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Perspectives in PDK1 evolution

Insights from photosynthetic and non-photosynthetic organisms

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Protein kinases belonging to the AGC group modulate many diverse cellular processes in all eukaryotes. One important way to regulate AGC kinases is through phosphorylation by the upstream kinase PDK1. PDK1 localization and activity usually depend on interactions with phospholipids, which are mediated by a conserved lipid-binding pleckstrin homology (PH) domain. We recently analyzed putative PDK1 sequences from 17 photosynthetic organisms, finding that PDK1s from vascular and nonvascular species seem to be distinguished by the presence or absence of a PH domain, respectively. The only other reported PDK1 lacking a PH domain is from yeast (*Saccharomyces cerevisiae*). These observations raise questions about how plant PDK1s and their lipid-binding capabilities have evolved in relation to other eukaryotes, and what this means for PDK1 function. Here we use 100 PDK1 sequences from diverse organisms to discuss possible evolutionary aspects of plant PDK1 structure and lipid binding.

Due to its phosphorylation of numerous substrate proteins, the eukaryotic 3-phosphoinositide dependent protein kinase-1 (PDK1) is a central coordinator of cellular metabolism, growth and death.[1,2] PDK1 and its substrates belong to the AGC group of protein kinases, which has been identified in five of the six hypothesized main groups of eukaryotes:[3-9] Amoebozoa, Opisthokonta, Excavata, Archaeplastida and the Rhizaria-Alveolates-Stramenopiles (RAS or SAR) group.[10-12] The majority of protein kinases, including many AGC kinases, are not conserved in diverse organisms, supporting the idea that kinases have undergone many lineage-specific expansions and reductions. In contrast, PDK1 is believed to be one of just 40–60 ancient and highly conserved kinases that probably predate the divergence of the eukaryotic groups and have been universally maintained since that time.[3,5,9] This evidence argues that the collective responsibilities of PDK1 and its substrates must be incredibly important for the survival of eukaryotic cells.

Though PDK1 is now recognized to be a fundamental regulator of many essential cellular processes, it was not identified until the late 1990s. The two groups first to describe PDK1[13,14] were specifically searching for an enzyme capable of phosphorylating the activation loop of protein kinase B (PKB, a.k.a. Akt), an AGC kinase that is activated in a phospholipid-dependent manner through the recognition of second messengers like insulin and growth factors.[15] PDK1's roles apart from PKB became an important focus of research efforts when it became apparent that a host of substrates contained a conserved PDK1 phosphorylation motif.[1,16-18]

Cloning of the human and fruit fly PDK1 genes[19] led to an initial understanding of how PDK1 may interact with substrates such as PKB. Both proteins possess a pleckstrin homology (PH) domain that enables binding to several phospholipids, particularly PtdIns(3,4,5)P_3 and facilitates plasma membrane colocalization for PDK1 phosphorylation of PKB. However, most PDK1 substrates lack PH domains and are commonly localized in the cytoplasm,[1] and thus require a mechanism to dock with PDK1 in order to be phosphorylated. This is typically achieved by interaction between a complementary pair of protein modules: the short PDK1-interacting fragment (PIF) found at the C-terminus of PDK1 substrates and its corresponding PIF-binding pocket within the N-terminal lobe of the PDK1 kinase domain.[20] This interaction is now a well-established mechanism for stimulating PDK1 phosphorylation on most substrates.[21]

Soon after the initial identification of PDK1, a flurry of reports described PDK1 homologues in mouse,[22] nematode,[23] budding yeast,[24] fission yeast,[25] and the flowering plants *Arabidopsis thaliana* and rice.[26] Together these early reports established that PDK1 has been maintained in organisms with dramatically different life strategies, and that sequence similarity within the catalytic domain could be used to accurately predict and identify PDK1 homologues. Of these proteins, only the budding yeast PDK1 homologue lacks a readily identifiable PH domain, suggesting that lipid regulation of PDK1 could also be highly conserved across most eukaryotes. Therefore, we were somewhat surprised to find that unlike previously reported plant PDK1s, the PDK1 homologue of the moss *Physcomitrella patens* lacks a PH domain.[27] This also appears to be true in putative PDK1s from several other species of Archaeplastida (consisting of land plants plus red, green, glaucophyte and charophyte algae).[27] To better understand the evolution of PDK1 structure and function, we have investigated putative PDK1 homologues from highly divergent eukaryotes, attempted to categorize them based on

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protein domain organization, and used this information to speculate about plant PDK1 function.

Analysis of PDK1 Sequences from Diverse Eukaryotes

Putative PDK1 protein sequences from 100 different species of eukaryotes, including 35 photosynthetic and 65 non-photosynthetic organisms, were obtained from BLAST searches of NCBI GenBank and genome databases (**Fig. S1**). Although some organisms possess multiple putative PDK1s, only the top BLAST hit from each species was selected for further analysis. We did not include an exhaustive list of putative PDK1s from each species for several reasons: (1) space considerations; (2) genome analysis tools, particularly for the most recently sequenced genomes, limit our ability to confidently assert the number of the putative PDK1s present in a given organism; (3) though abundant evidence suggests that every eukaryote is likely to have at least 1 PDK1,[3-5,7-9] not all organisms have more than 1 PDK1. Thus, we chose the simplistic strategy of analyzing only the top BLAST hit from each species, as we believe the highest-scoring sequence is most likely to represent a true PDK1 homologue. In our opinion, the true test of a PDK1 is its ability to phosphorylate AGC kinase substrates at the conserved activation loop site. This test must be experimentally performed with each putative PDK1, so we are hesitant to attempt to differentiate high-scoring BLAST hits from each other solely based on sequence analysis. Our approach is a conservative one, and it means we cannot assess instances of possible PDK1 functional divergence in organisms with multiple putative PDK1s, a topic that should be investigated in the future.

The phylogenetic analysis shown in **Figure 1** was produced as previously described in reference 27. The PIF-binding pocket regions of all proteins in this alignment were visually inspected for the presence or absence of nine amino acids demonstrated to participate in substrate interaction.[28] We found that six of these amino acids (corresponding to F82, K115, R131, F149, Q150 and L155 of human PDK1) are conserved in almost all putative PDK1 sequences, whereas 3 amino acids (corresponding to K76, I119 and F147 of human PDK1) are not well conserved (**Fig. S2**). Eighty-four of the putative PDK1 sequences have a conserved amino acid at all 6 highly-conserved PIF-binding pocket positions, while 16 putative PDK1 sequences contain a non-conserved amino acid at one or more positions. In addition, three putative PDK1 sequences were identified that might be incomplete at the N-terminus: *Trichoplax adhaerens* PDK1 does not start with methionine, and *Monosiga brevicollis* and Micromonas sp PDK1s appear to lack at least one amino acid in the N-terminal part of the PIF-binding pocket (**Fig. S2**).

After phylogenetic analysis was performed, sequence similarity within the most highly conserved regions of putative PDK1s, the catalytic domain and the PIF-binding pocket, was further assessed to determine whether either of these regions may have diverged. First, the NCBI Conserved Domain Database (CDD)[29] was used to search for a PDK1-like kinase domain (CDD domain cd05581) within each sequence as a measure of overall sequence similarity within the catalytic domain. Second, sequences were classified by the presence or absence of the six highly conserved PIF-binding pocket residues identified in the sequence alignment (**Fig. S2**). If a putative PDK1 lacked (1) a PDK1-like kinase domain or (2) greater than 2 of the highly conserved PIF-binding pocket residues, that sequence was classified as a more divergent PDK1. The kinase domain of the *Plasmodium vivax* putative PDK1 lacked the first feature, whereas the PIF-binding pocket regions of the *Ciona intestinalis*, *Cryptococcus neoformans*, *Cyanidioschyzon merolae*, *Mucor circinelloides*, *Rhizopus oryzae* and *Perkinsus marinus* putative PDK1s lacked the second feature. Thus, all seven of these sequences are shown in **Figure 1** as being more divergent than the other putative PDK1s. Most of the putative PDK1s investigated here share a good deal of similarity within the catalytic domain and PIF-binding pocket, but the lack of a particular conserved sequence within a putative PDK1 does not necessarily mean it lacks PDK1 function. Again, we believe this can best be determined by the ability to phosphorylate the AGC kinase activation loop.

The presence or absence of a conserved lipid-binding domain was also investigated by CDD search. Two potential lipid-binding domains, the PDK1-like PH domain (CDD domain cd01262) and the PH-like domain (CDD superfamily cl00273), were identified in 47 and 14 putative PDK1s, respectively (**Fig. 1**). Interestingly, an FYVE lipid-binding domain (CDD domain cd00065) was identified in putative PDK1s from both *Leishmania major* and *Trypanosoma brucei*, but not in any other sequences analyzed (**Fig. 1**). Finally, a region with very weak similarity (E-value ~ 10^{-3}) to the PH-like domain was identified in the putative PDK1s of four fungal species: *Malassezia globosa*, *Puccinia graminis*, *Saitoella complicata* and *Ustilago maydis* (**Fig. 1**). Because the similarity of this region to other PH and PH-like domains is so low, these proteins were classified as lacking a readily identifiable lipid-binding domain, for a total of 37 putative PDK1s without a conserved lipid-binding domain and 63 putative PDK1s possessing a PH, PH-like or FYVE domain (**Fig. 1**). It is important to note that the lipid binding capability of each protein should be experimentally investigated, as not all PH domains mediate lipid interactions.[30] In contrast, the *S. cerevisiae* PDK1 homologues Pkh1 and Pkh2 do not possess readily identifiable lipid-binding domains but are nevertheless regulated by sphingoid bases.[31] Because PH domain sequences are less conserved than their folded structure[32] they might not be identified by sequence similarity. Thus, a PH domain does not necessitate phospholipid binding, and lack of a detectable PH domain may not imply lack of regulation by lipids.

Lipid Binding by PDK1 Homologues

The most well established lipid regulators of mammalian PDK1 are PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, which are produced by class I phosphatidylinositol-3-kinases (PI3Ks) in response to growth factor or insulin perception in order to modulate the cellular environment through downstream signaling pathways like PDK1-PKB.[15] Both PDK1 and PKB are able to bind PtdIns(3,4,5)P_3 through high affinity interactions of their PH domains. Structural and

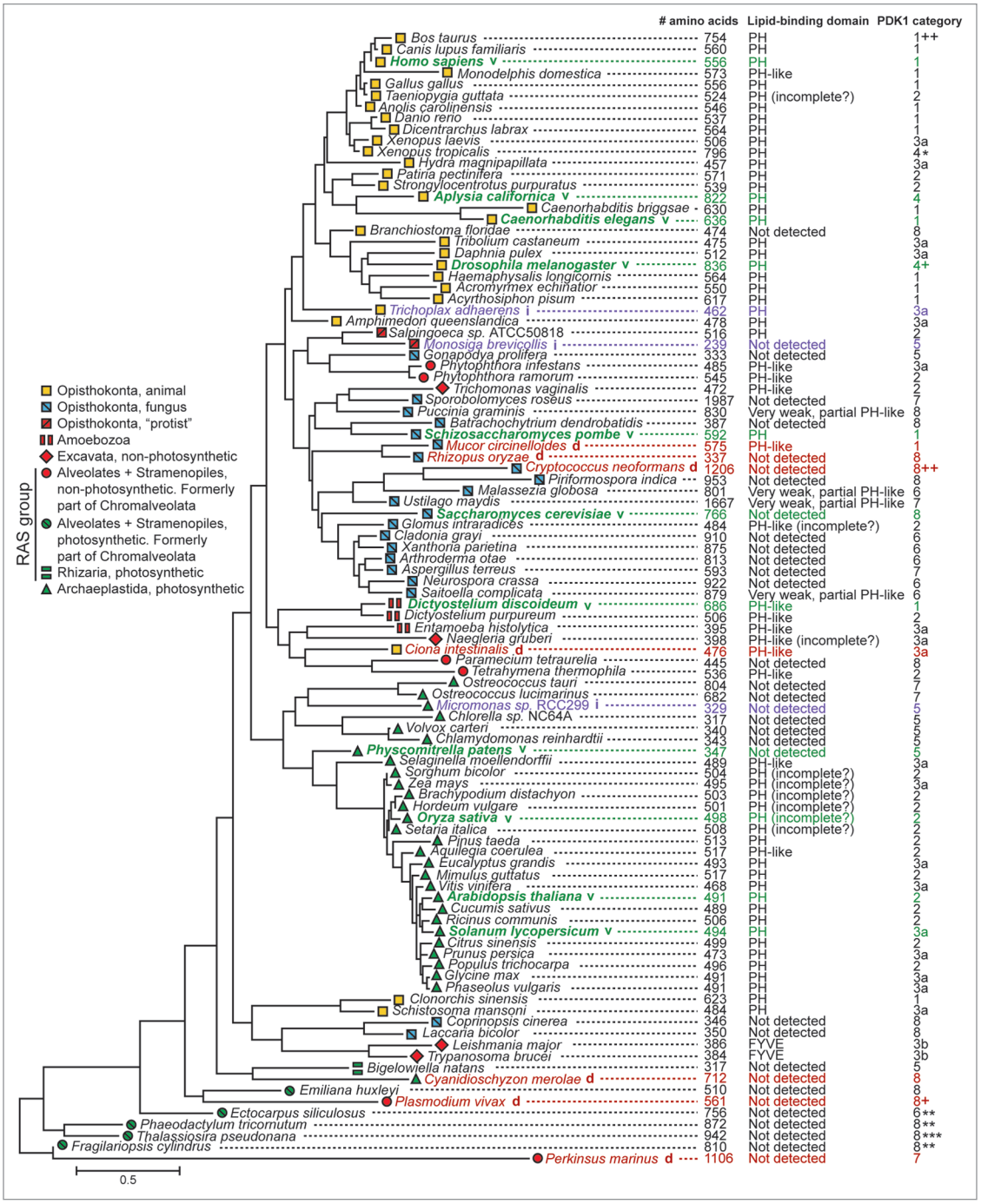

Figure 1. Phylogenetic relationship of putative PDK1 proteins from 100 different eukaryotes. A maximum-likelihood phylogenetic tree was created from MUSCLE-aligned sequences using MEGA version 5,[53] and the resulting phylogram was labeled in Adobe Illustrator. NCBI Conserved Domain Database (CDD),[29] was used to search for protein domains, including the PDK1-like kinase domain (CDD domain cd05581), the PDK1-like PH domain (CDD domain cd01262), the PH-like domain (CDD superfamily cl00273), and the FYVE domain (CDD domain cd00065). A "v" next to the species name indicates species with experimentally verified PDK1 homologues; "d" indicates species with more divergent PDK1 homologues; "i" indicates species whose PDK1 sequences may be incomplete. A+next to the PDK1 category number indicates an insertion within the kinase domain of <150 amino acids; ++indicates an insertion of >150 amino acids; *indicates the presence of a putative chromo domain (CDD domain cd00024) outside the kinase domain; ** indicates a putative tetratricopeptide repeat (TPR) domain (CDD domain cd00189); ***indicates a putative TPR-like domain (CDD superfamily cl02429).

functional studies on lipid-free and PtdIns(3,4,5)P_3-bound PDK1 PH domains have identified the residues that contribute to lipid binding and shown that, unlike for PKB, lipid binding does not induce a conformational change in the PDK1 PH domain.[33,34] Though lipid binding is now thought to facilitate PDK1 and PKB colocalization at the plasma membrane,[1,21] it has

been difficult to conclusively determine whether PtdIns(3,4,5)P_3 alters the localization of PDK1. A recent report has found that the abundant and constitutively present lipid phosphatidylserine (PS) recruits mammalian PDK1 to the plasma membrane via a small pocket within the PH domain that specifically mediates PS binding.[35] This result provides one possible mechanism for the earlier finding that a substantial fraction of PDK1 is membrane-bound even when PtdIns(3,4,5)P_3 production has not been stimulated.[36] In the future it will be interesting to test whether plant PDK1s are also recruited to membranes through interactions with abundant lipids.

In contrast to mammalian PDK1, *A. thaliana* PDK1 is capable of somewhat promiscuous lipid binding in vitro, displaying strong interactions with PtdIns3P, PtdIns(3,4)P_2, PtdIns(4,5)P_2, PtdIns(3,4,5)P_3 and phosphatidic acid (PA).[26] However, it is unlikely that either PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 play a role in plant PDK1 regulation for two reasons. First, plants lack a class I PI3K and thus cannot produce PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 as mammals do; second, neither of these lipids has been reliably documented in plant cells.[37] Plants do generate PtdIns3P, PtdIns(4,5)P_2 and PA,[37] so these lipids might participate in PDK1 regulation. Several reports have shown that PDK1 may act as a downstream effector of PA during a variety of circumstances, including both beneficial[38] and harmful[39] biotic interactions, and in the regulation of root hair growth[40] and auxin transport.[41] Unfortunately, few experiments have tested whether PtIns3P or PtdIns(4,5)P_2 contribute to plant PDK1 function. One study discovered that both PtdIns(4,5)P_2 and PA increase *A. thaliana* PDK1 activity on a peptide containing a PIF motif almost 2 fold, whereas PtIns3P does not.[40] Further understanding of how PDK1 PH domains mediate distinctive lipid interactions in different species will give valuable insight into PDK1 function in plants.

In agreement with a previous report in reference 26, our qualitative in vitro lipid-binding assays found that PDK1s from both *A. thaliana* and tomato (*Solanum lycopersicum*) strongly interact with phosphorylated phosphoinositides including PtdIns3P, PtdIns(4,5)P_2 and PA.[27] However, the *P. patens* PDK1 lacks a detectable lipid-binding domain and accordingly does not strongly bind either phospholipids or sphingolipids.[27] A search for PDK1s from divergent photosynthetic and non-photosynthetic organisms recovered 37 putative PDK1 proteins lacking any strongly identified lipid interaction domains (**Figs. 1 and 2**). This observation raises the possibility that lipid regulation of PDK1 is not nearly as widespread as might be expected based on published PDK1 sequences. A lack of lipid regulation seems most likely in the seven putative PDK1s we found whose protein domain structure resembles the *P. patens* PDK1, with fewer than ~50 amino acids on each side of the kinase domain (category 5 in **Fig. 2**). The majority of putative PDK1s without a known lipid-binding domain have more than ~50 amino acids on one or both sides of the kinase domain (categories 6–8 in **Fig. 2**). A more intriguing possibility for some of these proteins, like the *S. cerevisiae* PDK1 homologues,[24,31] is that cryptic lipid- or protein-interaction domains may exist for regulating PDK1 activity and/or localization. Sequences outside the kinase domain could also comprise additional auto-regulatory modules, as the N-terminal region of protein kinase C does.[1]

From the proteins mentioned in this review, a few general trends seem apparent regarding PDK1 lipid-binding capability. First, the majority of putative PDK1s with a PH or PH-like domain are found in animals and vascular plants (**Fig. 1**). Only 1 putative PDK1 lacking a detectable lipid-binding domain (from the lancelet *Branchiostoma floridae*) was identified from 50 species of animals and vascular plants (**Fig. 1**). This gives us some confidence that lipid regulation of PDK1 may be a characteristic feature of organisms in both clades. Second, putative PDK1s lacking any detectable lipid-binding domain most often belong to fungi and non-vascular members of the Archaeplastida (**Fig. 1**). No putative PDK1s with an obvious lipid-binding domain were identified from 8 nonvascular Archaeplastidal species, again suggesting that this may be a common situation, though more species must be analyzed to verify this trend. Only 3 of 21 fungal species possess a readily identifiable PH or PH-like domain within their putative PDK1: *Mucor circinelloides* (belonging to Mucoromycotina), *Glomus intraradices* (belonging to Glomeromycota), and the relatively unrelated yeast *Schizosaccharomyces pombe* (an ascomycete belonging to Taphrinomycotina).[42] Due to the small number of species discussed here we cannot confidently assert that fungal PDK1s are more likely to lack a PH domain, though this does seem to be the trend to date.

Because very few genomes are available from representatives of the RAS group (6 photosynthetic + 6 non-photosynthetic), Excavata (4 non-photosynthetic) and Amoebozoa (3 non-photosynthetic), trends regarding the lipid-binding abilities of these PDK1s are more difficult to ascertain, and less likely to remain true in the future. All seven species of Amoebozoa and Excavata have putative lipid-binding domains in their PDK1s. Two of the mitochondriate Excavates (*L. major* and *T. brucei*) have an FYVE domain at the C-terminus of the putative PDK1, possibly reflecting a PDK1 modification that occurred only within the ancestor of these organisms, since no other PDK1s investigated have a similar lipid-binding domain (**Fig. 1**). Interestingly, a draft kinome for *L. major* identified three putative PDK1 homologues, one with a FYVE domain (used in our analysis) and two lacking any obvious lipid-binding domain.[5] This is discussed further below.

Lipid-binding sequences are present within several unexpected RAS group PDK1s. For example, reasonably close relatives of diatoms and brown algae, the oomycetes *Phytophthora infestans* and *Phytophthora ramorum*, have putative PDK1s that appear more closely related to animal PDK1s and have PH-like domains. A second example comes from the ciliates *Paramecium tetraurelia* and *Tetrahymena thermophila*, only one of which seems to have a PH-like domain. PDK1s from three diatoms (*Phaeodactylum tricornutum*, *Thalassiosira pseudonana* and *Fragilariopsis cylindrus*) and one brown alga (*Ectocarpus siliculosus*) lack a PH-like domain but have putative tetratricopeptide repeat (TPR)-like domains near the N-terminus, again possibly reflecting a specific PDK1 modification in the ancestor of these organisms. In summary, PDK1s from all six photosynthetic RAS species investigated lack lipid-binding domains, whereas some PDK1s from

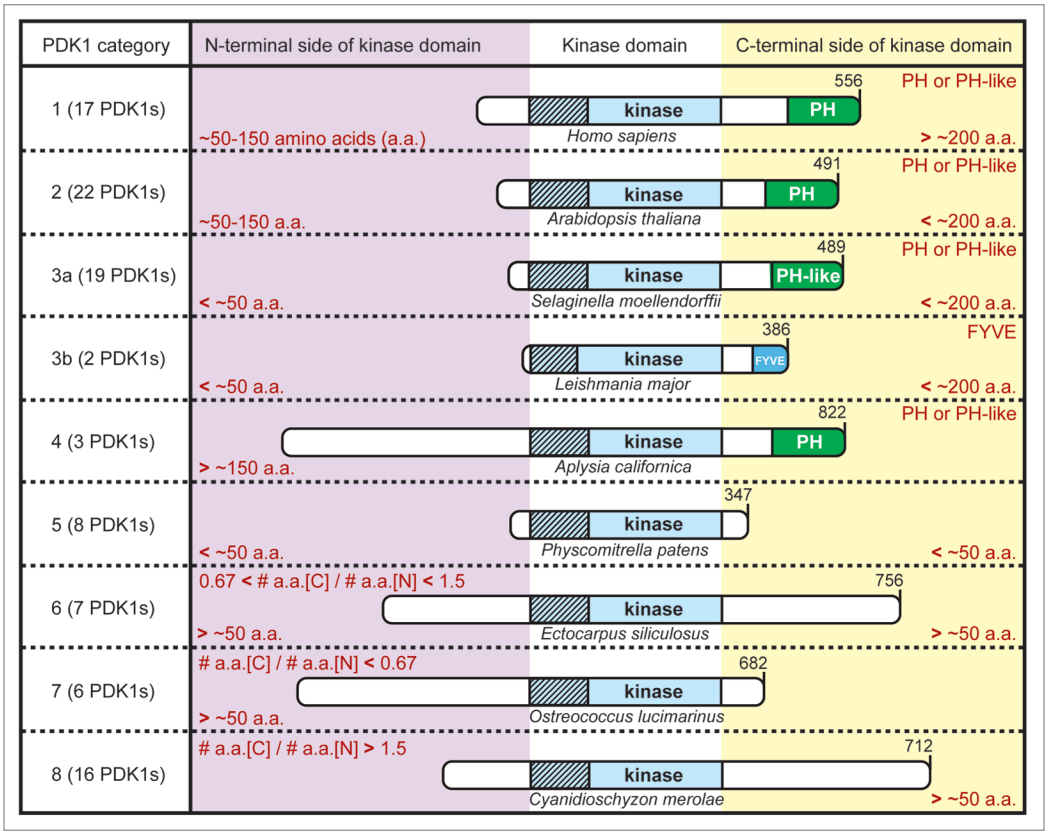

Figure 2. Categorization of PDK1s by protein domain structure. For ease of visualization, PDK1 sequences from Figure 1 were divided into categories using three criteria: first, the presence or absence of a lipid-binding domain identified by CDD; second, the number of amino acids on each side of the kinase domain; third, the ratio of amino acids on the C-terminal side of the kinase domain (# a.a.[C]) to amino acids on the N-terminal side of the kinase domain (# a.a.[N]). The left column shows the number of each category (1–8), indicating in parentheses the number of PDK1 sequences from Figure 1 belonging to that category. The right column contains diagrams of one representative PDK1 from each category, including the locations of the kinase domain, PIF-binding pocket (box with diagonal lines), and putative lipid-binding domain (if any) within each protein. Distinguishing characteristics of each category are found in the corners of each section of the right column: the upper-right corner shows the identified lipid-binding domain (if any); the lower-right corner shows the number of amino acids on the C-terminal side of the kinase domain; the lower-left corner shows the number of amino acids on the N-terminal side of the kinase domain; the upper-left corner shows the ratio of # a.a.[C] to # a.a.[N]. If no text is present in a given corner, then that characteristic was not used to distinguish PDK1s found in that category.

non-photosynthetic species have a lipid-binding domain and others do not.

More PDK1 sequences, particularly from the taxa underrepresented in **Figure 1**, must be identified before further investigating the predicted lipid-binding trends observed here and making sense of groups without a clear trend. It will also be interesting to test lipid binding specificity of diverse PDK1s and discover whether different clades regulate PDK1 via different lipid interactions, as animals, vascular plants, and at least some fungi seem to do.

PDK1 Evolution

Perhaps because the majority of experimentally verified PDK1 homologues[19,22-27,43-46] are relatively similar to each other in protein domain organization and broad functional conservation, few research reports discuss PDK1 in an evolutionary context. After finding unexpected diversity in nonvascular plant and algae PDK1s, we hoped to learn more about the features of distantly related PDK1 and gain some insight into how these PDK1s could have arisen. However, it is not fully resolved as to the phylogenetic grouping of eukaryotic speicies or where the root of the eukaryotic tree of life should be placed.[47] While several eukaryotic groups are well supported as monophyletic groups (Opisthokonta and Amoebozoa),[12] others are controversial (Archaeplastida),[11] and the placement of some species within particular groups has changed in recent years.[10-12,47] Thus, the ancestral origins of current PDK1 sequences can be difficult to distinguish. Species thought to be early-diverging representatives of each eukaryotic group should be aggressively investigated and sequenced to illuminate our understanding of possible ancestral species and the evolution of PDK1 sequences.

One aspect of PDK1 function that seems to differ between organisms is whether PDK1 is an essential gene. In animals and fungi, PDK1 null alleles have thus far proven to be lethal.[23-25,48,49] In contrast, loss of PDK1 in Amoebozoa[45] and plants[27,38] produces viable, though compromised organisms. In both *A. thaliana* and *P. patens*, all apparent PDK1

homologues (2 and 1 respectively) were knocked out[27,38] without lethality. Though we cannot exclude the possibility that highly divergent PDK1 sequences are able to perform the same functions as the PDK1s that were knocked out, this possibility seems unlikely to us. Additionally, presence or absence of a PH domian does not appear to correlate with whether any particular PDK1 is essential or not. Regardless, it is puzzling that a gene retained since the emergence of the earliest eukaryotes is at least sometimes non-essential. Despite the fact that distantly related PDK1s can perform the same basic cellular functions,[27] perhaps there are subtle differences in downstream components of PDK1 pathways, such as substrate AGC kinases, that make the difference between organism survival and death in the absence of PDK1. In the future perhaps PDK1 can be knocked out in genetically tractable organisms from different clades to better understand the circumstances that enable PDK1 to become a non-essential gene.

The only evident trends in protein size and domain structure within the 100 putative PDK1s we investigated are those discussed in the previous section regarding presence or absence of a PH-like domain (**Figs. 1 and 2**). Both the smallest PDK1s (with fewer than ~350 amino acids) and the largest (with more than 800 amino acids) are present in diverse groups of organisms, as are "typical" PDK1s of intermediate size with ~500–600 amino acids with a C-terminal PH domain (**Fig. 1**). This diversity complicates attempts to form a picture of PDK1 evolution, but might reflect rapid evolution in some lineages due to selective pressure, perhaps resulting in an unexpectedly wide variety of PDK1 activities. The very large PDK1s identified here do not contain conserved non-catalytic sequences identifiable by CDD search (**Fig. 1**), so it is difficult to discuss the possible origins and functions of the large regions of sequence outside the kinase domain. One possible approach to study the evolutionary and functional implications of these regions is to perform deletion and "domain"-swapping experiments, followed by biochemical analysis of lipid binding and substrate phosphorylation. Alternatively, it might be possible to perform a more sophisticated sequence analysis and arrive at possible functions of these large regions of sequence. Consquently, for the remainder of this section we focus on the possible contributions of PH-like domains to PDK1 evolution.

Animals and fungi are much closer relatives than animals and vascular plants,[12] so it seems counterintuitive that animal and vascular plant PDK1s typically possess obvious PH-like domains, whereas only three of 21 fungal species investigated (*S. pombe*, *M. circinelloides* and *G. intraradices*) do. Did PDK1 bind lipids in the ancestor of animals and fungi? One possibility is that PDK1s with PH domains arose separately in animals and fungi through convergent evolution. This explanation is reasonable, as PH domain promiscuity is well established in eukaryotes[50] and protein domain fusion is thought to occur more often than fission.[51] However, the three fungal PDK1s with PH-like domains are not from close relatives in a monophyletic group,[42] so these PDK1 PH domains must have been either added on several independent occasions or lost from multiple intervening lineages. Furthermore, four additional fungi (*M. globosa*, *P. graminis*, *S. complicata* and *U. maydis*) belonging to several different clades[42] have putative PDK1s with very weak PH-like regions on the C-terminal side of the kinase domain. We speculate that these regions of weak similarity to PH domains might be due to incomplete nonfunctionalization; at some point the PDK1s had more recognizable PH domains, but a lack of selective pressure neither maintained nor got rid of them completely. For these reasons we suggest that PDK1 in the Opisthokont ancestor may have had a PH domain that has been retained in almost all animals, but is in various stages of being lost in many lineages of fungi for unknown reasons (**Figs. 1 and 2**). Obtaining PDK1 sequences from more early-diverging Opisthokont species and performing lipid-binding tests with these PDK1s could be the first steps toward addressing these possibilities.

As mentioned previously, all vascular plants we investigated have putative PDK1s with a PH-like domain and are more similar to each other than PDK1s from *P. patens* and the algae within Archaeplastida, which is not particularly surprising since vascular plants are relatively recent innovations.[52] Similar to the fungi, putative PDK1s from nonvascular members of Archaeplastida vary substantially in size, with proteins ranging from ~300–800 amino acids (**Figs. 1 and 2**). However, none of these sequences contain a detectable lipid-binding domain. Before asserting that the trend we observe here is a general one, it will be particularly important to obtain PDK1 sequences from glaucophyte and charophyte algae, as well as more species of red algae and nonvascular land plants, such as liverworts and hornworts. If Archaeplastida are indeed monophyletic[12] and it remains true that the only representatives of Archaeplastida with PH-containing PDK1s are vascular plants, one possible explanation is that PDK1 in the Archaeplastidal ancestor lacked a PH domain, and then PDK1 in the ancestor of vascular plants acquired one from another gene.[50] This explanation seems less plausible if in the future PDK1s with a PH domain, or with regions of weak similarity to a PH-like domain (like those in several fungi), are identified in many distantly related species of algae. In any case, we find it intriguing that animal and vascular plant PDK1s are so similar in protein size, domain organization and lipid binding capabilities given their distant evolutionary relationship and the diversity of PDK1 sequences found in closer relatives of both clades. We hope in the future it will be possible to assess whether this remarkable similarity arose through convergent evolution due to selective pressure to efficiently regulate PDK1 or through some other mechanism, for example by many independent events of PH domain loss or nonfunctionalization.

Speculating about the nature of ancestral PDK1 sequences becomes increasingly difficult with more distantly related species. No groups of organisms (Amoebozoa, Opisthokonta, Excavata, Archaeplastida or RAS) have PDK1s that universally lack a lipid-binding domain; PDK1s from Amoebozoa and Excavata all have a lipid-binding domain, but since only seven representatives from these groups were investigated, this trend may not remain. Recently the kinomes of three Excavates were analyzed: two amitochondriate species (the Diplomonad *Giardia lamblia* and the Parabasalid *Trichomonas vaginalis*) and one mitochondriate species (the Euglenozoan *Leishmania*

major).[5] A single putative PDK1 was found in *G. lamblia*; it is extremely divergent from PDK1s discussed here and was not used in our analysis because its annotation is ambiguous[5] (**Fig. S1**). In contrast, draft kinomes from *T. vaginalis* and *L. major* revealed five and three putative PDK1s respectively, and their sequences vary widely.[5] A CDD search of all eight *L. major* and *T. vaginalis* putative PDK1s detected an FYVE domain only in the putative *L. major* PDK1 used in our analysis and a PH-like domain only in the putative *T. vaginalis* PDK1 used in our analysis (data not shown; see ref. 5 for putative PDK1 sequences from each kinome). Of course, it remains to be seen how many of these putative PDK1s are true homologues. Nevertheless, we bring our discussion to a close with a highly speculative thought about the origin of PDK1 diversity. If the kinomes of *L. major* and *T. vaginalis* do indeed contain several highly divergent PDK1 sequences, this suggests it is not beyond the realm of possibility for the ancestor of all eukaryotes to have had more than one PDK1-like gene in its genome. Ancient eukaryotes that were able to choose from several diverse PDK1-like proteins might have had more functional and regulatory possibilities, and thus a greater chance of surviving in different environments. It would be intriguing if the diversity of eukaryotic PDK1s could be at least partially explained by many instances of gene duplication and loss. In that case, it might be possible to find species whose genomes still contain multiple PDK1s with widely varying sizes and lipid-binding capabilities. Perhaps the *L. major* and *T. vaginalis* kinomes are just two examples of a phenomenon that was relatively common in the distant past, and could still be a feature of kinomes in some extant lineages.

Conclusions

The 100 putative PDK1s we have discussed here are much more varied than one might expect to find based on the small number of experimentally verified PDK1s, many of which are quite similar to each other. The diversity of putative PDK1s suggests that despite having a relatively conserved catalytic domain, PDK1 has nevertheless taken a number of different evolutionary paths since it first appeared, presumably in the ancestor of eukaryotes. The possible reasons for, and implications of, differences in PDK1 protein structure are still unknown, and in the future it will be interesting to test the degree of functional conservation amongst the most divergent PDK1 sequences. Our analysis suggests that at least some PDK1 proteins may not be regulated by lipid interactions. If this is the case, a future goal should be to elucidate novel PDK1 regulatory mechanisms. Perhaps unidentified protein-interaction domains, other functional domains, or tight control of PDK1 substrate availability have facilitated PDK1 diversification in many species. Finally, once eukaryotic evolutionary relationships have been determined with more certainty, PDK1 sequences from organisms close to major divergence points should be investigated to uncover more clues about the nature of ancestral PDK1s in each eukaryote group.

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Note

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www.landesbioscience.com/journals/psb/article/20038/

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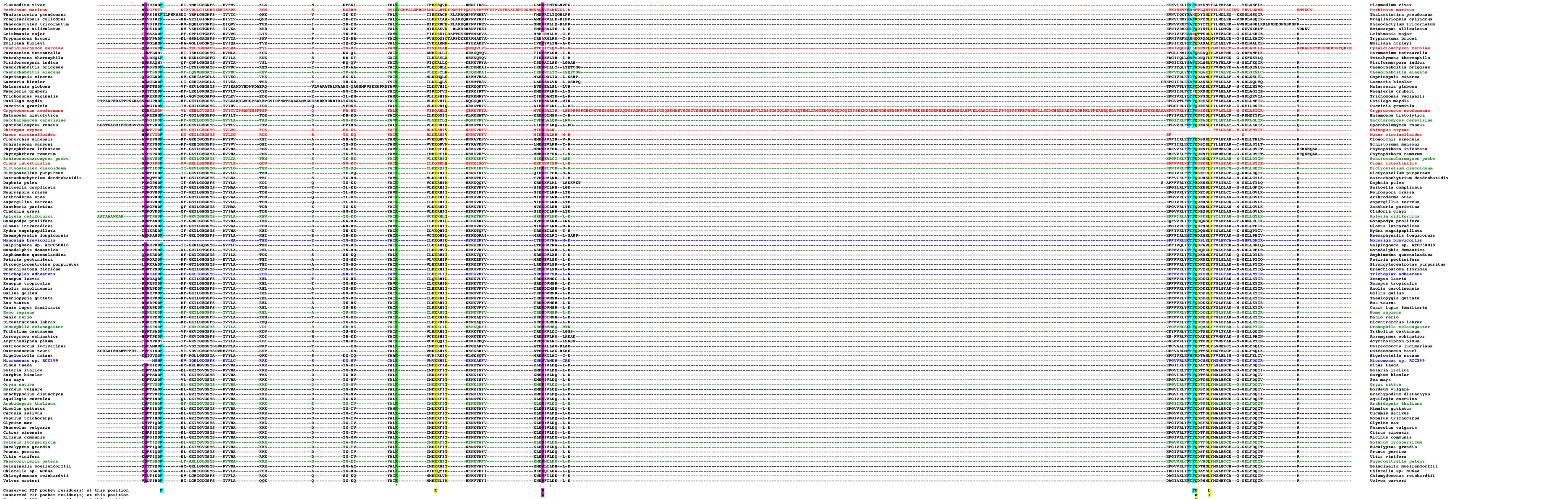

Species in green text = experimentally-confirmed PDK1
Species in red text = more divergent PDK1 (≥3/6 of the highly conserved PIF-binding pocket residues are either not conserved or not present)
Species in blue text = possibly incomplete PDK1

Purple highlight = Binds phosphate in PIF. Corresponds to K76 and R131 of human PDK1.
Blue highlight = Binds last phenylalanine in PIF. Corresponds to F82, F147, and F149 of human PDK1.
Yellow highlight = Binds first two phenylalanines in PIF. Corresponds to K115, I119, Q150, and L155 of human PDK1.
Green highlight = Conserved lysine required for catalytic activity in all kinases (not considered a conserved PIF-binding pocket residue here)

16/100 sequences do not have 6/6 of the highly conserved PIF-binding pocket residues:
P. vivax (4/6 conserved), P. marinus (1/6 conserved), T. pseudonana (5/6 conserved), L. major (4/6 conserved), T. brucei (4/6 conserved),
C. merolae (3/6 conserved), P. tetraurelia (5/6 conserved), T. thermophila (5/6 conserved), N. gruberi (5/6 conserved), C. neoformans (3/6 conserved),
R. oryzae (3/6 conserved), M. circinelloides (3/6 conserved), C. intestinalis (3/6 conserved), M. brevicollis (5/6 conserved),
A. pisum (5/6 conserved), B. natans (5/6 conserved).

Supplemental Figure 2. Alignment of the PIF-binding pocket region of all 100 putative PDK1s used in the Figure 1 phylogeny. All 9 PIF-binding pocket residues from Frodin et al, 2002 (EMBO J 21: 5396–5407) are highlighted. Purple highlight indicates this amino acid binds the phosphate in PIF, and corresponds to K76 and R131 of human PDK1. Blue highlight indicates this amino acid binds the last phenylalanine in PIF, and corresponds to F82, F147, and F149 of human PDK1. Yellow highlight indicates this amino acid binds the first two phenylalanines in PIF, and corresponds to K115, I119, Q150, and L155 of human PDK1. Finally, green highlight indicates the conserved lysine required for catalytic activity in all kinases (not considered a conserved PIF-binding pocket residue here). Species shown in green text have an experimentally confirmed PDK1 (see text for references), species in blue text may have an incomplete PDK1 sequence, and species in red text have a more divergent PDK1, as assessed by a lack of more than 2 of the 6 most highly conserved PIF-binding pocket residues.