Characterization of PDZ-binding kinase, a mitotic kinase

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hDlg, the human homologue of the Drosophila Discs-large (Dlg) tumor suppressor protein, is known to interact with the tumor suppressor protein APC and the human papillomavirus E6 transforming protein. In a two-hybrid screen, we identified a 322-aa serine/threonine kinase that binds to the PDZ2 domain of hDlg. The mRNA for this PDZ-binding kinase, or PBK, is most abundant in placenta and absent from adult brain tissue. The protein sequence of PBK has all the characteristic protein kinase subdomains and a C-terminal PDZ-binding T/SKV motif. In vitro, PBK binds specifically to PDZ2 of hDlg through its C-terminal T/SKV motif. PBK and hDlg are phosphorylated at mitosis in HeLa cells, and the mitotic phosphorylation of PBK is required for its kinase activity. In vitro, cdc2/cyclin B phosphorylates PBK. This evidence shows how PBK could link hDlg or other PDZ-containing proteins to signal transduction pathways regulating the cell cycle or cellular proliferation.

Multiple proteins, including some tumor suppressors, regulate the cell cycle machinery in a network of signaling pathways. hDlg, the human homologue of the Drosophila tumor suppressor Discs-large (Dlg), may be among these proteins. But how it interacts with signal transduction pathways is not fully understood.

hDlg is a membrane-associated guanylate kinase homologue (MAGUK) (1). Like other MAGUKs, it contains several protein–protein interaction domains: three PDZ domains, an SH3 domain, and a C-terminal guanylate kinase homology region that may act as a protein-binding domain (2–4). There are several MAGUKs in mammalian organisms. Some, like PSD-95 and Chapsyn-110, appear to be important for the function of synapses (5, 6). Others, such as ZO-1 and ZO-2, are part of the tight junction protein complex (7, 8). Yet another, p55, is a component of the erythrocyte membrane skeleton (9). Although all are homologues of the Drosophila tumor suppressor Dlg, it is unclear whether they also act as tumor suppressor proteins. A p55 mutation was identified in a leukemia patient, but it is not known whether this mutation was important for cellular transformation (10). ZO-1 protein levels are correlated with the differentiation level of breast and intestinal track tumors, but no causal effect of the lack of ZO-1 has been demonstrated in tumor formation (11, 12).

The identification of several proteins that bind to hDlg supports its involvement in cellular growth control. The adenomatous polyposis coli (APC) tumor suppressor protein binds the PDZ2 domain of hDlg in vitro, and the two proteins coimmunoprecipitate and colocalize in some cell types (13, 14). Furthermore, the PDZ2 domain of hDlg also interacts with the human papillomavirus E6 transforming protein and the 9ORF1 domain, and a C-terminal guanylate kinase homology region that regulates kinase activity. In vitro, cdc2/cyclin B phosphorylates PBK. This evidence shows how PBK could link hDlg or other PDZ-containing proteins to signal transduction pathways regulating the cell cycle or cellular proliferation.

Experimental Procedures

Two-Hybrid Screen. The two-hybrid screen was performed by using the MatchMaker system (CLONTECH). The bait, full-length hDlg cDNA, was subcloned in pGBT9 and used to screen a HeLa cell cDNA library in pGAD-GH (CLONTECH). A 750,000 independent clones were screened, of which 2,000 were HIs+ and 240 were positive for β-galactosidase activity. Two of those clones were partial cDNA sequences of PBK.

Cloning and Mutagenesis. The full-length PBK cDNA was obtained by a set of PCR amplifications from the pGAD-GH HeLa cDNA library (CLONTECH). To clone the 5’ end, the first amplification used a sense primer within the vector sequence (5’-CTGTCACCTGTGTTGACCGAC-3’) and an antisense primer within the original PBK clone (5’-GCGAAGCCACACTTCAGC-3’). The secondary PCR used nested primers with the sense primer within the vector sequence (5’-TACACTCAAATTGGAAT-3’) and the antisense primer within the original PBK clone (5’-CTCAGGGTGACTCAGCAGTC-3’). The PCR sequence was confirmed by cloning the same full-length cDNA from the λExo λ HeLa cDNA library (Novagen) and later by comparison to human expressed sequence tag (EST) sequences.

To generate constructs expressing PBK with a mutant C terminus, the PBK cDNA sequence was amplified by PCR using a wild-type 5’ primer sequence (5’-GCGGGATCCATGGAAGGGATCATGAAATTTCAAG-3’) and an antisense primer in which the appropriate bases were substituted (V322A: 5’-GCGCATGCTCAAGCATTCCAGGTCC-3’, T320A: 5’-GCGACTACGTCATGACATCGTGGCTTTCCAGGCTCAGGACTCAGCAGTC-3’). For deletion mutant, the same sense primer was used with an antisense primer that inserted a stop codon after base 936 (Δ5’-GCGACTACGTCATGACAGGAGCAGGATC-3’).

The construct used to express inactive PBK with mutations of lysines 64 and 65 to alanines was generated with the QuickChange Site-Directed Mutagenesis kit (Stratagene) and the primers: 5’-GGCTGTAGCAGCGATCAATCCTATAGTGTAATG-3’ and 5’-GGATGATCGTGCCTGTCAGCAGCCAGGAGCAGGAGCAGGATC-3’.

The underlined bases show where the sequences differ from that of wild-type kinase. The doubly underlined bases are silent mutations that remove a Vsp1 restriction site to facilitate

Abbreviations: GST, glutathione S-transferase; MAGUK, membrane-associated guanylate kinase homologue; PBK, PDZ-binding kinase; EST, expressed sequence tag.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF189722).

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screening of mutant sequences. All the constructs were verified by sequencing (Sequenase Ver. 2.0, United States Biochemical).

**Recombinant Protein Expression.** The pGEX expression system was used to express recombinant proteins in *Escherichia coli*. The coding sequences were subcloned in frame downstream of glutathione S-transferase (GST) in a pGEX-2T vector. The various hDlg constructs have been described elsewhere (1, 17). The proteins were expressed in the *E. coli* 71/18 bacterial strain and purified as described (18).

The proteins used in *in vitro* kinase assays were expressed in Sf9 cells by using the pAcGH LT B transfer vector and the BaculoGold baculovirus expression system (PharMingen). The vector was modified to remove the protein kinase A labeling site, the polyhistidine tag, and the thrombin cleavage site. Wild-type and inactive mutant PBK cDNAs were subcloned in frame downstream of GST. Baculovirus generation and protein expression were performed as recommended by the manufacturer, harvesting the cells 72 h after infection. The baculoviruses used for the expression cdc2 and cyclin B were a generous gift from Frederic Yarm and Ray Erikson (Harvard University).

To isolate active PBK from Sf9 cells, the cells were treated with 100 nM okadaic acid for 3 h before harvesting (19, 20). The cells were lysed in lysis buffer (10 mM Tris-Cl, pH 7.5/130 mM NaCl/1% Triton X-100/10 mM NaF/10 mM Na3VO4/10 mM Na2P2O7/1 mM Pefabloc/1 μg/ml pepstatin/1 μg/ml leupeptin). The cleared lysates were incubated with glutathione Sepharose-4B (Sigma), and the beads were washed with lysis buffer and then with storage buffer (50 mM Tris-Cl, pH 7.5/150 mM NaCl/10 mM NaF/1 mM Na3VO4/1 mM EDTA/1 mM DTT). Bound proteins were eluted in 50 mM Tris-Cl, pH 7.5, and 5 mM reduced glutathione, then dialyzed against storage buffer. A sample was precipitated with acetone and resuspended in 1% SDS to determine the protein concentration by using the BCA protein determination kit (Pierce).

The TNT SP6 coupled reticulocyte lysate expression system (Promega) was used to produce radiolabeled proteins for *in vitro* binding assays and blot overlay assays. The relevant cDNAs were subcloned into the pNB40 vector (21). 35S-methionine and 3H-labeled proteins were obtained from Amersham Pharmacia.

**In Vitro Binding Assays and Blot Overlay Assays.** For *in vitro* binding assays, fusion proteins (2 μg) on beads were incubated 2 h with 10 μl of reticulocyte lysate containing the 35S-labeled kinase with 5 μl RIPA buffer (50 mM Tris-Cl, pH 7.5/150 mM NaCl/1% Nonidet P-40/0.5% deoxycholate/0.1% SDS/1 mM EDTA/1 mM DTT). The beads were washed in RIPA buffer, and the bead pellet was resuspended in Laemmli sample buffer for SDS/PAGE analysis and autoradiography.

For blot overlay assays, equivalent amounts of each GST-PBK fusion protein were run on a 10% Laemmli gel and then transferred to nitrocellulose. The blots were probed with 35S-labeled hDlg or GST-PDZ1–2, washed extensively, and dried for autoradiography as described (22).

**Antibody Production, Immunoprecipitations, and Immunoblots.** A rabbit polyclonal antibody against PBK was generated as described (23) (Serasource, Royalston, MA) by using the *E. coli* expressed full-length PBK released from GST with thrombin, further purified on a Mono-Q column, and dialyzed into PBS. The anti-hDlg antibody has been described elsewhere (17).

For immunoprecipitations, about 106 HeLa cells were lysed in 1 ml of Nonidet P-40 lysis buffer (50 mM Tris-Cl, pH 7.5/150 mM NaCl/0.5% Nonidet P-40/10 mM β-glycerophosphate/10 mM NaF/10 mM Na3VO4/10 mM Na2P2O7/1 mM Na3VO4/1 mM Pefabloc/1 μg/ml pepstatin/1 μg/ml leupeptin/1 mM EDTA/1 mM DTT). The cellular debris were sedimented, and lysates (about 100 μl) with equal amounts of total protein were incubated with the appropriate serum. The resulting immunocomplexes were recovered with 10 μl of protein A-Sepharose CL-4B beads (Pharmacon Biotech), washed in Nonidet P-40 washing buffer (lysis buffer without β-glycerophosphate, Na3VO4, or Na2P2O7), then resuspended in Laemmli sample buffer for SDS/PAGE analysis and immunoblotting. Alternatively, the complexes were washed once more in kinase assay buffer (see below) and resuspended for kinase assays.

For immunoblots, anti-PBK serum was used at 1:500, and anti-hDlg serum (directed against residues 200–960) was used at 1:1,000. The primary antibodies were detected with an alkaline phosphatase conjugated goat anti-rabbit IgG antibody (1:1,000, Zymed) and NBT/BCIP detection reagents (Sigma).

**In Vitro Kinase Assays.** Proteins purified from Sf9 cells (1 or 2 μg) or immunoprecipitates containing endogenous PBK from about 106 HeLa cells were used in *in vitro* kinase assays. For auto-phosphorylation assays, the kinase was incubated in 30 μl kinase assay buffer (50 mM Tris-Cl, pH 7.5/150 mM NaCl/10 mM MgCl2/10 mM NaF/1 mM Na3VO4/1 mM EDTA/1 mM DTT) supplemented with 5 μCi of (32P)-ATP. For substrate phosphorylation assays, 2 μg of myelin basic protein, histone (Sigma) or GST-hDlg, and 100 μM nonradiolabeled ATP were also added. After 45 min at 30°C or 2 h on ice, the reactions were stopped by addition of Laemmli sample buffer. The samples were resolved on 10% tricine gels and analyzed by autoradiography.

For phosphorylation of PBK by cdc2/cyclin B, 2 μg of GST, or wild-type or inactive mutant GST-PBK, was incubated 45 min at 30°C with 5 units of cdc2/cyclin B (New England Biolabs) in the recommended cdc2/cyclin B assay buffer supplemented with 100 μM ATP and 5 μCi of (32P)-ATP for radiolabeling.

**Cell Culture and Cell Cycle Synchronization.** HeLa cells were grown in DMEM low glucose supplemented with 4 mM l-glutamine and 7.5% FBS (Life Technologies) under a 5% CO2 atmosphere at 37°C. To synchronize cells at S-phase, we performed a double thymidine block (24). Nine hours after releasing the cells from the second thymidine block, the mitotic cells were isolated by gently pipetting media over them. To isolate G1-phase cells, mitotic cells were replated and incubated another 1.5 h. By visual inspection, over 95% of the cells were then postmitotic.

**Results**

**Two-Hybrid Screen and Cloning of PBK.** A two-hybrid screen with full-length hDlg as bait identified many hDlg-interacting clones, two of which encoded the C-terminal half of a protein kinase (Fig. 1). The 5’ end of the PBK cDNA was cloned by PCR from the pGAD-GH HeLa cDNA library to complete the coding sequence. The entire sequence was confirmed by sequencing additional clones isolated from a phage AEX40 HeLa cDNA library. Several EST sequences in the human EST and UNIGENE databases corroborate the PBK sequence from HeLa cell cDNA libraries (Fig. 1). Mouse, zebrafish, and *Drosophila* homologues were also found in the EST and UNIGENE databases (Fig. 1).

The human PBK cDNA encodes a 322-aa protein (calculated molecular mass, 35 kDa) containing all the conserved subdomains and most of the nearly invariant residues found in kinases (Fig. 1). Its active site sequence corresponds to the consensus for serine/threonine or dual specificity kinases (D-X-K-X-X-N, residues 174 to 179 of the alignment in Fig. 1). Another feature of the human PBK sequence is the TEDV motif at the C terminus, which matches the binding specificity of the hDlg PDZ1 and PDZ2 domain determined by peptide screens (25).

Most of the mouse PBK amino acid sequence can be deduced from overlapping EST sequences (Fig. 1). Only a short piece of the sequence is still undetermined, corresponding to residues 232
to 258 of the alignment in Fig. 1. The mouse protein sequence shows 89% identity with the human sequence and also contains all of the appropriate kinase subdomains and conserved residues. All five identical ESTs encoding the mouse protein C terminus code for a C-terminal SSKH motif. As expected, the zebrafish and Drosophila homologues are more distantly related to human PBK than the mouse homologue, but still contain all the kinase domain features. The C-terminal sequences for both of these proteins have not been determined. The amino acid conservation across the four homologues (shaded residues in Fig. 1) is not restricted to kinase subdomains. Even the most distantly related homologue, from Drosophila, shows much higher identity with human PBK (35% over the entire available sequence, 44% over the sequence confirmed by multiple EST sequences) than any other known non-PBK kinase.

**Tissue Distribution of the PBK mRNA.** Unlike hDlg, which is ubiquitously expressed (1), the tissue distribution of PBK mRNA varies. It is most abundant in placenta and is also present in heart muscle and pancreas, and at low levels in skeletal muscle, kidney, liver, and lung (Fig. 2). No signal was detected from adult brain mRNA by Northern blot (Fig. 2) or by PCR amplification from a human adult brain cDNA library (data not shown). Thus, even although PBK and hDlg are both present in many tissues, their overall distribution differs.

**In Vitro Interaction of PBK with hDlg.** Because human PBK has a C-terminal T/SXV motif corresponding to the consensus sequence:  

![Fig. 1. The protein sequences of human PBK and its homologues from other species. Shaded residues show identity or conservation among at least two homologues. Boxed residues are conserved among most known kinases. Asterisks (*) mark residues that have a conserved hydrophobic character, size, or charge in the various kinase subdomains (identified with roman numerals above the sequences). The original clone of PBK encodes residues 185–334 of human PBK in this alignment. The sequence of PBK has been deposited in GenBank (accession no. AF189722).](image)

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![Fig. 2. The PBK mRNA is present in several human tissues and most abundant in placenta. Autoradiograph of a human multiple tissue mRNA blot (CLONTECH) probed by hybridization (26) with radiolabeled 966bp PBK cDNA ORF. The tissue source is indicated above each lane.](image)
quency known to interact with hDlg, we examined whether the two proteins could interact in vitro as well as in the two-hybrid system. PBK bound to a GST-hDlg fusion protein but not to GST alone. Furthermore, the three PDZ domains of hDlg were sufficient for PBK binding, whereas other domains of hDlg did not bind PBK in vitro (Fig. 3A). The binding of PBK’s C-terminal domain specifically required PDZ2 of hDlg, and it did not interact with PDZ1 or PDZ3 (Fig. 3B). The C-terminal motif of PBK was essential for its interaction with hDlg in vitro. Full-length hDlg or its PDZ1–2 domains bound to wild-type PBK but not to a mutant form of PBK lacking its C-terminal 10 residues (Fig. 3C). PBK mutants in which Thr-320 or Val-322 in the C-terminal motif were mutated to Ala-320 or Ala-322, respectively, showed greatly reduced binding to full-length hDlg and to hDlg’s PDZ1–2 domains (Fig. 3C).

Cell Cycle-Dependent Phosphorylation of PBK. Kinase assays with recombinant PBK or with PBK immunoprecipitated from asynchronous HeLa cells resulted in no detectable activity (data not shown). Therefore, we reasoned that PBK might be regulated in a cell cycle-dependent fashion. In fact, we observed that in M-phase HeLa cells, a subset of both PBK and hDlg molecules converted to slow migrating species by SDS-PAGE, whereas there was no change in asynchronous (less than 5% mitotic) cells or in S-phase and G1-phase cells (Fig. 4).

The change in PBK mobility at mitosis was attributable to phosphorylation. Simply omitting phosphatase inhibitors in lysate preparation was sufficient to cause the slower migrating species of PBK to disappear (Fig. 5).

In the case of hDlg, the absence of phosphatase inhibitors in the lysis buffer did not affect the pattern of bands observed on the immunoblot of S-phase or M-phase lysates, but treatment with calf intestinal phosphatase caused the fastest migrating species to shift upward in both S-phase and mitotic cell lysates (Fig. 5). The slower migrating species of hDlg at mitosis was unaffected. This suggests that some isoforms of hDlg are phosphorylated at both S-phase and mitosis. This does not exclude the possibility that other isoforms are specifically phosphorylated at mitosis because calf intestinal phosphatase may not be able to remove all phosphate groups from hDlg.

PBK Is a Mitotically Active Serine/Threonine Kinase. We examined whether PBK that was phosphorylated at mitosis was enzymatically active. We found that a protein band comigrating with PBK became phosphorylated in an immunocomplex assay (Fig. 6). Because this band was absent in immunoprecipitates made by using preimmune serum, we concluded that PBK itself was phosphorylated. This phosphorylation was cell cycle dependent because PBK immunoprecipitated from asynchronous cells, S-phase cells, or G1-phase cells was not phosphorylated (Fig. 6; G1 data not shown). To confirm that the observed activity was caused by PBK and not another kinase contaminant, we produced and isolated recombinant wild-type and mutant (K64 and K65 mutated to A64 and A65) PBK from mitotic insect cells by using a method specifically designed to isolate active mitotic kinases (19, 20). Sf9 cells expressing GST-PBK fusion proteins were treated with 100 nM okadaic acid for 3 h before their lysis. Okadaic acid, a phosphatase inhibitor, induces premature mitosis in insect cells. As expected, PBK fusion proteins isolated from Sf9 cells treated with okadaic acid migrated more slowly than those isolated from nontreated cells (data not shown).

The wild-type and mutant PBK were tested for activity with no substrate (autophosphorylation) and with myelin basic protein or histone as substrates. In all three cases, only wild-type kinase isolated from okadaic acid-treated Sf9 cells was active (Fig. 7). Because no activity was detected with PBK bearing mutations at lysines 64 and 65, the activity observed in these assays can be attributed only to PBK (Fig. 7).

The ability of PBK to phosphorylate myelin basic protein and histone in vitro together with the sequence of its active site implied that it is a Ser/Thr kinase. This expectation was confirmed by phosphoamino acid analysis (Fig. 8): autophosphorylation and histone phosphorylation occurred primarily on threonines, whereas myelin basic protein phosphorylation occurred on both threonines and serines.

PBK Is a Substrate of Cdc2/Cyclin B In Vitro. Finally, we attempted to reproduce the mitotic phosphorylation and activation of PBK by coinfesting Sf9 cells with viruses expressing human cdc2 and human cyclin B. Like PBK isolated from okadaic acid-treated cells, PBK isolated from cells coexpressing cdc2 and cyclin B was active. Because no histone phosphorylation was detected when kinase-deficient PBK is isolated from these cells, the phosphor-
PBK is a serine/threonine kinase. GST-PBK, myelin basic protein (MBP), and histone were phosphorylated in vitro by GST-PBK isolated from okadaic acid-treated Sf9 cells. Autoradiographs of the cellulose plates (EM Science) after separating the proteins on 10% tricine gel and transferred to nitrocellulose. The tracings outlines show where the nonradiolabeled phosphoamino acid standards migrated (phosphoserine, S, phosphothreonine, T, and phosphotyrosine, Y).

Discussion

Cloning of PBK and Its Interaction with PDZ Domain-Containing Proteins. Our results show that a 35-kDa PDZ-binding protein cloned in a two-hybrid screen is a cell cycle-regulated Ser/Thr kinase. In vitro assays confirmed the potential associations and sites of interaction between PBK and hDlg, although in vivo association could not be confirmed by immunoprecipitation (data not shown). Failure of interacting proteins to coimmunoprecipitate is not unprecedented (28) (human CASK, a protein containing PDZ domains, and syndecan-2, a protein with a T/SXV motif, also interact in vitro and colocalize but do not coimmunoprecipitate).

Because our anti-PBK antibodies gave inconsistent staining results in cells, whether hDlg and PBK colocalize remains to be investigated.

Cell Cycle-Dependent Phosphorylation and Activation. What is most interesting about PBK is that it is phosphorylated in a cell cycle-dependent manner at mitosis, and that this phosphorylation is required for its activation. Furthermore, it is most abundant in placenta, a highly proliferative tissue, and absent, or at very low levels, in normal adult brain tissue where there is virtually no cellular proliferation. Interestingly, one human PBK EST sequence (GenBank no. AI366737) is from a brain anaplastic oligodendroglioma, a rapidly proliferating brain tumor.
These results suggest that PBK may have a role in the regulation of cellular proliferation and progression of the cell cycle. We have shown that only PBK immunoprecipitated from mitotic HeLa cells is phosphorylated in an immunocomplex kinase assay. These data, combined with the fact that recombinant PBK is activated during the premature mitosis induced by treating insect cells with okadaic acid, lead us to believe that the activity observed in the immunocomplex is caused by the auto-phosphorylation of active PBK.

Furthermore, PBK is active when it is isolated from Sf9 cells coexpressing human cdc2/cyclin B. Yet in vitro cdc2/cyclin B-induced phosphorylation alone was not sufficient for activation of PBK. These results suggest that, whereas cdc2/cyclin B phosphorylation of PBK is important for its activation, PBK requires further posttranslational modifications or a cofactor to be activated.

PBK has a characteristic cdc2/cyclin B phosphorylation site (S/T-P-X-K/R) (29) at its N terminus, and this site is conserved across species, implying that it is important for the activity of the protein. Phosphorylation at this site could be required for a conformational change or a relocalization that allows PBK to be acted on by a second kinase in its activation loop.

Most cell cycle research focuses on the timing of cell cycle regulator activation, but such activation frequently depends on localization of the regulator. For example, the activity of Cdc14, a protein phosphatase that promotes the breakdown of cyclin, is regulated by activation, but such activation frequently depends on its localization in the cell. Phosphorylation at this site could be required for a conformational change or a relocalization that allows PBK to be activated during the premature mitosis induced by treating insect cells with okadaic acid, lead us to believe that the activity observed in the immunocomplex is caused by the auto-phosphorylation of active PBK.

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