Plk5, a Polo Box Domain-Only Protein with Specific Roles in Neuron Differentiation and Glioblastoma Suppression

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Received 26 May 2010/Returned for modification 22 July 2010/Accepted 9 January 2011

Polo-like kinases (Plks) are characterized by the presence of a specific domain, known as the polo box (PBD), involved in protein-protein interactions. Plk1 to Plk4 are involved in centrosome biology as well as the regulation of mitosis, cytokinesis, and cell cycle checkpoints in response to genotoxic stress. We have analyzed here the new member of the vertebrate family, Plk5, a protein that lacks the kinase domain in humans. Plk5 does not seem to have a role in cell cycle progression; in fact, it is downregulated in proliferating cells and accumulates in quiescent cells. This protein is mostly expressed in the brain of both mice and humans, and it modulates the formation of neuritic processes upon stimulation of the brain-derived neurotrophic factor (BDNF)/nerve growth factor (NGF)-Ras pathway in neurons. The human PLK5 gene is significantly silenced in astrocytoma and glioblastoma multiforme by promoter hypermethylation, suggesting a tumor suppressor function for this gene. Indeed, overexpression of Plk5 has potent apoptotic effects in these tumor cells. Thus, Plk5 seems to have evolved as a kinase-deficient PBD-containing protein with nervous system-specific functions and tumor suppressor activity in brain cancer.

Polo was originally identified in Drosophila as a mutant with mitotic and meiotic defects in the microtubule spindle (51). This mutation was later mapped to a serine-threonine protein kinase specifically concentrated in dividing cells (32). Polo-like kinases (Plks) are found in all eukaryotes and are characterized by a highly conserved kinase domain and one or two C-terminal polo box domains (PBDs). These include the Plk1 subfamily, containing Drosophila polo and mammalian Plk1; the SAK subfamily, containing Drosophila SAK and mammalian Plk4, both of which are widely distributed across the eukaryotes; and the Plk2 subfamily, containing vertebrate Plk2 and Plk3 and also including homologs from echinoderms (4, 8, 10, 35). The PBD participates in subcellular localization and partner interaction (16, 54). The founding member of the family, Plk1, localizes to the cytoplasm and centrosomes in interphase and concentrates to the kinetochores and the cytokinesis bridge during cell division. This protein has major functions in centrosome maturation, mitotic entry, and cytokinesis (4, 8, 40, 53).

The other members of the family are less studied. Plk4 (Sak) is a critical regulator required for centriole duplication both in Drosophila and in mammals (9, 21). Plk2 (also known as Snk) localizes to the centrosome and may also participate in centrosome biology and S-phase checkpoints (37). Plk3 (Pnk or Prk) activity peaks in G1 and localizes to the nucleolus in interphase. This protein may function in S-phase entry (64), and it is activated in response to replicative stress and genotoxic insults leading to apoptosis in a p53-dependent manner (56, 61, 62). The three Plk subfamilies have distinct functions and operate in multiple cell types. Their expression is regulated differentially in cells and tissues and in response to several cellular processes and stimuli (59). Whereas Plk1 and Plk2 are found only in dividing cells, Plk2 and Plk3 are also expressed in neurons and other, nondividing differentiated cells (47, 55). Plk2 and perhaps Plk3 seem to have a crucial function in modulating synaptic plasticity in neuronal dendritic spines through the phosphorylation of the spindle-associated protein SPAR (3, 46). Plk2 also phosphorylates the neuronal alpha-synuclein, a phospho-protein accumulated in several neurological diseases, such as Parkinson’s and dementia with Lewy bodies (23).

Given the critical roles of Plk1 during cell division and its frequent overexpression in human tumors (15), it has been considered a cancer therapeutic target (34, 50). Several small-molecule Plk1 inhibitors that compete with ATP at the kinase domain are in clinical trials (39), and efforts are ongoing to identify and validate small molecules that bind to the PBD to inhibit the interaction of Plk1 with its partners (39, 42). These efforts, however, must take into account that inhibition of Plk2, Plk3, or Plk4 may lead to tumor development, as these less-known Plks may function as tumor suppressors in specific cell types (28, 39, 52, 63).

The mammalian genome contains a fifth member of the Plk family, Plk5, initially described as a putative protein encoded by a pseudogene (12) and recently linked to DNA damage (2).
Although mouse cells express a full-length Plk5 similar in size to Plk2 or Plk3, human cells express a shorter PLK5 form in which the kinase domain is disrupted due to a stop codon in exon 6, which is followed by an in-frame ATG codon immediately downstream, in the boundary between exons 6 and 7. However, both the murine (long) and human (short) forms display similar cellular functions, and the kinase domain of the murine protein seems to be inactive in kinase assays. Plk5 is specifically expressed in the eye and the brain as well as the ovary in the mouse. Interference with Plk5 expression in PC12 cells and primary hippocampus neuroblasts results in reduced axon growth and neurite formation upon stimulation with neuronal growth factors, suggesting a role for Plk5 in the proper formation of neurite processes. Accordingly, the human PLK5 (hPLK5) protein is detected in the cytoplasm of neurons and glia. Interestingly, PLK5 is dramatically downregulated in human brain tumors, and its expression is inactivated by hypermethylation of the PLK5 promoter region. Reexpression of PLK5 in glioblastoma multiforme (GBM) cells results in the induction of apoptosis, suggesting a therapeutic potential of this protein.

MATERIALS AND METHODS

Plk5 sequences, cDNA constructs, and mutagenesis. All gene and protein sequences were taken from Ensembl (http://wwwensembl.org) or Uniprot (http://www.uniprot.org). Sequence alignment and comparison were performed using Clustal2 (http://www.ebi.ac.uk/Tools/clustalw2/). The mouse Plk5 (mPlk5; NM_183152) and human PLK5 (NR_026557) cDNAs were obtained from the Mammalian Gene Collection Consortium (http://mgc.nci.nih.gov/). Each cDNA was verified by sequencing and subsequently subcloned and fused either to enhanced green fluorescent protein (EGFP) (pDEST31-GFP) or to a triple c-myc tandem tag (pcDNA3.1/3×c-myc from Invitrogen) N-terminal to the mouse Plk5 (IMAGE Consortium cDNA Collection; clone 6480647) or to the predicted human PLK5 short form (IMAGE:40000565). mPlk5-ΔN was generated by PCR amplification of the mouse Plk5 cDNA from position 900 to the final stop codon. Point mutations in the cDNA were performed using the QuickChange site-directed mutagenesis kit (Stratagene) and verified by DNA sequencing. The human PLK1-PBD (residues 326 to 603), PLK2-PBD (residues 394 to 677), and PLK3-PBD (residues 372 to 646) were subcloned and fused to EGFP (pDEST31-GFP) for expression terminals.

Cell culture and transfection. NIH 3T3 mouse fibroblasts, B16F10 mouse melanoma cells, HeLa human cervical carcinoma cells, U2OS human osteosarcoma cells, 293T human kidney cells, and the glioblastoma-derived cell lines U251MG, U87MG, U373MG, LN18, A172 were grown in petri dishes at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. PC12 rat pheochromocytoma cells were cultured in gelatin-coated dishes with either 400 ng/ml of G418 (Sigma) or 200 μg/ml of G418 (Sigma) and soft agar cultures were performed as described previously (41, 49).

For Western blot analysis, cells were scraped from petri dishes, washed in PBS, and resuspended for 30 min in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 0.5% NP-40, and 10% glycerol) supplemented with protease inhibitor cocktail (Calbiochem), phosphatase inhibitor cocktail (Calbiochem), 0.5 mM dithiothreitol (DTT), and 50 units/ml of benzamidine. Protein extraction from mouse tissues was performed by grinding the tissue in liquid nitrogen using a ceramic mortar. The resulting powder was lysed using RIPA buffer for 30 min, and lysates were clarified by centrifugation for 20 min at 16,000 × g. Protein extracts from human tissues were obtained from frozen tissue samples in optimal lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) and stored at −80°C. Lysates were then separated in 12 to 15% Bis-Tris Criterion XT gels (Bio-Rad) and transferred onto a nitrocellulose membrane (Bio-Rad). Transfer efficiency was checked by red Porcine S staining, and the blot was then blocked with 5% nonfat milk in PBS-Tween (PBS plus 0.05% Tween 20) for 1 h at room temperature. After being blocked, the blot was incubated with primary antibodies at 4°C overnight. Thereafter, the blots were washed in PBS-Tween and incubated with either anti-rabbit or anti-mouse antibodies (Alexa Fluor 680 labeled from Invitrogen) for 45 min at room temperature. Finally, the fluorescent signal was detected by using the Odyssey infrared imaging system (Li-Cor Biosciences).

For immunohistochemistry, 5-μm sections were taken from human normal or tumor samples and put on glass slides. Slides were deparaffinized, rehydrated, and immersed in 10 mM citrate solution for antigen retrieval. Slides were washed in water, blocked in a 1:10 dilution of normal goat serum (Vector Labs), and incubated with primary antibodies. Slides were then incubated with secondary biotinylated antibodies followed by signal development with an immunoperoxidase reagent (ABC-HP; Vector Labs) and DAB (Sigma). Sections were lightly counterstained with hematoxylin and analyzed by light microscopy using a Nikon microscope. Human glioblastoma and brain samples were selected from the archives of the Virgen de la Salud Hospital (Toledo, Spain) and the Virgen del Rocio Hospital (Seville, Spain) between 2001 and 2004. Informed consent was required from patients according to the policies of the ethical committee of the hospital. Tissue macroarrays were constructed by getting 1-μm-thick cylinders from formalin-fixed and paraffin-embedded tissue blocks from 157 different glioblastoma samples. Five nontumor controls (four
normal brain tissue samples and one tonsil tissue sample) were included in each array. Hematoxylin and eosin-stained full sections from each donor block were used for morphological selections of the representative areas of each tumor.

**Kinase assays.** Protein lysates from 293T cells transfected with different constructs of c-myc-tagged Plk5 cDNA or Plk1 cDNA (0.5 to 1 mg of protein) were incubated for 2 h at 4°C in a total volume of 200 mL with 10 μl of anti-c-myc antibody agarose beads (Santa Cruz). One-third of the immunoprecipitates was collected by centrifugation, washed in RIPA lysis buffer, and subjected to SDS-PAGE and immunoblot analysis. The remnant two-thirds of the immunoprecipitation were used for the kinase activity assay. The immunoprecipitates were washed in RIPA buffer three times, with an extra wash in lysis buffer supplemented with 0.5 M NaCl and 5 mM DTT to remove possible nonspecific bound kinases. Then, beads were washed in kinase buffer (10 mM HEPES-NaOH [pH 7.4], 150 mM NaCl, 10 mM MgCl2, and 1 mM EGTA) supplemented with 0.5 mM DTT and phosphatase inhibitor cocktail (Calbiochem). The kinase reaction was initiated by the addition of the beads to 0.2 mM ATP, 2 μCi [γ-32P]ATP (Amersham) in 10 μl of kinase buffer, and either 3 μg of myelin basic protein (MBP) or 3 μg of histone H1 as the substrate. After incubation for 30 min at 30°C, the reaction was stopped with Laemmli sample buffer and analyzed by SDS-PAGE, and signal was obtained by using a Storm 820 phosphorimager scanner (Amersham).

**Culture and transfection of neuroblasts and neurite outgrowth assays.** Differentiation and neurite outgrowth were induced in PC12 cells either by incubating cells with neuronal growth factor (NGF) (Sigma) as described previously (19) or by expressing oncogenic Ras (20). After treatment, PC12 cells were kept in low serum medium (1% fetal bovine serum plus 0.5% horse serum) for 5 to 7 days, renewing the medium every 2 days. After the treatment, cells were fixed in 4% formaldehyde and stained with HCS CellMask blue stain (Invitrogen) and propidium iodide for nuclear staining. Neurite length quantification was performed using a specific Metamorph script (Molecular Probes). DNA was counterstained with 4',6-diamidine-2-phenylindole (DAPI; 2 μg/ml, H11032, Molecular Probes).

For analysis in vivo, hippocampal neurons were prepared from E18 rat embryos as described previously (7, 24). Dissociated neurons were seeded on coverslips coated with poly-l-lysine (1 mg/ml) in plating minimal essential medium supplemented with 0.5 M NaCl and 5 mM DTT to remove possible nonspecific bound kinases. Then, beads were washed in kinase buffer (10 mM HEPES-NaOH [pH 7.4], 150 mM NaCl, 10 mM MgCl2, and 1 mM EGTA) supplemented with 0.5 mM DTT and phosphatase inhibitor cocktail (Calbiochem). The kinase reaction was initiated by the addition of the beads to 0.2 mM ATP, 2 μCi [γ-32P]ATP (Amersham) in 10 μl of kinase buffer, and either 3 μg of myelin basic protein (MBP) or 3 μg of histone H1 as the substrate. After incubation for 30 min at 30°C, the reaction was stopped with Laemmli sample buffer and analyzed by SDS-PAGE, and signal was obtained by using a Storm 820 phosphorimager scanner (Amersham).

**RESULTS**

Plk5 is an antiproliferative Plk family member. The murine Plk5 gene contains 15 exons, as does Plk3, and has clear orthologs in all mammals, including humans (2) (see Fig. S1 in the supplemental material). Plk5 contains the key catalytic and ATP-binding residues but has unusual substitutions in certain regions, including the activation loop, which lacks the key activatory phosphorylation site (T210 in human Plk1) conserved as serine or threonine in all other Plks. Plk5 contains a clear polo box domain (PBD1) and has weaker sequence similarity in the PBD2 region, which lacks some key residues involved in substrate recognition in Plk1 to Plk3 (see Fig. S1 in the supplemental material), suggesting relevant differences in partner binding. Despite being of more recent origin than Plk2 and Plk3, Plk5 is more divergent in sequence: Plk2 and Plk3 share 68% identity in their kinase domains, 50% overall, while they are just 50% identical (kinase domain) and 37 to 41% identical (overall) to Plk5.

In humans, the corresponding PLK5 locus (ENSG00000185898; SgK384ps) has been considered a pseudogene since there is an in-frame stop codon in exon 6 disrupting the kinase domain (2, 12, 36). This stop codon is present in all human samples in public databases and in samples we have analyzed by genomic PCR in either tumor samples or healthy individuals (see Fig. S2 in the supplemental material). However, the human PLK5 contains a conserved ATG in frame in the boundary between exons 6 and 7, and it therefore has the potential to encode a short (about 36-kDa) protein containing the final fragment of the kinase domain, the PBDs, and the linker between these two domains. All human cDNAs skip the DNA region corresponding to mouse exon 13, which encodes the lesser conserved part of PBD1, although the genomic sequence appears to still be under evolutionary constraint and so may be expressed under some conditions. All other primate genomes sequenced have gaps within the Plk5 coding sequence. None have the frameshift or stop codon within the human sequence, but the chimera sequence has a distinct frameshift within the kinase domain confirmed by four individual reads.

We first tested the effects of Plk5 on the cell cycle by overexpressing the mouse Plk5 cDNA in NIH 3T3 cells. Plk5 induces a significant arrest in G1, and the subsequent reduction in S-phase and G2/M cells (Fig. 1A). This arrest is independent of kinase activity since expression of a D172A mutant (Plk5-DA) or the N terminus-deleted mutant (Plk5-NT) results in no or minor differences similar to Plk5-NT or PLK5) results in no or minor differences similar to Plk5-NT or PLK5).

**Statistical analysis.** Error bars and P values were obtained upon three different assays in each experiment. P values were obtained using the t test or one-way analysis of variance (ANOVA) with a Bonferroni posttest in order to compare individual points (GraphPad software).
were found using the corresponding mutant in the mouse sequence (Plk5-W421F; data not shown). These data indicate that the C-terminal region present in the human sequence is sufficient for inducing G1 arrest and that this function does not require the conserved tryptophan in the PBD.

The antiproliferative effect of the murine or human Plk5 proteins is accompanied by a clear induction of p21Cip1 in these fibroblasts (Fig. 1C and D), suggesting a role for the p53 pathway in these proliferative arrests. These results were obtained using both GFP- and myc-tagged proteins, thus excluding the remote possibility that these tags may modify the function of Plk5. In fact, overexpression of the murine Plk5 or the
mutant Plk5-DA or Plk5-$\Delta$N has no significant effect on the cell cycle of p53-null fibroblasts (Fig. 1E). These data suggest a p53-dependent $G_1$ arrest specifically mediated by Plk5 overexpression in primary fibroblasts.

**Murine Plk5 accumulates during quiescence and displays limited kinase activity.** To test the presence of Plk5 proteins in mouse cells, we generated antibodies targeting specific amino acid sequences at the N-terminal region of the murine Plk5 kinase domain (mPlk5-NT) or at the linker region (mPlk5-LK) (Fig. 2A). As a first validation for the antibodies, both mPlk5-NT and mPlk5-LK are able to recognize the EGFP-mPlk5 fusion transiently transfected in 293T cells but do not cross-react with EGFP-Plk1 (see Fig. S4 in the supplemental material). These antibodies are able to detect the endogenous mPlk5 protein in several cell lines, including NIH 3T3 fibroblasts and B16F10 melanoma cells (Fig. 2B). To validate specificity of the antibodies toward the endogenous mPlk5, we tested NIH 3T3 cells treated with specific short hairpin RNAs (shRNAs) against the mouse Plk5 sequence. These shRNAs provoke a significant reduction in Plk5 protein levels 48 h and 72 h after transfection (Fig. 2C). Despite this effect on Plk5 protein levels, treatment with these shRNAs did not result in any alteration in the cell cycle profile or cell proliferation of NIH 3T3 cells (Fig. 2D).

Using the mPlk5-NT antibody, we detected decreased levels of Plk5 in mitotic cells compared to those in asynchronous or confluent NIH 3T3 cultures (Fig. 2E). When NIH 3T3 cells were treated with doxorubicin to induce DNA damage, Plk5 protein levels were similar to those of asynchronous cultures (Fig. 2E). To further analyze the response of endogenous Plk5 levels, we cultured NIH 3T3 cells in the presence of reduced amounts of mitogenic signals (0.5% serum). As shown in Fig. 2F, serum deprivation induces the accumulation of Plk5 in about 9 h, and this level is maintained for 24 h after removal of serum. Restimulation of these cells with 10% serum results in downregulation of Plk5 in 9 to 24 h. We then next evaluated whether Plk5 levels fluctuate during cell cycle progression. Synchronization of cells in $S$-phase with thymidine shows a clear downregulation of Plk5 within 4 h of release from the thymidine block, at a time when cells exit from $S$-phase and enter into $G_2/M$ as detected by cell cytometry (see Fig. S5 in the supplemental material). Overall, these results suggest specific downregulation of Plk5 in the later stages of the cell cycle, in agreement with the downregulation of exogenous Plk5 during $G_2/M$ when expressed as a stable GFP fusion protein, as shown by video microscopy (Fig. 2G).

The absence of the typical activation residue in the T-loop of Plk5 (see Fig. S1 in the supplemental material) suggests some differences in the regulation of Plk5 activity. In fact, when tested in a typical kinase assay from immunoprecipitated proteins, wild-type or mutant mouse Plk5 only induces a residual signal on the myelin basic protein (MBP) and background activity on histone H1, two general substrates of many kinases (Fig. 2H). Plk1, however, displays a robust kinase activity on both substrates. Immunoprecipitated Plk5 tagged with GFP also failed to phosphorylate these substrates, while GFP-tagged Plk1 provided a significant signal (data not shown), suggesting that these different tags have no effect on the overall kinase activity of either Plk1 or Plk5.

**Plk5 suppresses malignant transformation by Ras oncoproteins.** The fact that Plk5 is downregulated in proliferating cells suggests it may be repressed by mitogenic signals or oncogenes. In fact, both the human and mouse Plk5 genes are repressed at the mRNA levels after transfection of primary human cells with oncogenes such as Ras, c-myc, and others (Fig. 3A and B).

We also tested whether the endogenous Plk5 may counteract the proliferative effect of oncogenes. Downregulation of endogenous Plk5 by specific shRNAs cooperates with Ras and E1A oncogenes in cell proliferation and malignant transformation in mouse fibroblasts, as measured by the focus assay as well as the formation of transformed colonies that are able to grow in soft agar (Fig. 3C and D). These results indicate that the endogenous Plk5 counters the proliferative effect of mitogenic pathways and may function as a tumor suppressor *in vitro*.

**Plk5 is expressed in differentiated cells, such as neurons and glia, both in mice and in humans.** We next tested the expression of Plk5 in several mouse tissues. As depicted in Fig. 4A, the Plk5 mRNA is highly expressed in differentiated tissues, such as the brain, ovary, or eye, but is not detectable in proliferating tissues, such as the colon, spleen, placenta, etc. This expression pattern was confirmed at the protein level, indicating significant levels of Plk5 in the murine central nervous system (cerebellum and brain cortex) as well as in the eye (Fig. 4B). These expression data are also confirmed by available massive cDNA microarray analysis or *in situ* hybridization in the mouse embryo or young mice (see Fig. S6 in the supplemental material).

We next analyzed whether human cells also express PLK5 at the protein level by generating two different antibodies. The hPLK5-LK antibody was generated against amino acid sequences in the region homologous to the linker of the murine protein (Fig. 4C). An additional antibody was generated using the predicted amino acid sequences at the N-terminal region (hPLK5-NT). The hPLK5-LK antibody clearly recognizes the human PLK5 protein when overexpressed as a GFP fusion protein, and this signal is significantly decreased by PLK5-specific shRNAs (see Fig. S7 in the supplemental material). Interestingly, the hPLK5-LK antibody recognizes a short, endogenous, ~40-kDa protein in brain tissue extracts, in agreement with the predicted sequence originated from the ATG codon at the boundary between exons 6 and 7. Human PLK5 is highly expressed in brain but not in other cell types, such as primary fibroblasts or tumoral HeLa or HEK293T cells (Fig. 4D). On the other hand, hPLK5-NT (designed against the human N terminus) detects the long Plk5 isoforms in mouse tissues, but it is not able to detect any peptide in human cells (data not shown).

To further analyze in detail the expression of the human PLK5 protein, we used the hPLK5-LK antibody in immunohistochemistry of a tissue macroarray containing a variety of human tissues. As represented in Fig. 4E, hPLK5 displays a characteristic pattern of expression, with polarized localization in neurons and glial cells. This staining is specific, and it is lost when the primary antibody is previously preabsorbed with the peptide used for its generation (Fig. 4F). The cytoplasmic localization observed by this antibody is in agreement with the cytoplasmic signal detected with the human PLK5-GFP fusion constructs in stable cell lines (see Fig. S8 in the supplemental material) as well as in primary neuroblasts (Fig. 4G). In addi-
FIG. 2. The mouse Plk5 is preferentially expressed in quiescent cells and has no detectable kinase activity. (A) Schematic representation of the mPlk5-NT (N terminus) and mPlk5-LK (linker) epitopes selected for generating antibodies against murine Plk5. (B) The mPlk5-NT antibody detects an endogenous protein of about 65 to 68 kDa in NIH 3T3 fibroblasts and B16F10 melanoma cells. An additional upper strong band is found in NIH 3T3 fibroblasts (asterisk). (C) Immunodetection of endogenous mPlk5 in NIH 3T3 cells transfected with vectors expressing short hairpin RNAs (shRNAs) against Plk5 (shPlk5) or scrambled sequences (shCtrl). The additional band (asterisk) found frequently in these cells is not modulated by these shRNAs, suggesting that it is an unspecific band. Protein levels of Plk5 were quantified and normalized to /H9251 -tubulin. (D) Expression of shRNAs against Plk5 does not result in major alterations in DNA content profile in these cells. (E) Quantification of Plk5 protein levels in different cellular conditions, such as asynchronous cultures (Asyn.), DNA damage induced by doxorubicin, full confluence during 48 h, or mitosis (nocodazole arrested). Cyclin D1 was used as an additional control for some of these culture conditions. (F) mPlk5 is induced during the exit from the cell cycle upon serum starvation, and its protein levels decrease during the entry into the cell cycle after serum stimulation. In panels E and F, α-tubulin was used as a loading control and cyclin D1 was used as an additional control for some of these culture conditions. Histograms represent means ± SEM. (G) mPlk5 was stably expressed in U2OS cells as a GFP fusion protein, and fluorescence was recorded by video microscopy. Green fluorescence of asynchronously growing cells (20 cells per assay) in two different assays was quantified and plotted at different time points before nuclear envelope breakdown (NEBD), showing that GFP-Plk5 expression levels decrease in the G2/M transition. The red line shows the fluorescence average intensity of all the recorded cells. Bars represent the standard deviations of the recorded fluorescence intensity. (H) Mouse Plk1 and Plk5, as well as Plk5 mutant isoforms, were immunoprecipitated using antibodies against the myc epitope or the endogenous proteins (not shown) and assayed for kinase activity on the myelin basic protein (MBP) or histone H1. The immunoprecipitation of the kinase was measured with antibodies against myc (top), whereas the loading of MBP or H1 was measured by Coomassie blue staining.
tion to these cells in the central nervous system, hPLK5 seems to be expressed in other highly differentiated cells, such as cells of the serous acini in the parotid gland, distal and proximal tubules of the kidney, tubules of the seminal gland, Kupffer cells and some hepatocytes in the liver, and some cells in the germinal center of lymph nodes (see Fig. S9 in the supplemental material).

**Plk5 is required for axonal growth in neuroblasts.** Since both mouse and human Plk5 proteins seem to be preferentially expressed in the central nervous system, we next tested the effect of modulating Plk5 levels on neuronal function. We first used rat pheochromocytoma PC12 cells, which can be differentiated into neuron-like cells in vitro by either Ras oncogenes or nerve growth factor (NGF). Overexpression of either mouse or human Plk5 per se does not induce cell differentiation (measured by the formation of neurites) but cooperates with Ras in this assay (Fig. 5A). Overexpression of mPlk5-D172A or mPlk5-D9004 resulted in similar effects, suggesting that Plk5 synergizes with Ras in neurite formation in a kinase-independent manner. Interestingly, the human PLK5 construct also resulted...
FIG. 4. Mouse and human Plk5 proteins are preferentially expressed in brain cells. (A) Northern blot of mouse tissues indicating a preferential expression of the murine Plk5 mRNA in brain, eye, and ovary. (B) Immunodetection of mPlk5 protein in different tissues, showing the preferential expression in the nervous system. (C) Schematic representation of the predicted hPLK5-NT (N terminus) and hPLK5-LK (linker) epitopes selected for generating antibodies against human PLK5. Note that the hPLK5-NT antibody has been designed against an epitope not present in the predicted 36-kDa short form. (D) The hPLK5-LK antibody is able to detect a band that runs at a position slightly higher than that of the predicted molecular size (values in kilodaltons at left). This protein is present in two different samples from human brain tissue, but it is barely detectable in different cell lines, including primary human foreskin fibroblasts. (E) Detection of PLK5 in human brain cortex and cerebellum sections by immunohistochemistry. PLK5 (brown signal) is present mostly in the cytoplasm of neurons and glial cells in the cortex and granular cells in the cerebellum. Eventual nuclear staining is also observed in some of these cells (asterisk), although it is not evident with this technique. (F) Preabsorption with the antigenic hPLK5-LK peptide used to generate the antibody eliminates the signal in the brain, suggesting the specificity of the hPLK5-LK antibody in these samples. (G) When expressed in neuroblasts as a GFP fusion protein, the human PLK5 is mostly confined to the somatic and proximal neuritic levels (arrowheads), localizing to the cytoplasm and eventually to the nucleus. In contrast, soluble GFP localizes all over the cell, including the nucleus, cytoplasm, and neuritic processes.
Rat PC12 cells were stably transfected with different shRNAs against the rat Plk5 transcript (shPlk5). In general, shPlk5 transfected cells display a significantly reduced number of neurites when cultured in rich medium with serum (Fig. 5B). In addition, NGF-induced neurite outgrowth was reduced in these cultures in the presence of the two different shRNAs against Plk5, suggesting that this protein is required for maintaining neurites or for de novo formation of these cellular extensions in these assays (Fig. 5C).

To analyze the relevance of Plk5 in primary neuroblasts, we next investigated the effect of Plk5 overexpression or inhibition in primary cells isolated from embryonic rat hippocampus. Overexpression of human PLK5 induces the formation of abundant F-actin-rich protrusions that are much less abundant in neuroblasts negative for the GFP-Plk5 construct (Fig. 6A). These Plk5-overexpressing neurons present a distorted phenotype with an abnormally thick neuritic initial segment at the basis of the neurites and an increased abundance of thin actin-positive protrusions. Transfection of shPlk5 shRNAs into these neuroblasts does not result in major effects in the existing neuron extensions. However, knockdown of Plk5 prevents the growth of axons induced by brain-derived neurotrophic factor (BDNF) (Fig. 6B). Whereas BDNF induces a significant growth of the axons, increasing their lengths by 20%, no axonal growth is observed in the presence of the shRNAs against Plk5, thus suggesting that Plk5 is involved in neuron differentiation and axonal growth induced by growth factors such as NGF or BDNF.

**PLK5 is downregulated in human brain tumors by promoter hypermethylation.** The presence of Plk5 in the central nervous system and its possible function in modulating neuron differentiation prompted us to analyze the expression of hPLK5 in human brain tumors. Our preliminary data using cDNA expression profiles suggested that hPLK5 is indeed downregulated at the mRNA level in human primary brain tumors, including oligodendrogliomas, oligoastrocytomas, and astrocytomas of different grades (see Fig. S10 in the supplemental material). To test PLK5 protein levels in these tumors, we used the hPLK5-LK antibody for immunohistochemistry in a panel of 144 gliomas of different grades. As represented in Fig. 7A, hPLK5 is readily detectable in the normal brain controls in this array, whereas this specific signal is reduced in tumor cells. We also analyzed specific tumors and normal brain samples by microarray, whereas this specific signal is reduced in tumor cells. We analyzed specific tumors and normal brain samples by microarray, confirming that hPLK5 protein levels are severely decreased in advanced tumors, such as glioblastoma multiforme (GBM) samples both in paraffin sections and in tissue lysates (Fig. 7B). Whereas hPLK5 is detected in neurons and glia cells of control brains (an average of 95% of control cells are positive), most tumors are negative or present reduced expression of hPLK5 staining (an average of 25 to 35% of positive cells in astrocytomas and 30 to 55% of positive cells in oligodendrogliomas). Higher-grade tumors tend to show a stronger reduction in PLK5 transcripts. Thus, whereas about 80% of grade II astrocytomas are positive for hPLK5, only 15 to 20% of advanced tumors (grade III and IV) maintain this signal (Fig. 7C and D; see also Fig. S10 in the supplemental material). PLK5 and PLK2 are the only Plk family members downregulated in these tumors, whereas Plk1 is upregulated and PLK3 and PLK4 display only minor changes in expression (see Fig. S10).

We then studied the mechanism that silences human PLK5 in brain tumors. Plk5 genes contain a CpG island, close to the transcription factor binding site both in mouse (not shown)
and in humans (Fig. 8A), that is susceptible to changes in methylation. Whereas most CpG dinucleotides are not methylated in control brains, we have detected a significant methylation of CpG dinucleotides in DNA isolated from human astrocytoma and GBM. Indeed, 16 out of 18 tumors tested by either methylation-specific PCR (MS-PCR) or DNA sequencing after bisulfite modification showed a significant methylation in F-actin-positive protrusions (asterisks). In the graph, the numbers of protrusions per 10 μm of axon length are compared between controls and GFP-hPLK5-transfected neurons. (B) Expression of shRNAs against Pkl5 (shPkl5) or scrambled sequences (shCtrl) (green signal) does not have a major impact on the morphology of non-treated primary neuroblasts. However, whereas the control cultures respond to stimulation with BDNF by elongating their axons, this response is impaired in cells in which Pkl5 is knocked down. Green reflects shRNA expression, red indicates F-actin staining, and blue shows α-tubulin staining. Results are means plus standard deviations of results from three independent experiments (25 cells/experiment).

We next tested whether the reexpression of hPLK5 has any effect in these tumor cells. We observed that several GBM cell lines, such as A172, T98, SW1088, U373MG, or LN18, contain methylated PLK5 promoters (Fig. 8C). Overexpression of hPLK5 in these cell lines (Fig. 8D; see also Fig. S11 in the supplemental material) results in a dramatic apoptotic response as measured by annexin V staining. Overexpression of the mouse wild-type Pkl5 or the Pkl5-ΔN mutant, which mimics the truncated human protein, has a similar apoptotic effect. Similarly, the conserved W207 in the PBD is not required for the apoptotic effect in GBM cells (see Fig. S3 in the supplemental material). The effect of the other Plk family members is

FIG. 6. Plk5 modulates cellular morphology in primary neuroblasts. (A) A GFP-hPLK5 fusion protein was overexpressed for 48 h in primary neuroblasts from rat hippocampus. High expression levels of GFP-hPLK5 (neuron 1, green) preclude normal axon development, inducing abnormally thick hillocks at the basis of the neurites (arrow) and abundant thin F-actin-positive protrusions (arrowheads). In the case of medium/low expression levels (neuron 2, green), axons develop normal length, but in contrast to control nontransfected neurons (neuron 3), they display axon tracts highly enriched in F-actin-positive protrusions (asterisks). In the graph, the numbers of protrusions per 10 μm of axon length are compared between controls and GFP-hPLK5-transfected neurons. (B) Expression of shRNAs against Pkl5 (shPkl5) or scrambled sequences (shCtrl) (green signal) does not have a major impact on the morphology of non-treated primary neuroblasts. However, whereas the control cultures respond to stimulation with BDNF by elongating their axons, this response is impaired in cells in which Pkl5 is knocked down. Green reflects shRNA expression, red indicates F-actin staining, and blue shows α-tubulin staining. Results are means plus standard deviations of results from three independent experiments (25 cells/experiment).
FIG. 7. PLK5 is downregulated in human brain tumors. (A) Human PLK5 expression is evaluated in 144 different gliomas by immunohistochemistry (IHC) on tissue microarrays. In some of these tumors, hPLK5 (brown signal; arrows) is expressed in normal areas but not in the tumor mass. (B) The reduction in hPLK5 expression is demonstrated by IHC and Western blot analysis with similar results. Three different control brains and four different glioblastomas (GBM) are represented. The glioblastoma marker EphA2 is overexpressed in three out of the four glioblastomas, but it is expressed at low levels in control brains. The relative hPLK5 protein levels were normalized versus the levels of α-tubulin. (C) Quantification of hPLK5 expression in oligodendrogliomas sorted by tumor type and by malignant grade. OA, oligoastrocytoma (grade II); AOA, anaplastic oligoastrocytoma (grade III); OG, oligodendroglioma (grade II); AOG, anaplastic oligodendroglioma (grade III). (D) Quantification of hPLK5 expression in astrocytomas sorted by tumor type and by malignant grade. DA, diffuse astrocytoma (grade II); AA, anaplastic astrocytoma (grade III); GBM, glioblastoma multiforme (grade IV). Cases with cytoplasmic staining of hPLK5 in more than 10% of the cells were scored as a positive tumor in this analysis. However, whereas the average number of positive cells was close to 90% in normal samples, this average ranged from 10% to 60% in the positive tumors, indicating that PLK5 levels in the tumors never reached the protein levels found in normal samples. Error bars represent SEM.
less dramatic, although overexpression of Plk1 and Plk2 also results in a moderate increase in apoptosis, at least in the p53-null U373MG cell line (see Fig. S3). All together, these data suggest that PLK5 silencing through epigenetic means may help brain tumors to escape from apoptosis.

**DISCUSSION**

Polo kinases are considered central modules in cell cycle regulation and have attracted much attention due to their therapeutic potential (4, 34, 39, 50). Most research efforts have...
been focused on the analysis of Plk1 as an essential protein in mitotic entry and cytokinesis (8, 40, 53) and, recently, on the relevant role of Plk4 in centriole biology and mitotic fidelity (9, 21, 27, 28, 43).

Whereas Plk1 and Plk4 orthologs are present in most eukaryotes, the Plk2 subfamily is first clearly seen in deuterostomes, duplicates to form Plk2 and Plk3 in vertebrates, and gives rise to Plk5 in mammals (see Fig. S1 in the supplemental material). Plk2 seems to share some functions with Plk1 and Plk4 in centriole function (13, 58), while Plk3 localizes either to the nucleolus or to the microtubules around the nuclear membrane and may be involved in several cellular processes, including S-phase progression and cell cycle checkpoints, Golgi fragmentation, cytokinesis, and apoptosis (5, 6, 14, 33, 44, 57, 60, 62). Mouse Plk5 is highly similar to Plk2 and Plk3 in sequence, and it may share similar functions in DNA damage, as recently reported (2). However, whereas both Plk2 and Plk3 are induced by mitogens, mouse Plk5 is downregulated in serum-stimulated cells and accumulates upon serum deprivation (Fig. 2). Downregulation during G2/M is likely to be due to protein degradation, as it is also observed in exogenous GFP-Plk5 proteins (Fig. 2G; see also Fig. S5 in the supplemental material). Proteasome-dependent degradation has also been observed for Plk3 (1).

The most striking feature of the human \textit{PLK5} gene is the presence of a stop codon in exon 6, close to the end of the kinase domain, and the presence of a new ATG in-frame codon in the boundary between exons 6 and 7. This stop codon is not observed in any primate sequence, suggesting a significant functional shift in this gene within the human lineage. Surprisingly, while we cannot detect the expression of an N-terminal-truncated protein, we do see strong expression of a product likely (by size and epitope) to initiate just downstream of the stop codon. Thus, as predicted from the sequence and also reported in reference 2, the human gene encodes a smaller, 40-kDa protein that lacks the kinase domain but contains the linker sequences and the PBD, which is in agreement with our results using specific antibodies against the N terminus or the linker of the predicted proteins. This downstream ATG is conserved in all Plk5 orthologs and in the other Plk family members, although we have not detected shorter Plk5 proteins in mouse extracts. All Plk5 orthologs lack an activation loop threonine, which is the activatory residue on Plk1 and conserved in all other Plks. In addition, the mouse Plk5 protein fails to efficiently phosphorylate typical kinase substrates, such as histone H1 or MBP, suggesting that Plk5 proteins may have lost some or all kinase catalytic activity and function in a PBD-dependent manner. Indeed, mouse Plk5 not only lacks the activatory threonine at the T-loop but also has a divergence in the highly conserved DFG motif present in the ATP binding pocket of almost all kinase domains of the kinome (see Fig. S1 in the supplemental material). This is carried further in humans, where only a truncated form lacking the kinase domain is expressed. The apparent deletion of the kinase domain in the human protein may be a logical extension of this trend. Several kinases are known to lack catalytic function and instead serve as scaffolds or kinase substrates. About 45 kinases lack at least one of the catalytic sites required for the kinase function (36). An almost identical set of pseudokinases is predicted in mice, supporting a conserved noncatalytic function for many mammalian kinases (12).

Both Plk2 and Plk3 participate in neuron structure and synaptic plasticity, and the conserved PBD is involved in this neuronal function (26). Plk2 is also required for plasticity of hippocampal neurons during epileptiform activity and chronically elevated activity (46, 48). The present study demonstrates that Plk5 is expressed mostly in the adult murine and human brain and may modulate neurite formation in established cell lines and primary neurons. In addition, Plk5 participates in the formation of neuritic processes in response to NGF/BDNF or Ras. The formation of these processes is not observed upon interference with the endogenous \textit{Plk5} transcript, suggesting a relevant role for Plk5 in this pathway. As suggested for Plk2 and Plk3, this function is also likely to be dependent on the PBD, as human PLK5 also modulates neurite formation (Fig. 5 and 6) and does not contain the kinase domain. The PBD is a relevant domain that confers specificity in partner binding (54). Thus, it is tempting to speculate that Plk5 has evolved as a kinase-deficient protein, where the PBD may function in protein-protein interaction without the subsequent phosphorylation. Similar gene duplication and subfunctionalization or neofunctionalization have been suggested for other kinase family members, such as VRK3 (45), a duplicate gene that loses catalytic activity but maintains a modification of the regulatory site, similar to the PBD here. Whether the Plk5 PBD recognizes specific substrates or may compete for common partners with other Plk family members is currently unknown. However, the fact that Plk5 lacks relevant residues involved in protein interaction in PBD2 (H538/K450 in Plk1) and the dispensability of the conserved tryptophan in PBD1 suggests that Plk5 may use different mechanisms than those reported for Plk1, as suggested for PLK2 and PLK3 (54).

Human PLK5 is significantly downregulated in brain tumors at both the mRNA and the protein level. In fact, the \textit{PLK5} gene is frequently silenced by promoter hypermethylation in astrocytomas and glioblastoma multiforme, the most common and most aggressive type of primary brain tumor, accounting for 52% of all primary brain tumor cases and 20% of all intracranial tumors. This epigenetic silencing suggests the relevance of this protein in either maintaining the differentiated phenotype or triggering cell death in neurons, glia, or their progenitors. Loss of heterozygosity (LOH) for \textit{PLK5} on chromosome 19p13.3, where \textit{PLK5} is located, is frequently found in several human cancers, including breast tumors, uterine cervical adenocarcinomas, carcinoma metastases to the brain, and ovarian tumors. Although the main candidate so far has been \textit{STK11/LKB1} (0.3 Mb away from \textit{PLK5}), recent evidence suggests additional targets in this region (25).

The roles of PLK1 in cell division and its overexpression in human tumors have suggested that inhibition of PLK1 activity may have beneficial effects in cancer. Indeed, current therapeutical strategies are directed to identify not only small molecular ATP competitors for PLK1 but also drugs that interact with the PBD (39, 42). However, both PLK2 and PLK3 have been suggested to have tumor suppressor functions, and PLK2 may be inactivated by promoter hypermethylation in B-cell malignancies or liver tumors (38, 52). On the other hand, Plk3-null mice displayed an increase in weight and developed tumors in multiple organs with age (63). Thus, Plk family
members may behave both as oncogenes and as tumor suppressors (30). As suggested by our results, the new member of the Plk family, PLK5, may also function as a tumor suppressor in the brain. In fact, PLK5 has a relevant antiproliferative effect, and it is quite potent in the induction of apoptosis. Thus, reexpression of PLK5 by gene therapy strategies or by using epigenetic drugs may have beneficial effects on these aggressive tumors. The detailed analysis of the PBD of PLK5 and its comparison to other Plk family members may provide a molecular basis for new compounds targeting PBD partners for cancer intervention.

ACKNOWLEDGMENTS

We thank Susana Temiño for technical assistance, Marta Cañamero for help in histology, and Jorge Martínbalbo and Eriech Nigg for preliminary assays on the overexpression of PLK5 constructs.

This work was funded by grants from the Association for International Cancer Research (AICR number 08-0188), Foundation Ramón Areces, Ministerio de Ciencia e Innovación (MICINN; SAF2009-07973), and NHGRI HG001641-04. The Cell Division and Cancer Group of the CNIO is supported by the OncoCycle Programme (S-BIF-0232-2006) from the Comunidad de Madrid, the OncoBIO Consolider-Ingenio 2010 Programme (CSD2007-0017) from the MICINN, Madrid, Spain, and the European Community’s Seventh Framework Programme (FP7/2007-2013) under grant agreement MitoSys (no. 241548).

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