the four isoforms of the p110 catalytic subunit using a well established apoptosis model in Rat1 fibroblasts in which Myc-overexpression has been shown to promote sensitization to apoptotic stimuli [Evan et al., 1992]. Substantial experimental evidence indicates that activation of the PI3K/Akt signaling pathway is able to rescue Rat1/cMyc cells from serum deprivation-induced apoptosis [Harrington et al., 1994; Kauffmann-Zeh et al., 1997]. In this study, we show that all class I PI3-kinase isoforms are capable of inducing Akt phosphorylation, inhibiting caspase 3-like activity, and suppressing apoptosis triggered by ectopic c-Myc expression. We provide an experimental system with molecular and biological readout suitable for testing specificity of PI3-kinase inhibitory drug candidates.

MATERIALS AND METHODS

Material

Phospho-Akt(Ser473) and Akt antibodies were supplied by Cell Signaling Technology (Beverly, MA), anti-p110α and p110δ antibodies were purchased from Transduction Laboratories Technology (Lexington, KY); anti-p110β and anti-c-myc monoclonal antibody 9E10 were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-p110γ from Alexis Biochemicals (San Diego, CA), anti-p85 and anti-p101 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY) and anti-α-tubulin from Sigma (St. Louis, MO). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were supplied by Promega (Madison, WI) and Calbiochem (Nottingham, UK), respectively. The chemiluminescence detection system (ECL), was supplied by Amersham Pharmacia Biotech (Little Chalfont, UK). Tissue culture Petri plates were purchased from Falcon (Plymouth, UK). Dulbecco's modified Eagle's medium, fungizone, and penicillin/streptomycin were from Gibco Invitrogen (Carlsbad, CA).

Plasmid Construction

The cDNA encoding human c-Myc protooncogene was subcloned into the *Eco*RI site of the retroviral vector pWZL-Blast which is based on the pBabe plasmid and encodes a blasticidin-resistance gene transcribed from the retroviral 3′-long terminal repeat. p110α, p110β, p110γ, and p110δ were cloned by PCR from cDNA library. We fused the myristoylation sequence from human c-src in frame to the N-terminus of the p110 proteins. Sequences encoding these fusion proteins were subcloned into the pBABE-puro retroviral vector. Akt K179M was kindly provided by Dr. Philip Tsichlis and subcloned in pcDNA3 vector. The authenticity of all constructs was confirmed by DNA sequencing.

Cell Culture Conditions and Drug Treatment

Rat1 fibroblasts and LinXE ecotropic retrovirus producer cells [Carnero et al., 2000] were grown in Dulbecco's modified Eagle's medium

with glutamax supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and fungizone. U937 cells were grown in RPMI 1640 supplemented with 10% FBS, penicillin, streptomycin, and fungizone. Retroviral constructs were introduced into packaging cells by standard calcium phosphate transfection. Retroviral-mediated gene transfer was performed as described earlier [Carnero et al., 2000]. After infection, stable colonies were selected in medium containing either 2 μg of blasticidin or 1 μg of both puromycin and blasticidin. After the selection individual clones were isolated and expanded. For PI3K inhibition cells were treated with 25 μM LY294002 (Calbiochem) or vehicle (DMSO) for 1 h.

RT-PCR

cDNA was synthesized from 10 μg of DNAseI-treated mRNA using MMLV reverse transcriptase (Promega) and oligo dT primer according to the manufacture's recommendations. Semiquantitative PCR amplification was performed using a dilution of the resulting cDNA. PCR products were removed at intervals (20, 30, 35, and 40 cycles). In order to amplify exclusively the ectopically expressed myristoylated derivates of the p110 isoforms, we used appropriate sets of primers combining a forward primer annealing within the myristoylation signal sequence with a reverse isoform-specific primer. The intensity of the amplified products was normalized to that of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western Blot Analysis

Subconfluent cells incubated under different conditions were washed twice with TBS prior to lysis. Lysis buffer containing 50 mM Tris HCl, 150 mM NaCl, 1% NP-40, 2 mM Na_3VO_4, 100 mM NaF, 20 mM $Na_4P_2O_7$, and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN), was added. Co-immunoprecipitation of p110α was performed combining cell lysate with 5 μg of an anti-p85 antibody. Proteins were resolved on 10% SDS–PAGE, and transferred to PVDF membrane (Immobilon-P, Millipore). The membranes were incubated with the first antibody overnight at 4°C, washed and incubated with anti-mouse (1:10,000) or anti-rabbit (1:5,000) horseradish peroxidase antibody. Bands were visualized by using the enhanced chemiluminescence (ECL) Western Blotting detection system (Amersham Pharmacia Biotech) exposed to Kodak-X-Omat LS film (Kodak).

Caspase Assay

Cells were seeded, grown to 70% confluence and incubated in the presence of 10% (controls) or 0.1% serum for 24 h. Plates were washed twice with TBS and then harvested by adding cold lysis buffer containing 50 mM Tris HCl, 150 mM NaCl, 1% NP-40, 100 mM DTT, and protease inhibitor cocktail (Roche Molecular Biochemicals). We used Promegas CaspACE assay systems (fuorometric and colometric). The assays were performed following the manufacture's instructions.

Clonogenic Survival Assay

Cell survival following transfection with empty pcDNA3 or pcDNA3 containing AKT K179M was determined by clonogenic assay. Transfection of Rat-1/c-Myc control cells and Rat-1/c-Myc cells expressing myristoylated forms of p110α, p110β, p110γ, or p110δ was achieved by a standard $CaPO_4$ precipitation method using 2 μg plasmid DNA and 18 μg sheared salmon sperm DNA carrier. Selection of geneticin-resistant colonies was obtained by adding 0.7 mg/ml G418 (Sigma) to the medium. After selection for 10 days, cells were rinsed in phosphate-buffered saline and fixed in 0.5% glutaraldehyde (Sigma). Fixed fibroblasts were stained for 30 min in 0.1% crystal violet solution.

RESULTS

Establishment of Rat-1/c-Myc Cells That Express Activated, Specific Class I PI3-Kinase Isoforms

To investigate the contribution of p110 isoforms in survival signaling we took advantage of the experimental system described by Evan et al. [1992]. Rat-1 fibroblasts were infected with retroviral constructs in which expression of the human c-Myc gene is constitutively driven by a LTR promoter. After drug selection we isolated stable transfectants. A Rat-1 cell line that stably expressed c-Myc (Rat-1/c-Myc) was then infected by retrovirus carrying myristoylated forms of human p110α, p110β, p110γ, and p110δ, respectively. Several Rat-1/c-Myc and Rat-1/c-Myc/Myr-p110 isoform clones were selected and the expression of the transgenes was analyzed. Intracellular level of c-Myc

protein was monitored by Western blot analysis using a specific antibody (Fig. 1A). These results show that the level of c-Myc expression in each of the cell clones is very similar. In order to specifically detect the expression of myristoylated p110 isoforms a semiquantitative RT-PCR strategy was designed. Figure 1B shows a time course of transgenic p110 isoform amplification. The expression of the recombinant p110 proteins was verified by immunoblot analysis (Fig. 1C) using specific antibodies against p110β, p110γ, and p110δ. Expression of p110α proteins was monitored after co-imunoprecipitation using an antibody against the p85 adaptor protein. We detected high level of endogenous p110α and p110β expression in Rat-1 fibroblasts whereas endogenous p110δ proteins are barely detectable and p110γ was virtually absent in these cells. Hence, ectopic expression of activated p110γ and p110δ proteins increased robustly while Myr-p110α and Myr-p110β expression is increased 2 to 4 fold over the high endogenous level of these isoforms. In order to test whether Rat1 cells contain accessory proteins needed for proper PI3Kγ signaling, we performed immunoblot analysis to detect the p101 non-catalytic subunit. We observed that p101 is abundantly expressed at levels comparable to that of U937 cells (data not shown), widely used to analyze signaling via the p101/p110γ complex [Baier et al., 1999]. Furthermore p101 protein level is unaltered in cells with an ectopic source of p110 (data not shown). In addition we monitored the expression of the adaptor subunit p85 in Rat1/c-Myc cells and its Myr-p110 isoform expressing derivates. We observed a very homogenous level of p85 expression among all cells tested (data not shown). To investigate if the expression of the endogenous p110 proteins is altered by the ectopic expression of activated isoforms we performed immunoblot analysis. We found that endogenous p110 levels were unaffected by overexpression of the myristoylated isoforms (data not shown).

Membrane Targeted p110 Isoforms Provide Survival Activity in the Absence of Serum

Consistent with previous reports, Rat-1 cells that constitutively express c-Myc underwent apoptosis upon serum withdrawal. Control Rat-1/c-Myc cells were cultured at various serum concentrations and the degree of cell death was assessed after 2 days by microscopic examination. Figure 2A, B, and C show that the number of cells with apoptotic phenotype is significantly increased when the serum concentration was reduced in the presence of c-Myc expression. To examine the effect of ectopic expression of the different p110 isoforms, representative cell clones were treated as described above. As shown in Figure 2D-O the anti-apoptotic capacity is shared by all membrane targeted PI3K isoforms. The degree of cell death in Rat-1/c-Myc cells expressing myristoylated forms of p110α, p110β, p110δ, or p110γ was dramatically reduced even in the complete absence of serum.

Caspase-3 Activity is Reduced in the Presence of Membrane Targeted p110 Isoforms

To further understand the mechanism by which the active PI3-kinase isoforms mediate cell survival in serum deprived Rat1/c-Myc cells, we examined the ability of p110α, p110β, p110δ, or p110γ to reduce caspase activity. We prepared cell lysates from control Rat-1/c-Myc cells and from representative cell clones expressing c-Myc together with one of the active p110 isoforms before and after serum deprivation. By using two different types of fluorogenic substrates, Ac-YVAD-MCA and Ac-DEVD-MCA we measured the caspase 1-like and the caspase 3-like protease activities, respectively. The cell lysates from Rat-1/c-Myc cells and Rat-1/c-Myc cells that overexpress the p110 isoforms had no significant ability to cleave Ac-YVAD-MCA and this caspase 1-like activity remained unchanged upon serum withdrawal (data not shown). In contrast, Ac-DEVD-MCA processing caspase 3-like activity was significantly induced after 12 h of serum starvation in Rat-1/c-Myc cells. The co-expression of membrane targeted p110 isoforms reduced caspase 3-like activity dramatically (Fig. 3). Regardless of the p110 isoform, the conferred protection against caspase 3 activation ranged from 60 to 80%.

Membrane Targeted p110 Isoforms Maintain Akt Activated in the Absence of Serum

Since Akt is one of the major downstream targets of PI3 kinase survival signaling, we analyzed the possible involvement of Akt activation in p110 isoform-mediated protection from serum deprivation induced apoptosis. We monitored Akt activity using a specific antibody against Ser473 phosphorylation. Figure 4 shows that membrane targeting of each of the class I PI3-kinase isoforms led to a significant

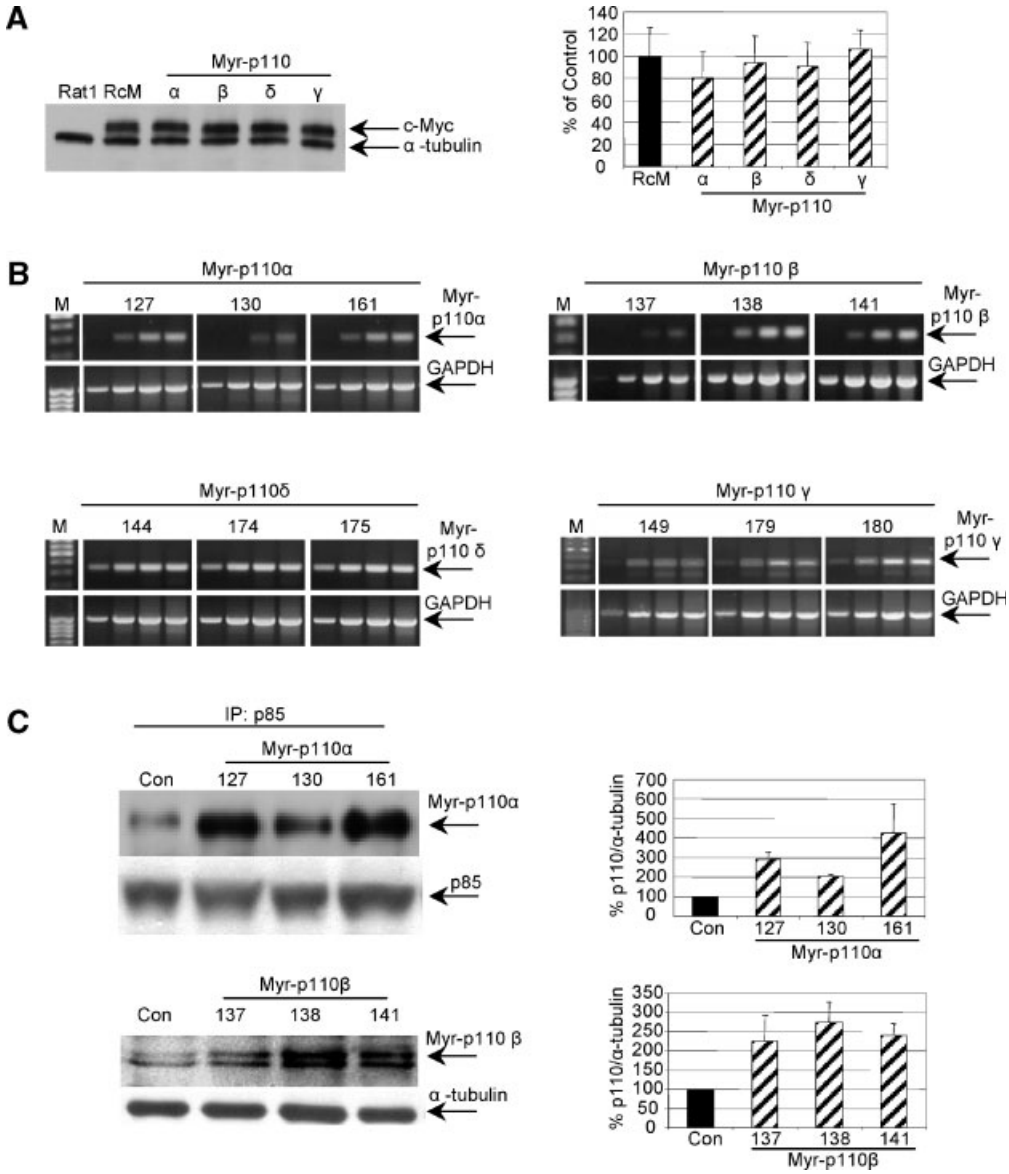

Fig. 1. Analysis of ectopic expression of c-Myc and myristoylated PI3K isoforms. **A**: Expression level of c-Myc proteins was detected in Rat-1 cell lines that stably express c-Myc (RcM) and in Myr-p110 isoform expressing derivates (Myr-p110α, β, δ, γ). Thirty micrograms of protein was loaded in each lane. Relevant proteins are indicated by arrows. Quantification of bands was performed using a BioRad GS-800 densitometer. Values were normalized against α-tubulin. Results were plotted as percentage of the RcM control values and are represented as means. **B**: Transcript level of ectopically expressed activated p110 isoforms in individual cell clones (Myr-p110α: 127, 130, 161; Myr-p110β: 137, 138, 141; Myr-p110δ: 144, 174, 175; Myr-p110γ: 149, 179, 180) was determined using semiquantitative PCR. **Lanes 1**, **2**, **3** and **4**: of each clone represent PCR amplification after 20, 30, 35, and 40 cycles, respectively. Lane M: DNA size marker. Control amplification was performed using a primer pair specific for GAPDH. Relevant amplification products are indicated by arrows. **C**: Immunoblot analysis of ectopically expressed proteins in established cell clones (see Fig 1 B). p110α proteins were co-immunoprecipitated with an antibody against the p85 adaptor protein and analyzed by immunoblotting with anti-p110α antibody. Values were normalized against p85. Lysates of Rat1/c-Myc cells overexpressing Myr-p110β, Myr-p110δ, or Myr-p110γ were evaluated by Western blotting using specific antibodies against p110β, p110δ, or p110γ. Values were normalized against α-tubulin. Relevant proteins are indicated by arrows. Quantification of bands was performed using a BioRad GS-800 densitometer. The data were presented as the percentage relative to the expression of the endogenous isoforms in control Rat1/c-Myc cells. In the case of the absence of endogenous isoforms (p110δ and p110γ) the mean value obtained from all evaluated clones was defined as 100%.

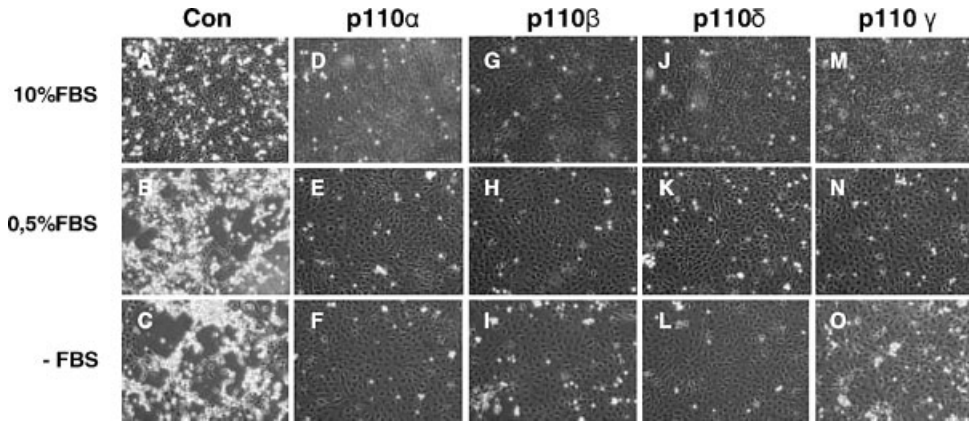

Fig. 2. Degree of apoptosis in Rat1 cells expressing c-Myc alone (Con) or cells that co-express c-Myc and one of the activated PI3K isoforms. Results are shown from representative clones (Myr-p110α: 127; Myr-p110β: 138; Myr-p110δ: 144; Myr-p110γ: 180). Photos were taken at 48 h after transferring the cells to medium containing 10; 0.5, or 0% of FBS.

increase in Akt phosphorylation both in the presence or absence of serum. Whereas the reduction of Akt phosphorylation upon serum deprivation was about 50% in the Rat-1/c-Myc control cells, overexpression of the activated p110 isoforms abolished this reduction almost completely in most of the clones. By contrast, total levels of Akt did not change (Fig. 4A). Interestingly, cells that overexpressed myristoylated p110δ displayed higher levels of Ser473 phosphorylation compared to cells expressing active forms of p110α, p110β, or p110γ.

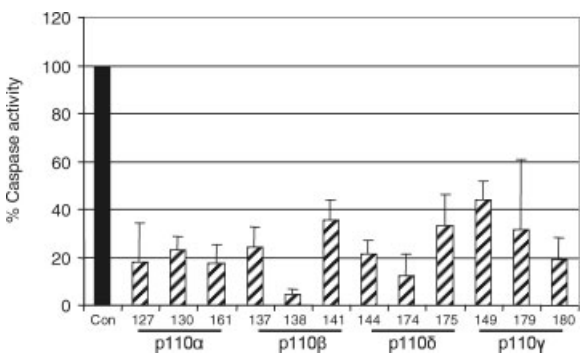

Fig. 3. Expression of activated p110 isoforms protects from c-Myc-induced apoptosis after serum deprivation. Effect was measured by caspase 3 activation in Rat1 cells expressing c-Myc alone (Con) or cells that co-express c-Myc and one of the activated PI3K isoforms using the CaspACE Assay System Fluorometric (Promega) according to the manufacturer's instructions. Three different cell clones from each Myr-p110 are shown (Myr-p110α: 127, 130, 161; Myr-p110β: 137, 138, 141; Myr-p110δ: 144, 174, 175; Myr-p110γ: 149, 179, 180). The data were normalized against values obtained from the same clone in the presence of 10% serum and expressed as percentage of control cells. Values are means based on three independent experiments.

The Different Activated p110 Isoforms Still Respond to PI3K Inhibition

In order to investigate if our engineered cell lines of defined signaling properties can be used to validate candidate p110 inhibitors, we tested whether the widely used specific PI3K inhibitor LY294002 is still able to reduce Akt activation triggered by the ectopic expression of myristoylated p110 isoforms. LY294002 competes with ATP for binding to the ATP-binding site of all class I p110 isoforms [Walker et al., 2000]. In keeping with this concept exposure to 25 μM LY294002 for 1 h resulted in a significant decrease of Akt phosphorylation regardless of the p110 isoform ectopically expressed in the cell lines (Fig. 5). In contrast, we did not detect changes in the levels of total Akt (Fig. 5A). These results were similar to those obtained for Rat-1/c-Myc control cells.

The Protection From c-Myc Induced Apoptosis Conferred by the Myr-p110s Is Akt-Dependent

In order to further analyze the implication of Akt activation in p110 mediated protection from c-Myc-induced apoptosis, we performed clonogenic survival assays in the presence of an Akt derivate that carries a K179M point mutation resulting in a loss of kinase activity [Franke et al., 1995]. We monitored the clonogenic survival of Rat-1/c-Myc control cells and Rat-1/c-Myc cells expressing myristoylated forms of p110α, p110β, p110δ, or p110γ after transfection with Akt K179M or with the pcDNA3 vector alone by a crystal violet colorimetric test.

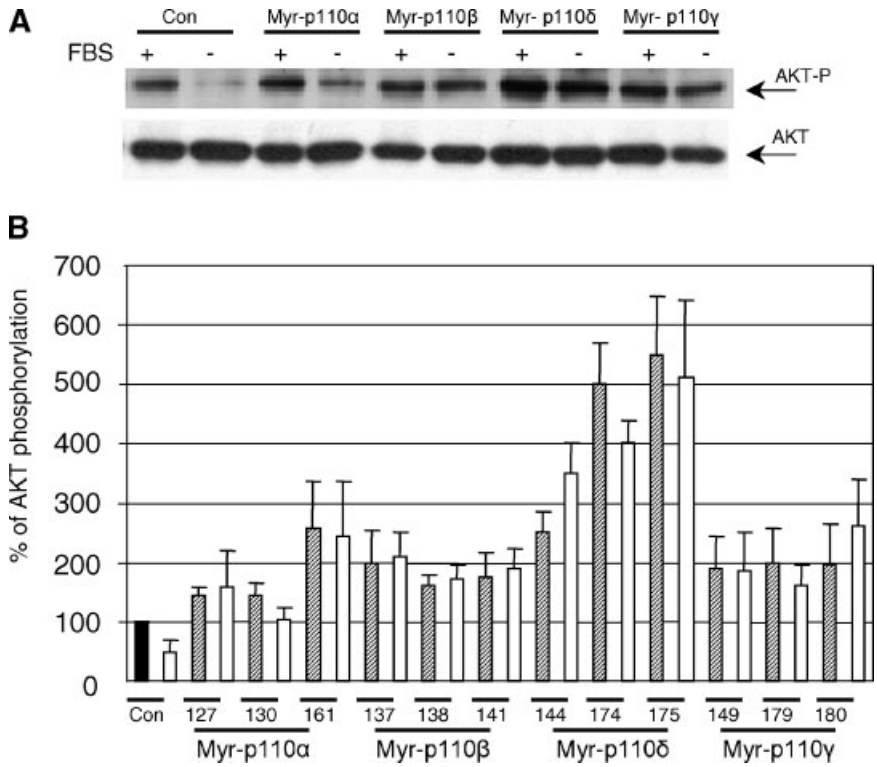

Fig. 4. Akt phosphorylation is enhanced upon expression of activated p110 isoforms. **A**: Immunoblot analysis of total lysates from Rat1 cells expressing c-Myc alone (Con) or cells that co-express c-Myc and one of the activated PI3K isoforms. A representative experiment with one individual cellular clone for each isoform (Myr-p110α: 127; Myr-p110β: 138; Myr-p110δ: 144; Myr-p110γ: 180) is shown. Cells were seeded, grown to 80% confluence and starved for 12 h in serum-free medium (FBS−) or maintained in medium containing 10% FBS (FBS+). Relevant proteins are indicated by arrows. **B**: Quantification of bands was performed using a BioRad GS-800 densitometer. Values were normalized against total Akt and are represented as percentage of AKT phosphorylation compared to control cells in the presence of serum (black bar). The different cellular clones that co-express c-Myc and one of the activated PI3K isoforms were cultured either in the absence of serum (white bars), or in the presence of 10% FBS (hatched bars). Results represent the average ± standard deviations of three independent experiments.

Figure 6 shows that the expression of the myristoylated p110 isoforms abolished the reduction of clonogenic survival in the absence of serum confirming data shown above. However, co-expression of the kinase-inactive form of Akt blocked the survival of Myr-p110 isoform expressing Rat-1/c-Myc cells suggesting an essential role of Akt activation in the suppression of c-Myc-induced apoptosis upon ectopic expression of all myrisistoylated p110 isoforms.

DISCUSSION

In this study, we have used retroviral-mediated gene transfer to assess the role of the four class I PI3-kinase isoforms in survival signaling in c-Myc overexpressing Rat1 fibroblasts. We show that targeting of p110α, p110β, p110δ, or p110γ to the cell membrane, by incorporating a myristoylation signal, is sufficient to induce Akt phosphorylation, to inhibit caspase 3 like activity and to reduce cell death triggered by ectopic c-Myc expression under low serum conditions.

Constitutively Active p110 Isoforms

Fusing the myristoylation consensus of a src-type tyrosine kinases to the N-terminus of the p110 proteins allows for a postranslational modification that directs proteins to the plasma membrane. It has been shown that membrane targeting of p110α by the attachment of heterologous localization signals produces constitutively elevated PI(3,4,5)-P_3 content, increase in Akt phosphorylation and p70 S6 kinase activation in the absence of growth factor stimulation [Didichenko et al., 1996; Klippel et al., 1996; Marte et al., 1997; Egawa et al., 1999]. A p110γ variant with a myristoyl group at its N terminus has been shown to be constitutively active

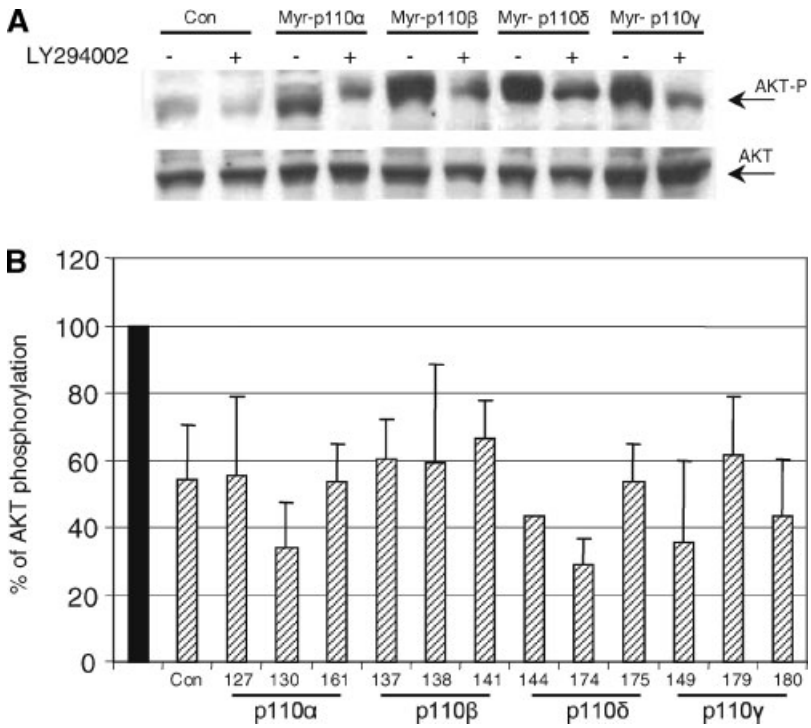

Fig. 5. Akt phosphorylation is reduced upon LY294002 treatment. **A**: Immunoblot analysis of total lysates from Rat1 cells expressing c-Myc alone (Con) or cells that co-express c-Myc and one of the activated PI3K isoforms. A representative experiment with one individual cellular clone for each isoform (Myr-p110α: 127; Myr-p110β: 138; Myr-p110δ: 144; Myr-p110γ: 180) is shown. Cells were seeded, grown to 80% confluence, and treated with 25 μM LY294002 or vehicle (DMSO) for 1 h. Relevant proteins are indicated by arrows. **B**: Quantification of bands was performed using a BioRad GS-800 densitometer. Values were normalized against total Akt in the same sample for each clone. Data are expressed as percentage of the values obtained from untreated cells from the same clone. Black bar, vehicle treated; hatched bars, treated with 25 μM LY294002. Values are means based on three independent experiments.

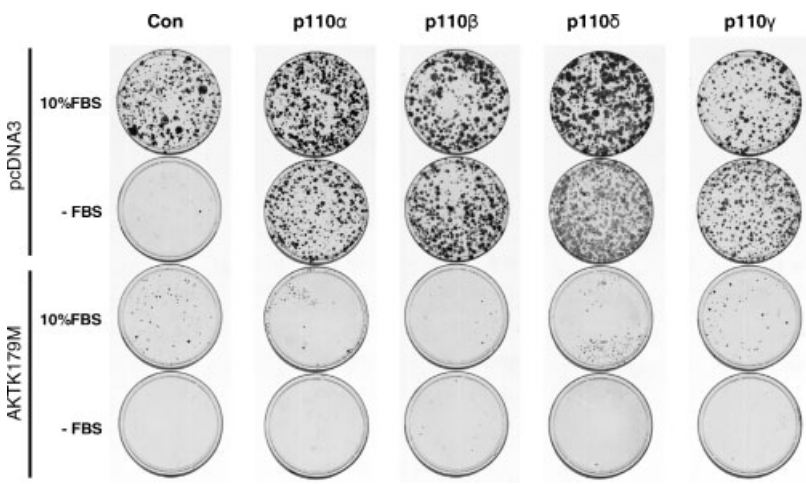

Fig. 6. Akt K179M blocked the survival of Myr-p110 isoform expressing Rat-1/c-Myc cells. Degree of clonogenic survival in the presence or absence of serum was analyzed in Rat1 cells expressing c-Myc alone (Con) or in cells that co-express c-Myc and one of the activated PI3K isoforms. Results are shown from representative clones (Myr-p110α: 127; Myr-p110β: 138; Myr-p110δ: 144; Myr-p110γ: 180). Transfection with empty pcDNA3 or pcDNA3 containing AKT K179M using $CaPO_4$ precipitation was followed by G418 selection. Survival was assessed by crystal violet assay.

biochemically [Lopez-Ilasaca et al., 1997; Brock et al., 2003]. Our results are consistent with a model in which the localization of all class I PI3-kinase isoforms to the membrane is sufficient to trigger downstream signaling events.

Membrane Targeted p110 Isoforms and Apoptosis

Anti-apoptotic properties have been documented only for the α-isoform of p110 [Kauffmann-Zeh et al., 1997; Khwaja et al., 1997]. The data presented in this manuscript provide evidence that all class I PI3-kinase isoforms are able to suppress apoptosis in c-Myc overexpressing Rat1 fibroblasts under low serum conditions. c-Myc has been shown to promote apoptosis of fibroblasts by causing the caspase-independent release of cytochrome c from mitochondria into the cytosol [Juin et al., 1999; Iaccarino et al., 2003]. Furthermore, caspase-3-like proteases play a critical role in c-Myc mediated apoptosis [Kagaya et al., 1997; Kangas et al., 1998]. Sensitisation to apoptotic stimuli is an intrinsic activity of c-Myc which under normal cell culture conditions, is suppressed by survival factors present in FBS. Thus, c-Myc induces apoptosis when the counteracting survival signaling is attenuated by serum deprivation [Evan et al., 1992]. Myc-induced apoptosis in low serum is suppressed by PDGF and IGF-1 via PI3K signaling [Harrington et al., 1994; Kauffmann-Zeh et al., 1997]. Hence, introducing active p110 isoforms in this well established apoptosis model appears to be a suitable strategy for testing their efficiency in survival signaling. Interestingly, the degree of protection from c-Myc induced apoptosis conferred by the different membrane targeted p110 isoforms was very similar. According to these data the reduction of caspase-3-like activity was quite homogenous in the different p110 isoform expressing Rat1-c-Myc cell lines.

Akt Phosphorylation by Different PI3K Isoforms

The serine/threonine kinase Akt is thought to be the major effector that determine the anti-apoptotic mechanism initiated by PI3-kinase activation. Akt exerts its anti-apoptotic activity by preventing release of cytochrome *c* from mitochondria. Among the growing list of Akt substrates several are implicated in apoptosis [Datta et al., 1997; Cardone et al., 1998; Brunet et al., 1999; Romashkova and Makarov, 1999]. We demonstrate here the capability of all four PI3K isoforms to stimulate Akt phosphorylation at serine 473. The increase in Akt phosphorylation upon expression of myristoylated p110 isoforms was observed both in the presence and absence of serum, suggesting that stimulation with 10% FBS fails to reach the threshold for maximum Akt activation. Most importantly, the expression of all activated p110 isoforms almost completely abolished the reduction of Akt phosphorylation upon serum deprivation. These results suggest that membrane localization of the p110 isoforms is able to substitute for growth factor mediated activation of PI3K/Akt signaling. Interestingly, clones expressing the myristoylated δ-isoform of p110 trigger Akt phosphorylation much more efficiently than activated p110α, p110β, or p110γ. The reason for this differential response in different cell lines is currently unknown. Differences in the expression level of the exogenous p110 proteins may account for this effect. Alternatively, membrane localization may induce the activity of the p110δ-isoform to a higher degree compared to the other p110 isoforms. However, there is no evidence available for isoform-specific differences in enzymatic activity. Since there is no endogenous p110δ protein detectable in Rat1/c-Myc cells, it is possible that ectopic expression of the activated p110δ-isoform elicits the activity of downstream effectors more potently. Interestingly, the different levels of Akt phosphorylation did not lead to any significant differences in the reduction of caspase 3-like activity or in the protection from c-Myc induced apoptosis under low serum conditions. These data support the view that a modest increase of Akt activity is sufficient to shut down the apoptosis machinery and confer survival. An additional increment in Akt phosphorylation does not seem to provide further protection. Clonogenic survival assays after blockage of Akt function with a kinase dead version of Akt revealed the critical role of Akt activation in the protection from c-Myc induced apoptosis conferred by all PI3K isofoms.

p110 Isoform Specificity

The results presented here are consistent with the fact that class I PI3-kinase isoforms produce the same lipid reaction products serving as docking sites for lipid binding proteins which mediate downstream signaling events.

However, there is also convincing evidence for non-overlapping functions of different class I PI3K isoforms coming from class I gene knockouts and microinjection of inhibitory antibodies specific for the p110 subunits [Stein and Waterfield, 2000]. An intriguing question that remains to be clarified is how the product of PI3-kinase activity, PI(3,4,5)-P_3, can trigger different cellular events depending on which p110 isoform produced the lipid signaling molecule. In our system PI3-kinase signaling is constitutively enhanced independent of extracellular stimulation supporting the view that upstream regulatory events are able to confer p110 isoform specificity. The reversible PI3K inhibitor LY294002, which has been shown to inhibit all p110 isoforms to the same extent [Vanhaesebroeck et al., 2001] proved to block also the activity of membrane targeted PI3K derivates in our cell based system. LY294002 treatment produced similar suppression of Akt phosphorylation in control cells and in cells expressing the activated p110 isoforms, indicating that the level of ectopic p110 expression did not exceed the critical threshold for effective PI3K inhibitory drug activity. Recent published studies and patent specifications describe compounds that exhibit selectivity for specific p110 isoforms [Ward and Finan, 2003]. Based on the data presented here, it may be of benefit to selectively target the different PI3K isoforms as a potential strategy for restoration of apoptotic signaling in human cancers minimizing the potential side effects resulting from unspecific inhibition of all PI3K isoforms.

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