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# **Role of Rho GTPases and PTEN in the Migration of Human Glioma Cells**

**Myrto Raftopoulou**

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MRC Laboratory for Molecular Cell Biology and Cell Biology Unit  
University College London  
Gower Street  
London WC1E 6BT, UK



## Abstract

Rho GTPases play a key role in regulating the migration of many cell types including astrocytes. Astrocytes are relatively poor migrating cells, whereas, gliomas, can be highly invasive, infiltrating the surrounding tissue and spreading diffusely in the brain due, in part, to their highly motile behaviour. This thesis investigates the aberrant migration of three human glioma cell lines (U373, U138, U87) and, using microinjection techniques, it is shown that the small GTPase Rac is essential for the migration of astrocytes as well as for the three glioma cell lines. In agreement with a higher rate of migration, it was demonstrated that the level of active Rac (Rac-GTP) is higher in U373 than in astrocytes. Surprisingly, however, the levels of Rac-GTP in the more motile cell lines, U138 and U87, are much lower than U373 cells.

Rac activity in cells can be stimulated through increases in the levels of PI(3,4,5)P<sub>3</sub> generated by the enzyme PI 3- kinase. PI(3,4,5)P<sub>3</sub> levels are negatively controlled by the dual specificity phosphatase PTEN, which dephosphorylates PI(3,4,5)P<sub>3</sub>. In *in vitro* assays, PTEN has also been shown to have protein phosphatase activity. All three glioma cell lines lack PTEN. This work demonstrates that re-expression of wtPTEN inhibits the migration of all three glioma cell lines, without affecting the migration of astrocytes. Moreover, data is presented showing that the protein, not the lipid, phosphatase activity of PTEN, is essential for inhibiting migration. Most unexpectedly, however, additional mutagenesis studies reveal that it is the C2 domain of PTEN that mediates the inhibitory effect on the migration of these tumour cell lines, and that in the full-length protein, the phosphorylation of residue Thr383 controls the anti-migratory activity of the C2 domain. Finally, using the carboxy terminus of PTEN as bait to screen a brain yeast two-hybrid library, new potential binding partners of PTEN are identified.

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# Chapter 1

## Introduction

### 1.1 Summary

Tumour cells possess a plethora of mechanisms to escape from their site of origin and invade normal neighbouring tissue or metastasize to distant organs. This property of tumour cells is the major cause of the ineffectiveness of the current treatment therapies. Cancers of the central nervous system (CNS) in particular, are considered to be some of the most invasive tumours, and although they lack the ability to use the lymphatic system to metastasize to sites outside the CNS, they acquire properties that allow them to infiltrate normal brain tissue very effectively (Le et al., 2003). The most common brain tumours originate from glial cells and are termed gliomas. Glial cells are subdivided into microglia (cells that function as circulating leukocytes of the brain) and into macroglia. Macroglia cells include astrocytes, oligodendrocytes and ependymal cells. Gliomas can arise from all glial cell types, but the most common and aggressive are thought to derive from astrocytes (Kleihues et al., 1995).

Tumour cells, regardless of their tissue of origin, share a variety of signal transduction pathways and biochemical properties that enable them to migrate and disseminate within or across tissues and organs. Migrating cells need to break the extracellular matrix, which provides the first physical barrier for the advancing cell. Gliomas possess a unique set of proteolytic enzymes that makes them particularly successful in spreading diffusely in the CNS. In addition, unlike other cancers, gliomas exhibit a mesenchymal type movement, which enables them to infiltrate the brain with greater ease (Friedl and Wolf, 2003). For a

cell to migrate, it needs to elongate a protrusion (spread) towards the direction of movement, form new adhesion sites with the substratum at the front, contract the cell body, and move the rear by dissolving the extracellular –matrix adhesions at the back. Signaling cascades are activated to coordinate cell spreading and migration, most notably, pathways that regulate actin cytoskeleton remodeling. At the centre of the actin rearrangement lies the Rho family of small GTPases that regulate many cellular processes involved in cell movement (Ridley, 2001a).

Rho GTPases are tightly regulated and can be activated by a variety of upstream signals, including growth factors binding to tyrosine kinase receptors, agonists binding to seven-pass membrane receptors, or integrin-matrix interactions. Although there is little evidence to implicate Rho GTPase dysregulation directly with cellular transformation, aberrant upstream signaling to Rho GTPases may play an important role in human cancer formation, particularly with respect to cell invasion and metastasis (Jaffe and Hall, 2002; Malliri and Collard, 2003). Gliomas, for example, upregulate EGF and PDGF tyrosine kinase receptor signaling, which can lead to Rac activation (Ridley et al., Cell 1992, Nobes et al., Cell 1995). Phosphatidylinositol 3-kinase (PI 3-kinase) is also regulated downstream of receptor tyrosine kinases and the PI 3-kinase signaling pathway is frequently a target in tumors, as it confers a cell growth and proliferative advantage. PTEN, the primary antagonist to PI 3-kinase signaling, is mutated in many primary human cancers, including gliomas. It plays a role in the insulin signaling pathway and has been shown to regulate cell growth, apoptosis, migration and invasion (Knobbe et al., 2002). PTEN inactivation lies therefore at the heart of human tumour formation.

This thesis addresses the aberrant signaling pathways of glioma cells that affect cell migration, with particular emphasis on the roles played by the Rho family of GTPases and PTEN.

## **1.2 Gliomas**

### **1.2.1 Classification and general characteristics**

Gliomas, and in particular astrocytomas, are the most common primary glial-derived brain tumours in humans. They rarely metastasize outside the CNS and are therefore defined by their clinical grade classification. Grade I tumours are biologically benign, cystic astrocytomas, grade II tumours are low-malignancy tumours, histologically

characterised by nuclear atypia and cellular pleomorphism. Grade III tumours are known as anaplastic astrocytomas and exhibit a high mitotic rate. Finally, grade IV tumours are highly malignant and are referred to as glioblastoma multiforme (GBM). GBMs have a high proliferation rate and are accompanied by increased angiogenesis and tissue necrosis (Figure 1.1) (Maher et al., 2001; Rao, 2003). Malignant gliomas are highly invasive and are termed diffuse gliomas because of their property to infiltrate normal brain tissue and quickly spread in the CNS. This makes them incurable by surgery and glioblastomas are invariably fatal.

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**FIGURE 1.1**

Two examples of GBMs with different clinical history. Reproduced from Maher et al., 2001. (A) Grade II astrocytoma from a young woman whose tumour progressed to GBM 5 years after diagnosis. Arrows indicate neoplastic astrocytes with hyperchromatic nuclei. (B) GBM from an older man with a short clinical history. N:necrosis, P: palisading nuclei, MVP: microvasculature proliferation.

The heterogeneity of glioblastoma morphology makes it difficult to trace the cell of origin. They are grossly subdivided according to their histological characteristics into astrocytomas, oligodendrogliomas and oligoastrocytomas (Dai and Holland, 2003). Most GBMs, however, display astrocytic differentiation markers, e.g. glial fibrillary acid



protein (GFAP) and astrocytes are therefore regarded as the most common cell of origin. Astrocytes perform a variety of functions in the CNS, including the regulation of neuronal homeostasis, growth and survival, guidance of neuronal axonal migration and formation of synapses. They are also involved in immune responses and in repair of tissue after injury, in a process known as gliogenesis. Some astrocyte precursor cells retain the ability to proliferate well after CNS development, a property that may make them more prone to transformation compared to other cells of the CNS (Wechsler-Reya and Scott, 2001).

Astrocytes are produced from multipotent neural stem cells (NCSs) that have the capacity to self renew and generate neurons, astrocytes and oligodendrocytes. As differentiation progresses along the cell lineages, more restrictive precursor cells are generated. First, NCSs produce neural precursor cells that only give rise to neurons, and glial precursor cells that can only give rise to astrocytes and oligodendrocytes (Rao and Mayer-Proschel, 1997; Rao et al., 1998). Glial-precursor cells then generate astrocyte or oligodendrocyte progenitor cells that produce astrocytes or oligodendrocytes respectively. The proliferation and differentiation of these progenitor cells are tightly controlled by extracellular stimuli. Astrocyte differentiation, for example, is promoted by EGF (Bachoo et al., 2002) and EGF receptor (EGFR) knockout mice exhibit a delay in astrocyte differentiation and have a fewer number of astrocytes in several parts of the brain (Kornblum et al., 1998). Other astrocyte differentiation factors include the cytokines of the ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) that use the Jak/Stat signaling pathway, and bone morphogenetic proteins (BMPs) that can promote astrocyte differentiation on their own or in combination with LIF (Wechsler-Reya and Scott, 2001).

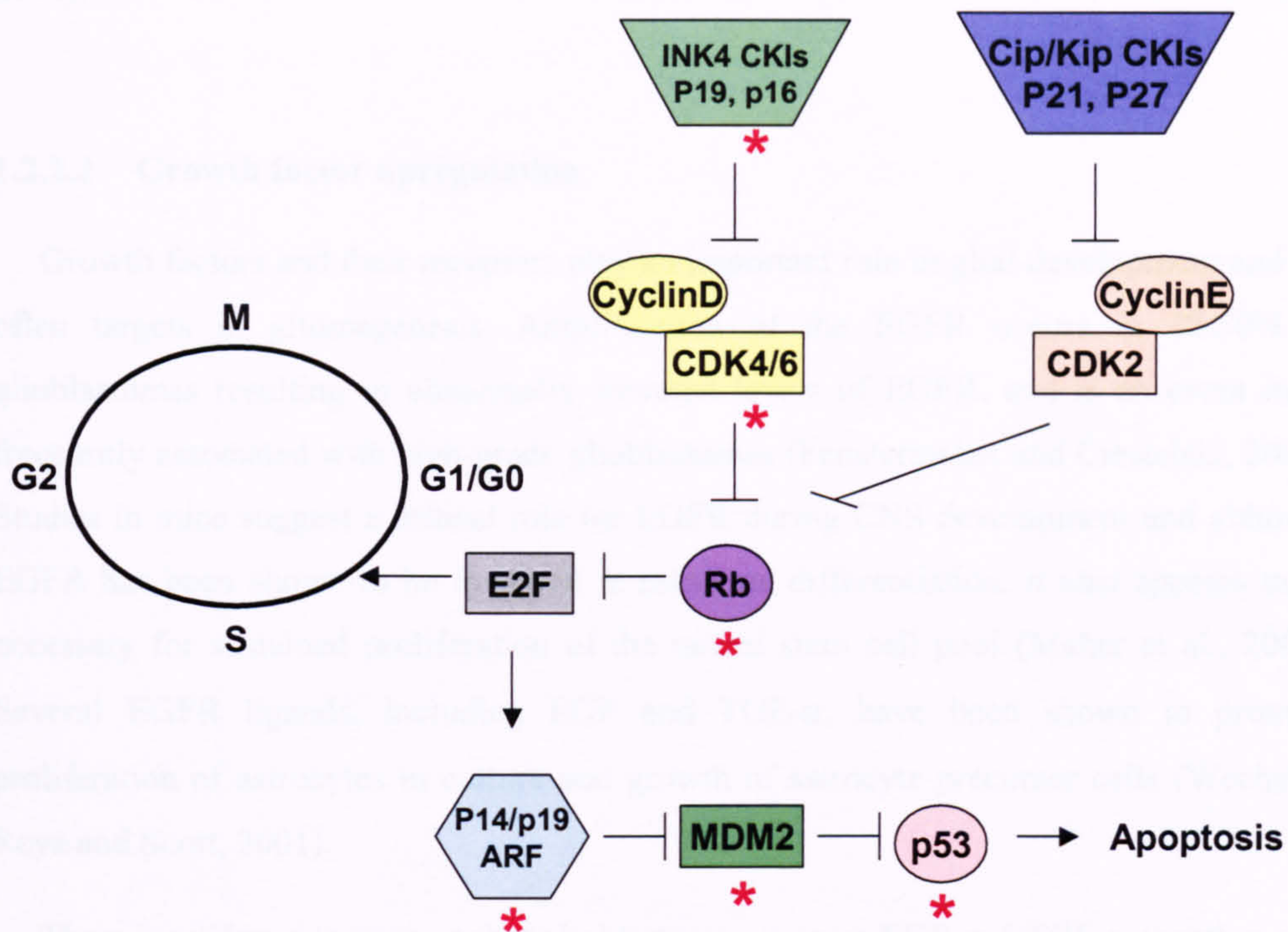
Gliomagenesis is a process likely to involve a combination of events leading to the formation of the highly malignant phenotype of GBM. A popular hypothesis is that genetic mutations cause mature astrocytes or oligodendrocytes to dedifferentiate to an earlier glial cell type, for example a precursor cell, and thus acquire all the migratory and proliferative properties that this cell possessed during CNS development (Maher et al., 2001). The acquisition of further mutations either in a progenitor cell or in a cell that has undergone dedifferentiation in signal transduction pathways that control growth, proliferation and apoptosis, would then result in the formation of increasingly aggressive tumours with a high invasive capacity (Zhou et al., 2003).

## 1.2.2 Glioma mutations in signaling pathways

Gliomas arise either *de novo* (primary) or as low-grade tumours that progress to a more malignant phenotype (secondary). It is thought that they develop along different genetic pathways and are thus clinically distinct. Gliomas exhibit losses of parts of chromosomes or amplification of genes and most of the genetic mutations identified target either cell cycle arrest and apoptotic pathways or signal transduction cascades downstream of receptor tyrosine kinases.

### 1.2.2.1 Cell cycle dysregulation

Alterations in genes encoding Rb, cyclins, CDKs or CDK inhibitors (CKIs) are frequent in gliomas. The transition of low-grade to intermediate-grade in gliomas is usually characterised by an increase in proliferation rate and is accompanied by losses of parts of chromosomes 9 and 13 that encode for key components of the Rb signaling pathway. Mutations in Rb have been described in about 25% of malignant astrocytomas. Mutations have also been described in the cyclin-dependent kinases CDK4 and CDK6. It has been reported that the CDK4 gene is amplified 10- to 100-fold in 15% of glioblastomas and overexpression of CDK4 in cultured astrocytes results in immortalization (Holland et al., 1998). The most common mutation that disrupts the cell cycle pathway in gliomas is in the INK4A-ARF gene, which encodes for the proteins p16<sup>INK4a</sup> and p14<sup>ARF</sup> (or p19<sup>ARF</sup> in mice) (Arap et al., 1997). CKIs prevent the binding of D-type cyclins to CDK4/6 and displace the inhibitor p27<sup>KIP1</sup>, which in turn inhibits CDK2 (Figure 1.2). This prevents CDK2-dependent hyperphosphorylation of Rb and subsequent progression into S phase. Loss of p16<sup>INK4a</sup> promotes the activation of CDK4/6 and results in the phosphorylation of Rb and entry into S phase. Mutation of p16<sup>INK4a</sup> occurs in around 60% of high-grade gliomas (Maher et al., 2001).



**FIGURE 1.2**

Cell cycle components mutated in gliomas. Proteins involved in the regulation of G1 to S phase transition are depicted. INK4 family CKIs inhibit cyclinD/CDK4/6 complexes, whereas Cip/Kip CKIs act on both CyclinD/CDK4/6 and Cyclin E/CDK2 complexes. This prevents phosphorylation of Rb and subsequent release of E2F and entry into S phase. p14/p19ARF negatively regulates the p53 inhibitor MDM2, and promotes apoptosis. A red asterisk indicates the proteins frequently mutated in gliomas.

The p14/p19<sup>ARF</sup> gene is a tumour suppressor that acts on the MDM2-p53 signaling pathway. Expression of p19<sup>ARF</sup> inhibits the growth of human gliomas in culture (Arap et al., 1997) probably by binding directly to MDM-2 and inhibiting the degradation of p53 (Figure 1.3). It also promotes p53-dependent apoptosis and induces G1 cell cycle arrest. The p53 gene is another common target of inactivating mutations in human gliomas (Evans et al., 2003; Fei and El-Deiry, 2003). It is primarily an early event in gliomagenesis and mutations mostly affect residues required for DNA binding. It has been reported that around 25% of glioblastomas have inactivating mutations or deletions in the p53 gene and around 5%-12% have amplification of the gene that encodes the p53

regulator MDM-2. MDM-2 promotes the proteosomal degradation of p53 by directly binding to p53 and acting as an E3 ubiquitin ligase (Kubbutat et al., 1997; Lohrum et al., 2000).

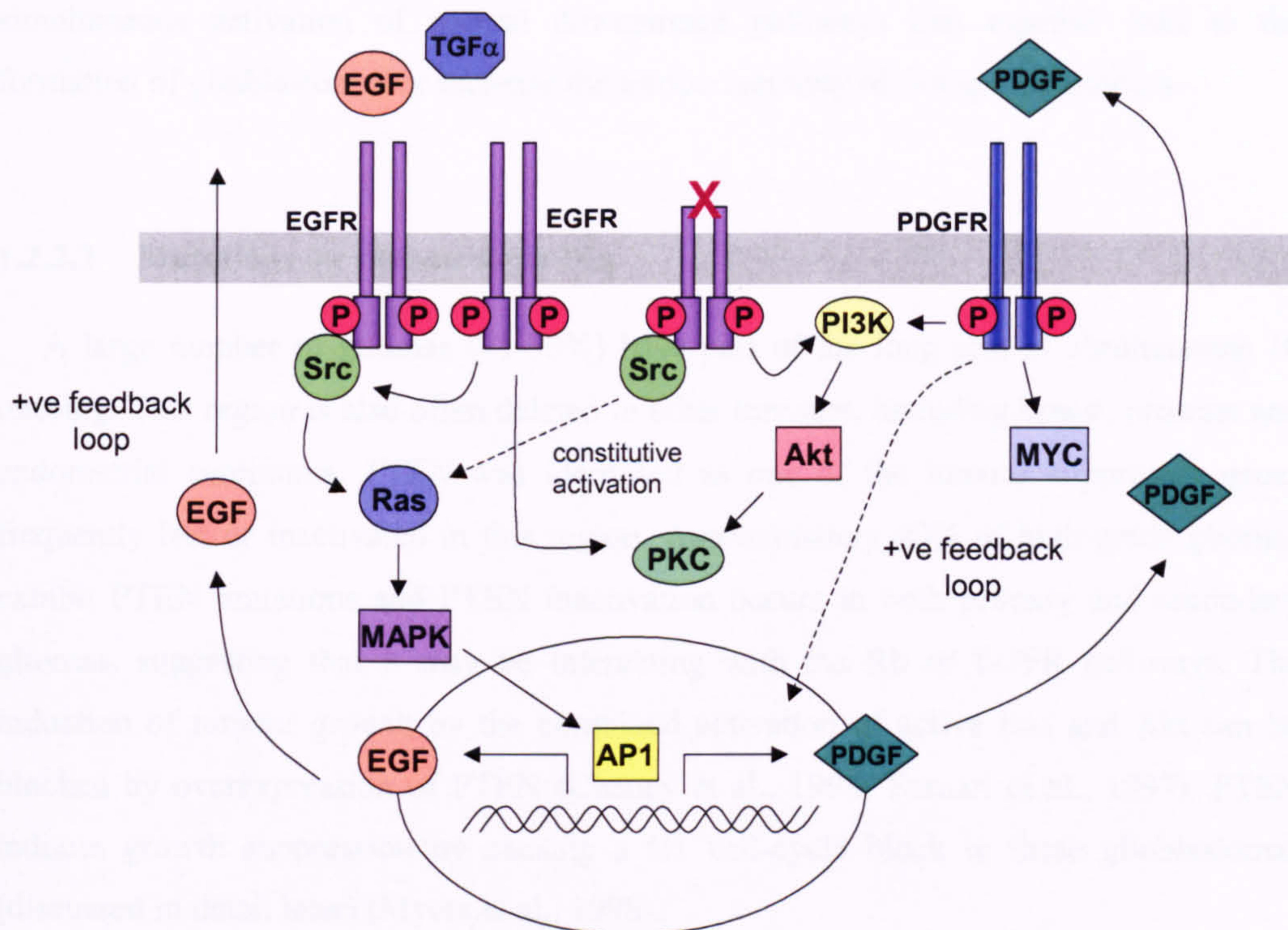
### **1.2.2.2 Growth factor upregulation**

Growth factors and their receptors play an important role in glial development and are often targets in gliomagenesis. Amplification of the EGFR occurs in 40-50% of glioblastomas resulting in abnormally elevated levels of EGFR, and is an event more frequently associated with high-grade glioblastomas (Fenstermaker and Ciesielski, 2000). Studies in mice suggest a critical role for EGFR during CNS development and although EGFR has been shown to be involved in astrocyte differentiation, it also appears to be necessary for sustained proliferation of the neural stem cell pool (Maher et al., 2001). Several EGFR ligands, including EGF and TGF- $\alpha$ , have been shown to promote proliferation of astrocytes in culture and growth of astrocyte precursor cells (Wechsler-Reya and Scott, 2001).

There is evidence to suggest that glioblastomas express EGF and TGF- $\alpha$  together with EGFR and thus growth of these tumours can be sustained through both paracrine and autocrine stimulatory loops of the EGFR (Figure 1.3). Another consequence of receptor amplification (which occurs in 40-60% of GBMs) is that often this results in the expression of mutated forms of the receptor. Approximately 60% of glioblastomas express truncated EGFR, which lacks part of the extracellular domain and can no longer bind ligand, but is constitutively tyrosine phosphorylated and is thus always active (Ekstrand et al., 1994; Nagane et al., 2001; Nishikawa et al., 1994). The constitutively active EGFR is not efficiently downregulated, unlike the wild-type form, and introduction of the truncated active EGFR into glioma cells, dramatically enhances their tumorigenicity (Ciesielski and Fenstermaker, 2000), proliferative capacity and resistance to apoptosis (Nagane et al., 1996). In addition, mice expressing the viral oncogene derived from the EGFR, v-erbB, have been shown to develop oligodendrogliomas (Weiss et al., 2003).

A second growth factor suggested to be involved in an autocrine stimulatory loop with its receptor is PDGF (Figure 1.3). Increased expression levels of both PDGF and PDGFR have been reported in low-grade astrocytomas, and tumours that express both ligand and receptor have also been identified (Lokker et al., 2002). Transgenic mice expressing

PDGF in neural progenitors and astrocytes developed oligodendrogliomas and oligoastrocytomas respectively (Dai et al., 2001). In addition, the pattern of PDGF expression closely correlates with p53 mutations in low-grade tumours, suggesting a possible genetic interaction (Hermanson et al., 1996).



**FIGURE 1.3**

Growth factor receptor upregulation in gliomas. A few of the signaling pathways downstream of EGFR and PDGFR are depicted. EGFR amplification results in EGF upregulation and the subsequent autocrine and paracrine loop of receptor activation (similarly for PDGF/PDGFR). A truncated EGFR (denoted by a red cross) is constitutively active. The Ras/MAPK and PI 3-kinase/PKB are two of the many pathways activated downstream of the PDGF and EGF receptors. PKC and MYC are also targets of the tyrosine kinase receptor signaling.

Constitutive activation or amplification of EGF/EGFR and PDGF/PDGFR leads to the activation of downstream signaling pathways including the PI3-kinase/Akt pathway, the RAS/MAPK pathway, the protein kinase C pathway and the C-MYC pathway (Figure 1.3). Consistent with this, elevated levels of Ras and Akt have been reported in human

gliomas. Studies with transgenic mice showed that expression of active Ras or v-Src in astrocytes induced the formation of glioblastomas and astrocytomas respectively. Mice with germline genetic mutations, however, resulting in active Ras and Src, develop normally and any gliomas formed are due to secondary mutations (Guha et al., 1997). In addition, although the combined activation of Ras and Akt signaling pathways have been reported to induce glioblastomas, no activating mutations in either protein have been identified in human gliomas (Dai and Holland, 2001). It is likely that the abnormal activation of the receptor tyrosine kinase receptors EGFR, PDGFR and FGF results in the simultaneous activation of several downstream pathways that together lead to the formation of glioblastomas or increase the tumorigenicity of low-grade tumours.

### 1.2.2.3 Mutations on chromosome 10q

A large number of gliomas (75-90%) have part of the long arm of chromosome 10 missing. This region is also often deleted in other tumours, including breast, prostate and endometrial carcinoma. *PTEN* was identified as one of the tumour suppressor genes frequently lost or inactivated in this region. Approximately 40% of high-grade gliomas exhibit *PTEN* mutations and *PTEN* inactivation occurs in both primary and secondary gliomas, suggesting that it may be interacting with the Rb or EGFR pathways. The induction of tumour growth by the combined activation of active Ras and Akt can be blocked by overexpression of *PTEN* (Cheney et al., 1998; Furnari et al., 1997). *PTEN* induces growth suppression by causing a G1 cell-cycle block in these glioblastomas (discussed in detail later) (Myers et al., 1998).

Another important tumour suppressor candidate in this region is the antagonist of the MYC oncoprotein, Mxi1 (Max interactor 1) (Meroni et al., 2000). Mxi1 is a cell growth regulator and is mutated in prostate carcinomas and in 15% of high-grade glioblastomas. Re-introduction of Mxi1 in glioblastoma cells inhibits proliferation and induces cell cycle arrest at the G2 to M phase transition (Manni et al., 2002). Mice lacking Mxi1 develop prostatic tumours but do not develop any gliomas, suggesting that Mxi1 is not an important target in gliomagenesis. *DMBT1* (deleted in malignant brain tumours) is another gene frequently mutated on chromosome 10q and is deleted in 50-80% of glioblastomas and anaplastic astrocytomas. *DMBT1* is an extracellular matrix protein that belongs to the scavenger-receptor cysteine-rich superfamily with a role in epithelial cell differentiation and in immune responses and polarity. Its function in the CNS, however, is unknown.

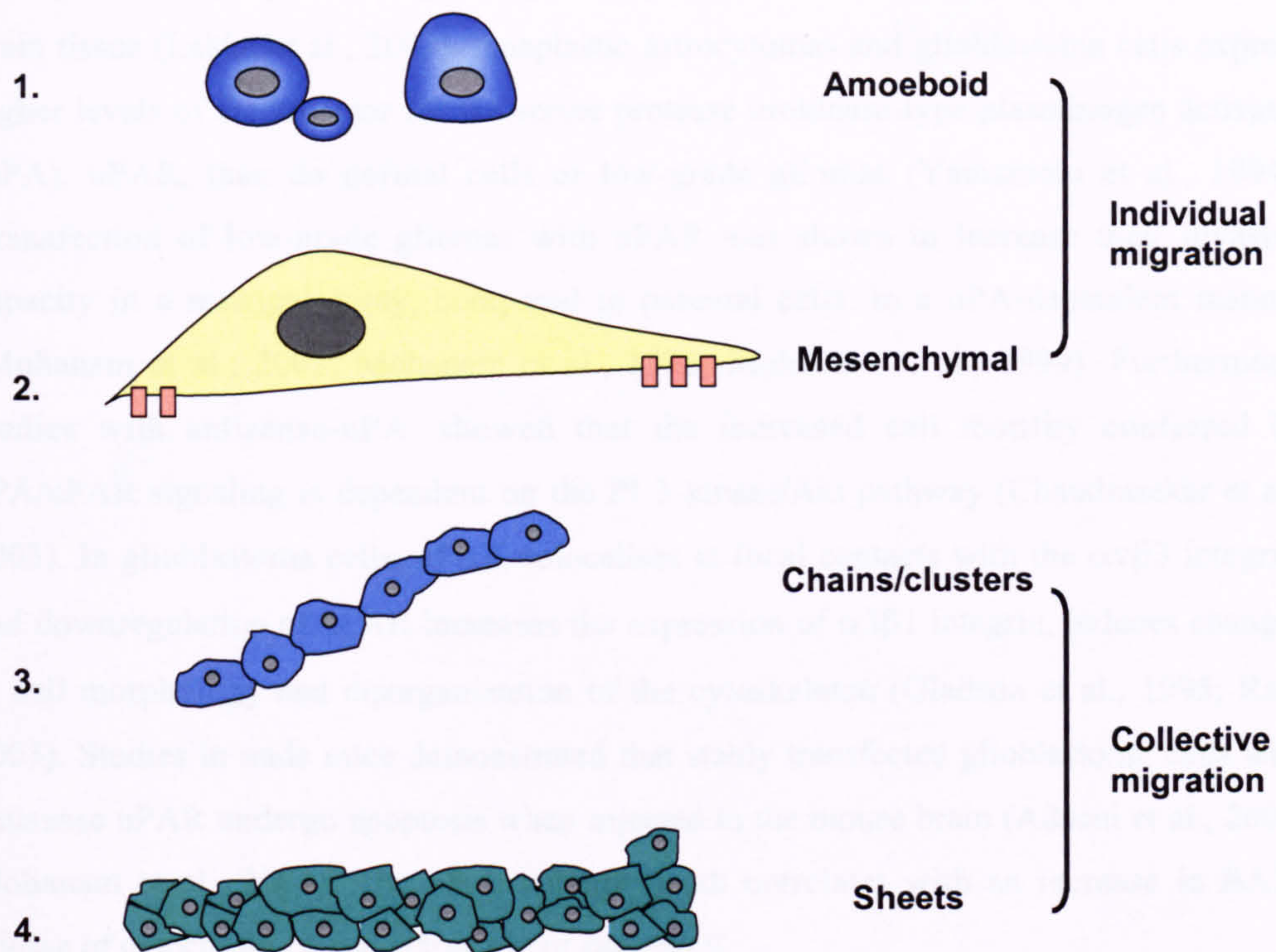
### 1.2.3 Tumour invasion

#### 1.2.3.1 Migratory phenotype

The migratory behaviour of gliomas and their ability to invade normal tissue and spread diffusely is a key component of their malignant phenotype. Invasion is a property of both low-grade astrocytomas and high-grade glioblastomas and resembles the migratory phenotype of glial cells during CNS development. It is possible that glioma invasion follows the same mechanisms of early glial migration during embryogenesis through the reactivation of the same signaling pathways. In the adult, neurons, oligodendrocytes and astrocytes do not migrate. However, it has been shown that in response to injury, nestin-positive cells, most probably neural stem cells and glial progenitors, migrate to the site of injury and there undergo terminal differentiation into astrocytes. It is not clear whether these pathways can be disrupted during tumorigenesis, but they provide a likely source for malignant transformation. It was demonstrated that neural stem cells and glioma cells co-cluster following *in vivo* injection, suggesting that they might use the same routes when migrating in the CNS (Johansson et al., 1999; Magavi et al., 2000). Migration and invasion of gliomas is very different to other tumours that do not spread locally to the extent that gliomas do, but rather use the lymphatic system and the blood vessels to metastasize to distant sites. Gliomas lack the ability to cross the blood brain barrier, but rather have acquired properties that enable them to spread in the surrounding normal tissue (Rao, 2003).

Tumour cells infiltrate neighbouring tissue in diverse patterns that usually reflect their differentiation stage (Figure 1.4). They can migrate as individual cells or as clusters, strands or sheets, known as 'collective migration'. Collective cell invasion is predominantly a feature of highly differentiated tumours such as prostate and large-cell lung carcinomas. Cells either protrude as sheets that still remain in contact with the primary site and disseminate only locally, or clusters of cells detach from their site of origin and enter tissue gaps or metastasize via the lymphatic system. Single cell migration can be subdivided into mesenchymal, amoeboid and formation of cell chains. Myoblasts and melanomas move in a chain-like manner, still maintaining cell-cell contacts and forming junctions at the tip of the cell (Friedl and Wolf, 2003). Amoeboid migration is characteristic of the amoeba, *Dictyostelium discoideum*. In higher eukaryotes, this type of movement is retained by leukocytes and some tumour cells, such as lymphoma and

myeloid leukemia cells (Farina et al., 1998). Leukocytes and *Dictyostelium* use a type of movement that is generated by cortical filamentous actin and is based on short-lived interactions with the substratum. Small-cell lung and prostate carcinomas use this type of movement to undergo early detachment and spread from a small primary tumour.



**FIGURE 1.4**

The different types of tumour cell migration. Individual cells display either an amoeboid type of migration (*Dictyostelium*, leukemia or lymphoma cells) or a mesenchymal migration (gliomas, fibrosarcomas). Collective migration is subdivided into chain-like movement (melanoma cells, epithelial tumours) and migration as sheets (epithelial tumours, vascular tumours).

Gliomas show an elongated, spindle-like morphology and exhibit a mesenchymal-type movement that is dependent on integrin attachments to the substratum to produce the high traction forces necessary to propel the cell forward. Mesenchymal migration is slower



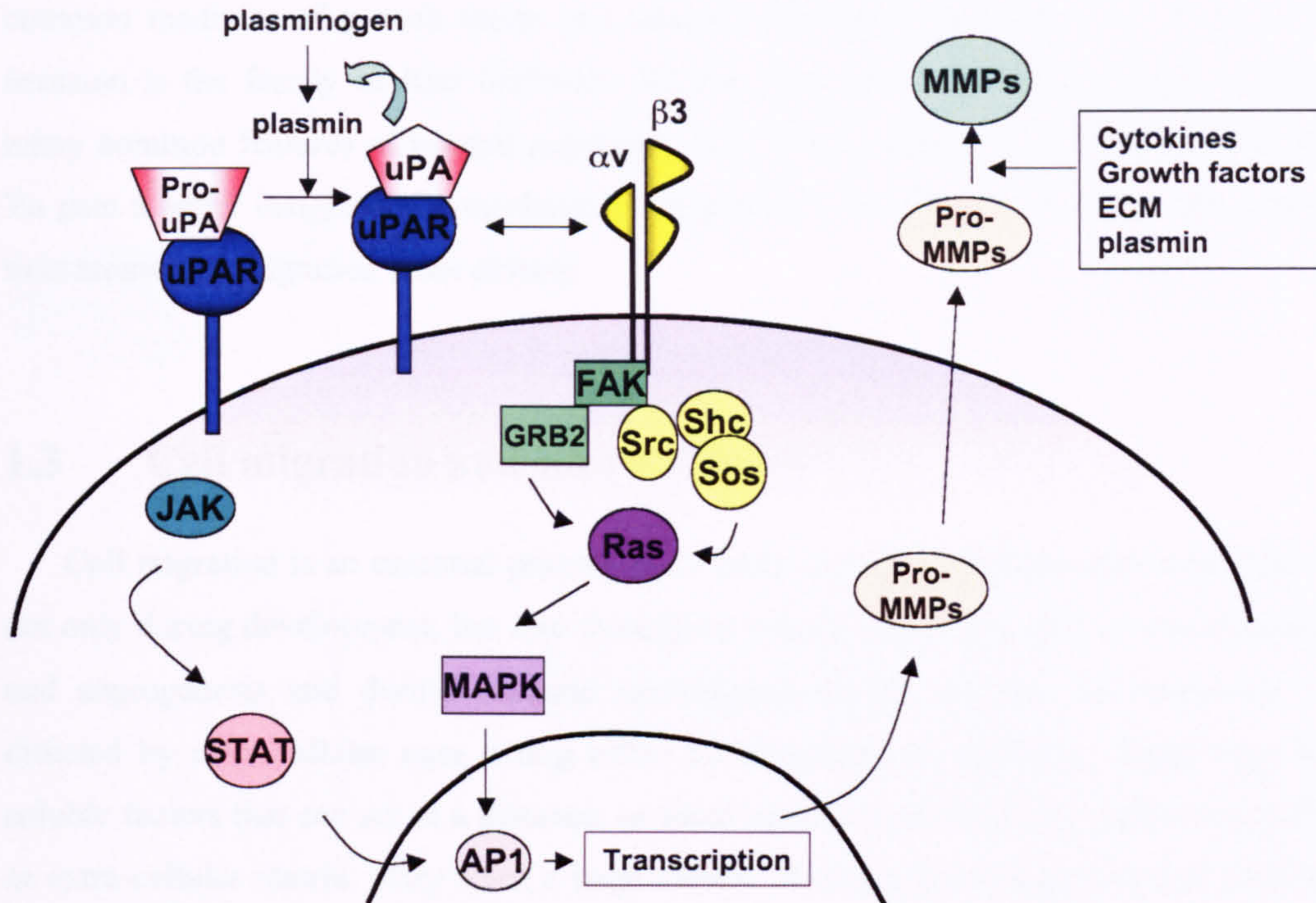
than amoeboid migration and one of the reasons is that focal adhesion turnover is relatively slow (Webb et al., 2002).

### 1.2.3.2 Factors promoting invasion

The extracellular matrix is the main component being destroyed in a tissue during tumour cell invasion and differs for each cell type within an organ. Tumour cells of the CNS possess a unique set of proteolytic enzymes that enables them to infiltrate normal brain tissue (Lakka et al., 2003). Anaplastic astrocytomas and glioblastoma cells express higher levels of the receptor for the serine protease urokinase-type plasminogen activator (uPA), uPAR, than do normal cells or low-grade gliomas (Yamamoto et al., 1994). Transfection of low-grade gliomas with uPAR was shown to increase their invasive capacity in a matrigel assay, compared to parental cells, in a uPA-dependent manner (Mohanam et al., 2002; Mohanam et al., 1998; Mohanam et al., 1999). Furthermore, studies with antisense-uPA, showed that the increased cell motility conferred by uPA/uPAR signaling is dependent on the PI 3-kinase/Akt pathway (Chandrasekar et al., 2003). In glioblastoma cells, uPAR colocalises at focal contacts with the  $\alpha v\beta 3$  integrin, and downregulation of uPAR increases the expression of  $\alpha 3\beta 1$  integrin, induces changes in cell morphology and disorganization of the cytoskeleton (Gladson et al., 1995; Rao, 2003). Studies in nude mice demonstrated that stably transfected glioblastoma cells with antisense uPAR undergo apoptosis when injected in the mouse brain (Adachi et al., 2002; Mohanam et al., 2001). The observed cell death correlates with an increase in BAX, release of cytochrome c and activation of caspase-9.

A second key factor in glioma invasiveness is the production of MMPs (Le et al., 2003). MMPs are a family of secretory-independent endopeptidases with proteolytic activity against ECM components. Expression of MMPs is regulated by a variety of factors including growth factors, cytokines, cell-matrix and cell-cell interactions. MMPs are kept in inactive state by their specific tissue inhibitors (TIMPs) (Nuttall et al., 2003). In addition to degrading the ECM, which also leads to the release of matrix-bound growth factors, they activate signal transduction pathways that promote migration and proliferation. Human glioma cells express MMP-2 and MMP-9 and the expression levels of these MMPs closely correlate with tumour progression (Hu et al., 2003; Lakka et al., 2003; Wang et al., 2003). In gliomas, the PI 3-kinase signaling pathway is often constitutively activated due to the loss of the PI 3-kinase antagonist PTEN or due to

EGFR overexpression. EGFR signaling was shown to induce MMP-9 activation, an effect dependent on PI 3-kinase, and hyaluronic acid-mediated secretion of MMP-9 and subsequent activation of the MAPK pathway in human gliomas, was inhibited by re-introduction of PTEN (Choe et al., 2002; Park et al., 2002).



**FIGURE 1.5**

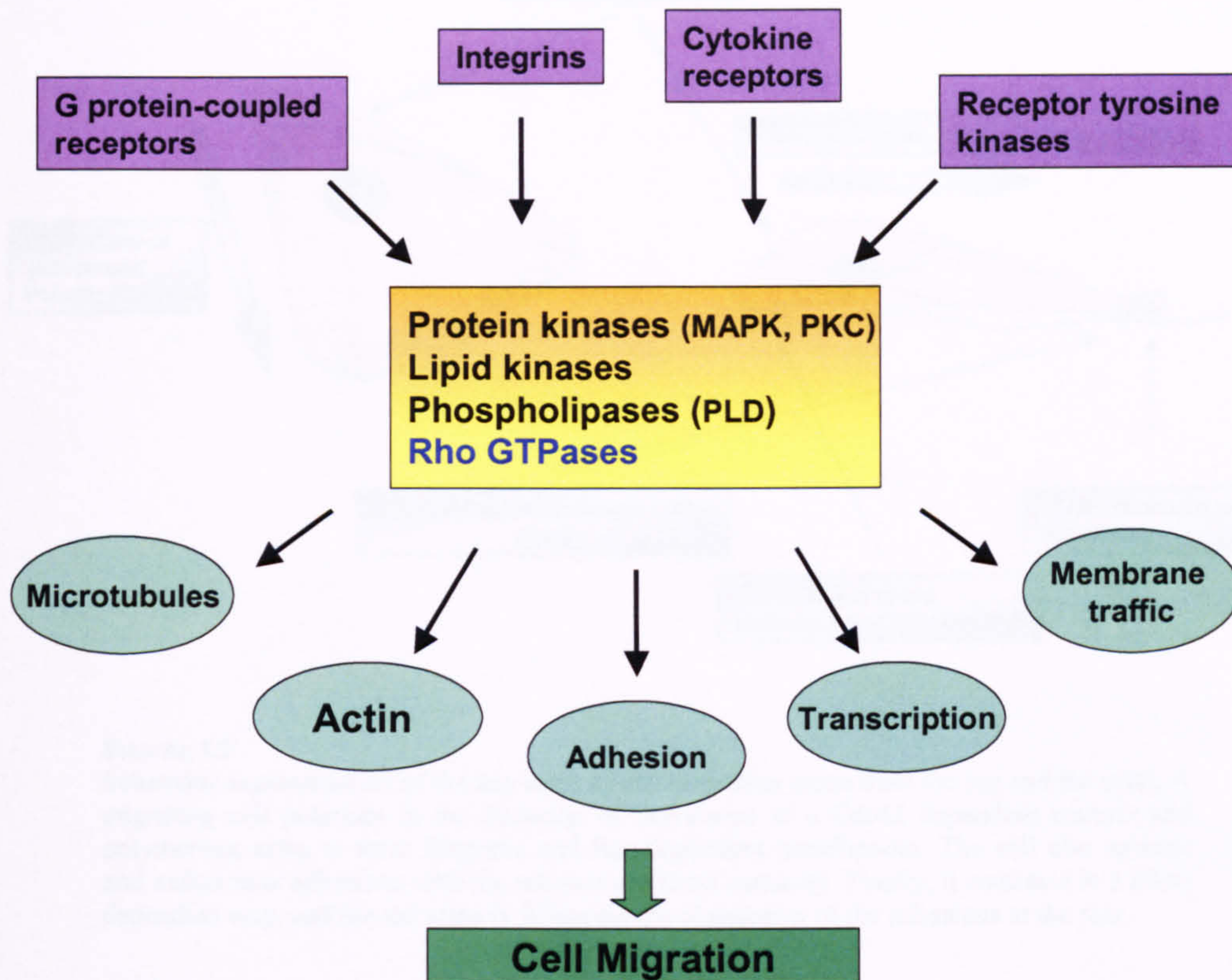
The uPA/uPAR and MMP pathways. Pro-uPA bound to its receptor is cleaved more efficiently by plasmin to produce uPA. uPA, in turn, cleaves plasminogen into plasmin in a positive feedback loop. The tail of uPAR can activate transcription via the Jak/Stat pathway. uPAR colocalises with integrins at focal contacts and is involved in the activation of several pathways downstream of integrins, like the Ras/MAPK pathway. MMPs are released as inactive pro-MMPs and are cleaved into active MMPs by a variety of components, including growth factors and plasmin. ECM: extracellular matrix.

A multitude of environmental factors promote, regulate and direct tumour cell migration and consequently tumour invasion. In addition to MMPs and other proteases

that facilitate movement within the tissue, growth factors, such as EGF, PDGF and IGF1 also promote cell migration and invasion through the activation of several signal transduction pathways (Lakka et al., 2002)). EGF induces migration and process extension of both normal astrocytes and astrocytic tumour cells, whereas IGF-1 promotes chemotaxis of breast cancer cells in an integrin-dependent mechanism (Hu et al., 2003; Wang et al., 2003). PDGF, on the other hand, has been shown to be important in low-grade glioma migration through activation of PI 3-kinase and PLC- $\gamma$  pathways. A common mediator of growth factor and integrin stimulated migration and tumour cell invasion is the family of Rho GTPases. Gliomas and tumour cells in general, display many common features of normal migrating cells, when migrating during development. To gain a better insight in the mechanisms of glioma invasion, it is important, therefore, to examine cell migration more closely.

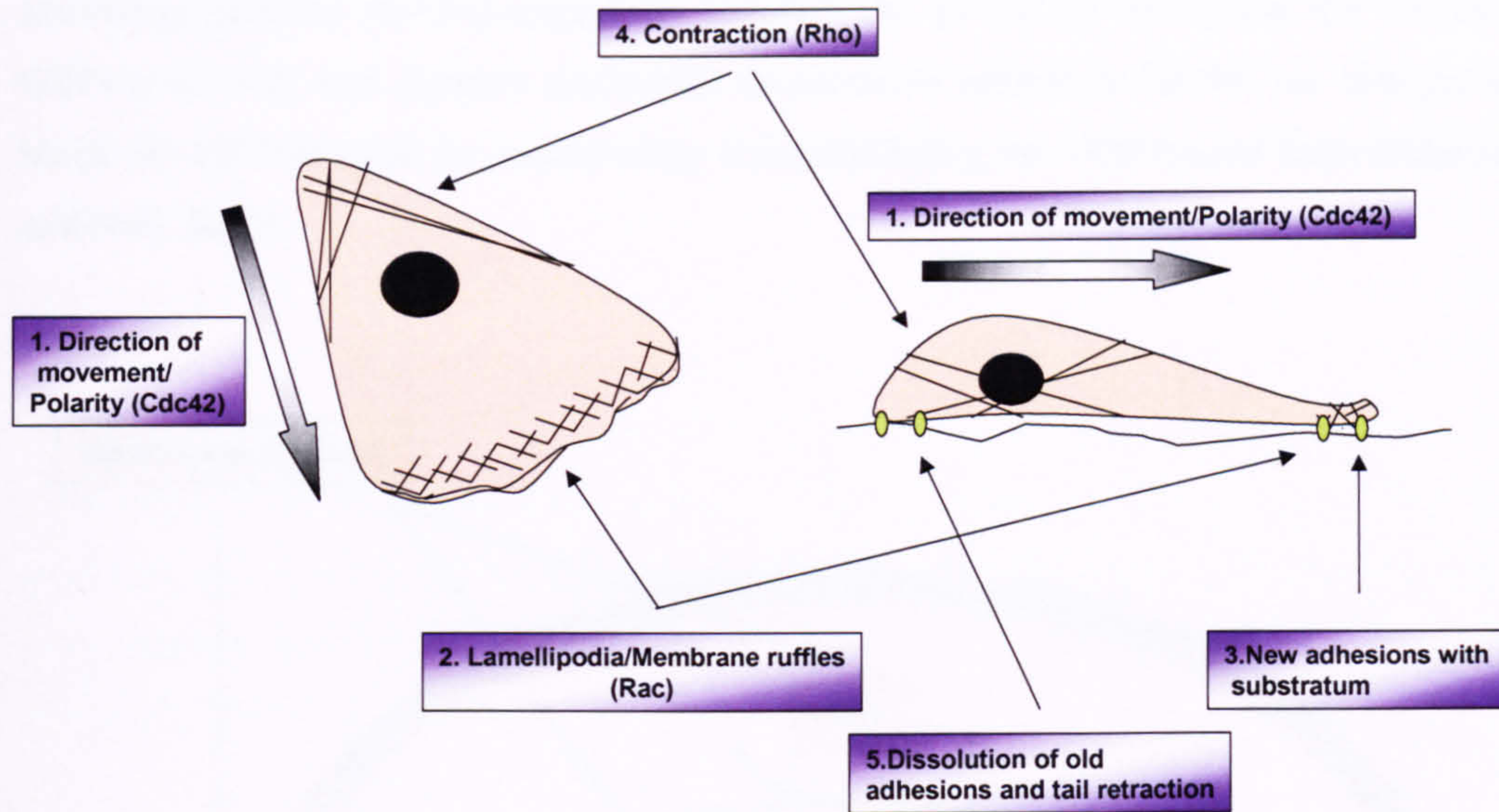
### **1.3 Cell migration and Rho GTPases**

Cell migration is an essential process in all multi-cellular organisms and is important not only during development, but also throughout life for responses such as wound repair and angiogenesis and during immune surveillance. In the animal, cell migration is directed by extra-cellular cues acting either as attractants or repellants. These may be soluble factors that can act at a distance, or local signals received from neighboring cells or extra-cellular matrix. They elicit a large variety of intracellular responses that include changes in the organization of the actin and microtubule cytoskeletons, vesicular transport pathways and gene transcription (Figure 1.6).

**FIGURE 1.6**

Regulation of cell migration. The main pathways that regulate cell migration are shown. These include PKCs, PLC, PI 3-kinase, MAPK and Rho GTPases.

The process of cell migration has been extensively studied in tissue culture, where the environment can be controlled and easily manipulated. However, the genetic analysis of whole organisms is making significant and novel contributions (Lehmann, 2001). It is now widely accepted that the central mechanism driving cell migration is the extension of a leading edge protrusion or lamellipodium, the establishment of new adhesion sites at the front, cell body contraction, and detachment of adhesions at the cell rear (Figure 1.7). All these steps involve the assembly, the disassembly or the reorganization of the actin cytoskeleton and each must be coordinated both in space and time to generate productive, net forward movement.



**FIGURE 1.7**

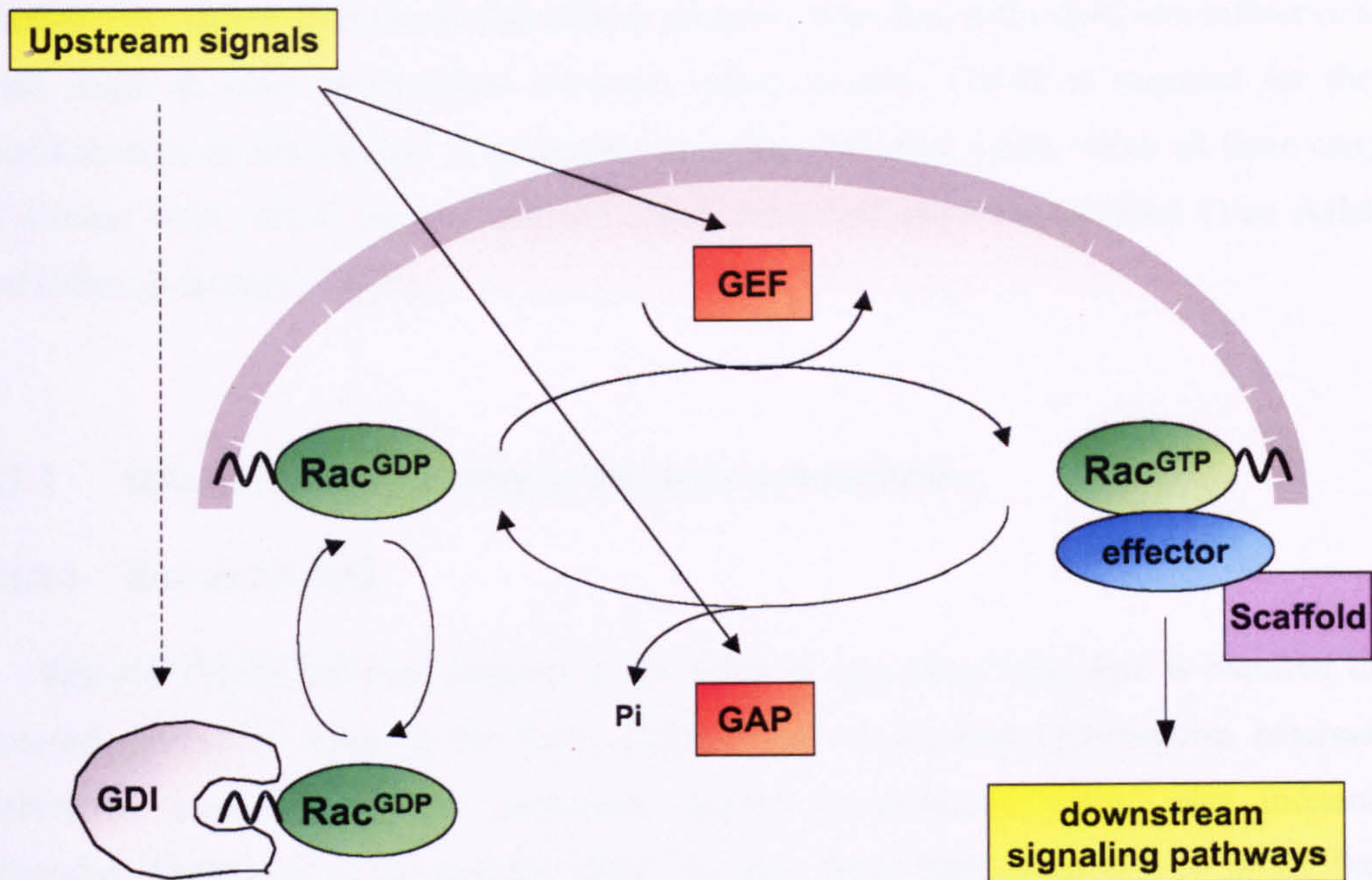
Schematic representation of the key steps of cell migration (seen from the top and the side). A migrating cell polarises in the direction of movement in a Cdc42 dependent manner and polymerises actin to form filopodia and Rac-dependent lamellipodia. The cell also spreads and makes new adhesions with the substratum (focal contacts). Finally, it contracts in a Rho-dependent way, and the tail retracts following the dissolution of the adhesions at the rear.

Multiple intracellular signaling molecules have been implicated in cell migration, MAPK cascades, lipid kinases, phospholipases, Ser/Thr kinases (such as protein kinase Cs [PKC] and p65PAK) and tyrosine kinases (such as Abl and Src). However, one particular family of proteins seems to play a pivotal role in regulating the biochemical pathways most relevant to cell migration; Rho GTPases (Figure 1.7) (Ridley, 2001a).

### 1.3.1 The Rho GTPase cycle

Rho GTPases are ubiquitously expressed and approximately 20 members have been identified in mammals, seven in *Drosophila melanogaster*, five in *Caenorhabditis elegans* and 15 in *Dictyostelium discoideum* (Schultz et al., 1998). They act as molecular switches to control signal transduction pathways by cycling between a GDP-bound, inactive form and a GTP-bound, active form (Figure 1.8). The RhoGTPases cycle is tightly regulated by three groups of proteins. Guanine nucleotide exchange factors

(GEFs), promote the exchange of GDP for GTP to activate the GTPase, GTPase-activating proteins (GAPs) negatively regulate the switch by enhancing the intrinsic GTPase activity and guanine nucleotide dissociation inhibitors (GDIs) are thought to block the GTPase cycle by sequestering and solubilizing the GDP-bound form (Schmidt and Hall, 2002).



**FIGURE 1.8**

The Rho GTPase cycle. Rho GTPases cycle between an inactive GDP-bound form and an active GTP-bound form. The cycle is tightly regulated mainly by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). In their active form, Rho GTPases can bind to effector molecules and mediate their effect, for example, on the actin cytoskeleton.

In their GTP-bound state, the Rho GTPases interact with a variety of downstream targets (effectors), to elicit a variety of intracellular responses. The signaling specificity is thought to be established through the binding of scaffold proteins (Buchsbaum et al., 2002; Buchsbaum et al., 2003). The best-characterised function of Rho GTPases is in the regulation of actin dynamics. Tissue culture studies (carried out originally in fibroblasts,

but later in many other cell types) using constitutively active and dominant negative, interfering forms, have shown that Rho regulates the assembly of contractile, actin:myosin filaments, while Rac and Cdc42 regulate the polymerisation of actin to form peripheral lamellipodial and filopodial protrusions, respectively (Hall, 1998). In addition, all three GTPases promote the assembly of integrin-based, matrix adhesion complexes (Nobes and Hall, 1995; Ridley and Hall, 1992). It is perhaps not surprising, therefore, that these three regulatory proteins play such an important part in controlling cell migration. Furthermore, and in addition to their effects on actin, Rho, Rac and Cdc42 can influence a wide range of other biochemical activities. Most notably, Cdc42 is required for the establishment of cell polarity in all eukaryotic cells, including yeast, while all three can, in distinct ways, affect the microtubule cytoskeleton and gene transcription (Van Aelst and D'Souza-Schorey, 1997).

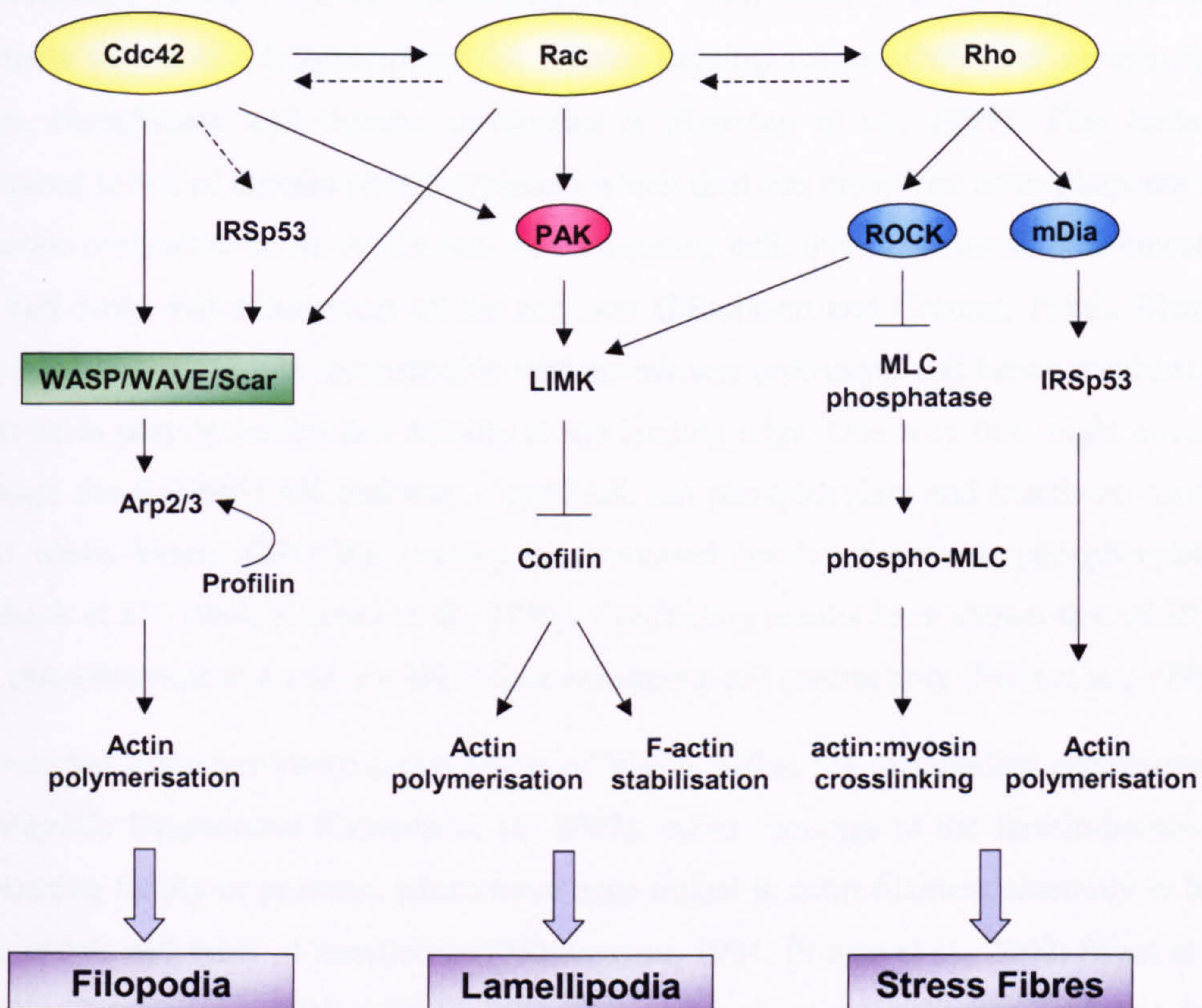
### **1.3.2 Rho GTPase signaling to the actin cytoskeleton**

#### **1.3.2.1 Rac and Cdc42**

Rac and Cdc42 are both required at the front of migrating cells. Rac is required to generate protrusive force at the front, while Cdc42 is required to recognize external directional cues and polarise protrusive activity accordingly. Cdc42 also induces filopodia. Their role is not entirely clear, but they have been suggested to probe the extracellular milieu. The cellular targets of Rac and Cdc42 that promote changes to the actin cytoskeleton have been the subject of intense investigation. The Ser/Thr kinase p65PAK is commonly activated upon either Rac or Cdc42 activation and is believed to play an important rôle in regulating actin dynamics during cell adhesion and migration. p65PAK regulates focal adhesion turnover with the help of PIX and GIT1 (GRK interactor 1) but how it does so is not known (Manabe Ri et al., 2002; Obermeier et al., 1998). In addition, p65PAK1 phosphorylates and activates LIM kinase (LIMK), which in turn phosphorylates and inactivates cofilin (Figure 1.9) (Arber et al., 1998; Edwards et al., 1999). Cofilin, which facilitates depolymerisation at the pointed end of actin filaments, cycles between an active and inactive form, and this is essential for promoting filament treadmilling at the front of migrating cells (Bamburg, 1999).

Members of the WASp/SCAR/WAVE family of scaffold proteins are key regulators of actin polymerisation and WASp interacts directly with the GTP-bound form of Cdc42 (Takenawa and Miki, 2001). In their activated state, the WASp/SCAR/WAVE proteins

are able to activate the Arp2/3 complex, which can initiate actin polymerisation, either *de novo* or at the tips or sides of pre-existing filaments. In this way the dendritic morphology of lamellipodial actin is thought to be generated (Amann and Pollard, 2001; Weaver et al., 2003). WASp/WAVE can also bind to profilin, which acts synergistically with Arp2/3 to speed-up actin polymerisation (Blanchoin et al., 2000; Yang et al., 2000). Rac and Cdc42 are thought to activate members of this family directly (Eden et al., 2002; Rohatgi et al., 2000), although activation could also be indirect through the Rac and Cdc42 effector IRSp53 (Figure 1.9) (Krugmann et al., 2001; Miki and Takenawa, 2002; Miki et al., 2000).



**FIGURE 1.9**

Schematic representation of some of the main components that mediate the effects of Rho GTPases on the actin cytoskeleton. Rac and Cdc42 both induce actin polymerisation through the activation of IRSp53 and WASp/WAVE/Scar family members that subsequently activate Arp2/3 to initiate actin nucleation. Alternatively, they bind and activate the kinase p65PAK that inhibits cofilin downstream of LIMK. Rho, on the other hand, activates IRSp53 through its effector mDia. Rho also leads to actin:myosin crosslinking by inhibiting myosin light chain (MLC) phosphatase through p160ROCK. There is substantial cross-talk between the GTPases in order to coordinate the responses on the actin cytoskeleton.



### 1.3.2.2 Rho

Rho activity in migrating cells is associated with focal adhesion assembly and cell contractility. One important Rho target involved in stimulating actin:myosin filament assembly and therefore contractility is the Ser/Thr kinase p160ROCK. In migrating leukocytes, for example, Rho and p160ROCK are required for proper rear cell detachment (Ablas et al., 2001). ROCK plays an essential role during migration of P cells in the larval development of *C. elegans* and during dorsal closure and gastrulation in *Drosophila* (Barrett et al., 1997; Magie et al., 1999; Spencer et al., 2001). In its active state, p160ROCK, like p65PAK, can phosphorylate and activate LIMK, which in turn phosphorylates and inactivates cofilin leading to stabilization of actin filaments within the actin:myosin filament bundles (Maekawa et al., 1999; Sumi et al., 2001). p160ROCK interacts with and phosphorylates the myosin binding subunit (MBS) of myosin light chain phosphatase and thereby inactivates it (Kawano et al., 1999). This leads to increased levels of myosin phosphorylation which then can cross-link actin filaments and generate contractile force. At the rear of a migrating cell, this can promote movement of the cell body and detachment of the cell rear (Mitchison and Cramer, 1996). Clearly, however, this activity is incompatible with membrane protrusion and hence mechanisms must be in play to inhibit this activity at the leading edge. One way this might occur is through the Rac/p65PAK pathway – p65PAK can phosphorylate and inactivate myosin light chain kinase (MLCK), leading to decreased levels of myosin phosphorylation (Kaback et al., 1984; Kiosses et al., 1999). Conflicting results have shown that p65PAK can phosphorylate and activate MLC thus enhancing cell contractility (Sells et al., 1999).

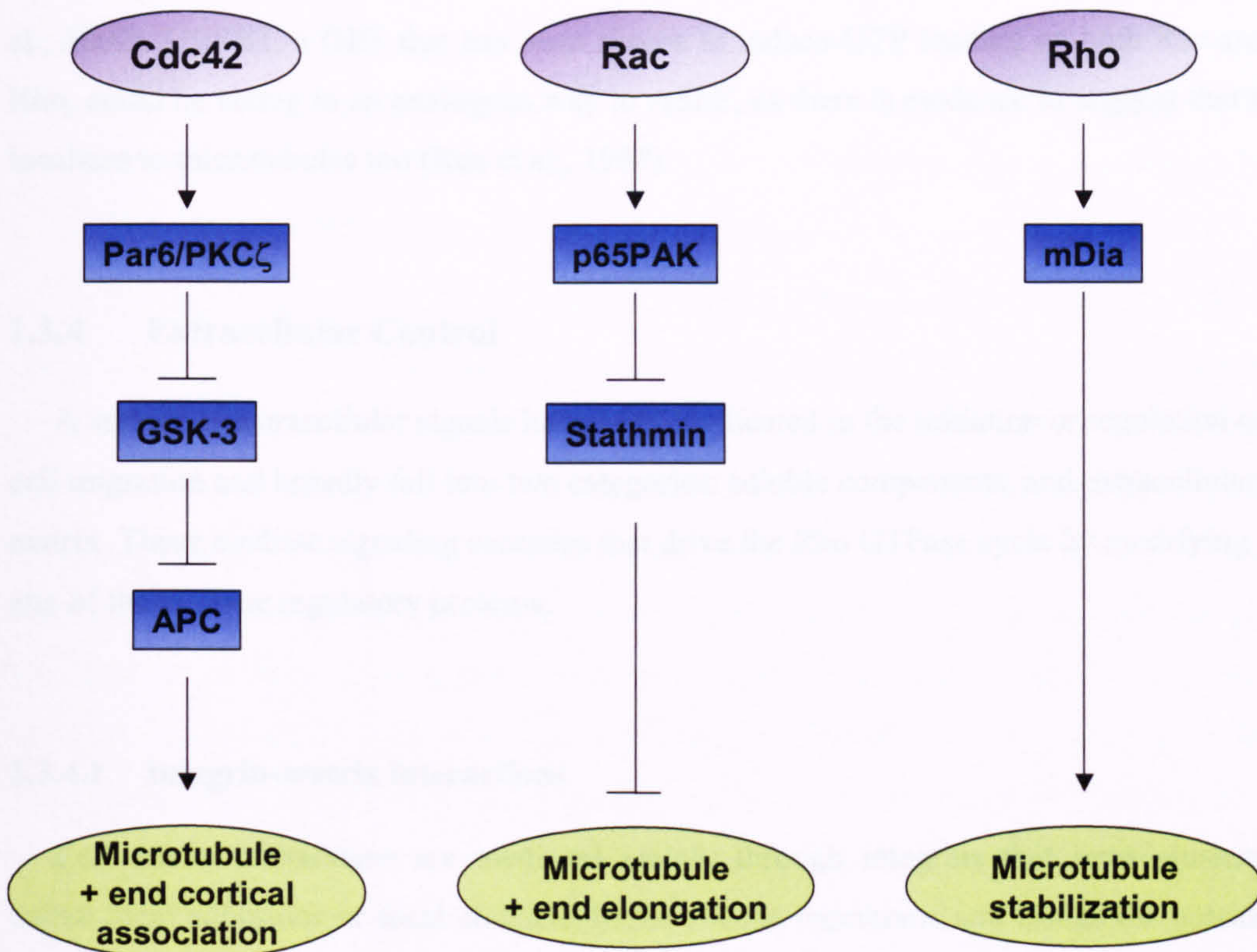
Another important downstream target of Rho is mDia, the mammalian orthologue of *Drosophila* Diaphanous (Geneste et al., 2002). mDia belongs to the formin-homology containing family of proteins, which have been linked to actin filament assembly in both *Drosophila* and yeast (Castrillon and Wasserman, 1994; Pruyne et al., 2002; Sagot et al., 2002). The binding of RhoGTP to mDia opens up and activates this scaffold protein. It cooperates with p160ROCK in the assembly of actin:myosin filaments, but its biochemical contribution is unclear (Uehata et al., 1997; Watanabe et al., 1999). Interestingly, mDia can also bind to the Rac and Cdc42 effector IRSp53 in a Rho-dependent manner and this could provide yet another way for Rho GTPase cross-talk (Fujiwara et al., 2000).

### 1.3.3 Rho GTPases and microtubules

Although the effects of Rho GTPases on the actin cytoskeleton have received most attention to date, it is now clear that they can also modulate the microtubule cytoskeleton and this may play an important role during the migration of at least some cell types (Wittmann and Waterman-Storer, 2001). The first clue for a link was the observation that disruption of microtubules with nocodazole activates Rho, but when washed out of cells it leads to Rac activation (Liu et al., 1998). Later Rho was shown to promote the stabilization of microtubules through its target mDia that directly interacts with microtubules and promotes their capping (Figure 1.10) (Ishizaki et al., 2001). Another possible pathway to regulate microtubule dynamics in mammalian cells could well be the one that is known to operate in yeast (Gundersen, 2002). It has been shown, by direct imaging studies of the budding cortex, that yeast regulate capture and shrinkage of microtubules by a mechanism that involves the formin Bni1, Kar9 and Bim1/Yeb1, a homologue of the mammalian microtubule tip protein EB1 (Adames and Cooper, 2000; Schuyler and Pellman, 2001). Rac, on the other hand, has been shown to modify microtubule dynamics through p65PAK-dependent phosphorylation and inactivation of the microtubule destabilising protein stathmin (Figure 1.10) (Daub et al., 2001; Kuntziger et al., 2001).

Cdc42 plays a crucial role in defining cell polarity with respect to the external environment. Inhibition of Cdc42 in macrophage cells, for example, blocks their ability to undergo chemotaxis towards a gradient of CSF-1, although it does not inhibit their ability to move (which is Rac dependent) (Ridley, 2001b). The mechanism by which Cdc42 regulates chemotaxis is not known. However, work with neutrophils, suggests that there exists a positive feedback loop between the Rho GTPases and PI(3,4,5)P<sub>3</sub> in order to establish cellular asymmetry and polarity (discussed later) (Weiner et al., 2002). Polarised cell migration is often reflected in the organization of the microtubule cytoskeleton and the centrosome, which usually face the direction of migration. In migrating astrocytes and fibroblasts, Cdc42 regulates this reorientation of the microtubules and centrosome and recent work has revealed some of the mechanisms involved (Nobes and Hall, 1999). In its GTP-bound state, Cdc42 activates a target complex of Par6 (a scaffold protein) and the atypical protein kinase C, PKC $\zeta$  (Figure 1.10) (Etienne-Manneville and Hall, 2001). The specific activation of this complex at the leading edge of the migrating cells is essential for determining the polarity and direction of migration. Recently it has been shown that Cdc42-dependent phosphorylation of GSK3 releases APC to bind to plus ends

of microtubules and contribute to the regulation of cell polarisation (Etienne-Manneville and Hall, 2003).



**FIGURE 1.10**

Simplified representation of the effects of Rho GTPases on microtubules. Cdc42 regulates microtubule dynamics by becoming recruited to a Par6/ PKC $\zeta$  complex. Formation of the complex leads to the inhibition of GSK-3 and the release of APC, which can associate with the plus-end of microtubules at the leading edge. Rac also regulates microtubules by activating p65PAK to phosphorylate and inactivate stathmin, a microtubule-destabilising protein. Little is known on how Rho activation stabilises microtubules, other than it involves its effector mDia.

Interestingly APC has recently been shown to interact with ASEF, a Rac-specific GEF, again pointing to cooperation between Rac and Cdc42 at the leading edge (Kawasaki et al., 2000). APC, however, has also been shown to promote microtubule polymerisation in vitro and in cultured mammalian fibroblasts and in *Xenopus* epithelial cells, there is

evidence to indicate that APC can bind to microtubules and move along them probably through binding to the microtubule-associated protein EB1 and the kinesin KAP3, thus playing a similar role to the yeast protein Kar9 (Gundersen, 2002; Jimbo et al., 2002; Nakamura et al., 2001). The movement of APC towards the plus ends of microtubules in migrating cells could serve to localise ASEF to sites of actin-rearrangement, where Rac could be recruited in a similar way and be activated only locally (Bienz, 2002; Jimbo et al., 2002). GEFH1, a GEF that has been shown to induce GTP loading on both Rac and Rho, could be acting in an analogous way to ASEF, as there is evidence to suggest that it localises to microtubules too (Ren et al., 1998).

### 1.3.4 Extracellular Control

A variety of extracellular signals have been implicated in the initiation or regulation of cell migration and broadly fall into two categories: soluble components, and extracellular matrix. These mediate signaling cascades that drive the Rho GTPase cycle by modifying any of the GTPase regulatory proteins.

#### 1.3.4.1 Integrin-matrix interactions

Cell-matrix interactions are mediated mainly through integrins that form clusters, called focal adhesions or focal contacts, in the plasma membrane and bridge the outside world to the intracellular milieu particularly the actin cytoskeleton (Figure 1.11) (DeMali et al., 2003; Hynes, 2002). In *Drosophila* for example, position-specific (PS) integrins, which are most similar to the  $\beta 1$  vertebrate integrins, are needed for proper migration of the endodermal cells during midgut development (Martin-Bermudo et al., 1999). Real time imaging by a number of groups has revealed the assembly of small, Rac-dependent focal contacts associated with the protruding front of a migrating cell, followed by their Rho-dependent maturation into larger focal adhesions in the cell body and eventually disassembly at the rear (Rottner et al., 1999; Webb et al., 2002). It is clear, therefore, that Rho GTPases must be activated precisely in space. In migrating endothelial cells, for example, Rac induces the localisation of integrin  $\alpha v \beta 3$  to lamellipodia as visualized with an antibody specific to the high-affinity state of the integrin (Kiosses et al., 2001). In addition to promoting integrin clustering, Rho GTPases are themselves activated by integrin engagement with matrix, making this a particularly intimate relationship (Nguyen

et al., 2001). Thus during spreading of fibroblasts on fibronectin, which has some similarities to migration, Cdc42 and Rac are activated upon engagement of the integrins to the substratum (as observed by looking at the phosphorylation of several downstream kinases like FAK or Src), leading to actin polymerisation at the periphery and protrusion of the membrane (Berrier et al., 2002; Clark et al., 1998; O'Connor and Mercurio, 2001). At later times, these two GTPases are downregulated and Rho activity is increased, as the cells stabilize their morphology and put down firm focal adhesions (Arthur and Burridge, 2001; Ren et al., 1999). Activation of Rac and transient inhibition of Rho seem to be critical events at the front of migrating cells, whereas activation of Rho is required in the cell body and at the rear.

The mechanisms by which these two GTPases are differentially activated within a single cell are not clear, however recent studies have given us some insights (Sander et al., 1999). Integrin  $\alpha3\beta1$  engagement by laminin (10/11) preferentially activates Rac through phosphorylation of p130Cas and activation of the CrkII / DOCK180 pathway but engagement of the integrin  $\alpha5\beta1$  to fibronectin seems to activate Rho (Gu et al., 2001). Elegant FRET studies by the Schwartz lab have shown that integrins control the translocation of Rac to the plasma membrane at the front of the migrating cell and enhance its association with effectors by causing its dissociation from Rho-GDI (Del Pozo et al., 2002). Finally, activation of integrin  $\alpha6\beta4$  by laminin has been shown to play a role in cell migration of carcinoma cells by inducing the activation and translocation of Rho (rather than Rac) to ruffles, in a cAMP dependent way, presumably for the formation of new adhesions (O'Connor et al., 2000).

#### 1.3.4.2 Soluble factors

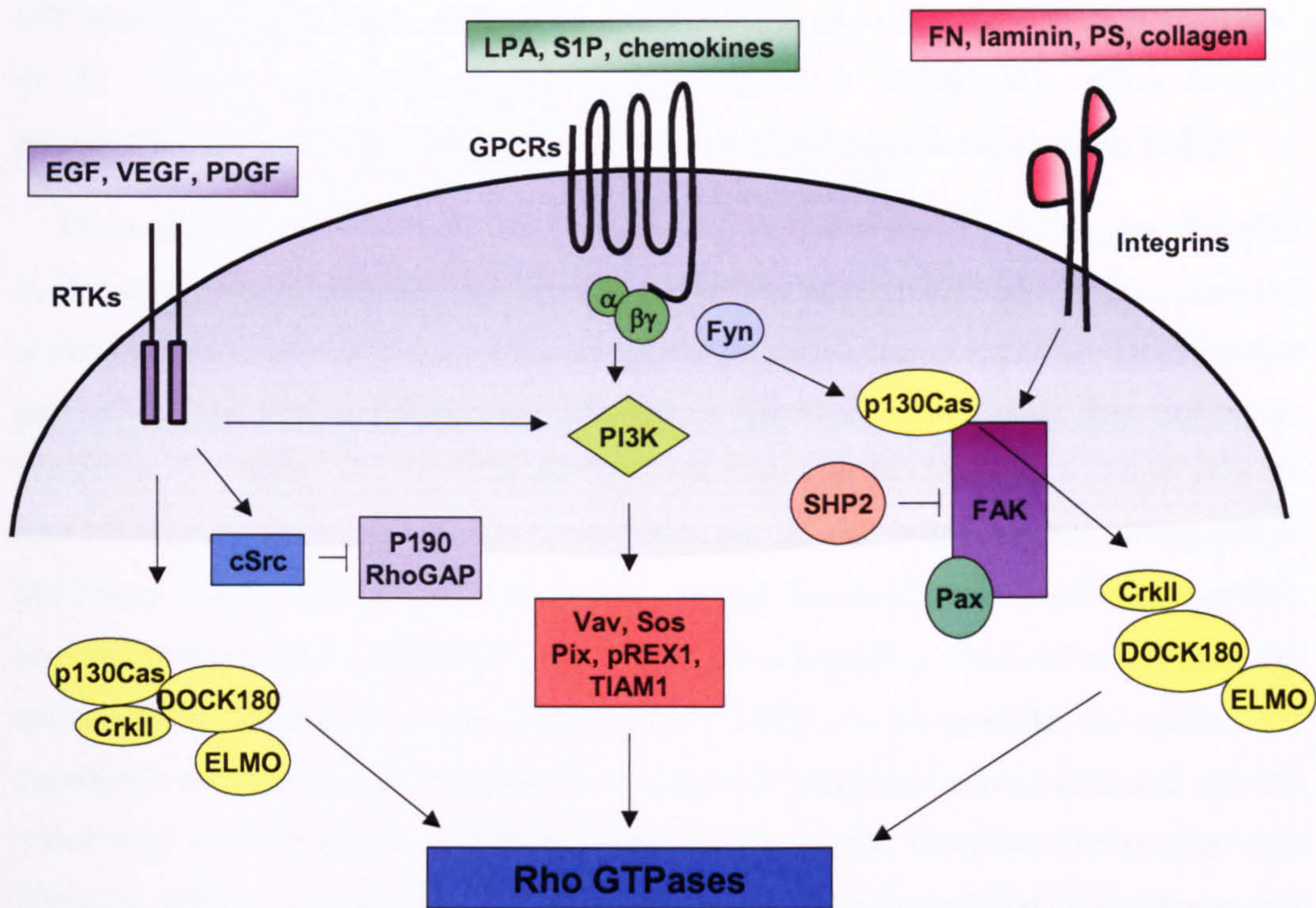
A huge range of proteins and lipids have been found to affect the migratory behaviour of cells (Figure 1.11). Growth factors acting on tyrosine kinase receptors are important regulators of cell migration. They have been studied extensively in tissue culture assays and in genetically tractable organisms such as *Drosophila* and *C. elegans*, particularly during development (Forbes and Lehmann, 1999; Lehmann, 2001). Studies first carried out in mammalian cells showed that many growth factors capable of promoting cell migration are strong activators of Rac (Nobes and Hall, 1995; Ridley et al., 1992). Addition of PDGF to fibroblasts, for example, stimulates Rac-dependent lamellipodia formation and membrane ruffling reflecting the induction of actin polymerisation at the

cell periphery. Recently a new technique for visualizing activated Rac in living cells, FLAIR (fluorescence activation indicator for Rho proteins), has been used with cells induced to migrate along a gradient of PDGF. In this case, PDGF induces an intracellular gradient of active Rac, the highest levels being at the leading edge and the lowest at the rear of the migrating cell (Kraynov et al., 2000).

Interestingly, PDGF also leads to the activation of Rho, apparently through the inactivation of p190RhoGAP, a negative regulator of Rho GTP levels (Chiarugi et al., 2000). It is expected that active Rho will be excluded from the leading edge of migrating cells, but this has not yet been shown directly. PDGF signaling provides a nice example of how the same stimulus activates different GTPases to produce a coordinated response; the mechanisms by which the two GTPase are differently localised is, however, completely unknown.

Two peptide factors acting through tyrosine kinase receptors, EGF and Pvf1 (homologous to VEGF and PDGF) are thought to act as chemoattractants directing border cell migration during oocyte maturation in *Drosophila*. The PVR (the receptor for Pvf1) is thought to control F-actin accumulation in the border cells by signaling to Rac via *mbc* (myoblast city), the fly orthologue of mammalian DOCK180 (Duchek and Rorth, 2001; Duchek et al., 2001).

Lipids, acting externally through seven-pass trans-membrane receptors, can also influence cell migration through the modulation of GTPase pathways. In fibroblasts, lysophosphatidic acid (LPA) is a strong activator of Rho and might be expected to inhibit cell migration (Nobes and Hall, 1995; Ridley and Hall, 1992). However, in vascular smooth muscle cells, LPA acting through a different receptor leads to the rapid activation of the Ser/Thr kinase p65PAK, a target of Rac. It appears that this is mediated by activation of the Tiam-1 (a Rac GEF) in a G $\alpha$ i and PI 3-kinase dependent mechanism and this leads not only to the activation of Rac but also to the inhibition of Rho signaling (Sander et al., 1999; Schmitz et al., 2002; Van Leeuwen et al., 2003). A recent paper has shown that Rho can activate Rac and when p160Rho kinase is inhibited, this leads to lamellipodia formation rather than stress fibers (Tsuji et al., 2002). This cross-talk between Rho GTPases is clearly an important feature of their ability to act coordinately.



**FIGURE 1.11**

Simplified representation of some of the upstream regulatory pathways of Rho GTPases. Ligand binding to either tyrosine kinase receptors or G-protein coupled receptors or integrins results in the activation of signaling components like FAK, Src, Shc, Fyn, PI 3-kinase that ultimately lead to the activation or inactivation of GEFs and GAPs respectively. Activation of PH-domain GEFs (e.g. TIAM-1, pREX, Vav) leads to the activation of Rac and Cdc42.

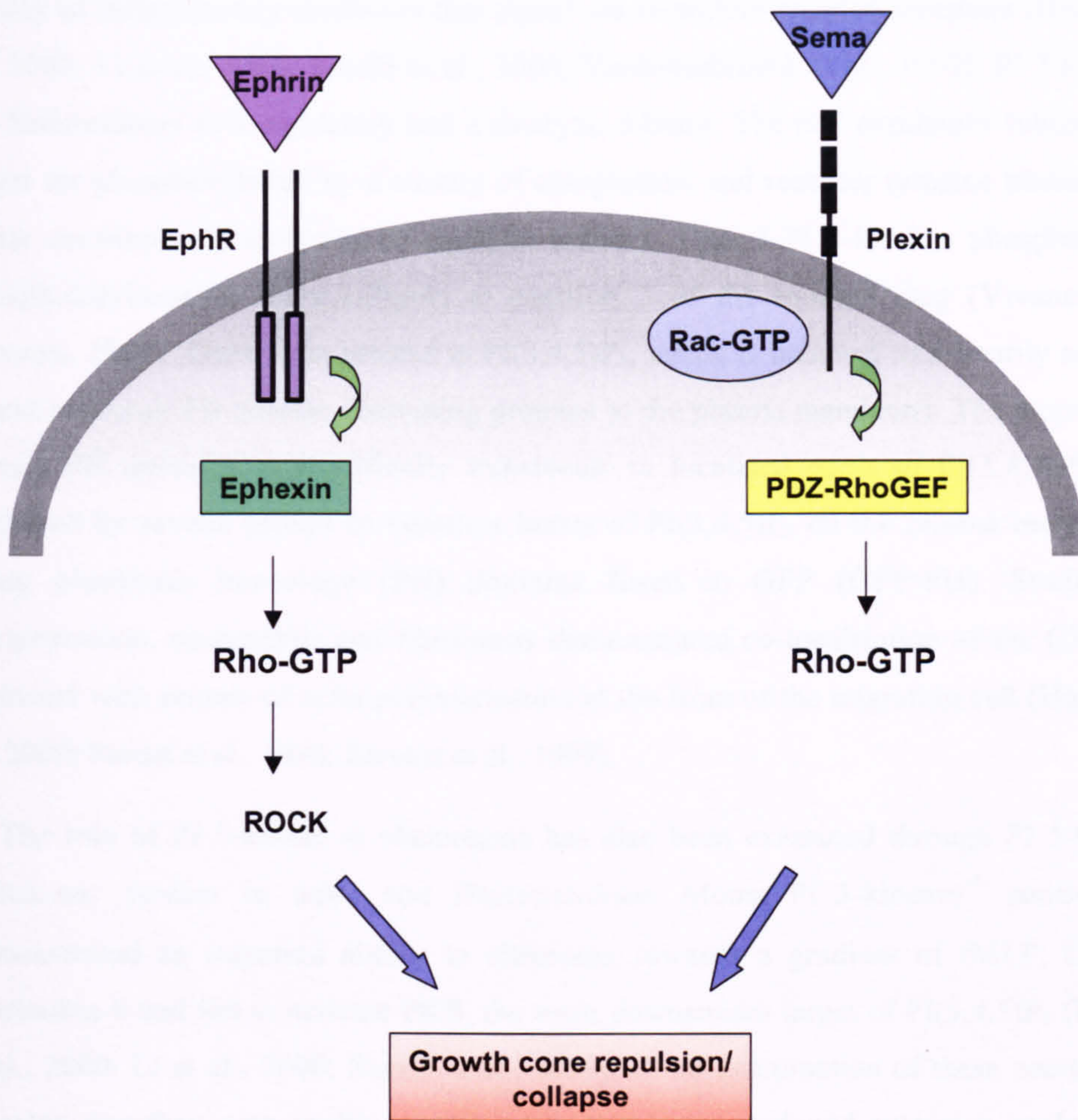
*In vivo* experiments have pointed to the importance of another lipid, sphingosin-1-phosphate (S1-P), in migration processes. In zebrafish, S1-P is required for embryonic myocardial precursor cell migration, while a gene knockout for the S1P receptor EDG1/S1P<sub>1</sub> in mice is embryonic lethal due to a vascular maturation defect because the mural cells, vascular smooth muscle cells and pericytes fail to migrate to the arteries and capillaries (Liu et al., 2000; Pyne and Pyne, 2000). The S1P receptor is a G-protein coupled receptor and in response to S1P, Rac is most likely activated via the heterotrimeric G protein, Gi leading to phosphorylation of p130Cas by the Src-like kinase

Fyn. p130Cas translocates to the plasma membrane and activates Rac (Okamoto et al., 2000a; Okamoto et al., 2000b; Vouret-Craviari et al., 2002). It has also been reported that S1P stimulates endothelial cell migration and cortical actin assembly through activation of PI 3-kinase and subsequently protein kinase B (PKB/Akt), which in turn phosphorylates the S1P receptor EDG1 and promotes Rac activation (Lee et al., 2001).

In addition to attractive cues, there is growing evidence that repulsive cues also play an important role in directing cell migration. This has been studied best in the context of neuronal axon extension and growth cone guidance, which shows many similarities to the protrusive front of a migrating cell, although in this case the cell body does not move. Studies in *C. elegans*, *Drosophila* and *Xenopus* have clearly identified a crucial role for Rho GTPases in these aspects of neuronal behavior, as well as in neuronal cell migration itself (Luo, 2000). Work carried out by many groups has come to the conclusion that Rho acts antagonistically to Rac/Cdc42, the former causing growth cone and axon retraction and the latter causing extension (Kozma et al., 1997). As an example, the ephrins are membrane-bound repulsive ligands for dorsal root ganglion-derived neuronal growth cones that act through the family of Eph tyrosine kinase receptors (Schmucker and Zipursky, 2001). Upon ligand binding, EphA activates Rho through the GEF ephexin and promotes growth cone collapse, in part through activation of its downstream Ser/Thr kinase target, p160ROCK (Figure 1.12) (Shamah et al., 2001; Wahl et al., 2000).

Another family of repulsive growth cone guidance molecules, the semaphorins act through the plexin family of receptors. Here too, activation of Rho appears to be essential for repulsion, though in this case Rac activity is also required (Driessens et al., 2001; Hu et al., 2001). Further analysis has revealed a complicated relationship between the plexin B receptor and GTPases; the cytoplasmic tail of the receptor can interact directly with RacGTP (suggesting it might be a Rac target) and with PDZ-RhoGEF, a Rho-specific GEF (Figure 1.12) (Driessens et al., 2002; Perrot et al., 2002).





**FIGURE 1.12**

Rho GTPases are regulators of growth cone migration. Rho is activated downstream of the repulsive growth cone cues, ephrins and semaphorins. Ephrins bind to their receptor EphR to activate the Rho-GEF ephexin, whereas semaphorins bind to plexins to activate PDZ-Rho GEF. Rac-GTP is also known to bind to the cytoplasmic tail of plexin.

### 1.3.5 PI 3-kinase signaling in cell migration

#### 1.3.5.1 PI(3,4,5)P<sub>3</sub>, a signal for polarised chemotaxis

PI 3-kinases (PI 3-kinases) have been widely implicated in controlling cell migration and polarity [Stephens, 2002 #3198]. During leukocyte chemotaxis, type IA PI 3-kinases are required for lamellipodium extension and migration towards colony-

1 (CSF-1), whereas type IB PI 3-kinases are required for neutrophil chemotaxis to a variety of inflammatory mediators that signal via G-protein coupled receptors (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000; Vanhaesebroeck et al., 1999). PI 3-kinases are heterodimers of a regulatory and a catalytic subunit. The p85 regulatory subunit is a target for phosphorylation by a variety of cytoplasmic and receptor tyrosine kinases and binds constitutively to the p110 catalytic subunit. Type I PI 3-kinases phosphorylate phosphatidylinositol phospholipids at position 3 of the inositol ring (Vivanco and Sawyers, 2002). Their main product is PI(3,4,5)P<sub>3</sub>, which is believed to primarily act as a ligand to recruit PH domain containing proteins to the plasma membrane. The property of certain PH domains to specifically translocate to localised pools of PI(3,4,5)P<sub>3</sub> was exploited by several groups to visualize bursts of PI(3,4,5)P<sub>3</sub> on the plasma membrane, using pleckstrin homology (PH) domains fused to GFP (GFP-PH). Studies in *Dictyostelium*, neutrophils and fibroblasts demonstrated co-localisation of the GFP-PH construct with centers of actin polymerisation at the front of the migrating cell (Haugh et al., 2000; Parent et al., 1998; Servant et al., 1999).

The role of PI 3-kinase in chemotaxis has also been examined through PI 3-kinase knock-out studies in mice and *Dictyostelium*. Mouse PI 3-kinase<sup>-/-</sup> neutrophils demonstrated an impaired ability to chemotax towards a gradient of fMLP, C5a or interleukin-8 and fail to activate PKB, the main downstream target of PI(3,4,5)P<sub>3</sub> (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000). Close examination of these neutrophils revealed that they were unable to efficiently sustain a pseudopod extension exclusively towards the chemotactic gradient. Similarly, *Dictyostelium* cells that had the *PI 3-kinase1* and *PI 3-kinase2* genes inactivated, showed an impaired directionality, migrated more slowly and in a less polarised manner towards a gradient of either cAMP or folate, compared to parent cells (Funamoto et al., 2002; Funamoto et al., 2001). The importance of PI 3-kinases was supported further by studies in macrophages using antibodies raised specifically against class IA PI 3-kinase $\beta$  and PI 3-kinase $\delta$  isoforms and several studies using the PI 3-kinase inhibitors wortmannin and LY294002 (Chung et al., 2001; Sadhu et al., 2003; Vanhaesebroeck et al., 1999).

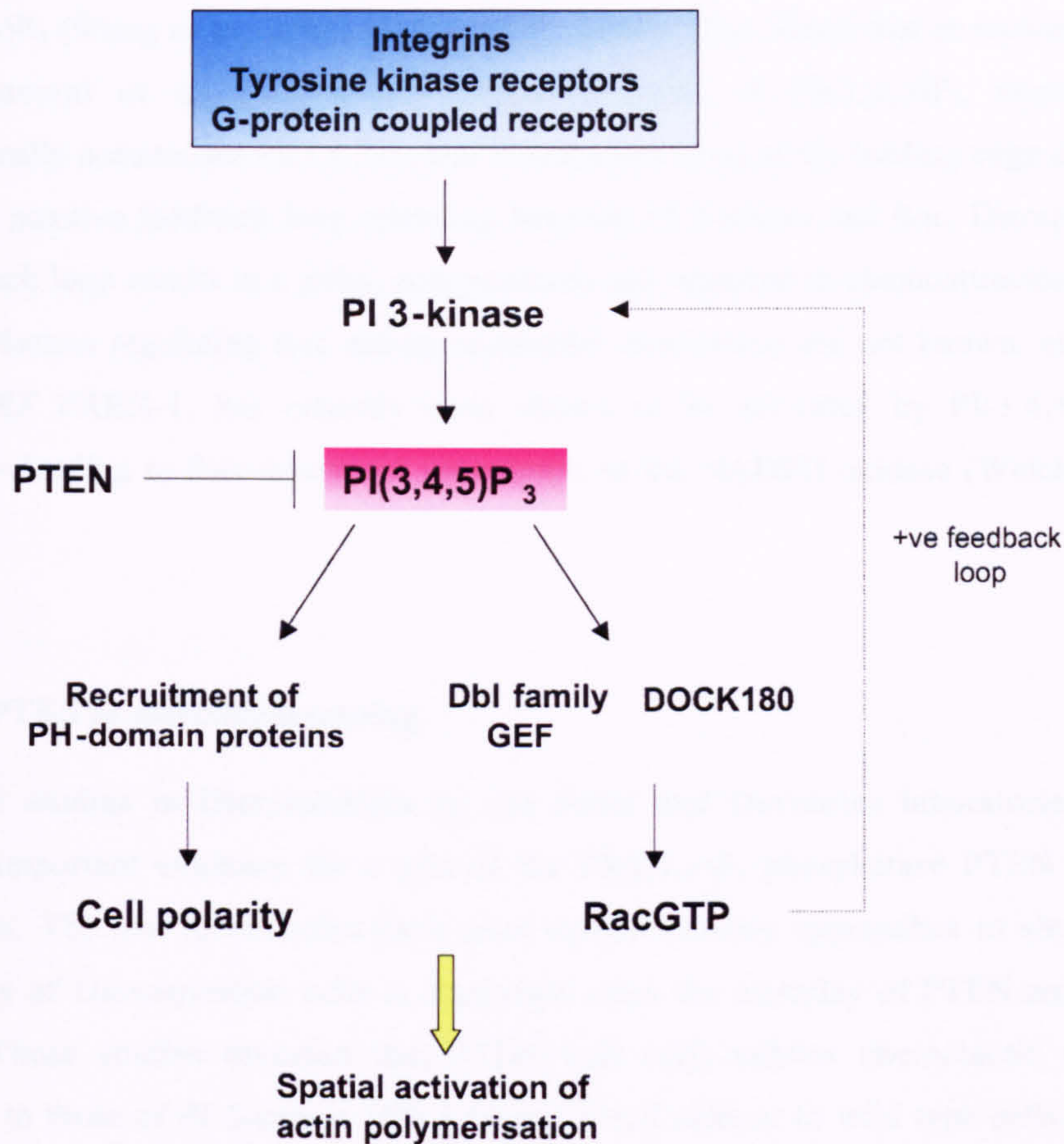
Recent studies in *Dictyostelium* demonstrated that PI 3-kinases are responsible for the establishment and maintenance of the response to a chemotactic gradient through the localised production of PI(3,4,5)P<sub>3</sub> (Funamoto et al., 2002; Iijima and Devreotes, 2002). Funamoto et al., showed that PI 3-kinases rapidly translocate to the plasma membrane in response to the global application of a chemoattractant, and that their localisation is

restricted to the leading edge of migrating cells during chemotaxis. The kinetics of relocalisation of PI 3-kinases closely resembles the relocalisation kinetics of PH domain proteins (Funamoto et al., 2002). Importantly, the same group demonstrated that relocalisation of PI 3-kinases to the plasma membrane is independent of their activation. These results suggest that although PI 3-kinases are key regulators of cell polarity, additional mechanisms that lie upstream of PI3-kinases must be responsible for establishing the initial cell asymmetry, in response to a chemotactic gradient.

### 1.3.5.2 Downstream targets of PI(3,4,5)P<sub>3</sub> signaling

The production of PI(3,4,5)P<sub>3</sub> leads to an increase in GTP-bound Rac in many cell types. Expression of a constitutively activated PI 3-kinase in fibroblasts, for example, generates extensive lamellipodia and membrane ruffling through Rac activation, though interestingly not other Rac-mediated signals such as JNK activation (Reif et al., 1996). The mechanism by which this lipid promotes GTP loading on Rac is thought to be through a direct interaction with Rac GEFs. All members of the Dbl family of GEFs contain a PH domain and at least some of these can bind phospholipids. In Vav, for example, PI(3,4,5)P<sub>3</sub> binding relieves an interaction between the PH and DH (catalytic) domains to stimulate activity, though this is unlikely to be the whole story since tyrosine phosphorylation also occurs (Abe et al., 2000; Han et al., 1998; Liu and Burrige, 2000; Sachdev et al., 2002). Deletion of the PH domain in the GEFs Dbs and Lfc, on the other hand, results in a loss of activity, but this can be restored by addition of a CAAX motif to target the protein to the plasma membrane (Whitehead et al., 1995; Whitehead et al., 1999). In this case, a major role of PI(3,4,5)P<sub>3</sub> is thought to be in inducing membrane translocation. Tiam-1, a Rac specific GEF, provides another example where the PH domain regulates targeting to the plasma membrane as it can be functionally replaced by a myristoylation signal (Michiels et al., 1997). PIX has been shown to be regulated by PI(3,4,5)P<sub>3</sub>. When localised at the front of a migrating cell, PIX activates Rac and promotes actin polymerisation, but it also interacts with the Rac target p65PAK and is thought to regulate focal adhesion turnover (Bagrodia et al., 1999; Koh et al., 2001; Manser et al., 1998; Yoshii et al., 1999). Finally, members of a new class of unconventional GEFs, including the Rac specific GEF, DOCK180/DOCK2, and the Cdc42 specific GEF, DOCK9/zizimin, also contain domains that would allow their regulation through binding to PI(3,4,5)P<sub>3</sub> (Brugnera et al., 2002; Cote and Vuori, 2002). DOCK180 contains a basic domain that has been shown to bind PI(3,4,5)P<sub>3</sub> and zizimin

contains a PH domain (Cote and Vuori, 2002; Meller et al., 2002). Studies in *Drosophila* and lymphocytes have revealed a role for DOCK180 in cell migration and chemotaxis and these effects might be modulated through PI(3,4,5)P<sub>3</sub> targeting and subsequent activation of Rac (Duchek and Rorth, 2001; Duchek et al., 2001).



**FIGURE 1.13**

PI(3,4,5)P<sub>3</sub> is a key regulator of polarised chemotaxis and cell migration. Activation of PI 3-kinase leads to increased levels of cellular PI(3,4,5)P<sub>3</sub>. PI(3,4,5)P<sub>3</sub> regulates the spatial recruitment of PH domain containing proteins involved in cell polarity and chemotaxis. It also activates Rac to induce actin polymerisation required for cell migration, by recruiting and activating Dbl family GEFs and unconventional GEFs like DOCK180. PTEN is a negative regulator of PI(3,4,5)P<sub>3</sub> and hence cell polarity and migration.

The relationship between Rac and PI 3-kinase during cell migration may be more interesting, since the two are able to interact directly with each other and Rac activation stimulates PI 3-kinase leading to the production of PI(3,4,5)P<sub>3</sub> (Benard et al., 1999; Bokoch et al., 1996; Genot et al., 2000; Hawkins et al., 1995; Servant et al., 2000). This would provide an opportunity for a positive feedback loop. To explore this possibility, Bourne and co-workers made use of neutrophils expressing the PH domain of Akt coupled to fluorescent green protein (PH-Akt-GFP) as a probe for the spatial distribution of PI(3,4,5)P<sub>3</sub> (Wang et al., 2002; Weiner et al., 2002). They found that in response to a chemoattractant or an exogenously delivered bolus of PI(3,4,5)P<sub>3</sub>, neutrophils asymmetrically accumulate PI(3,4,5)P<sub>3</sub> and filamentous actin at the leading edge and this involves a positive feedback loop operating between PI 3-kinase and Rac. Disruption of this feedback loop results in a jerky, non-polarised cell response to chemoattractants. The exchange factors regulating Rac during neutrophil chemotaxis are not known, although the RacGEF PREX-1, has recently been shown to be activated by PI(3,4,5)P<sub>3</sub> in neutrophils leading to Rac-dependent stimulation of the NADPH oxidase (Welch et al., 2002).

### 1.3.5.3 PTEN in directional sensing

Elegant studies in *Dictyostelium* by the Firtel and Devreotes laboratories have provided important evidence for a role of the PI(3,4,5)P<sub>3</sub> phosphatase PTEN in cell chemotaxis. The two laboratories have used complementary approaches to show that chemotaxis of *Dictyostelium* cells is dependent upon the interplay of PTEN and PI 3-kinases. These studies revealed that PTEN null cells exhibit chemotactic defects reciprocal to those of PI 3-kinase 1/PI 3-kinase 2 null cells or to wild type cells treated with PI 3-kinase inhibitors. PTEN null cells have numerous filopodia and pseudopodia, move more rapidly and appear to have multiple polarity axes thus exhibiting a slower overall response to the chemoattractant compared to wild type cells (Iijima and Devreotes, 2002). On the other hand, PI 3-kinase 1/PI 3-kinase 2 null cells have a reduced number of pseudopodia compared to wild type cells and had chemotactic defects due to an overall loss in polarity (Funamoto et al., 2002). In addition, PTEN knock-in cells displayed a slower rate of chemotaxis and a decrease in cell polarity.

Both groups observed that PH domain proteins were still able to broadly localise at the front of the migrating cells and the tips of membrane protrusions, suggesting that although polarity is impaired, it is not completely lost. PTEN knock-out cells, however,

although polarity is impaired, it is not completely lost. PTEN knock-out cells, however, had a more sustained accumulation of PH domains at the plasma membrane upon chemoattractant stimulation (Iijima and Devreotes, 2002). Funamoto et al., observed that PTEN had a reciprocal localisation to PI 3-kinase. PTEN transiently translocates from the plasma membrane to the cytosol with kinetics that mirror those of PI 3-kinase relocalisation. In chemotaxing cells, PTEN is localised mostly at the edges and the posterior part of the cells, whereas PI 3-kinase accumulates at the leading edge of the migrating cell, towards the chemotactic gradient (Funamoto et al., 2002). In addition, PTEN null cells, displayed a prolonged accumulation of F-actin relative to wild type cells, suggesting that actin polymerisation is a direct downstream event in PI 3-kinase signaling. Overall these observations demonstrate that PI 3-kinases and PTEN through their antagonistic effects on PI(3,4,5)P<sub>3</sub> are key players in the maintenance, and also possibly in the establishment of cell polarity.

## 1.4 PTEN

### 1.4.1 Discovery of PTEN

PTEN is a tumour suppressor protein that was independently identified by three groups in 1997 (Li and Sun, 1997; Li et al., 1997a; Steck et al., 1997). It maps to the chromosomal region 10q, which had previously been described as the most frequently mutated region in prostate and endometrial cancers and glioblastomas. Two groups, Li et al. and Steck et al. used positional cloning approaches to map the PTEN gene to region 10q23 (Li et al., 1997a; Steck et al., 1997). Sequence analysis revealed a region that was homologous to tensin and auxillin and that coded for a tyrosine-phosphatase domain, hence the name PTEN (phosphatase and tensin homologue deleted on chromosome 10), also known as MMAC1 -for mutated in multiple advanced cancers. A third group, Li and Sun was searching for novel protein phosphatases when they identified TEP1, for transforming growth factor (TGF) – regulated and epithelial cell-enriched phosphatase, which proved to be identical to PTEN. TEP1 was shown to dephosphorylate phosphotyrosyl-RCML, a substrate frequently used to test for *in vitro* tyrosine phosphatase activity. Furthermore, when the critical cysteine residue in the active site was mutated to a serine residue (C124S), a mutation present in Cowden's disease patients, the ability of PTEN to act as a phosphatase was abolished (Li and Sun, 1997). The major physiological substrate of PTEN, however, was later shown to be PI(3,4,5)P<sub>3</sub>, and several

groups demonstrated the ability of PTEN to dephosphorylate position D3 of phosphoinositol phospholipids, with highest affinity for PI(3,4,5)P<sub>3</sub> (Maehama and Dixon, 1998; Myers et al., 1998).

#### 1.4.2 PTEN homologues

The *PTEN* gene is ubiquitously expressed in all eukaryotic cells, and the mammalian gene encodes for a 403 amino acid protein comprised of a phosphatase and a C2 domain (Lee et al., 1999). PTEN orthologues in *Saccharomyces cerevisiae* and *Schizosacharomyces pombe*, however, lack the C2 domain (Figure 1.15) (Heymont et al., 2000; Li et al., 1997b; Sulis and Parsons, 2003). Although a single PTEN gene has been identified in *Xenopus laevi*, *Caenorhabditis elegans* and *Drosophila melanogaster*, several mammalian homologues of PTEN have now been characterised, and these are TPTE, PTEN2 and TPIP (Figure 1.14) (Chen et al., 1999; Marfatia et al., 2000; Walker et al., 2001; Wu et al., 2001). The expression patterns of PTEN2 and TPTE are restricted to the testis, whereas TPIP is expressed in the testis, stomach and brain. In addition to the phosphatase and C2 domains, these PTEN homologues have multiple transmembrane (TM) domains in their amino termini and differ in their subcellular distribution and phosphatase activities with respect to PTEN. In contrast to the cytosolic distribution of PTEN, TPTE is localised to the plasma membrane but lacks any detectable phosphatase activity (Walker et al., 2001). The murine homologue PTEN2 and the human homologue TPIP display similar phosphoinositide-3-phosphate activity to PTEN *in vitro*, but are localised to the Golgi apparatus and the endoplasmic reticulum, respectively (Walker et al., 2001; Wu et al., 2001). It seems, therefore, that there is no functional redundancy of PTEN in the downregulation of cellular PI(3,4,5)P<sub>3</sub> levels, at least at the plasma membrane, which might explain its importance as a tumour suppressor and why it is a key target in tumourigenesis.

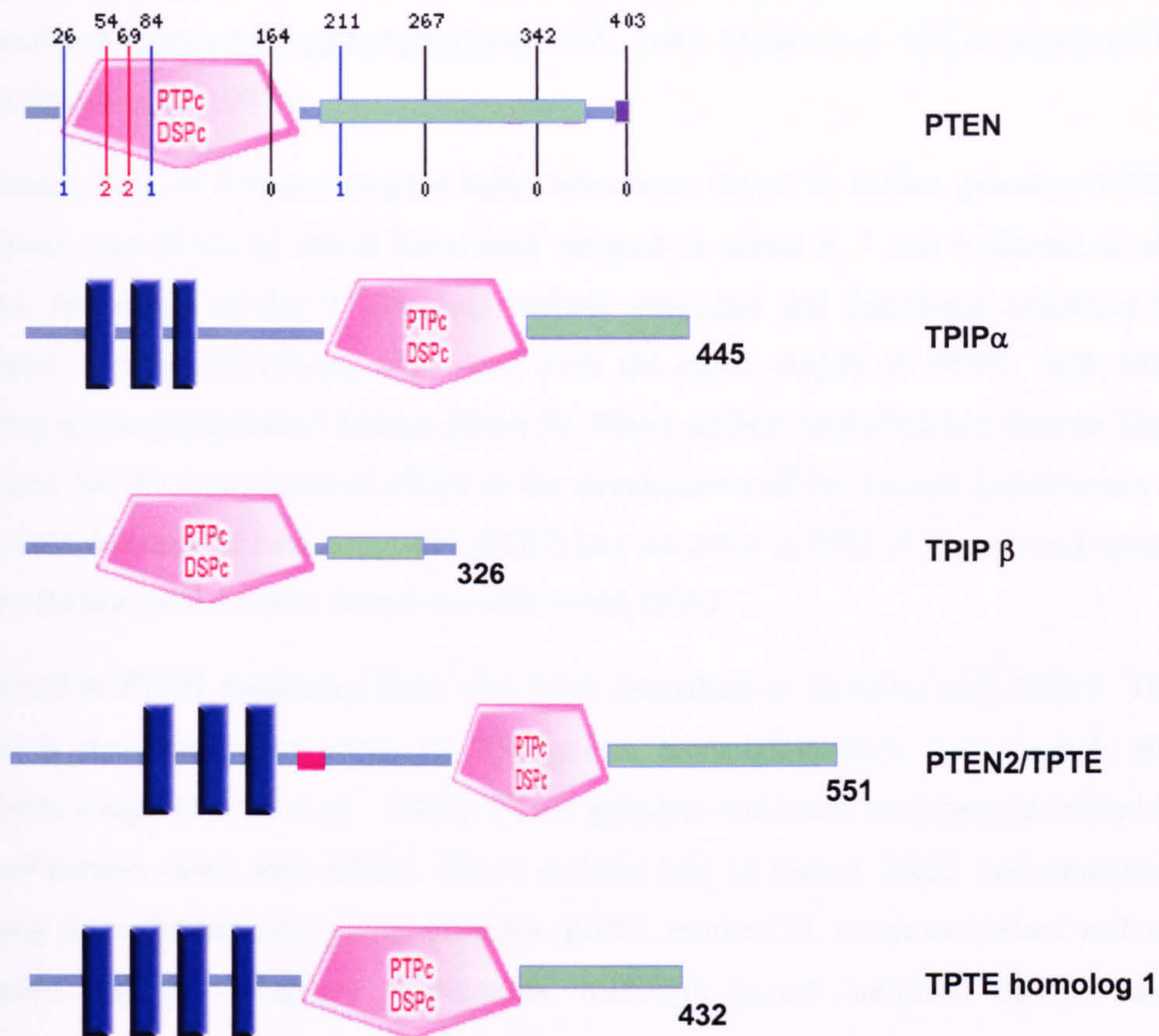


FIGURE 1.14

Domain structure of the mammalian PTEN homologues PTEN2, TPIP, TPTE. The domain predictions were performed using the SMART (Simple Modular Architectural Tool) website and modified according to Walker et al., 2001. Phosphatase domain: pink, C2 domain: green, TM domain: blue, PDZ binding motif: purple.

## 1.4.2 PTEN: a tumour suppressor

### 1.4.3.1 PTEN is the major susceptibility gene in two human hamartoma syndromes

Deletions of the whole or parts of chromosome 10q23 were shown to be associated, at a high frequency with various human sporadic tumours and at least two hamartoma syndromes. *PTEN* is the major susceptibility gene in Cowden disease (CD) and for Bannayan-Riley-Ruvalcaba syndrome (BRRS). Cowden disease is an autosomal dominant familial cancer syndrome with an estimated incidence of more than 1 in 200,000. It is characterised by the development of malignant carcinomas of the breast and



epithelial thyroid glands, as well as the presence of hamartomas of the skin, oral mucosa, intestinal and endometrial epithelium (Liaw et al., 1997; Marsh et al., 1998a; Marsh et al., 1997a; Marsh et al., 1997b).

Around 80% of Cowden disease cases have been found to harbor germline PTEN mutations, two thirds of which have been mapped to exons 5, 7 and 8 (Marsh et al., 1998a). Mutations of the PTEN gene include deletions and insertions, resulting in nonsense or missense events, and occur over the entire length of PTEN, with 43% mapping to the phosphatase domain (exon 5). When patients with Cowden disease were examined for the importance of PTEN in the development of the various hamartomas, it was found that loss of heterozygosity (LOH) had occurred in 25% of thyroid and breast cancers (Gimm et al., 2000a; Simpson and Parsons, 2001).

Germline PTEN mutations have also been described in families with BRRS. The disease is characterised by polyposis, lipomatosis, hemangiomas, macrocephaly and high birth weight (Marsh et al., 1998b). PTEN germline mutations have been identified in 60% of patient cases with BRRS. These include loss of region 10q23 and mutations resulting from chromosome rearrangements. BRRS, unlike CD, is not associated with an increased risk in malignant carcinomas, although breast tumours, lipomas and fibroadenomas have all been associated with the disease. The clinical manifestations of CD and BRRS are very similar and their mutational patterns seem to overlap. They are therefore believed to represent variable penetrance of the same disorder. Thus, the syndromes that harbor PTEN mutations have all been grouped together and referred to as the PTEN Hamartoma Tumour Syndrome (PHTS), and these also include Proteus syndrome (PS) and Lhermitte-Duclos syndrome (LDS) (Gimm et al., 2000a; Waite and Eng, 2002).

#### 1.4.3.2 PTEN is mutated in many sporadic cancers

The importance of PTEN's tumour suppressor function has been highlighted in a range of human sporadic malignancies, including glioblastoma, melanoma, and cancers of the thyroid, breast, endometrium, prostate and ovary (Li et al., 1997a; Steck et al., 1997). The frequency of PTEN mutations and the most likely cause of PTEN loss are summarized in Table 1.1. Interestingly, PTEN inactivation can affect very different stages of tumour progression. In endometrial carcinomas, loss of PTEN expression can occur very early on, even in the premalignant stages of the disease, and appears at highest

frequency in those adenocarcinomas that exhibit a non-aggressive phenotype (Mutter, 2001). The same is also true in some ovarian cancers (Sato et al., 2000). Endometrioid endometrial carcinomas display PTEN mutations in 45% of the cases, of which 61% show complete loss of PTEN expression and more than 90% show reduced protein expression, whereas endometrioid ovarian tumours exhibit PTEN mutations at a frequency of 21%.

<b>Tissue</b>	<b>Tumor type</b>	<b>Frequency of PTEN Inactivation Average/ Range</b>	<b>Primary cause</b>
Brain	Glioblastoma	48% (17-70%)	Mostly LOH
Breast	Ductal carcinoma	37% (15-48%)	Mostly LOH
Endometrium	Endometrioid carcinoma	42% (34-83%)	LOH and mutation
Prostate	Adenocarcinoma	33% (17-41%)	Mostly LOH
Ovary	Cyst adenocarcinoma	33% (6-45%)	LOH and mutation
Skin	Melanoma	33% (32-33%)	Mostly LOH
Thyroid	Carcinoma	37%	Moslty LOH

**TABLE 1.1**

PTEN mutations and deletions in sporadic human cancers. Adapted from Mutter, AJP 2001.

In contrast, melanoma, prostate carcinoma and glioblastoma show loss of PTEN expression in more advanced stages of the disease, usually associated with the metastatic forms of these tumours. PTEN loss most likely occurs before metastasis, however, since in most prostate tumours that have metastasized, PTEN expression is also absent in the primary site. It was found that, in 50% of the prostate tumours studied the region lost was 10q23 and in 10-15% of the cases, PTEN had been inactivated by homozygous deletion. Furthermore, studies examining PTEN expression revealed that the mRNA and protein

levels of PTEN were reduced in 50% of the cases, and that this occurred predominantly in advanced cancers. Similarly, in melanomas, the frequency of PTEN mutations in the primary tumour is much lower than that in which the cancer has metastasized. Examination of 4 primary and 30 metastatic melanomas revealed very low or no expression of PTEN in 65% of the cases (Zhou et al., 2000). Therefore, it is likely that loss of PTEN is associated with progression of these tumours to a more malignant phenotype.

Gliomas that have lost PTEN are primarily the aggressive glioblastomas, and it has been shown that PTEN deletion is not a frequent occurrence among tumours that undergo a progressive increase in malignancy grade, but is rather a characteristic of primary glioblastomas. Indeed, the mutation rate of PTEN in glioblastomas was found to be 44%, whereas in low grade gliomas and astrocytomas PTEN mutations are rare (Duerr et al., 1998). It seems, therefore, that PTEN loss plays an important role in the progression of this and other tumours to a more aggressive phenotype.

#### **1.4.3.3 PTEN controls cell growth, cell cycle arrest and apoptosis**

In order to examine the role of PTEN in development and tumour progression, mice were generated that were homozygous or heterozygous deficient for PTEN. These experiments demonstrated an important role for PTEN during development. Homozygous PTEN deficient mice were not viable and died within embryonic days 6.5 and 9.5, and the embryos appeared disorganised. One group targeted exons 3 to 5 of the PTEN gene, another group targeted exons 4 to 6, whereas a third group targeted exon 5, which coded for the phosphatase domain of PTEN. In all cases the mutant mice showed abnormal patterning with expanded cephalic and caudal regions, and developed a variety of tumours, including prostate endometrium and thyroid carcinomas (Di Cristofano et al., 1998; Podsypanina et al., 1999; Suzuki et al., 1998). Some of the observed neoplasias and hamartomas resembled those observed for CD and BRRS, but these mice also developed a variety of other tumours that were not characteristic of human hamartoma syndromes (Podsypanina et al., 1999).

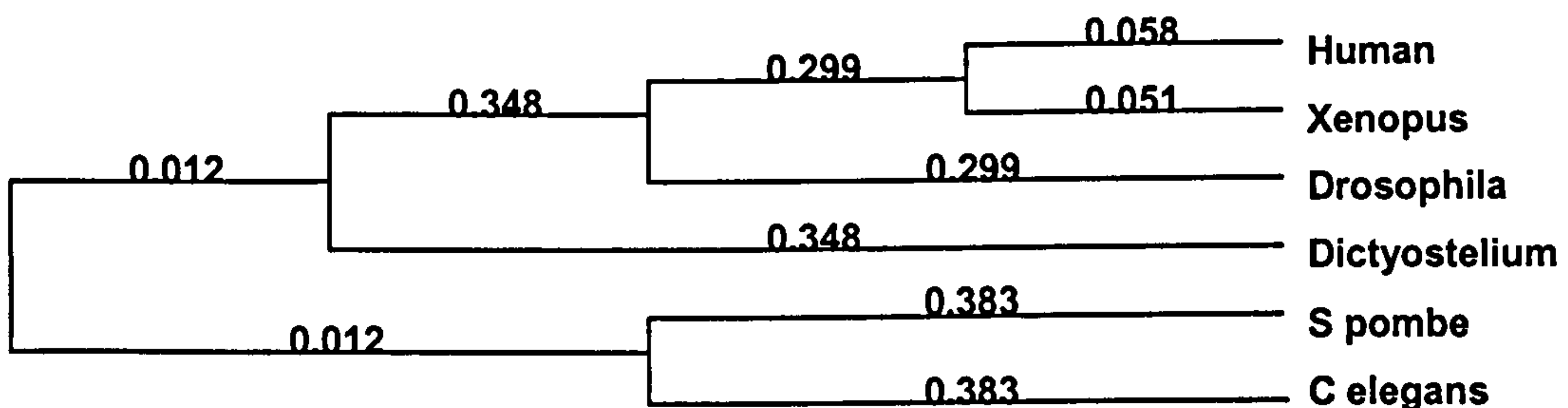
Recently, several groups have used tissue-specific promoters to express Cre recombinase, and conditionally mutate both alleles of PTEN by incorporating lox recombination sites that flank specific PTEN exons. Cre recombination was used to target functionally inactive PTEN in mouse T and B cells, cardiac myocytes, mammary

epithelium cells, primordial germ cells, keratinocytes and neurons (Backman et al., 2001; Crackower et al., 2002; Kwon et al., 2001; Suzuki et al., 2001). Surprisingly, loss of PTEN in these cell types did not induce carcinomas immediately, but after a long follow-up, a subpopulation of PTEN<sup>-/-</sup> cells was observed to transform into malignancies. When Cre-LoxP was used to inactivate PTEN in the mouse brain, mice developed enlarged brains, and in particular, the cerebellum and neuronal cell bodies were almost double the size of their wild type counterparts, thus contributing to the observed brain phenotype. However, no tumours were observed, and depending on which promoter was used to inactivate PTEN, there were significant differences in the observed phenotypes (Morrison, 2002). Groszner et al., used the nestin promoter to delete PTEN throughout the brain, which resulted in an increase in proliferation of neural stem cells and a decrease in cell death of PTEN<sup>-/-</sup> neurons (Groszer et al., 2001). However, when Kwon et al., and Backman et al., used the glial fibrillary acidic protein (GFAP) promoter to induce a more restricted deletion of PTEN and avoid targeting of neuronal stem cells, the levels of proliferation and apoptosis appeared normal (Backman et al., 2001; Kwon et al., 2001). These groups did, however, observe a progressive increase in neuronal soma cell size.

To analyse the role of PTEN more closely in cellular processes such as cell growth and proliferation, mouse embryonic stem cells and fibroblasts lacking PTEN expression were generated. PTEN<sup>-/-</sup> cells exhibited elevated levels of PI(3,4,5)P<sub>3</sub>, phosphorylated-PKB and P70 S6 kinase (S6K). Furthermore, an increased rate in proliferation and a reduced sensitivity to apoptotic stimuli was also observed for PTEN<sup>-/-</sup> T and B cells, neurons, keratinocytes and mammary epithelium. An increase in cell size was also observed in T cells, neurons and cardiac myocytes, which also exhibited defects in contractility (Backman et al., 2001; Crackower et al., 2002). Reintroduction of PTEN in PTEN<sup>-/-</sup> fibroblasts or carcinoma cells restored the cells' ability to respond to apoptotic stimuli and inhibited cell cycle progression in PTEN<sup>-/-</sup> glioblastoma cells (Cheney et al., 1998; Furnari et al., 1997; Li et al., 1998; Stambolic et al., 1998). Most of the effects associated with loss of PTEN appear to be due to the high levels of cellular PI(3,4,5)P<sub>3</sub> generated by these cells. PTEN<sup>+/-</sup> T and B cells were impaired in their ability to undergo Fas-dependent apoptosis, because of the abnormally high levels of phosphorylated active Akt/PKB generated by the elevated levels of cellular PI(3,4,5)P<sub>3</sub>. Accordingly, treatment of these cells with wortmannin restored the ability of these cells to degrade Akt in a caspase-dependent way and to respond to Fas-induced apoptosis (Di Cristofano et al., 1999).

The effect of PTEN has also been examined in several model organisms, including *Drosophila* and *C. elegans*. The *Drosophila* PTEN orthologue, dPTEN shares 44% overall amino acid identity with the mammalian PTEN, with most of sequence similarities lying in the amino terminal half of the protein (Figure 1.15a). Similar to mice, *Drosophila* dPTEN mutants die during embryogenesis. Tissue specific inactivation of dPTEN in the wing and eye revealed PTEN<sup>-/-</sup> clones to be larger than wild type clones, and this was found to be cell autonomous, whereas overexpression of dPTEN during development results in inhibition of cell proliferation (Gao et al., 2000; Huang et al., 1999). Interestingly, overexpression of PTEN in differentiated cells in the eye induces apoptosis, suggesting different roles of PTEN according to the developmental stages (Huang et al., 1999). Finally, a distant *C. elegans* homologue of PTEN, DAF-18, has been shown to control lifespan, and to be an important component of the insulin signaling pathway (Figure 1.15). Deletion of the *daf-18* gene dramatically reduces lifespan and rescues the *daf-2* (the worm PI 3-kinase orthologue) phenotype, which normally promotes longevity (Mihaylova et al., 1999).

**a.**



b.

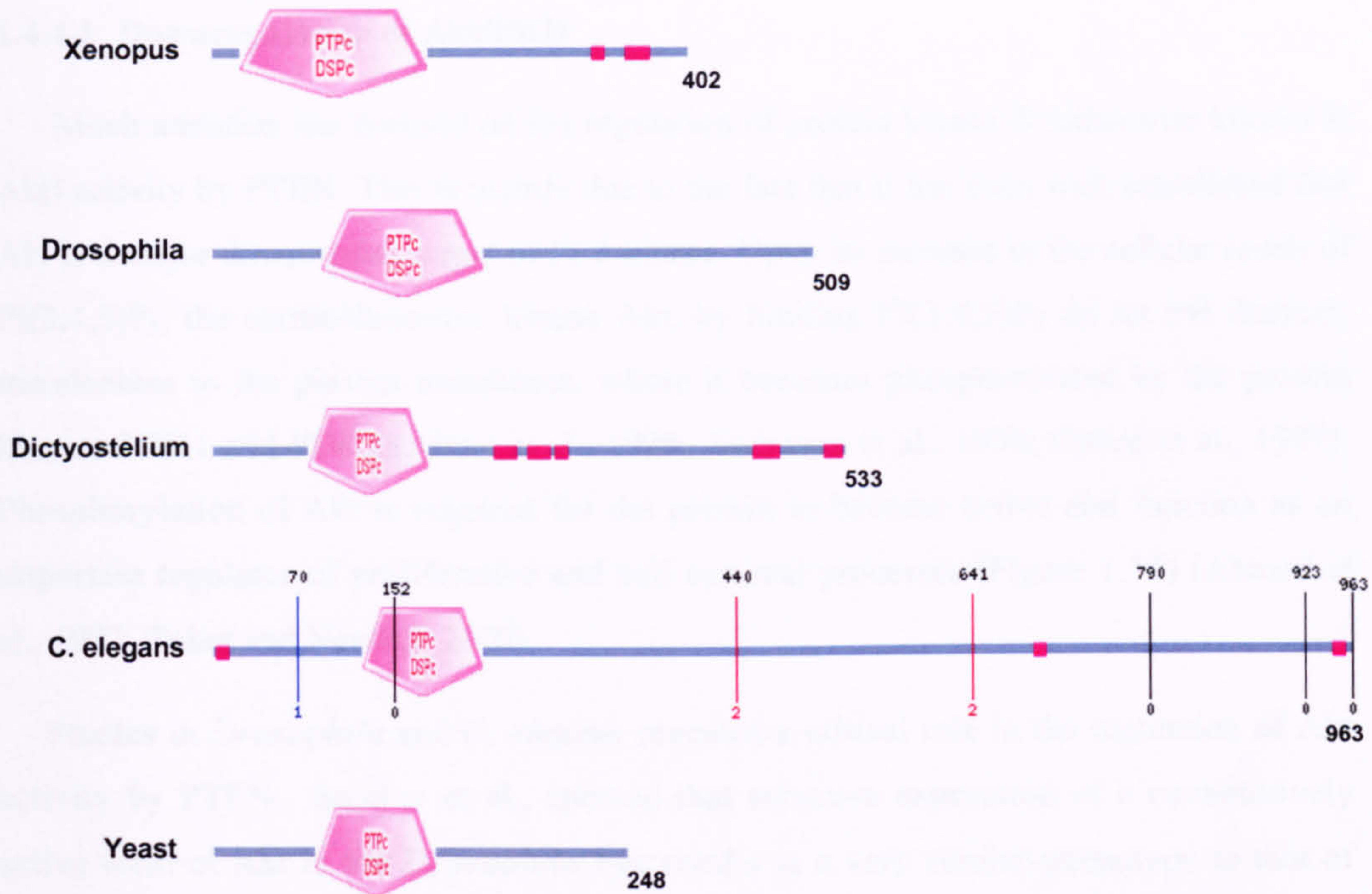


FIGURE 1. 15

PTEN orthologues. (a) Homology tree. Numbers refer to the proportion of differences between sequences. (b) Domain structure predicted using the SMART website. The catalytic domain is depicted with a pink pentagon. Regions of low compositional complexity are depicted as dark pink.

#### 1.4.4 An antagonist to PI 3-kinase

Shortly after the discovery of PTEN and its characterization as an important tumour suppressor belonging to the family of tyrosine phosphatases, work by several groups led to the significant finding that PTEN dephosphorylates  $\text{PI}(3,4,5)\text{P}_3$  both *in vitro* and *in vivo* (Li and Sun, 1997; Maehama and Dixon, 1998; Myers et al., 1998). The isolation of another PTEN mutant (G129E) from tumor and Cowden disease samples that abolished PTEN's phospholipid phosphatase activity, but retained activity against phospho-peptide substrates, strengthened the link between PTEN's tumour suppression function and its lipid phosphatase activity.

#### 1.4.4.1 Downregulation of Akt/PKB

Much attention has focused on the regulation of protein kinase B (otherwise known as Akt) activity by PTEN. This is mainly due to the fact that it has been well established that Akt is a major downstream target of PI 3-kinase. Upon an increase in the cellular levels of PI(3,4,5)P<sub>3</sub>, the serine/threonine kinase Akt, by binding PI(3,4,5)P<sub>3</sub> on its PH domain, translocates to the plasma membrane, where it becomes phosphorylated by the protein kinases PDK1 and PDK2 (Alessi et al., 1998; Anderson et al., 1998; Cohen et al., 1997). Phosphorylation of Akt is required for the protein to become active and function as an important regulator of proliferative and cell survival processes (Figure 1.16) (Ahmed et al., 1997; Toker and Newton, 2000).

Studies in *Drosophila* and *C. elegans* revealed a critical role in the regulation of Akt activity by PTEN. Stocker et al., showed that selective expression of a constitutively active form of Akt in the *Drosophila* eye results in a very similar phenotype to that of PTEN mutant cells, whereas overexpression of a hypomorphic allele of Akt rescues PTEN<sup>-/-</sup> fly embryos from lethality (Stocker et al., 2002). In *C. elegans*, some of the PTEN<sup>-/-</sup> phenotypes are rescued by double stranded RNA interference of Akt1 and Akt2 (Ogg and Ruvkun, 1998). Similarly, studies in mammalian cells have shown that deletion of PTEN results in elevated levels in phospho-Akt. In addition, tumour cell lines that lack PTEN and PTEN null mouse fibroblasts have higher levels of phospho-Akt than PTEN positive cells. Pre-treatment of serum-starved PTEN<sup>-/-</sup> and PTEN<sup>+/-</sup> cells with the PI 3-kinase inhibitor wortmannin abolished the basal and PDGF-induced increase in Akt phosphorylation. Finally, reintroduction of PTEN in these cells lowered the levels of phosphorylated Akt (Davies et al., 1998; Li et al., 1998; Li and Smithgall, 1998; Ramaswamy et al., 1999).

#### 1.4.4.2 Control of cell cycle

Several groups have now shown that PTEN controls the cell cycle by inducing G1 arrest in glioblastoma cells and thyroid carcinoma cells. This correlated with an upregulation in the cell cycle inhibitor p27<sup>KIP1</sup> and a concomitant downregulation in the activities of the G1-dependent cyclin kinases, such as CDK2, cyclin-A and cyclin-E

(Bruni et al., 2000; Cheney et al., 1998; Li et al., 1998). The D cyclins are key regulators of the G1 to S transition, whereas p27 is an inhibitor of the G1 cyclin-dependent kinases. It was later shown that PTEN controls the ubiquitin-dependent degradation of p27 through the ubiquitin E3 ligase SCF<sup>SKP2</sup> complex (Mamillapalli et al., 2001). For PTEN to induce cell cycle arrest, a functional phosphatase activity is required, though using two phosphatase mutants (C124S and G129E), Weng et al. showed that it is the protein phosphatase activity that mediates regulation of cyclin D1 expression. In contrast, the levels of the cyclin-dependent inhibitors p27, p21 and p57 were upregulated downstream of PTEN's lipid-phosphatase activity following a decrease in the levels of active Akt (Weng et al., 2001). More recently, PTEN has been reported to prevent cyclin D1 from localizing to the nucleus during the G1 to S phase transition and this could be rescued by expression of a constitutively active form of Akt (Radu et al., 2003). An interesting mechanism by which PTEN might also control cyclin D1 levels is through the regulation of  $\beta$ -catenin. Recently, a study by Persad et al., revealed that in prostate cancer cells that lack PTEN expression, there is a constitutively high accumulation of  $\beta$ -catenin in the nucleus. The authors propose that PTEN negatively regulates GSK-3 activity by downregulating integrin-linked kinase (ILK) activity, which would stabilize  $\beta$ -catenin to translocate to the nucleus and activate gene transcription (Persad et al., 2000; Persad et al., 2001; Stambolic, 2002).

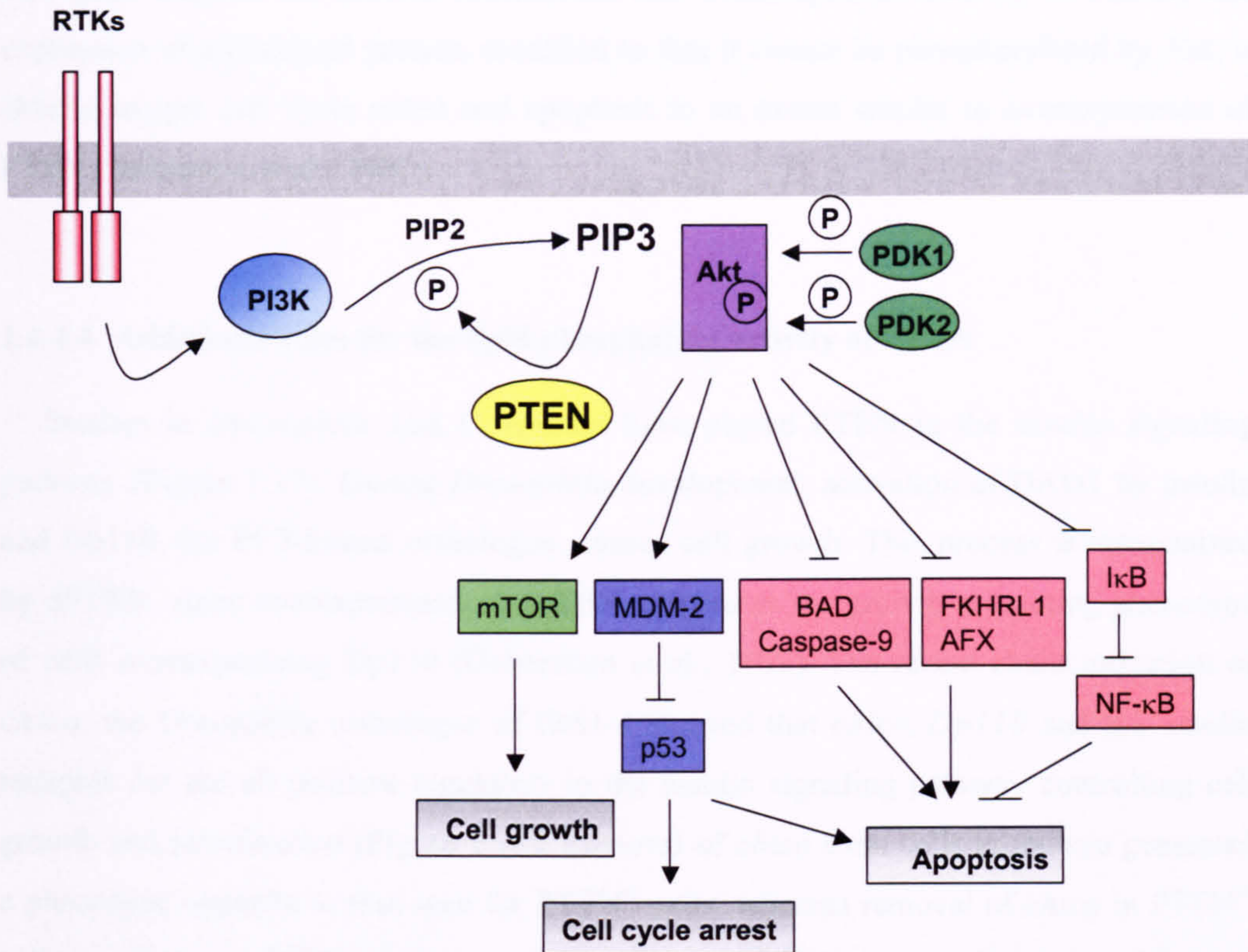
Another way, however, by which PTEN might control cell cycle arrest is through the inhibition of the retinoblastoma protein Rb (Paramio et al., 1999). Paramio et al., showed that PTEN is unable to mediate cell cycle arrest in Rb-deficient cells, an effect rescued by reintroduction of Rb. Moreover, PTEN overexpression inhibits the hyperphosphorylation of Rb and reduces cyclin D1 protein expression, which is restored by coexpression of a constitutively active form of PI 3-kinase. Finally, mice in which Rb had been selectively deleted under the GFAP promoter displayed aberrant proliferation of astrocytes and the development of astrocytomas, a process that was accelerated in a PTEN<sup>+/-</sup> background (Xiao et al., 2002).

#### 1.4.4.3 Regulation of apoptosis

The PI 3-kinase/Akt pathway is an important regulator of apoptosis. Akt has been shown to phosphorylate the proapoptotic protein Bad, a Bcl-2 family member, and the protease Caspase-9. Dephosphorylated Bad inactivates pro-survival signals, such as Bcl-



$X_L$ , and promotes apoptosis.  $PTEN^{-/-}$  ES cells exhibit increased levels of phosphorylated Akt and phosphorylated Bad (Sun et al., 1999). Reintroduction of PTEN in  $PTEN^{-/-}$  glioma cells or mouse embryonic fibroblasts (MEFs) resulted in a reduction in the levels of phosphorylated Akt and in the inhibition of Bad phosphorylation. Interestingly, however, the cells were not induced to undergo apoptosis, but instead they underwent PTEN-dependent anoikis (Davies et al., 1998). Finally, several studies have demonstrated that the PI 3-kinase/Akt pathway provides cell survival signals through the activation of the transcription factor NF- $\kappa$ B. NF- $\kappa$ B is sequestered in the cytosol as an inactive complex bound by its inhibitor I $\kappa$ B. Degradation of I $\kappa$ B allows the translocation of NF- $\kappa$ B to the nucleus where it initiates transcription of target genes. Activation of NF- $\kappa$ B is also regulated by phosphorylation, which upregulates its transactivation potential of the NF- $\kappa$ B subunits (Ghosh et al., 1998; Zandi et al., 1998; Zandi et al., 1997). Recently, PTEN was shown to block the transcriptional activity of NF- $\kappa$ B (Koul et al., 2001b; Mayo et al., 2002). Reintroduction of PTEN in prostate cells inhibited the TNF-stimulated NF- $\kappa$ B-dependent transcription, without affecting I $\kappa$ B degradation, DNA binding or nuclear translocation, but by down-modulating the transactivation potential of NF- $\kappa$ B (Mayo et al., 2002). Another study, however, showed that PTEN blocks TNF or IL-1 induced NF- $\kappa$ B transcriptional activity by affecting its DNA binding (Koul et al., 2001b). These conflicting results might be explained by the differences in the assays or the cells used.

**FIGURE 1.16**

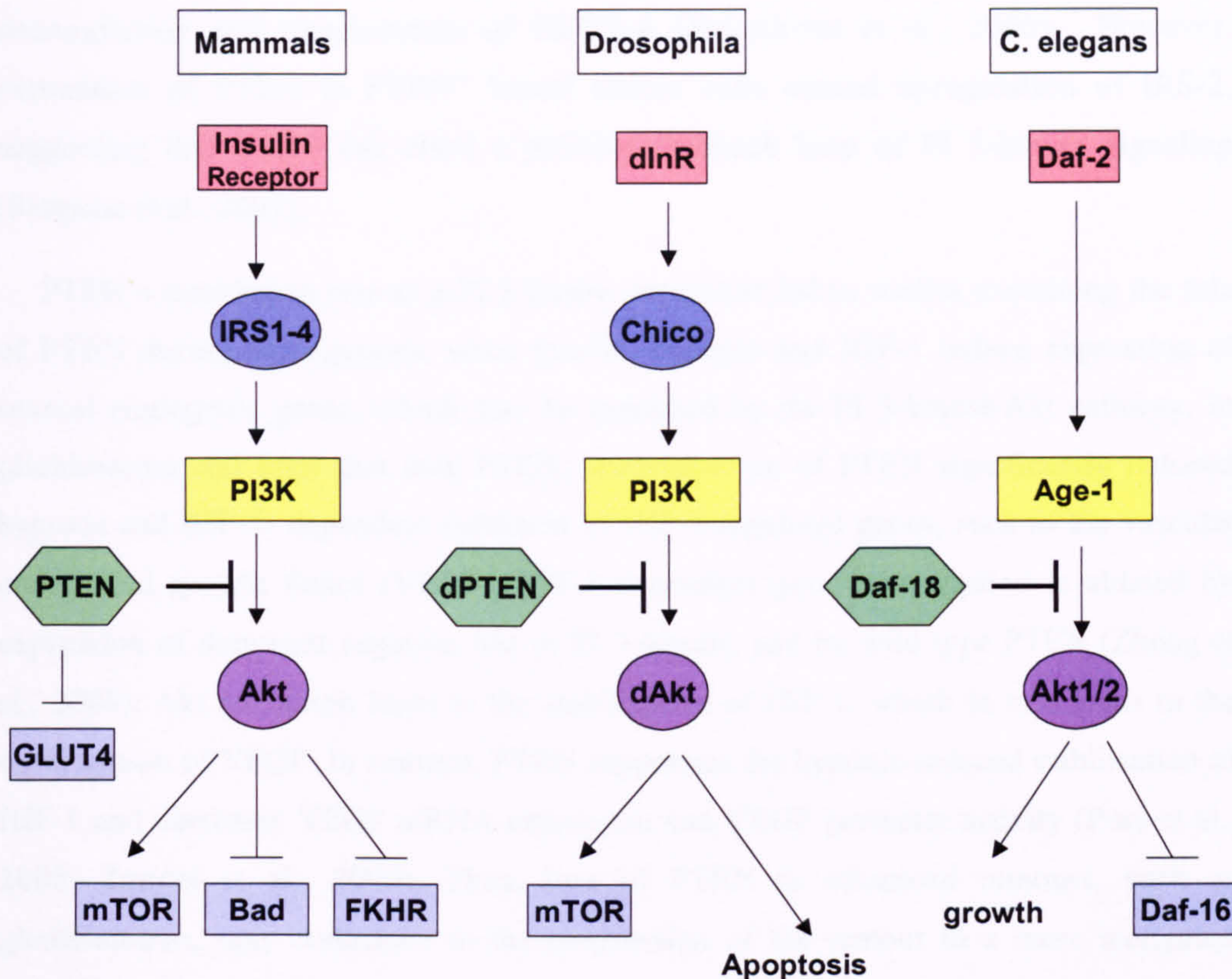
PTEN regulates apoptosis and cell growth by downmodulating phospho-Akt levels. Akt promotes cell growth through the activation of mTOR and the inactivation of p53 by MDM-2. It inhibits apoptosis by negatively regulating apoptotic mediators such as Bad, Caspase-9 and FKHRL1. Concomitantly, it causes the release of NF- $\kappa$ B from its inhibitor I $\kappa$ B to inhibit apoptosis.

Another important substrate for the kinase Akt is the Forkhead family of transcription factors. Dephosphorylated FKHRL1 can translocate to the nucleus and induce transcription of target genes, such as Fas ligand, and trigger apoptosis. Several studies have now shown that Akt promotes cell survival by phosphorylating and inactivating FKHRL1 (Brunet et al., 1999; Tang et al., 1999; Tang et al., 2002). AFX, another forkhead transcription factor family member, is also a target of Akt/PKB phosphorylation (Kops and Burgering, 1999). AFX has been shown to mediate cell cycle arrest by transcriptionally inactivating the cyclin-dependent kinase inhibitor p27<sup>KIP1</sup> (Medema et al., 2000). PTEN, by controlling the levels of active Akt in a cell, could thus regulate both cell cycle arrest and apoptosis (Figure 1.16). Indeed, reintroduction of PTEN into PTEN-

null cells induces the nuclear localisation and transcriptional activity of FKHR, and expression of a Forkhead protein, modified so that it cannot be phosphorylated by Akt, is able to trigger cell cycle arrest and apoptosis to an extent similar to overexpression of PTEN (Nakamura et al., 2000).

#### 1.4.4.4 Additional roles for the lipid phosphatase activity of PTEN

Studies in *Drosophila* and *C. elegans* have placed PTEN in the insulin signaling pathway (Figure 1.17). During *Drosophila* development, activation of DAkt1 by insulin and Dp110, the PI 3-kinase orthologue, causes cell growth. This process is antagonized by dPTEN, since overexpression of dPTEN suppresses the growth-promoting phenotype of cells overexpressing Dp110 (Goberdhan et al., 1999). The recent characterization of *chico*, the *Drosophila* orthologue of IRS1-4 showed that *chico*, *Dp110* and the insulin receptor *Inr* are all positive regulators in the insulin signaling pathway controlling cell growth and proliferation (Figure 1.17). Removal of *chico* from cells in the eye generated a phenotype opposite to that seen for PTEN<sup>-/-</sup> cells, whereas removal of *chico* in PTEN<sup>-/-</sup> cells resulted in a PTEN phenotype, suggesting that PTEN acts to antagonize *chico* and thus confirmed PTEN's role as a negative regulator of the insulin pathway. Overexpression of *Inr* in the eye induces overproliferation and results in lethality. Cooverexpression of dPTEN, however, completely rescued lethality and the overproliferation phenotype (Huang et al., 1999). *C. elegans* studies revealed that DAF-18 (the *C. elegans* PTEN homologue) acts as a negative regulator of the DAF-2 (insulin) and AGE-1 (PI 3-kinase) signaling pathways (Ogg and Ruvkun, 1998; Rouault et al., 1999). DAF-2 and AGE-1 are key regulators of lifespan and dauer formation (a state of hibernation in unfavourable growth conditions) in *C. elegans* (Gil et al., 1999). Deletion of the *daf-2* or *age-1* genes induces dauer formation and dramatically increases lifespan. Deletion of *daf-18*, however, completely suppresses the *daf-2* or *age-1* phenotype (Mihaylova et al., 1999; Paradis et al., 1999).



**FIGURE 1.17**

PTEN's role in the insulin pathway is evolutionarily conserved. In mammals, the insulin pathway regulates growth and apoptosis through the activation of Akt, which activates mTOR to promote growth, and inhibits mediators of apoptosis, like Bad and FKHR. In *Drosophila*, the insulin pathway controls growth and cell proliferation through the activation of chico and dAkt. In *C. elegans*, the insulin pathway homologues Daf-2 and Age-1 regulate longevity and dauer formation. They activate Akt that normally antagonizes the function of Daf-16, a forkhead transcription factor. In mammals, *Drosophila* and *C. elegans*, PTEN acts as negative regulator of the insulin signaling pathway.

Mammalian studies have reinforced the role of PTEN in the insulin pathway (Figure 1.17). IRS-1, IRS-2, IRS-3 and IRS-4 are cellular insulin signaling substrates that become phosphorylated when insulin binds to its receptor (Figure 1.17). Upon tyrosine phosphorylation, these proteins can bind substrates that contain Src homology 2 domains, including PI 3-kinase. Upon activation, PI 3-kinase increases the levels of PI(3,4,5)P<sub>3</sub> in the cell and activates Akt that induces the translocation of the membrane transporter

GLUT4 to the plasma membrane and results in higher glucose uptake. Expression of PTEN in 3T3-L1 adipocytes inhibited the insulin-stimulated production of 2-deoxyglucose and translocation of GLUT-4 (Nakashima et al., 2000). However, expression of PTEN in PTEN<sup>-/-</sup> breast cancer cells caused upregulation of IRS-2, suggesting that PTEN can elicit a positive feedback loop of PI 3-kinase signaling (Simpson et al., 2001).

PTEN's established role as a PI 3-kinase antagonist led to studies examining the role of PTEN during angiogenesis, since insulin, hypoxia and IGF-1 induce expression of several angiogenic genes, which may be regulated by the PI 3-kinase/Akt pathway. In glioblastoma cell lines that lack PTEN, reintroduction of PTEN significantly reduced hypoxia and IGF-1-dependent induction of HIF-1-regulated genes, such as the vascular endothelial growth factor (VEGF). HIF-1-dependent gene transcription is ablated by expression of dominant negative Akt or PI 3-kinase, and by wild type PTEN (Zhong et al., 2000). Akt activation leads to the stabilization of HIF-1, which in turn leads to the upregulation of VEGF. In contrast, PTEN suppresses the hypoxia-induced stabilization of HIF-1 and decreases VEGF mRNA expression and VEGF promoter activity (Pore et al., 2003; Zundel et al., 2000). Thus, loss of PTEN in advanced tumours, such as glioblastomas, may contribute to the progression of the tumour to a more malignant phenotype through the upregulation of HIF-1 gene expression and the induction of angiogenesis.

When PTEN's effect on the regulation of Akt was examined in cells of the immune system, it was shown that it regulates proliferation and activation of T cells, and also plays a role in FC $\gamma$ -receptor phagocytosis. PTEN expression in Jurkat T cells induced apoptosis. This effect could be rescued by coexpression of a membrane-bound constitutively active form of Akt. Similar to the effects seen by inhibiting PI 3-kinase, PTEN expression decreased TCR-activation of the extracellular signal-related kinase 2 (ERK2) (Wang et al., 2000). The T-cell specific PH-domain containing kinase Itk is found constitutively associated with the plasma membrane. PTEN expression, however, resulted in the redistribution of Itk to the cytosol. In addition, PTEN null cells were found to be hyper-responsive to TCR stimulation as they displayed an increased Itk activation and phosphorylation of phospholipase C- $\gamma$ , as well as an increased activation of ERK (Shan et al., 2000). Finally, a study by Kim et al., showed that expression of PTEN completely abrogates phagocytosis of IgG-sensitized red blood cells, and this is dependent on PTEN's lipid phosphatase activity (Kim et al., 2002). These studies indicate

that PTEN, in addition to regulating the insulin pathway and angiogenesis, plays an important role in phagocytosis and in TCR signaling in T cells.

#### 1.4.5 A role for PTEN in cell spreading, migration and invasion

##### 1.4.5.1 PTEN and cell spreading

PTEN's role as a tumour suppressor is thought to be due to its ability to act as a lipid phosphatase and dephosphorylate PI(3,4,5)P<sub>3</sub> and thus antagonize the PI 3-kinase signaling pathway. PTEN, however, has also been shown, at least *in vitro*, to dephosphorylate protein substrates, and this may also contribute to PTEN's tumour suppressor role (Myers et al., 1997). Indeed, several groups have made use of two naturally occurring mutants of PTEN isolated from tumours, to show that PTEN's protein phosphatase activity contributes to a role for PTEN in cell spreading, migration and tumour invasion. Tamura et al., showed that restoring PTEN expression in PTEN<sup>-/-</sup> fibroblasts or glioma cells delays or inhibits integrin-dependent spreading of these cells. This effect seems to be associated with a downregulation in the phosphorylation of the integrin-signaling molecules, focal adhesion kinase (FAK), p130Cas and Shc (Tamura et al., 1998). Using a mutant of PTEN (G129E) that was inactive in its ability to dephosphorylate lipid substrates but still retained activity towards protein substrates and a mutant of PTEN (C124S) that displayed no catalytic activity towards lipid or protein substrates, they showed that the effects on cell spreading were dependent on protein phosphatase activity, perhaps acting through FAK, p130Cas or Shc. In support of this, PTEN directly interacts with and reduces adhesion-dependent tyrosine phosphorylation of FAK (Haier and Nicolson, 2002; Tamura et al., 1998).

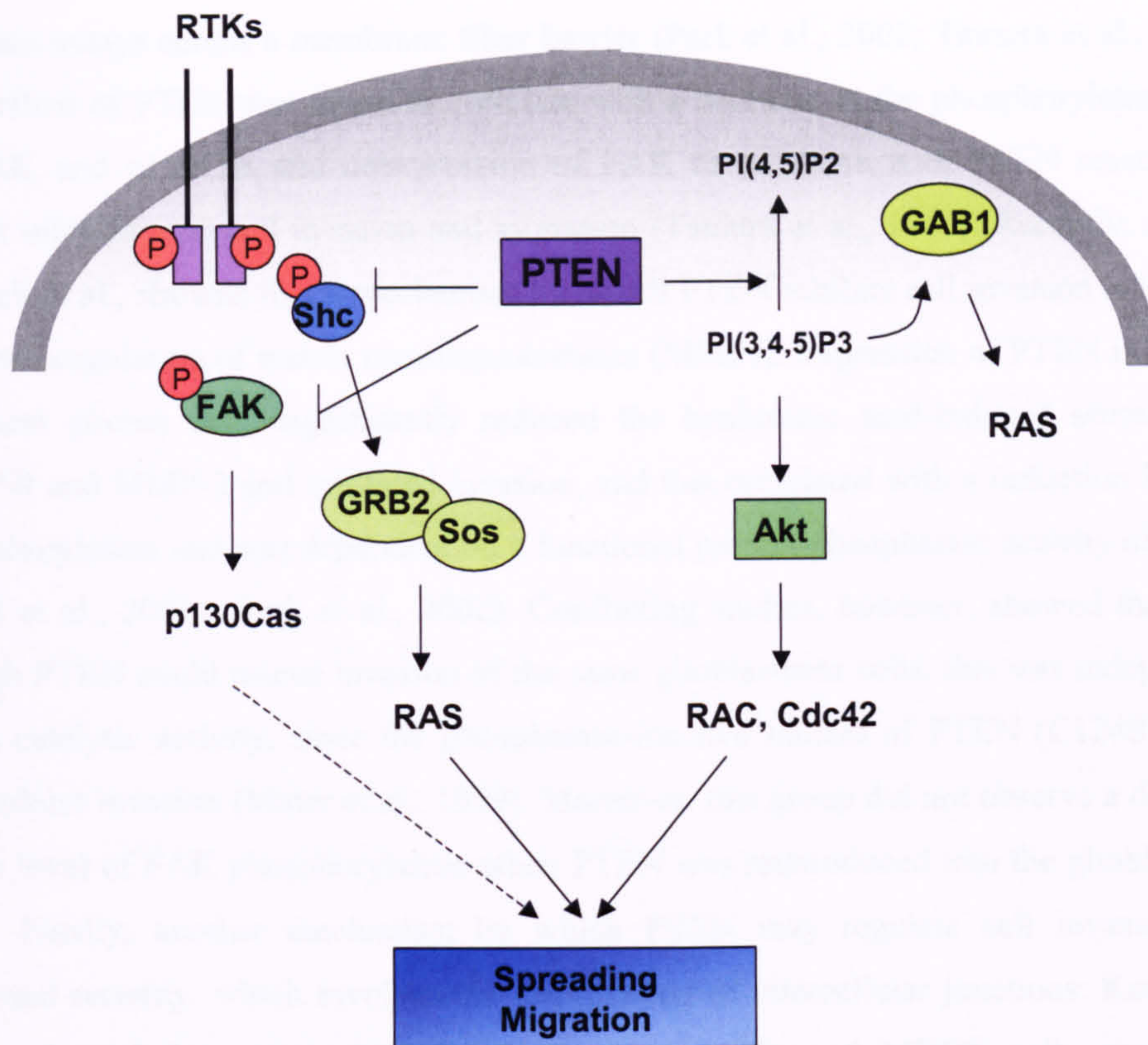
Further investigation by Gu et al., led to the finding that PTEN can downregulate Shc phosphorylation, which inhibits the recruitment of the Grb2/Sos complex, and downregulates the EGF-stimulated activation of the MAPK pathway (Gu et al., 1999). This group proposed two pathways by which PTEN might lead to the inhibition of cell spreading and growth, both dependent on PTEN's protein phosphatase activity. In the first pathway, PTEN downregulates integrin-mediated activation of FAK and Shc and thus inhibits Ras activation and the formation of focal contacts. Dephosphorylation of FAK by PTEN, however does not seem to be universal since it was shown that the phosphorylation status of FAK in PTEN<sup>-/-</sup> ES cells is not higher than that in PTEN<sup>+/+</sup> ES cells (Sun et al., 1999). The second pathway involves the inhibition of EGF or PDGF-

stimulated Shc phosphorylation and recruitment of the Grb2/Sos complex by PTEN, which in turn inhibits activation of Ras and the MAPK signaling pathway. It is likely, however, that the lipid phosphatase activity of PTEN also plays a role in the inhibition of the MAPK pathway since PTEN, by downmodulating the levels of cellular PI(3,4,5)P<sub>3</sub>, can inhibit the translocation of Gab1 to the plasma membrane. Gab1 is an adaptor molecule that has been shown to move to the plasma membrane through the binding of PI(3,4,5)P<sub>3</sub> on its pleckstrin homology domain (PH), recruit Ras, and hence activate the MAPK pathway (Liu and Rohrschneider, 2002).

#### 1.4.5.2 PTEN and cell migration

Several studies have reported an important role for PTEN in cell migration particularly neuronal and glial cell migration (Marino et al., 2002). Furthermore, reexpression of PTEN in PTEN-deficient fibroblast or human glioma cells significantly inhibits their ability to migrate in an *in vitro* wound-healing assay, while inactivation of PTEN by expression of antisense enhances fibroblast cell migration (Tamura et al., 1998). It seems, however, that PTEN can potentially control cell migration through different mechanisms.

In PTEN<sup>-/-</sup> fibroblasts, neither the C124S or G129E mutants could reduce cell motility, suggesting that migration is affected through lipid phosphatase activity. This effect of PTEN seemed to depend on the downregulation of the GTPases Rac and Cdc42, as PTEN<sup>-/-</sup> cells were found to have increased levels of active Rac and Cdc42 compared to PTEN<sup>+/+</sup> cells (Figure 1.18) (Liliental et al., 2000). Interestingly, it has been reported that Rac and Cdc42 can activate Akt and colocalise with Akt at the leading edge of fibroblasts (Higuchi et al., 2001). Expression of a constitutively active form of Akt increased cell motility of fibroblasts, whereas a dominant negative form suppressed cell motility mediated by active Rac and Cdc42. Finally, dominant negative Akt was shown to reduce the enhanced cell motility of PTEN<sup>-/-</sup> mouse embryonic fibroblasts (Higuchi et al., 2001). Thus, it seems that PTEN can also regulate cell motility by downmodulating the levels of cellular phospho-Akt.

**FIGURE 1.18**

PTEN controls cell spreading and migration. PTEN directly dephosphorylates and inactivates FAK and Shc, key mediators of cell spreading and migration. By downmodulating PI(3,4,5)P<sub>3</sub> levels, PTEN also inhibits activation of Rac and Cdc42 by PKB and blocks recruitment of Gab1 to the plasma membrane, where it can activate Ras.

Another mechanism by which PTEN may be regulating cell migration is by regulating the phospho-tyrosine activation of FAK, Shc and p130Cas (Figure 1.18) (Tamura et al., 1999). Work by Gu et al., proposes a mechanism by which PTEN can inhibit both a pathway regulating random cell migration promoted by Shc, MEK and ERK MAP kinase signaling, and a pathway regulating a more persistent, directed cell motility, which depends on FAK and P130Cas and involves extensive cytoskeletal rearrangements and an increase in focal adhesions (Gu et al., 1999).

### 1.4.5.3 PTEN and cell invasion

Importantly, PTEN also exerts its tumour suppressor function by regulating cell invasion. Interestingly, the protein phosphatase activity of PTEN was shown to be required for inhibition of cell invasion of glioblastoma cells, as measured by *in vitro*



invasion assays across a membrane filter barrier (Park et al., 2002; Tamura et al., 1999). This effect of PTEN was found to correlate with a decrease in the phosphorylation state of FAK and p130Cas and coexpression of FAK or p130Cas with PTEN rescued the PTEN inhibition on cell invasion and migration (Tamura et al., 1999). Recently, a study by Park et al., showed that a mechanism by which PTEN inhibits cell invasion is through the downregulation of matrix metalloproteinases (MMPs). Expression of PTEN in PTEN-deficient glioma cells significantly reduced the hyaluronic acid-induced secretion of MMP-9 and MMP-2 and inhibited invasion, and this correlated with a reduction in FAK phosphorylation and was dependent on a functional protein-phosphatase activity of PTEN (Koul et al., 2001a; Park et al., 2002). Conflicting studies, however, showed that even though PTEN could rescue invasion of the same glioblastoma cells, this was independent of its catalytic activity, since the phosphatase-inactive mutant of PTEN (C124S) could also inhibit invasion (Maier et al., 1999). Moreover, this group did not observe a decrease in the level of FAK phosphorylation when PTEN was reintroduced into the glioblastoma cells. Finally, another mechanism by which PTEN may regulate cell invasion was proposed recently, which involves the stabilization of intercellular junctions. Kotelevets et al., showed that expression of PTEN in Src-transformed MDCK cells reverts the morphological transformation of these cells, forces them to aggregate and inhibits cell scattering. PTEN inhibits the invasive phenotype of these cells in an E-cadherin, lipid-phosphatase activity-dependent manner (Kotelevets et al., 2001).

#### 1.4.6 Linking structure to function

PTEN contains a signature motif HCXXGXXR present in the active site of dual specificity and protein tyrosine phosphatases, but aside from this motif, has little sequence homology to these phosphatases. Instead, its amino terminal half was shown to have some similarity to tensin and auxillin. Nevertheless, PTEN was shown to exhibit phosphatase activity *in vitro* against highly acidic phospho-tyrosine, phospho-serine and phospho-threonine containing peptide substrates (Myers et al., 1997). As a result of PTEN needing acidic substrates, Maehama and Dixon were prompted to test phospholipids as potential substrates and found that the protein could dephosphorylate phosphoinositol substrates at the D3 position both *in vitro* and in cells (Maehama and Dixon, 1998; Maehama and Dixon, 1999). This was later confirmed by Myers *et al.*, who linked the ability of PTEN to act on phospholipids with its tumour suppressor function (Myers et al., 1998).

Little was known about the carboxy-terminal half of PTEN until the crystal structure was solved in 1999 (Lee et al., 1999) (Figure 1.19). This revealed that PTEN contained a 170 amino acid C2 domain. C2 domains are independently folding structural modules of about 130 residues, believed to serve a membrane-targeting role (Murray and Honig, 2002; Rizo and Sudhof, 1998). They were first identified in protein kinase C, hence the name C2 for PKC conserved-2, and were shown to bind  $\text{Ca}^{2+}$  and phospholipids. Most C2 domains identified belong to proteins involved in signal transduction (e.g., cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>), phospholipase C (PLC), protein kinase C (PKC), and PI 3-kinase) or in vesicle trafficking (e.g., synaptotagmin and rabphilin-3). C2 domains share little sequence identity, which may explain their functional diversity. They primarily bind membranes in a  $\text{Ca}^{2+}$ -dependent way (e.g., PLC, PI 3-kinase), although there are many C2 domains that bind membranes in a  $\text{Ca}^{2+}$ -independent way (e.g., PKC $\delta,\epsilon$ ) or are involved in protein-protein interactions (e.g., synaptotagmin) (Rizo and Sudhof, 1998).

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**FIGURE 1.19**

Crystal structure of PTEN. Reproduced from Lee et al., 1999. PTEN is composed of an N-terminal catalytic domain and a C-terminal C2 domain. The first 6 residues and the last 50 residues are missing from the crystal structure.

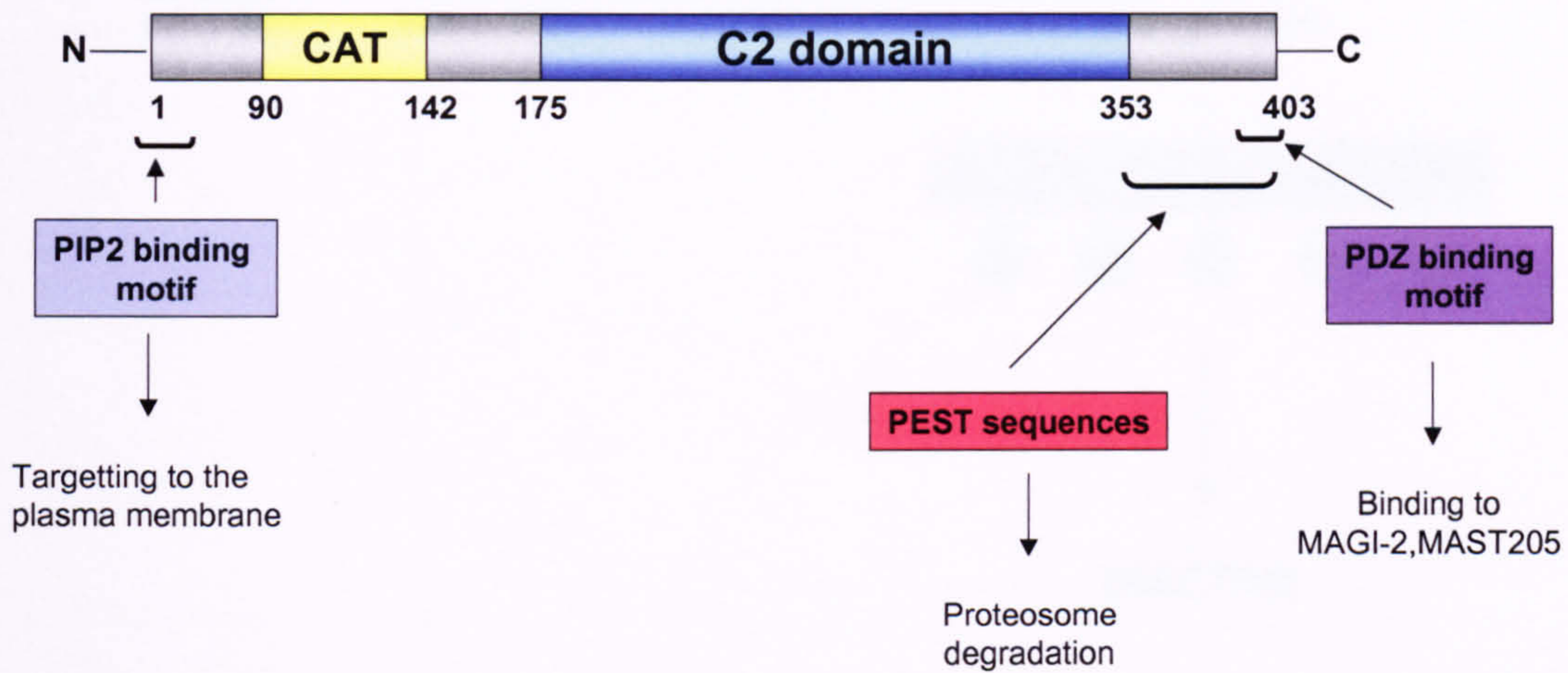
The C2 domain of PTEN is a  $\text{Ca}^{2+}$ -independent domain that binds phospholipids *in vitro*, initially thought to function as a membrane-targeting domain for PTEN (Lee et al., 1999) but in fact, it seems that the catalytic domain of PTEN is more directly involved with membrane binding. The C2 domain is most likely required for a higher affinity interaction with the plasma membrane, possibly to stabilize PTEN and to productively position the catalytic domain (Das et al., 2003; Georgescu et al., 2000). A variety of human tumours have been described with mutations that map to the C2 domain or C-terminus of PTEN and these either completely or partially abrogate PTEN's ability to dephosphorylate inositol-1,3,4,5-tetraphosphate *in vitro*, or have no effect on its catalytic activity (Table 1.2) (Waite and Eng, 2002). Since there are several missense mutations that do not directly target PTEN's phosphatase activity, although they might affect it, this suggests that PTEN's tumour suppressor function is not always dependent on its ability to reduce the levels of cellular PI(3,4,5)P<sub>3</sub>.

Mutation	Phosphatase activity
S227F	20%
G251C	<5%
K289E	60%
D331G	30%
F341V	<5%
K342N	50%
V343E	<5%
L345Q	<5%
F347L	10%
V369G	90%
T401I	130%

**TABLE 1.2**

PTEN mutations that map to the C2 domain. Adapted from Han et al., 2000. Phosphatase activity of PTEN measured against Ins(1,3,4,5)P<sub>4</sub> and results were normalized to wild type PTEN (100%).

In addition to the C2 domain, PTEN contains a PDZ binding motif at its extreme C-terminus. PDZ-binding motifs are short consensus sequence motifs (ITKV) present at carboxy termini of proteins. They bind to PDZ-domains, so named for first being identified in the junctional proteins PSD-95, DLG and ZO-1 (Hung and Sheng, 2002). Yeast-two hybrid studies isolated several potential PTEN binding partners that mapped to the PDZ binding motif of PTEN. These include MAGI-2 (membrane associated guanylate kinase inverted-2) and hDLG (human Disc-large), which belong to the membrane-associated guanylate kinase (MAGUK) family of proteins, and MAST205 (microtubule-associated serine-threonine kinase) (Adey et al., 2000; Wu et al., 2000a; Wu et al., 2000b). MAGI-2 is the only protein so far to have been shown to interact with endogenous PTEN. MAGI-2 enhances PTEN's ability to suppress phospho-Akt levels in a cell, whereas deletion of the PDZ binding motif from PTEN, reduces its ability to do so (Wu et al., 2000a). On the contrary, deleting the PDZ binding motif, by introducing a stop codon, did not abrogate PTEN's ability to act as a lipid phosphatase and lower the levels of cellular phospho-Akt and FKHRL1, nor did it reduce its ability to inhibit anchorage-independent cell growth (Koul et al., 2002; Leslie et al., 2000). It did, however, abolish PTEN's ability to inhibit PDGF-stimulated ruffling of fibroblasts (Leslie et al., 2000). Interestingly, *Drosophila* and *C. elegans* PTEN does not contain a PDZ binding motif but this sequence is highly conserved among the mammalian orthologues. It is likely that PTEN has evolved to control additional signaling pathways that might or might not include PI(3,4,5)P<sub>3</sub> regulation. In several proteins, it has been suggested that phosphorylation of a threonine or serine residue in the PDZ binding motif, regulates its association with PDZ domain-containing proteins. It was shown, with the use of peptides, that phosphorylation of Thr401 in the carboxy terminus of PTEN regulates its interaction with PDZ domain proteins (Adey et al., 2000). Phosphorylation of PTEN on Thr401, however, has not yet been reported. A variety of human tumours have been identified that contain missense mutations in the PDZ binding sequence, including Thr401, and it is therefore likely that the PDZ motif enhances PTEN activity, possibly by more efficient targeting through the binding of PDZ domain proteins. Recently, a PI(4,5)P<sub>2</sub> binding motif was also identified at the extreme N-terminus of PTEN. Studies in *Dictyostelium* showed that it was important for PTEN localisation to the membrane during cell chemotaxis (Iijima and Devreotes, 2002; Iijima et al., 2002).

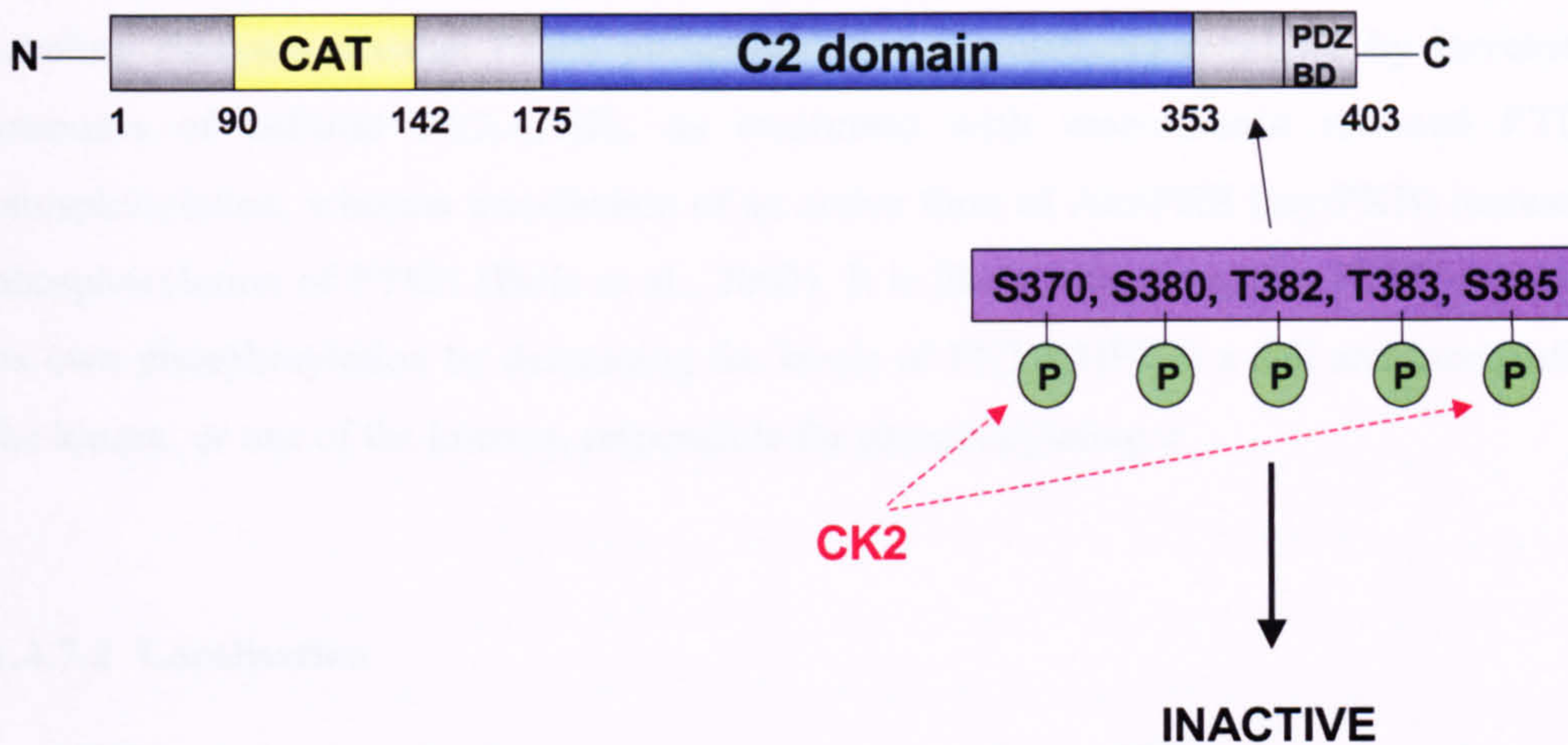
**FIGURE 1.20**

Schematic representation of the PTEN protein structure. In addition to a catalytic domain and a C2 domain, PTEN contains a PDZ binding motif at its extreme C-terminus and a PI(4,5)P<sub>2</sub> binding motif at its extreme N-terminus. Both these motifs are thought to have a targeting role.

## 1.4.7 Regulation of PTEN

### 1.4.7.1 Phosphorylation

PTEN is extensively phosphorylated on a cluster of five residues (three serine and two threonine residues) in its carboxy terminal tail, after the C2 domain (Figure 1.21) (Birle et al., 2002; Tolkacheva et al., 2001; Torres and Pulido, 2001; Vazquez et al., 2000). Radiolabelling experiments of overexpressed or endogenous PTEN with [<sup>32</sup>P] in cells have revealed that PTEN becomes phosphorylated on both serine and threonine residues. Although the extent of phosphorylation of PTEN at individual residues is not yet clear, it appears that PTEN is constitutively phosphorylated in cells. The kinase responsible for phosphorylating each site is unknown, although there has been some *in vitro* evidence to suggest that CK2 phosphorylates PTEN on at least two of the five sites (Torres and Pulido, 2001; Vazquez et al., 2000).

**FIGURE 1.21**

PTEN is extensively phosphorylated on its carboxy terminus. PTEN is primarily phosphorylated on residues S370, S380, T382, T383 and S385. Some of these residues are substrates for casein kinase 2 (CK2). When phosphorylated, PTEN is inactive, as measured by its ability to dephosphorylate phospholipids.

Mutational and deletional analysis has shown that phosphorylation regulates PTEN activity and stability. Mutation of the phosphorylation sites to alanine residues or deletion of the last 50 residues of PTEN seems to make the protein more unstable and prone to degradation, possibly through a proteasome-dependent mechanism (Birle et al., 2002; Tolkacheva et al., 2001; Torres and Pulido, 2001; Vazquez et al., 2000). This agrees with early studies showing that PTEN contains two PEST sequences, which have been known to target proteins for proteolytic degradation, and with studies showing that C-terminally truncated PTEN mutants have reduced expression levels compared to full-length PTEN (Georgescu et al., 1999). On the other hand, substitution of the phosphorylation sites to aspartic or glutamic acid residues (to mimic phosphorylation), reduced phosphatase activity as measured by their ability to induce G1 arrest and transcription of FKHR, but had a higher half-life compared to the wild type protein (Tolkacheva et al., 2001; Vazquez et al., 2000).

Phosphorylation, therefore, seems to stabilize PTEN in an inactive conformation, whereas dephosphorylation activates it. To date the phosphatase responsible for dephosphorylating PTEN remains unknown and until recently, there was no data to suggest any conditions under which the phosphorylation levels of endogenous PTEN would be regulated. Studies by Birle et al., however, have shown that, following polyclonal activation of human blood T lymphocytes, PTEN expression increases and

PTEN becomes phosphorylated on at least one residue. In addition, [<sup>32</sup>P] metabolic labeling data suggest that PTEN phosphorylation is positively regulated by increasing amounts of cellular PI(3,4,5)P<sub>3</sub>, as treatment with wortmannin reduced PTEN phosphorylation, whereas transfection of an active form of Akt/PKB (myrPKB) increased phosphorylation of PTEN (Birle et al., 2002). It is likely, therefore, that PTEN regulates its own phosphorylation by decreasing the levels of PI(3,4,5)P<sub>3</sub> in a cell and inactivating the kinase, or one of the kinases, responsible for phosphorylating it.

#### 1.4.7.2 Localisation

PTEN predominantly localises to the cytosol. Since PTEN has been shown to dephosphorylate PI(3,4,5)P<sub>3</sub> levels at the plasma membrane, it must somehow translocate to the site of PI(3,4,5)P<sub>3</sub> production, in response to stimuli. Recent studies by Das et al., using a PTEN mutant that lacks the last 52 amino acids fused to green fluorescent protein (GFP), demonstrated that PTEN relocates to the plasma membrane and that phosphorylation of the PTEN tail seems to negatively regulate membrane association (Das et al., 2003). In addition, in *Dictyostelium*, expression of GFP-PTEN has been shown to localise uniformly on the plasma membrane and relocate in response to cell chemotaxis (Funamoto et al., 2002). Changes in the subcellular localisation of PTEN have also been identified in several human tumours. In normal follicular thyroid cells, PTEN is mostly found in the nucleus, whereas in thyroid carcinomas, PTEN cytoplasmic staining was stronger than nuclear staining (Gimm et al., 2000b). Similarly, in normal pancreatic islets, PTEN is predominantly nuclear, but in 19 of 23 endocrine pancreatic tumours, it was shown to relocate to the cytoplasm (Perren et al., 2000).

Immunohistochemical studies have demonstrated that PTEN localisation varies among tissues. In neurons, fibroblasts and cells of the thyroid and adrenal medulla, PTEN is predominantly in the nucleus, whereas in epithelial cells of the skin, colon, prostate and breast, most of PTEN is cytoplasmic. It is not yet clear what controls the subcellular distribution of PTEN. Work by Ginn-Pease and Eng in breast cancer cells showed that the level of PTEN in the nucleus varies with the cell cycle. An increased accumulation of PTEN was found associated with the G<sub>0</sub>-G<sub>1</sub> phase, which was significantly reduced in S phase (Ginn-Pease and Eng, 2003). PTEN does not contain any nuclear localisation signals and the mechanism of PTEN accumulation in the nucleus is unknown. Localisation of PTEN in the cytoplasm or the plasma membrane may, however, be regulated by its C-terminus. In polarised MDCK cells, PTEN was shown to localise at

tight junctions, possibly through the binding of the protein MAGI-2 through its PDZ binding motif (Wu et al., 2000a). Whether the specific cellular distribution of PTEN closely correlates with its differing roles as a tumour suppressor, remains to be seen.

### 1.4.7.3 Expression

Although the study of PTEN function has been intense since its discovery in 1997, little is known about the regulation of its expression. PTEN is a ubiquitously expressed gene but its expression pattern varies during development (Gimm et al., 2000a). It has been proposed that one of the causes of PTEN inactivation is promoter methylation and the timing in loss of PTEN expression might correlate with the frequency in the occurrence of certain carcinomas (Baeza et al., 2003; Podsypanina et al., 1999). PTEN has been linked to suppression of tumour growth and loss of PTEN in ES cells results in the gain-of-function for anchorage-independent growth (Di Cristofano et al., 1998). In human glioma and breast cancer cells, expression of PTEN leads to anoikis (cell death due to loss of adhesion) and recently it was shown that loss of cell-matrix interactions increased the expression levels of endogenous PTEN (Koul et al., 2001a; Lu et al., 1999; Tamura et al., 1999; Wu et al., 2002).

Three groups have recently identified key regulators of PTEN transcriptional activation. Virolle et al., demonstrated that PTEN and Egr-1 (early growth response-1) transcription factor expression are upregulated during ultraviolet irradiation. Egr-1 directly binds and transactivates expression of the *PTEN* gene in response to irradiation or etoposide treatment, both *in vitro* and *in vivo* and is required for PTEN-induced apoptosis (Virolle et al., 2001). Mouse embryonic fibroblasts (MEFs) that have either null for PTEN or Egr-1 are resistance to radiation-stimulated apoptosis. Reintroduction of Egr-1 into *egr-1*<sup>-/-</sup> MEFs restored PTEN mRNA transcription and abolished resistance to apoptosis (Virolle et al., 2001).

PPAR $\gamma$  (peroxisome proliferator-activator receptor  $\gamma$ ), is a tumour suppressor gene involved in the regulation of cell growth and proliferation and in anti-inflammatory responses (Kersten et al., 2000; Sarraf et al., 1999). Activation of PPAR $\gamma$  was shown to lead to upregulation in PTEN expression in macrophages, colorectal cancer cells (Caco2) and in breast cancer cells (MCF7). Similarly to Egr-1, PPAR $\gamma$  can directly bind to a genomic sequence upstream of the PTEN promoter and thus activate PTEN transcription. Conversely, disruption of PPAR $\gamma$ -mediated PTEN expression using an antisense



oligonucleotide, reduced the extent of monocyte differentiation into macrophages and decreased the number of macrophages undergoing apoptosis (Patel et al., 2001).

Finally, Stambolic et al., have shown that the tumour suppressor p53 is also responsible, at least in part, in activating PTEN transcription during cell death. p53 directly induces the transactivation of the *PTEN* gene and, in turn, PTEN expression is required to mediate p53-dependent apoptosis, possibly by shutting down the cell survival machinery (Stambolic et al., 2001). The same group, in addition to identifying the p53 DNA binding sequence, uncovered a positive regulatory element upstream of the PTEN promoter that drives constitutive activation of the *PTEN* gene. Very recently, Freeman et al., showed that PTEN also regulates transcription of p53, suggesting that there exists a positive feedback loop between these two very important tumour suppressors (Freeman et al., 2003).

## 1.5 Conclusions

It is clear that PTEN controls many critical cellular functions and therefore merits the attention it has had so far. Significant advances have been made in delineating the PTEN signaling pathways both as a lipid and a protein phosphatase. Several pressing questions, however, still remain. For example, how are the lipid and protein phosphatase activities regulated and under which conditions, if any, does PTEN act as a protein phosphatase *in vivo*? Regulation of PTEN through phosphorylation is a compelling possibility, but little is known about the extent of PTEN phosphorylation *in vivo*, and although CK2 has been proposed as the kinase, no antagonizing phosphatase has been discovered so far. Even though the basic concepts of PTEN structure and function have been established, it is not entirely clear what the role of the C2 domain is. In addition, mammalian PTEN contains a PDZ binding motif that has been shown to bind to several PDZ-domain containing proteins, but the significance of this is not known.

This thesis addresses PTEN's function in glioma cell migration and spreading and identifies a new role for its C2 domain.

## Chapter 2

### Material and Methods

#### 2.1 Molecular Biology

##### 2.1.1 Polymerase chain reactions

PCR reactions were carried out in a reaction volume of 100 $\mu$ l with 100ng of DNA template, 50pmol of each primer, 10mM dNTPs and with 1 $\mu$ l of High fidelity PCR reaction system (3.5U/ $\mu$ l, ROCHE), in the provided Mg<sup>2+</sup>- containing buffer. Cycling conditions were as follows: 1 cycle at 94°C for 3 min, followed by 30 cycles at [94°C for 1 min, 52°C for 2 min and 72°C for 3 min] and 1 cycle at 72°C for 10 min. All primers were ordered from MWG-Biotech, unless otherwise indicated.

##### 2.1.2 DNA constructs

cDNA constructs used in this study are described in table 2.1. Where necessary, constructs were verified by sequencing (CYTOMYX).

Plasmids	Characteristics and Source
pRK5myc::N17Rac	Encodes for dominant negative Rac, Hall lab.
pRK5myc::L61Rac	Encodes for constitutively active Rac, Hall lab.

pRK5myc::N17Cdc42	Encodes for dominant negative Cdc42, Hall lab.
pRK5myc::L61Cdc42	Encodes for constitutively active Cdc42, Hall lab.
pCGN-HA::PTENwt	Encodes for full-length wt PTEN, a gift from N. Tonks (Myers et al., PNAS 1998).
pCGN-HA::PTEN (C124S)	Encodes for full-length PTEN (C124S), a gift from N. Tonks (Myers et al., PNAS 1998).
pCGN-HA::PTEN (G129E)	Encodes for full-length PTEN (G129E), a gift from N. Tonks (Myers et al., PNAS 1998)
pRK5myc::PTENwt	Encodes for full-length wt PTEN. Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc. Template: pCGN-HA::PTENwt  Primer 1: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3' Primer 2: 5' GCCGAATTCCTCAGACTTTTGTAATTTGTG 3'
pRK5myc::PTEN (C124S)	Encodes for full-length PTEN, with a point mutation in the catalytic domain at C124. Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc. Template: pCGN-HA::PTEN (C124S)  Primer 1: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3' Primer 2: 5' GCCGAATTCCTCAGACTTTTGTAATTTGTG 3'
pRK5myc::PTEN (G129E)	Encodes for full-length PTEN, with a point mutation in the catalytic domain at G129. Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc. Template: pCGN-HA::PTEN (G129E)  Primer 1: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3' Primer 2: 5' GCCGAATTCCTCAGACTTTTGTAATTTGTG 3'
pRK5flag::PTEN $\Delta$ C	Encodes for the N-terminal domain of PTEN (residues 1- 179). Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5flag.  Primer 1: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3' Primer 2: 5'GCGAATTCCTCAGCTATAATAATACACATAGCG 3'

pRK5flag::PTEN $\Delta$ N	<p>Encodes for the C-terminus of PTEN lacking the PI(4,5)P2 binding motif (residues 86-403). Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5flag.</p> <p>Primer 1: 5' GCAGGGATCCGCACAATATCCTTTTGAAGAC 3'</p> <p>Primer 2: 5' GCCGAATTCTCAGACTTTTGTAATTTGTG 3'</p>
pRK5myc::PTEN $\Delta$ CAT	<p>Encodes for the C-terminal domain of PTEN (residues 179-403). Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc.</p> <p>Primer 1: 5' GCGGGATCCAGCTACCTGTAAAGAATCAT 3'</p> <p>Primer 2: 5' GCCGAATTCTCAGACTTTTGTAATTTGTG 3'</p>
pRK5myc::PTEN $\Delta$ PDZBM	<p>Encodes for full-length wt PTEN lacking the PDZ binding motif (residues 1- 394). Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc.</p> <p>Primer 1: 5'GCAGGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 2: 5' GC GAATTCTCATT CATCAA 3'</p>
pRK5myc::PTEN $\Delta$ TAIL	<p>Encodes for full-length wt PTEN lacking the last 50 amino acids (residues 1- 354). Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc.</p> <p>Primer 1: 5'GCAGGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 2: 5' GC GAATTCTCATGACGGCTC 3'</p>
pRK5myc::PTEN $\Delta$ CAT $\Delta$ PDZBM	<p>Encodes for PTEN<math>\Delta</math>CAT minus the PDZ binding motif (residues 179-394). Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc.</p> <p>Primer 1: 5' GCGGGATCCAGCTACCTGTAAAGAATCAT 3'</p> <p>Primer 2: 5' GC GAATTCTCATTCATCAA 3'</p>
pRK5myc::PTEN-C2	<p>Encodes for the C2 domain of PTEN (residues 179 – 353). Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc.</p> <p>Primer 1: 5' GCGGGATCCAGCTACCTGTAAAGAATCAT 3'</p> <p>Primer 2: 5' GC GAATTCTCATGACGGCTC 3'</p>

pRK5myc::Syt-C2A	<p>Encodes for the C2A domain of Synaptotagmin (a gift from Dr. Dan Cutler) (residues 80-182). Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc. Primers were purchased from SIGMA Genosys.</p> <p>Primer 1: 5'GCGGATCCCTGGATTATGATTTCCAGAATAAC 3'</p> <p>Primer 2: 5'GCGAATTC TCACTCTTCCTTCTCTGCACTTT GCAG 3'</p>
PRK5myc::Syt-C2B	<p>Encodes for the C2B domain of Synaptotagmin (a gift from Dr. Dan Cutler) (residues 211-325). Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc.</p> <p>Primer 1: 5' GCGGATCCCTTCGCTATGTACCTACTGCTGGC 3'</p> <p>Primer 2: 5'GCGAATTCTCACTCCTCTACCTGCAGGGTGT GCCA 3'</p>
pRK5flag::PTEN (C124S) $\Delta$ C	<p>Encodes for PTEN<math>\Delta</math>C with a point mutation in the catalytic domain at C124 (residues 1- 179). Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5flag.</p> <p>Primer 1: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 2: 5'GCGAATTC TCAGCTATAATAATACACATAGCG 3'</p>
pRK5flag::PTEN (C124S)-(86-147)	<p>Encodes for the active site of PTEN with a point mutation at C124 (residues 86 – 147). Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5flag.</p> <p>Primer 1: 5' GCAGGATCCGCACAATATCCTTTTGAAGAC 3'</p> <p>Primer 2: 5'GCGAATTC TCACTTTAAAAATTTGCCCCGATG 3'</p>
pRK5myc::PTEN (C124S)-A4	<p>Encodes for PTEN (C124S) with point mutations at S380, T382, T383, S385 to alanine residues. Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc.</p> <p>Primer 1: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 2: 5'GCGAATTC TCAGACTTTTGTAATTTGTGTATGCTG ATCTTCATCAAAGGTTTCATTCTCTGGATCAGCGTCAGCGGCG TCAGCATATCTATAATG 3'</p>

pRK5myc::PTEN (C124S)-S380A	<p>Encodes for PTEN (C124S) with a point mutation at S380 to an alanine residue. Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc.</p> <p>Primer 1: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 2: 5'GCGAATTC TCAGACTTTTGTAATTTGTGTATGCTG ATCTTCATCAAAGGTTTCATTCTCTGGATCAGAGTCAGTGGTG TCAGCATATCTATAATG 3'</p>
pRK5myc::PTEN (C124S)-T382A	<p>Encodes for PTEN (C124S) with a point mutation at T382 to an alanine residue. Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc.</p> <p>Primer 1: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 2: 5'GCGAATTC TCAGACTTTTGTAATTTGTGTATGCTG ATCTTCATCAAAGGTTTCATTCTCTGGATCAGAGTCAGTGGCG TCAGAATATCTATAATG 3'</p>
pRK5myc::PTEN (C124S)-T383A	<p>Encodes for PTEN (C124S) with a point mutation at T383 to an alanine residue. Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc.</p> <p>Primer 1: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 2: 5'GCGAATTC TCAGACTTTTGTAATTTGTGTATGCTG ATCTTCATCAAAGGTTTCATTCTCTGGATCAGAGTCAGCGGTG TCAGAATATCTATAATG 3'</p>
pRK5myc::PTEN (C124S)-S385A	<p>Encodes for PTEN (C124S) with a point mutation at S385 to an alanine residue. Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc.</p> <p>Primer 1: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 2: 5'GCGAATTC TCAGACTTTTGTAATTTGTGTATGCTG ATCTTCATCAAAGGTTTCATTCTCTGGATCAGCGTCAGTGGTG TCAGAATATCTATAATG 3'</p>

pRK5myc::PTEN $\Delta$ CAT-A4	<p>Encodes for PTEN<math>\Delta</math>CAT with point mutations at S380, T382, T383, S385 to alanine residues. Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc.</p> <p>Primer 1: 5' GCGGGATCCAGCTACCTGTTAAAGAATCAT 3'</p> <p>Primer 2: 5'GCGAATTCAGACTTTTGTAATTTGTGTATGCTG ATCTTCATCAAAAGGTTTCATTCTCTGGATCAGCGTCAGCGGCG TCAGCATATCTATAATG 3'</p>
pRK5myc::PTEN $\Delta$ CAT-DDTD	<p>Encodes PTEN<math>\Delta</math>CAT with point mutations at S380, T382, S385 to aspartic acid residues. Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc.</p> <p>Primer 1: 5' GCGGGATCCAGCTACCTGTTAAAGAATCAT 3'</p> <p>Primer 2: 5' GCGAATTCAGACTTTTGTAATTTGTGTATGCT GATCTTCATCAAAAGGTTTCATTCTCTGGATCATCGTCAGTG TCGTCATCATATCTATAATG 3'</p>
pRK5myc::PTENwt-T383D	<p>Encodes for full-length wt PTEN with a point mutation at T383 to an aspartic acid residue. Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc. A gift from Annette Self. Primer 2 purchased from SIGMA Genosys.</p> <p>Primer 1: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 2: 5'GCGAATTCAGACTTTTGTAATTTGTGTATGCTG ATCTTCATCAAAAGGTTTCATTCTCTGGATCAGAGTCATCGGTG TCAGAATA 3'</p>
pRK5myc::PTEN $\Delta$ CAT-T383D	<p>Encodes PTEN<math>\Delta</math>CAT with a point mutation at T383 to an aspartic acid residue. Made by PCR and subcloned as a BamHI/EcoRI fragment into pRK5myc. A gift from Annette Self. Primer 2 purchased from SIGMA Genosys.</p> <p>Primer 1: 5' GCGGGATCCAGCTACCTGTTAAAGAATCAT 3'</p> <p>Primer 2: 5'GCGAATTCAGACTTTTGTAATTTGTGTATGCTG ATCTTCATCAAAAGGTTTCATTCTCTGGATCAGAGTCATCGGTG TCAGAATA 3'</p>

pRK5myc::PTENwt-D4	<p>Encodes for full-length wt PTEN with point mutations at S370, S380, T382, S385 to aspartic acid residues. Made by overlapping PCR using as template pRK5myc::PTENwt-D3, and subcloned as a BamHI/EcoRI fragment into pRK5myc. A gift from Sarah Nicholls. Primers 2, 4 and 6 purchased from SIGMA Genosys.</p> <p><u>PCR1:</u></p> <p>Primer 1: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 2: 5' CAGGTTTCATTGTCAGAAACATCTGGTGTTAC 3'</p> <p><u>PCR2:</u></p> <p>Primer 3: 5' GTAACACCAGATGTTGATGACAATGAACCTG 3'</p> <p>Primer 4: 5' GCCTCAGAAGGTACCTAAC 3'</p> <p><u>PCR3:</u> Template; products of PCR1 and PCR2.</p> <p>Primer 5: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 6: 5' GCCTCAGAAGGTACCTAAC 3'</p>
pRK5myc::PTEN (C124S)-D4	<p>Encodes for PTEN (C124S) with point mutations at S370, S380, T382, S385 to aspartic acid residues. Made by overlapping PCR using as template pRK5myc::PTEN(C124S)-D3, and subcloned as a BamHI/EcoRI fragment into pRK5myc. A gift from Sarah Nicholls. Primers 2, 4 and 6 purchased from SIGMA Genosys.</p> <p><u>PCR1:</u></p> <p>Primer 1: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 2: 5' CAGGTTTCATTGTCAGAAACATCTGGTGTTAC 3'</p> <p><u>PCR2:</u></p> <p>Primer 3: 5' GTAACACCAGATGTTGATGACAATGAACCTG 3'</p> <p>Primer 4: 5' GCCTCAGAAGGTACCTAAC 3'</p> <p><u>PCR3:</u> Template; products of PCR1 and PCR2.</p> <p>Primer 5: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 6: 5' GCCTCAGAAGGTACCTAAC 3'</p>



<p>pRK5myc::PTENwt-S370D-A4</p>	<p>Encodes for full-length wt PTEN with point mutations at S370 to an aspartic acid residue and S380, T382, T383, S385 to alanine residues. Made by overlapping PCR using as template pRK5myc::PTENwt (for PCR1) and pRK5::PTEN(C124S)-A4 (for PCR2), and subcloned as a BamHI/EcoRI fragment into pRK5myc. A gift from Sarah Nicholls.</p> <p>Primers 2, 4 and 6 purchased from SIGMA Genosys.</p> <p><u>PCR1:</u></p> <p>Primer 1: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 2: 5' CAGGTTTCATTGTCAGAAACATCTGGTGTTAC 3'</p> <p><u>PCR2:</u></p> <p>Primer 3: 5' GTAACACCAGATGTTGATGACAATGAACCTG 3'</p> <p>Primer 4: 5' GCCTCAGAAGGTACCTAAC 3'</p> <p><u>PCR3:</u> Template; products of PCR1 and PCR2.</p> <p>Primer 5: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 6: 5' GCCTCAGAAGGTACCTAAC 3'</p>
<p>pRK5myc::PTEN (C124S)-S370D-A4</p>	<p>Encodes for PTEN (C124S) with point mutations at S370 to an aspartic acid residue and S380, T382, T383, S385 to alanine residues. Made by overlapping PCR using as template pRK5::PTEN(C124S)-A4 and subcloned as a BamHI/EcoRI fragment into pRK5myc. A gift from Sarah Nicholls. Primers 2, 4 and 6 purchased from SIGMA Genosys.</p> <p><u>PCR1:</u></p> <p>Primer 1: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 2: 5' CAGGTTTCATTGTCAGAAACATCTGGTGTTAC 3'</p> <p><u>PCR2:</u></p> <p>Primer 3: 5' GTAACACCAGATGTTGATGACAATGAACCTG 3'</p> <p>Primer 4: 5' GCCTCAGAAGGTACCTAAC 3'</p> <p><u>PCR3:</u> Template; products of PCR1 and PCR2.</p> <p>Primer 5: 5'GCAGGATCCACAGCCATCATCAAAGAGATC 3'</p> <p>Primer 6: 5' GCCTCAGAAGGTACCTAAC 3'</p>

pYTH9-HA:: PTENΔNΔPDZBM	<p>Encodes for PTENΔCAT minus the PDZ binding motif (residues 179-394). Made by PCR and subcloned as an NCOI/EcoRI fragment into pYTH9-HA. Primer 1 purchased from SIGMA Genosys</p> <p>Primer 1: 5' TACCGG<b>CCATGG</b>TAAGCTACCTGTTAAA 3'</p> <p>Primer 2: 5' GCG<b>GAATTC</b>TCATTCATCAAA 3'</p>
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**TABLE 2.1**

Cloning strategy and list of primers. Restriction sites are indicated in red. Templates used were pRK5myc:PTENwt or pRK5myc:: PTEN(C124S) accordingly, unless otherwise stated.

### 2.1.3 Restriction digests and purification of DNA fragments

PCR products were purified prior to digestion using phenol:chloroform extraction or the Quiagen PCR Extraction method according to the manufacturer's instructions. Plasmid DNA (2-5μg) and PCR products were normally digested in a volume of 40μl with 1μl of each restriction enzyme (New England Biolabs) in New England Biolabs buffers, for 1h at 37°C. The resulting digest products were added to 1x loading buffer [6x: 30% glycerol (v/v), 0.25% bromophenol blue (w/v), 0.25% xylene cyanol FF (w/v)] and routinely run on 1% TAE agarose gels (10 x TAE: 0.4M Tris acetate, 10mM EDTA) containing 0.8μg/ml ethidium bromide. Bands of interest were excised and purified using the Quiagen Purification method according to the manufacturer's instructions.

### 2.1.4 Ligations

Ligation reactions were carried out using a molar ratio of vector:insert of 1:6 or 1:8 in a final volume of 10μl containing 1μl of T4 DNA ligase (1U/ μl, Invitrogen) and 2μl of the 5x DNA ligase buffer supplied with the ligase by Invitrogen. Reactions were incubated at room temperature for 3-4hrs and the entire ligation mix used to transform CaCl<sub>2</sub> competent *E. Coli*.

### 2.1.5 Preparation of CaCl<sub>2</sub> competent *E. Coli*

Competent *E. Coli* were prepared by inoculating 25mls of L-Broth with a single colony of DH5 $\alpha$  and incubating overnight at 37°C with vigorous shaking. The overnight culture was diluted into 500mls of L-Broth and incubated at 37°C with vigorous shaking until an OD<sub>600</sub> of 0.6 was reached. The cells were chilled on ice for 5 min and centrifuged for 15 min at 4000rpm at 4°C. The pellet was resuspended into 250mls of 0.1M CaCl<sub>2</sub> and left on ice for 30 min prior to centrifugation at 4000rpm for 10 min at 4°C. The pellet was then resuspended into 25mls of cold buffer composed of 15% glycerol and 50mM CaCl<sub>2</sub> in ddH<sub>2</sub>O and aliquoted into 200 $\mu$ l aliquots that were snap frozen in liquid nitrogen and stored at -80°C.

### 2.1.6 Transformation of competent *E. Coli*

Competent *E. Coli* (100 $\mu$ l/DNA sample) were thawed on ice, mixed gently with the DNA and incubated for 30 min on ice. The *E. Coli* were induced to take up the DNA by 'heat shocking' the transformation mix for 2 min at 42°C, followed by immediate addition of 1ml of L-Broth. Each *E. Coli* transformation mix was then incubated for 45 min at 37°C with vigorous shaking. Typically, 200 $\mu$ l of each sample was plated onto L-Broth/ Agar plates containing the required antibiotic (100 $\mu$ l/ml ampicillin or 50 $\mu$ l/ml kanamycin) for selection of growth of the transformed *E. Coli*, and the plates incubated overnight at 37°C to allow colony growth.

### 2.1.7 Purification of DNA

Minipreps of DNA were prepared by inoculating 2mls of L-Broth, containing the appropriate antibiotic (100 $\mu$ g/ml ampicillin or 50 $\mu$ g/ml kanamycin), with a single colony of *E. Coli* and incubating overnight at 37° with vigorous shaking to allow growth. 1.5 mls of each culture were transferred to an eppendorf tube and centrifuged at 13000rpm for 3 min at room temperature. The supernatant was discarded, except for 50 $\mu$ l, and the pellet resuspended by vortexing. The cells were lysed by adding 300 $\mu$ l of STET buffer (8% sucrose, 5% TritonX-100, 50mM EDTA pH 8.0, 50mM TrisHCl pH8.0, ~1mg/ml lysozyme) and boiling for 1 min at 100°C. The samples were centrifuged at 13000rpm for

15 min at 4°C. The resulting pellet was removed using a sterile Gilson pipette tip and discarded. DNA was precipitated by addition of 300µl of cold isopropanol and centrifugation at 13000rpm for 10 min at room temperature. The pellet containing the DNA was washed with 70% ethanol, air dried and resuspended in 50µl TE pH 8.0. Each DNA sample (5µl) was digested and run on an agarose gel to check for purity and the size of the insert.

Maxipreps of DNA were prepared by growing overnight cultures in 250mls of L-Broth at 37°C with vigorous shaking. The DNA was purified using the QIAFilter Plasmid Maxi kit (Quiagen) purification method, according to the manufacturer's instructions.

## **2.2 Cell Biology**

### **2.2.1 Cell lines and culture conditions**

#### **2.2.1.1 COS-7 and MDA-MB-435 cells**

The monkey fibroblast cell line COS-7 and the human mammary epithelial cell line MDA-MB-435 (received from Michael O'Hare) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with a penicillin/streptomycin solution mix (penicillin 100U/ml, streptomycin 100µg/ml, Gibco) and 10% of fetal calf serum (PAA laboratories) that had been heat inactivated by incubating for 45 min at 52°C (hi-FCS). Both cell lines were grown in a 10% CO<sub>2</sub> and 37°C temperature controlled incubator.

#### **2.2.1.2 U373, U138 and U87**

The human glioma cell lines U373, U138 and U87 were maintained in Modified Eagle's Medium [MEM (Eagle)] with Glutamax (Gibco) supplemented with a penicillin/streptomycin solution mix (penicillin 100U/ml, streptomycin 100µg/ml, Gibco), 5mls of MEM 100x non-essential amino acids (Gibco), 20mM HEPES [pH 7.5] (Gibco) and 10% of heat-inactivated fetal calf serum (PAA laboratories). All three cell lines were grown in a 5% CO<sub>2</sub> and 37°C temperature controlled incubator and were kept in culture until passage 20 (P20) had been reached, at which point they were discarded and a new vial of low-passage cells was thawed.

### 2.2.1.3 Astrocytes

Primary cultures of astrocytes were obtained as follows: Striata were dissected out from the brains of E17 CD rat embryos and mechanically dissociated in serum-free medium. The resulting cells were plated on (poly)-L-ornithine (1.5 $\mu$ g/ml) coated dishes (60 mm diameter) in DMEM containing 1 g/l glucose, 10mM HEPES [pH 7.5] (Gibco) and 10% FCS (The dissection was performed by Dr. Sandrine Etienne-Manneville). The cells were grown in a 5% CO<sub>2</sub> and 37°C temperature controlled incubator. After 21 days in culture, more than 95% of the cells were routinely positive for glial fibrillary acidic protein (AMERSHAM). Cultures were further enriched for astrocyte cells by washing once in calcium- and magnesium-free PBS-A, trypsinisation (0.25% trypsin, 0.02% EDTA) and subsequent plating on new (poly)-L-ornithine coated dishes or on (poly)-L-ornithine coated glass coverslips. Cells were used 10-15 days later for experimental manipulations.

### 2.2.2 The wound-healing assay

Astrocytes, U373, U138 and U87 cells were plated on glass coverslips and allowed to grow in a 5% CO<sub>2</sub> and 37°C temperature controlled incubator until they formed a monolayer or reached ~90% confluency in the case of the U138 and U87 cells. A sterile glass pipette or a Gilson tip was used to scratch the monolayer (wound) in the shape of a cross and the coverslip was moved to a dish containing fresh medium. The cells were allowed ~1h to recover from wounding prior to manipulation by microinjection or analysis by time-lapse microscopy (see below). Microinjected cells on coverslips were typically allowed to migrate for 8h or 16h (for U373, U138, U87) or 24h (astrocytes) before washing once with PBS-A and fixing with 4% (w/v) paraformaldehyde (SIGMA) for 15 min.

### 2.2.3 Microinjection of U373, U138, U87 and astrocytes

Just prior to microinjection, cells on coverslips were transferred to a 6cm culture dish, containing 5mls of the corresponding culture medium that had been allowed to equilibrate for 30 min in a 5% CO<sub>2</sub> and 37°C temperature controlled incubator. Cells were

microinjected using an Eppendorf micromanipulator 5171 and transjector 5240 system on a Zeiss Axiovert 135M microscope in a 5% CO<sub>2</sub> and 37°C controlled chamber. Maxiprep-prepared DNA was diluted into PBS-A at a final concentration of 0.1mg/ml and microinjected directly in the nuclei of 100 cells for each coverslip. The cells were returned to the incubator to allow for expression and completion of the wound-healing assay.

#### **2.2.4 Transfection of COS-7 cells**

Small-scale transfection of COS-7 cells was performed using the GeneJuice (Novagen) method according to the manufacturer's instructions. Typically, cells were seeded on 6-well culture plates at a density of  $1 \times 10^5$  cells/ well or  $2 \times 10^5$  cells/ well in 2ml culture medium (DMEM containing 10 % FCS and penicillin/streptomycin), 16h to 18h prior to transfection. Cells in each well were transfected with a total of 1µg of maxiprep DNA diluted in 100µl DMEM containing 3µl of GeneJuice reagent. Cells were then incubated for 24h or 48h in 5% CO<sub>2</sub> at 37°C a to allow for expression of the DNA, before being harvested for biochemistry.

#### **2.2.5 Immunofluorescence staining**

Cells on coverslips were fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature prior to permeabilisation with 0.2% Triton X-100 (TX-100)/ PBS-A for 10 min and quenching in sodium borohydride/ PBS-A (0.2mg/ml) for 15 min. Immunostaining was performed by incubating cells with primary antibodies diluted in PBS-A for 1h at room temperature, followed by 9 washes in PBS-A and incubating with fluorescent-conjugated antibodies for 45 min at room temperature. Cells were washed 6 times in PBS-A and 2 times in ddH<sub>2</sub>O prior to mounting on slides using 7µl of Mowiol mountant (Calbiochem). Images were captured using a Hammamatsu C5985 CCD camera and processed with OpenLab software.

## 2.2.6 Reagents

### 2.2.6.1 Antibodies used for immunofluorescence staining

Myc-epitope tagged constructs were visualised with mouse anti-Myc 9E10 antibody (prepared in the lab by Dr. Annette Self) used at a dilution of 1:100 in PBS-A followed by TRITC or FITC – conjugated anti-mouse IgG (Jackson Labs). Flag-epitope tagged constructs were visualised with Mouse anti-Flag M2 IgG (SIGMA) used at 10µg/ml in PBS-A followed by TRITC or FITC – conjugated anti-mouse IgG (Jackson Labs) and HA-epitope tagged constructs were visualized with rat anti-HA IgG (Roche, clone 3F10) used at 1:150 dilution (100µg/µl) followed by FITC – conjugated anti-rat IgG (Jackson Labs).

### 2.2.6.2 Inhibitors

Cytochalasin D (SIGMA) was used at a concentration of 500nM or 2µM.

Wortmannin (SIGMA) was used at a concentration of 100nM or 500nM.

## 2.2.7 Video time-lapse microscopy

Time-lapse movie recordings were captured using a CCD camera (SONY SSC-M370CE) and time-lapse controller (Openlab Software), attached directly to a Zeiss Axiovert 135M microscope. Cells were maintained in sealed flask in a temperature - controlled chamber (37°C). Images were captured under phase-contrast, at 1 frame/ 5 min directly by Openlab Software.

## 2.3 Protein Biochemistry

### 2.3.1 Preparation of GST fusion proteins

#### 2.3.1.1 Purification of recombinant Rac/ Rho/ Cdc42 for GEF assays

GST-Rac, GST-RhoA and GST-Cdc42 (pGEX-2T-Rac, pGEX-2T-RhoA, pGEX-2T-Cdc42 in B121 *E-Coli*) were prepared by inoculating 100ml of L-Broth (containing 100µg/ml ampicillin) and incubating overnight at 37°C with vigorous shaking. The

overnight culture was diluted into 900ml of L-Broth/ ampicillin and incubated for a further 2h at 37°C with vigorous shaking. Expression of the fusion protein was induced by adding isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) to a final concentration of 0.1mM for 3h followed by vigorous shaking at 37°C. Cells were collected by centrifugation at 4000rpm for 10min at 4°C and resuspended in 3ml of cold lysis buffer [50mM TrisHCl pH7.6, 50mM NaCl, 5mM MgCl<sub>2</sub>, 1mM dithiotreitol (DTT), 1mM phenylmethylsulfonyl fluoride (PMSF)]. Cells were lysed by sonicating on ice (3 bursts of 1 min with 30sec intervals, using small probe on an MSE Soniprep 150 sonicator at an amplitude of 14 $\mu$ m) and the lysates cleared by centrifugation at 4000rpm for 10 min at 4°C. The supernatant was transferred to a clean 15ml Falcon tube and incubated with 1ml of Glutathione-agarose beads (prepared by prewashing 5 times with an equal volume of lysis buffer and kept as a 1:1 suspension) on a rotating wheel for 1h at 4°C. The beads were pelleted by centrifugation at 4000rpm for 1 min and washed 6 times with 5ml of cold Buffer A (50mM TrisHCl pH7.6, 50mM NaCl, 5mM MgCl<sub>2</sub>) to remove any unbound protein. The GST-fusion proteins were eluted from the beads by adding an equal volume of freshly prepared release buffer (50mM TrisHCl pH8, 150mM NaCl, 5mM MgCl<sub>2</sub>, 1mM DTT, 5mM reduced glutathione) and incubated for 2 min at 4°C on a rotating wheel. The beads were pelleted and the supernatant kept in a separate clean tube. The elution step was repeated and the two supernatants pooled. Alternatively, the protein was released from the beads through cleavage of the GST tag by incubating the beads with 10U of human thrombin in digestion buffer (50mM TrisHCl pH7.6, 150mM NaCl, 5mM MgCl<sub>2</sub>, 2.5mM CaCl<sub>2</sub>, 1mM DTT) overnight, on a rotating wheel at 4°C. After thrombin digestion, the beads were pelleted by centrifugation at 13000rpm for 1 min and the supernatant removed to a clean microfuge tube. The beads were incubated with a further 0.5 ml of high salt buffer (50mM TrisHCl pH7.6, 150mM NaCl, 5mM MgCl<sub>2</sub>, 1mM DTT) for 2 min on a rotating wheel at 4°C. The beads were pelleted once again and the two supernatants were pooled. In both cases (elution or thrombin digestion), the protein was concentrated to a volume of ~150 $\mu$ l (using an Amicon Centricon 10 filter device) by centrifugation in a fixed angle rotor at 7000rpm (~2h), aliquoted in 10 $\mu$ l samples and snap frozen in liquid nitrogen and stored at -80°C.



### 2.3.1.2 Purification of GST-PAK CRIB and GST-Rhotekin for pull-down assays

GST-PAK CRIB (pGEX-2T-PAK CRIB in B121 *E.Coli*) was prepared by inoculating 100ml of L-Broth (containing 50µg/ml ampicillin and 25µg/ml chloramphenicol) and allowing growth overnight at 37°C with vigorous shaking. The overnight culture was diluted into 900ml of L-Broth/ampicillin/chloramphenicol and incubated for 2h at 30°C with vigorous shaking. Protein expression was induced by adding IPTG to a final concentration of 0.5mM and allowing growth for a further 5h at 30°C with vigorous shaking. The cells were collected by centrifugation at 4000rpm for 20 min at 4°C and the pellet resuspended in 20ml of cold sucrose buffer [50mM TrisHCl pH8, 40mM EDTA, 25% (w/v) sucrose, 5mM DTT, 1mM PMSF, 1 complete inhibitor tablet/50mls of buffer (Boehringer)] and incubated for 20 min on a rotating wheel at 4°C. The cells were lysed by addition of 8ml of cold lysis buffer (50mM TrisHCl pH8, 100mM MgCl<sub>2</sub>, 0.2% (w/v) Triton X-100, 5mM DTT, 1mM PMSF, 1 complete inhibitor tablet/50mls of buffer) and rotating for a further 10 min at 4°C prior to sonication (as described previously). The suspension was cleared by centrifugation at 10000rpm for 45 min and the supernatant transferred to a 15ml Falcon tube prior to the addition of 1ml of a 50% Glutathione-agarose bead slurry (kept as a 1:1 suspension in lysis buffer). The GST-fusion protein was allowed to bind the beads on a rotating wheel at 4°C for 1h. The beads were pelleted by centrifugation (at 2500rpm for 30 sec) and washed 6 times with 5mls of cold wash buffer (50mM TrisHCl pH 7.6, 50mM NaCl, 5mM MgCl<sub>2</sub>). Finally, the beads were resuspended in 500µl of wash buffer containing 25% glycerol and aliquoted in 50µl aliquots, snap frozen in liquid nitrogen and stored at -80°C.

GST-Rhotekin was prepared in the same way as GST-PAK CRIB with the following modifications: pGEX-2T-PAK CRIB *E.Coli* (B121) were grown for 2h at 22° prior to induction with IPTG.

### 2.3.2 GTPase Pull-down assays

U373, U138 and U87 cells were each grown to confluency in 9mm dishes in culture medium (as described in 2.2.1). Each dish was washed twice with cold TBS (50mM TrisHCl pH8, 150mM NaCl) prior to lysing the cells with 200µl / dish of cold lysis buffer (50mM TrisHCl pH 7.5, 1% Triton X-100, 500mM NaCl, 50mM MgCl<sub>2</sub>, 0.5% sodium deoxycholate, 1mM PMSF, 20µg/ml aprotinin, 20µg/ml leupeptin, 5µg/ml pepstatin) on

ice. The cells were scraped off the dish immediately and the lysates cleared by centrifugation at 13000rpm for 10 min at 4°C. 20µl of the supernatant were transferred to a clean eppendorf and boiled for 5 min in 2x Laemli sample buffer. The rest of the supernatant was added to 50µl of GST-PAK CRIB coupled to Glutathione agarose beads (see 2.3.1) in order to precipitate the active forms of Rac (Rac-GTP) and Cdc42 (Cdc42-GTP). The samples were incubated on a rotating wheel at 4°C for 1h. As a positive control, one of the samples was incubated with GTPγS (which binds to Rac and Cdc42 and mimics the activated state) and 50µl of GST-PAK CRIB beads and placed on a rotating wheel for 30 min at room temperature and 30 min at 4°C. The beads were then precipitated by centrifugation at 13000rpm for 15sec at 4°C and washed 4 times with cold wash buffer (50mM TrisHcl pH 7.5, 150mM NaCl, 1%Triton X-100, 10mM MgCl<sub>2</sub>, 0.2 mM PMSF). The samples were split in half and analysed by SDS-PAGE followed by immunoblotting using either mouse monoclonal anti-Cdc42 IgG or mouse monoclonal anti-Rac IgG (clone 23A8). The relative levels of Rac and Cdc42 were analysed for each cell line relative to the corresponding levels of Rac and Cdc42 in total cell lysates by quantification using Quantity One software (Biorad).

### 2.3.3 GEF assays (ON-Rates)

The different GTPases (2 µg) were incubated at 30°C for 15 min in 320 µl loading buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 100µM AMP-PNP, 0.2 mM DTT, 2 mM EDTA) containing 10µM [<sup>3</sup>H]-GDP (AMERSHAM), then placed on ice, followed by addition of 5µl of 0.1M MgCl<sub>2</sub> was added. Exchange reactions were allowed to take place at 30°C in the presence of 1mM cold GTP and 0.5mg/ml BSA, and in the presence or absence (control buffer) of 20µg of each of the cell lysates. At the indicated times, 40µl aliquots of the reaction were removed and diluted into 1 ml of cold Buffer A (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, and 5 mM MgCl<sub>2</sub>) and passed through pre-soaked nitrocellulose filters (NC45 Schleicher & Schuell 0.4µm). Filters were washed once with 10 ml cold Buffer A, transferred to vials, left to dry and counted in a scintillation counter following addition of 8 ml of scintillation fluid per vial.

### 2.3.4 Immunoprecipitation assays

Cells were rinsed on ice with cold PBS-A containing 1mM orthovanadate (SIGMA) and lysed on ice with RIPA buffer (10 mM Tris/HCl [pH 7.5], 140 mM NaCl, 1 mM orthovanadate, 1% Nonidet P-40, 2 mM PMSF, 5 mM EDTA, 20 µg/ml aprotinin, 20 µg/ml leupeptin) 48h post-transfection. The nuclei were discarded following centrifugation at 13000rpm for 2 min and the supernatant lysates incubated at 4°C for 2h with specific antibodies and 20-50µl of 50% Glutathione-sepharose beads. The beads with the immunoprecipitates were centrifuged at 13000rpm for 30 sec and washed 5 times with RIPA buffer. The immunoprecipitated proteins were eluted with Laemli sample buffer and analyzed by 12% SDS-PAGE (see below). Mouse Anti-Myc mAb 9E10 or anti-flag IgG (SIGMA) was used for detection of the MYC or FLAG tagged constructs. Rabbit anti-PTEN IgG (Cell Signalling) or rabbit anti-phospho-PTEN (Ser380/Thr382/383) IgG (Cell Signaling Technology) was used respectively for detection of PTEN or the phosphorylated forms of the PTEN expressed constructs.

### 2.3.5 Kinase assays

#### 2.3.5.1 PAK kinase assay

Cells were plated to confluency in 9cm dishes, washed 2x with PBS-A containing 10mM orthovanadate and lysed with 200µl lysis buffer (25mM HEPES [pH 7.3], 300 mM NaCl, 1.5mM MgCl<sub>2</sub>, 1 mM sodium vanadate, 0.5% Triton X-100, 5% glycerol, 0.5 mM EGTA, 0.5 mM PMSF and 1µg/ml each of pepstatin, aprotinin, leupeptin). Cells were cleared by centrifugation at 13000rpm for 10min and the supernatant lysates incubated at 4°C for 2h with 2µg of anti-PAK (Santa Cruz, CA) IgG and 50µl of 50% Glutathione-sepharose beads. The beads containing the immunoprecipitated PAK protein were washed twice with 1ml PBS-A containing 0.1% Triton and once with kinase buffer (50mM HEPES [pH7.3], 10mM MgCl<sub>2</sub>, 10mM NaF, 2mM MnCl<sub>2</sub>, 1mM DTT, 0.05% Triton). PAK activity was assayed by incubating the PAK protein containing beads in 25µl of pre-warmed kinase buffer containing 10µM ATP, 20µg/ml MBP (SIGMA) and 3µCi/sample of  $\gamma^{32}\text{P}$ -ATP (AMERSHAM) at 37°C for 15min and the reactions stopped by boiling in 5X Laemli sample buffer. Proteins were analysed by 12% SDS-PAGE and transferred to a nitrocellulose membrane by Western blotting. The levels of phosphorylated MBP were assessed by exposure to film (KODAK) for the required

lengths of time and the immunoprecipitated PAK levels monitored by probing the nitrocellulose membrane with anti-Pak IgGs.

### 2.3.5.2 PKC $\zeta$ Kinase assay

Same as for PAK, with the following modifications: RIPA buffer (10 mM Tris/HCl [pH 7.5], 140 mM NaCl, 1 mM orthovanadate and 1% Nonidet P-40, 2 mM PMSF, 5 mM EDTA, 20  $\mu$ g/ml aprotinin, 20  $\mu$ g/ml leupeptin) was used to lyse the cells, anti-PKC $\zeta$  (Santa Cruz, CA) IgG was used to immunoprecipitate PKC $\zeta$  from the cell lysates and the kinase reaction was carried out in 50 $\mu$ l kinase buffer (1mM DTT, 10mM PNPP, 10mM sodium vanadate, 10 $\mu$ M ATP, 20 $\mu$ g/ml MBP, 3 $\mu$ Ci/sample of  $\gamma^{32}$ P-ATP).

### 2.3.6 PTEN Phosphatase assay

COS-7 cells were transfected with the required Myc-epitope tagged PTEN constructs (2 wells of a 6-well dish / construct) with GeneJuice (as described in 2.2.4). Cells were lysed 24h post-transfection with cold lysis buffer (1% NP40, 10mM TrisHCl pH7.5, 140mM NaCl, 2mM MgCl<sub>2</sub>, 2mM PMSF, supplemented with 1 complete inhibitor tablet / 50ml of buffer) and the supernatants, which had been transfected with the same construct, pooled. Lysates were cleared by centrifugation at 13000rpm for 2 min and Myc-PTEN immunoprecipitated with Mouse anti-Myc 9E10 antibody as described in 2.3.4. Following immunoprecipitation, the immunocomplexes were washed twice in cold lysis buffer and 3 times in phosphatase buffer (50 mM HEPES [pH 7.0], 150 mM NaCl, 10mM DTT) before being incubated in a phosphatase buffer (60 $\mu$ l final volume) at 30°C or 37°C for the indicated lengths of time. The reactions were terminated by boiling in 5x sample buffer. Proteins were analysed by 12% SDS-PAGE and transferred to a nitrocellulose membrane, followed by Western blotting with a phospho-specific PTEN antibody (Cell signaling).

### 2.3.7 SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were boiled in 5x sample buffer (250mM Tris-HCl pH 6.8, 0.5M DTT, 10% SDS, 50% glycerol, 0.1% bromophenol blue) at 100°C for 5-10 min prior to being loaded onto a polyacrylamide gel (stacking gel: 5% acrylamide/ bisacrylamide solution, 125mM TrisHCl pH6.8, 0.1% SDS; resolving gel: 10 – 15 % acrylamide/ bisacrylamide solution as required, 375mM TrisHCl pH8.8, 0.1% SDS). 10µl of Rainbow markers (RPN800V, Amersham) were also loaded on each gel and the protein samples resolved by electrophoresis at 90-120V using a Biorad minigel apparatus. Proteins were detected by staining the resolving gel with coomassie blue stain (0.5g brilliant blue R-250, 10% acetic acid, 50% methanol) for ~1h, followed by destaining with 10% acetic acid (v/v) / 25% methanol (v/v), until the protein bands were visible and the gel almost clear. Alternatively, proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell) for Western blotting.

### 2.3.8 Immunoblotting/ Western blotting

Proteins were transferred from a polyacrylamide gel to nitrocellulose membranes in 1x cold transfer buffer (10x stock: 30g/L Trizma base, 144g/L glycine) at 0.35A for 2h or 0.25A overnight at 4°C, using a Biorad 'wet' transfer apparatus. The nitrocellulose membrane was incubated in block [5% Marvel (w/v), 1M glycine, 5% FCS (v/v), 1% albumin (w/v)] for 1h at room temperature on a shaker and washed 3 x 5 min with 5% Marvel (w/v) / 0.2% TWEEN-20 (v/v) in PBS-A, prior to incubation with antibodies. Primary antibodies were prepared in 2% Marvel/ 0.2% TWEEN-20/ PBS-A to the required dilution and applied to the membrane for 1h at room temperature or overnight at 4°C, on a shaker. The membrane was washed 3 x 10 min with 2% Marvel/ 0.2 % TWEEN-20/ PBS-A at room temperature prior to incubation for 45 min at room temperature with the secondary HRP-conjugated antibody, prepared in 2% Marvel/PBS-A. Finally, the membrane was washed 3 x 15 min with 0.4 % TWEEN-20/ PBS-A and developed using ECL reagent detection kit (AMERSHAM) according to the manufacturer's specifications. The membrane was exposed to ECL hyperfilm (AMERSHAM), as necessary.

All HRP - conjugated antibodies for immunoblotting were purchased from PIERCE and used at a dilution of 1:2000 unless otherwise indicated (in 2%Marvel/ 0.2 % Tween10/ PBS-A).

### 2.3.9 Labelling of cells with [<sup>32</sup>P]-orthophosphate and phosphatase assay

COS-7 cells were plated on 6cm dishes at a density of  $2 \times 10^5$  cells/ dish and transfected 16h later with the required PTEN constructs by GeneJuice. The cells were washed once with 5ml of Phosphate-free DMEM (Gibco) and labeled with 125 $\mu$ Ci/ dish of <sup>32</sup>P-orthophosphate (Amersham) for 4h in 5%CO<sub>2</sub>, at 37°C. Cells were washed once with 5ml of cold PBS-A and lysed for 5min with 400 $\mu$ l/ dish of cold lysis buffer (40mM TrisHCl pH7.6, 150mM NaCl, 1mM EDTA, 1% Triton X-100), supplemented with one complete inhibitor tablet/ 50ml of buffer. Lysates were cleared by centrifugation at 13000rpm for 3 min and incubated with 50 $\mu$ l of 50% Glutathione-sepharose beads for 30min at 4°C on a rotating wheel. The beads were pelleted (13000rpm, 1 min) and the lysates were transferred to clean tubes containing 50 $\mu$ l of fresh 50% Glutathione-sepharose beads. The Myc epitope-tagged PTEN constructs were precipitated by incubating the samples with 5 $\mu$ l of anti-Myc 9E10 antibody for 2h at 4°C on a rotating wheel. The immunocomplexes were washed twice with cold lysis buffer, twice with wash buffer (40mM TrisHCl pH7.6, 150mM NaCl, 1mM EDTA) and finally twice with cold phosphatase buffer (25mM HEPES pH7.2, 50mM NaCl, 2.5mM EDTA, 10mM DTT). The phosphatase reaction was initiated by addition of 60 $\mu$ l of phosphatase buffer to each sample (final reaction volume of 100 $\mu$ l) followed by incubation at 30°C for the required times. The reaction was stopped by addition of 5x sample buffer and boiling at 100°C for 10 min. The protein samples were analysed by SDS-PAGE on a 10% polyacrylamide resolving gel. The gel was stained (with coomassie blue stain) and destained in order to fix the proteins and visualize the molecular weight markers (Rainbow, RPN800V). Finally, the gel was dried using a Biorad gel drier and radioactive proteins visualized by exposing the gel to film (Biomax-MR Kodak) or to the Biorad Imaging screen K, as required (usually overnight). The Biorad Imaging screen K was 'read' using a phosphorimager (Biorad Molecular Imager FX) and the data were analysed using Quantity One software (Biorad).

### 2.3.10 Peptide maps and phosphoamino acid analysis

The required radioactive protein bands were cut out of the gel, washed 3 x 30 min in destain, 2 x 30 min in 50% methanol (v/v) and 2 x 30 min in ddH<sub>2</sub>O prior to drying for 2h in a Speed-vac. The proteins in the gel pieces were digested with 500 $\mu$ l of 0.3mg/ml Trypsin in 100mM NH<sub>4</sub>HCO<sub>3</sub> for 24h at 37°C. The supernatant was removed to a clean

tube and the gel pieces were incubated with 500 $\mu$ l of 100mM  $\text{NH}_4\text{HCO}_3$  for 2h at 37°C to remove any remaining peptide fragments from the gel. The two supernatants were combined and lyophilized by drying in a Speed-vac.

The peptide fragments were analysed by thin layer chromatography (TLC) as follows: digested dried samples were resuspended in 10 $\mu$ l of  $\text{H}_2\text{O}$ , followed by heating at 65°C for 1 min and centrifugation at 13000rpm for 15 min, prior to spotting (1 $\mu$ l at a time, followed by drying with a hair-dryer) in the middle of a chromatography paper (Macherey); 4cm up, 10cm over. One microgram each of basic fuchsin and phenol red were also spotted on the sample and the chromatography paper was overlaid with electrophoresis buffer pH3.5 (acetic acid: pyridine:  $\text{H}_2\text{O}$  in a 19:1:89 ratio). Electrophoresis was performed at 500V (in buffer pH3.5) until the dye was within 4cm of the opposite side (~2h). The chromatograph was allowed to dry thoroughly in the fume hood and the peptides were resolved in the second dimension using an ascending chromatography tank containing 150ml of buffer (pyridine: butanol: acetic acid:  $\text{H}_2\text{O}$  in a 15:10:3:12 ratio) until the buffer reached to within 3cm of the top of the chromatograph (~4h). The chromatograph was once again allowed to dry thoroughly under the fume hood, the covered with saran wrap and exposed to film (Biomax-MR, Kodak).

Alternatively, the digested peptide fragments were subjected to total phosphoamino acid analysis. The dried samples were resuspended in 50 $\mu$ l of dd $\text{H}_2\text{O}$  and incubated with 6M HCl, for 1h at 105°C, followed by lyophilisation in a Speed-vac. The dried samples were resuspended in 10 $\mu$ l dd $\text{H}_2\text{O}$ , heated at 65°C for 1 min and centrifuged at 13000rpm for 15min. The samples were spotted (1 $\mu$ l at a time, followed by drying with a hair-dryer) onto chromatography paper; 4cm up and 4cm over. One microgram each of phosphoserine, phosphothreonine and phenol red were spotted on each sample and the chromatography paper was overlaid with electrophoresis buffer pH1.9 (formic acid: acetic acid:  $\text{H}_2\text{O}$  in a 1:10:89 ratio). Electrophoresis was allowed to proceed at 500V (in buffer pH1.9) until the dye had moved ~5cm. The chromatograph was then moved to an electrophoresis tank containing buffer pH3.5 and electrophoresis was allowed to proceed until the dye had moved ~7cm. The chromatograph was allowed to dry and phosphoserine and phosphothreonine standards were visualized as purple spots by application of a few drops of 1% ninhydrin in acetone (w/v). The chromatograph was wrapped in saran wrap and exposed to film (Biomax-MR, Kodak).

## 2.4 Yeast Two-Hybrid

### 2.4.1 Yeast Plates

YEPD plates: 20g peptone (Difco, No.0118-01-8), 10g yeast extract (Difco, No. 0127-01-7), 20g Bacto-agar (Difco No.0140-01) in 900ml of H<sub>2</sub>O + 100ml 20% glucose.

SC plates: 6.7g yeast nitrogen base (Difco, No. 0919-15-3), 20g Bacto-agar (Difco No.0140-01), 2g of drop-out amino acid mix in 900ml of H<sub>2</sub>O + 100ml 20% glucose. According to the selection conditions, one or more of the following supplements were added: 0.1g tryptophan (Trp), 0.2g leucine (Leu), 0.1g histidine (His).

3-AT Plates: Same as for Sc-Trp-Leu-His plates + 25ml of a 1M sterile-filtered 3-amino-1,2,4-triazole solution (SIGMA) per litre of SC medium.

### 2.4.2 Cloning

A cDNA encoding PTEN $\Delta$ CAT $\Delta$ PDZBM was fused to the sequence encoding the GAL4 DNA-binding domain (GAL4DB) in the pYTH9 vector. The construct was subsequently transformed into the yeast strain Y190. To facilitate integration of the construct into the yeast genome (through homologous recombination), the plasmid was linearised with Xba1 (New England Biolabs).

### 2.4.3 Yeast transformations

5mls of YEPD [20g peptone (Difco, No.0118-01-8), 10g yeast extract (Difco, No. 0127-01-7), 20% glucose, in a final volume of 1lt] were inoculated with one colony of yeast and incubated overnight at 30°C with vigorous shaking. The overnight culture was diluted into 50ml YEPD and allowed to grow until the OD<sub>600</sub> had reached approximately 0.6. The cells were centrifuged at 3000rpm for 5min and washed once with 5ml TE [pH8.0], followed by a single wash with 5ml 1X LiAc/TE (10X LiAc/TE: 1M lithium acetate, 100mM TrisHCl [pH 7.5], 5mM EDTA). The cells were resuspended in 500 $\mu$ l 1X LiAc/TE. To 100 $\mu$ l of yeast cells were added 1.5 $\mu$ g of transforming DNA + 50 $\mu$ g of carrier DNA (QBiogene Inc.) and the mixture was incubated at 30°C for 30min. The yeast cells were then induced to take-up DNA by heat shocking at 42°C for 10min in the presence of 110 $\mu$ l of DMSO. The cells were pelleted at 13000rpm for 1min, washed



Yeast colonies were allowed to grow by placing the plates in a n incubator at 30°C for 2 days.

#### 2.4.4 Yeast lysates

2mls of SC medium were inoculated with each of the yeast colonies and incubated overnight in a 30°C shaker-incubator. The following day, 1.5mls of each culture were transferred to a microfuge tube and centrifuged at 13000rpm for 1 min at room temperature. The cell-containing pellet was re-suspended in 1ml of ddH<sub>2</sub>O and cells were pelleted for 1 min at 13000rpm at room temperature. The pellet was re-suspended in 100µl of 1x sample buffer and boiled for 5 min at 95°C. The samples were once again centrifuged at 13000rpm for 5 min and 25µl of each lysate were loaded on an SDS-polyacrylamide gel.

#### 2.4.5 Yeast two-hybrid screen

10ml of YEPD were inoculated with Y190[pYTH9:PTENΔCATΔPDZ] and allowed to grow overnight at 30°C with vigorous shaking. The overnight culture was diluted into 100ml YEPD and incubated at 30°C for a further 24h with vigorous shaking. The overnight culture was diluted further in 1L YEPD and incubated at 30°C with vigorous shaking until it reached an OD<sub>600</sub> of approximately 0.6. The culture was then centrifuged in a sterile tube at 4000rpm at room temperature for 7min. The cells were resuspended in 500ml of TE [pH 7.5] and centrifuged at 4000rpm at room temperature for 7min. The cells were then resuspended in 100ml of 1X LiAc/TE (10X LiAc/TE: 1M lithium acetate, 100mM TrisHCl [pH 7.5], 5mM EDTA) and incubated in a shaking incubator at 30°C for 1h. The cells were pelleted at 4000rpm at room temperature for 7min and resuspended in 10ml 1X LiAc/TE. 500µl carrier DNA (a gift by Steve Moss) and 450µl of brain library cDNA were added to the yeast cells and the mixture was incubated in a 30°C waterbath for 30min. 100ml of 40% PEG [polyethylene glycol 3,350 (w/v)] in 0.1M LiAc/TE were added to the mixture and incubated in a 30°C waterbath for a further 30min. Cells were induced to take up the DNA by heat shocking at 42°C for 10min in the presence of 11ml of DMSO. The cells were pelleted at 4000rpm at room temperature for 7min, resuspended in 500ml YEPD and incubated at 30°C for 1h in a shaking incubator. The culture was centrifuged at 4000rpm at room temperature for 7min and the pelleted cells were washed

3x with 200ml of TE [pH 7.5], prior to being resuspended in 10ml of TE [pH 7.5]. The yeast were then plated out on 10 large 3-AT plates (1ml/plate) and 10 $\mu$ l, 50 $\mu$ l and 100 $\mu$ l were each plated out on SC-Trp-Leu plates in order to calculate the transformation efficiency. The 3-AT plates were incubated at 30°C and yeast colonies were picked every 2 days for 9 days, starting at day 3 and streaked onto Sc-Trp-Leu plates. The freshly grown yeast clones were tested for a positive interaction by re-streaking on 3-AT plates. The DNA from the positive colonies was then isolated and transformed into DH5 $\alpha$  *E.Coli* and the presence of an insert was examined by restriction digest mapping.

#### 2.4.6 Isolation of yeast DNA

2ml of SC-Trp-Leu solution were incubated with individual yeast colonies and incubated overnight at 37°C with vigorous shaking. Cells were pelleted at 13000rpm for 3min at room temperature and the pellets were resuspended in 200 $\mu$ l breaking buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM TrisHCl [pH 8.0], 1mM EDTA [pH8.0]. 200 $\mu$ l of glass beads (SIGMA) and phenol/chloroform solution were added to each tube, before vortexing at full speed for 5 min. To isolate the yeast DNA, which was present in the aqueous layer, the samples were spun down at 13000rpm for 5min at room temperature.

#### 2.4.7 Transformation of yeast DNA into DH5 $\alpha$

5 $\mu$ l of yeast DNA were added to electro-competent DH5 $\alpha$  cells on ice and transferred to an electroporation cuvette (2mm) on ice. The DNA was transformed into the DH5 $\alpha$  cells by an electric pulse at 200 Ohm, 25 $\mu$ F, 1.8kV and 1ml of L-Broth was added to the cells in the cuvette and the mixture was transferred to a 1.5ml microfuge tube. The bacteria were incubated for 1h at 37°C, pelleted, resuspended in 100 $\mu$ l L-Broth and plated on ampicillin-plates. The plates were then incubated at 37°C overnight, to allow for bacteria growth.

#### 2.4.8 Minipreps and digests

2 x 2ml of L-Broth containing 100 $\mu$ l/ml ampicillin were inoculated with a single colony of each bacterial clone and allowed to grow at 37°C overnight. The DNA was purified as described in section 2.1.7 and digested in the following way to check for the presence of an insert: 5 $\mu$ l DNA + 1.5 $\mu$ l RNase A (1mg/ml) + 1.5 $\mu$ l Buffer B (ROCHE) 7 $\mu$ l H<sub>2</sub>O + 1 $\mu$ l of each XhoI and ECOR I / per 12 constructs, at 37°C for 1h.

#### 2.4.9 Fast transformation of yeast

Half of a YEPD plate was densely streaked with Y190[pYTH9:PTEN $\Delta$ CAT $\Delta$ PDZ] and incubated at 30°C overnight. Cells were scraped off the plate using a sterile inoculation loop and resuspended in 1ml sterile water. Cells were pelleted at 13000rpm for 1 min and resuspended in 1ml of 1X LiAc/TE (10X LiAc/TE: 1M lithium acetate, 100mM TrisHCl [pH 7.5], 5mM EDTA). Cells were pelleted once again and resuspended in 500 $\mu$ l of 1X LiAc/TE. To each 50 $\mu$ l of cell suspension were added 1 $\mu$ g of transforming DNA, 50 $\mu$ g of carrier DNA (QBiogene) and 300 $\mu$ l of 40% PEG [polyethylene glycol 3,350 (w/v)] in 0.1M LiAc/TE. The yeast were incubated at 30°C for 30min and induced to take-up the DNA by heat shocking at 42°C for 10min in the presence of 30 $\mu$ l of DMSO. Cells were then washed 3x with 1ml TE [pH7.5], resuspended in 50 $\mu$ l TE [pH 7.5] and allowed to grow on Sc-Trp-Leu plates at 30°C for 2 days.

#### 2.4.10 $\beta$ -galactosidase filter transfer assay

Yeast cells grown on SC-Trp/-Leu plates containing bait and prey constructs were replica plated on to Whatmann number 1 filter discs (9cm), which were then dipped three times into liquid nitrogen for 20s and allowed to thaw to induce cell lysis. The discs were placed in 10cm plates already containing a filter disc pre-soaked in 2ml of buffer Z (60mM Na<sub>2</sub>HPO<sub>4</sub>.7H<sub>2</sub>O, 40mM Na<sub>2</sub>HPO<sub>4</sub>.4H<sub>2</sub>O, 10mM KCl, 1mM MgSO<sub>4</sub>. H<sub>2</sub>O) with 50mM  $\beta$ -mercaptoethanol and 0.5mg/ml 5-bromo-4-chloroindolyl- $\beta$ -D-galactoside (X-gal). Positive bait and prey protein interactions were detected by the presence of a blue

coloured product released by the action of  $\beta$ -galactosidase on X-Gal after incubation between 5h and 16h at 30°C.

## **Chapter 3**

### **Results – Glioma migration and the Rho GTPases**

#### **3.1 Summary**

Rho GTPases play a key role in regulating cell migration. Astrocytes, the principal macroglial cells of the CNS, migrate relatively slowly, while gliomas, which are highly invasive astrocyte-derived tumours, are highly motile and able to infiltrate the surrounding tissue and spread diffusely in the brain. In this chapter, the aberrant migration of three increasingly malignant human glioma cell lines (U373, U138, U87) was characterised using a wound-healing assay and compared to the migration of primary rat astrocytes. Furthermore, the contribution of Rho GTPases to the motility of these gliomas cell lines was investigated. Microinjection experiments showed that Rac and Cdc42 are essential for the migration of both astrocytes and the glioma cell lines. Interestingly, however, the higher migration rate observed for the gliomas does not correlate with the levels of active Cdc42 or Rac. Nevertheless, the activity of the Rac downstream effector kinase, p65PAK, was increased in the higher malignant cell lines U138 and U87. In contrast, the activation levels of a Cdc42 target kinase, PKC $\zeta$ , remained unchanged across the three glioma cell lines.

## 3.2 Introduction

Cell migration is a complex process that is driven primarily through the remodeling of the actin cytoskeleton. A large variety of signaling molecules have been implicated in the control of cell migration, including MAPK cascades, lipid kinases, phospholipases, Ser/Thr and Tyr kinases and scaffold proteins, and in particular, one family of proteins seems to play a critical role in regulating the biochemical pathways central to cell migration, the Rho GTPases (Ridley, 2001a). In order for the cell to migrate in the correct direction and to know where to stop and differentiate the activity of these signaling molecules must be tightly regulated both in time and space. In most aggressive tumours, however, this tight regulation has been lost, resulting in tumour invasion and metastasis.

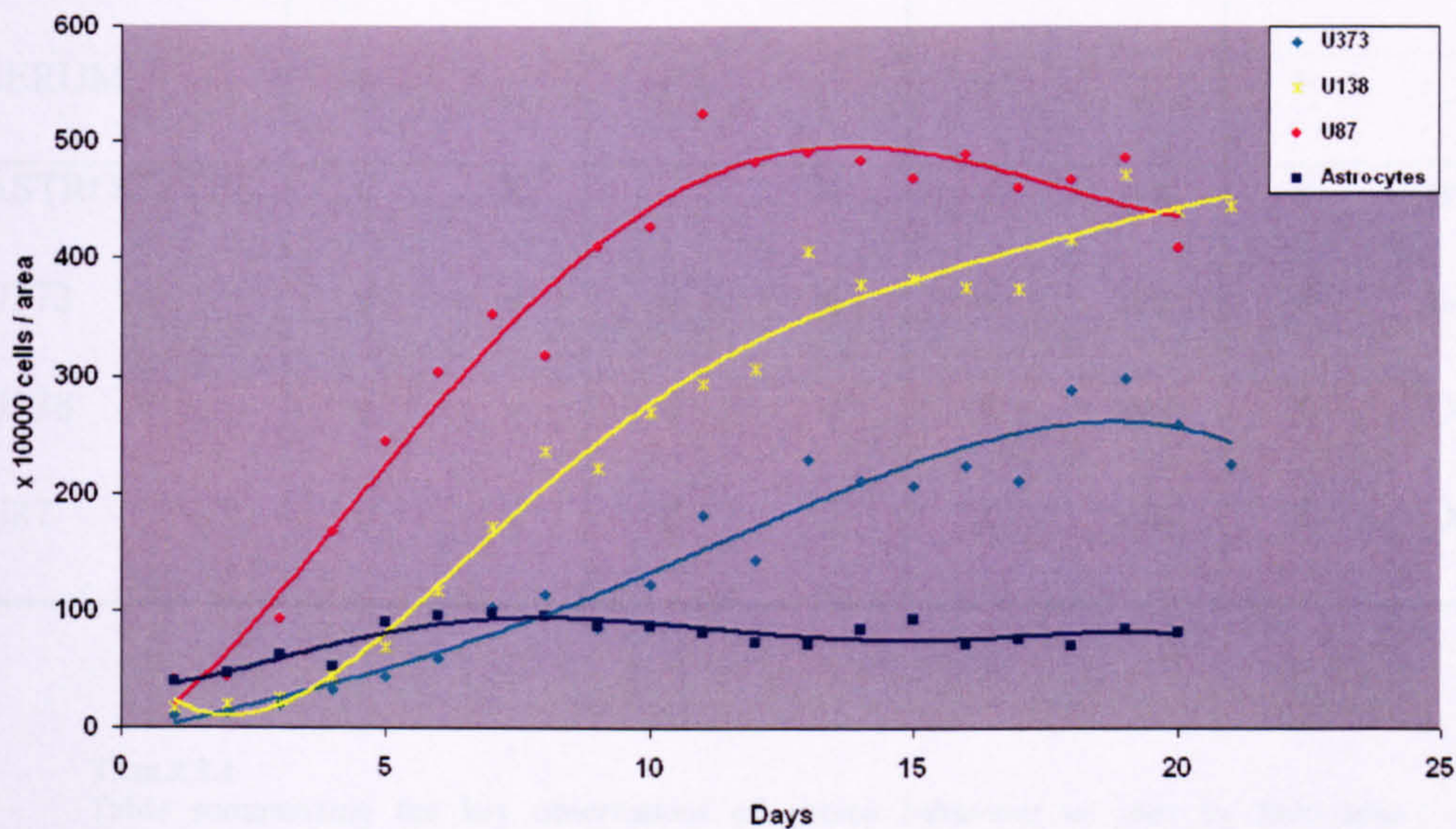
Genetic mutations identified in human gliomas often target receptor tyrosine kinase signaling pathways (e.g. EGFR or PDGFR), resulting in their constitutive activation. Rho GTPases have been shown to be activated downstream of several such receptor tyrosine kinases and their activities are thus potentially misregulated in these tumours and this may contribute to their aberrant migratory behaviour. To gain a better understanding into how glioma cell migration is regulated, the migration of three human glioma cell lines (U373, U138 and U87) and of primary astrocytes was examined using an *in vitro* wound-healing assay. Furthermore, the activity state of Rho GTPases in these gliomas as well as their contribution to the glioma migration behaviour was investigated.

## 3.3 Results

### 3.3.1 General characteristics of glioma behaviour

To characterise the behaviour of the gliomas in culture, the migration, polarity and proliferation rate of three increasingly malignant human glioma cell lines (U373, U138, U87) was analysed under conditions when the cells were grown with (10% FCS) or without any serum. The results are summarized in table 3.1. Unsurprisingly, astrocytes are the only cells that cannot divide in the absence of serum. All three gliomas, are known to have acquired several mutations in genes regulating cell cycle arrest and apoptosis, and can by-pass the requirement for serum in order to grow and divide (Maher et al., 2001). Furthermore, when the rate of proliferation of the gliomas is compared to that of

astrocytes in figure 3.1, astrocytes are much slower and their proliferation rate plateaus after day 5. The rate of proliferation of gliomas reflects their malignancy grade, with the highly aggressive U138 and U87 cells dividing much faster than U373 cells. Even though U373 cells seem to initially form a monolayer, they do not seem to be contact inhibited for division as they keep dividing long after they have reached confluency at day 7. U138 and U87 cells, on the other hand, do not form a monolayer and cells keep dividing on top of each other, resulting in the formation of foci.



**FIGURE 3.1**

Quantitation of cell growth relative to time. A confluent 9cm dish of cells was split 1:5 and 1ml was split equally into a 24-well dish, resulting in a cell density of  $2 \times 10^4$  cells / well at day 0. Everyday, cells in one of the wells were trypsinised and counted using a haemocytometer. The experiment was performed in duplicate for each cell line.

When the migratory behaviour of these cells was analysed, as indicated in table 3.1, astrocytes and U373 do not migrate in the absence of serum, but instead grow long extensions/protrusions and stop (data not shown). U87 and U138, however, can still migrate in the absence of serum, albeit more slowly. A plausible explanation for these differences could be that U138 and U87 secrete the necessary growth factors and matrix proteins required for cell migration. Gliomas have previously been shown to acquire mutations such as gene duplications of the EGFR and PDGFR and also to upregulate the

corresponding receptor ligands thus resulting in a positive feedback loop (Fenstermaker and Ciesielski, 2000; Lokker et al., 2002). Both EGFR and PDGFR are key regulators of cell migration and have been shown to activate the small GTPase Rac (discussed in section 1.3.4.2).

	DIVISION		MIGRATION		CONTACT INHIBITION		POLARITY	
	+	-	+	-	+	-	+	-
SERUM	+	-	+	-	+	-	+	-
ASTROCYTES	√	X	√	X	√	n/a	√	n/a
U373	√	√	√	X	√	n/a	√	n/a
U138	√	√	√	√	X	n/a	X	X
U87	√	√	√	√	X	n/a	X	X

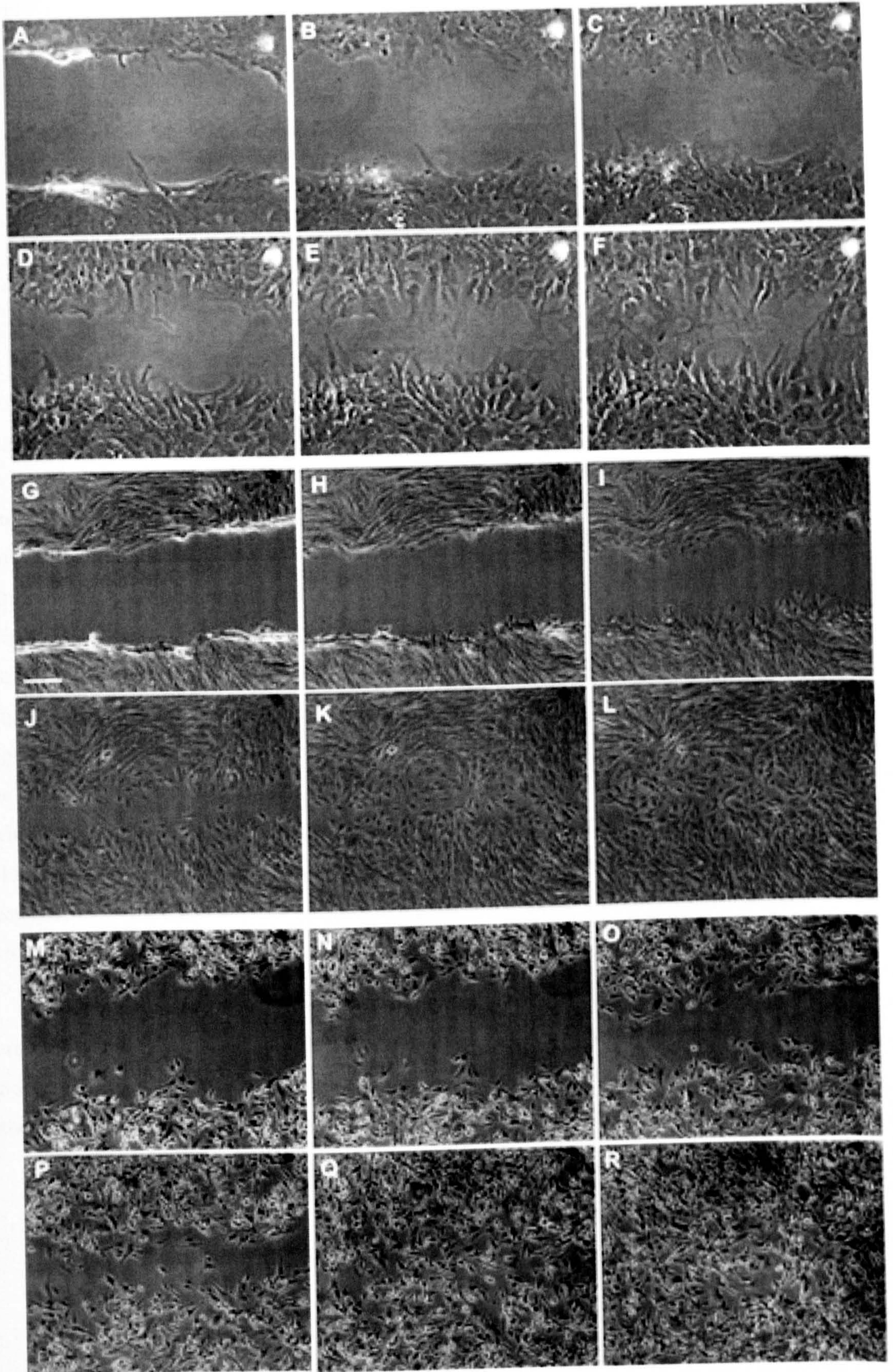
**TABLE 3.1**

Table summarizing the key observations of glioma behaviour as seen by time-lapse microscopy recordings of cells migrating in a wound-healing assay. N/a stands for non-applicable.

### 3.3.2 Increased malignancy of glioma cells corresponds to an increase in their migration rate

In order to investigate the migration behaviour of the gliomas more closely, time-lapse movies of U373, U138 and U87 cells migrating in a wound-healing assay were recorded over a period of 16 hours (Figure 3.2 and Table 3.1). These were then compared to time-lapse recordings of migrating astrocytes.





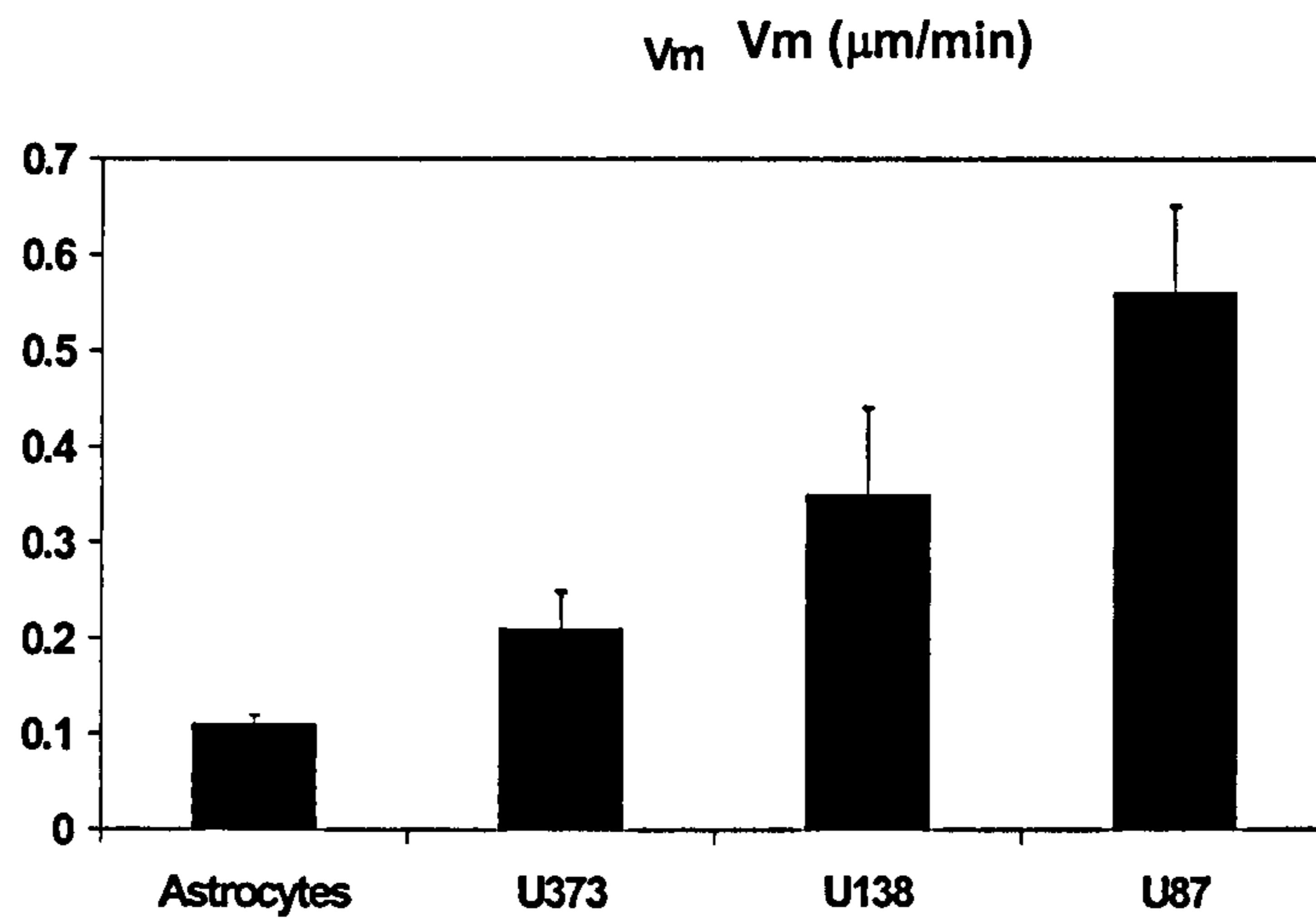
**FIGURE 3.2**

The migration rate of glioma cells increases and polarity of movement decreases with an increase in malignancy. Astrocytes (A-F) and U373 (G-L) cells migrate as a sheet in a polarised way, whereas the U87 (M-R) migrate as individual cells and are non-polarised. Movie stills are shown for astrocytes migrating at  $t=0\text{min}$  (A),  $t=3\text{h}$  (B),  $t=10\text{h}$  (C),  $t=16\text{h}$  (D),  $t=24\text{h}$  (E) and  $t=30\text{h}$  (F), and for U373 and U87 cells migrating at  $t=0\text{min}$  (G, M),  $t=1\text{h}$  (H, N),  $t=4\text{h}$  (I, O),  $t=8\text{h}$  (J, P),  $t=12\text{h}$  (K, Q), and  $t=16\text{h}$  (L, R) of time-lapse movies taken in phase at 1 frame / 5 minutes, using a 10x objective. The scale bar represents  $150\mu\text{m}$ .

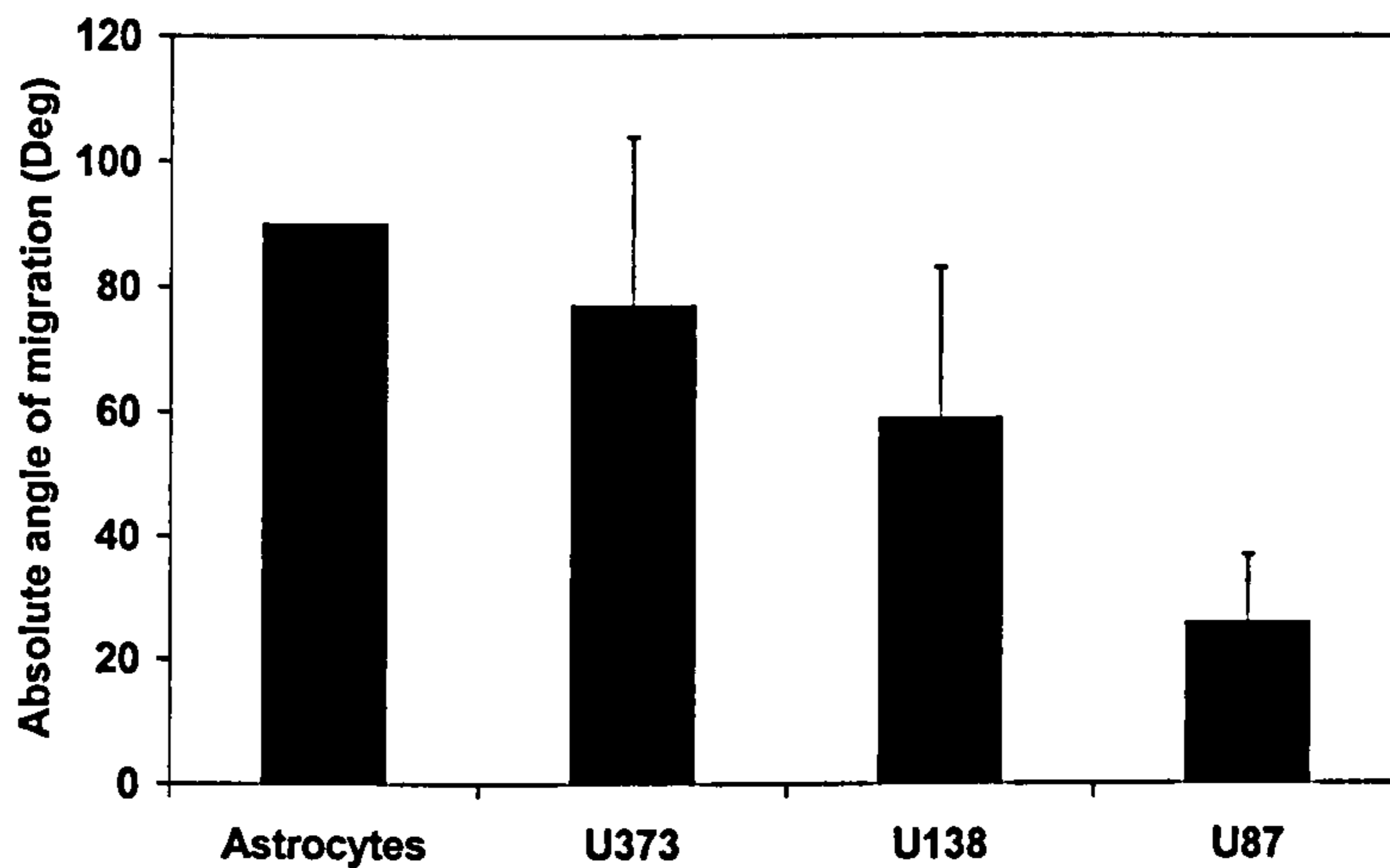
Similarly to primary rat astrocytes, U373 cells, the least malignant tumour cells, migrate as a sheet and are mainly polarised in their migration, i.e. migrate in a direction perpendicular to the wound. However, they migrate faster than primary astrocytes ( $0.21\ \mu\text{m}/\text{min}$  compared to  $0.11\ \mu\text{m}/\text{min}$ ) and are able to close the wound, taken as the time-point when cells from each side of the wound touch each other, in approximately 12h, while astrocytes typically take more than 24 hours to close a wound of the same size [Figure 3.2 (A-F)]. In contrast, the more malignant cells U87 and U138 do not form a monolayer; cells migrate independently of each other and exhibit a non-polarised, random migratory behaviour. Although the overall rate of migration for the U87 and U138 is higher ( $0.56\ \mu\text{m}/\text{min}$  and  $0.35\ \mu\text{m}/\text{min}$ , respectively) than that of U373, they also close the wound in approximately 12h, presumably because of their non-directed migration behaviour (Figure 3.3a). If the average absolute angle of migration is calculated, by taking as  $90^\circ$  the angle when the migration is perpendicular to the wound, then it can be seen that U87 and U138 cells do not exhibit a polarised migratory behaviour, like the one observed for astrocytes and U373 cells (Figure 3.3b).

In addition, compared to primary astrocytes or U373 cells, the highly malignant tumour cells U138 and U87 are not contact inhibited for migration, i.e. when meeting another cell they do not stop and change direction, but frequently crawl on top of each other. A summary of the migration behaviour of these cells is presented in Table 3.1.

a.



b

**FIGURE 3.3**

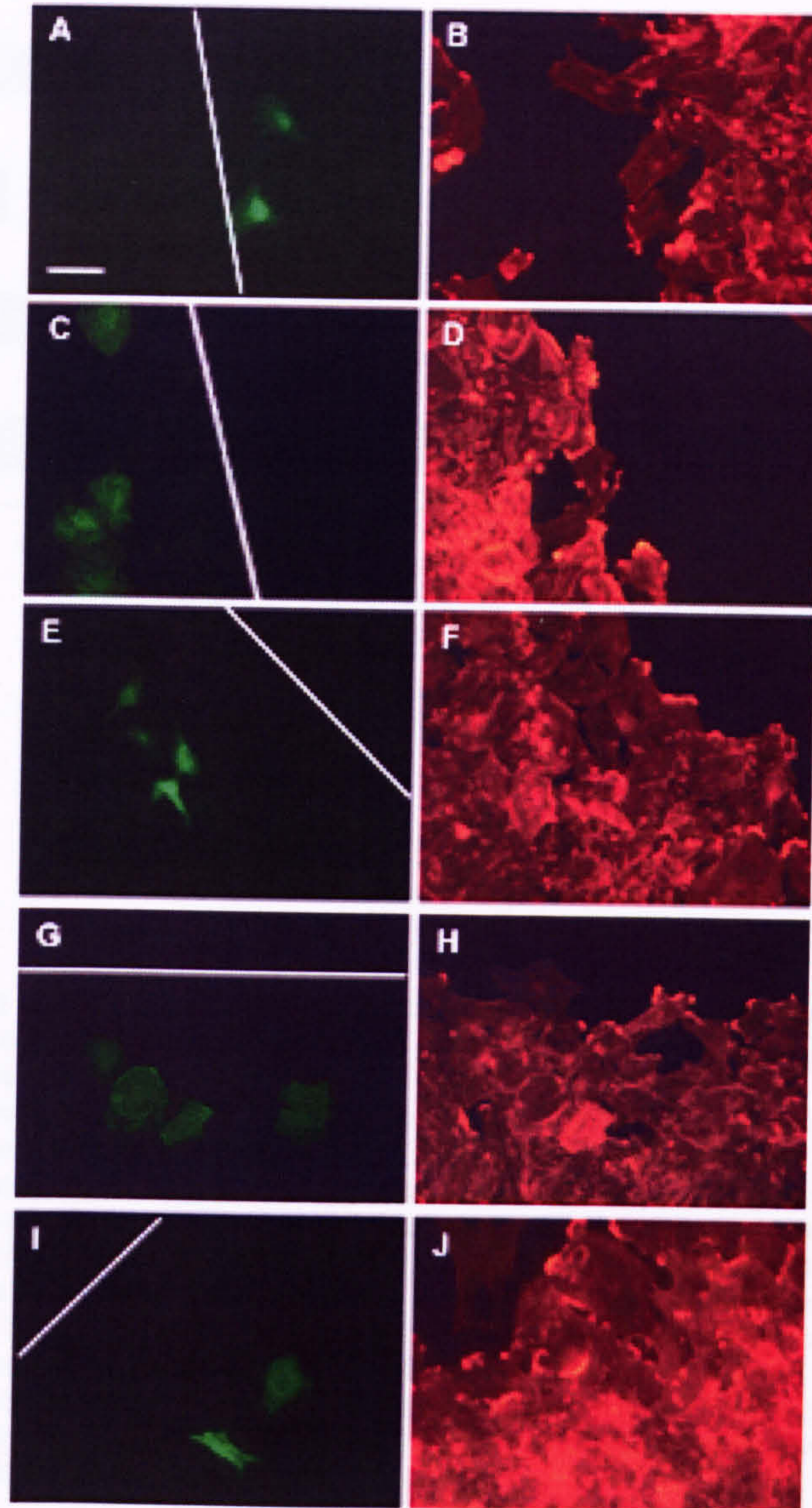
(a) The higher malignancy U87 and U138 cells migrate faster than the lower malignancy cells U373 or primary astrocytes. The graphs were drawn using the measurements acquired with the Metamorph software by tracking 10 individual cells from each time-lapse movie of the U373, U138 and U87. The measurements for the primary astrocytes were provided by Dr. Sandrine Etienne-Manneville. The y-axis represents the mean velocity ( $V_m$ ) / speed of migration measured as  $\mu\text{m}/\text{min}$ . (b) U138 and U87 cells do not exhibit a polarised directed migration and do not migrate at a  $90^\circ$  angle to the wound like astrocytes. The angle measurements were acquired with the Metamorph software.

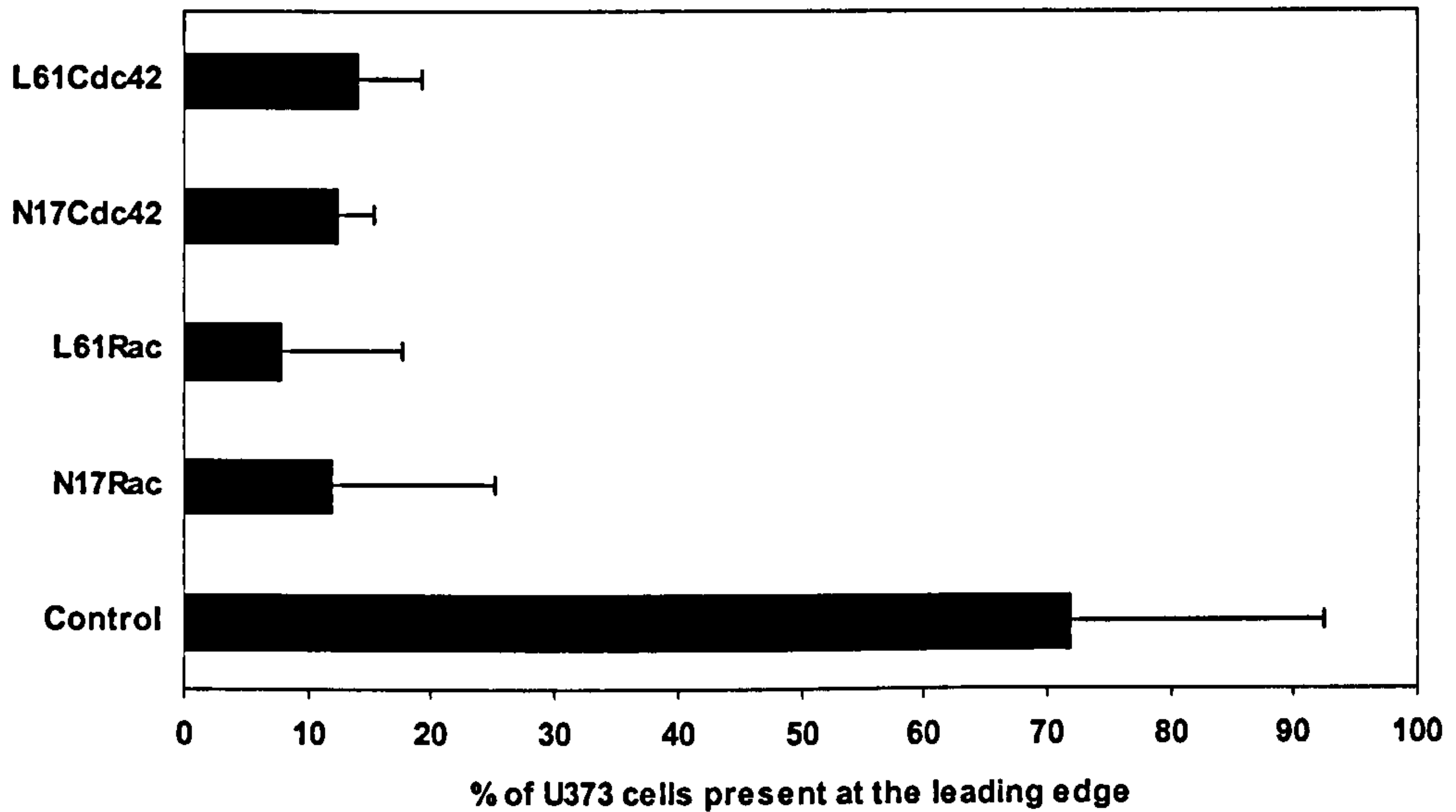
### 3.3.3 Analysis of cell migration after microinjection - Role of Rac and Cdc42

Rac and Cdc42 have been shown to be essential for cell movement and cell polarisation respectively, in astrocytes (Etienne-Manneville and Hall, 2001). Since all three gliomas have a higher rate of migration than primary astrocytes, and the U138 and U87 are no longer polarised in their migration, the requirement of Rac and Cdc42 for their migration behaviour was assessed. Due to the different migratory behaviours of the three gliomas, slightly different assays were used in each case.

#### 3.3.3.1 Assay for U373 cells

For U373 cells, cDNA expression constructs of dominant negative and constitutively active forms of Rac and Cdc42, as well as GFP as a negative control, were injected into the nucleus of cells in the front row of a wound. To examine the effects of the various constructs on migration, the cells were allowed to migrate for 8h and were then fixed and stained for expression of the injected constructs and in order to visualise the position of the injected cells relative to the leading edge of the wound. Phalloidin was used to visualise filamentous actin in all the cells. Since U373 cells, like astrocytes, migrate as a sheet and in a polarised manner, cells in the front row of the wound not inhibited in their migration, are still present at the leading edge after 8h, and are thus scored positively. Cells that have been inhibited in their ability to migrate, on the other hand, are overtaken by the back rows of cells and therefore are no longer present at the leading edge of the wound and these are scored negatively. Figure 3.4 shows that expression of a dominant negative Rac (N17Rac) construct inhibits migration of U373 cells by more than 80% compared to control GFP - injected cells. Interestingly, expression of a constitutively active form of Rac (L61Rac) also inhibits migration of U373 cells to a similar extent as N17Rac. This could be due to the fact that overexpressed L61Rac is no longer specifically localised and activated at the front of the cell and that the cell is thus unable to form a polarised protrusion and specify new adhesion sites at the front. Indeed, as seen in figure 3.4b E,F, U373 cells overexpressing L61Rac extend lamellipodia all around the cell, 'pulling' the cell in all directions at once. Similarly to L61 Rac, microinjection of constitutively active Cdc42 also inhibits migration and cells show lamellipodia all around, presumably because Cdc42 can activate Rac (see above). Interestingly, microinjection of dominant negative Cdc42 also inhibits migration.



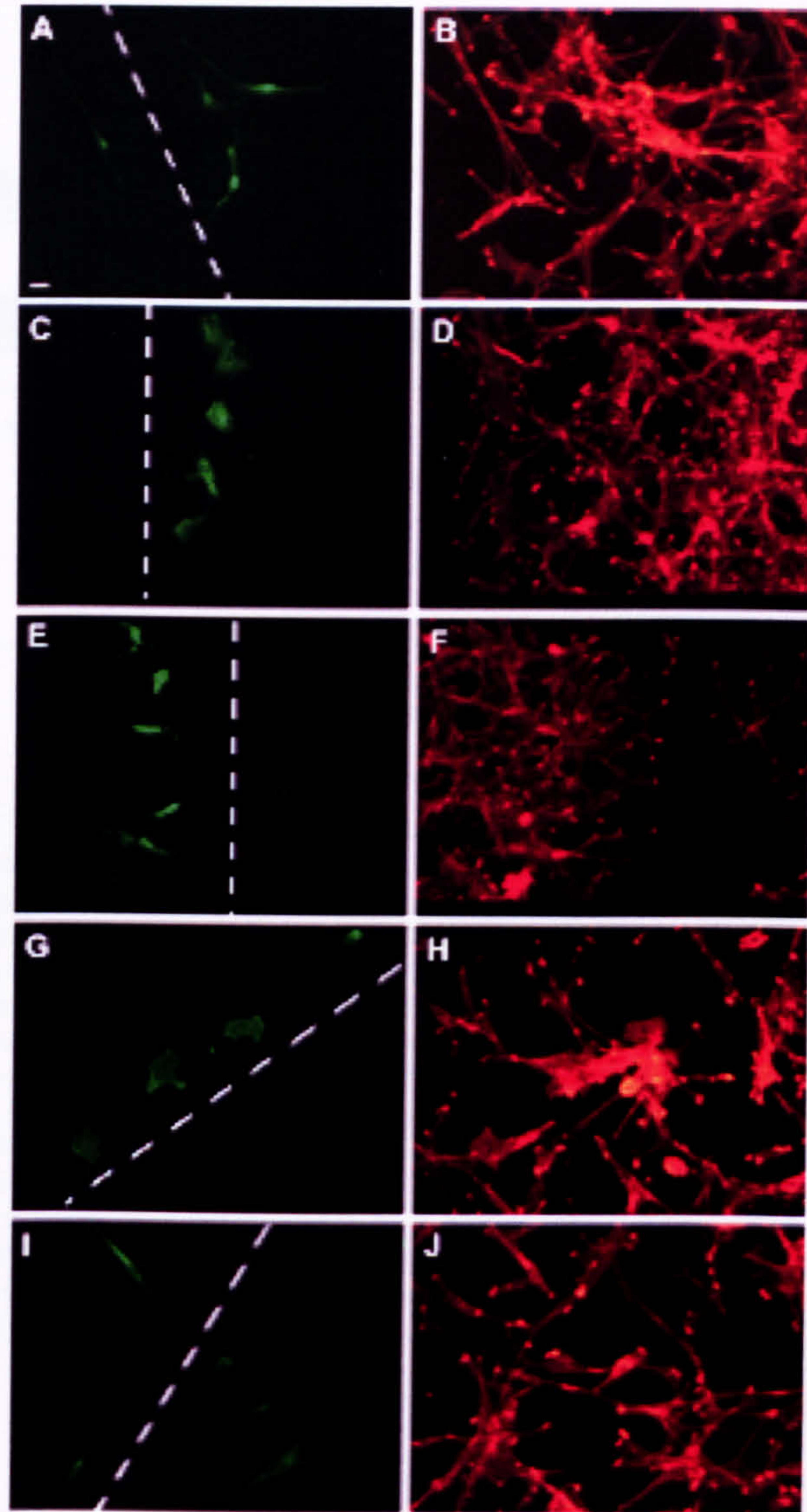
**FIGURE 3.4**

Migration of U373 is dependent on Rac and Cdc42. (a) Representative examples are shown of U373 cells microinjected in a wound-healing assay with cDNA constructs encoding, control GFP (A, B), Myc-epitope tagged L61Rac (C, D), N17Rac (E, F), L61Cdc42 (G, H) or N17Cdc42 (I, J). Microinjected cells were fixed and detected by staining with 9E10 anti-Myc antibody. The actin was visualized using Rhodamine-conjugated phalloidin. Scale bar, 50 $\mu$ m. A white line is drawn to indicate the leading edge of the wound. (b) Quantification of the number of U373 microinjected cells that are found at the leading edge of the wound after migration for 8h. The effect of microinjected cDNA constructs encoding Myc-epitope tagged dominant negative Rac (N17Rac), constitutively active Rac (L61Rac), dominant negative Cdc42 (N17Cdc42) or constitutively active Cdc42 (L61Cdc42) is shown. Results are represented as a percentage of the number of cells found at the leading edge of the wound over the total number of cells injected. Data shown are the mean + SEM of three independent experiments where an average of 100 cells (20cells in each of 5 separate wound edges) were injected for each construct.

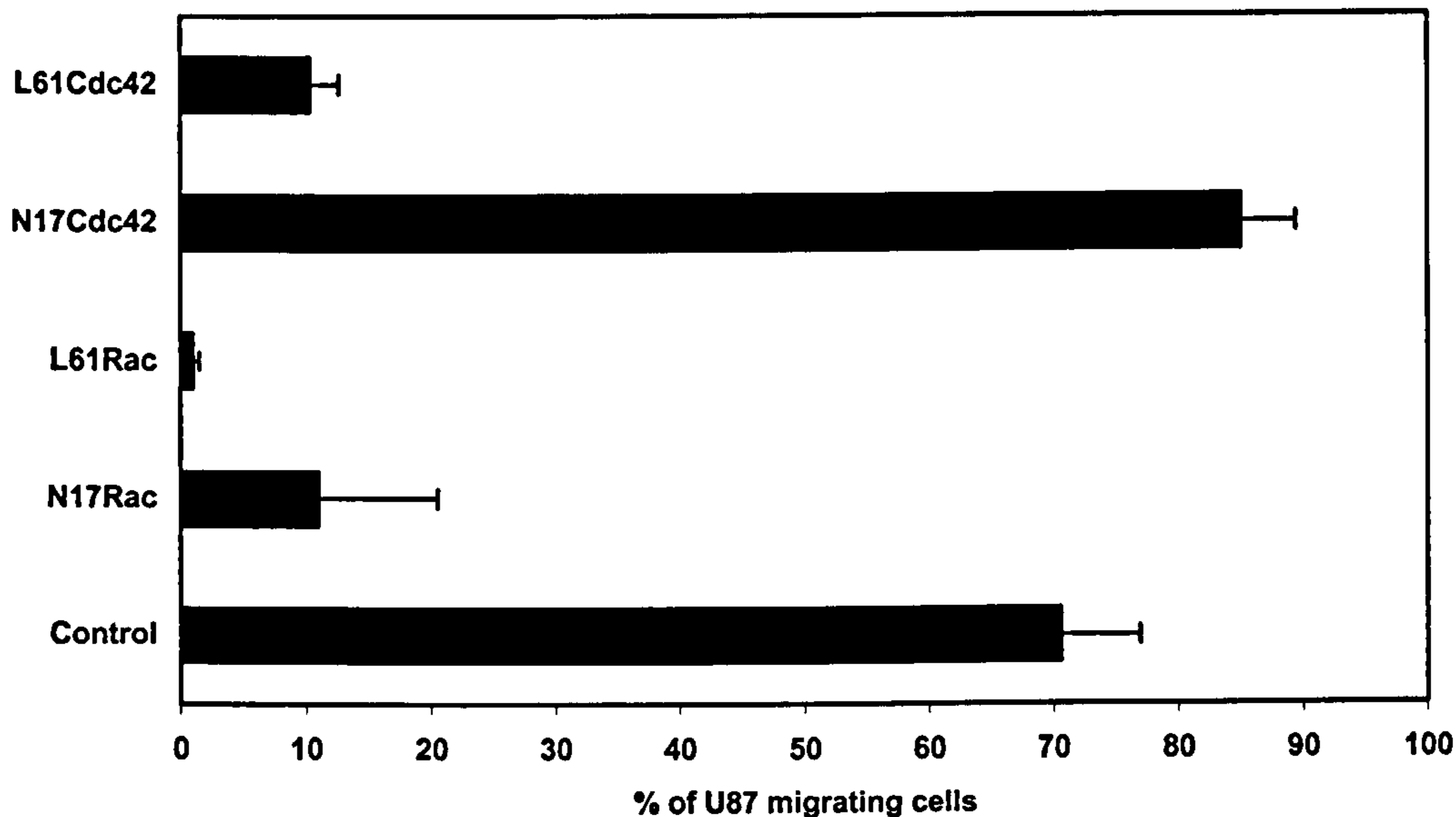
### 3.3.3.2 Assay for U87 cells

Since U138 and U87 cells do not migrate as a sheet but as individual cells, the assay used to examine the effects of microinjecting cDNA expression constructs of dominant negative and constitutively active forms of Rac and Cdc42, as well as GFP as a negative control on the migration of these cells was the following. The various constructs were microinjected into the nucleus of cells in a straight line parallel to that of the 'scratch'. The cells were left to migrate for a total of 8h and were fixed and stained in order to visualize the microinjected cells. Phalloidin was used to visualize filamentous actin of all cells. Cells expressing the various constructs and that were still found in a straight line parallel to the original wound were scored as non-migratory, whereas cells that exhibited a random distribution were scored as positive, i.e. still retaining the ability to migrate.

Similarly to U373 cells, the migration of U87 and U138 cells was found to be dependent on Rac, as overexpression of both dominant negative and constitutively active forms of Rac inhibited their migration by more than 90% (Figure 3.5, and data not shown). In contrast to astrocytes (data not shown) and U373 cells however, microinjection of dominant negative Cdc42, had no effect on the migration of U87 and U138 cells. U87 and U138 cells are not polarised and would therefore be expected to already have very low endogenous levels of active Cdc42, or to have acquired mutations downstream of Cdc42. In contrast, a constitutively active form of Cdc42 inhibited migration of U87 cells, but this could simply reflect the subsequent activation of Rac, as discussed earlier for the U373 cells. The cells acquire a much more spread, flattened morphology, with a lamellipodium extending all around the cell, reminiscent of an L61Rac phenotype [Figure 3.5b, (C, G)].





**FIGURE 3.5**

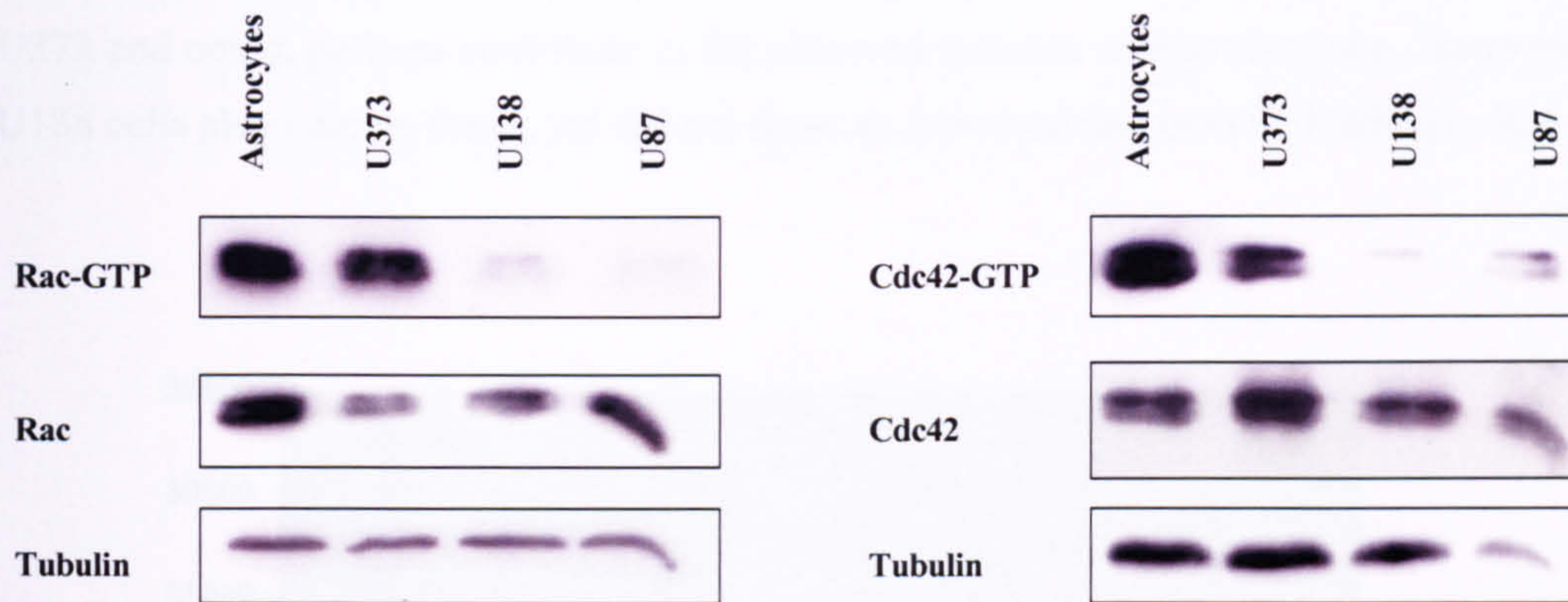
Migration of U87 cells is dependent on Rac and is not inhibited by N17Cdc42. (a) Representative examples are shown of U87 cells microinjected in a wound-healing assay with vectors encoding, control GFP (A, B), Myc-epitope tagged L61Rac (C, D), N17Rac (E, F), L61Cdc42 (G, H) or N17Cdc42 (I, J). Microinjected cells were fixed and detected by staining with 9E10 anti-Myc antibody. The actin was visualized using Rhodamine-conjugated phalloidin. Scale bar, 50 $\mu$ m. A dotted white line is drawn to indicate the direction of the wound parallel to which cells were microinjected in a straight line. (b) Quantitation of the number of U87 microinjected cells that are found still aligned in the direction of the wound after migration for 8h. The effects of vectors encoding Myc-epitope tagged cDNAs of dominant negative Rac (N17Rac), constitutively active Rac (L61Rac), dominant negative Cdc42 (N17Cdc42) or constitutively active Cdc42 (L61Cdc42) are shown. Results are represented as a percentage of the number of cells still aligned in the direction of the wound over the total number of cells injected. Data shown are the mean + SEM of three independent experiments where an average of 100 cells (20 cells in each of 5 separate wound edges) were injected for each construct.

### 3.3.4 Relative levels of active Rac and Cdc42 in gliomas

To examine whether the endogenous levels of active Rac and Cdc42 could account for the observed increase in migration rate and loss in polarity, pull-down assays were performed to investigate the GTP-loading on each of the GTPases. These assays take advantage of the fact that effector proteins bind specifically only to the active, GTP-bound form of the small GTPases and can be used to precipitate only the activated GTPases directly from cell lysates. In the case of Rac and Cdc42, the Cdc42/Rac interacting binding (CRIB) region of the effector protein p65PAK was used to specifically precipitate Rac-GTP and Cdc42-GTP.

Figure 3.6 shows that while the levels of active Rac are higher in U373 than primary astrocytes, they are lower in U87 and U138 cells. In fact they are almost undetectable in these two cell lines, even though the total levels of Rac protein are similar. This is very surprising given that the migration rate of these gliomas is much higher than that of U373 cells or astrocytes. There are several possible explanations for this finding: First, although the total cellular levels of active Rac are decreased, the amount of active Rac at the leading edge of the cell where it is required for migration, could still be increased (this could, for example, be measured with the use of FRET). Secondly, there could be an increase in the stable downstream association of Rac with targets, which prevent it to be accessible in the 'pull-down' assay. Thirdly, the pull down assay might not be a true representation of the activation of Rac in cells, if Rac is rapidly cycling between its GTP and GDP forms (Lin et al., 1997).

Using the pull-down assay to look at levels of active Cdc42, it was found that U87 and U138 cells have lower levels of active Cdc42 compared to the U373 and primary astrocytes. This correlates with the loss in polarity observed previously in the migration assay. However, given the similar results found for Rac (see above), interpretation of this result is not straightforward.



**FIGURE 3.6**

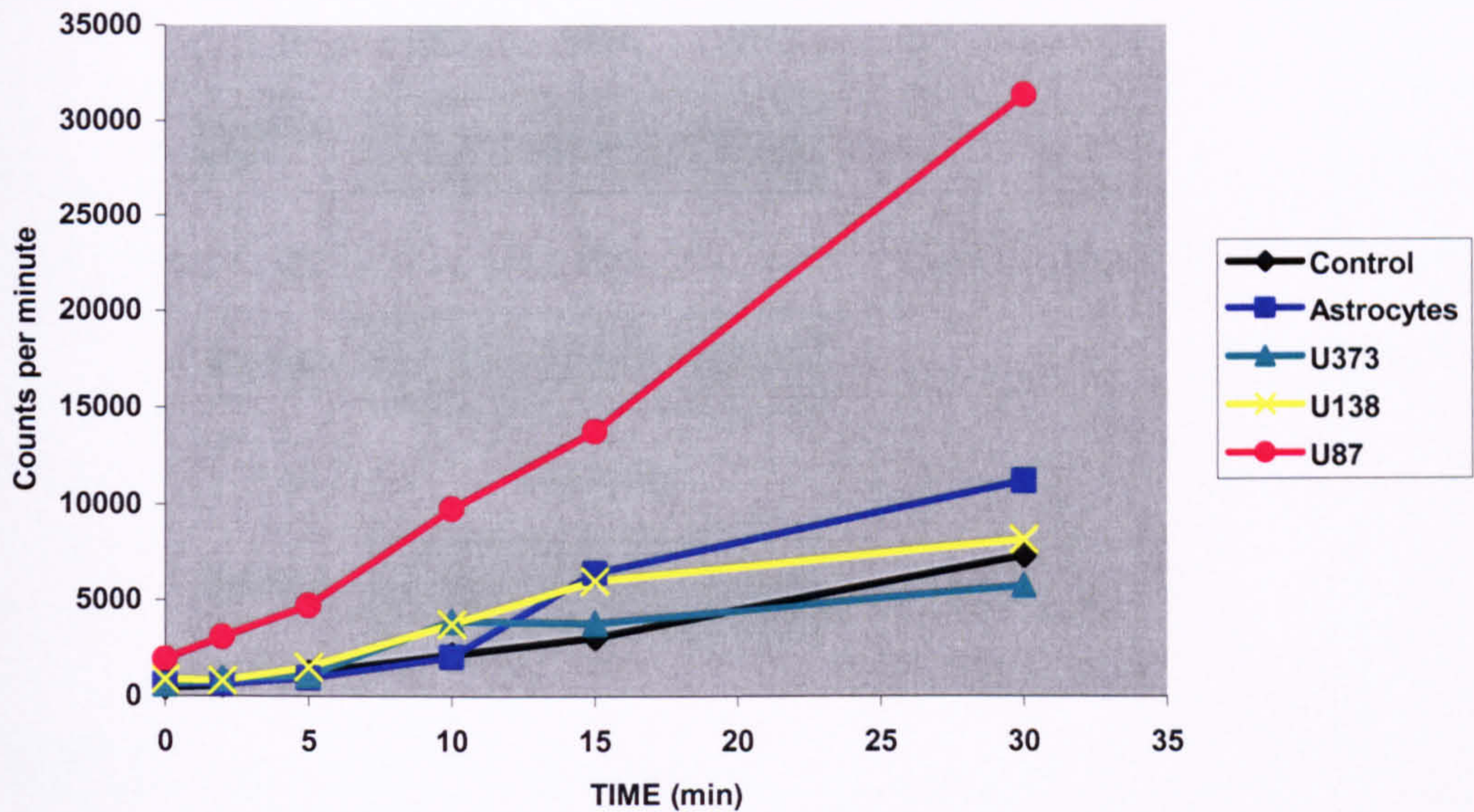
Comparison of the levels of active Rac and Cdc42 in astrocytes, U373, U138 and U87. U87, U138, U373 cells and primary astrocytes were lysed and incubated with GST-PAK-CRIB to isolate GTP-bound Rac and Cdc42. Levels of Rac and Cdc42 were monitored by Western blot analysis using anti-Rac and anti-Cdc42 antibodies. Total protein levels were monitored using an anti-tubulin antibody. U87 and U138 cells have lower levels of active Rac and Cdc42.

In order to investigate further the cellular activity of Cdc42 and Rac, two approaches were taken. The first was to look at the cellular GEF activity (discussed in section 3.3.5) and the second was to look at the activation level of downstream effectors of the Rac and Cdc42 (see section 3.3.6).

### 3.3.5 Determination of cellular GEF activity

To investigate the activation rate of Rac and Cdc42, *in vitro* GEF assays were performed with lysates from each of the three glioma and astrocytes, using bacterially purified GST-tagged Rac protein. Rac is loaded *in vitro* with GDP and subsequently incubated with control buffer or with cell lysate and the exchange reaction is allowed to proceed in the presence of [<sup>3</sup>H]-GTP (ON-rates). Aliquots are taken at fixed time-points and the reaction is terminated by diluting in cold buffer. The time-course of exchange of GDP for [<sup>3</sup>H]-GTP is assessed by scintillation counting (see Materials and Methods).

As shown in figure 3.7, GTP-loading of Rac with extracts from U87 cells is significantly higher compared to U138, U373 and astrocyte lysates. This supports the possibility of an active GEF promoting fast cycling of Rac compared to the astrocytes and U373 and could, perhaps contribute to the observed increase in migration rate. However, U138 cells also migrate faster, yet did not show an increased rate of GTP-binding to Rac.



**FIGURE 3.7**

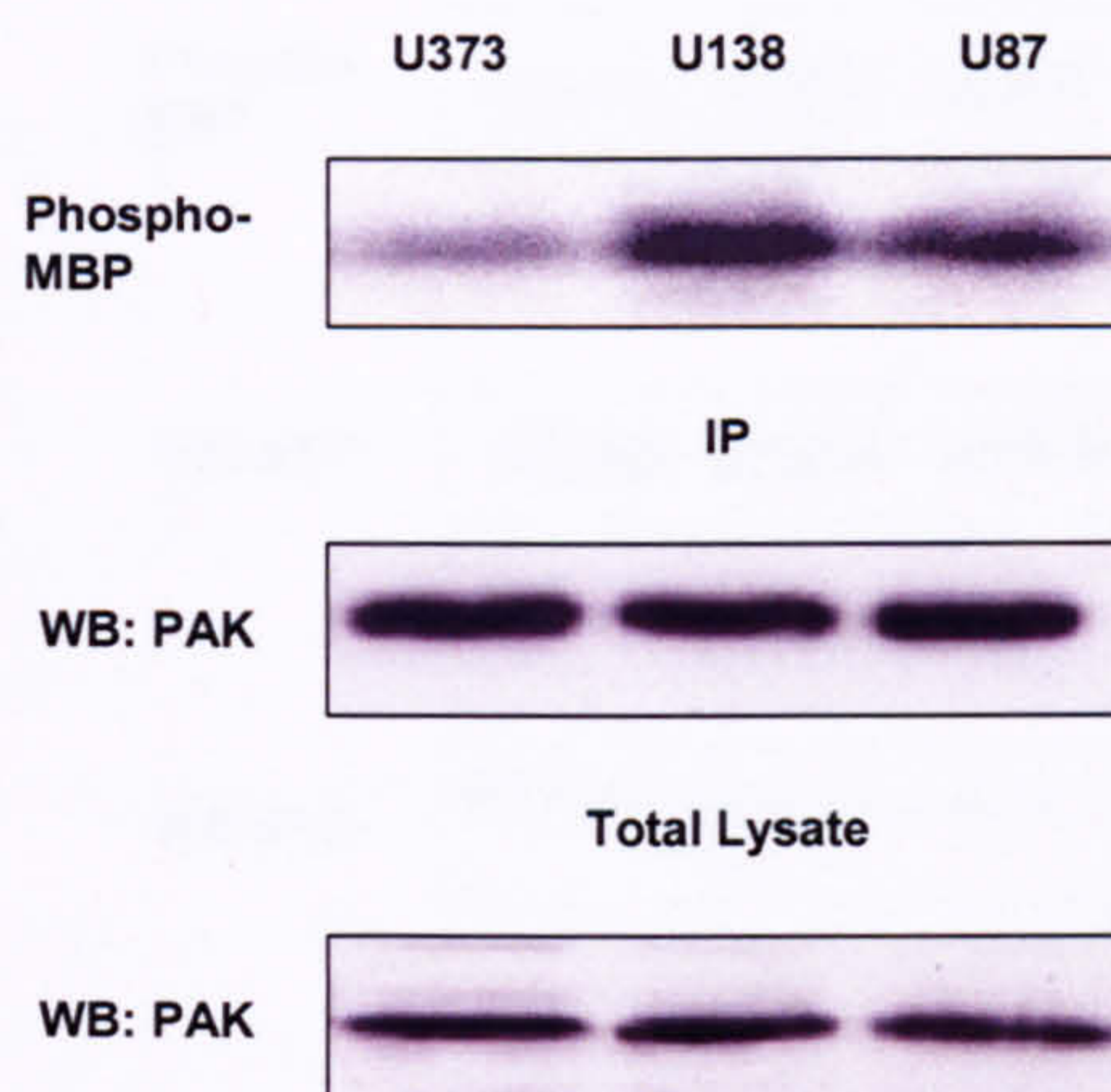
The overall GEF activity towards Rac in U87 cells is increased compared to the other gliomas and astrocytes. The exchange rate of GDP-loaded Rac for [<sup>3</sup>H]-GTP was measured for every cell lysate or control buffer by taking aliquots at t=0, 5, 10, 15, 20 and 30 minutes, followed by scintillation counting. The graph represents the change in the number of counts per minute for each sample over time.

### 3.3.6 PAK and PKC $\zeta$ activity as downstream effectors of Rac and Cdc42

To gain further insight into the signaling mechanisms involved in the faster migration rates and loss of polarity observed in the gliomas, the activation state of downstream effectors of Rac and Cdc42 was examined. PAK, a Ser/Thr kinase, is believed to be one of the main targets of active Rac and/or active Cdc42 and an important player in the regulation of actin dynamics during cell adhesion and migration.

In order to look at the activation levels of endogenous PAK in the gliomas, PAK was immunoprecipitated from cell lysates and its activity towards myelin basic protein (MBP)

was assessed in an *in vitro* kinase assay. As shown in figure 3.8, MBP phosphorylation is significantly higher in U138 and U87 cells (~3.8 and 3.3 fold respectively) compared to U373 cells. This suggests an increase in PAK activation in U138 and U87 probably reflecting an increase in the activation levels of Rac and/or Cdc42, despite the very low levels of Rac and Cdc42 found in the 'pull-down' assays.

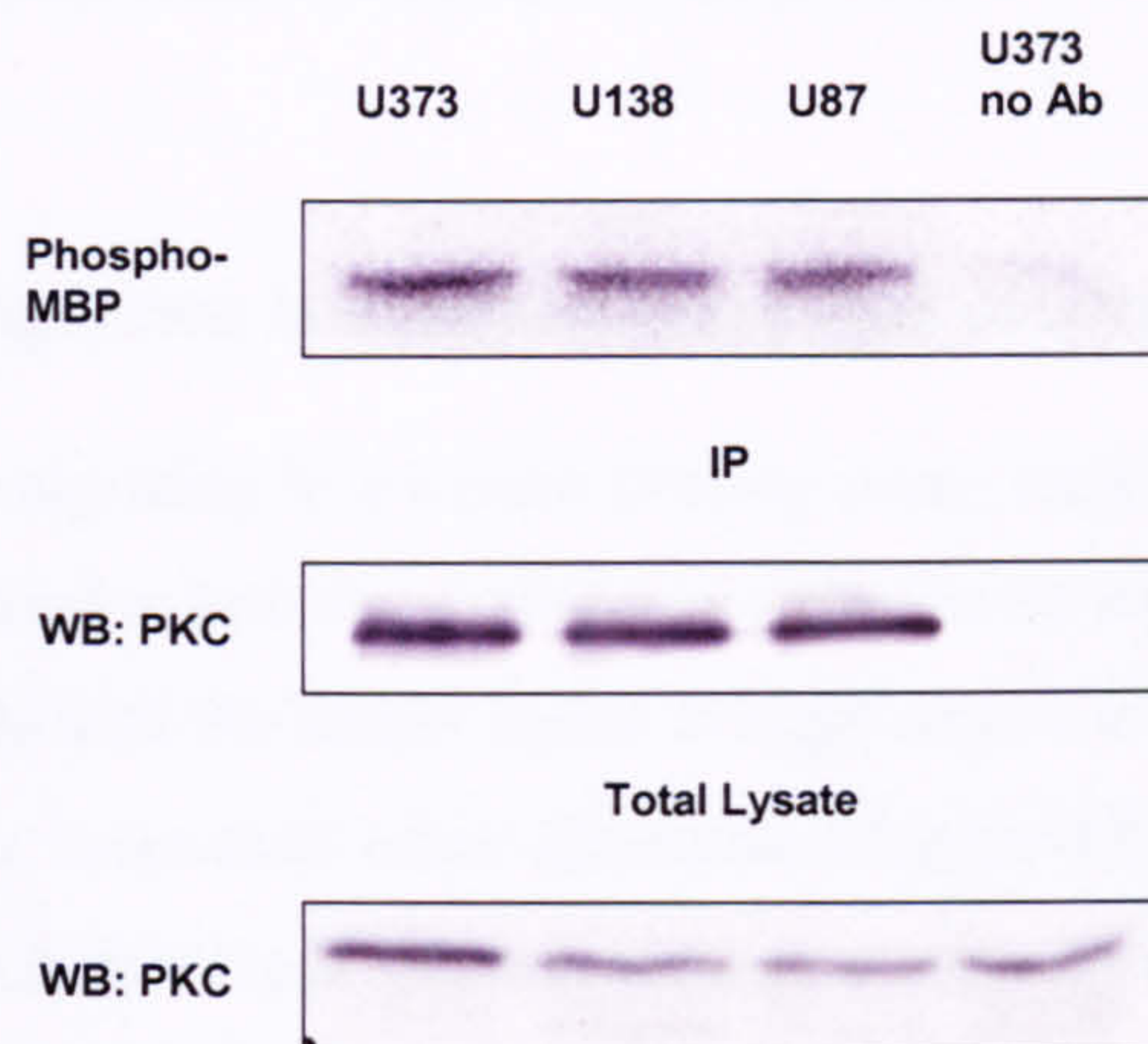


**FIGURE 3.8**

PAK activity is higher in U138 and U87 compared to U373. Endogenous PAK was immunoprecipitated from cell lysates and incubated for 15min at 37°C with myelin basic protein (MBP) in the presence of [<sup>32</sup>P]-ATP. The phosphorylation of MBP was assessed by SDS-PAGE and subsequent Western blot analysis and exposure to film. The levels of PAK in the IP and in the total lysates was analysed by blotting with an anti-PAK antibody.

In its active state, Cdc42 can also activate the atypical protein kinase C  $\zeta$  (PKC $\zeta$ ) when complexed with Par6. The specific activation of this complex at the leading edge of migrating cells is required to determine the polarity and direction of movement of primary astrocytes (Etienne-Manneville and Hall, 2001). Therefore, the activation of endogenous PKC $\zeta$ , as a direct downstream target of Cdc42-GTP, was assessed in an *in vitro* kinase assay. MBP was used as a substrate. As shown in figure 3.9, there is no significant difference in the phosphorylation of MBP by PKC $\zeta$  when immunoprecipitated from the various glioma lysates. This suggests that deregulation in the overall activation of PKC $\zeta$  is not the main cause for the observed loss in polarity of the U138 and U87

cells, although it is still possible that the mislocalisation or mislocalised activation of PKC $\zeta$  in the gliomas, could contribute to loss of polarity.



**FIGURE 3.9**

PKC $\zeta$  activity does not reflect the decrease observed in the levels of Cdc42-GTP or the subsequent loss in polarity. Endogenous PKC $\zeta$  was immunoprecipitated from cell lysates and incubated for 15min at 37°C with MBP in the presence of [<sup>32</sup>P]-ATP. The phosphorylation of MBP was assessed by SDS-PAGE and subsequent Western blot analysis and exposure to film. The levels of PKC in the IP and in the total lysates was analysed by blotting with an anti-nPKC $\zeta$  antibody.

### 3.4 Discussion

Migration is a complex process that requires the precise co-ordination of a large number of signaling pathways. In human gliomas, most of the genetic mutations identified to date result either in the disruption of cell cycle regulation pathways, or in the disruption of signaling cascades downstream of receptor tyrosine kinases. These mutations account for the increase in proliferation, cell survival and invasion characteristics acquired by these tumours. For example, amplification of the EGFR or PDGFR and/or over-expression of their corresponding ligands, results in constitutive activation of the corresponding downstream signaling pathways, which lead among other

things, to cell de-differentiation and tumour invasion by reactivation of the mechanisms underlying early glial migration during development.

In this chapter, the aberrant migration of three human glioma cell lines (U373, U138 and U87) was characterised by use of a wound-healing assay and, consistent with previous studies in other cell types, the small GTPases Rac and Cdc42 were shown to also play a role in the regulation of tumour cell migration.

### **3.4.1 Glioma migration is different from that of astrocytes**

Astrocytes, when migrating in a wound-healing assay, migrate as a sheet and begin by extending a long protrusion into the wound perpendicular to the direction of the 'scratch'. The cells then migrate into the empty space at right angles to the wound and stop when the two wound edges meet each other (Etienne-Manneville and Hall, 2001). The results in section 3.3.2 show that gliomas exhibit a very different migratory behaviour. The lowest grade glioma cell line U373 exhibits a mesenchymal form of migration. The cells still migrate as a sheet, they polarise a lamellipodium into the wound and when the two wound edges meet and the wound closes they stop. Compared to astrocytes, however, their migration rate is higher (almost 2-fold) and the protrusion at the front of the cell is much smaller. The more malignant U138 and U87 cells both show a similar mode of migration that differs greatly from that of astrocytes and U373 cells. U138 and U87 cells do not form a confluent monolayer and do not migrate as a sheet but as individual cells and display a much more characteristic amoeboid movement (Friedl and Wolf, 2003). The cells are not polarised for migration at right angles to the wound but instead their movement is much more random. They eventually close the wound, but they are not contact inhibited in their migration, i.e. they do not stop when they meet each other. They also have a much faster rate of migration compared to that of U373 and astrocytes.

### **3.4.2 A role for Cdc42?**

Cdc42 has been shown to play a crucial role in specifying cell polarity in various cell types, including primary astrocytes and fibroblasts, where it most likely specifies the location of the cell protrusion or lamellipodium (Etienne-Manneville and Hall, 2001; Nobes and Hall, 1999). Furthermore, inhibition of Cdc42 has been shown to block macrophage chemotaxis towards a gradient of CSF-1, probably by disrupting the cells'

ability to sense the gradient, since the macrophages are still able to move (Vanhaesebroeck et al., 1999). The mechanism of how Cdc42 regulates chemotaxis is not known. Cdc42 promotes localised actin polymerization to form filopodia, which are thought to be important in probing the extracellular milieu and this might contribute to its role in chemotaxis and the specification of the cell protrusion/lamellipodium.

A role for Cdc42 in the aberrant migration of glioma cells was demonstrated here by expressing a dominant negative form of Cdc42 (N17Cdc42). As had previously been shown for primary rat embryonic fibroblasts, microinjection of N17Cdc42 in a wound-healing assay significantly impaired the migration of the normally polarised U373, as the cells were no longer found at the leading edge of the wound in contrast to control-injected cells. N17Cdc42, however, did not seem to affect the migration of the already non-polarised U87 cells. An investigation in the levels of active Cdc42 in this chapter showed that a decrease in the levels of Cdc42-GTP is consistent with a loss in polarity and confirmed the role of Cdc42 as a key regulator in this process. The highly malignant U138 and U87 cells, which were shown not to be polarised in their migration in a wound-healing assay, unlike the lower malignancy grade U373 and primary astrocytes, exhibited very low levels of active Cdc42 as seen by pull-down assay. Care should be taken however in interpreting pull-down assays, since, the levels of Rac-GTP were clearly not consistent with the observed rates in migration. Pull-down assays are typically a reflection of steady-state levels of GTP loading on the GTPases and may not account for differences in the GTPase rates of cycling. Therefore, even though U138 and U87 cells displayed lower levels of Cdc42-GTP compared with the polarised U373 and primary astrocytes, the possibility of a faster cycling of the GTPase (see also section 3.4.3 on Rac), cannot be excluded, especially when an increased GEF activity for Cdc42 was demonstrated for the U87 and U138 cells, by *in vitro* GEF assays (data not shown).

The precise mechanism of how Cdc42 regulates polarity is not known, but work in neutrophils and astrocytes has identified some of the players involved. In astrocytes, Cdc42 regulates the re-orientation of the MTOC and hence the direction of movement by specifying the formation of the protrusion at the front of the cell, in this case at right angles to the wound. It is thought to do so by binding and activating, in its GTP-bound state, a complex of Par-6 and PKC $\zeta$ . The specific activation of this complex at the front of these cells is crucial for determining the direction of movement (Etienne-Manneville and Hall, 2001). It is therefore not surprising that overexpression of a constitutively active form of Cdc42 (L61Cdc42) inhibited the migration of U373 cells, as shown in section



3.3.3. U87 cells, even though already not polarised, were also inhibited in their migration. When the activation levels of PKC $\zeta$  were assessed by an *in vitro* kinase assay, however, no difference was observed between the levels of active PKC $\zeta$  in the U373 and the U138 and U87 cells. Recently, it was shown that the Cdc42/Par-6/PKC complex regulates cell polarity by promoting the phosphorylation of the kinase GSK3 $\beta$  specifically at the leading edge of migrating cells and thus allowing the scaffold protein APC to associate with the plus ends of microtubules (Etienne-Manneville and Hall, 2003). Therefore, the observed loss in polarity of the U87 and U138 glioma cells could be at the level of GSK3 or APC.

### 3.4.3 A role for Rac?

Rac has previously been shown to be a key regulator of cell migration (Nobes and Hall, 1999; Ridley et al., 1992). In *Drosophila*, it regulates border cell migration during oocyte maturation (Duchek et al., 2001). In this chapter, the role of Rac in glioma migration was investigated and, in agreement with what is known for the migration of other cell types, Rac was found to be essential for the migration of all three gliomas. Overexpression of dominant negative and constitutively active forms of Rac inhibited the migration of U373, U138 and U87 cells. Interestingly, - looking by pull down assay - less activated Rac was found in the highly malignant U138 and U87 glioma cells compared to primary astrocytes. This is very different from what has been found in other tumours; in breast cancer tumours, an increase in cell migration corresponds to an increase in Rac activation through overexpression of the Rac-specific GEF TIAM-1 (Bourguignon et al., 2000). One explanation for this could be that other additional genetic mutations acquired by these aggressive gliomas lead to the downregulation of active Rac. Alternatively, the lower levels of Rac-GTP could be a reflection of a faster cycling of the GTPase or that the GTPases are more efficiently localised at the front of the migrating cells in the gliomas (as discussed in section 3.3.4). A closer look at the total GEF activity in the various cell lysates revealed a very high GEF activity towards Rac in the U87 cell lysate, whereas the U138, and U373 cell lysate GEF activity was not significantly affected. There have been approximately 60 GEFs identified in humans and the GEF activity seen in U87 cells could be attributed to either a single GEF or a mixture of GEFs (Schmidt and Hall, 2002). Isolation of the active GEF(s) from U87 lysate would be a huge challenge, and instead, an alternative approach was chosen, in order to examine possible regulatory

pathways upstream of GEFs, focusing on the PI3-kinase/PTEN pathway (see section 3.4.4 and chapters 4, 5).

Investigation into the activation state of PAK, a common downstream effector of Rac and Cdc42, revealed that PAK was significantly more active in both U87 and U138 cells compared to U373 cells. PAK had previously been shown to lie downstream of the receptor tyrosine kinases PDGFR and EGFR, which are frequently upregulated in gliomas. Activation of PAK in U138 and U87 is therefore consistent with a pathway leading from PDGFR/EGFR to the activation of Rac and the subsequent activation of PAK (He et al., 2001; Nobes et al., 1995; Ridley et al., 1992).

#### 3.4.4 Regulation of Rac and Cdc42

This chapter presented a more thorough investigation of the key features that define the aberrant migration of three human gliomas and confirmed the role of Rac and Cdc42 as key regulators of cell polarity and migration. Still, however, very little is known about the mechanisms by which Rac and Cdc42 control cell movement and especially how they control directed cell migration. Recent work in neutrophils suggests the establishment of a cellular asymmetry that involves the interplay of Rho GTPases and the lipid PI(3,4,5)P<sub>3</sub>, a product of PI 3-kinase, in the establishment of cell chemotaxis. Whether PI3K and PI(3,4,5)P<sub>3</sub> lie upstream or downstream of the Rho GTPases in the regulation of cell movement is not yet clear. It has previously been shown, however, that several GEFs, like Vav, TIAM-1, SWAP-70 and p-Rex-1, are subject to PI(3,4,5)P<sub>3</sub> activation (Schmidt and Hall, 2002). PTEN, a lipid and protein phosphatase, is believed to be the main antagonist of the PI 3-kinase signaling pathway by down-regulating the levels of PI(3,4,5)P<sub>3</sub> in the cell and is absent in all three glioma cell lines studied here. Increased levels of PI(3,4,5)P<sub>3</sub> in the gliomas could therefore account for the increase in GEF activity observed in U138 and U87 cells. Indeed, treatment of U138 and U87 cells with wortmannin (100nM), inhibited their ability to close the wound in a wound-healing assay, compared to non-treated control cells (section 4.3.3), confirming therefore a role of PI(3,4,5)P<sub>3</sub> in the migration of these cells. The role of PTEN in glioma cell migration was therefore more closely investigated in the results chapters that follow.

## **Chapter 4**

# **Results – The Role of PTEN in Glioma Cell Migration**

### **4.1 Summary**

Many GEFs for Rac are regulated by PI(3,4,5)P<sub>3</sub> levels, and the results in chapter 3 suggest that there might be an increased GEF activity towards Rac in at least the U87 cells. The levels of PI(3,4,5)P<sub>3</sub> in the cell are controlled primarily by PI3-kinase and PTEN. Interestingly, PTEN is a tumour suppressor protein that is frequently deleted in many human cancers, including gliomas. In this chapter the role of PTEN in the migration of the glioma cells was investigated. It was confirmed that all three glioma cell lines studied (U373, U138, U87) lack PTEN and have thus higher levels of PI(3,4,5)P<sub>3</sub>. This suggests that the resultant increase in PI(3,4,5)P<sub>3</sub> levels could account for the higher rate of migration described in the previous chapter, possibly through the activation of Rac. Microinjection of wtPTEN inhibits the migration of these gliomas, but does not affect astrocyte migration. Interestingly, however, it was revealed that the protein, but not lipid phosphatase activity is needed to inhibit migration. Furthermore, deletion analysis revealed that PTEN inhibits glioma cell migration through a previously uncharacterized mechanism.

## 4.2 Introduction

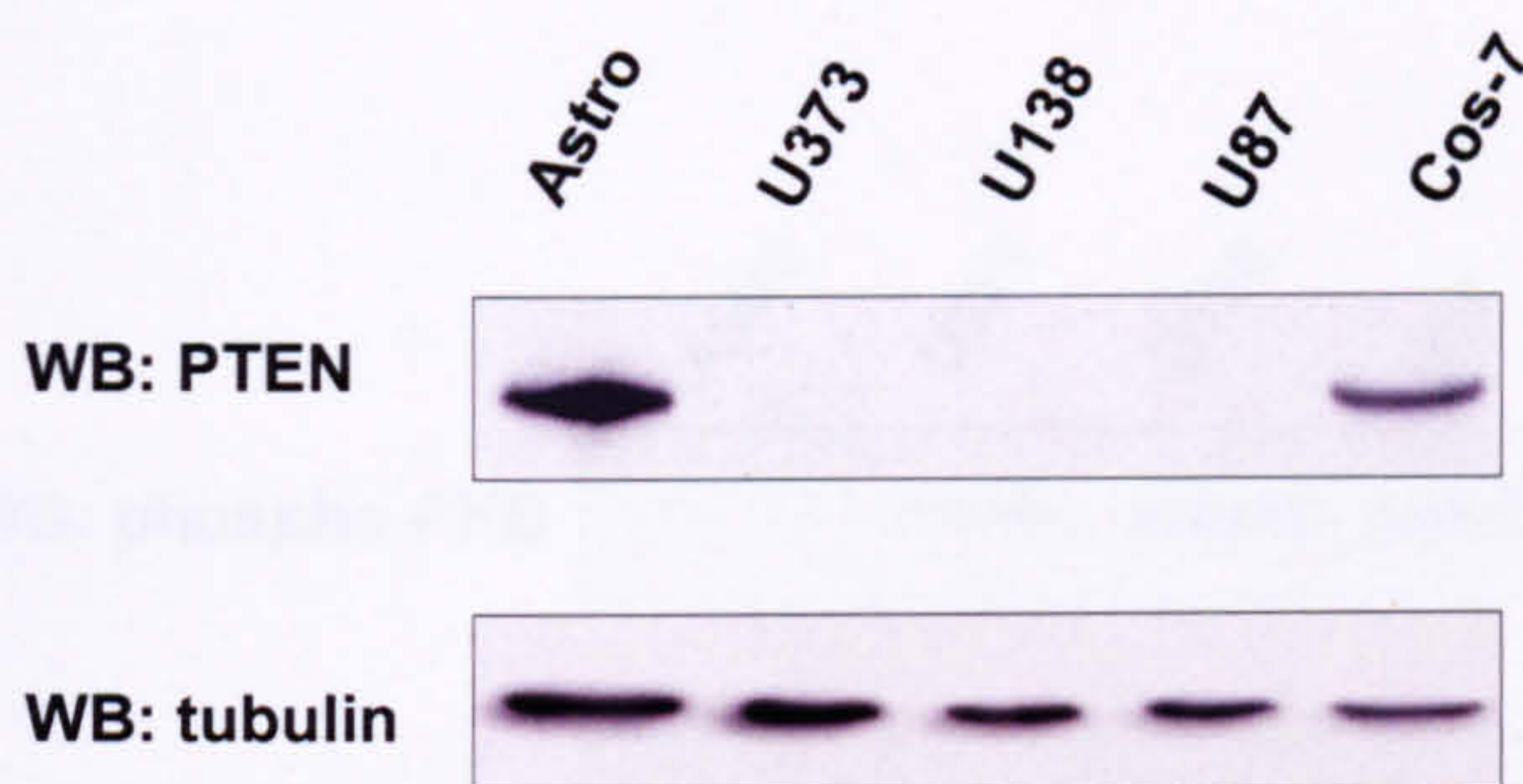
The dual lipid/protein phosphatase PTEN is a tumour suppressor frequently deleted in multiple human advanced cancers, including astrocytomas and glioblastomas (Li et al., 1997a). The major physiological substrate of PTEN is PI(3,4,5)P<sub>3</sub> and to a lesser extent PI(3,4)P<sub>2</sub> and PI(3,5)P<sub>2</sub>, and it acts, therefore, as an antagonist of the PI 3-kinase signaling pathway (Lee et al., 1999; Maehama and Dixon, 1998). Studies in *C. elegans*, *Drosophila* and mammalian cells have revealed that PTEN and PI 3-kinase play major roles both during development and in the adult to control cell size, growth and survival (Huang et al., 1999; Mihaylova et al., 1999). The loss of PTEN found in human tumors leads to an increase in PI(3,4,5)P<sub>3</sub> and the uncontrolled stimulation of growth and survival signals (Downes et al., 2001).

The PTEN protein consists of a PI(4,5)P<sub>2</sub> binding motif at the start of its N-terminus, followed by the catalytic phosphatase domain, which can act on both protein and lipid substrates, a C2 domain and a PDZ binding motif at its extreme C-terminus. In this chapter the role of PTEN in the migration of the gliomas was examined and the contribution of the various PTEN domains on cell migration was investigated.

## 4.3 Results

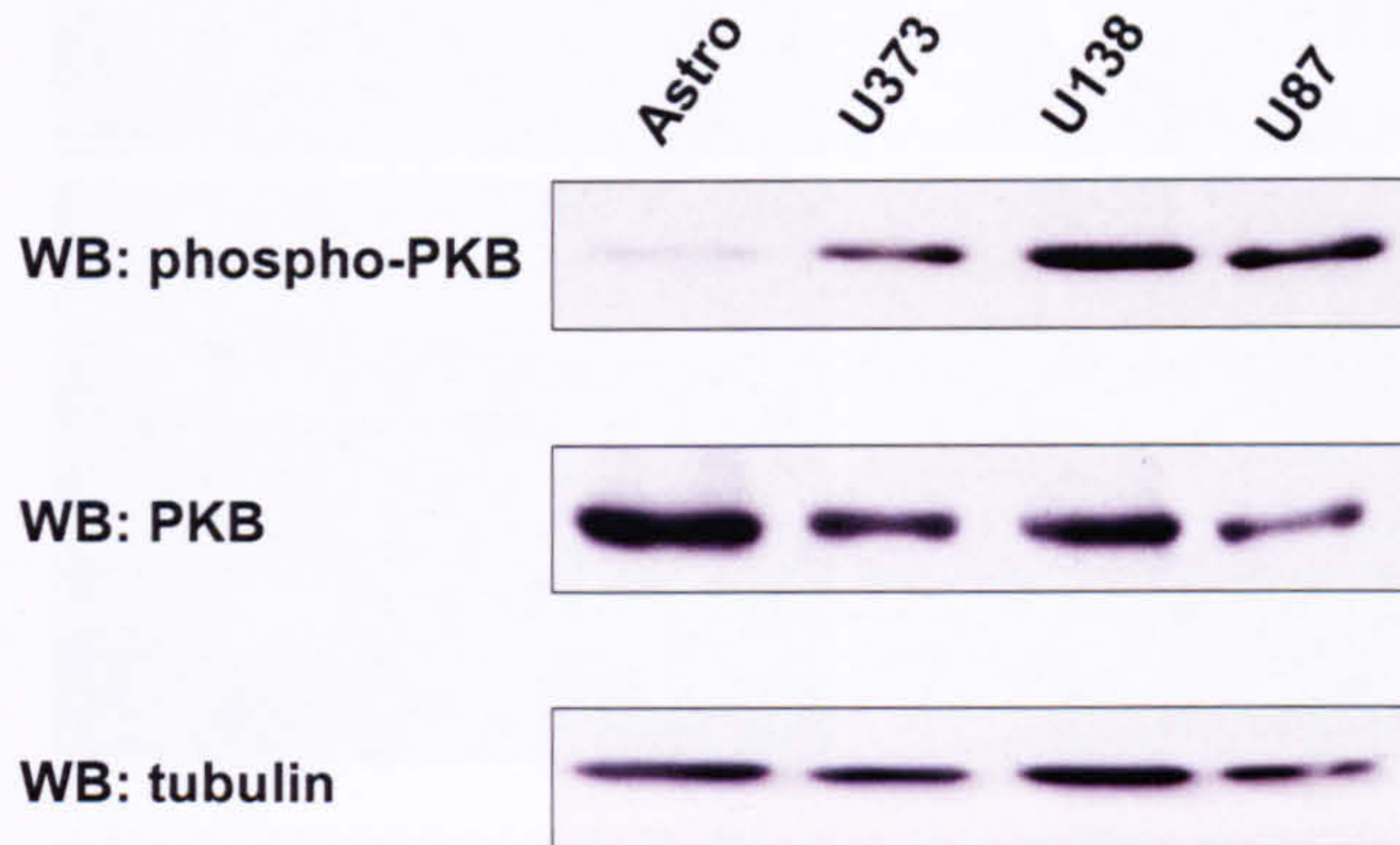
### 4.3.1 PTEN is not expressed in U373, U138 and U87 cells

The U373, U138 and U87 gliomas have been reported to have mutations in the PTEN gene that are predicted to abolish PTEN protein expression (Chiariello et al., 1998; Wang et al., 1997). This was confirmed by Western analysis of glioma cell extracts using a commercially available antibody specific for PTEN. As expected, no signal was detected in the U373, U138 and U87 cell lysates, while PTEN was expressed both in primary astrocytes and COS-7 cells (Figure 4.1).

**FIGURE 4.1**

PTEN is expressed in primary astrocytes and COS-7 cells, but not in U373, U138 or U87 cells. 20 $\mu$ g of each cell lysate were analysed by SDS-PAGE and Western blot analysis using an antibody raised against the C-terminus of PTEN. An antibody against tubulin was used as a loading control.

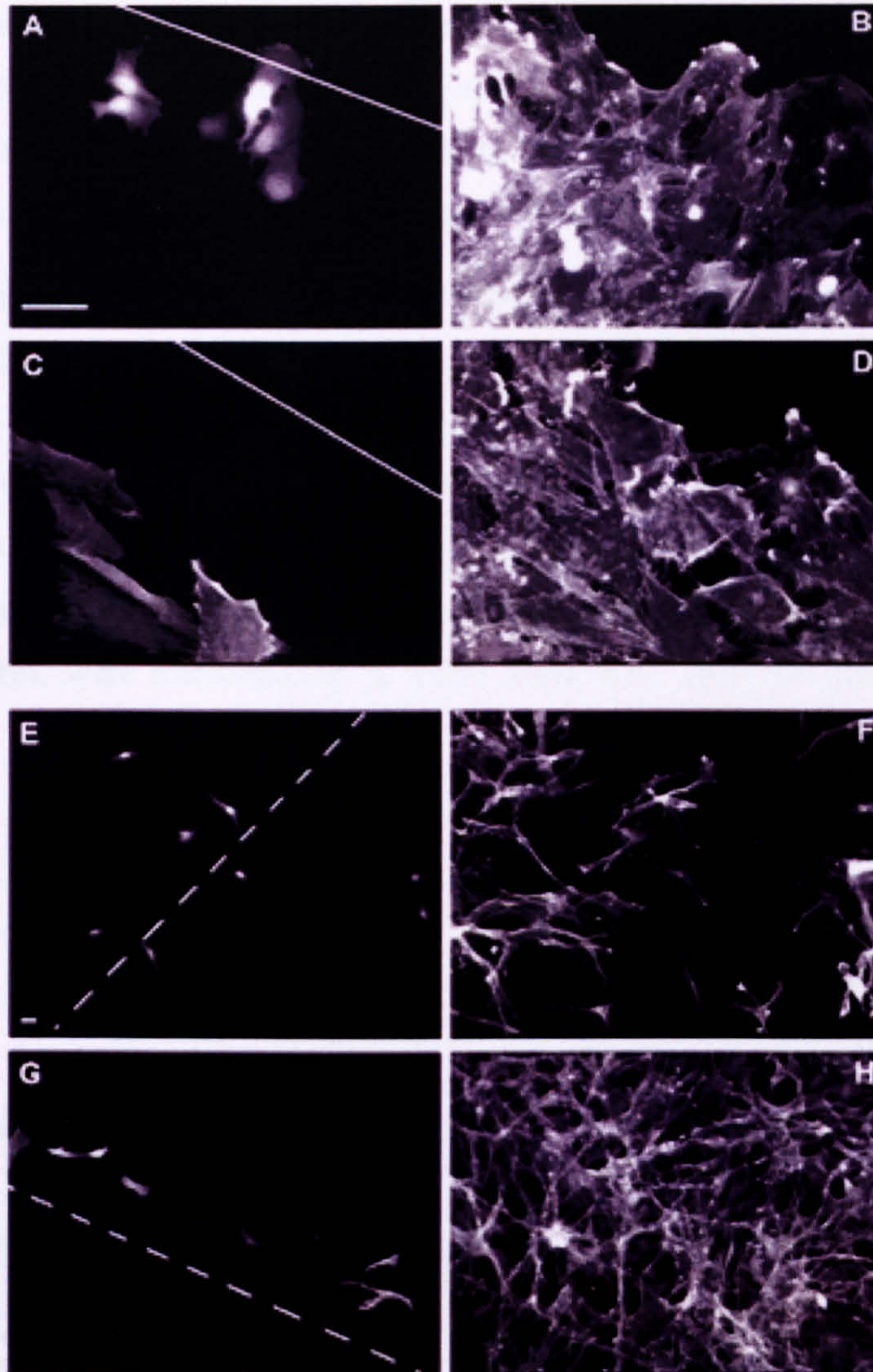
As mentioned above, PTEN has been shown to be a direct antagonist of the PI 3-kinase pathway by directly dephosphorylating PI(3,4,5)P<sub>3</sub> leading to decreased levels of PI(3,4,5)<sub>3</sub> in the cell. To investigate if the absence of PTEN in the glioma cells indeed leads to increased levels of PI(3,4,5)P<sub>3</sub>, the phosphorylation state of protein kinase B(PKB)/Akt was assessed. PKB is a key regulator of cell survival and is activated in a PI 3-kinase-dependent mechanism by binding PI(3,4,5)P<sub>3</sub> and translocating to the plasma membrane where it becomes phosphorylated (Vanhaesebroeck and Alessi, 2000). Its phosphorylation state, therefore, can serve as an indirect readout of cellular PI(3,4,5)P<sub>3</sub> levels, and previous studies have shown that in cells that lack PTEN, PKB phosphorylation is higher than in cells where PTEN is present (Higuchi et al., 2001; Myers et al., 1998). Protein extracts of astrocytes, U373, U138 and U87 cells were prepared, subjected to SDS-PAGE and Western analysis using an anti-phospho-PKB antibody. As shown in figure 4.2, the lack of PTEN expression correlates well with an increase in phospho-PKB levels. U373, U138 and U87 cells all have higher levels of phospho-PKB compared to primary astrocytes, which express PTEN normally.

**FIGURE 4.2**

Lack of PTEN expression in U373, U138 and U87 cells leads to increased levels of phospho-PKB compared to the levels observed in PTEN-expressing astrocytes. 20  $\mu$ g of each cell lysate were analysed by SDS-PAGE and blotted for levels of phospho-PKB, PKB or  $\alpha$ -tubulin (as loading control).

### 4.3.2 PTEN overexpression inhibits the migration of gliomas but not astrocytes

It has previously been shown that PTEN overexpression in PTEN null fibroblasts, as well as in the glioma cell line U87, inhibits their migration (Tamura et al., 1998). To confirm these results, and to extend the analysis to the other gliomas cell lines, wtPTEN was microinjected into the gliomas cells in a wound-healing assay. As shown in figures 4.3 and 4.4, both U373 and U138 glioma cells are inhibited for migration when PTEN is re-introduced by about 75 %. Overexpression of PTEN in primary astrocytes, however, does not inhibit their migration (figure 4.4).



**FIGURE 4.3**

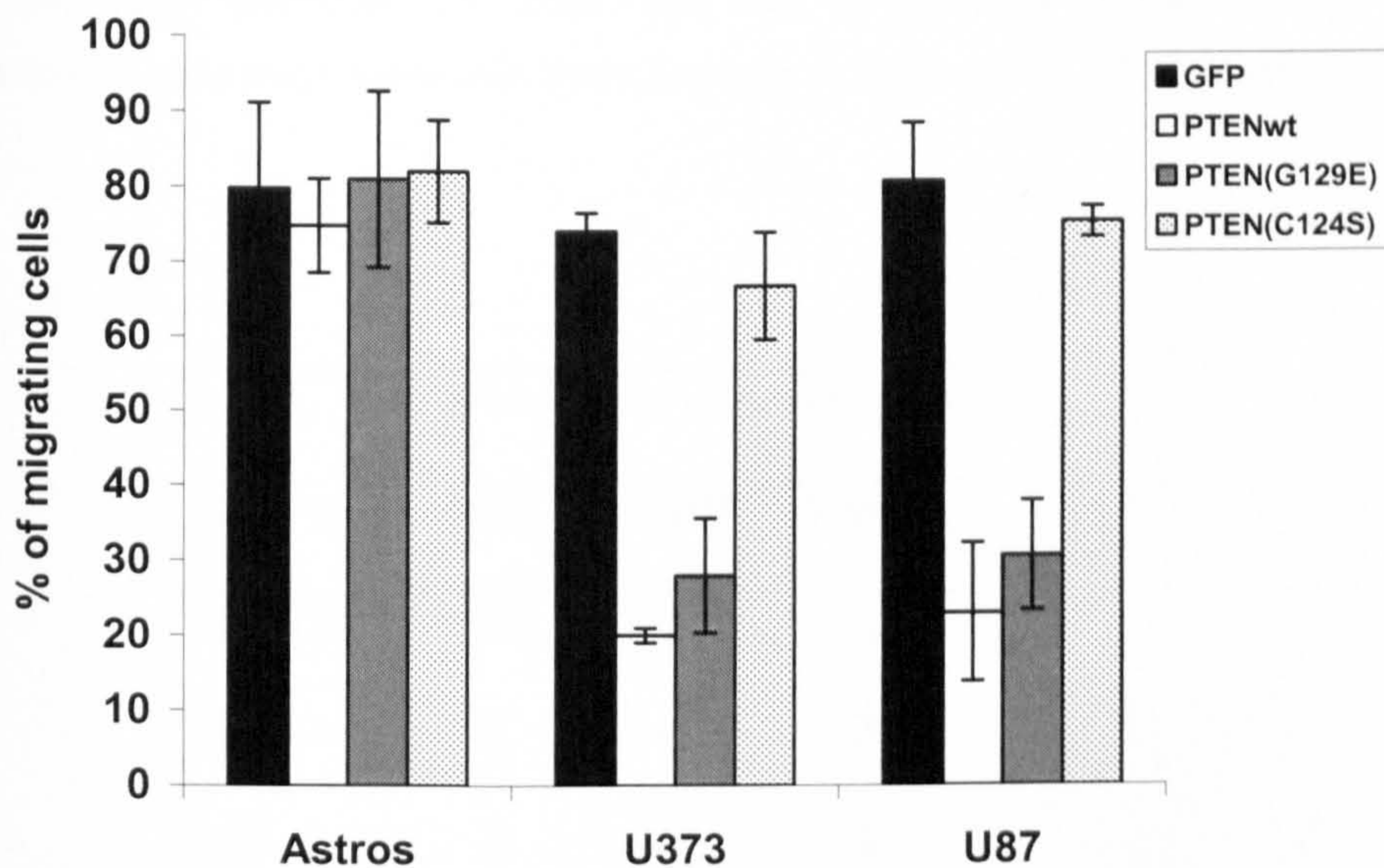
PTEN inhibits migration of gliomas. U373 cells (A-D) or U87 cells (E-H) microinjected with control GFP vector (A, B, E, F) or with a vector encoding HA-epitope-tagged PTEN (C, D, G, H) are shown. Cells were left to migrate for 16h (A-H). Expressing cells were detected after fixation by staining with rat anti-HA antibody (A, C, E, G) and all cells were visualized using rhodamine-conjugated phalloidin (B, D, F, H). A white line is drawn to indicate the leading edge of the wound (A-D) in U373 cells, or the direction of the wound (E-H) parallel to which U87 cells were microinjected in a straight line. Scale bar 50 $\mu$ m.

### 4.3.3 The protein phosphatase activity of PTEN is necessary to inhibit migration

PTEN is an unusual phosphatase in that it has been shown to exhibit phosphatase activity towards both lipids and protein substrates. *In vitro* studies using highly acidic

phosphopeptides demonstrated an activity of PTEN towards phospho-tyrosine and phospho-serine/threonine substrates (Myers et al., 1997). However, due to its unusually large active site, PTEN's major physiological substrate is thought to be PI(3,4,5)P<sub>3</sub> and to a lesser extent PI(3,5)P<sub>2</sub> (Maehama and Dixon, 1998).

To test whether the ability of PTEN to inhibit migration of gliomas was dependent upon its lipid or its protein phosphatase activities, two naturally occurring catalytic mutants were used: PTEN(C124S) lacks both lipid and protein phosphatase activity, while PTEN(G129E), a mutant first isolated from a Cowden disease patient, lacks detectable activity towards lipids but retains its ability to dephosphorylate peptide substrates (Han et al., 2000; Myers et al., 1998). Both mutants, as well as GFP as a negative control, were microinjected in U373 cells, U87 cells and primary astrocytes (Figure 4.4).



**FIGURE 4.4**

The protein, but not lipid, phosphatase activity of PTEN is required to inhibit glioma cell migration. Quantitation of the microinjection results. The number of cells expressing the various constructs that are still found at the leading edge of each wound 16h (for U373 or U87 cells) or 24h (for astrocytes) after injection was counted. Results are shown as a percentage of the total number of expressing cells. Data shown are the mean + SEM of three to five independent experiments where an average of 100 cells (20 cells in each of 5 separate wound edges) were injected for each construct.



As expected, the catalytically dead mutant, PTEN(C124S), is no longer able to inhibit migration of U373 and U87 cells. Surprisingly, however, PTEN(G129E), which still retains catalytic activity towards phospho-peptides, inhibits the migration of both U373 and U87 cells similarly to the wild type protein. This is in agreement with previous studies by Tamura et al., demonstrating that PTEN's ability to inhibit migration of U87 cells or PTEN<sup>-/-</sup> fibroblasts is dependent on its protein and not its lipid phosphatase activity (Tamura et al., 1998; Tamura et al., 1999).

This finding is further supported by studies using the PI 3-kinase specific inhibitor, wortmannin. The results are summarized in table 4.1. Wortmannin does not inhibit migration of astrocytes or U373 cells. PI 3-kinase does not, therefore, seem to play a role in the migration of these cells. It is thus not surprising that a lipid phosphatase-inactive mutant of PTEN (G129E) can still inhibit migration of U373 cells independently of PTEN's effect on the PI 3-kinase pathway. PI 3-kinase does, however, seem to regulate migration of the highly malignant U87 cells. This may be explained by the fact that additional mutations have made these cells more sensitive to varying PI(3,4,5)P<sub>3</sub> levels.

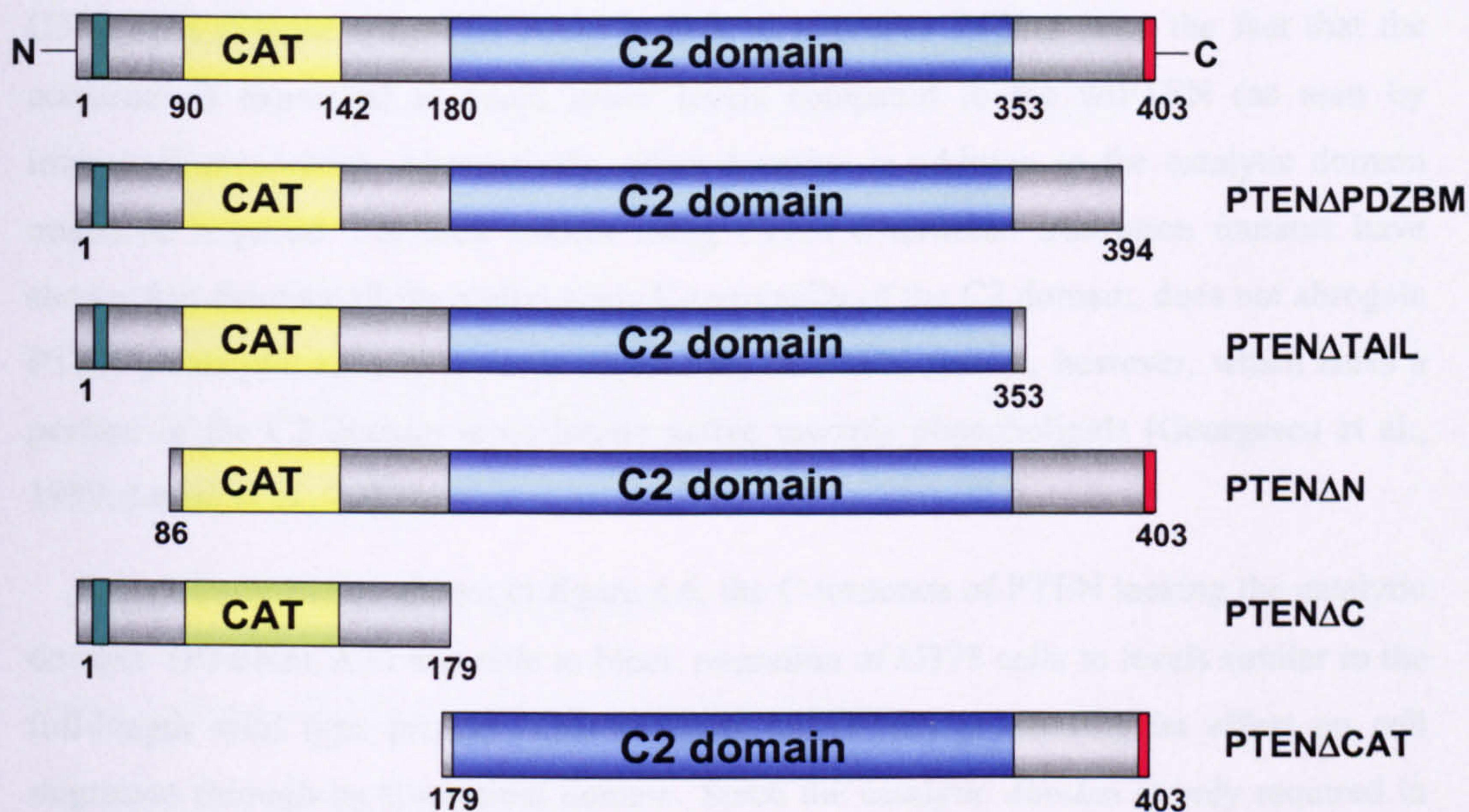
Cell Type	Wortmannin concentration		
	0nM	100nM	500nM
Astrocytes	√	√	√
U373	√	√	X
U87	√	X	X

**TABLE 4.1**

Effect of wortmannin on cell migration as measured in a wound healing assay. Cells were scratched 30 min after addition of wortmannin and left to migrate for as long as it took control cells (0nM wortmannin) to close the wound. The ability of cells to close the wound is indicated by a tick mark (√), whereas inhibition of wound closure by addition of wortmannin is indicated by a cross (X).

#### 4.3.4 Additional domains in PTEN are required for inhibition of glioma migration

Deletion analysis was performed to test whether, in addition to the catalytic domain, any other domains present in PTEN contributed to the effect on cell migration. PTEN fragments (Figure 4.5) were cloned into mammalian expression vectors encoding a Myc or Flag-epitope and microinjected into U373 and U87 cells in a wound-healing assay. Microinjection of GFP and wtPTEN was used as positive and negative control, respectively, for inhibition of migration.



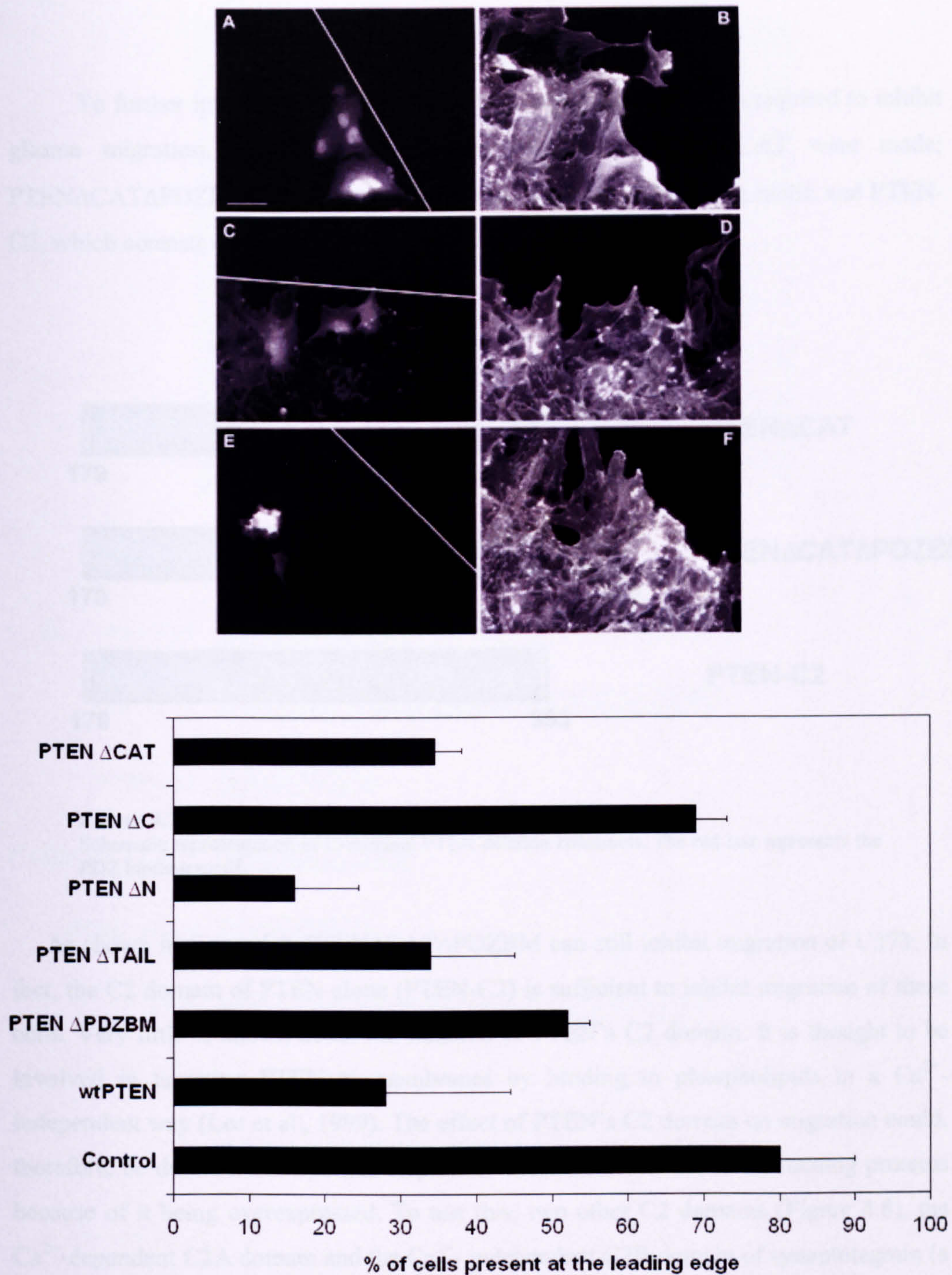
**FIGURE 4.5**

Schematic representation of the PTEN deletion constructs. The PI(4,5)P<sub>2</sub> and PDZ binding motifs are represented by a green box or a red box respectively.

As shown in figure 4.6, deletion of the PI(4,5)P<sub>2</sub> (PTEN $\Delta$ N) or PDZ binding motifs (PTEN $\Delta$ PDZBM) or of the last 50 residues (PTEN $\Delta$ Tail) has no significant effect on the ability of PTEN to inhibit migration in U373 cells. It is possible that the PI(4,5)P<sub>2</sub> binding

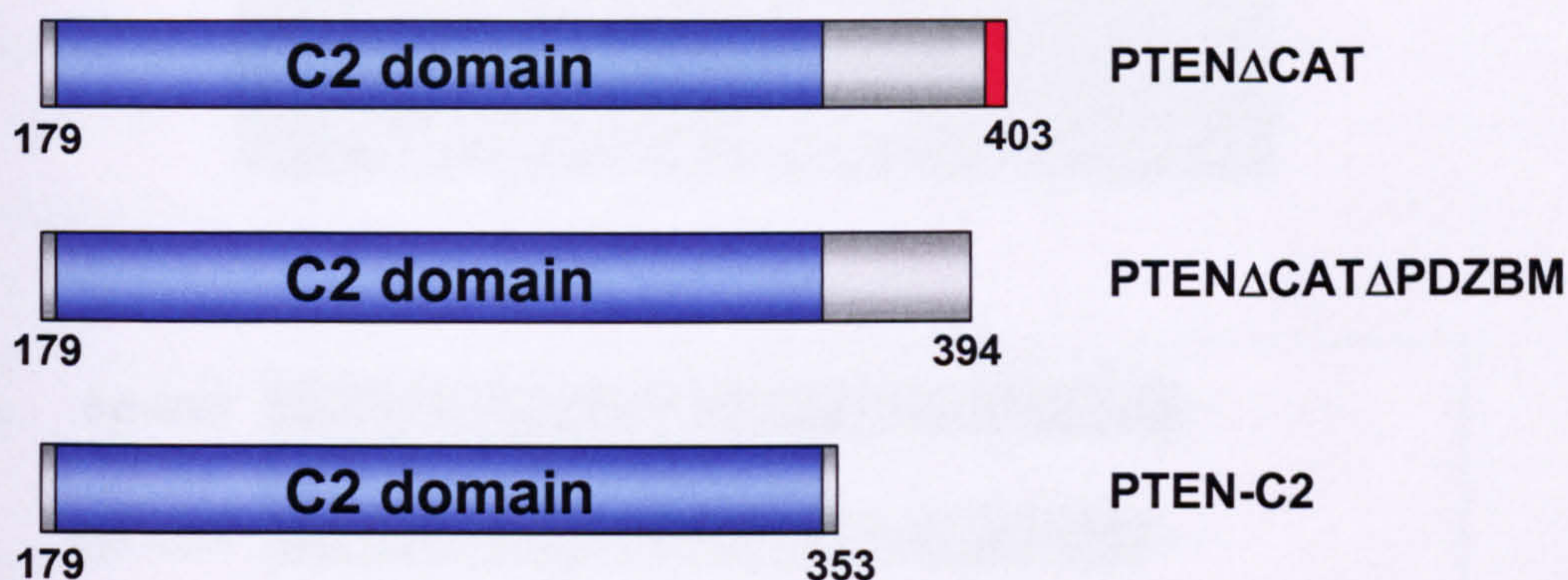
motif serves to localise the endogenous PTEN protein to PI(4,5)P<sub>2</sub>-enriched regions on the plasma membrane, as suggested previously by Iijima et al. Similarly, the PDZ binding motif might serve to localise the protein through the binding to PDZ containing proteins to sites where PTEN's action is needed. The finding that these domains are not required here could be due to the fact that both PTEN $\Delta$ N and PTEN $\Delta$ PDZBM constructs are overexpressed and this bypasses the need for proper localisation. Alternatively, proper PTEN localisation has been shown to be essential for gradient sensing during chemotaxis, but not for migration per se, and therefore deleting the putative PI(4,5)P<sub>2</sub> binding motif would not be predicted to disrupt its mode of action regarding migration (Iijima and Devreotes, 2002). The catalytic domain of PTEN alone is unable to inhibit migration of U373 cells (Figure 4.6). This could be due to improper folding or to the fact that the construct is expressed at much lower levels compared to the wtPTEN (as seen by immunofluorescence). Alternatively, other domains in addition to the catalytic domain might be required. Previous studies using PTEN C-terminal truncation mutants have shown that deleting all the amino acids C-terminally of the C2 domain, does not abrogate PTEN's catalytic activity towards PI(3,4,5)P<sub>3</sub>. A PTEN mutant, however, which lacks a portion of the C2 domain is no longer active towards phospholipids (Georgescu et al., 1999; Leslie et al., 2000).

Surprisingly and as shown in figure 4.6, the C-terminus of PTEN lacking the catalytic domain (PTEN $\Delta$ CAT) was able to block migration of U373 cells to levels similar to the full-length wild type protein. This suggests that PTEN is exerting its effect on cell migration through its C-terminal domain. Since the catalytic domain is only required in the full-length protein, it is likely that its role is to regulate the activity or accessibility of the C-terminal domain.

**FIGURE 4.6**

Effect of PTEN deletion constructs on U373 migration. (a) Representative examples of the effect of microinjecting Myc-epitope tagged PTEN $\Delta$ C (A, B) and PTEN $\Delta$ CAT (C, D) into U373 is shown. Microinjected cells were fixed and detected by staining with 9E10 anti-Myc antibody. The actin was visualized using Rhodamine-conjugated phalloidin. Scale bar, 50 $\mu$ m. A white line is drawn to indicate the leading edge of the wound. (b) Quantitation of the microinjection results. The number of U373 cells expressing control vector or the various Myc-epitope tagged constructs that are still found at the edge of each wound 16h after injection of U373 cells was counted. Results are shown as a percentage of the total number of expressing cells. Data shown are the mean + SEM of three to five independent experiments where an average of 100 cells (20 cells in each of 5 separate wound edges) were injected for each construct.

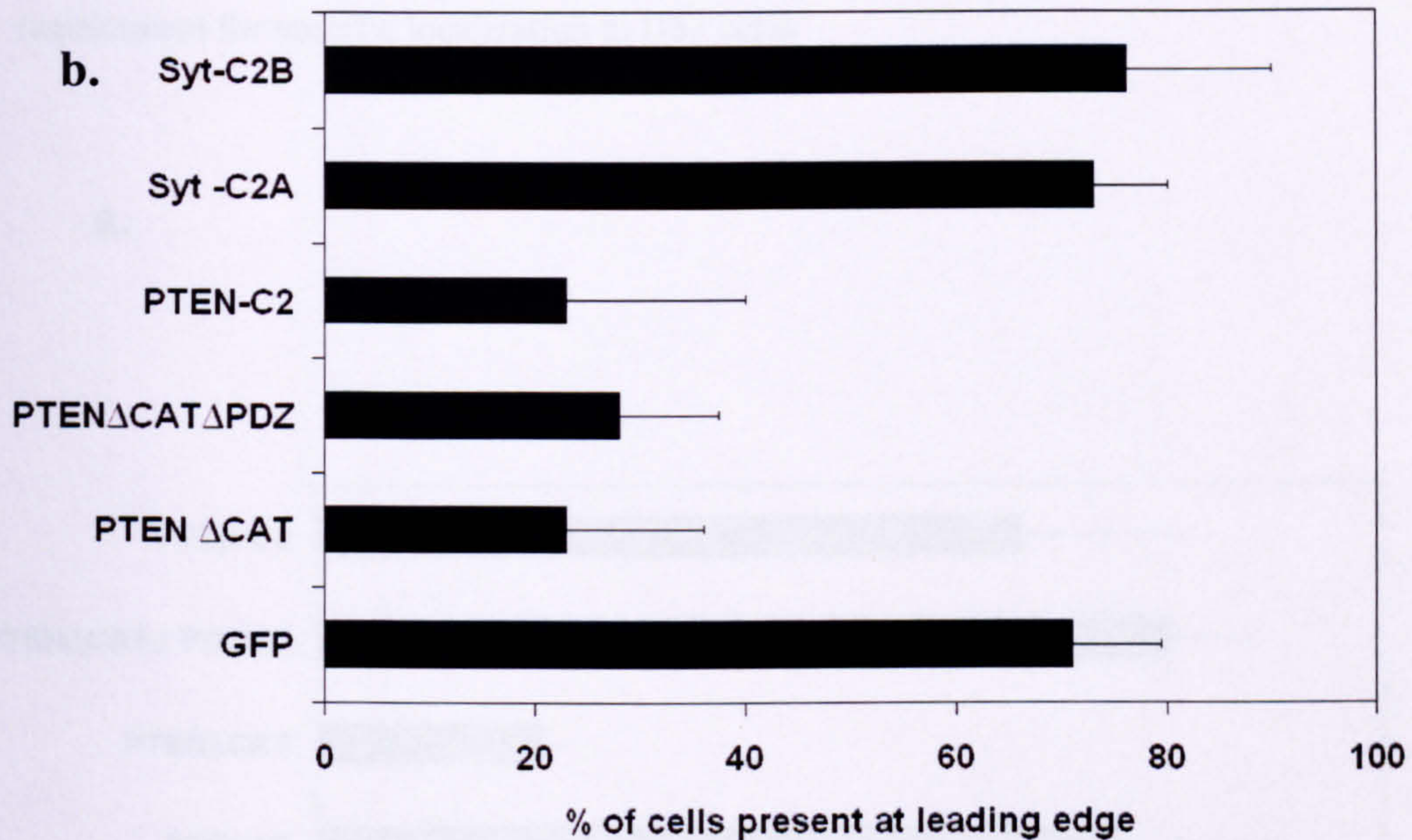
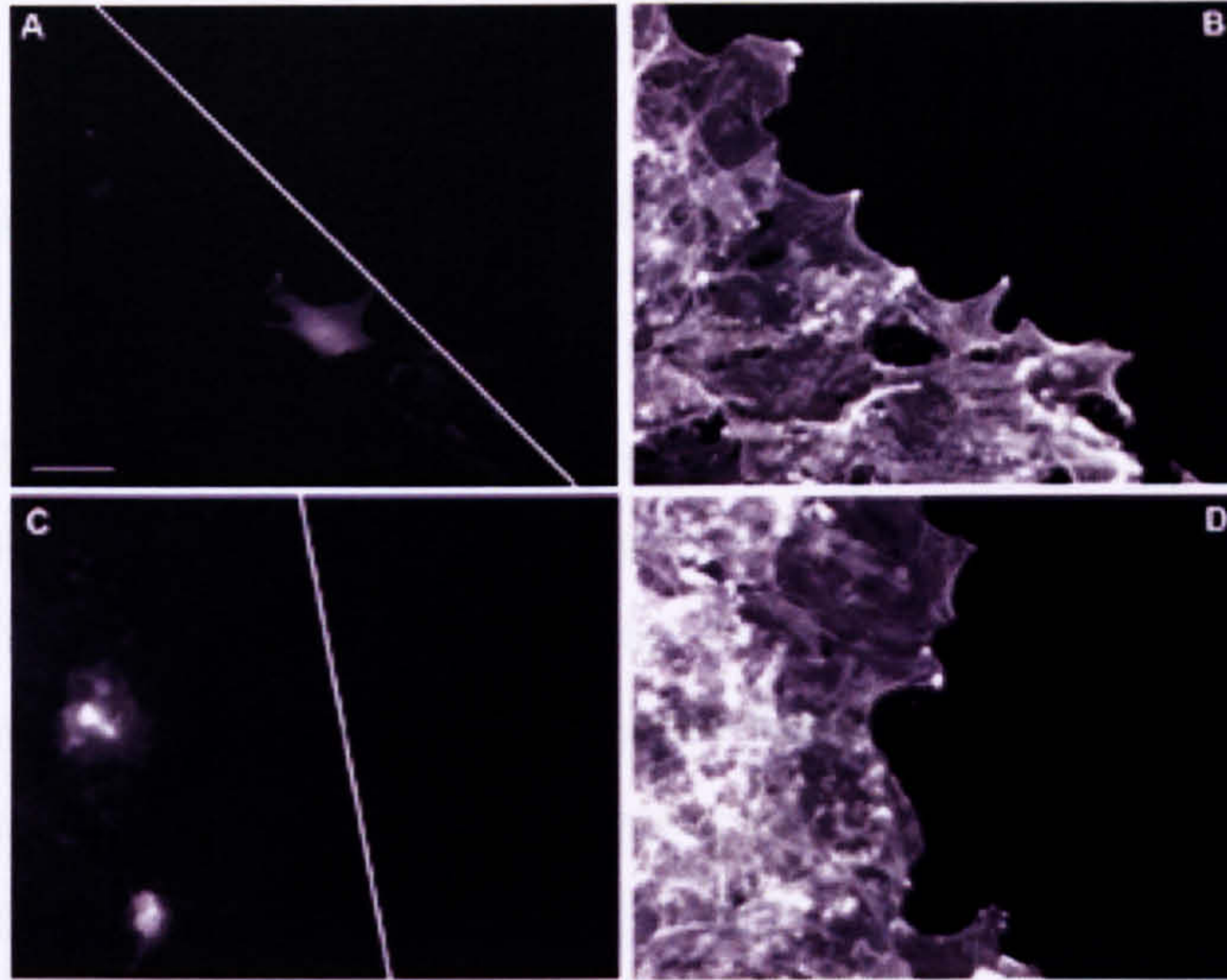
To further investigate which part of the C-terminus of PTEN is required to inhibit glioma migration, the following deletion mutants of PTEN $\Delta$ CAT were made; PTEN $\Delta$ CAT $\Delta$ PDZBM, a C-terminal domain lacking the PDZ binding motif, and PTEN-C2, which consists only of the C2 domain of PTEN (Figure 4.7).



**FIGURE 4.7**  
Schematic representation of C-terminal PTEN deletion constructs. The red box represents the PDZ binding motif.

As shown in figure 4.8, PTEN $\Delta$ CAT $\Delta$ PDZBM can still inhibit migration of U373. In fact, the C2 domain of PTEN alone (PTEN-C2) is sufficient to inhibit migration of these cells. Very little is known about the function of PTEN's C2 domain. It is thought to be involved in targeting PTEN to membranes by binding to phospholipids in a Ca<sup>2+</sup>-independent way (Lee et al., 1999). The effect of PTEN's C2 domain on migration could, therefore, be due to a non-specific displacement of other C2-domain containing proteins because of it being overexpressed. To test this, two other C2 domains (Figure 4.8), the Ca<sup>2+</sup>-dependent C2A domain and the Ca<sup>2+</sup>-independent C2B domain of synaptotagmin (a protein involved in vesicle trafficking) were also tested for their ability to inhibit migration. Unlike the C2 domain of PTEN, these had no effect on U373 cell migration (Murray and Honig, 2002).

a.

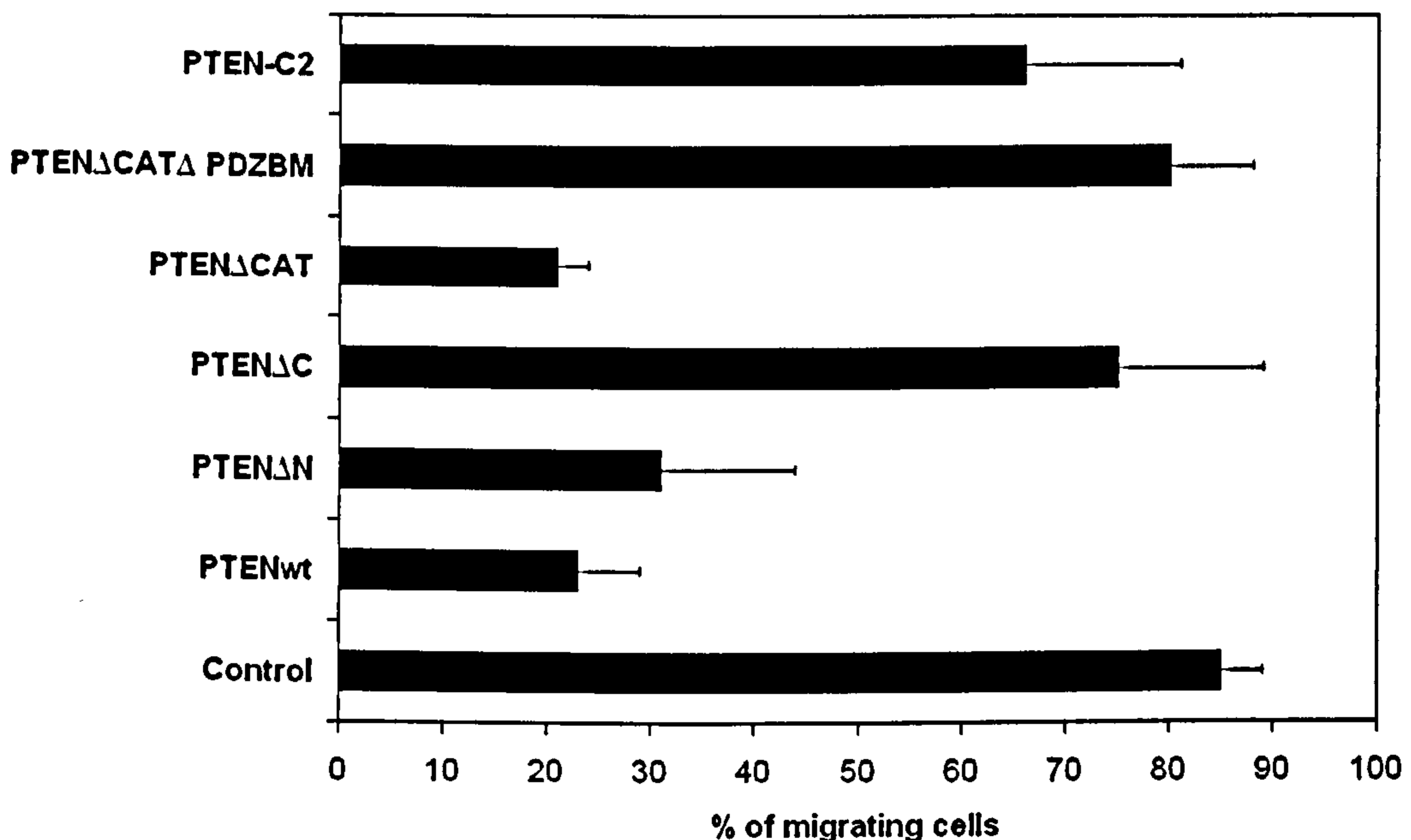
**FIGURE 4.8**

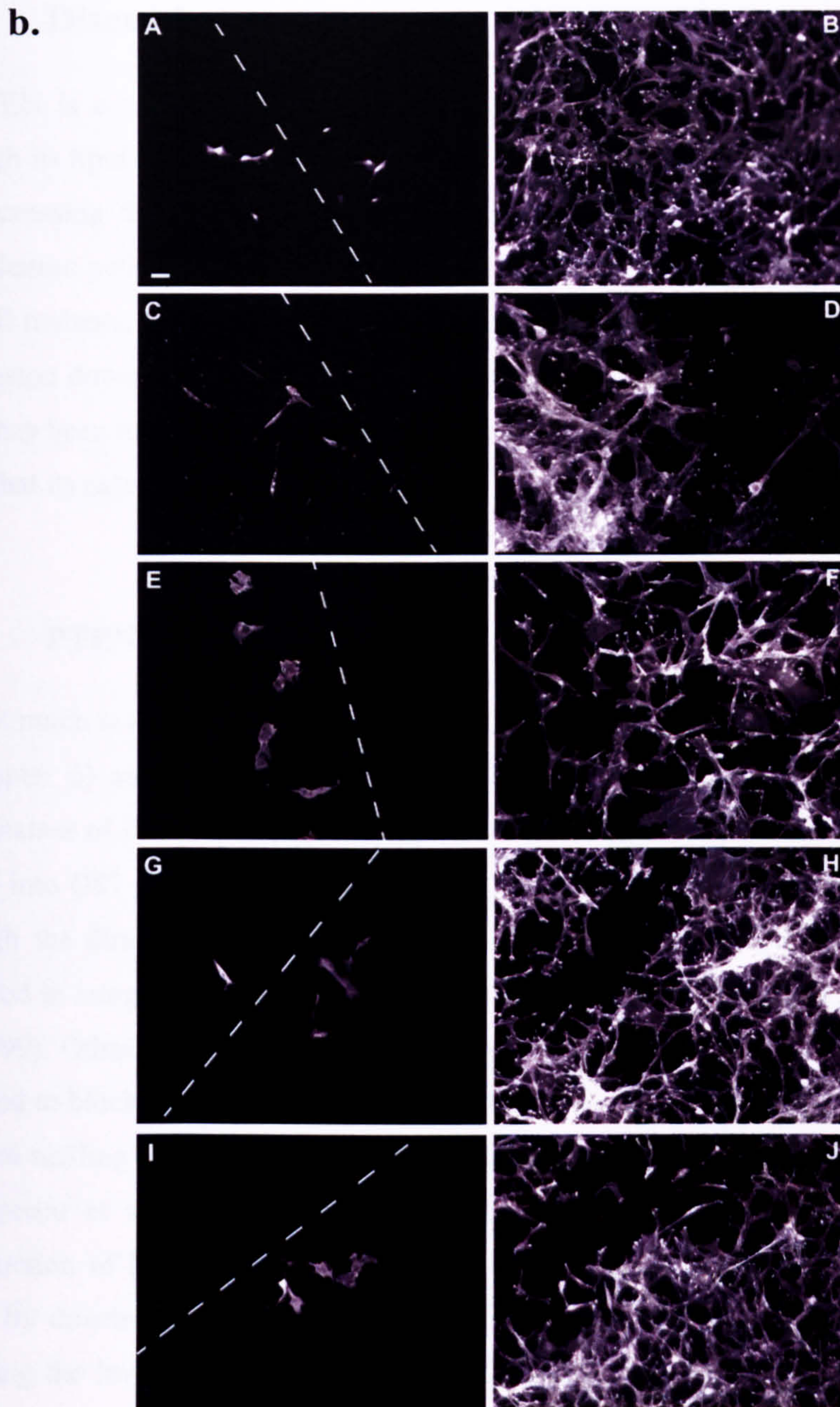
PTEN's C2 domain is sufficient to inhibit migration of U373. (a) Representative examples of the effect of microinjecting Myc-epitope tagged Syt-C2 (A, B) and PTEN-C2 (C, D) into U373 is shown. Microinjected cells were fixed and detected by staining with 9E10 anti-Myc antibody. The actin was visualized using Rhodamine-conjugated phalloidin. Scale bar, 50 $\mu$ m. (b) Quantitation of the microinjection results. The number of U373 cells expressing control vector or the various Myc-epitope tagged constructs that are still found at the edge of each wound 8h after injection of U373 cells was counted. Results are shown as a percentage of the total number of expressing cells. Data shown are the mean + SEM of three to five independent experiments where an average of 100 cells (20 cells in each of 5 separate wound edges) were injected for each construct.

#### 4.3.5 The inhibition of migration by the C-terminus of PTEN is not unique to U373 cells

To see whether these results were specific to U373 cells, the PTEN deletion constructs were also microinjected into U87 cells. As shown in figure 4.8, similar to what was seen for U373 cells, the C-terminus of PTEN lacking the catalytic domain (PTEN $\Delta$ CAT) also inhibits migration of U87 cells, while the catalytic domain on its own is not sufficient to inhibit migration (PTEN $\Delta$ C). However, as shown in figure 4.9, a C-terminal construct that lacks the PDZ binding motif (PTEN $\Delta$ CAT $\Delta$ PDZBM) is no longer able to inhibit migration of U87 cells, whereas it did inhibit U373 cells. Since the PDZ motif is likely to affect sub-cellular localization of PTEN, this could be due to a more stringent requirement for specific localization in U87 cells.

a.





**FIGURE 4.9**

Effect of the PTEN deletion constructs on U87 migration. **(a)** Quantitation of the microinjection results. The number of U87 microinjected cells expressing control vector or the various Myc-epitope tagged constructs that are found still aligned in the direction of the wound after migration for 8h was counted. Results are represented as a percentage of the number of cells still aligned in the direction of the wound over the total number of cells injected. Data shown are the mean + SEM of three to five independent experiments where an average of 100 cells (20 cells in each of 5 separate wound edges) were injected for each construct. **(b)** Representative examples of the effect of microinjecting Myc-epitope tagged PTEN $\Delta$ C (A, B), PTEN $\Delta$ CAT (C, D), PTEN $\Delta$ CAT $\Delta$ PDZBM (E,F) and PTEN-C2 into U87 cells is shown. Microinjected cells were fixed and detected by staining with 9E10 anti-Myc antibody. The actin was visualized using Rhodamine-conjugated phalloidin. Scale bar, 50 $\mu$ m. A white line is drawn to indicate the direction of the wound parallel to which cells were microinjected.



## 4.4 Discussion

PTEN is a tumour suppressor protein that is thought to exert its effects primarily through its lipid phosphatase activity, which directly antagonizes the PI 3-kinase pathway by decreasing the cellular pool of PI(3,4,5)P<sub>3</sub>. PTEN has also been shown to exhibit phosphatase activity towards phospho-peptides and with the use of both the C124S and G129E mutants, Weng et al., were able to show that PTEN can downregulate cyclinD1 expression downstream of its protein-phosphatase activity (Weng et al., 2001). However, there has been much controversy as to the importance of the protein phosphatase activity and what its natural substrate might be.

### 4.4.1 PTEN's role in cell migration

Not much is known about how PTEN controls cell migration and spreading (discussed in chapter 6) and there is conflicting evidence as to whether the lipid or the protein phosphatase of PTEN is required. Early studies in PTEN<sup>-/-</sup> fibroblasts and transfection of PTEN into U87 glioma cells suggested that PTEN regulates cell spreading and migration through the direct dephosphorylation of the signaling proteins FAK and Shc, proteins involved in integrin-mediated cell spreading and motility (Tamura et al., 1998; Tamura et al., 1999). Other studies, however, showed that the lipid phosphatase activity of PTEN is required to block anchorage-independent cell growth of U87 cells in soft agar and PDGF-induced ruffling of fibroblasts, by acting as a direct antagonist to the PI3-kinase pathway (Georgescu et al., 1999; Leslie et al., 2001; Leslie et al., 2000). Furthermore, re-introduction of PTEN into PTEN<sup>-/-</sup> fibroblasts reduced their speed of migration, most likely by downregulating the activation of the small GTPases Rac and Cdc42 through lowering the levels of PI(3,4,5)P<sub>3</sub> in the cell (Liliental et al., 2000). The results in this chapter reveal a completely different mechanism of action, at least in glioma cells.

### 4.4.2 Distinct functions for the different PTEN domains

PTEN is comprised primarily of two domains: an N-terminal catalytic domain and a C-terminal C2 domain. Its PDZ binding motif, at its extreme C-terminus, has been shown to bind several PDZ domain-containing proteins, including those that belong to the MAGUK superfamily, like hDLG and MAGI-2, and to MAST205 (Adey et al., 2000; Wu et al., 2000b). It is thought that these proteins may serve to localize PTEN to the

necessary sites where PTEN is active. Work in this chapter has revealed that the PDZ binding motif is dispensable for inhibition of U373 glioma migration by PTEN. This is supported by previous studies by Leslie et al., which have shown that the PDZ binding motif is not needed for PTEN to lower the levels of PI(3,4,5)P<sub>3</sub> in a cell. On the other hand the same group also showed that the PDZ binding motif is necessary for inhibition of cell spreading and PDGF-induced membrane ruffling (Leslie et al., 2001; Leslie et al., 2000). Recently, studies in *Dictyostelium*, revealed a putative PI(4,5)P<sub>2</sub> binding motif on the N-terminus of PTEN, which was required for membrane localisation and proper cell chemotaxis (Iijima and Devreotes, 2002). In this chapter, however, it was found that this PI(4,5)P<sub>2</sub> binding motif was dispensable for PTEN's ability to inhibit migration of gliomas.

A third domain implicated in regulating the localisation of PTEN is the C2 domain. When the crystal structure of PTEN was solved, it was shown that the catalytic and the C2 domains associate with each other over an extensive surface and that the C2 domain may be required for the correct positioning of the catalytic domain onto the membrane (Lee et al., 1999). Interestingly, the studies reported in this chapter have demonstrated that the C-terminus of PTEN, including the C2 domain, is both necessary and sufficient to inhibit glioma migration.

Further investigation into the minimal domain required for inhibition of migration, revealed that the mechanism of PTEN function is different between the two glioma cell lines studied. The PDZ binding motif is dispensable and the C2 domain is both necessary and sufficient to inhibit migration of U373 cells. This appears to be specific to the PTEN C2 domain, since overexpression of other C2 domains does not inhibit their migration. In U87 cells, however, deletion of the PDZ binding motif from the C-terminal fragment of PTEN, abrogates this mutant's ability to inhibit cell migration.

#### 4.4.3 A function for the C2 domain

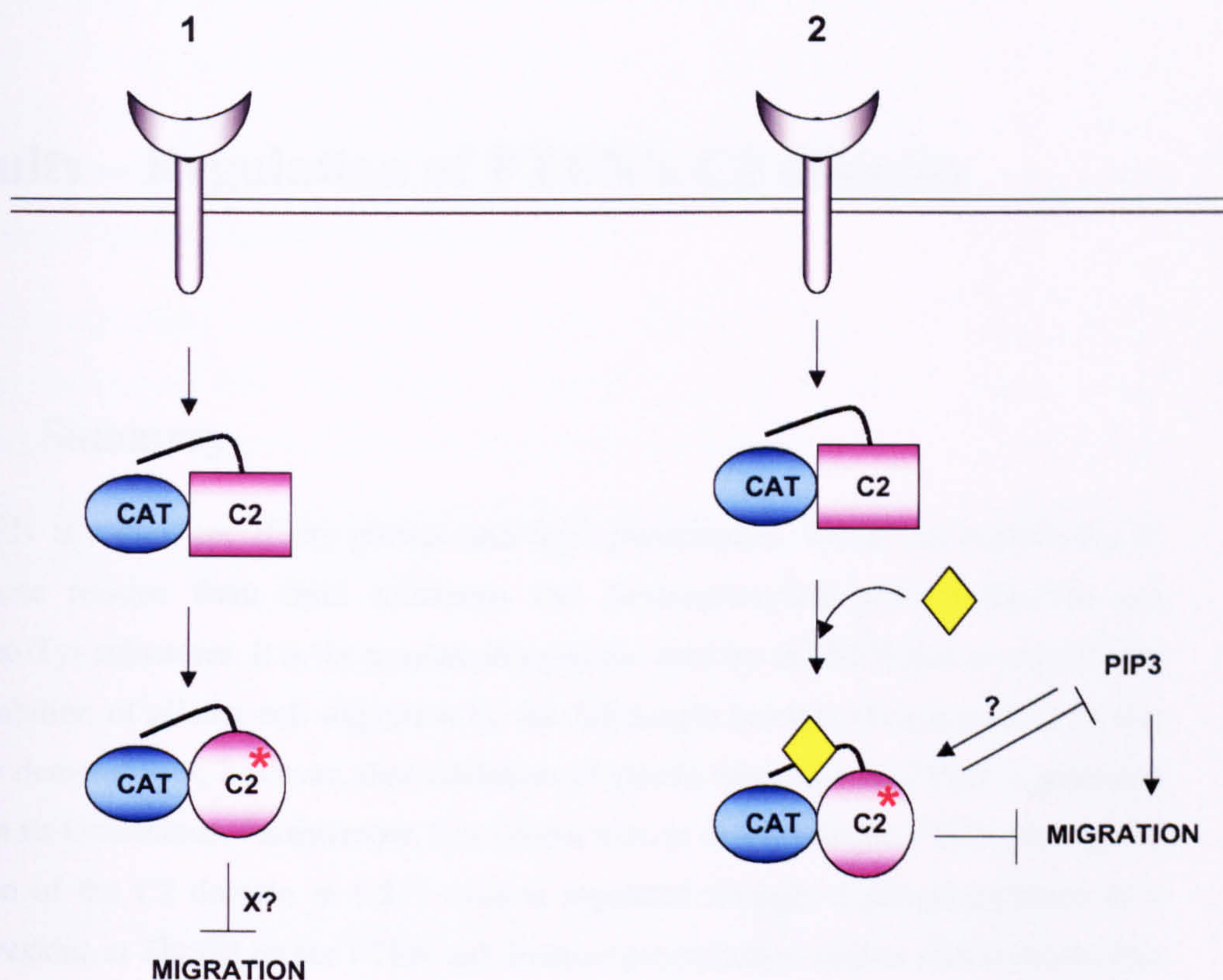
Little is known about C2 domains and their precise cellular function. C2 was first identified as a structural Ca<sup>2+</sup>-binding domain in conventional PKCs. Since then it has been identified in several signaling proteins, including PLCs and PI 3-kinases. C2 domains have a characteristic fold composed of a sandwich of eight antiparallel  $\beta$  strands connected by variable loops and they are primarily believed to bind membranes in a Ca<sup>2+</sup>-dependent way, like the C2A domain of synaptotagmin (Murray and Honig, 2002; Rizo

and Sudhof, 1998). Originally, C2 domains were believed to be  $\text{Ca}^{2+}$ -dependent membrane-targeting domains. Meanwhile it has emerged, that many C2 domains bind membranes in a  $\text{Ca}^{2+}$ -independent way and some of them have no membrane-binding potential at all, but rather are involved in protein-protein interactions.

The C2 domain of PTEN has been shown to bind membranes *in vitro*, in a  $\text{Ca}^{2+}$ -independent way, though there is also some evidence for a  $\text{Ca}^{2+}$ -dependent mechanism. Work by Das et al., showed that, even though the isolated PTEN C2 domain can bind vesicle membranes *in vitro*, it does so with a much lower affinity compared to the full-length protein and that the C2 domain's membrane affinity is probably not high enough to target PTEN to the membrane. The same group showed that it is the phosphatase domain that is most directly involved in the electrostatic interaction of PTEN with the membrane and that the C2 domain is required for high-affinity binding (Das et al., 2003). This data agrees with previous studies by Georgescu et al., which demonstrated that forcing the membrane recruitment of a C2 membrane-binding deficient mutant of PTEN (M-CBR3), by adding a myristoylation signal, does not fully restore the tumour suppressor function of PTEN (Georgescu et al., 2000). This suggests that the C2 domain of PTEN cannot simply be playing a membrane-targeting role. The same group went further to suggest that C2 might serve to stabilise the PTEN protein and to productively position the catalytic domain on the membrane for it to gain better access to its substrate.

In this chapter, evidence is presented for a novel role for the C2 domain of PTEN. Microinjection analysis revealed that the C2 domain alone can inhibit migration of U373 cells, and, in contribution with an intact PDZ binding motif, U87 cells. This supports the findings of Freeman et al. for an additional role of the C2 domain. Freeman et al. recently showed that the C2 domain of PTEN can bind the tumour suppressor protein p53 and enhance its transcriptional activity, albeit to a lesser extent than the full-length protein (Freeman et al., 2003). Currently it is not clear how the C2 domain affects cell migration. Any mechanism, however, is most likely to require the binding of the C2 domain to another protein –rather than to the membrane (especially since no particular membrane localization was observed when the C2 domain was overexpressed on its own in U373 cells). A model to explain why the C2 domain alone can inhibit migration, while in the full-length protein, the protein phosphatase activity is also essential, is presented in figure 4.10. In U373 cells, an extracellular stimulus might lead to the activation of the protein phosphatase activity of PTEN, which leads to the subsequent activation of the C2 domain (probably through a conformational change, or 'opening up' of the protein) that inhibits

migration possibly via the recruitment of other cellular proteins (model 1). In U87 cells, an extracellular stimulus leads to the activation of the protein phosphatase activity of PTEN. This is followed by the recruitment of a protein to the PDZ binding motif, which would then localize the C2 domain to inhibit migration (model 2). PI(3,4,5)P3 could play a role in this or a parallel pathway to regulate migration, since wortmannin inhibits U87 cell migration.



**FIGURE 4.10**

Proposed model for the inhibition of U373 and U87 cell migration by PTEN. See discussion for explanation. Model 1 proposes a mechanism for the regulation of U373 cell migration, whereas Model 2 proposes a mechanism for U87 cells.

This model, however, does not address how the protein phosphatase activity of PTEN regulates the activity of the C2 domain, and this is examined in the next chapters.

## **Chapter 5**

### **Results – Regulation of PTEN's C2 domain**

#### **5.1 Summary**

PTEN is a dual specificity protein and lipid phosphatase, which can remove the 3' phosphate residue from lipid substrates and dephosphorylate phospho-Ser/Thr and phospho-Tyr substrates. It is the protein phosphatase activity of PTEN that is required for the inhibition of glioma cell migration by the full-length protein. Data presented in this chapter demonstrates, however, that inhibition of glioma migration by PTEN is mediated through its C-terminus. Furthermore, it is shown that, in the full-length PTEN protein, the function of the C2 domain in U373 cells is regulated through dephosphorylation of a single residue at Thr383 on the PTEN tail. Immunoprecipitation studies also revealed that the N-terminus of PTEN binds to the C-terminus. A mechanism is proposed whereby PTEN negatively regulates cell motility through its C2 domain, and this depends on the protein phosphatase activity of PTEN to mediate dephosphorylation at Thr383.

#### **5.2 Introduction**

Domain analysis has revealed that PTEN has a number of clearly defined regions in addition to an N-terminal catalytic domain and a C-terminal C2 domain (discussed in detail in chapter 4). Adjacent to the C2 domain, at the C-terminus, lies a stretch of about 50 amino acid residues, the tail, which has been shown to be extensively phosphorylated on 5 major sites (Birle et al., 2002; Torres and Pulido, 2001; Vazquez et al., 2000). The

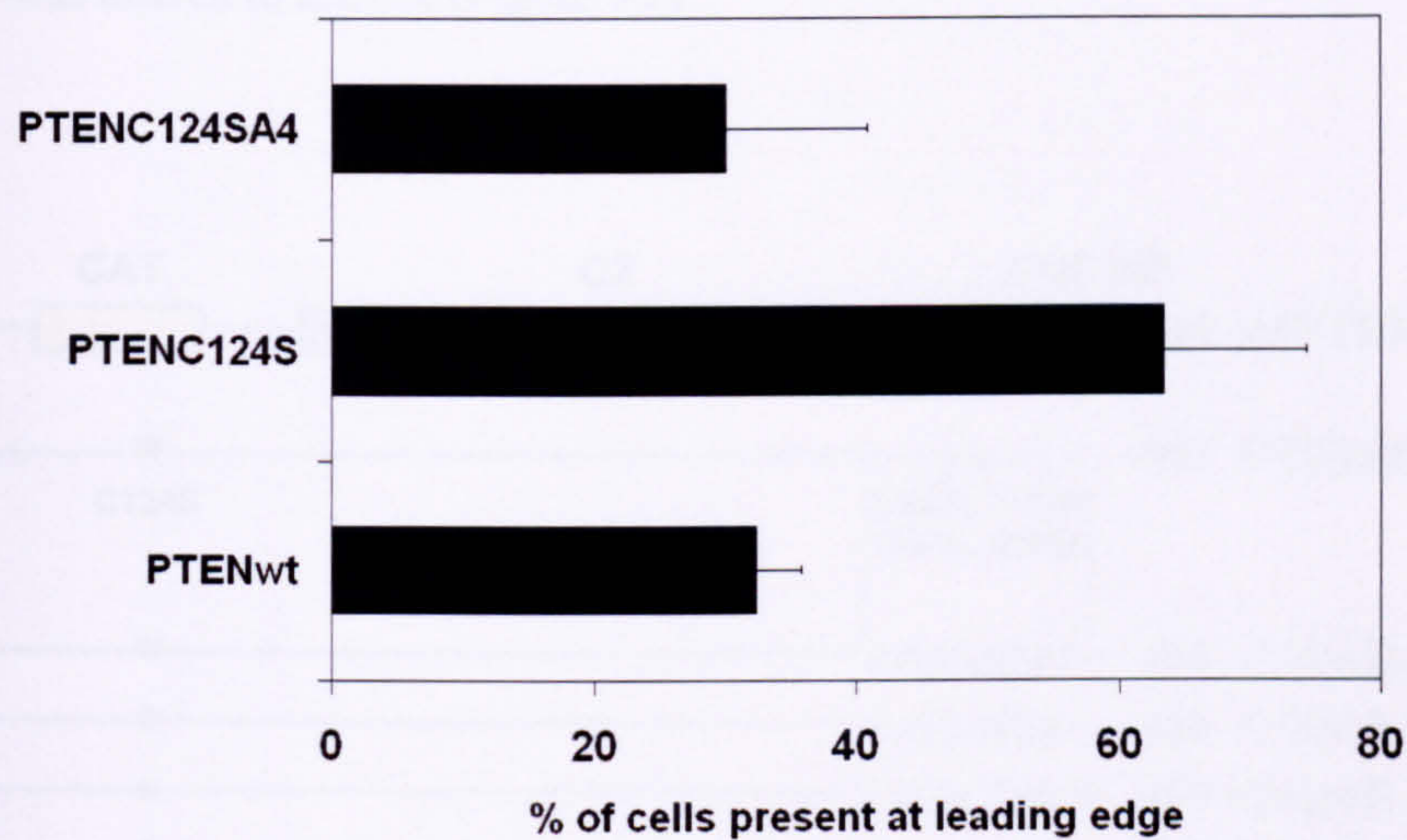
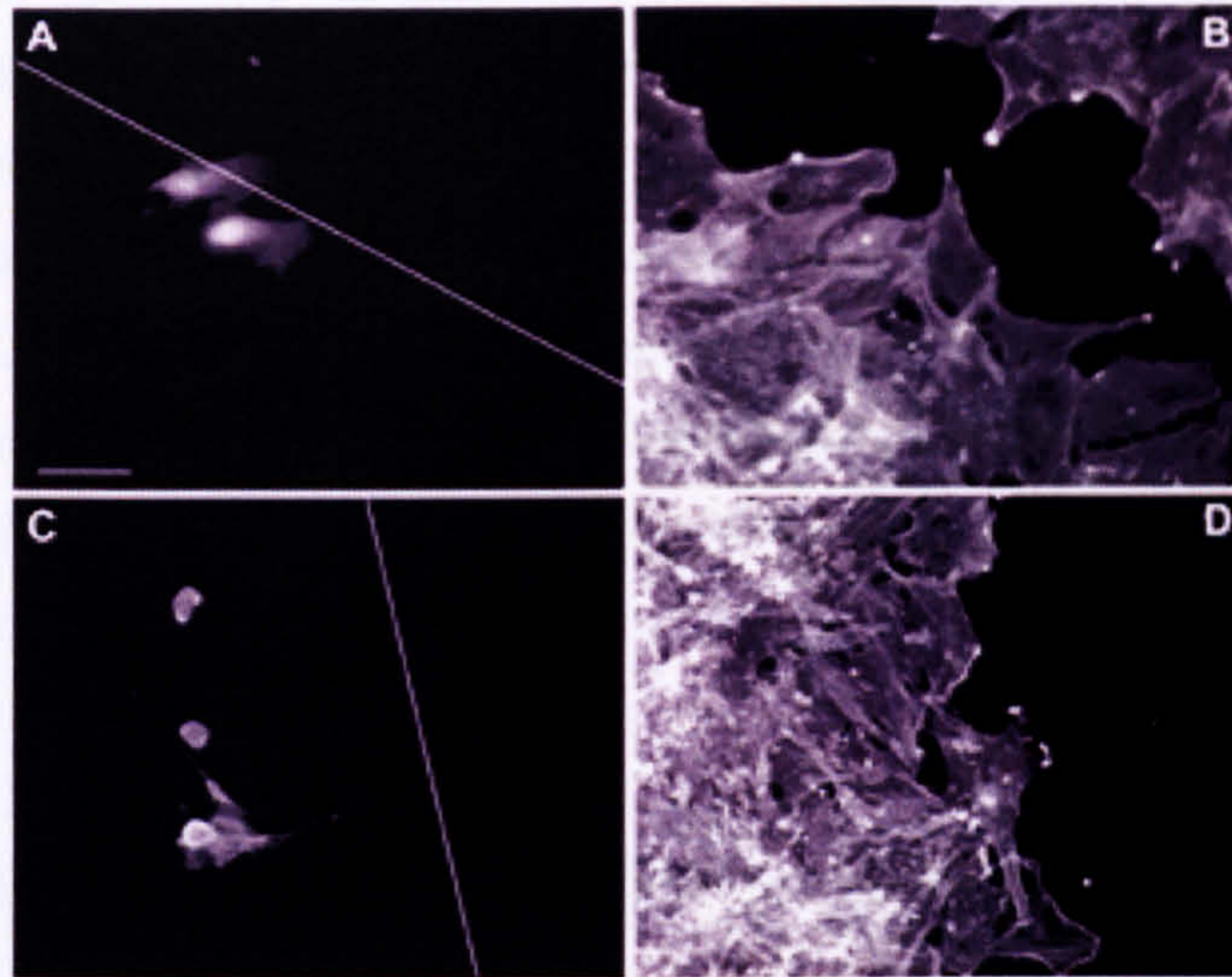
tail also contains two putative PEST sequences, implicated in PTEN protein stability, probably by targeting PTEN for proteasome-dependent degradation (Torres et al., 2003). In cells, PTEN is believed to be constitutively phosphorylated on its tail (Torres and Pulido, 2001; Vazquez et al., 2000). When cells are labeled with  $^{32}\text{P}$ -orthophosphate, endogenous and overexpressed PTEN molecules become radioactively labeled. PTEN was shown to become phosphorylated on Ser370, Ser385, and to a lesser extent Ser380, and on Thr382 and Thr383. Based on *in vitro* data, a kinase that is responsible for phosphorylating PTEN on its tail is believed to be casein kinase 2 (CK2), although exactly which sites CK2 targets is unclear (Birle et al., 2002; Miller et al., 2002; Torres and Pulido, 2001). In any case, phosphorylation seems to downregulate PTEN's activity as assessed by examining the induction of cell growth arrest by overexpressing PTEN phosphorylation site mutants (Vazquez et al., 2000).

Having established a role for the C2 domain of PTEN in U373 cell migration, this chapter investigates how this domain is regulated in the full-length protein. Many questions arise. For example, what is the role of the phosphatase activity in the full-length protein? Does PTEN phosphorylation or protein stability play a role in the regulation of PTEN function during cell migration?

## 5.3 Results

### 5.3.1 Post-translational modification of PTEN regulates its function

From previous studies, it appears that PTEN is constitutively phosphorylated and this might regulate its biological activity. To address whether phosphorylation of PTEN specifically plays a role in PTEN's ability to control cell migration, four alanine substitutions were introduced at four of the major phosphorylation sites at Ser380, Thr382, Thr383 and Ser385 in full length, phosphatase-dead PTEN [PTEN(C124S)-A4, Figure 5.1, 5.2]. This construct was then microinjected in U373 cells in a wound-healing assay and its ability to inhibit migration was examined. As shown in figure 5.1, removal of these four phosphorylation sites completely rescues the ability of catalytically-dead PTEN to inhibit migration of U373 cells.



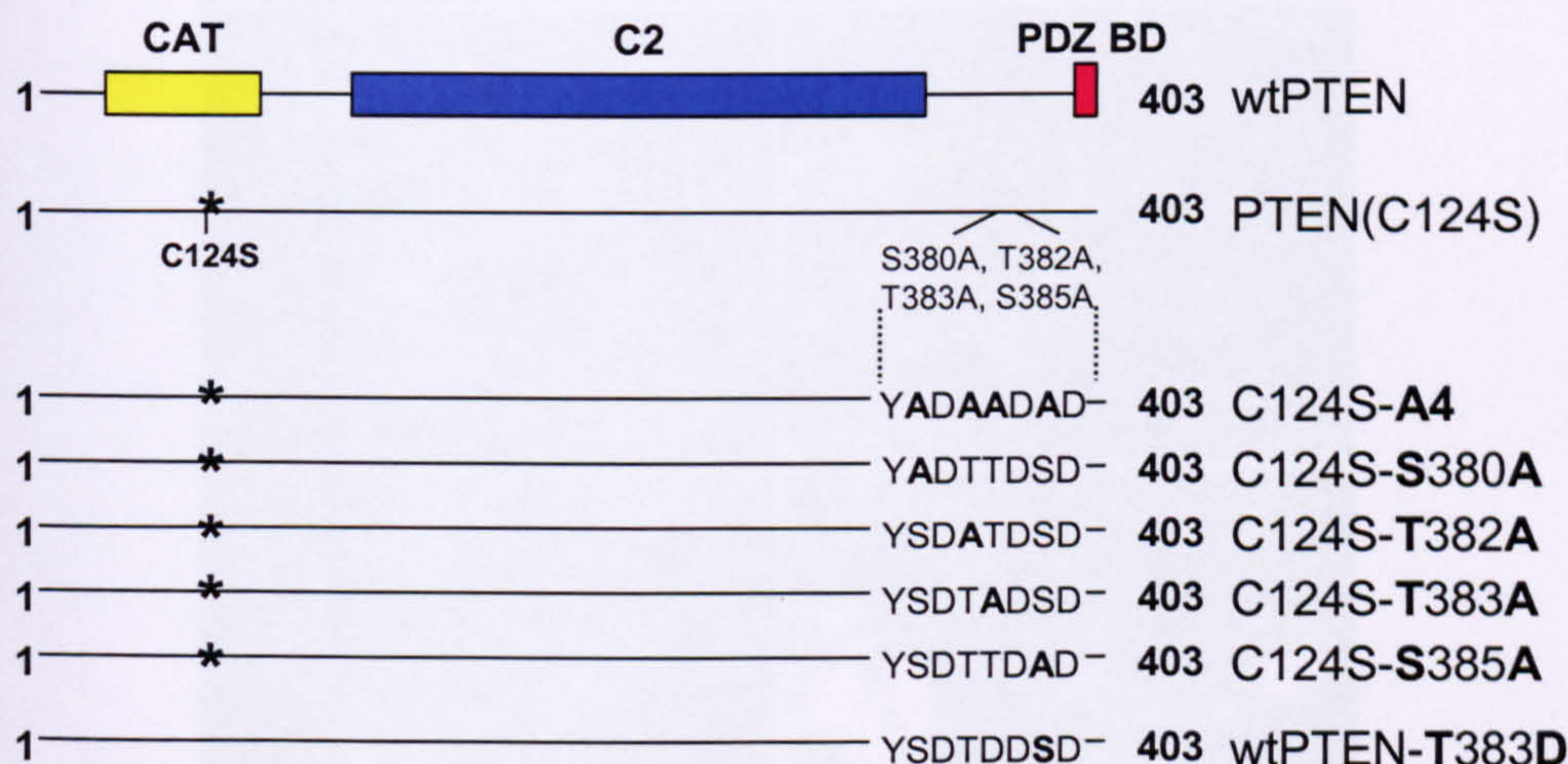
**FIGURE 5.1**

The ability of PTEN to regulate U373 migration is dependent on its phosphorylation state. (a) U373 cells microinjected with vectors encoding GFP (A, B) or Myc-epitope tagged PTEN(CS)-A4 (C, D). Microinjected cells were fixed and detected by staining with 9E10 anti-Myc antibody (A, C). Actin was visualized using rhodamine-conjugated phalloidin (B, D). Scale bar, 50 $\mu$ m. A white line is drawn to indicate the leading edge of the wound. (b) Quantitation of the number of microinjected U373 cells that are found at the edge of the wound after migration for 8h. Data shown are the mean + SEM of three to five independent experiments where an average of 100 cells (20 cells in each of 5 separate wound edges) were injected for each construct.

Interestingly, a significant fraction of the PTEN(CS)-A4 protein seemed to localize predominantly to the nucleus in around 60% of the injected cells. Although nuclear import/ export sequences could not be found in PTEN, it has recently been shown to be able to localize to the nucleus of cells in a complex with p53 and this interaction has been mapped to the C2 domain of PTEN (Freeman et al., 2003). Mutation of these four potential phosphorylation sites of PTEN to alanine residues might therefore uncover the interaction site of PTEN's C2 domain and cause it to be retained in the nucleus through its binding to p53, but this has not been further investigated.

### 5.3.2 Phosphorylation of a single PTEN residue renders the protein inactive

In order to pinpoint which of the four phosphorylation sites are involved in the regulation of the C2 function, a series of constructs were made in which only one residue at a time was altered to alanine (Figure 5.2).



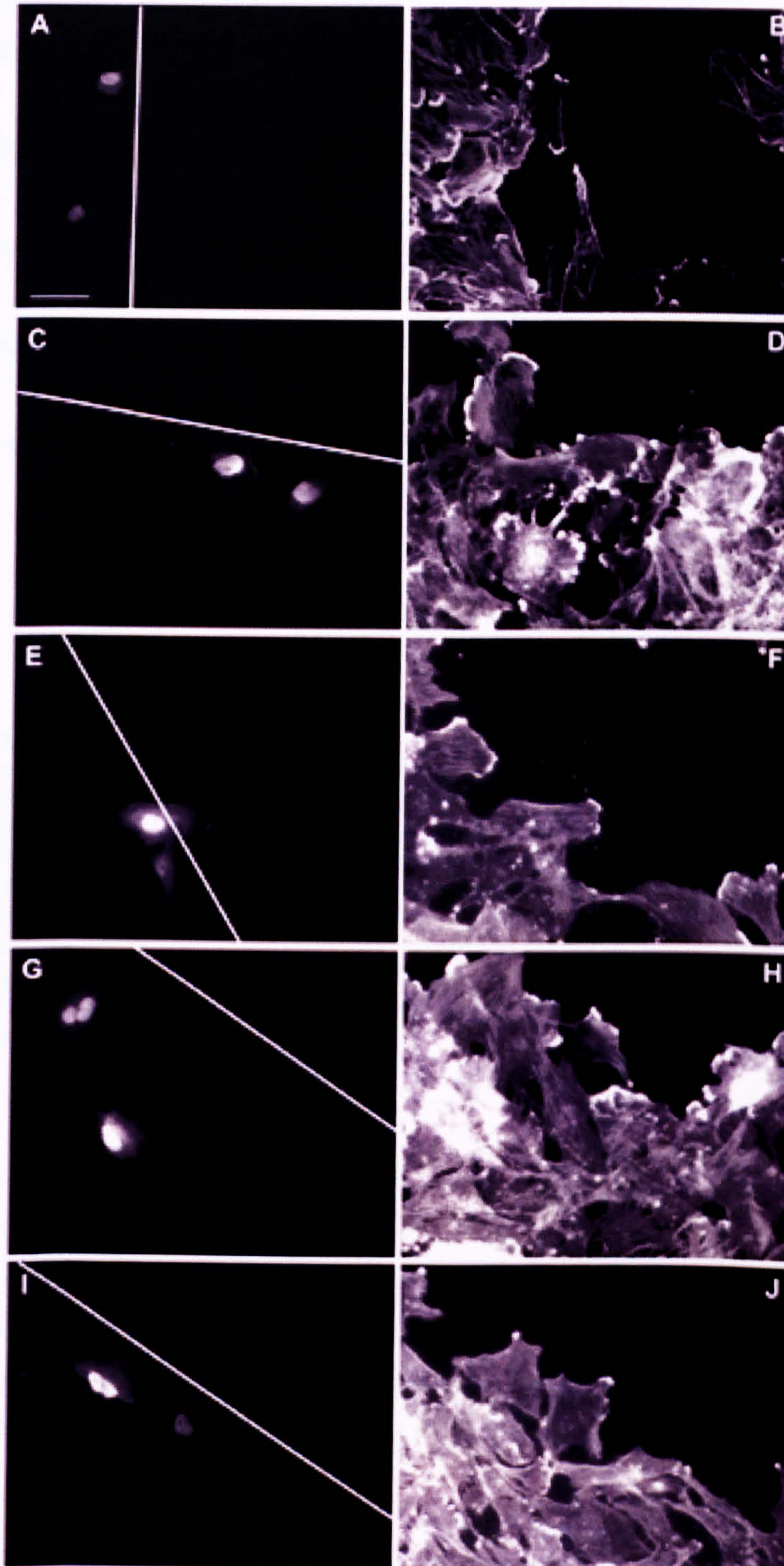
**FIGURE 5.2**

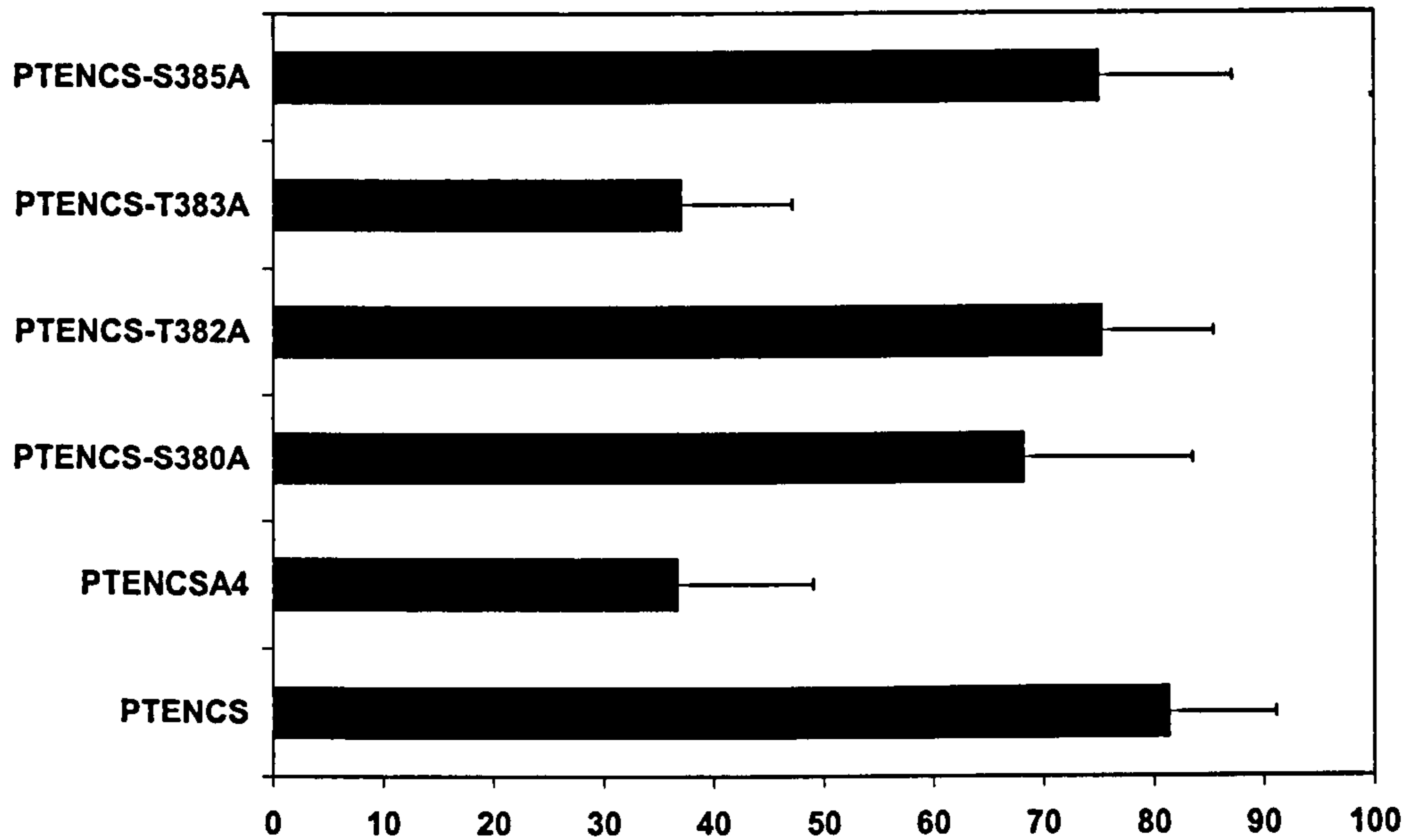
Schematic representation of the PTEN phosphorylation site mutants used.

Interestingly, injection of these constructs in a wound-healing assay revealed that mutation of one residue, Thr383, was sufficient to reverse the phenotype of catalytically dead PTEN [PTEN(C124S)] and inhibit migration to levels comparable to those of the PTEN(C124S)-A4 mutant (Figure 5.3). The other three constructs [PTEN(C124S)-



S380A, PTEN(C124S)-T382A and PTEN(C124S)-S385A] had no statistically significant effect on migration. This strongly suggests that, in the full-length PTEN protein, Thr383 needs to be dephosphorylated in order for the C2 domain to regulate migration of U373 cells.

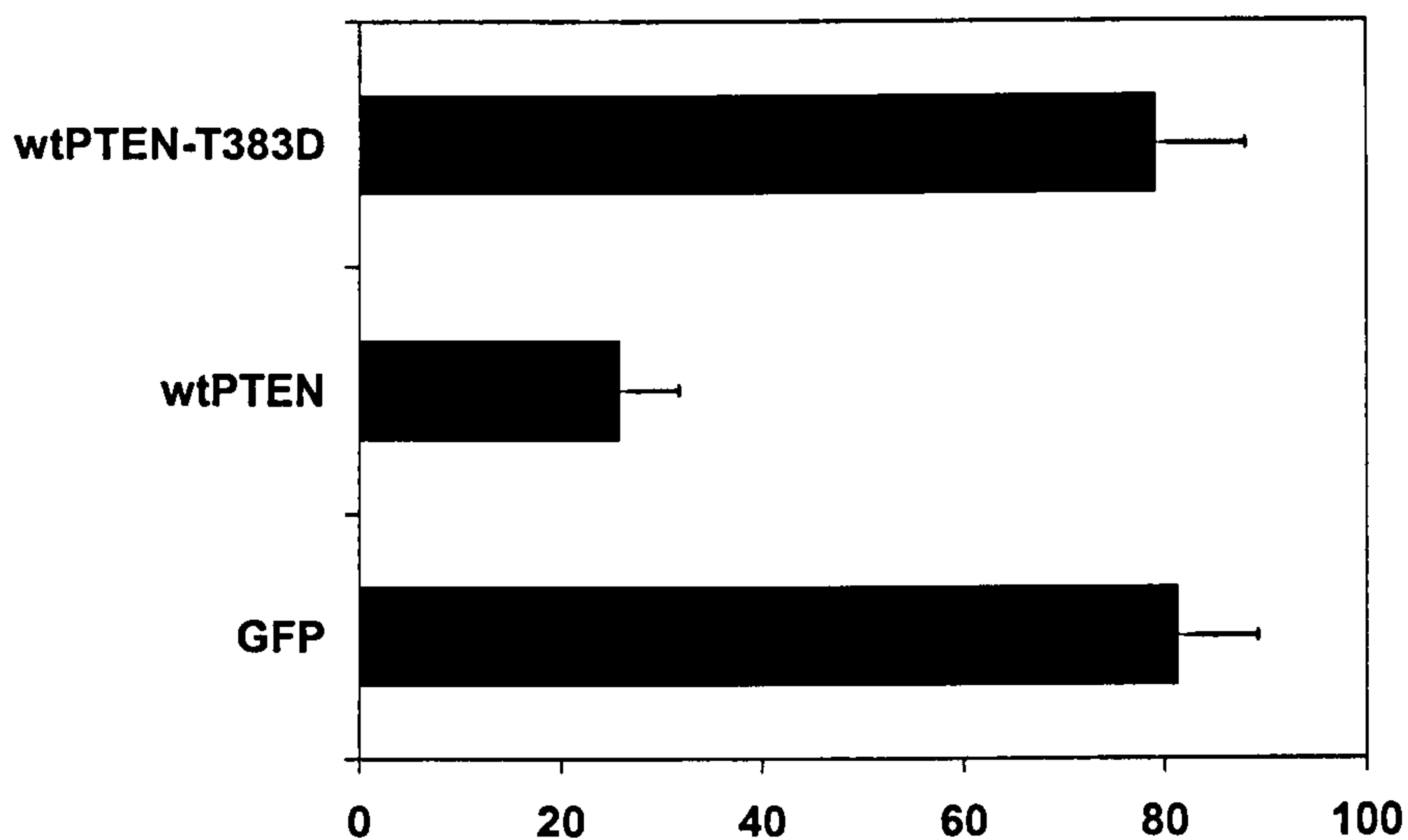




**FIGURE 5.3**

Dephosphorylation of Thr383 is critical for PTEN's ability to inhibit migration of U373. (a) U373 cells microinjected with vectors encoding GFP (A, B) or Myc-epitope tagged PTEN(CS)-S380A (C, D), PTEN(CS)-T382A (E, F), PTEN(CS)-T383A (G, H) or PTEN(CS)-S385A (I, J). Microinjected cells were fixed and detected by staining with 9E10 anti-Myc antibody (A, C, E, G, I). Actin was visualized using rhodamine-conjugated phalloidin (B, D, F, H, J). Scale bar, 50 $\mu$ m. A white line is drawn to indicate the leading edge of the wound. (b) Quantitation of the number of microinjected U373 cells that are found at the leading edge of the wound after migration for 16h. Data shown are the mean + SEM of three to five independent experiments where an average of 100 cells (20 cells in each of 5 separate wound edges) were injected for each construct.

In order to confirm that regulation of phosphorylation at Thr383 is critical for PTEN's biological activity, the converse experiment was performed where the same residue was mutated to an aspartic acid in the full-length, wild type protein (PTENwt-T383D). When microinjected in U373 cells, this phospho-mimic mutant could no longer inhibit migration of U373 cells (Figure 5.4).



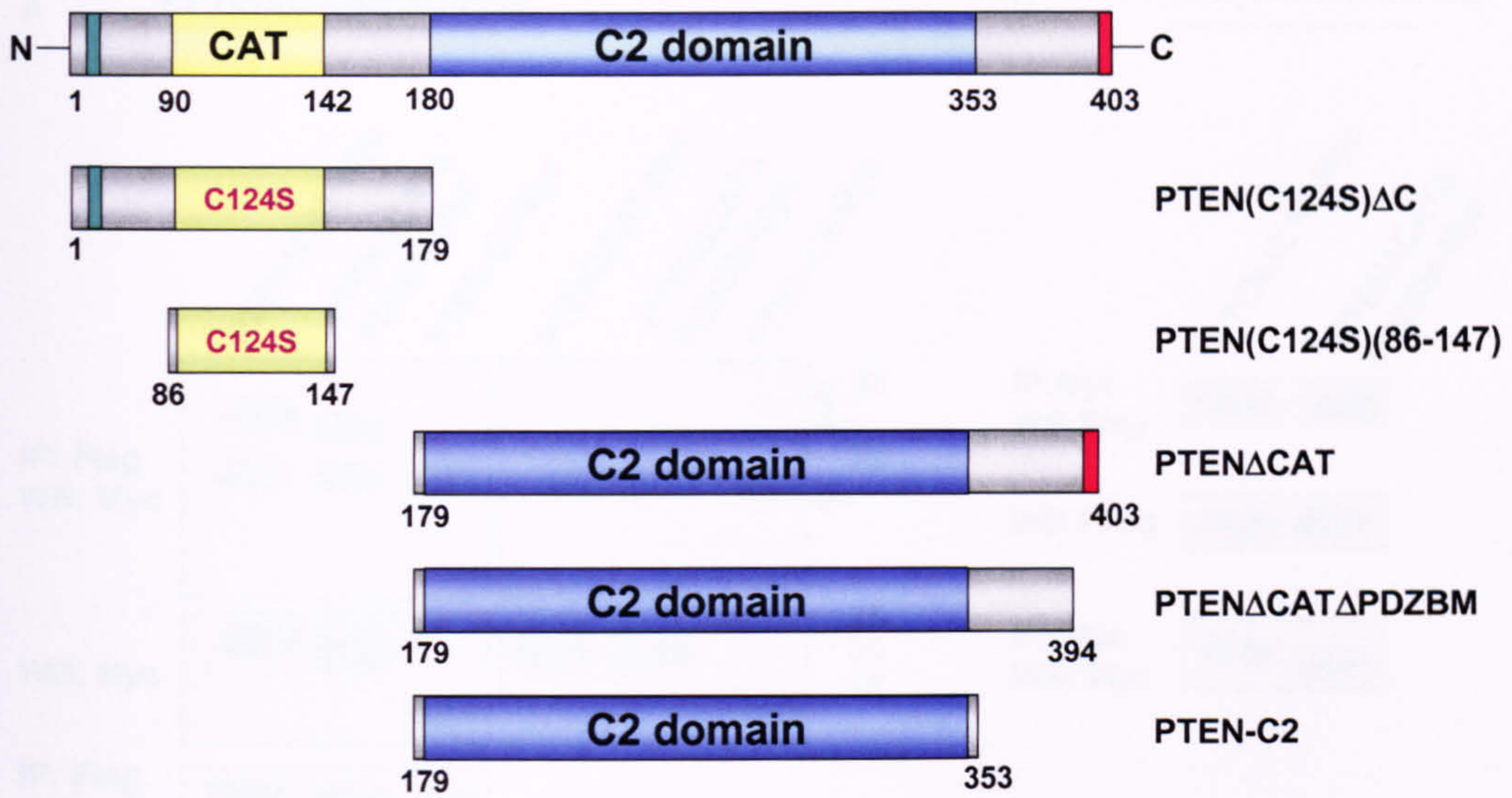
**FIGURE 5.4**

A PTEN phospho-mimetic mutation at Thr383 abrogates PTEN's ability to inhibit migration. (a) Quantitation of the number of microinjected U373 cells that are found at the edge of the wound after migration for 8h. Data shown are the mean + SEM of three to five independent experiments where an average of 100 cells (20 cells in each of 5 separate wound edges) were injected for each construct.

In conclusion, the data indicates that Thr383 must be dephosphorylated to allow the C2 domain to inhibit migration and that this depends on the protein, but not lipid, phosphatase activity of PTEN.

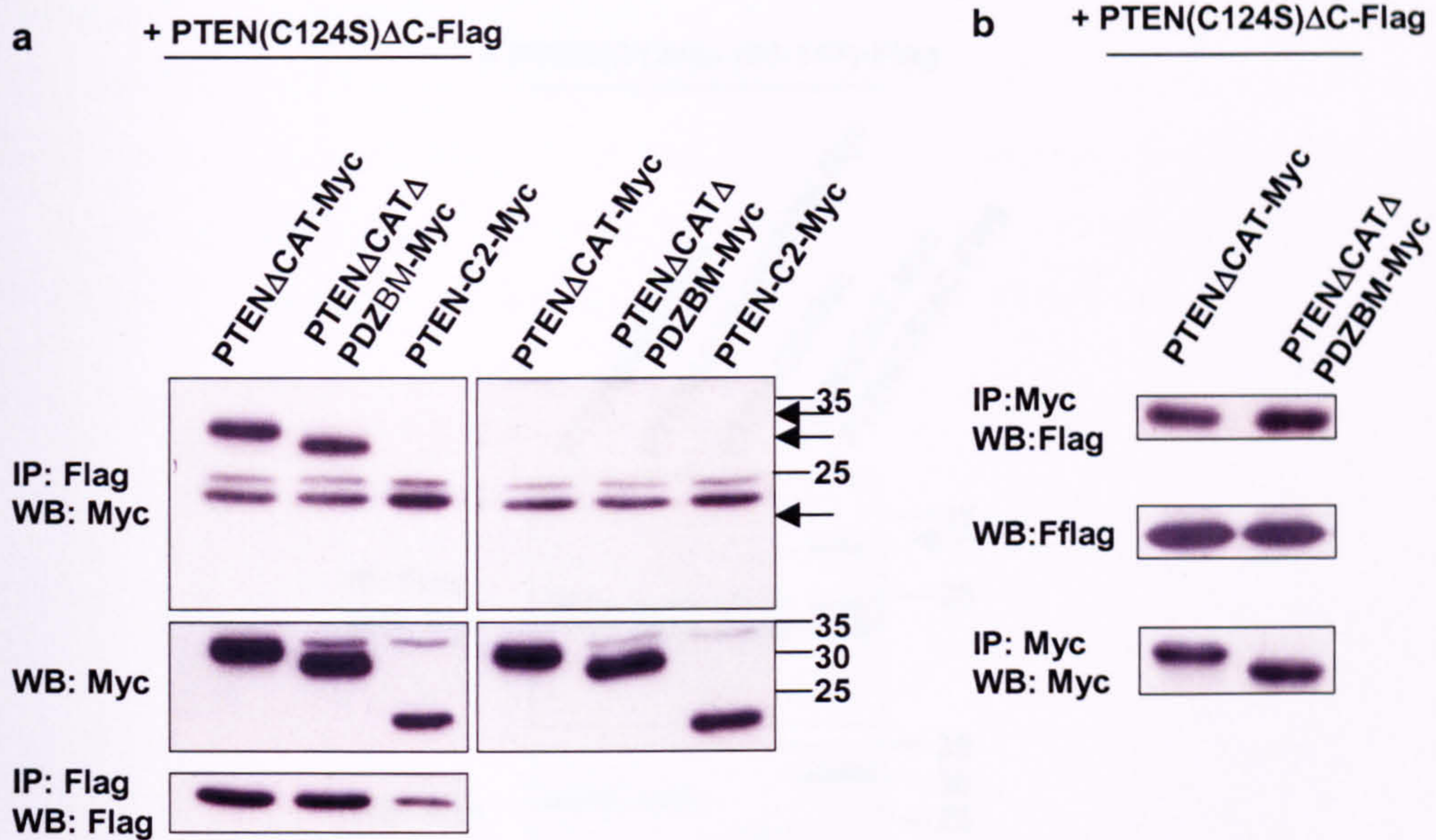
### 5.3.3 The N-terminus of PTEN binds its C-terminus

As inferred from the crystal structure, there is a significant overlap between the catalytic domain and the C2 domain of PTEN, showing that PTEN exists in a folded state. To gain further insight into the possible mode of regulation of C2 activity, PTEN's intramolecular interactions were explored. We first examined whether the C-terminus of PTEN could be co-precipitated with the N-terminus. The following constructs were cloned into pRK5-Myc or pRK5-Flag mammalian expression vectors.

**FIGURE 5.5**

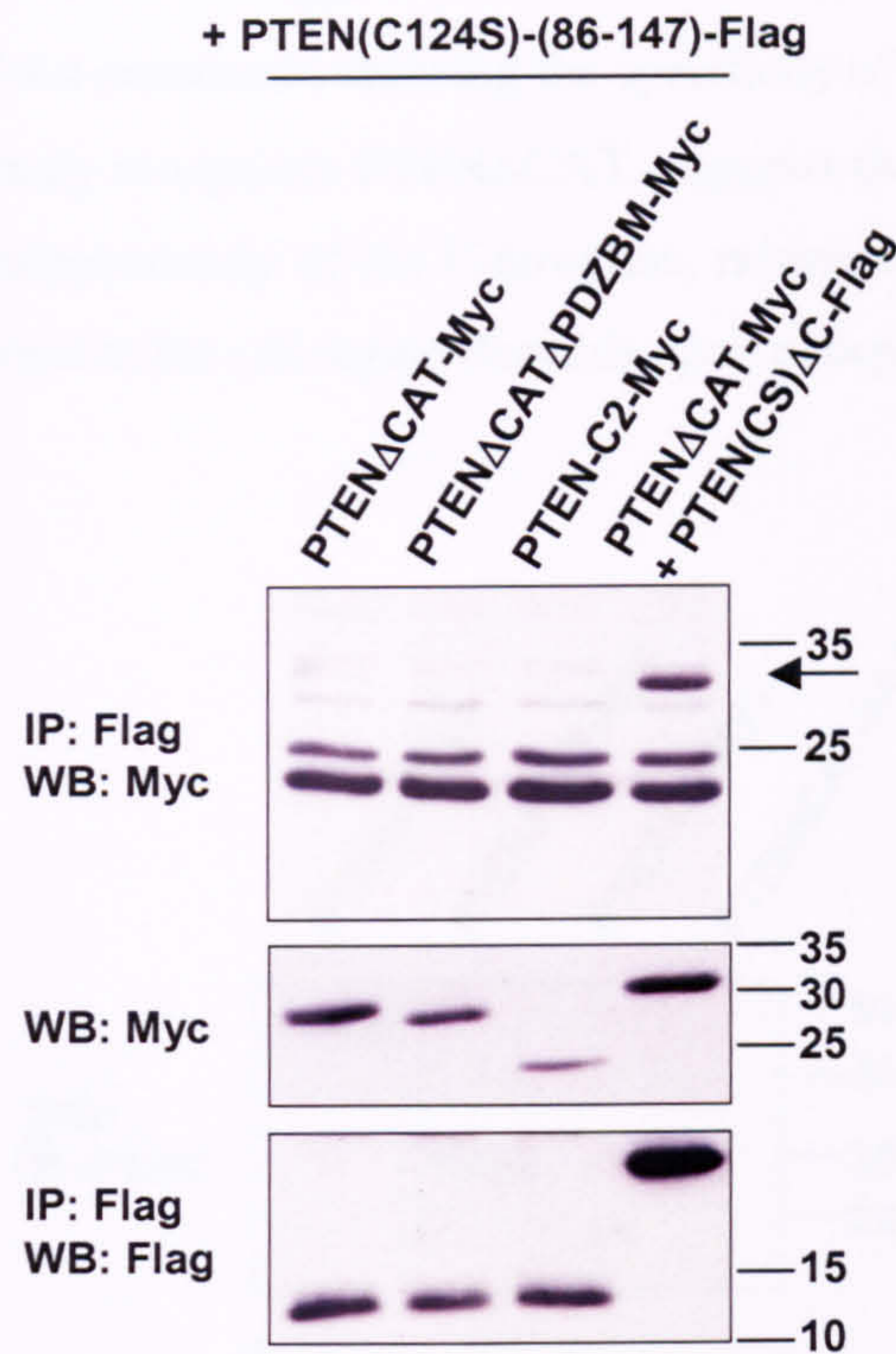
Schematic representation of the PTEN truncation mutants used in the immunoprecipitation assays.

COS-7 cells were co-transfected with the catalytically inactive N-terminus of PTEN [PTEN(C124S) $\Delta$ C] and either the C-terminus (PTEN $\Delta$ CAT), the C-terminus minus the PDZ binding motif (PTEN $\Delta$ CAT $\Delta$ PDZBM), or the C2 domain of PTEN (PTEN-C2) (see Figure 5.5). In each case an anti-Flag antibody was used to immunoprecipitate the N-terminus and an anti-Myc antibody to detect for binding of the different C-terminal constructs (Figure 5.6). Immunoprecipitation of the N-terminus [residues 1-175; PTEN(C124S) $\Delta$ C] efficiently precipitated the C-terminus (residues 175-403; PTEN $\Delta$ CAT). However, despite the large surface of interaction revealed in the crystal structure, the C2 domain alone (residues 175-353; PTEN-C2) was not present in immunoprecipitates of the N-terminus (Figure 5.6a), indicating that the C-terminal 50 amino acid tail is essential for strong interaction. The interaction was also confirmed by immunoprecipitating with an anti-Myc antibody and blotting with an anti-Flag antibody to detect the N-terminus (Figure 5.6).

**FIGURE 5.6**

The N-terminus of PTEN binds the C-terminus of PTEN. (a) COS-7 cells were co-transfected using Myc-epitope tagged PTENΔCAT, PTENΔCATΔPDZBM or PTEN-C2 with, (lanes 1-3), or without, (lanes 4-6), Flag-epitope tagged, catalytically inactive N-terminus of PTEN [PTEN(C124S)ΔC]. PTEN(C124S)ΔC was immunoprecipitated (IP) with anti-Flag antibody and the precipitates analysed on Western blots with 9E10 anti-Myc antibody or anti-flag antibody. One tenth of the total lysate was also immunoblotted with 9E10 anti-Myc antibody. (b) COS-7 cells were co-transfected using Myc-epitope tagged PTENΔCAT, PTENΔCATΔPDZBM with Flag-epitope tagged, catalytically inactive N-terminus of PTEN [PTEN(C124S)ΔC-flag]. PTENΔCAT and PTENΔCATΔPDZ were immunoprecipitated (IP) with anti-myc antibody and the precipitates analysed on western blots with anti-Flag antibody or 9E10 anti-Myc antibody. One tenth of the total lysate was also immunoblotted with anti-Flag antibody.

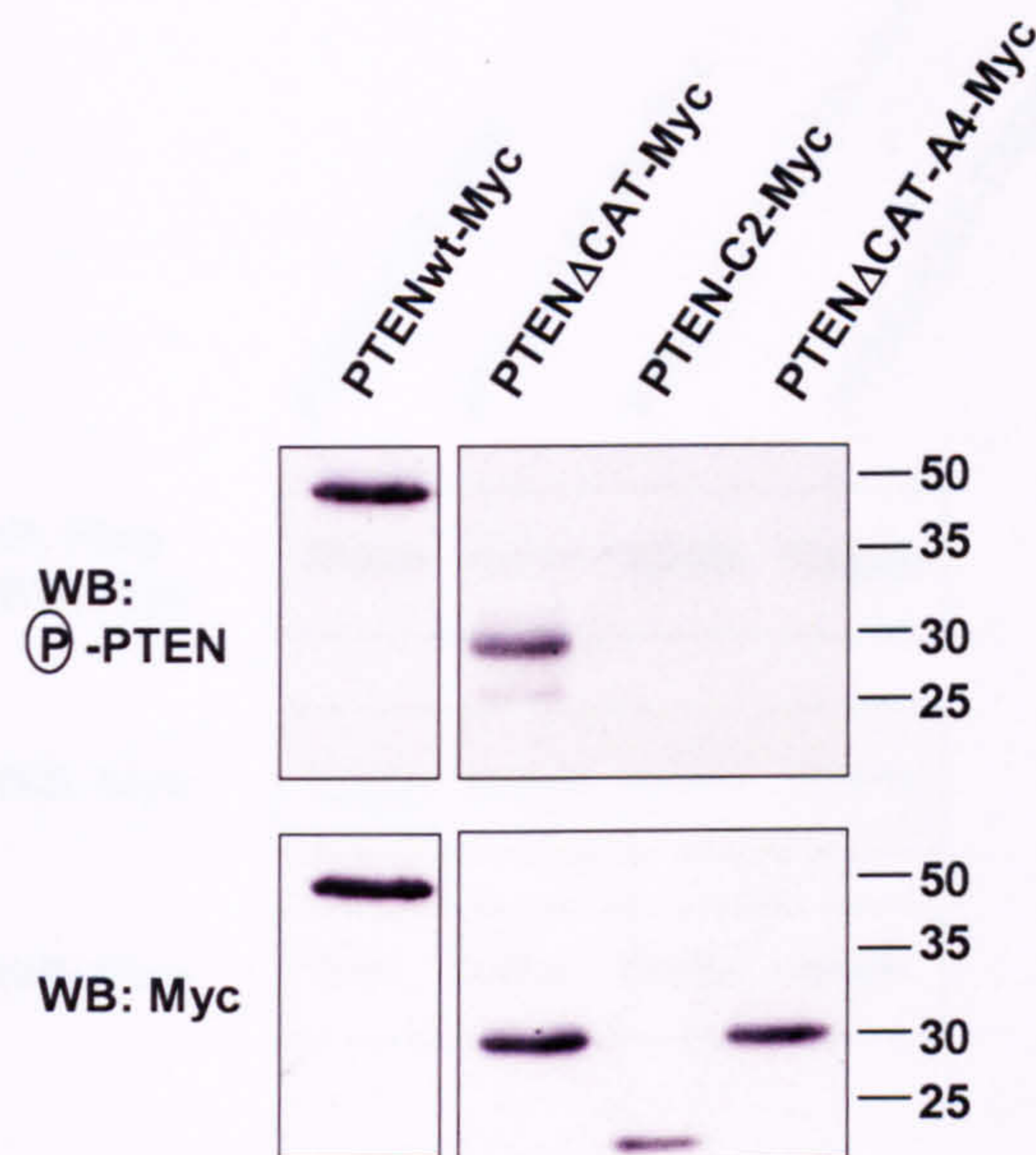
To test whether the minimal catalytic active site pocket of PTEN is required for the binding of the N-terminus to the C-terminus, a PTEN(C124S)(86-147) construct was co-transfected in COS-7 cells with each of the C-terminal constructs (figure 5.7). An anti-Flag antibody was used to immunoprecipitate the catalytic domain and an anti-Myc antibody to detect binding of the C-terminal constructs. None of the C-terminal constructs co-immunoprecipitated with PTEN(C124S)(86-147), suggesting that the site where the C-terminus tail interacts with the N-terminus lies outside the 86 to 147 region of the catalytic domain.

**FIGURE 5.7**

The C-terminus of PTEN interacts with the N-terminus in a region outside residues 86 to 147. COS-7 cells were co-transfected using Myc-epitope tagged PTEN $\Delta$ CAT, PTEN $\Delta$ CAT $\Delta$ PDZ or PTEN-C2 with flag-epitope tagged, catalytically inactive N-terminus of PTEN residues 90-142 [PTEN(CS)-(90-142), (lanes 1-3), or using Myc-epitope tagged PTEN $\Delta$ CAT with flag-epitope tagged PTEN(C124S) $\Delta$ C, (lane 4), to control for the immunoprecipitation. PTEN(C124S)(86-147) was immunoprecipitated (IP) with anti-flag antibody and the precipitates analyzed on western blots with 9E10 anti-myc antibody or anti-flag antibody. One tenth of the total lysate was also immunoblotted with 9E10 anti-myc antibody.

To determine whether this interaction is dependent on the PTEN tail being phosphorylated on one or more residues, the phosphorylation state of PTEN $\Delta$ CAT was examined by transfection in COS-7 cells and blotting with a commercial phospho-specific antibody raised against a synthetic PTEN-derived peptide triply phosphorylated at Ser380, Thr382, Thr383 (also used by (Das et al., 2003)). To control for the specificity of the antibody, full-length PTEN, PTEN-C2 and PTEN $\Delta$ CAT-A4, a C-terminal domain construct containing alanine substitutions that remove the four previously described phosphorylation sites at Ser370, Thr380, Thr382 and Ser385, were also transfected.

Figure 5.8 shows that the antibody specifically recognizes the full length PTEN construct as expected, whereas there is no signal detected in the lanes transfected with the PTEN-C2 and PTEN $\Delta$ CAT-A4 constructs, showing the specificity of the antibody. Furthermore, the fact that the antibody recognizes PTEN $\Delta$ CAT, suggests that the truncated protein can be phosphorylated independently of the C-terminus, raising the possibility that binding of the PTEN N-terminus to the tail region depends upon phosphorylation.

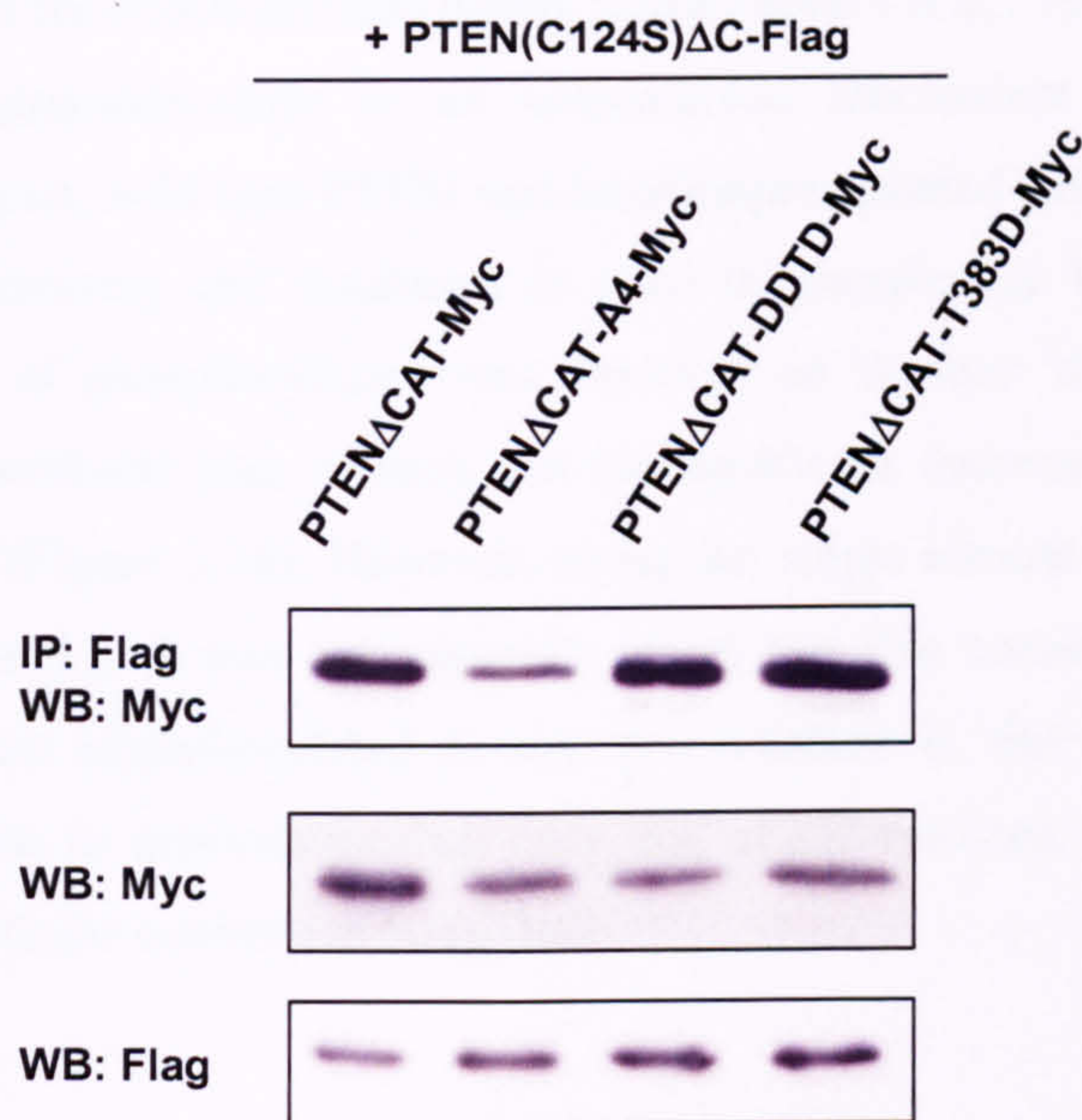


**FIGURE 5.8**

The C-terminus of PTEN when expressed alone in cells is still a substrate for phosphorylation. COS-7 cells were transfected with Myc-epitope tagged PTENwt, PTEN $\Delta$ CAT, PTEN-C2 and PTEN $\Delta$ CAT-A4. Anti-Myc 9E10 antibody was used to immunoprecipitate the constructs and this was followed by SDS-PAGE and Western blot analysis. Phosphorylation of these constructs was examined by blotting with an anti-phospho-PTEN specific antibody.

To examine whether phosphorylation affects the intermolecular interaction between the N- and C-termini, the N-terminal catalytic domain [PTEN(C124S) $\Delta$ C] was co-expressed with PTEN $\Delta$ CAT-A4 or the phospho-mimetic constructs substituted at Thr383 (PTEN $\Delta$ CAT-T383D) or Ser380, Thr382 and Ser385 (PTEN $\Delta$ CAT-DDTD) to aspartic acid residues. As shown in figure 5.9, all mutant C-terminal constructs, PTEN $\Delta$ CAT-A4, PTEN $\Delta$ CAT-T383D and PTEN $\Delta$ CAT-DDTD, and the wild type C-terminus interact

equally well with the N-terminal phosphatase domain in this assay. It appears, therefore, that the interaction of the N- and C-termini is neither promoted nor inhibited by phosphorylation, at least at these four sites.



**FIGURE 5.9**

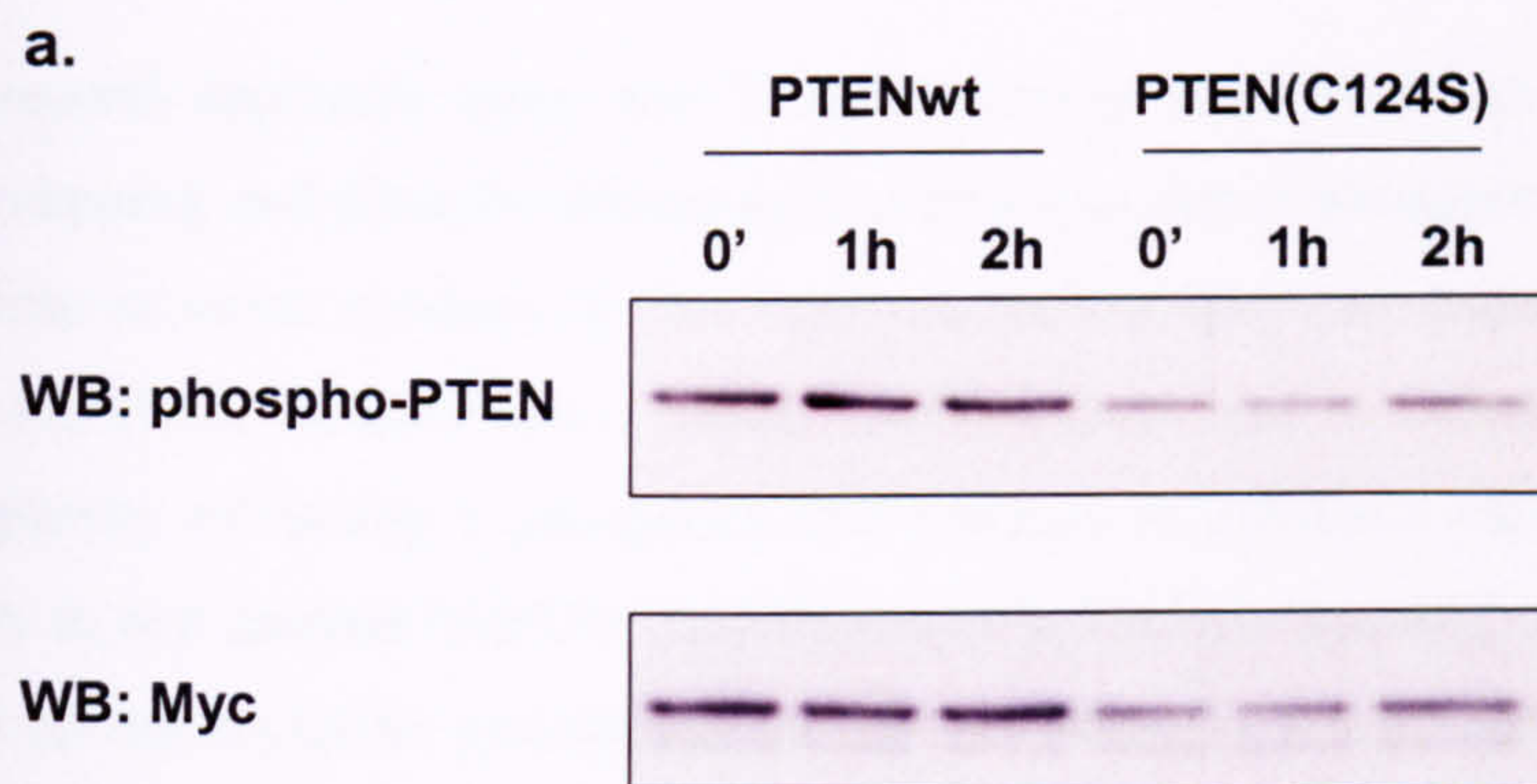
The intramolecular interaction of the N- and C-termini of PTEN is independent of the phosphorylation state of the C-terminus. COS-7 cells were co-transfected with the Flag-tagged, catalytically inactive N-terminus of PTEN [PTEN(C124S) $\Delta$ C-Flag] and either Myc-tagged PTEN $\Delta$ CAT (lane 1) or PTEN $\Delta$ CAT with residues Ser380, Thr382, Thr383 and Ser385 all mutated to alanine (PTEN $\Delta$ CAT-A4) (lane 2), or PTEN $\Delta$ CAT with residues S380, Thr382, and Ser385 all mutated to aspartic acid (PTEN $\Delta$ CAT-DDTD) (lane 3), or PTEN $\Delta$ CAT with residue Thr383 mutated to aspartic acid (PTEN $\Delta$ CAT-T383D). The N-terminus of PTEN was immunoprecipitated (IP) using an anti-Flag antibody and the precipitates analysed on Western blots with 9E10 anti-Myc antibody or anti-Flag antibody. One tenth of the total lysate was also immunoblotted with 9E10 anti-Myc antibody.

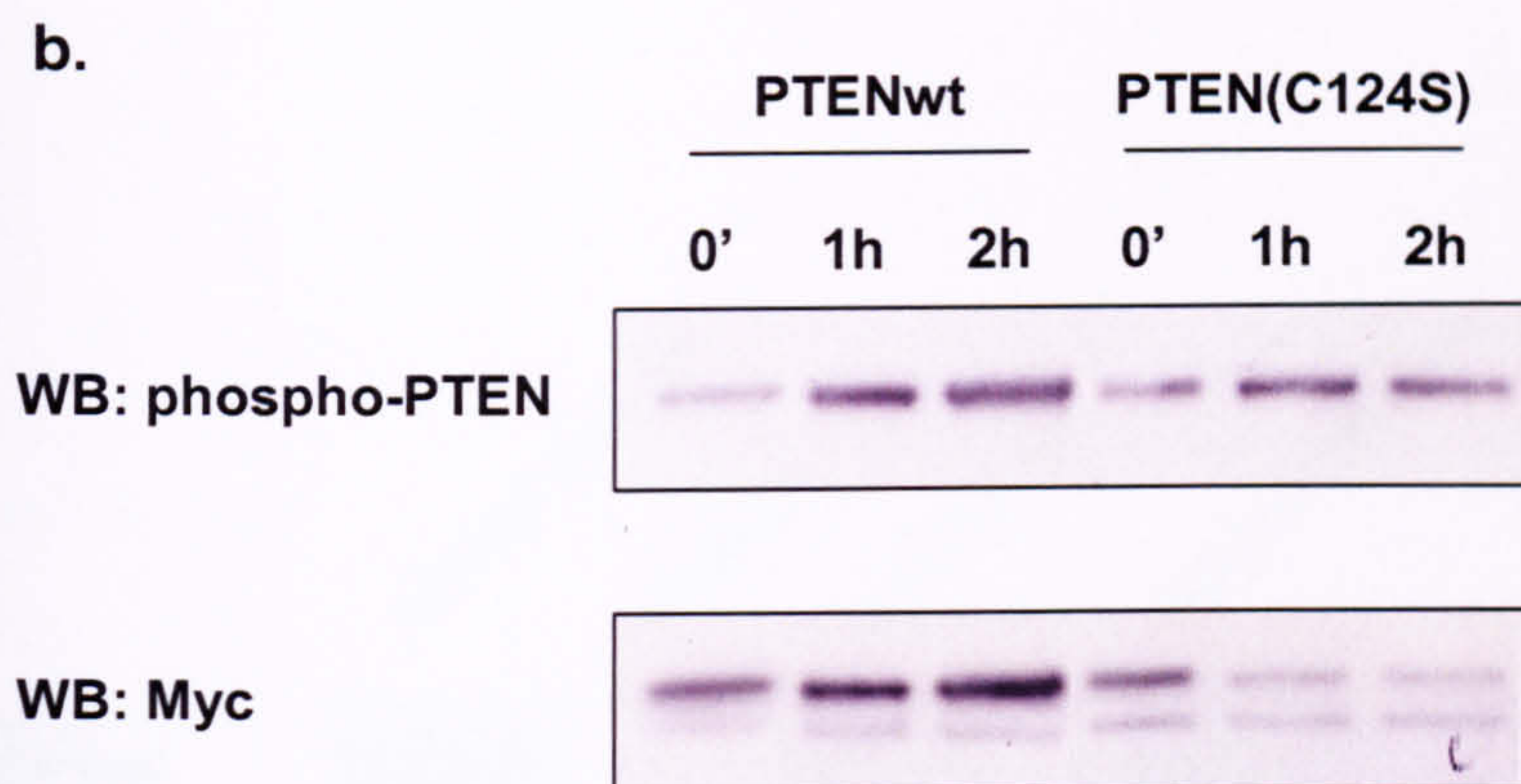
In conclusion, the immunoprecipitation data presented here, along with the information from the crystal structure, suggests that PTEN most probably exists in a folded conformation. Furthermore, there appears to be a physical interaction between the PTEN carboxyl-tail and the N-terminus, which is independent of the PDZ binding motif and does not appear to be regulated by phosphorylation.



### 5.3.4 PTEN regulates its own phosphorylation on Thr383

PTEN is reported to be a very poor protein phosphatase. Interestingly, however, the 12-residue peptide sequence surrounding Thr383 (YSDTTDSDPENE) is extremely acidic (5 Asp/Glu and 4 phosph-Ser/Thr residues) and the optimal peptide substrates previously reported for PTEN are also highly acidic (Myers et al., 1997). To test whether PTEN can act *intramolecularly* in an autocatalytic mechanism to dephosphorylate residues in this region, wild type PTEN was immunoprecipitated from transfected COS-7 cells, washed extensively and incubated *in vitro* in phosphatase buffer (Myers et al., 1997). The level of phosphorylation was observed on Western blots using the PTEN phospho-specific antibody (see earlier), but no significant decrease in phosphorylation could be detected (Figure 5.10). However, using the single alanine substitution mutants described in figure 5.2, it was subsequently found that this commercial antibody also detects PTEN when phosphorylated at any two residues in this sequence. If PTEN, therefore, were able to dephosphorylate only one of the residues, this would not have been detectable with the current antibody used.

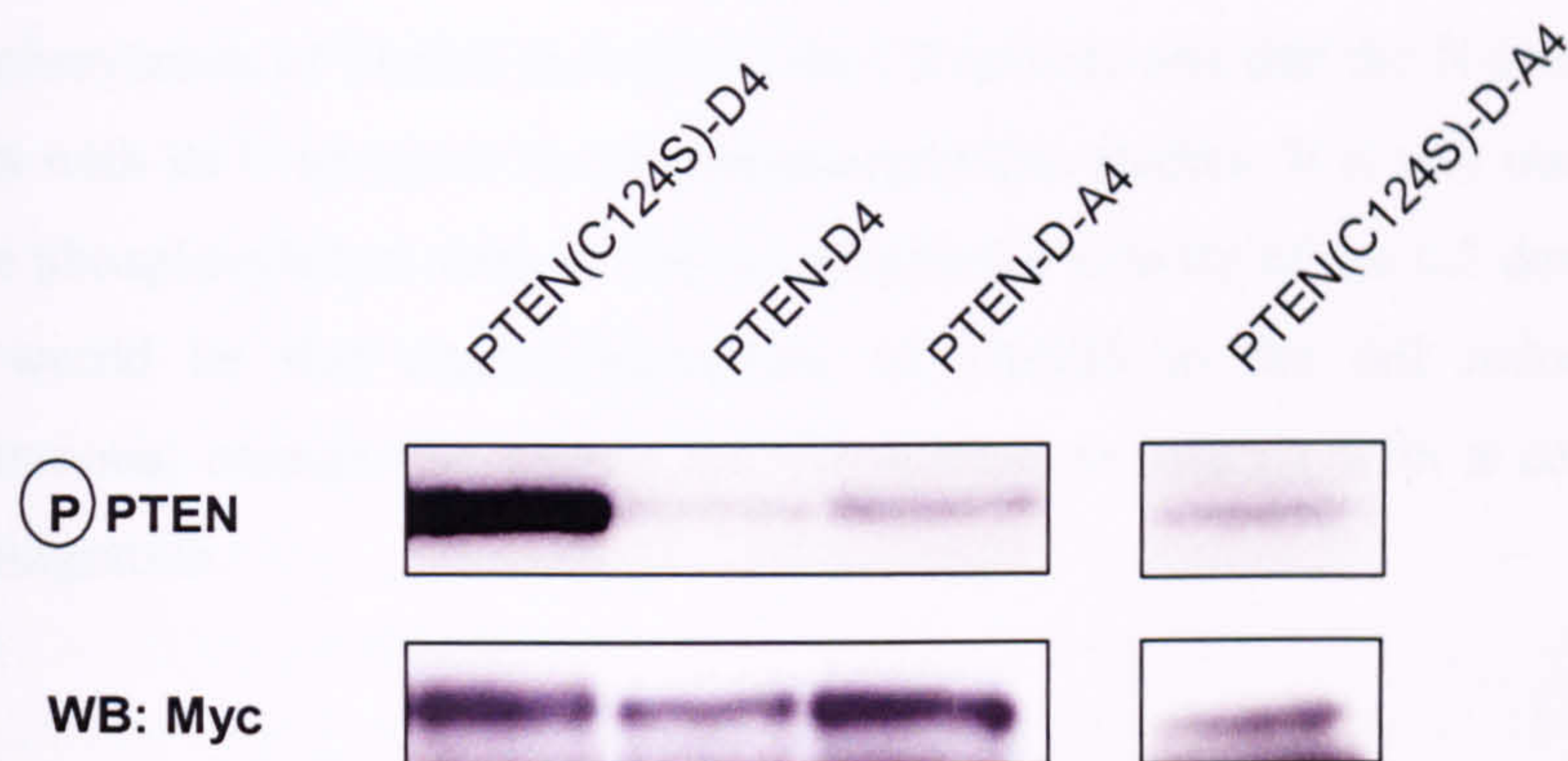


**FIGURE 5.10**

Comparison of PTEN phosphorylation in a wild type and phosphatase-dead background. COS-7 cells were seeded in 6-well dishes and transfected with the Myc-epitope tagged PTENwt or PTEN(C124S) constructs. Anti-Myc 9E10 antibody was used to immunoprecipitate the proteins and following extensive washing, the proteins were incubated in phosphatase buffer at 30°C (**a**) or 37°C (**b**) for the indicated times. Following analysis by SDS-PAGE and Western blot, an anti-phospho-specific PTEN antibody was used to detect for phosphorylated PTEN.

The second approach taken was to radioactively label PTEN in cells followed by peptide mapping and phospho-amino acid analysis to detect phosphorylation specifically at threonine or serine residues by thin layer chromatography (see Materials and Methods, (Birle et al., 2002; Vazquez et al., 2000)). Unfortunately, due to technical difficulties, and the complexity of having 5 phosphorylated residues at unknown stoichiometry in close proximity to one another (Ser370, Ser380, Thr382, Thr383, Ser385), it was impossible to compare phosphorylation specifically at Thr383 between a wild type and catalytically dead PTEN background (data not shown). To get around this problem, aspartic acid substitutions were introduced at four of the five major phosphorylated residues at Ser370, Ser380, Thr382 and Ser385, in the full length catalytically-active (PTEN-D4) and full-length catalytically-dead protein [PTEN(C124S)-D4]. As a control for phosphorylation at other sites, all five residues (Ser370, Ser380, Thr382, Thr383 and Ser385) were also mutated in both the catalytically-active (PTEN-S370D-A4) and catalytically-dead [PTEN(C124S)-S370D-A4] protein. The PTEN constructs were transfected in COS-7 cells and immunoprecipitated following [<sup>32</sup>P] orthophosphate - labeling. The proteins

were then analysed by SDS-PAGE and exposed to a phosphorimager screen (Figure 5.11).



**FIGURE 5.11**

PTEN is phosphorylated on Thr383 in a catalytically dead background but not in the wild type protein. COS-7 cells grown in [ $^{32}\text{P}$ ] orthophosphate were transfected with Myc-epitope-tagged versions of (i) catalytically-dead PTEN with residues Ser370, Ser380, Thr382, and Ser385 mutated to aspartic acid [PTEN(C124S)-D4], (ii) wild type PTEN with residues Ser370, S380, Thr382, and Ser385 mutated to aspartic acid (PTEN-D4), (iii) wild type PTEN with residues Ser370, Ser380, Thr382, Thr383 and Ser385 all mutated (PTEN-S370D-A4), (iv) catalytically-dead PTEN with residues Ser370, Ser380, Thr383, Thr383 and Ser385 all mutated [PTEN(C124S)-S370D-A4]. Anti-Myc 9E10 antibody was used to immunoprecipitate the PTEN constructs. Phosphorylation of the constructs was detected and quantitated by autoradiography using a phosphorimager. Anti-Myc 9E10 antibody was also used to control for the expression levels of the constructs immunoprecipitated.

Catalytically-inactive PTEN [PTEN(C124S)-D4] incorporated approximately 10 times more label than the catalytically-active PTEN (PTEN-D4). Furthermore, the residual phosphate incorporated into PTEN-D4 is not on Thr383, since the levels of phosphate incorporated into catalytically-active PTEN in which all five major phosphorylation sites (i.e. including Thr383) had been mutated, are the same as PTEN-D4. This background phosphorylation is also present in the catalytically-dead PTEN protein with all five major phosphorylation sites mutated [PTEN(C124S)-S370D-A4] (Figure 5.11). These data therefore indicate that while the catalytically inactive PTEN is phosphorylated at Thr383, wild type PTEN is not phosphorylated at this site, following immunoprecipitation from COS-7 cells.

## 5.4 Discussion

Work in chapter 4 identified a novel mechanism by which PTEN inhibits the migration of human glioma cells through its C2 domain, independently of its effects on the PI 3-kinase pathway. This chapter revealed that in wild type PTEN, dephosphorylation of Thr383 is required for C2 activity and that the N-terminus of PTEN interacts with its C-terminus in immunoprecipitation studies. It is still unclear, however, how the phosphorylation state of Thr383 affects the activity of the C2 domain. A simple model would be that dephosphorylation of Thr383 in the tail induces a specific conformational change that allows the C2 domain to interact with a cellular target to inhibit migration.

### 5.4.1 PTEN phosphorylation as key regulator of its function

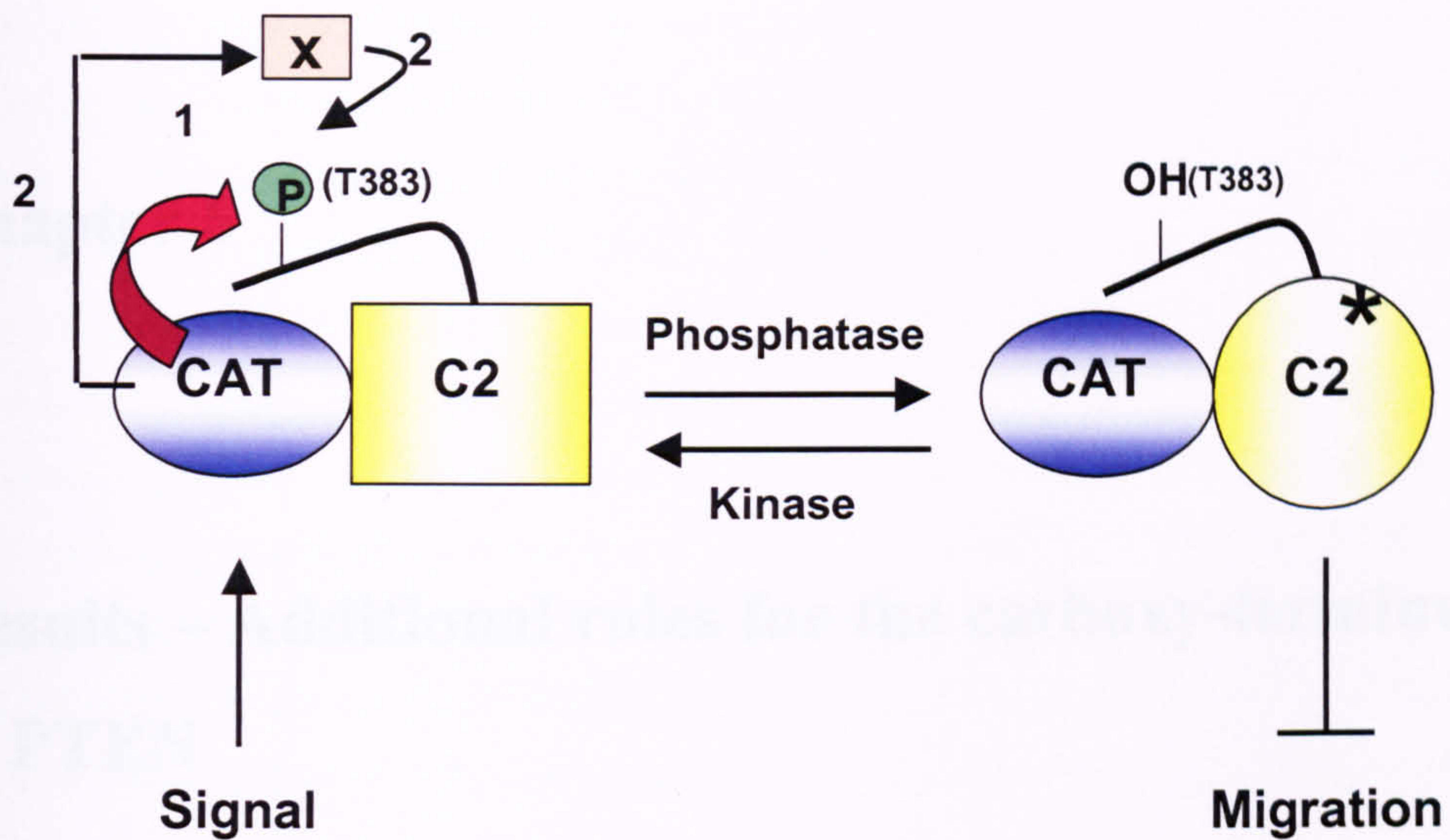
Several studies have now shown that PTEN undergoes extensive phosphorylation at its C-terminus and this affects its activity as a phosphatase and also its protein stability. Work by Tolkacheva et al. has shown that PTEN phosphorylation also affects its binding to PDZ-domain containing proteins, and could thus also regulate PTEN localisation (Tolkacheva et al., 2001). Most of these studies, however, have been carried out *in vitro* and there is so far no evidence to suggest what the biological significance of PTEN phosphorylation would be *in vivo*.

In this chapter, it was demonstrated that phosphorylation of only a single site, Thr383, is responsible for regulating the activity of the C2 domain in the full-length protein. The kinase responsible for phosphorylation of Thr383 is unclear; along with Ser370, Thr380, Thr382 and Ser385 it has been reported to be a site for phosphorylation by CK2, however others have suggested that CK2 only phosphorylates Ser370 and Ser385, but not Thr383 (Miller et al., 2002; Torres and Pulido, 2001). It is possible that phosphorylation of Thr383 requires priming through phosphorylation at these surrounding sites. Most intriguing, however, is the mechanism of dephosphorylation, which was shown here to be essential for activation of the C2 domain. The environment surrounding Thr383 is very acidic, making this an ideal substrate for the protein phosphatase activity of PTEN itself.

#### 5.4.2 Is PTEN a protein phosphatase acting autocatalytically?

Even though PTEN is reported to be a poor protein phosphatase, the 12-residue peptide sequence surrounding Thr383 (YSDTTDSDPENE) provides for an extremely acidic (5 Asp/Glu and 4 phospho-Ser/Thr residues) environment, which very closely resembles the optimal peptide substrates previously reported for PTEN (Myers et al., 1997). Moreover, the crystal structure predicts that the C-terminal tail of PTEN would be in close proximity to the active site (Lee et al., 1999). Work in this chapter showed that regulation of phosphorylation at Thr383 is dependent upon the protein catalytic activity of PTEN and that the catalytic domain of PTEN interacts with the carboxyl tail region of the protein where Thr383 is found, even though this interaction seems to be independent of any phosphorylation event. There is therefore a strong possibility that PTEN acts as an intramolecular phosphatase removing the phosphate residue from Thr383, and thereby activates the C2 domain (Figure 5.12). This is further supported by the finding that catalytically-active PTEN, when isolated from cells, is not phosphorylated on Thr383, whereas the catalytically-dead protein is. This, however, does not provide complete proof for a direct, intramolecular dephosphorylation of PTEN. Formal proof will require isolated wild-type PTEN phosphorylated on Thr383, followed by an *in vitro* phosphatase assay.

Data in this chapter suggest a model in which PTEN acts intramolecularly to dephosphorylate Thr383 (model 1, Figure 5.12). This induces a conformational change that activates the C2 domain to inhibit cell migration. An alternative would be that the protein phosphatase activity of PTEN activates another protein phosphatase to dephosphorylate Thr383 (model 2, Figure 5.12), or inactivates a kinase. In any event, it is likely that the competition between the kinase and the phosphatase acting at Thr383 will play a major part in controlling cell migration and work in this study suggests that in addition to controlling the intracellular levels of PI(3,4,5)P<sub>3</sub>, the ability of PTEN to regulate cell migration through its C2 domain, may be a key feature of its tumour suppressor activity and help explain why the PTEN gene is so frequently mutated in human cancer.

**FIGURE 5.12**

PTEN's phosphatase activity is required for dephosphorylation of Thr383 and inhibition of migration. Model 1, PTEN acts intramolecularly to dephosphorylate Thr383, induce a conformational change and activated the C2 domain to inhibit migration. Model 2, PTEN's protein phosphatase activity is required to activate protein X that dephosphorylates Thr383 and activates PTEN's C2 domain to inhibit migration.

## **Chapter 6**

### **Results – Additional roles for the carboxy-terminus of PTEN**

#### **6.1 Summary**

Having established a role for PTEN in cell migration, the research presented in this chapter addresses the role of PTEN in cell spreading. In the previous chapters, it was shown that PTEN controls migration of the U87 glioma cells in a protein-phosphatase-dependent mechanism. This chapter reveals that the C-terminus of PTEN induces a morphological change in U87 cells, from an elongated, spindle-like shape to a more flat, spread phenotype. This induction of cell spreading is independent of the catalytic domain, and in contrast to the effect seen on cell migration discussed in chapter 3, is also independent of the PDZ binding motif. The same C-terminal region of PTEN also causes spreading of the breast cancer cells MDA-MB-435. In an effort to elucidate the possible mechanisms involved in the contribution of cell migration and the induction of cell spreading by the C-terminus, a yeast-two hybrid screen was performed. This identified three potential novel binding partners of PTEN, namely; hVPS41p, a protein involved in vesicle docking and fusion, Ash2, a regulator of transcription, and a novel Zinc-finger containing protein of unknown function.

## 6.2 Introduction

In addition to controlling cell growth, arrest and migration, PTEN is also known to be a negative regulator of integrin-dependent cell spreading. Similar to the control of cell migration, there is conflicting evidence whether the protein or the lipid phosphatase is required for the inhibition of spreading. Initial studies suggested that the protein-phosphatase activity is needed for PTEN to inhibit spreading of U87 cells and PTEN<sup>-/-</sup> fibroblasts, probably by directly dephosphorylating the proteins Shc and FAK, two important regulators of integrin-dependent signaling (Gu et al., 1998; Tamura et al., 1998). Work by Leslie et al., revealed that mutations that abrogate the lipid- but not the protein-phosphatase activity of PTEN, or mutations that target the C2 domain (such as the M-CBR3 mutant) abolish PTEN's ability to inhibit spreading on fibronectin or collagen of U87 cells (Leslie et al., 2001). Furthermore, a C-terminal truncation mutant that lacks the PDZ binding motif only partially inhibits spreading of U87 cells, suggesting that the interaction with a PDZ domain containing protein might be required to inhibit spreading.

The precise mechanism of how PTEN inhibits cell spreading is currently unclear. The experiments described in this chapter demonstrate a role for PTEN in the activation of cell spreading. The C-terminus of PTEN induces spreading of U87 cells and this effect is dependent on the C2 domain, the tail region containing the phosphorylation sites, but not the PDZ binding motif. To further elucidate the role of the C-terminus in the induction of cell spreading as well as cell migration and to identify proteins involved in mediating these effects, a yeast-two hybrid screen was performed using PTEN $\Delta$ CAT $\Delta$ PDZBM as bait. This revealed three novel potential PTEN interacting proteins.

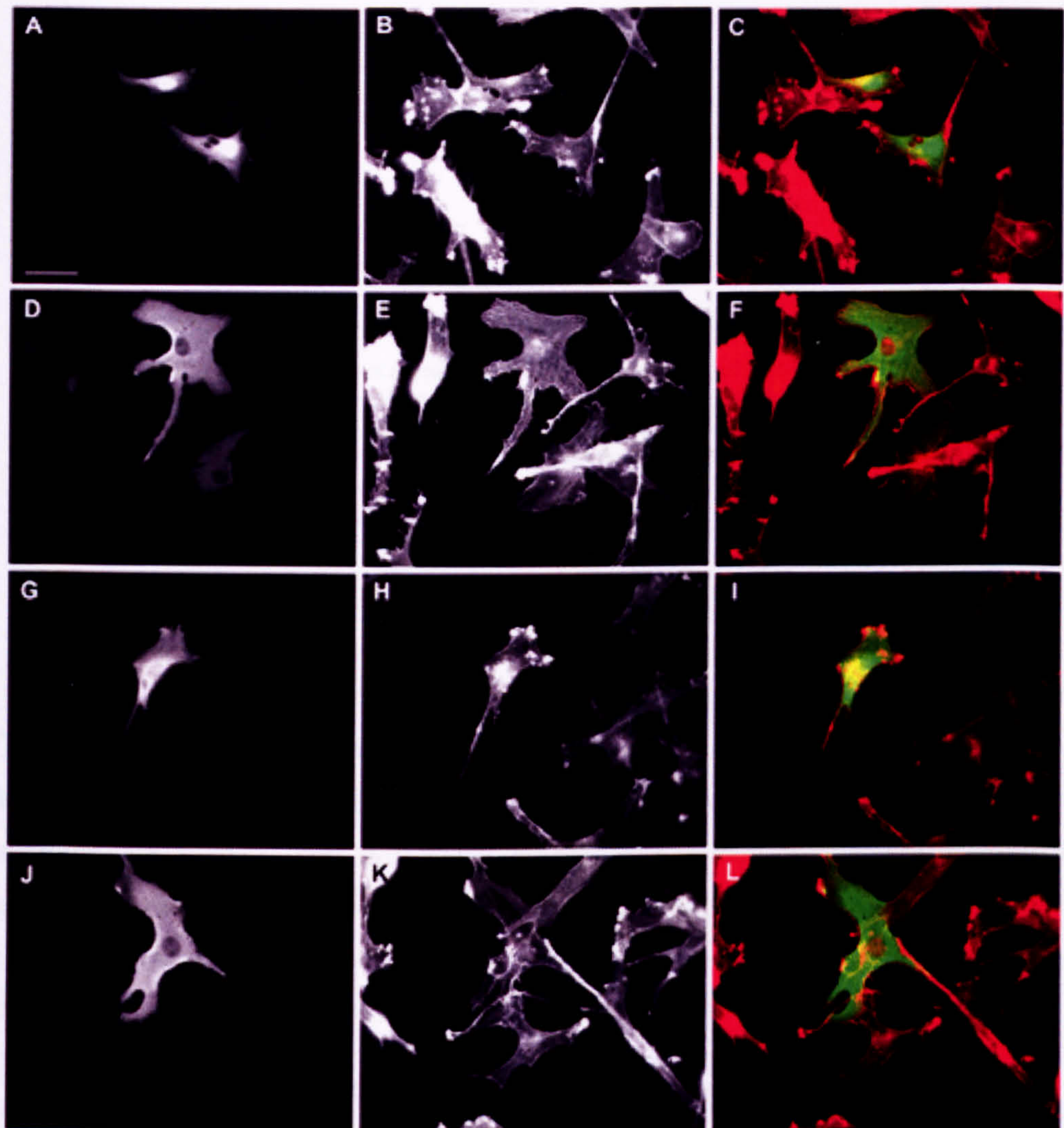
## 6.3 Results

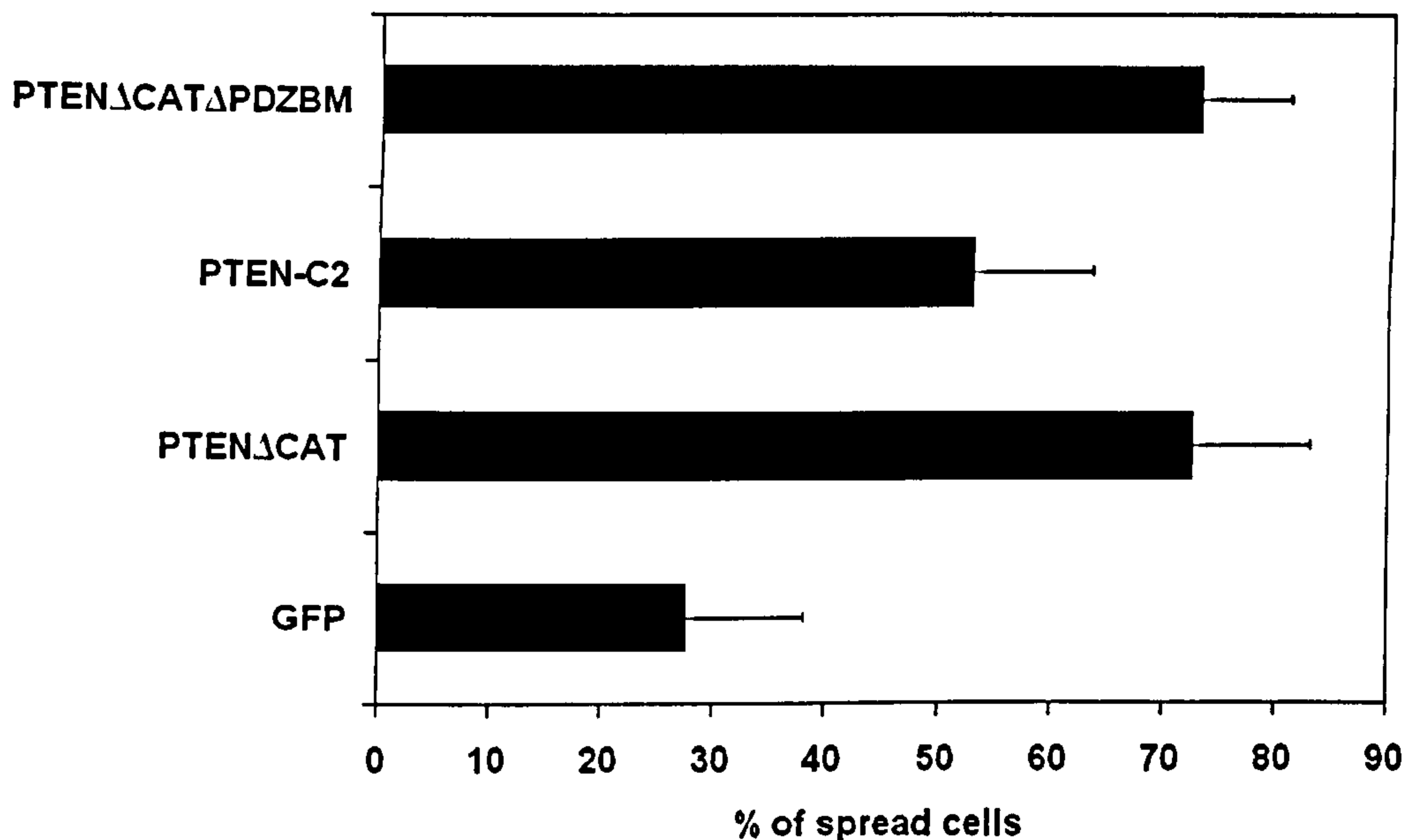
### 6.3.1 The role of the C-terminus of PTEN in cell spreading

While investigating the role of PTEN in cell migration, it was observed that the C-terminus of PTEN not only inhibited the migration of U87 cells, but also changed their morphology from a spindle-like elongated shape to a much more flat and spread phenotype (Figure 6.1). This suggests that PTEN can also have a positive effect in the induction of cell spreading, and is in contrast to previous experiments showing that wt PTEN inhibits cell spreading.



In order to determine the minimal region responsible for this effect, two C-terminal deletion mutants, lacking the PDZ binding motif (PTEN $\Delta$ CAT $\Delta$ PDZBM) and one consisting of just the C2 domain of PTEN (PTEN-C2) (see Figure 4.8), were introduced into sub-confluent U87 cells (Figure 6.1).





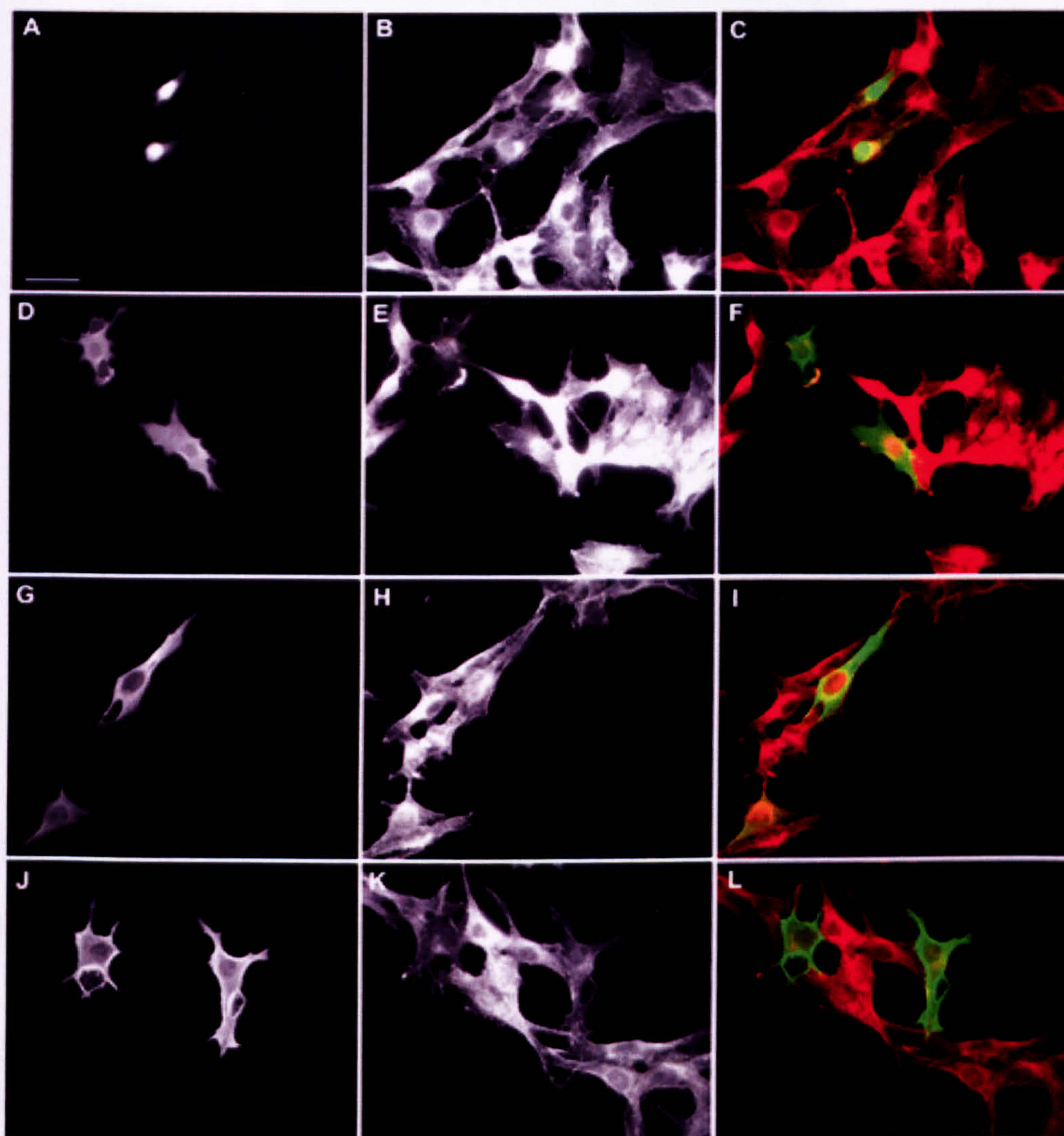
**FIGURE 6.1**

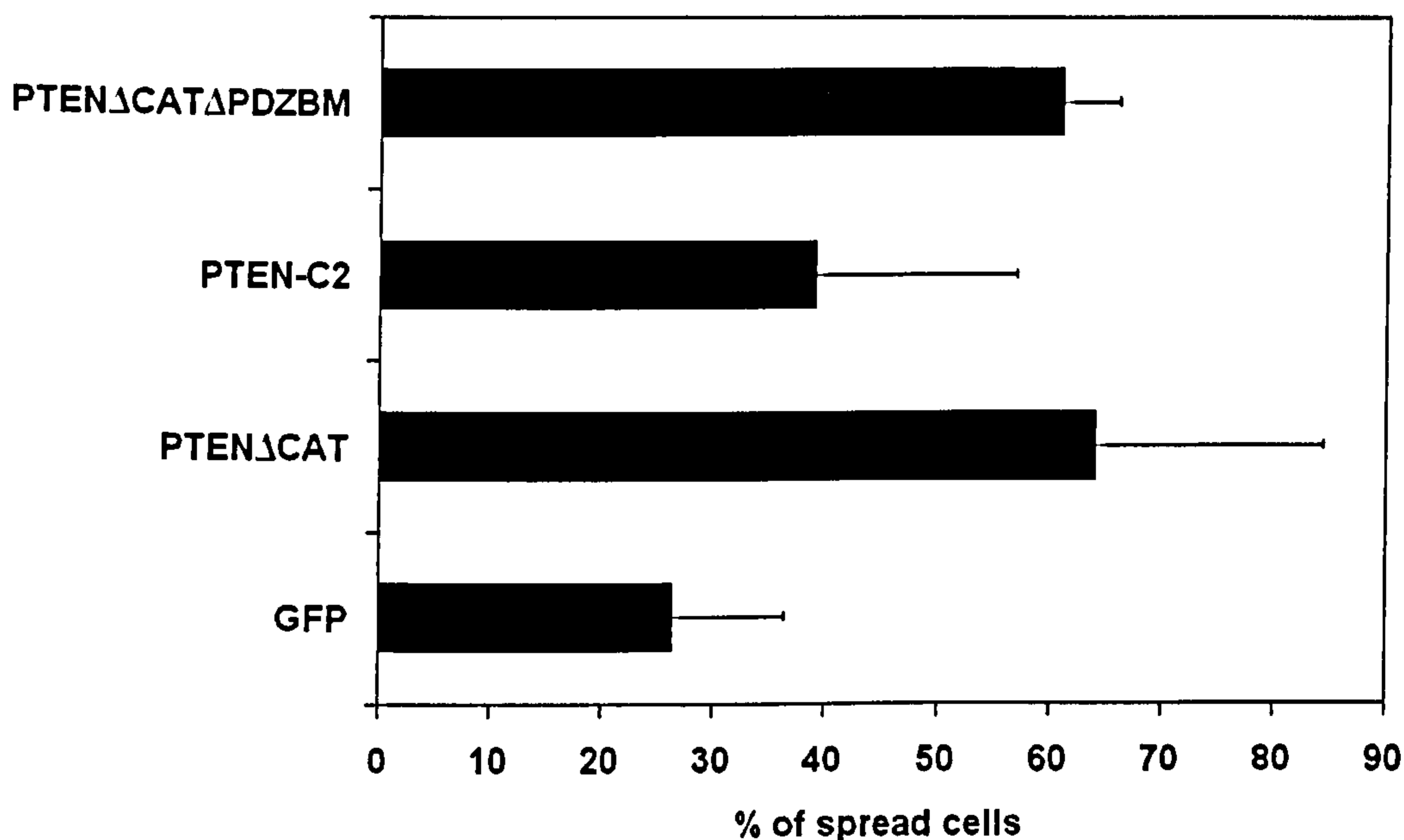
The C-terminus of PTEN induces spreading of U87 cells. (a) Representative examples of subconfluent U87 cells injected with control GFP (A-C) or Myc-epitope tagged cDNAs of PTEN $\Delta$ CAT (D-F), PTEN-C2 (G-I) and PTEN $\Delta$ CAT $\Delta$ PDZBM (J-L) is shown. Cells were fixed and stained with anti-Myc antibody to visualize the injected cells (A, D, G, J) and with Rhodamine-conjugated phalloidin to visualise the actin cytoskeleton (B, E, H, K). Scale bar 50 $\mu$ m. (b) Quantitation of the number of injected cells that exhibit a spread morphology.

As shown in figure 6.1, around 75% of U87 cells injected with the C-terminus of PTEN are spread compared to only 28% of control injected cells. This effect is independent of the PDZ binding domain, as PTEN $\Delta$ CAT $\Delta$ PDZBM induced spreading to a similar level. The C2 domain of PTEN (PTEN-C2) also induces spreading, albeit to a slightly lesser extent (around 50% of cells are spread), suggesting that the C2 domain and the region between the C2 domain and the PDZ binding motif, containing the phosphorylation sites, is required for the spreading effect.

To examine whether this effect is unique to U87 cells or represented a more general phenomenon, the above PTEN constructs were also tested for their ability to induce spreading in the breast cancer cells MDA-MB-435, which also exhibit an elongated – spindle-like morphology (the previously described U373 glioma cells could not be used because they already exhibit a spread phenotype). As can be seen in figure 6.2, the C-

terminal domain of PTEN also induces spreading of these cells, and as shown for the U87 cells, the PDZ binding motif, but not the tail region (residues 350-398) is dispensable for this effect.





**FIGURE 6.2**

The C-terminus of PTEN induces spreading of MDA-MB435 cells. (a) Representative examples of sub-confluent U87 cells injected with control GFP (A-C) or Myc-epitope tagged cDNAs of PTENΔCAT (D-F), PTEN-C2 (G-I) and PTENΔCATΔPDZBM (J-L) is shown. Cells were fixed and stained with anti-Myc antibody to visualize the injected cells (A, D, G, J) and with Rhodamine-conjugated phalloidin to visualize the actin cytoskeleton (B, E, H, K). Scale bar 50μm. (b) Quantitation of the number of injected cells with the various Myc-epitope tagged constructs that exhibit a spread morphology.

## 6.3.2 Binding partners for the C-terminus of PTEN

### 6.3.2.1 Yeast-two hybrid screen

The results so far indicate a role for the C-terminus of PTEN both in cell migration and cell spreading of glioma cells, however, the mechanisms involved are unclear. Therefore a yeast-two hybrid screen was performed in order to identify potential binding partners of the PTEN C-terminus that might be involved in mediating these effects. The PTENΔCATΔPDZBM was used as bait, firstly, because this region was identified as the minimal region required for cell spreading and because it includes the tail region (residues 350-398) shown to contain critical residues for the regulation of the C2 function

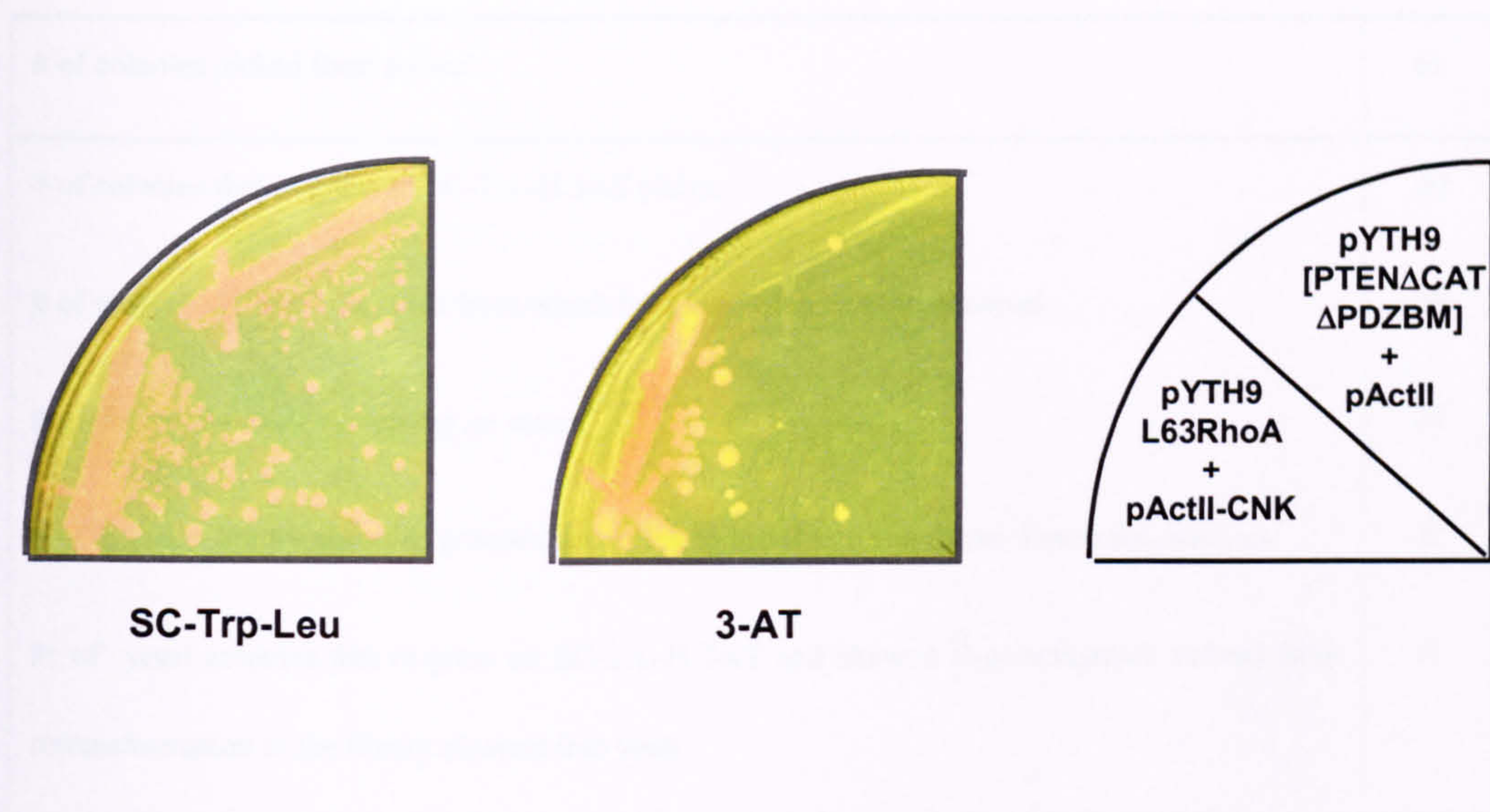
in U373 cells. Secondly, several groups had previously performed yeast-two hybrid screens using either full-length PTEN or the entire C-terminus and all of the proteins identified were shown to bind to the PDZ binding motif of PTEN and therefore, to avoid pulling out the same proteins, a C-terminal truncation mutant that lacked the PDZ binding motif, was used.

The PTEN $\Delta$ CAT $\Delta$ PDZBM construct was cloned into the yeast expression vector pYTH9 and integrated into the yeast strain Y190. To check that the construct was suitable as bait in a yeast – two hybrid system, we first investigated the expression of the HA-Gal4BD-PTEN $\Delta$ CAT $\Delta$ PDZBM fusion protein. Total yeast extract of five independent Y190 pYTH9::PTEN $\Delta$ CAT $\Delta$ PDZBM transformants was prepared, subjected to SDS-PAGE and Western analysis using an anti-HA antibody. As shown in figure 6.3, transformants #2 and #4 express the fusion protein. Colony #2 was chosen for further analysis. Secondly, this construct was tested for whether it was capable of self-activating transcription of the *HIS3* reporter gene (Figure 6.3). Yeast cells expressing Gal4BD-PTEN $\Delta$ CAT $\Delta$ PDZ BM were streaked out on selection medium lacking histidine and containing 3AT (SC-T-L-H 3AT). As seen in figure 6.4, Gal4BD-PTEN $\Delta$ CAT $\Delta$ PDZBM expressing cells were not growing on SC-T-L-H 3AT plates, while control cells were growing. Thus, since Gal4BD-PTEN $\Delta$ CAT $\Delta$ PDZBM did not self-activate transcription, it was deemed suitable for the yeast-two hybrid screen.



**FIGURE 6.3**

The PTEN $\Delta$ CAT $\Delta$ PDZBM construct is expressed in yeast. Yeast lysates were prepared as described in Materials and Methods and analysed by SDS-PAGE followed by Western blotting. An anti-HA antibody was used to detect expression of the construct. Colonies 2 and 4 express the construct.

**FIGURE 6.4**

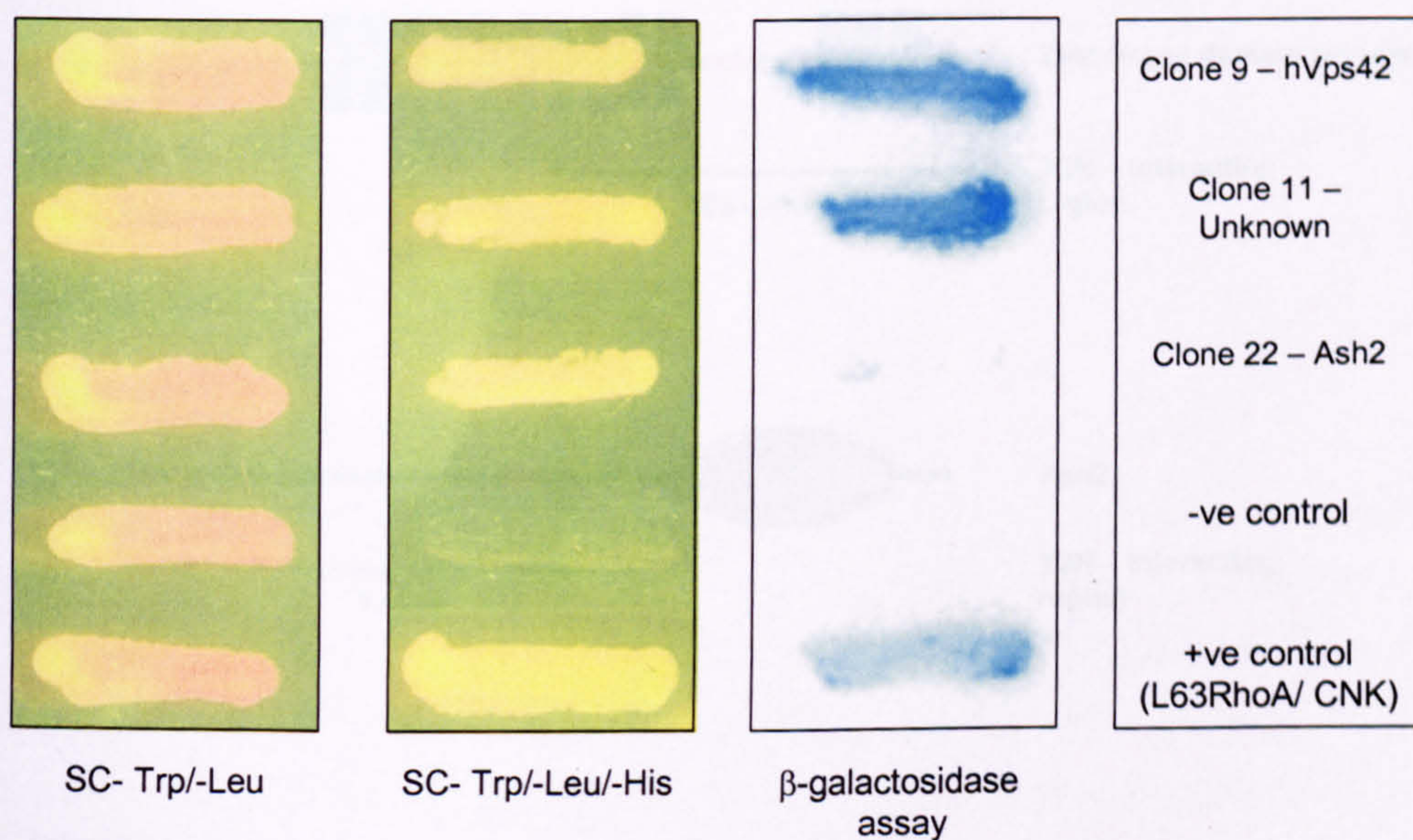
The PTEN $\Delta$ CAT $\Delta$ PDZBM construct does not self-activate transcription of the *HIS3* gene. Y190 pYTH9::PTEN $\Delta$ CAT $\Delta$ PDZBM strain with empty pACTII as well as Y190 pYTH9::L63 RhoA and pACTII::CNK1 as positive control were streaked on SC-T-L and SC-T-L-H 3AT plates to check for the absence of growth of Y190 pYTH9::PTEN $\Delta$ CAT $\Delta$ PDZBM strain on SC-T-L-H 3AT plate.

The Y190 pYTHP::PTEN $\Delta$ CAT $\Delta$ PDZBM strain was then transformed with a human brain cDNA library cloned into pACTII, and a total of  $9 \times 10^5$  colonies were screened for growth on SC-T-L-H 3AT medium. After 9 days, 61 growing colonies were picked, and re-streaked on SC-T-L-H 3AT plates (see Table 6.1). 30 yeast colonies were growing and the library plasmid of each of these was recovered and transformed into *E.coli*. Bacteria colonies from 26 clones were obtained. These were minipreped and analyzed by restriction digest. 24 library plasmids contained an insert and were grouped according to size and restriction digest pattern, revealing 6 different genes, encoding 6 potential PTEN interactors. To verify the interaction in the two-hybrid system, these plasmids were re-transformed into the original Y190 pYTH::PTEN $\Delta$ CAT $\Delta$ PDZBM strain and analyzed for growth on SC-T-L-H 3AT medium as well as for  $\beta$ -galactosidase activity on medium containing x-gal. As seen in figure 6.5, transformants of three library plasmids re-grew on SC-T-L-H 3AT plates and displayed strong  $\beta$ -galactosidase activity.

# of colonies picked form screen	61
# of colonies that re-grew on SC-T-L-H 3AT plates	30
# of recovered library plasmids from which bacterial colonies were obtained	26
# of library plasmids containing an insert	24
# of different library plasmids grouped according to insert size and further restriction analysis	6
#r of yeast colonies that re-grew on SC-T-L-H 3AT and showed $\beta$ -galactosidase activity after retransformation of the library plasmid into yeast	3

**TABLE 6.1**

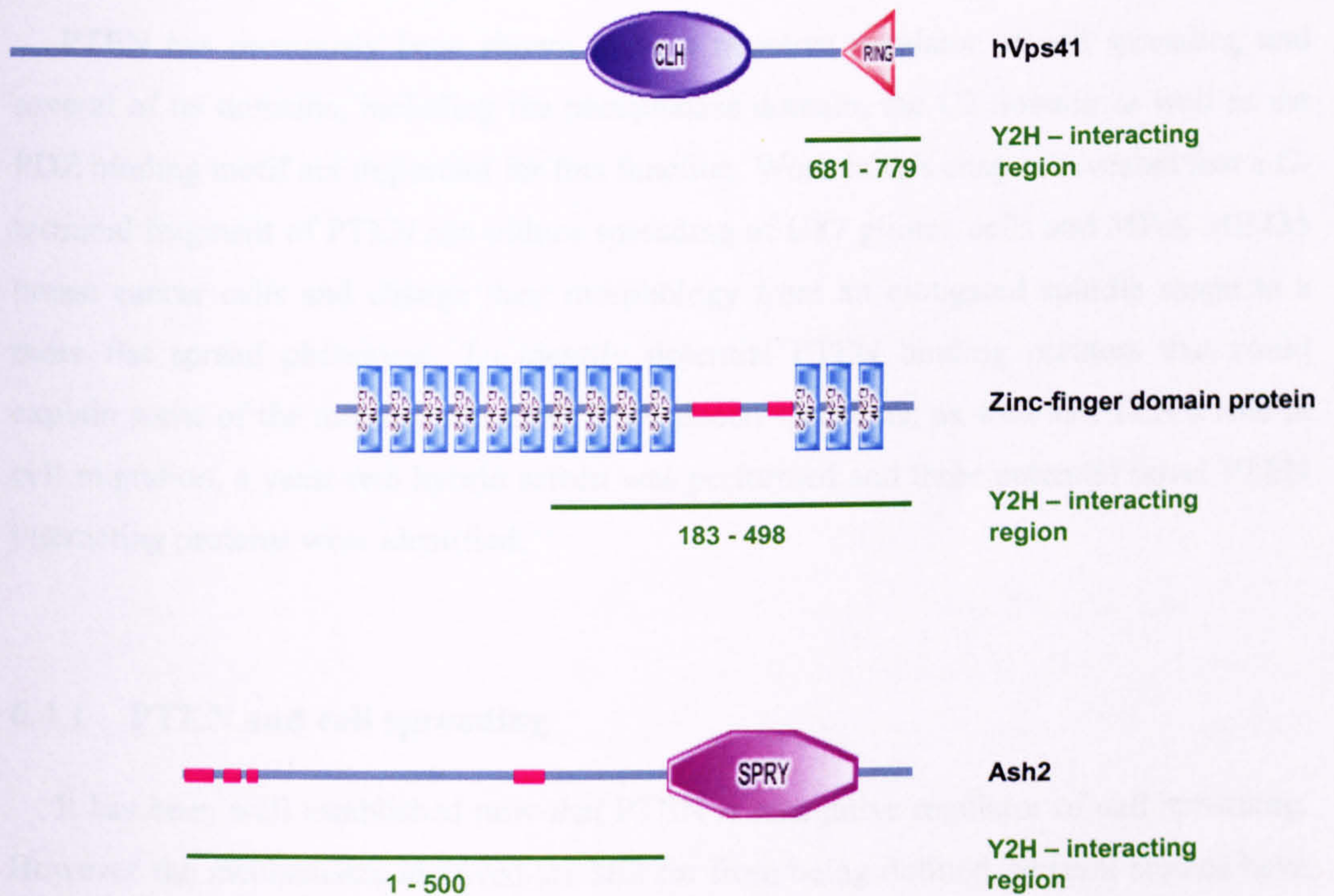
The details of the yeast – two hybrid screen are summarized here.

**FIGURE 6.5**

Three potential PTEN interactors identified in the yeast-two hybrid screen. (a) Y190 pYTH9::PTEN $\Delta$ CAT $\Delta$ PDZBM strain transformed with library plasmids #9, #11, #22 as well as empty pACTII as negative control, and Y190 pYTH9::L63 RhoA and pACTII::CNK1 as positive control were streaked out on SC-T-L, SC-T-L-H 3AT, and SC-T-L Xgal plates.

### 6.3.2.2 PTEN interactors found in the yeast-two hybrid screen

Sequencing of the DNA inserts of these three library plasmids and *Blast* analysis of the sequences, revealed three different genes encoding for three potential PTEN protein interactors: the vesicular protein hVps4, a novel zinc finger containing protein of unknown function and the transcription factor ash2. Figure 6.6 shows the predicted domain structure of these proteins and the PTEN-interacting region that was pulled out from the yeast-two hybrid screen for each of these proteins is indicated with a line.



**FIGURE 6.6**

Domain structure of the proteins identified by yeast-two hybrid as PTEN interactors. Sequence alignments indicate that PTEN interacts with the RING domain of hVps41, the C-terminus of the zinc-finger domain protein and the N-terminus of Ash2. The domain structures have been obtained from the SMART (Simple Modular Architectural Tool) website (Schultz et al., 1998).



Sequence alignments of the regions pulled out in the screen with the full-length proteins indicate that PTEN most probably interacts with the RING finger domain of hVps41 and the N-terminal of Ash2, which excludes the SPRY domain. In the case of the zinc-finger domain protein, PTEN seems to interact with the C-terminal half of the protein that includes 7 zinc-finger domains. The interaction of PTEN with these proteins is currently being confirmed biochemically and their contribution to PTEN function is being investigated.

## 6.4 Discussion

PTEN has previously been shown to be a negative regulator of cell spreading and several of its domains, including the phosphatase domain, the C2 domain as well as the PDZ binding motif are important for this function. Work in this chapter revealed that a C-terminal fragment of PTEN can induce spreading of U87 glioma cells and MDA-MB435 breast cancer cells and change their morphology from an elongated spindle shape to a more flat spread phenotype. To identify potential PTEN binding partners that could explain some of the mechanisms of PTEN-induced spreading, as well as PTEN's role in cell migration, a yeast-two hybrid screen was performed and three potential novel PTEN interacting proteins were identified.

### 6.4.1 PTEN and cell spreading

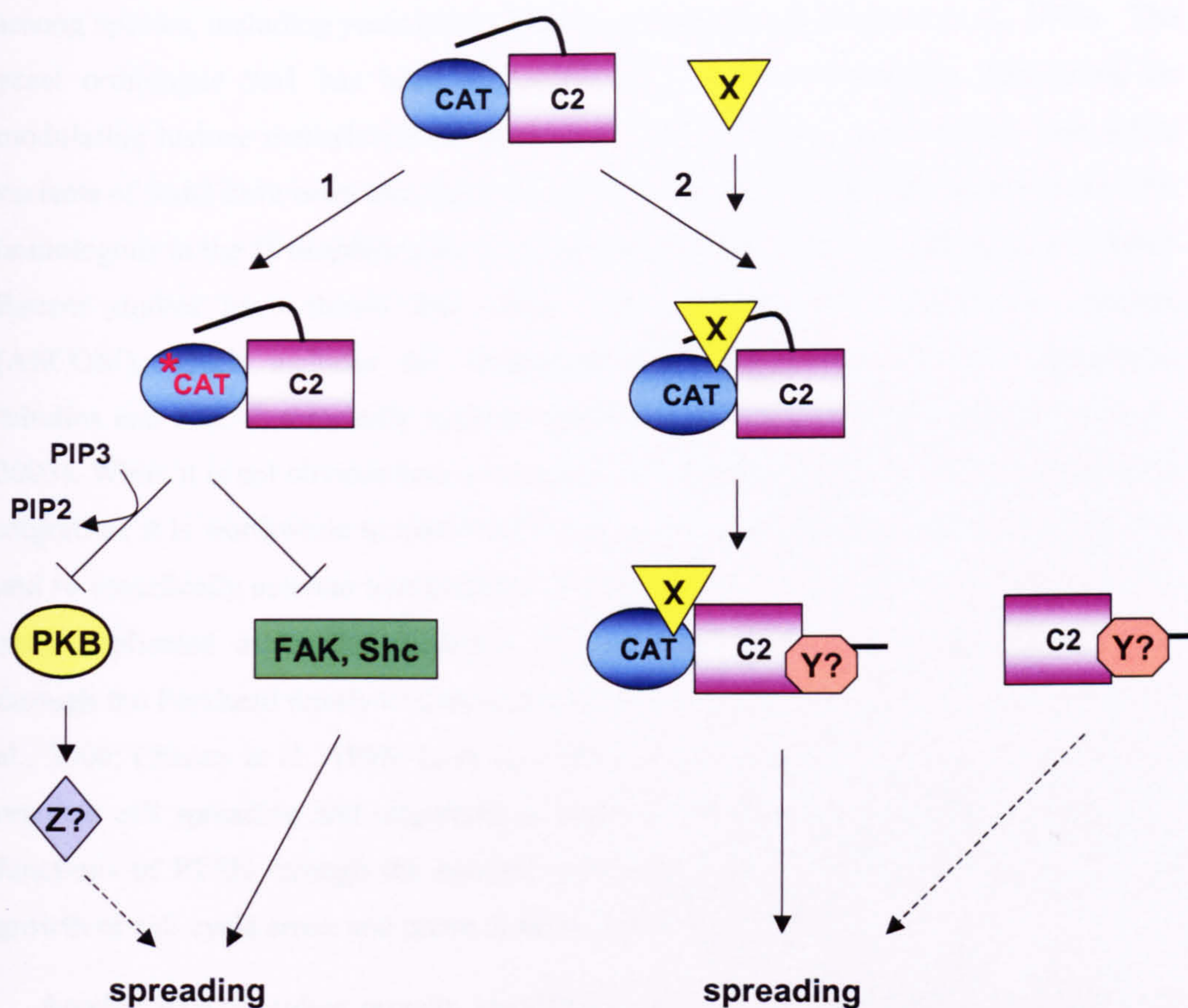
It has been well established now that PTEN is a negative regulator of cell spreading. However the mechanisms involved are still far from being defined. Several studies have suggested that the phosphatase activity of PTEN is required for the inhibition of cell spreading, but there is still some controversy over whether this involves the protein or the lipid phosphatase activity (Gu et al., 1998; Leslie et al., 2001). Work by Leslie et al., has shown that the PDZ binding motif of PTEN is required for efficient inhibition of cell spreading and PDGF-induced membrane ruffling by PTEN (Leslie et al., 2000).

The molecules that have so far been proposed to function downstream of PTEN-dependent inhibition of cell spreading include the integrin-regulated proteins FAK and

Shc. PTEN has been shown to inactivate these proteins by directly binding to them and dephosphorylating phospho-Tyr residues (Gu et al., 1998; Tamura et al., 1998). Dephosphorylation of Shc by PTEN prevents the recruitment of the adaptor protein Grb2 and this inhibits subsequent activation of the MAPK pathway. The inhibition of MAPK activation by PTEN, however, can also be attributed to downregulation of PI(3,4,5)P<sub>3</sub> levels and the subsequent inhibition of Gab1 recruitment to the plasma membrane (Rodrigues et al., 2000). Furthermore, the ability of PTEN to dephosphorylate FAK appears to be cell-type specific. Studies by Sun et al., have shown that there is no difference in the phosphorylation levels of FAK in PTEN<sup>-/-</sup> compared to PTEN<sup>+/+</sup> ES cells, and we have not been able to detect any difference in the levels of phosphorylated FAK between U373 cells and astrocytes ((Sun et al., 1999) and data not shown). In any case, more work is needed in order to delineate the mechanisms of the regulation of cell spreading by PTEN.

Work in this chapter, suggests a novel mechanism by which PTEN can induce cell spreading, and this requires the C-terminus, but not the catalytic domain. Introduction of a C-terminal fragment of PTEN into U87 or MDA-MB435 cells induced a shape change in these cells from an elongated –spindle-like morphology to a more flat and spread phenotype. The tail region of PTEN, containing the various phospho-serine and threonine residues, was required for this process to occur, while the PDZ binding motif was dispensable. How the C-terminal domain of PTEN induces cell spreading and whether this is dependent on Shc or FAK remains to be seen.

In order to explain why data in this chapter implicates PTEN in the induction –rather than the inhibition- of cell spreading seen by previous studies, a model is proposed where PTEN can act in one of two ways depending on the stimulus (Figure 6.7). In the first pathway, a stimulus activates the catalytic activity of PTEN to deplete the pool of PI(3,4,5)P<sub>3</sub> in the cell and thus downregulate PKB and/or dephosphorylate FAK/Shc and thus inhibit cell spreading. In the second pathway, a stimulus leads to the recruitment of a protein that probably either relieves an auto-inhibition or stabilises the C-terminus of PTEN in an active conformation. The active C-terminus induces cell spreading, probably through the recruitment of another protein.



**FIGURE 6.7**  
Model of PTEN function on cell spreading. Pathway 1, PTEN inhibits cell spreading.  
Pathway 2, PTEN induces cell spreading. See discussion for details.

#### 6.4.2 Novel, potential binding partners of PTEN

To further elucidate the mechanism by which PTEN controls spreading and migration, a yeast-two hybrid screen was performed and three potential binding partners of the PTEN C-terminus were isolated. None of these proteins have been implicated in cell spreading or migration as yet, and their role in PTEN function remains to be determined.

Ash2 is a transcription factor encoded by the absent, small, or homeotic disc 2 (*ash2*) gene, and was identified as being a positive regulator of homeotic genes in *Drosophila* (Adamson and Shearn, 1996). It belongs to the trithorax group of regulatory genes and has been implicated in playing a role early in development in the patterning of the various

discs in the fruit-fly, including the imaginal disc. The Ash2 gene is highly conserved among species, including yeast (Set1), *C. elegans* and mouse (Ikegawa et al., 1999). The yeast orthologue Set1 has been shown to play a role in chromatin remodeling by modulating histone methylation (Roguev et al., 2003; Roguev et al., 2001). Two splice variants of Ash2 have been identified in humans (ASH2L1 and ASH2L2), which are 60% homologous to the *Drosophila* gene and implicated in hematopoiesis (Wang et al., 2001). Recent studies have shown that human Ash2 belongs to a steady-state complex (ASCOM), which includes the retinoblastoma-binding protein RBQ-3, alpha/beta-tubulins and ASC-2, a recently isolated transcriptional coactivator molecule (Goo et al., 2003). While it is not obvious how a nuclear protein might play a role in cell spreading or migration, it is worthwhile to note that PTEN has also been found to exist in the nucleus and to specifically activate transcription of p53 (Freeman et al., 2003). Moreover, it has been implicated in the up-regulation of cyclin-dependent kinase inhibitors possibly through the Forkhead family of transcription factors, and thus regulate G1 arrest (Bruni et al., 2000; Cheney et al., 1998; Li et al., 1998). While Ash2 binding to PTEN might not regulate cell spreading and migration, it could be important in mediating these nuclear functions of PTEN through the specific switching off or on of genes required for cell growth or cell cycle arrest and prove to be an important interactor.

Another PTEN-binding protein, identified in this study, could also be contributing to PTEN's role in the nucleus, possibly through the regulation of gene transcription. This protein is a novel protein of unknown function that contains multiple zinc-finger domains (also present in Ash2), which have been shown to be nucleic acid binding proteins (see Figure 6.6). A zinc finger domain is a 25-30 amino acid self-folding domain, which contains two conserved cysteine and two conserved histidine residues and one atom of Zn. These domains have been shown to bind to both RNA and DNA. A *Blast* search of the amino acid sequence of this protein revealed high homology to the zinc-finger domain transcription factor Egr-1. Egr-1 belongs to the family of early growth response genes and is upregulated in response to mitogens in various cell types, including glioma cells (Al-Sarraj and Thiel, 2002). A direct role for Egr-1 regulation of proliferation of primary astrocytes has also been proposed and recently it has been shown to directly activate expression of PTEN (Virolle et al., 2001). Therefore, PTEN could be acting through zinc-finger domain transcription factors in order to regulate cell growth and proliferation.

The third protein identified by the yeast two-hybrid screen in this study, was the human orthologue of the yeast protein Vps41 (vacuolar protein sorting mutant 41). Vps41

has been shown to be required in vacuolar assembly in yeast and in the protein sorting of vesicles budding from the Golgi (Cowles et al., 1997; Nakamura et al., 1997). Subsequently, it was shown to play a role in the formation AP-3 transport vesicles by binding to AP-3 through its N-terminus and driving homo-oligomerization through its C-terminal clathrin heavy-chain repeat (CHCR) domain and to facilitate lysosomal vesicle docking and fusion (Darsow et al., 2001; Rehling et al., 1999; Stepp et al., 1997). The human orthologue of Vps41 contains a RING-H2 motif at its C-terminus, which is absent in yeast and is responsible for membrane tethering of the protein (McVey Ward et al., 2001). RING - finger domains, however, have also been shown to be involved in protein-protein interactions and this could be the domain interacting with PTEN, as it was belonged to the region that was pulled out in the yeast-two hybrid screen. Furthermore, various RING domains have been shown to bind to E2-ubiquitin conjugating enzymes and the RING domain of c-Cbl has intrinsic E3 ubiquitin-ligase activity. Previous studies have demonstrated the existence of two PEST sequences on the PTEN tail, which modulate protein stability most probably by targeting PTEN to a proteasome-dependent degradation pathway (Georgescu et al., 2000; Torres et al., 2003). hVPS41 could therefore potentially play a role in the sorting of PTEN to lysosomes and its subsequent degradation in the proteasome.

In summary, while it is not clear if and how these novel PTEN binding partners might be involved in the regulation of cell spreading and migration, their interaction with PTEN might still play an important contribution to the regulation of other PTEN functions.

# Chapter 7

## Discussion

### 7.1 Rho GTPases

Tumour cell migration and invasion are key features of tumour malignancy. The mechanisms that drive tumour cell migration, are believed to share similarities with early cell migration during development, but are still poorly understood. This thesis examined the role of the Rho family of GTPases, which are key regulators of most processes involved in cell migration, and explored the role of an important tumour suppressor, PTEN, in the control of the aberrant migration of brain tumours (gliomas).

Results presented in Chapter 3 demonstrated that both Rac and Cdc42 play an essential role in regulating the migration of three glioma cell lines (U373, U138 and U87) and of primary astrocytes. Interestingly, both dominant negative and constitutively active forms of Rac and Cdc42 inhibited the migration of U373 cells, suggesting that the precise activation in both space and time of these GTPases is crucial for their function. This is further supported by pull-down studies that showed that the higher migration rates of the gliomas did not correlate with the levels of active Rac or Cdc42 observed. A plausible explanation is that Rac and Cdc42 are more efficiently activated precisely at the front of the faster migrating U138 and U87 cells compared to U373 cells or astrocytes, something which cannot be measured by using pull-down assays. Further investigation into the activation levels of the kinases p65PAK and PKC $\zeta$ , downstream effectors of Rac and Cdc42 respectively, revealed that p65PAK was more active in the highly malignant U138

and U87 cells, compared to the less aggressive U373 cells. The levels of active PKC $\zeta$ , however, remained unchanged.

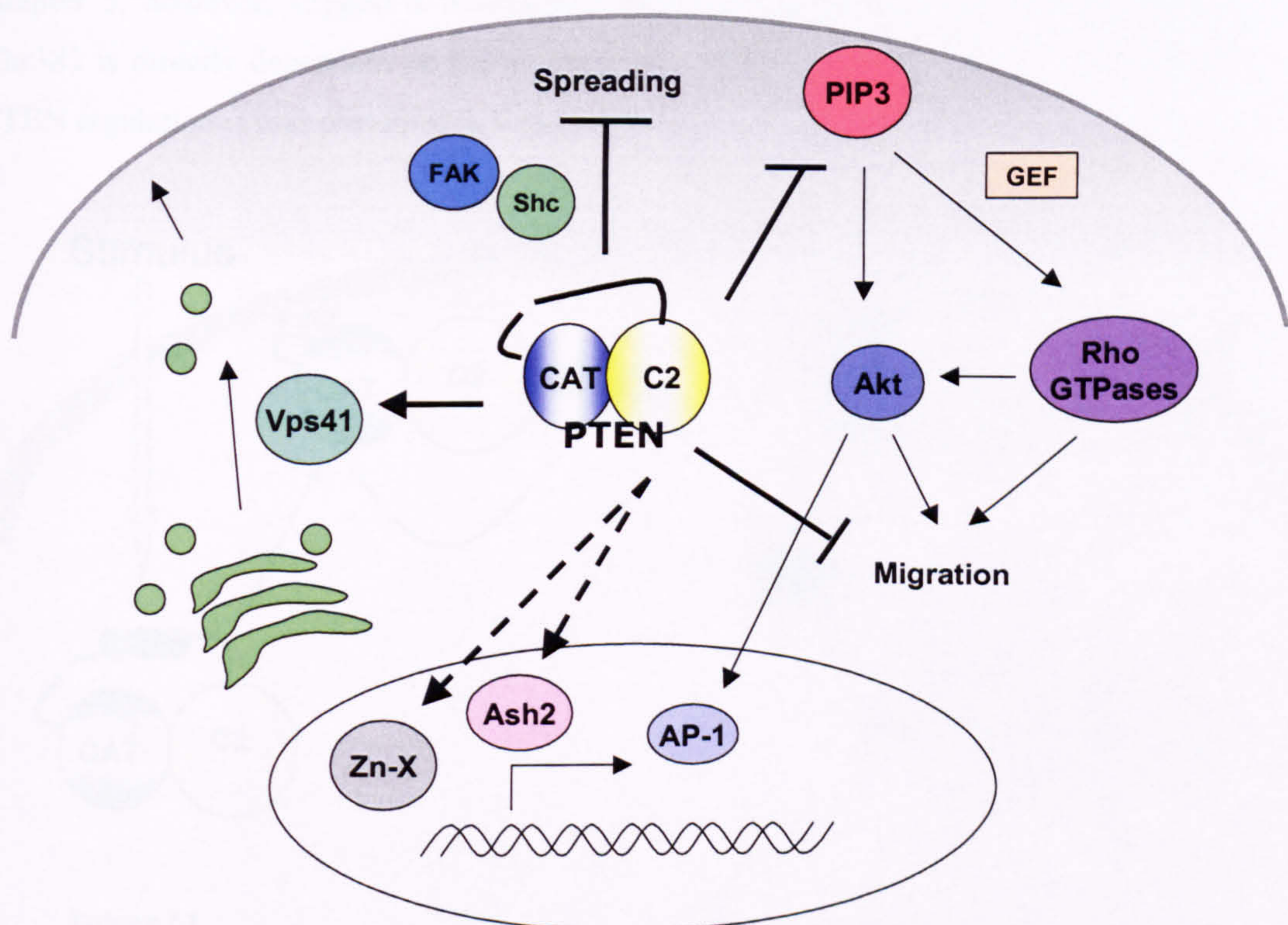
In addition to cell migration, the temporal and spatial activation of Rho GTPases is essential for the regulation of many other cellular processes, including morphogenesis (Etienne-Manneville and Hall, 2002). During *S. cerevisiae* and *C. elegans* morphogenesis, the activation of Cdc42 in the right place is crucial for the establishment of the bud site and cellular asymmetry respectively (Gotta et al., 2001; Gotta and Ahringer, 2001; Kay and Hunter, 2001; Pruyne and Bretscher, 2000). Thus, the aberrant glioma migration observed in chapter 3 could be accounted for, in part, by deregulation of the spatial and temporal activation of Rac and Cdc42, through, for example the upregulation of GEF activity (an effect observed in U87 cells for Rac).

## 7.2 PTEN

Chapters 4 and 5 focused on the controversial role of the lipid and protein phosphatase PTEN in the regulation of cell migration. Work in these chapters showed that PTEN inhibits cell migration of gliomas through a previously unrecognized activity of its C2 domain. This effect is dependent on PTEN's protein phosphatase activity and on the dephosphorylation specifically at residue Thr383 (Figure 5.12). It is likely that PTEN acts autocatalytically to dephosphorylate Thr383, although a more indirect pathway has not been formally ruled out. Chapter 6 explored the role of PTEN in cell spreading and revealed that, in addition to a previously reported inhibitory role for PTEN of cell migration, PTEN can also have a positive effect by inducing cells to spread, a process that depends on its C-terminus. Finally, a yeast-two hybrid screen identified three novel binding partners for the C-terminus of PTEN and suggested a role for PTEN in the nucleus (Figure 7.1).

The mechanisms that regulate PTEN activation remain unclear, as most studies have focused on what inactivates PTEN. It was recently demonstrated that PTEN becomes inactivated directly in response to oxidative stress and that this is concomitant with an increase of phospho-Akt levels in the cell (Leslie et al., 2003). The precise mechanism of inactivation, however, is unknown. It was shown by several groups that PTEN becomes phosphorylated on several serine and threonine residues at its C-terminus and that this reduces its lipid phosphatase activity as measured by examining several downstream signaling pathways (Birle et al., 2002; Georgescu et al., 1999; Torres and Pulido, 2001).

Furthermore it was recently demonstrated that phosphorylation at residues S380 and S385 protects proteolytic cleavage of PTEN by caspase-3 (Torres et al., 2003). The last 50 amino acids (tail) of PTEN seem to play a critical role in regulating protein stability. Deletion of the PTEN tail or mutation of residues S380, T382 and T383 found in that region to alanine, has been reported to reduce the protein half-life and cellular levels of PTEN (Vazquez et al., 2000). Dephosphorylated PTEN is degraded at a faster rate in what has been proposed to be a proteasome-dependent mechanism (Torres and Pulido, 2001; Torres et al., 2003). It was also shown that phosphorylation of PTEN at residues S380, T382 and T383 reduces its binding affinity to PDZ-domain containing proteins, probably through a conformational change that masks the PDZ binding motif (Tolkacheva et al., 2001; Vazquez et al., 2001; Vazquez et al., 2000). The binding of PDZ-domain proteins to the tail of PTEN might stabilize and protect it from degradation (Torres et al., 2003).

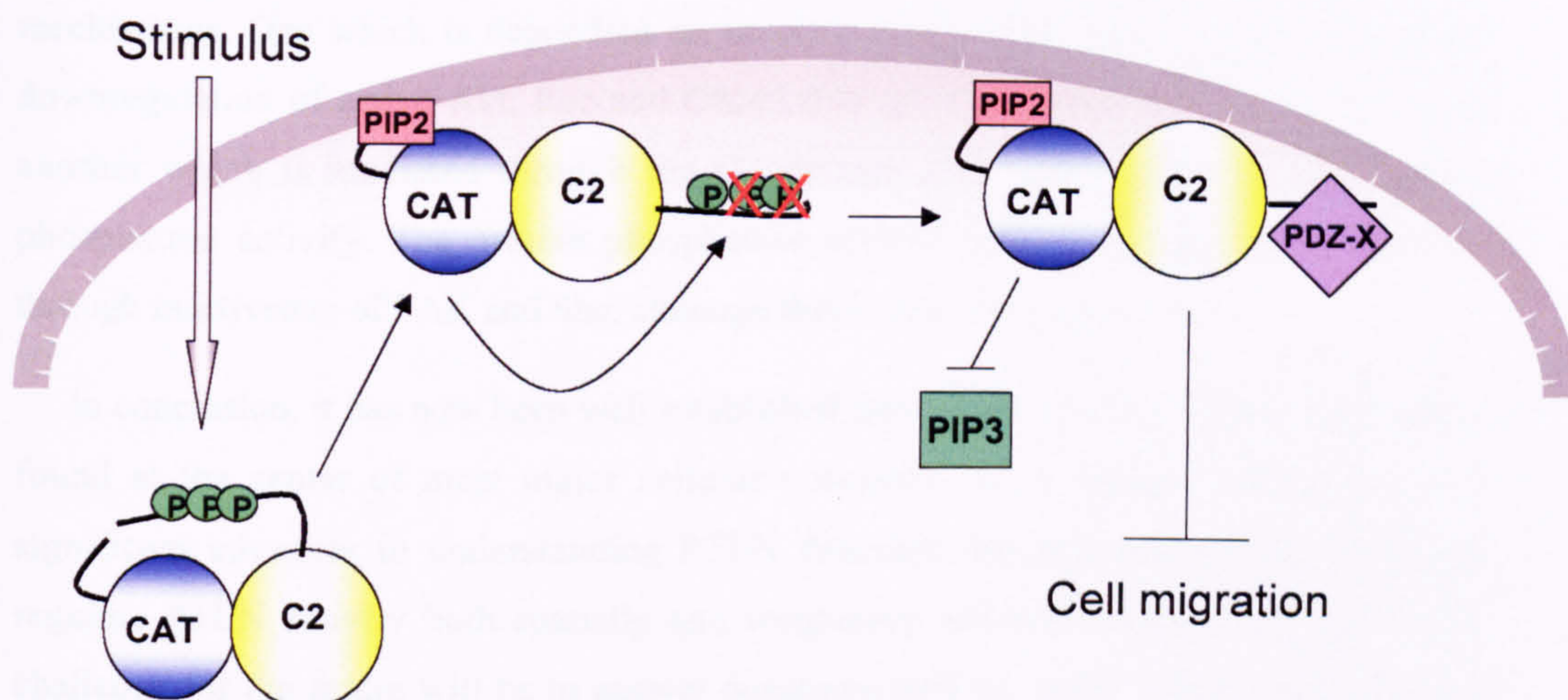


**FIGURE 7.1**

PTEN regulates a variety of cellular processes. Schematic representation of the PTEN functions investigated in this thesis. PTEN regulates spreading, migration, vesicle transport and transcription. The isolated Zn-finger protein of unknown function is depicted as Zn-X.



The only kinase identified so far as being able to phosphorylate PTEN is CK2. Several groups have now shown that both endogenous and exogenous CK2 phosphorylates PTEN on several residues at its C-terminus, thus enhancing its stability and acting as a negative regulator of PTEN activity (Birle et al., 2002; Georgescu et al., 2000; Torres and Pulido, 2001; Torres et al., 2003). Most intriguing, however, is what regulates PTEN activation. Campbell *et al.* recently demonstrated that PI(4,5)P<sub>2</sub> allosterically activates PTEN. A likely mechanism is that PTEN recruitment to the plasma membrane promotes the binding of PTEN's PI(4,5)P<sub>2</sub> binding motif to PI(4,5)P<sub>2</sub> present on the membrane, which induces a conformational change and activates the protein (Campbell et al., 2003). Data in chapter 4 provide evidence for an intramolecular interaction of the N- and C-termini of PTEN. PI(4,5)P<sub>2</sub> binding could thus out-compete the interaction of the C-terminus to the N-terminus and 'open up' the protein to interact with its substrate. This 'opening up' and activation of PTEN could be stabilised further through the binding of PDZ-domain proteins to the C-terminus of PTEN. No phosphatase has been identified so far that can antagonize the action of CK2. Results presented in chapter 5, however, suggest a mechanism whereby dephosphorylation specifically at Thr383 is directly dependent on the protein phosphatase activity of PTEN. A model for PTEN regulation is thus presented in Figure 7.2.



**FIGURE 7.2**

Model for the regulation of PTEN. PTEN is constitutively phosphorylated and inactive in the cell cytosol. Following stimulation, PTEN gets recruited to the plasma membrane where it binds PI(4,5)P<sub>2</sub> on its N-terminus. This activates PTEN to mediate dephosphorylation of residues on its C-terminus and promotes the binding of PDZ-domain proteins, which stabilise PTEN in an 'open' and active conformation. The C2 domain is then free to interact with cellular targets and mediate its role, for example, on cell migration (chapter 4).

### 7.3 Regulation of cell migration

Results presented in this thesis demonstrate a role for both the Rho GTPases and PTEN in the regulation of glioma cell migration. It has been reported that PTEN<sup>-/-</sup> fibroblasts have a higher migration rate compared to parental cells and exhibit an increase in the activation levels of Rac and Cdc42 (Liliental et al., 2000). The motility and activation levels of Rac and Cdc42 were reduced upon re-introduction of a lipid-phosphatase active form of PTEN. Even though the same increase in GTP-loading on Rac and Cdc42 was not observed in the gliomas (chapter 3) PTEN could still be playing a role in their regulation by decreasing the pool of cellular PI(3,4,5)P<sub>3</sub>. PI 3-kinase and PI(3,4,5)P<sub>3</sub> have been shown to activate Rac and Cdc42 and this is believed to be mediated through the activation of Rho GTPase upstream regulators, namely GEFs (Schmidt and Hall, 2002). In chapter 3, an increase in total GEF activity against Rac (and Cdc42, data not shown) was observed for the highly motile U87 cells, suggesting a correlation between the lack of PTEN expression and the activation of these GTPases. Furthermore, the absence of PTEN expression and the upregulation of active Rac and Cdc42 lead to an increase in the activation of Akt, a known regulator of cell motility (Higuchi et al., 2001).

In summary, PTEN seems to be regulating cell motility through at least two different mechanisms. One which is dependent on its lipid phosphatase activity and leads to the downregulation of active Akt, Rac and Cdc42 through the reduction of PI(3,4,5)P<sub>3</sub>, and another which is mediated through the C2 domain and is dependent on the protein phosphatase activity. The protein phosphatase activity may also affect cell migration through inactivation of FAK and Shc, although this is less clear (Figure 7.1).

In conclusion, it has now been well established that PTEN is a key tumour suppressor, found at the center of most major cellular processes. Even though there have been significant advances in understanding PTEN function, the mechanisms that precisely regulate PTEN activity both spatially and temporally are still poorly understood. The challenge for the future will be to answer questions such as, under which circumstances does PTEN act as a lipid or as a protein phosphatase and how does a cell control the various PTEN functions? PTEN has proven to be an intriguing molecule and future research will no doubt add to its complexity before it clarifies it.

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