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Regulation of Breast Cancer Cell Chemotaxis by the Phosphoinositide 3-Kinase p110δ[1]

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ABSTRACT

Class IA phosphoinositide 3′-kinases (PI3Ks) regulate many cellular processes downstream of tyrosine kinases and Ras. Despite a clear implication of PI3K in cancer, little is known about the distribution of the different PI3K isoforms in malignant cells. We screened a large panel of tissues and cell lines for expression of class IA PI3Ks, and document a ubiquitous expression of the p110α and p110β isoforms but a variable and more restricted tissue distribution of the p110δ isoform. Originally found in WBCs, p110δ was also detected in some nonhematopoietic cell types especially those of breast or melanocytic origin, both in the untransformed and transformed state. Isoform-specific neutralization of PI3K isoforms in breast cancer cell lines (by PI3K antibody microinjection or a p110δ-selective pharmacological inhibitor) demonstrated that p110δ is the most important class IA PI3K in the regulation of epidermal growth factor-driven motility *in vitro*, controlling the directionality and, to a lesser extent, the speed of migration. In contrast, p110β was required for the direction but not the speed of migration, whereas p110α did not impact on either of these parameters. These results show a nonredundant function of PI3K isoforms downstream of the epidermal growth factor receptor and indicate that the presence of p110δ may confer breast cancer cells with selective migratory capacities. The potential clinical implications of p110δ expression in non-WBC-derived tumors are discussed.

INTRODUCTION

PI3Ks[4] generate 3-phosphoinositide lipids in cell membranes. A variety of intracellular target proteins interact with these lipids via specific lipid-binding modules and, as a consequence, undergo changes in their localization and/or activity. In this way, PI3Ks participate in the regulation of mitogenesis, differentiation, survival, intracellular vesicular transport, cytoskeletal reorganization, and motility (reviewed in Refs. 1–4).

Tyrosine kinases and Ras, which are frequently overexpressed or mutationally activated in cancer (5, 6), use PI3Ks as essential intracellular signal relay molecules (7, 8). In addition to deregulation of signaling pathways upstream of PI3K, downstream targets of these enzymes are also often altered in cancer. Overexpression of Akt/protein kinase B, a broad-spectrum protein kinase of which the activity is controlled by 3-phosphoinositides, has been documented in gastric, ovarian, breast, and prostate tumors (reviewed in Refs. 9–12), where it contributes to increased cell growth and survival. The realization that the tumor suppressor protein PTEN, initially thought to be a tyrosine phosphatase, is a phosphatase for 3-phosphoinositides, has provided one of the strongest indications for a role of PI3K in cancer. PTEN inactivation, because of deletions or mutations in the *PTEN* gene, occurs in a large proportion of cancers and results in an accumulation of PI3K lipid products, leading to an up-regulation of the many PI3K-regulated cellular activities (reviewed in Refs. 13–15).

The class IA subgroup of PI3Ks comprises heterodimeric enzymes made up of an SH2-domain-containing regulatory subunit in complex with a p110 catalytic subunit (16).[5] Class IA PI3Ks are activated on interaction of the regulatory subunit with phosphorylated tyrosines and/or by direct binding of the heterodimer to Ras (17, 18). Mammals have genes encoding three distinct catalytic subunits (p110α, p110β, and p110δ) and three regulatory subunits (p85α, p85β, and p55γ). All of the p110 isoforms are capable of interacting with each type of regulatory subunit. They are also similarly recruited to phosphotyrosine complexes and have, at least *in vitro*, the same lipid substrate specificity. However, it is becoming increasingly clear that PI3K isoforms differ in their interaction with Ras and regulation of lipid kinase activity, and in their protein kinase activities (reviewed in Refs. 3, 19). Several groups have provided evidence that p110 isoforms have nonredundant functions in the regulation of cell proliferation, survival, actin cytoskeleton reorganization, and migration downstream of given receptors (20–27).

Increased expression of the p110α isoform, as a consequence of gene amplification, has been reported in ovarian (28), cervical (29), and head and neck (30) cancer, whereas somatic activating mutations in the p85α gene (*PIK3R1*) were reported in some colon and ovarian tumors (31). Additionally, mutant forms of p110α and p85α can, under certain conditions, induce cell transformation (32–34). A prerequisite of understanding how class IA PI3Ks participate in signaling pathways deregulated in cancer is to determine which PI3K isoforms are expressed in different cell types. Little information is currently available on the distribution and relative expression levels of PI3K isoforms in healthy and malignant tissues. Progress in this area is mainly hindered by a shortage of good quality, commercially available antibodies to the PI3K catalytic subunits. In this study, we have used EST database searches and a combination of commercially

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[4] The abbreviations used are: PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog, deleted on chromosome 10; BLAST, basic local alignment search tool; CSF-1, colony stimulating factor-1; EGF, epidermal growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EST, expressed sequence tag; polyoma mT, polyoma middle T antigen; FCS, fetal calf serum; nt, nucleotide; D000, 3-(2-chlorophenyl)-2-(9H-purin-6-ylsulfanylmethyl)-3H-quinazolin-4-one; Ig, immunoglobulin; HRP, horseradish peroxidase.

[5] See also K. Okkenhaug and B. Vanhaesebroeck, internet address: http://www.stke.org/cgi/content/full/OC_sigtrans:2001/65/pel.

available and "in-house" antibodies to assess the expression of PI3K p110 isoforms in a large and diverse panel of normal and tumor cells. We document that p110α and p110β are present in every tissue and cell line investigated. In addition to its expression documented previously in leukocytes (35, 36), we also detected a frequent and abundant expression of p110δ in breast cells and in melanocytes. We find that p110δ plays an important role in the EGF-driven *in vitro* migration of breast cancer cell lines, similar to that found previously in a macrophage cell line stimulated with CSF-1 (24). The implications for p110δ-regulated chemotaxis in tumor progression are discussed.

MATERIALS AND METHODS

GenBank Database Searches for PI3K mRNA Expression. ESTs for human and mouse p110α, p110β, and p110δ were retrieved from GenBank (June 2001 version) using the BLAST (37).[6] The BLAST "queries" were composite p110 cDNA nt sequences, consisting of the originally submitted GenBank sequences (accession numbers given below) maximally extended at the 5′ and 3′ ends with contiguous EST sequences that have become available subsequently. The details of the query sequences are as follows: human p110α, 4450 nt [accession no. U79143 (3207 nt) extended 5′ and 3′ (279 nt and 964 nt, respectively) with human EST sequences]; human p110β, 4966 nt [accession no. S67334 (3213 nt) extended 5′ and 3′ (352 nt and 1401 nt, respectively) using sequence from a 4966 nt p110β cDNA isolated from a U937 cDNA library];[7] human p110δ, 4943 nt [accession no. U86453 (5220 nt) with the removal of a repetitive sequence element (nt 4110–4393)]; mouse p110α, 4828 nt [accession no. U03279 (3207 nt) extended 3′ (1621 nt) with mouse ESTs]; mouse p110β, no cDNA sequence is available in GenBank and, therefore, the p110β contig used here (4817 nt) is a combination of mouse ESTs (nt 1–1040 and nt 1769–4817) and rat p110β cDNA (accession no. AJ012482, nt 1041–1768) sequences; and mouse p110δ, 4649 nt [accession no. U86587 (3130 nt) extended 3′ (1519 nt) with mouse ESTs]. Complete query sequence contigs are available on request.

ESTs retrieved from the GenBank database required extensive curation to filter out ESTs with poor quality sequence. ESTs with a low degree of identity with the query sequence (BLAST score $>7e^{-41}$) were not included in our analysis. The tissue of origin of ESTs was often incorrectly summarized in the EST section of GenBank, and more precise information was obtained by referring to the record of the relevant cDNA library in GenBank.

Northern Blotting. A multiple cell line polyadenylated + mRNA blot (Clontech) was probed with the 3.8 kb *Eco*RI fragment II of the p110δ cDNA as described previously (36).

Tissue Samples, Cell Culture, and Cell Stimulation. Normal human breast epithelial cells isolated from reduction mammoplasty tissue were prepared as described previously (38). Primary breast tumor cells were obtained from pleural effusions (minimizing the risk of contamination from nontumor cells) of patients with primary breast tumors. Established breast tumor cell lines were obtained from the American Type Culture Collection or European Collection of Cell Cultures. Normal human melanocytes, derived from neonatal foreskin, were provided by Mary K. Cullen (Washington University School of Medicine, Department of Cell Biology and Physiology, St. Louis, MO). Derivation and culture of mouse melanocyte and melanoma cell lines have been described elsewhere (39, 40). Ras- and polyoma middle T-transfected mel-ab melanocytes were gifts from John Marshall (Richard Dimbleby/Imperial Cancer Research Fund Department of Cancer Research, St. Thomas' Hospital, London, United Kingdom; Refs. 41, 42). All of the other cell lines were cultured in DMEM (Life Technologies, Inc.) or RPMI 1640 (in the case of Jurkat T cells), supplemented with 10% heat-inactivated FCS (Sigma), penicillin, and streptomycin (Sigma).

To stimulate PI3K activity in MDA-MB-231 cells, subconfluent cells were starved in DMEM containing 0.1% FCS for 24 h then treated with 15 ng/ml recombinant human EGF (R&D Systems) at 37°C. For chemical inhibition of PI3Ks, cells were pretreated with 20 μM LY294002 (Calbiochem) or 0.5 μM D000 (Labotest, Niederschoena, Germany) for 1 h at 37°C before the addition of EGF. Control cells were pretreated with 1:1000 dilution of DMSO, the vehicle for LY294002 and D000.

Antibodies. The following commercial antibodies were used in this study: rabbit polyclonal antibody to p110β (for Western blotting only; Santa Cruz Biotechnology; sc-602); mouse monoclonal antibody to β-actin (Sigma; clone AC-15); mouse monoclonal antibody to GAPDH (Abcam, Cambridge, United Kingdom); mouse monoclonal antibody to phosphotyrosine (Upstate Biotechnology; clone 4G10); HRP-conjugated sheep antimouse Ig and HRP-conjugated donkey antirabbit Ig (Amersham Pharmacia). Inhibitory antibodies to the COOH terminus of p110β and p110δ, used for microinjection and Western blotting, were made in-house and have been described elsewhere (24, 36). Antibodies to p110α for these studies were provided by Roya Hooshmand-Rad and Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden. Rabbit IgG (Sigma) was used for control microinjections.

Cell Lysis and Western Blotting. Cells were washed three times in ice-cold PBS and then lysed on ice in 50 mM Tris-HCl (pH 7.4), 1% (w/v) Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 0.09 trypsin inhibitor units/ml aprotinin (Sigma), 10 μM leupeptin (Sigma), 0.7 μg/ml pepstatin A (Sigma), 27 μM sodium p-tosyl-L-lysine chloromethyl ketone (Sigma), and 1 mM DTT. Insoluble material was removed by centrifugation at 13,000 $\times$ *g* for 15 min at 4°C, and a 10-μl sample of the supernatant was assayed for total protein content using Bio-Rad protein assay reagent. Proteins (50–100 μg per lane) were electrophoresed in 8% polyacrylamide-SDS gels and then transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore) by wet transfer (200 mA for 6 h). Membranes were blocked for 2 h in 5% (w/v) milk powder in wash buffer [PBS, 0.1% (v/v) Tween 20, and 0.02% (w/v) sodium azide] and probed with primary antibody diluted in blocking buffer overnight at room temperature. After washing, blots were incubated for 1 h with HRP-conjugated antibodies (diluted 1:5000), and bound antibody was detected with enhanced chemiluminescence reagents (Amersham Pharmacia).

Cell Staining and Microscopy. Cells grown on glass coverslips were fixed in 2% (w/v) paraformaldehyde in PBS, and permeabilized with 0.2% (v/v) Triton X-100 in PBS followed by blocking with 0.5% (w/v) BSA. Filamentous actin was stained with tetramethylrhodamine isothiocyanate-conjugated phalloidin (Sigma), and cells were viewed and photographed on a Zeiss LSM 510 confocal microscope.

Microinjection and Chemotaxis Assay. Glass coverslips (Chance Propper Nº 3, 18 $\times$ 18 mm), scratched with a diamond pen to mark the area for injection, were placed in 30-mm dishes and coated with either growth factor-reduced Matrigel (Becton Dickinson) for MDA-MB-231 cells or with laminin (Invitrogen) for MDA-MB-435 cells. Cells (5×10^4/dish) were seeded on to the coverslips and allowed to adhere overnight. The next day the cells were starved for 24 h in DMEM containing 0.1% FCS followed by pretreatment with DMSO, LY294002, D000, or microinjection with antibody. Antibodies (2–3 mg/ml in PBS) were centrifuged for 10 min at room temperature at 14,000 rpm in a microcentrifuge immediately before loading into a microinjection needle (Femtotips; Eppendorf) using a microloader (Eppendorf). Cells were injected using an Eppendorf automated injector and micromanipulator fitted with a heated stage (37°C), and a CO_2-enriched atmosphere. After a 1-h recovery period at 37°C, injected cells were mounted on a Dunn chemotaxis chamber (Hawksley Technology, Lancing, United Kingdom) as described previously (43, 44). For each experiment, two Dunn chambers were used in parallel, one being the test chamber and the other an appropriate control. EGF (15 ng/ml in DMEM containing 0.1% FCS) was added to the outer well of the chamber with DMEM-0.1% FCS alone in the inner well. Cell motility was digitally recorded by video microscopy using a time-lapse interval of 10 min over a 4–5 h period. Cells were tracked manually, and the trajectories were statistically analyzed using Mathematica software, as described previously (44, 45). Cell displacement was evaluated by calculating the percentage of the total cells analyzed that had migrated a minimum distance of 20 μm (MDA-MB-435) or 50 μm (MDA-MB-231) in each experiment. The IgG or DMSO control value for each experiment was then normalized to give a value of 100, and the mean displacement for the PI3K antibody injections/inhibitor treatments relative to the control value was calculated.

[6] Internet address: http://www.ncbi.nlm.nih.gov/BLAST.
[7] C. Paneretou, S. Volinia, and M. Waterfield, personal communication.

KinaseProfiler Assay. The activity of D000 was tested in an *in vitro* kinase assay (KinaseProfiler; Upstate, Milton Keynes, United Kingdom)[8] using a panel of 29 protein kinases and an ATP concentration of 100 μM.

RESULTS

Tissue Distribution of Class IA PI3K mRNAs. Previous studies indicated that p110α and p110β mRNAs are broadly but not uniformly expressed (36, 46, 47), whereas p110δ mRNA expression appeared to be largely restricted to WBCs (35, 36). However, long exposures of p110δ Northern blots revealed a weak mRNA signal for this PI3K isoform in most nonhematopoietic tissues (36). This could be because of blood "contamination" of these tissues but it could also indicate a low level of p110δ expression in nonleukocyte cell types. All of the leukocytes, except for some erythroleukemia cell lines such as K562 and UT7, have been documented to express p110δ protein (36).[9]

To gain additional insight into the tissue distribution of class IA PI3Ks, we retrieved sequences representing p110α, p110β, or p110δ genes from the EST section of the GenBank database, and compiled information on their tissue source. This approach enabled us to screen a much larger array of tissues than would be practical using conventional techniques such as Northern or Western blotting. Similar total numbers of ESTs were retrieved for each p110 isoform, and a summary of the relative tissue distribution of the curated EST sequences is presented in Fig. 1*A* (see Data Supplement 1 for additional details).

The tissue distribution of p110δ differed in two main aspects from that of p110α and p110β. Firstly, and as expected, the largest cluster of p110δ ESTs was found in blood/immune cells (39% of all of the ESTs). This contrasts with the lower but still significant clusters of p110α and p110β ESTs in these cell types (15 and 10%, respectively). Secondly, a relatively small proportion of the p110δ ESTs (18%) fell into the category "other" (18 different tissues, each representing $<$1% of all p110δ ESTs; see Data Supplement 1 for a list of these tissues) whereas the majority of p110α (41%) and p110β (54%) ESTs fell into this category (representing 26 and 24 different tissues, respectively). These observations indicate that there is a broader tissue distribution of p110α and p110β mRNAs compared with that of p110δ.

Outside of the hemopoietic system, p110δ ESTs were most frequently detected in cells of melanocytic and breast origin (both untransformed and tumor-derived), and in embryonic tissue (8% each), with additional smaller clusters (3–7%) in, among others, brain, kidney, lung, eye, and testis. The proportion of these nonblood cell EST clusters is similar for all of the p110 isoforms, with the exception of a larger cluster (15%) of p110β ESTs in the brain and a smaller representation of p110β in mouse embryos (2%).

Most of the ESTs represented in Fig. 1*A* were derived from tissues that had not been fractionated into different cell types and could have contained leukocytes. For this reason, we investigated the expression of p110δ by Northern blotting of mRNA derived from cell lines, a strategy that enables the exclusion of contaminating WBCs as a source of p110δ signals and which could provide information on the relative level of expression of p110δ in different cell types. An example of such a blot is shown in Fig. 1*B*, revealing a positive but rather weak p110δ mRNA signal in cell lines of nonleukocyte origin (colorectal and lung carcinoma) and a slightly stronger signal in a melanoma cell line.

p110α and p110β, but not p110δ, Proteins Are Ubiquitously Expressed in Mammalian Cells. It is not clear at present whether PI3K mRNA levels correlate with protein expression. Therefore, we analyzed cell lysates for the expression of p110 proteins by immunoblotting using isoform-specific antibodies (26, 31). We first investigated expression of p110δ protein in cells of breast and melanocyte origin, given that these cell types formed the largest clusters of p110δ ESTs outside of the hemopoietic system.

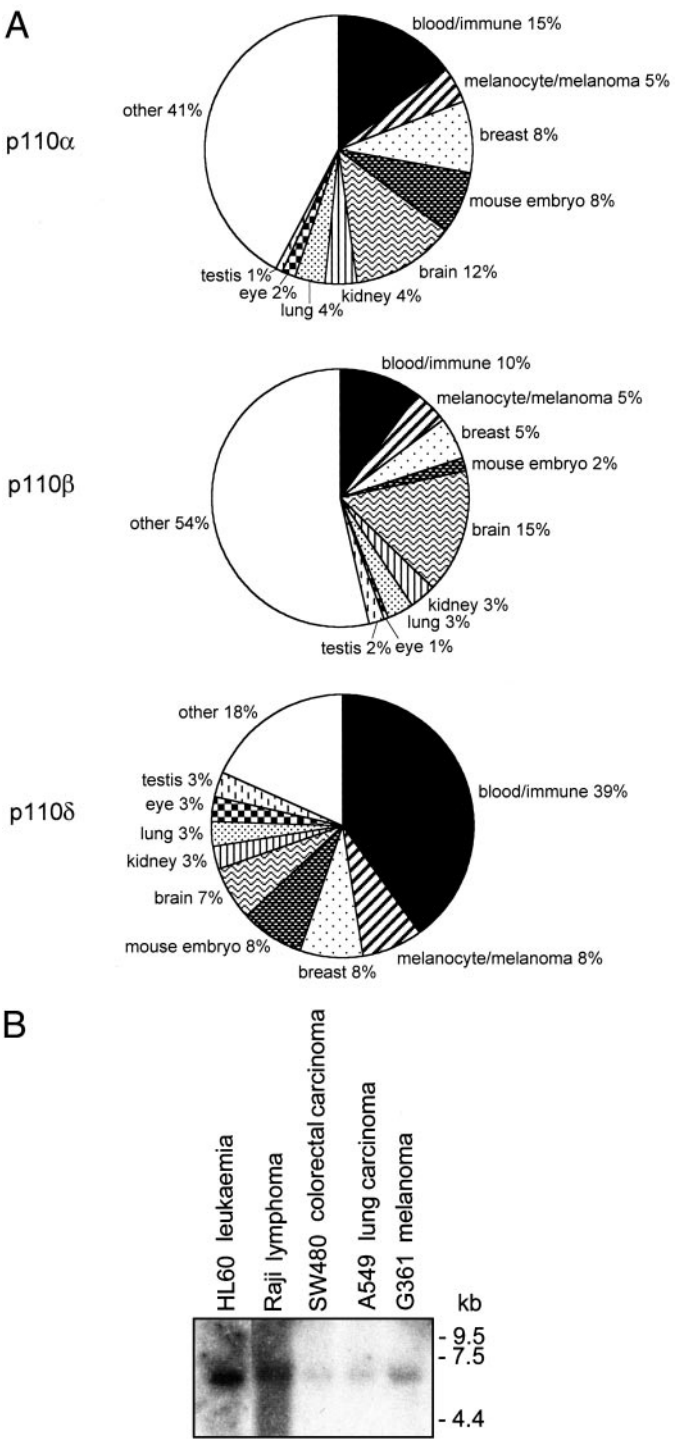

Fig. 1. *A*, relative tissue distribution of class IA PI3K ESTs. ESTs for each PI3K isoform were retrieved from GenBank and categorized into the indicated tissue groups. The abundance of the ESTs is presented as a percentage of the total number of ESTs retrieved for each p110 isoform. Tissue types that represented $<$3% of the total p110δ ESTs are presented as "other." These tissue types along with the actual numbers of ESTs in each category are listed in Data Supplement 1. *B*, Northern blot for p110δ mRNA in human tumor cell lines. A multiple cell line Northern blot was probed with a radiolabelled fragment of the p110δ cDNA.

[8] Internet address: http://www.upstate.com/features/kinaseprofiler.asp.
[9] B. Vanhaesebroeck, F. Verdier, and C. Sawyer, unpublished observations.

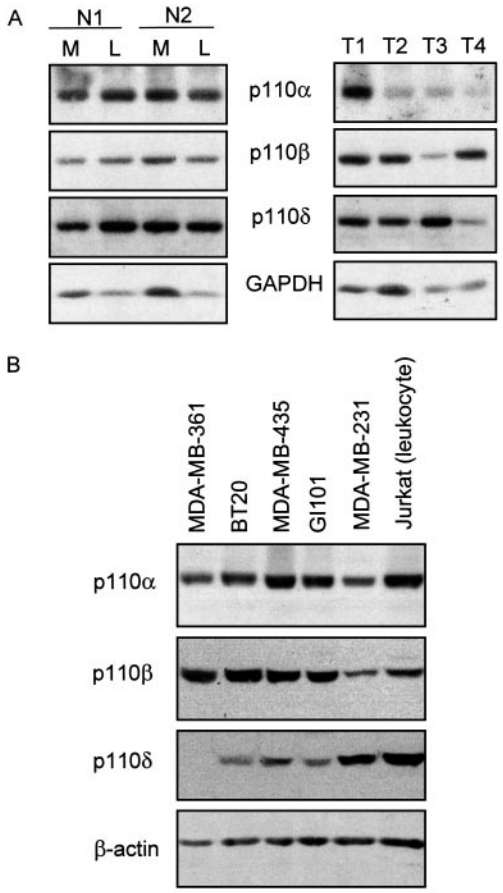

Fig. 2. Expression of class IA p110 isoforms in human breast cancer cells. p110 isoforms in total lysates of (*A*) primary normal (*N*) breast myoepithelial (*M*) or luminal epithelial (*L*) cells, or primary breast tumor (*T*) cells, and in total lysates of (*B*) breast tumor cell lines, as revealed by Western blotting using isoform-specific antibodies. Western blotting using antibodies to β-actin or GAPDH was used to check for equal protein loading of cell lysates.

Normal human breast cells (luminal and myoepithelial) and primary breast tumor cells were found to express all of the class IA PI3K isoforms (representative samples are shown in Fig. 2*A*), with frequently low levels of p110α in the tumor cells. In a panel of breast cancer cell lines, p110α and p110β were invariably detected, whereas p110δ expression was found in 9 of 15 lines tested (Fig. 2*B;* Data Supplement 2, I). Of the cell lines that lacked p110δ protein, MDA-MB-361 was found to also lack p110δ mRNA, as determined by reverse transcripton-PCR.[10]

All of the melanocyte and melanoma cell lines investigated contained p110α and p110β, whereas p110δ was expressed in a large subset of these (Fig. 3; see also Data Supplement 2, II). Human neonatal melanocytes and the majority (13 of 18) of human melanoma cell lines expressed high levels of p110δ (Data Supplement 2, II).[10] In these melanoma lines, expression of p110δ protein did not correlate with pigmentation or any aspect of tumor progression, including tumorigenicity in nude mice (Data Supplement 3). In mouse melanocytes, p110δ was either absent or expressed at low levels compared with mouse melanoma cells (representative data shown in Fig. 3; see also Data Supplement 2, II). Transformed derivatives of the mouse melanocyte cell line mel-ab, which expressed either Ha-Ras (Ras-cl1 and Ras-cl2) or polyoma mT, expressed all three of the PI3K p110 isoforms at elevated levels, relative to the parental cell line (Fig. 3, *right panel*). It is interesting to note that mel-ab cells are not tumorigenic in nude mice (41) unless they are transformed by expression of either Ras or polyoma mT (41, 42).

p110δ protein was additionally found in 5 of 23 cell lines of nonbreast/melanocyte/leukocyte origin (Table 1). No correlation of p110δ expression with a specific tissue origin could be demonstrated. The reason for the variation in p110δ expression in different cell types is unclear, but it indicates that expression of p110δ is dispensable for *in vitro* cell propagation.

Role of PI3K in Breast Cancer Cell Migration. We have demonstrated previously that p110δ and p110β are required for CSF-1-stimulated chemotaxis of the BAC1.2F5 macrophage cell line, whereas p110α did not play a role in this response (24). We tested whether such nonredundancy also existed in a nonleukocyte model of cell migration, namely EGF-induced chemotaxis of the MDA-MB-231 breast cancer cell line. In addition to p110α and p110β, these cells express p110δ at levels similar to those seen in WBCs (Fig. 2*B*). MDA-MB-231 cells are highly motile *in vitro* and metastatic *in vivo* (48), and respond to EGF by intracellular PI3K activation (49). In breast cancer, EGF receptor family members show a high frequency of aberrant expression or mutation (50, 51), correlating with increased or constitutive activation of intracellular signaling pathways including the PI3K cascade.

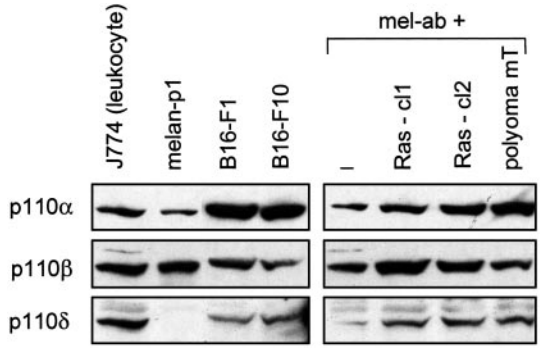

Fig. 3. Expression of class IA p110 isoforms in mouse melanocytes and melanoma cell lines. p110 isoforms in total cell lysates were revealed by Western blotting using isoform-specific antibodies. Melan-p1 and mel-ab are melanocytes, and B16-F1 and B16-F10 are melanoma cell lines.

Table 1 *Cells of non-leukocytic/breast/melanocytic origin which have been tested for expression of p110δ*

p110δ was detectable (+) or undetectable (−) by western blotting of 100 μg of total cell lysate. All cells are human unless stated otherwise.

Cell/tissue origin	Cell line	p110δ expression
aortic endothelial (pig)	PAE	−
astrocytes (mouse)	(primary)	−
cervical carcinoma	HeLa	−
colon adenocarcinoma	LS174T	−
colon adenocarcinoma	COLO 320HSR	−
embryonic stem cell, differentiated (mouse)	(primary)	−
embryonic stem cell, undifferentiated (mouse)	(primary)	−
endometrial carcinoma	ECC-1	−
fibroblast	(primary)	−
fibroblast (mouse)	Swiss 3T3	−
fibroblast (rat)	Rat-1	−
glioma (rat)	C6	−
kidney (canine)	MDCK	−
kidney (monkey)	Cos	−
larynx carcinoma	HEp-2	−
microglia (mouse)	BV2	+
microglia (mouse)	N11	+
microglia (mouse)	C8/B4	+
ovarian carcinoma	OVCAR3	+
ovary (chinese hamster)	CHO	−
small-cell lung cancer	POC	−
smooth muscle cells	(primary)	−
synoviocytes	(primary)	+

[10] C. Sawyer and B. Vanhaesebroeck, unpublished observations.

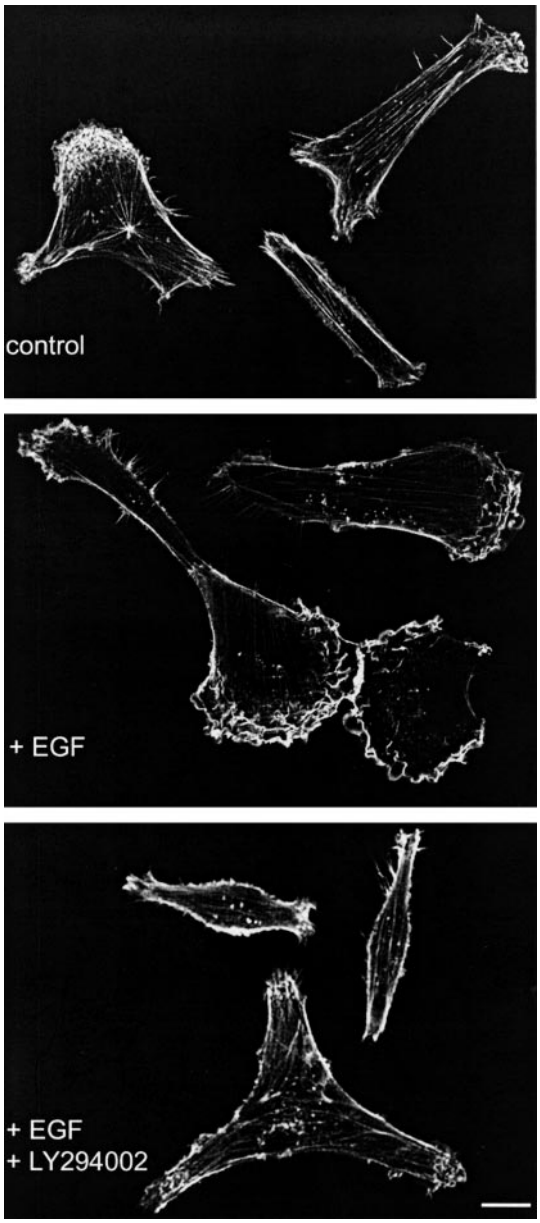

Fig. 4. Effect of EGF and LY294002 on the actin cytoskeleton of MDA-MB-231 cells. Serum-starved cells were preincubated with DMSO (*control*) or LY294002 then treated with EGF for 2 min, as indicated. After fixation and permeabilization, polymerized actin was visualized with a tetramethylrhodamine isothiocyanate-phalloidin conjugate. *Bar* represents 10 μm.

MDA-MB-231 cells are heterogeneous in size and shape (representative cells are shown in Fig. 4). Short-term treatment (2 min) with EGF induced extensive membrane ruffling and a reduction in the number of actin stress fibers. EGF-treated cells also adopted a polarized morphology with the generation of a distinct leading edge, whereas untreated cells have multiple sites of cell protrusion without a dominant leading edge. Incubation with the PI3K inhibitor LY294002 (which inhibits all of the class IA PI3K isoforms to the same extent) completely blocked EGF-induced membrane ruffles, prevented the loss of stress fibers, and inhibited the creation of a stable leading edge (Fig. 4). These findings indicate that, in MDA-MB-231 cells, PI3K activity is required for EGF-induced actin cytoskeletal changes and a motile cell morphology.

We next investigated directional cell migration (chemotaxis) induced by long-term (4–5 h) EGF treatment. For these experiments we used Dunn chemotaxis chambers and time-lapse video microscopy (43) to monitor the movement of individual cells and quantify distinct parameters of cell migration, such as directionality, displacement, and speed (see legend to Fig. 5 for more details). Serum-starved MDA-MB-231 cells exposed to a gradient of EGF in a Dunn chamber displayed positive chemotaxis in contrast to cells in the control chamber (Fig. 5*A*). This coincides with increases in cell speed (Fig. 5*A*) and cell displacement (the ability of cells to migrate beyond a given distance from their starting point; Fig. 5*B*). LY294002 treatment inhibited the increase in displacement (Fig. 5*B*), prevented directional migration, and had a slight inhibitory effect on cell speed (Fig. 5*C*) indicating a requirement for PI3K in EGF-induced MDA-MB-231 cell motility. It is interesting to note that a p110δ-negative breast tumor cell line, MDA-MB-361, does not migrate or chemotax when exposed to a gradient of EGF in a Dunn chamber.[10]

The role of PI3K isoforms in breast cancer cell line migration LY294002 is equally effective in inhibiting all of the class IA PI3K isoforms, and also inhibits enzymes such as DNA-dependent protein kinase, casein kinase-2, and mammalian target of rapamycin (mTOR) at doses at which it inhibits PI3Ks (reviewed in Ref. 3). To selectively target individual PI3K isoforms, we microinjected isoform-specific

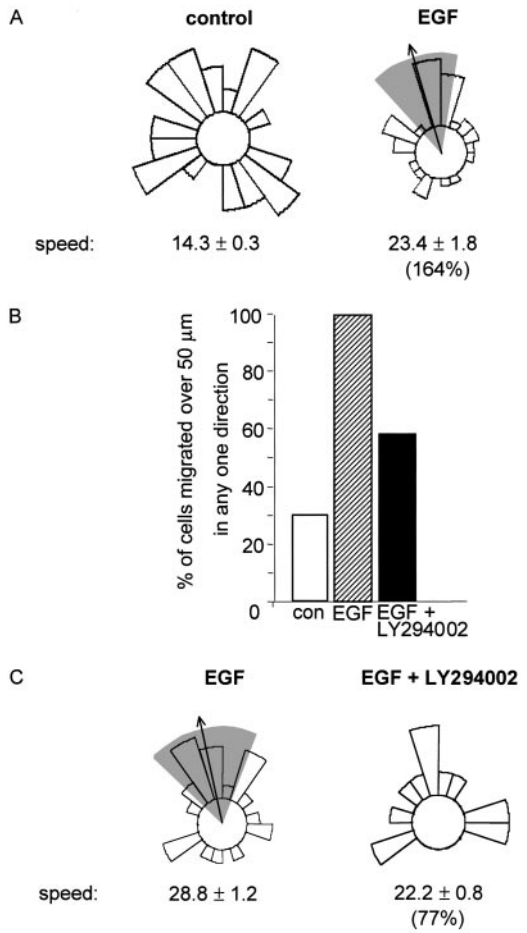

Fig. 5. Effect of EGF and LY294002 on the migration of MDA-MB-231 cells. Cells were placed in Dunn chemotaxis chambers in the presence or absence of EGF and DMSO/LY294002, as indicated. The direction of migration is depicted in horizon plots (*A* and *C*) in which the *bar height* represents the proportion of cells moving in that particular direction and where the chemoattractant source is located at the *top* of the plot. An *arrow* and a *shaded wedge* indicate the mean direction of cell migration and its 95% confidence interval, respectively. Therefore, positive chemotaxis is depicted by an *arrow pointing upwards, surrounded by a shaded wedge*. The mean speed (μm/h) and relative mean speed (*in parentheses*) are shown under each horizon plot (*A* and *C*). Cell displacement (*B*) was analyzed as described in "Materials and Methods." Experiments were repeated at least three times and representative experiments are shown. *Con* = control.

antibodies into MDA-MB-231 cells or used a recently developed p110δ-selective small molecule inhibitor (see below). The antibodies used are all equally effective at inhibiting the enzyme activity of their cognate antigen *in vitro* (24). MDA-MB-231 cells were serum-starved overnight and then microinjected with either antibodies to a specific p110 isoform or control rabbit IgG. After a short recovery period, the cells were exposed to a gradient of EGF.

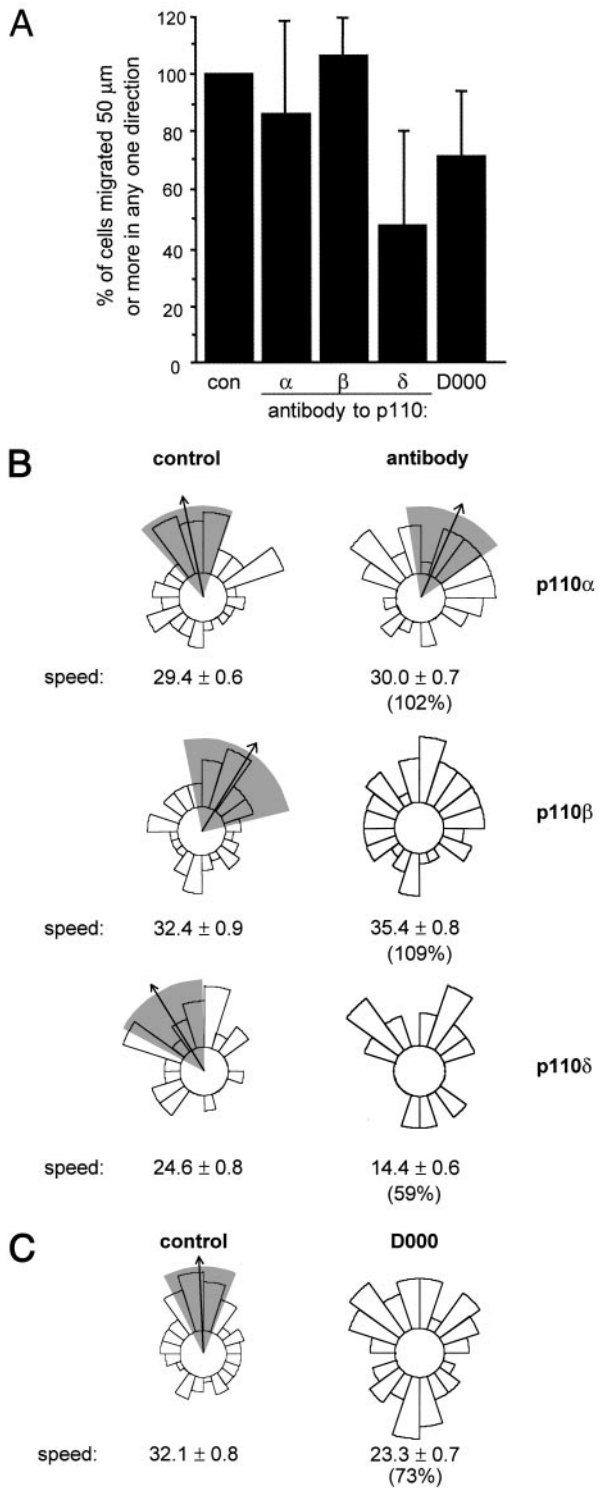

Fig. 6. Effect of neutralization of p110 isoforms on EGF-stimulated cell displacement, speed, and chemotaxis of MDA-MB-231 cells. Cells were microinjected with the indicated antibodies (*A* and *B*), or treated with D000 (*C*), and placed in a gradient of EGF. For each treatment, a parallel group of cells injected with control IgG or treated with DMSO was filmed alongside. Cell displacement (*A*), and the direction and mean speed of migration (μm/h; *B* and *C*) were analyzed as described in "Materials and Methods." The results presented are the pooled data from at least two experiments; *bars*, ±SD.

Antibodies to p110δ, but not those to p110α or p110β, were found to inhibit cell displacement (Fig. 6*A*), similar to cells that had been treated with LY294002 (Fig. 5*B*). Other parameters of cell migration were also found to be affected in a p110 isoform-specific manner. Antibodies to p110α, which effectively blocked CSF-1-induced cell proliferation in Bac1.2F5 cells (24), did not affect the direction or speed of migration of MDA-MB-231 cells (Fig. 6*B*). Neutralization of p110β blocked directional migration toward EGF but did not affect cell speed (Fig. 6*B*). These results indicate that p110β is not critical for random migration (chemokinesis) but is required for chemotaxis. Antibodies to p110δ most significantly impaired the motile response: chemotaxis was abolished and chemokinesis was reduced to 59% of that recorded in IgG-injected cells (Fig. 6*B*). The effects of antibodies to p110δ on cell migration were similar to effects seen with LY294002 (Fig. 5, *B* and *C*), which indicates that p110δ is the most important class IA PI3K isoform involved in migration of these cells. This conclusion was corroborated by the finding that coinjection of antibodies to p110β and p110δ did not have an effect above that of p110δ antibodies alone.[10] It appears that on neutralization of p110β its function in chemotaxis cannot be taken over by p110δ and *vice versa*, indicating that both p110 isoforms are essential for chemotaxis but may have nonoverlapping functions in this biological response.

To additionally assess the role of p110δ in directional cell migration, we used D000, a novel pharmacological agent that selectively inhibits p110δ (Patent WO 01/81346).[11] The relative IC_{50}s of this drug, as determined in an *in vitro* lipid kinase assay in the presence of 20 μM ATP, were reported to be 0.33 μM and 7.7 μM for p110δ and p110γ (a G-protein-coupled receptor linked class IB PI3K), respectively, whereas an inhibition of 50% could not be attained for p110α or p110β, even at 100 μM D000.[11] D000 (5 μM) was also unable to achieve appreciable inhibition of any of 29 protein kinases, some of which are downstream targets of PI3K activation such as Akt/protein kinase B α, Erk2, S6 kinase, and GSK-3β (Table 2). The reported effect of D000 on the proliferation of human B and T lymphocytes[11] was similar to the impaired proliferation of lymphocytes observed in $p110\delta^{D910A/D910A}$ mutant mice expressing a catalytically inactive form of p110δ (52).

When incubated with D000, the migratory behavior of MDA-MB-231 cells was comparable to that of cells microinjected with inhibitory antibodies to p110δ: cell displacement was inhibited (Fig. 6*A*), cell speed was reduced to ~70% of the control, and chemotaxis was abolished (Fig. 6*C*). These effects were seen even at very low concentrations (0.5 μM) of this drug.

We next tested the effect of D000 on the migration of another breast cancer cell line, MDA-MB-435. These cells express all of the class IA p110 isoforms (Fig. 2*B*) and display positive chemotaxis toward EGF. When incubated with 0.5 μM D000, cell displacement was reduced to 47% of the control (Fig. 7*A*), and the cells no longer chemotaxed toward EGF (Fig. 7*B*). Similar to observations with MDA-MB-231 cells, D000 only caused a small decrease in the speed of migration (83% of the control) suggesting that p110δ makes a smaller contribution to the maintenance of cell speed.

Taken together, the abolition of chemotaxis seen in D000-treated MDA-MB-231 and MDA-MB-435 cells indicates that p110δ is required by breast tumor cell lines to mount a directional response to a chemoattractive stimulus.

[11] Internet address: http://l2.espacenet.com/espacenet/viewer?PN=WO0181346&CY=gb&LG=en&DB=EPD.

Table 2 *Activity of D000 against a panel of protein kinases*

The selectivity of D000 (5 μM) was tested against a panel of 29 protein kinases by *in vitro* kinase assay (KinaseProfiler™, Upstate) in the presence of 100 μM ATP. SD = standard deviation.

Kinase	Activity (% of control) ± SD
Akt/PKBα	102 ± 5.9
AMPK	78 ± 6.0
CDK2/cyclin A	101 ± 8.5
CHK1	98 ± 2.8
CK1	90 ± 1.3
CK2	96 ± 0.2
CSK	97 ± 8.2
DYRK1a	112 ± 1.5
GSK3β	90 ± 0.0
JNK/SAPK1c	97 ± 11
Lck	105 ± 5.0
MAPK2/ERK2	113 ± 0.3
MAPKAP-K1a	132 ± 4.0
MAPKAP-K2	107 ± 7.0
MKK1	110 ± 6.2
MSK1	86 ± 10
PDK1	92 ± 2.0
Phosphorylase kinase	111 ± 5.0
PKA	121 ± 12
PKCα	104 ± 2.7
PP2a	102 ± 0.0
PRAK	93 ± 4.0
ROCK-II	106 ± 8.3
S6K1	97 ± 2.8
SAPK2a/p38	107 ± 11
SAPK2b/p38β2	107 ± 2.1
SAPK3/p38γ	114 ± 6.6
SAPK4/p38δ	104 ± 6.4
SGK	115 ± 12

DISCUSSION

PI3Ks are critical effectors of tyrosine kinase and Ras signaling pathways, which are commonly deregulated in cancer. Relatively little information is available on the expression pattern of PI3K isoforms in cancer cells, and we have therefore screened a large panel of tissues and cell lines at the mRNA and protein level for expression of class IA PI3K isoforms. p110α and p110β proteins were detected in every tissue and cell line investigated. By contrast, p110δ protein was not expressed ubiquitously. As expected, p110δ protein was found predominantly in leukocytes but was also detected in cells of melanocytic or breast origin. This finding concurs with a previous report of p110δ mRNA expression in adult human breast tissue (Ref. 53. wherein p110δ is referred to as "T119"). Untransformed mouse melanocytes were frequently devoid of p110δ, whereas primary human melanocytes and the majority of melanoma cell lines were p110δ-positive. Normal breast epithelial cells and primary breast cancers, as well as many breast tumor cell lines were found to express p110δ.

p110δ has been implicated previously in the regulation of actin cytoskeleton dynamics and chemotaxis of the BAC1.2F5 macrophage cell line stimulated with CSF-1 (24). We report here that p110δ can also regulate cell migration in breast cancer lines. Neutralization of p110δ using antibodies or a small molecule inhibitor, D000, blocked *in vitro* chemotaxis toward EGF, and inhibited increases in cell displacement and partially blocked cell speed. Antibodies to p110δ failed to inhibit platelet-derived growth factor-induced proliferation in p110δ-negative NIH 3T3 cells,[12] whereas D000 had no activity against p110α, p110β, or a panel of cellular protein kinases, demonstrating the selectivity of these inhibitory tools. Neutralization of p110β only blocked chemotaxis without having an effect on migration speed or cell displacement. Remarkably, antibodies to p110α had no impact on cell migration, despite their capacity to block fibroblast[12] and macrophage proliferation (24). Blocking p110β and p110δ simultaneously, or treatment with the broad-spectrum PI3K inhibitor LY294002, had fundamentally the same impact on migration as neutralization of p110δ alone. These results indicate that p110δ is the most important p110 isoform for the regulation of *in vitro* chemotactic migration in response to EGF and provides support for a shared biological function of p110δ in breast cancer cells and macrophages.

It is interesting to note that, in addition to expression of p110δ, breast and melanocytic cell types share functional characteristics with regard to migration. The breast is a highly dynamic tissue that undergoes successive rounds of growth and regression in response to hormonal cycles, and has a unique ability to accommodate a remarkable amount of extracellular matrix remodeling especially during puberty and after pregnancy (54, 55). During embryonic development, melanocytic precursors migrate as individual cells from the neural crest, through the developing embryo to populate, among other tissues, the skin. Therefore, cell motility and chemotaxis are likely to be critical components of normal breast growth and of melanocytes during development. Such migratory properties, including the crossing of basement membranes, are also shared by leukocytes in the adult organism. There is growing evidence for a role of chemotaxis in the regulation of breast cancer and melanoma metastasis, as these types of tumors share a similar pattern of metastatic sites, namely the lymph nodes, lung, liver, and bone marrow. It has been suggested that this phenomenon is linked to the pattern of chemokine receptors expressed by these tumors, with the chemokine ligands for these receptors being expressed specifically in the shared destination organs of the metastatic cells (56, 57). The expression of p110δ in breast cells and melanocytes might be causally related to their shared migratory activities under normal conditions and in the transformed state.

Precisely how PI3Ks regulate chemotaxis and the organization of the actin cytoskeleton is not known; however, it is likely to involve a polar distribution of PI3K lipid products after cell stimulation. In cells confronted with an external, directional stimulus, an intracellular anterior-posterior gradient of 3-phosphorylated lipids is created, which is steeper than that of the extracellular gradient of chemoattractant and results in cell polarization] (Refs. 58–60 and reviewed in Refs. 61–64). It is not clear how a localized production of lipids by

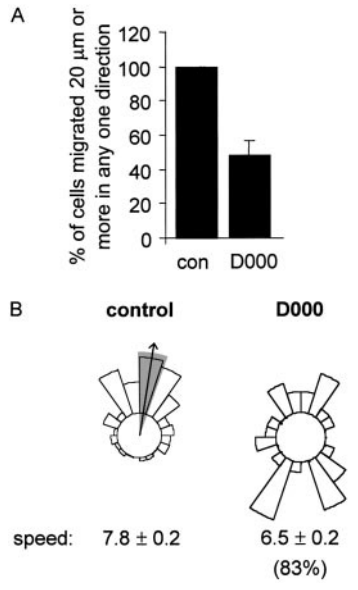

Fig. 7. Effect of neutralization of p110δ on the migration of MDA-MB-435 cells. Cells were preincubated with D000 or DMSO (*con*) before being placed in a gradient of EGF. Cell displacement (*A*), speed (μm/h), and chemotaxis (*B*) were assessed over a distance of 20 μm. The results presented are the pooled data of two experiments. *Con* = control; *bars*, ±SD.

[12] Y. Leverrier and A. Ridley, personal communication.

PI3K is brought about. Neither is it apparent how different p110 isoforms play distinct roles in regulating the actin cytoskeleton and cell migration. All of the class IA isoforms are capable of producing the same lipids *in vitro*, and there is no evidence for a differential recruitment to phosphotyrosine complexes. However, p110 isoforms can be differentially regulated in receptor complexes. For example, CD28-mediated recruitment of p110δ results in a down-regulation of its lipid kinase activity, whereas the activity of p110β in these complexes shows a time-dependent increase (65). Class IA PI3Ks can also differentially interact with Ras as was demonstrated in leukocytes under redox stress, which induces an interaction with Ras of p110β and p110δ, but not of p110α (66). More recent studies with Ras effector mutants support this observation (Ref. 67 and reviewed in Ref. 3). In addition to lipid kinase activity, class IA PI3Ks possess protein kinase activity with distinct substrate specificities being displayed by different p110 isoforms (65, 68, 69). Additional work, including the determination of the subcellular localization of the distinct p110 isoforms, will be required to uncover the detailed molecular mechanisms that underlie the differential functions of class IA PI3K isoforms in chemotaxis.

An appealing hypothesis arising from our observations is that cells expressing all three of the p110 isoforms have additional biological capabilities to cells expressing p110α and p110β only. PI3Ks are attractive targets for small molecule inhibitors, and isoform-specific PI3K antagonists are being developed in the pharmaceutical industry. At present p110δ is considered to be a prime target for anti-inflammatory drugs based on its prevalence in WBCs and on the immunological phenotype of p110δ kinase-dead mice (52). The finding that p110δ is also prominent in melanomas and breast cancer cells, and is a major regulator of EGF-induced migration in the latter cells, broadens the scope of these therapeutic endeavors and identifies p110δ as a potential target for antimetastatic agents.

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