C-terminal truncation and Parkinson’s disease-associated mutations down-regulate the protein serine/threonine kinase activity of PTEN-induced kinase-1

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The Parkinson’s disease (PD) causative \textit{PINK1} gene encodes a mitochondrial protein kinase called PTEN-induced kinase 1 (PINK1). The autosomal recessive pattern of inheritance of \textit{PINK1} mutations suggests that PINK1 is neuroprotective and therefore loss of PINK1 function causes PD. Indeed, overexpression of PINK1 protects neuroblastoma cells from undergoing neurotoxin-induced apoptosis. As a protein kinase, PINK1 presumably exerts its neuroprotective effect by phosphorylating specific mitochondrial proteins and in turn modulating their functions. Towards elucidation of the neuroprotective mechanism of PINK1, we employed the baculovirus-infected insect cell system to express the recombinant protein consisting of the PINK1 kinase domain either alone [PINK1(KD)] or with the PINK1 C-terminal tail [PINK1(KD + T)]. Both recombinant enzymes preferentially phosphorylate the artificial substrate histone H1 exclusively at serine and threonine residues, demonstrating that PINK1 is indeed a protein serine/threonine kinase. Introduction of the PD-associated mutations, G386A and G409V significantly reduces PINK1(KD) kinase activity. Since Gly-386 and Gly-409 reside in the conserved activation segment of the kinase domain, the results suggest that the activation segment is a regulatory switch governing PINK1 kinase activity. We also demonstrate that PINK1(KD + T) is \(~6\)-fold more active than PINK1(KD). Thus, in addition to the activation segment, the C-terminal tail also contains regulatory motifs capable of governing PINK1 kinase activity. Finally, the availability of active recombinant PINK1 proteins permits future studies to search for mitochondrial proteins that are preferentially phosphorylated by PINK1. As these proteins are likely physiological substrates of PINK1, their identification will shed light on the mechanism of pathogenesis of PD.

\section*{INTRODUCTION}

Parkinson’s disease (PD) is the most common neurodegenerative movement disorder affecting humans. Clinical symptoms of PD develop when dopamine levels in corpus striatum of PD patients falls below 70\% of the healthy level (1). Autopsy studies on idiopathic PD patients revealed that the movement disorder usually correlated with progressive preferential loss of dopaminergic neurons in substantia nigra pars compacta of the mid-brain (1,2). However, the cause of progressive preferential cell death of dopaminergic neurons in PD is still poorly understood.

Neurotoxins such as the mitochondrial complex I inhibitor, 1-methyl-4-phenylpyridium ion (MPP\textsuperscript{+}) are capable of inducing PD, indicating involvement of mitochondrial dysfunction in the pathogenesis of PD. This notion is further supported by identification of the \textit{PINK1} gene encoding a mitochondrial protein kinase as a causative PD gene in several pedigrees of autosomal recessive familial PD (3,4). Patients carrying the PD-associated \textit{PINK1} mutations exhibited early onset of
the disease (before 50 years of age) (5,6). Their clinical phenotypes are also somewhat different from those of patients suffering sporadic late-onset Parkinsonism—patients suffering from the early-onset Parkinsonism exhibit slow progression of the disease, good response to L-dopa treatment, dystonia at onset and lesser cognitive decline. Exactly how alteration of PINK1 functions by PD-associated PINK1 mutations contributes to the aforementioned clinical phenotypes remains unknown.

PTEN-induced kinase 1 (PINK1, the numbers in PINK1 constructs denote PINK1 residue numbers of the N- and C-terminal boundaries) is expressed throughout the brain (3,7). Bioinformatics analysis reveals that PINK1 contains an N-terminal mitochondrial targeting sequence and a protein kinase domain (Fig. 1A) (3,8,9). Silvestri et al. (8) also proposed the segment containing residues 101–107 as a putative transmembrane domain. Both endogenous and recombinant PINK1 expressed in mammalian cells are localized to the mitochondria. In addition to the presence of the full-length PINK1 (63 kDa), several proteolysed forms of PINK1 with molecular masses ranging from 30 to 50 kDa also exist (3,8–10), the functional role of these fragments is currently unclear.

![Figure 1. Putative functional domains and motifs of PINK1 and conserved subdomains of the PINK1 kinase domain.](https://academic.oup.com/hmg/article-abstract/15/21/3251/626128)
Down-regulation of PINK1 expression by siRNA in SH-SY5Y neuroblastoma cells increased the mortality of the cells by 3-fold when the mitochondrial complex I was inhibited with a neurotoxin (11). Furthermore, PINK1-deficient Drosophila mutants display mitochondrial defects leading to degeneration of flight muscles (12–14) and more importantly show loss of dopaminergic neurons (13,14). Overexpression of PINK1 in SH-SY5Y neuroblastoma cells resulted in a significant decrease (33–68%) in its susceptibility to neurotoxin-induced cell death (3,15). Collectively, these data suggest that PINK1 is neuroprotective—it protects neuronal cells against neurotoxin- and cell stress-induced cell death.

How might PINK1 exert its neuroprotective function? Treatment of SH-SY5Y neuroblastoma cells with neurotoxins causes loss of mitochondrial membrane potential and release of cytochrome c (3,15). Both events are typically triggered by apoptotic signals to initiate cell death (reviewed in 16). Overexpression of PINK1 in these cells suppresses this loss of mitochondrial membrane potential and subsequent release of cytochrome c induced by neurotoxins (3,15). These data indicate that PINK1 prevents cell death by inhibiting the release of cytochrome c and maintaining the mitochondrial membrane potential. Relevant to this notion, Petit et al. (15) reported that fibroblasts from PINK1 familial PD patients have caspase-3 activity at levels 33% higher than theiragematched controls. Since caspase-3 can be activated by cytochrome c released from mitochondria, this result implies that these PD patients may have a higher level of released cytochrome c. The mechanism by which PINK1 suppresses mitochondrial cytochrome c release and maintains mitochondrial membrane potential remains an important outstanding question. Presumably, PINK1 governs these two events by phosphorylating specific cellular proteins in the mitochondria. Thus, identifying the physiological substrates of PINK1 will be important in answering this question.

Sequence analysis reveals that the majority of PD-associated PINK1 mutations map to the protein kinase domain (Fig. 1A) (3–5,17–24). These mutations may significantly reduce or abrogate kinase activity and in turn abolish the neuroprotective function of PINK1. Previous biochemical studies performed by two groups of investigators on PINK1 kinase activity showed that the recombinant protein kinase domain of PINK1 (residues 112–496) expressed in E. coli was capable of undergoing autophosphorylation and of phosphorylating casein (8,9). Unexpectedly, introduction of PD-associated mutations into the recombinant PINK1 enzyme gave conflicting effects on kinase activity: (i) several missense mutations resulted in a slight reduction in autophosphorylation and kinase activity towards casein, but (ii) the C-terminal truncating W437X non-sense mutation leads to activation (8). Thus, it is uncertain from these observations whether PD-associated mutations indeed inactivate PINK1 kinase activity in PINK1 familial PD. These recombinant PINK1 proteins were generated with the E. coli expression system. Recombinant mammalian proteins are prone to misfolding and/or forming insoluble aggregates when expressed in E. coli (25). Furthermore, prokaryotic E. coli lacks the enzymatic machinery for proper post-translational modifications of mammalian proteins such as fatty acid acylation and glycosylation. For these reasons, the eukaryotic baculovirus-infected insect cell system has been widely used to express recombinant mammalian protein kinases for functional and structural studies (26–29). It is possible that recombinant PINK1 proteins expressed in E. coli were not properly folded and the data obtained may not truly reflect the effects of PD-associated mutations on PINK1 kinase activity. Since PINK1 is expressed in metazoans ranging from nematodes to mammals, it is more appropriate to use a eukaryotic expression system such as the baculovirus-infected insect cell system to generate recombinant PINK1 proteins for functional studies (25,30).

Protein kinase domains are folded into a bilobal structure. Catalysis of the phosphorylation reaction occurs within the cleft between the two lobes (reviewed in 31–33). Conserved regions within the kinase domain supply catalytically critical residues for binding of substrates and for the phosphorylation reaction (Fig. 1B). Mutations in the second PD-associated kinase gene, leucine-rich repeat kinase 2 (LRRK2) cause autosomal late-onset familial and sporadic disease with disease phenotype similar to typical PD (34,35). The most common disease causing mutation G2019S for this large protein kinase maps to the critical kinase activation loop DYG motif; the effect of this change on kinase activity for LRRK2 function is controversial requiring further investigation. Two PD-associated mutations also map to the activation loop of PINK1—Gly-386 and Gly-409 in the activation segment of PINK1 were recently found mutated in two pedigrees of autosomal recessive familial PD (4). Given that the activation segment is a likely regulatory switch governing PINK1 kinase activity and substrate selectivity, we investigated how these two PD-associated mutations affect PINK1 kinase activity.

Herein we show that active recombinant PINK1 proteins can be expressed in baculovirus-infected insect Spodoptera frugiperda (SF9) cells. Using these active recombinant PINK1 proteins, we have developed an in vitro assay to measure PINK1 kinase activity, allowing us to assess the impact of PD-associated mutations on PINK1 kinase activity. With the active PINK1 constructs, we were able to examine the substrate amino acid preference for phosphorylation by PINK1 and define the regions in PINK1 that govern its kinase activity.

RESULTS

Expression of intact recombinant PINK1 proteins in baculovirus-infected SF9 cells

To study the enzymatic properties of the PINK1 kinase domain, it is important to establish the N- and C-terminal boundaries of this domain. The start of a kinase domain is usually marked by a hydrophobic residue located about seven residues upstream of the P-loop (GXXGXXGXXV) (Fig. 1B) (36). The end of a kinase domain is usually represented by a loosely conserved His-X-aromatic-hydrophobic motif (Fig. 1B) (36). This C-terminal boundary motif is located 9–13 residues downstream of an invariant arginine (Arg-497 of PINK1) that salt-bridges with the glutamate (Glu-417) in the conserved APE motif of the activation segment to maintain the structural integrity of the kinase
domain (36). On the basis of sequence alignment of PINK1 with other protein kinases, we postulate that the Tyr-156 and Leu-511 of PINK1 correspond to N- and C-terminal boundaries of the kinase domain, respectively.

To study PINK1 kinase activity in the absence of possible influence posed by its adjacent domains, we generated the recombinant protein kinase domain of PINK1 (residues 148–515). We termed this construct as PINK1 kinase domain [PINK1(KD)]. PD-associated G386A and G409V mutations were introduced into PINK1(KD) to study their effects on kinase activity. PINK1(KD) bearing G386A or G409V mutations are referred to as KD(G386A) and KD(G409V), respectively. Figure 1C depicts the schematics of recombinant full length PINK1, the truncated PINK1 constructs and mutants produced for this study. These recombinant proteins were purified as intact proteins of the predicted size of ~42 kDa (Fig. 2A and B).

To investigate the influence of the C-terminal tail on PINK1 kinase activity, we also created a recombinant PINK1 protein, named as PINK1[KD + T], containing the protein kinase domain and the C-terminal tail of PINK1 (residues 148–581). The purified PINK1[KD + T] has the predicted size of ~48 kDa (Fig. 2A).

We also attempted to investigate the influence of regulatory motifs located N-terminal to the kinase domain on PINK1 kinase activity. Sequence analysis of PINK1 reveals that the segment (residues 121–147) located at the N-terminal end of the kinase domain has the propensity to adopt an amphipathic α-helix similar to the A-helix segment preceding the kinase domain of Protein Kinase A (PKA) (37). To examine the influence of this segment on PINK1 kinase activity, we tried generation of a recombinant PINK1 protein (residues 121–581), which lacks the mitochondrial targeting sequence and the putative transmembrane domain but retains the putative α-helix segment preceding the kinase domain. Upon expression in Sf9 cells, almost all the recombinant PINK1(121–581) was degraded. In addition, expression of full length PINK1 was also tried. Again, upon expression in Sf9 cells, almost all the recombinant PINK1 protein was degraded to the degradation product of ~30 kDa (data not shown).

**PINK1(KD) is a protein serine/threonine kinase capable of phosphorylating histone H1 and casein *in vitro***

Histone H1 and casein have been employed as *in vitro* artificial substrates of many protein serine/threonine kinases. For protein tyrosine kinases, poly(Glu,Tyr) copolymer was chosen as the *in vitro* substrate (38). To examine if recombinant PINK1(KD) is active and to assess the *in vitro* substrate selectivity of PINK1, histone H1, casein and poly(Glu,Tyr) were used as the substrates and the extent of their phosphorylation by PINK1(KD) was compared. As shown in Figure 3, PINK1 is catalytically active, and it prefers to phosphorylate the lysine-rich histone H1 to the acidic residue-rich casein, suggesting that PINK1 prefers basic proteins as *in vitro* substrates. Furthermore, PINK1(KD) is unable to phosphorylate poly(Glu,Tyr) (data not shown), indicating that PINK1 is not a protein tyrosine kinase. Phosphoamino acid analysis of PINK1-phosphorylated histone H1 reveals that histone H1 was phosphorylated exclusively at serine and threonine residues, confirming for the first time that PINK1 is a protein serine/threonine kinase (Fig. 3B).

**The PD-associated G386A and G409V mutations significantly reduce PINK1 kinase activity**

Most PD-associated mutations map to the conserved catalytically critical regions of the PINK1 kinase domain (5). It is therefore plausible that the loss of kinase activity is the common cause of PINK1 familial PD. To test this hypothesis, we introduced PD-associated G386A and G409V mutations into PINK1(KD) to examine the effects of these mutations on its activity.

Gly-386 and Gly-409 are located in the activation segment of the PINK1 kinase domain (Fig. 1B). The functional significance of these two residues of PINK1 can be predicted by examining the functions of homologous residues in PKA. As shown in Figure 4A, Gly-386 is within the conserved DFG motif responsible for chelating Mg$^{2+}$—its mutation is likely to disrupt the Mg$^{2+}$-chelating of ATP and consequently perturb the orientation of γ-phosphate of ATP for the phosphorylation reaction. The G409V mutation maps to the
protein substrate binding loop near the C-terminal end of the activation segment. This mutation may upset the binding of the protein substrates. Therefore, KD(G386A) and KD(G409V) are likely to be much less active than the wild-type PINK1(KD). In agreement with the prediction, the specific kinase activity of PINK1(KD) is about 7- and 20-fold higher than those of KD(G409V) and KD(G386A), respectively (Fig. 4), supporting the notion that PD-associated mutations cause PD by significantly reducing PINK1 kinase activity.

Figure 3. Analysis of the substrate preference and amino acid selectivity of PINK1(KD) and its mutants. (A) Comparison of the extent of phosphorylation of histone H1 and casein by PINK1(KD) and its PD-associated mutants. Autoradiogram of casein phosphorylated by PINK1(KD) and its PD-associated mutants (left). Autoradiogram of histone H1 phosphorylated by PINK1(KD) and its PD-associated mutants (right). On the basis of densitometry comparison, the level of histone H1 phosphorylation by PINK(KD) is 8-fold higher than that of casein. (B) Phosphoamino acid analysis of histone H1 phosphorylated by PINK1(KD). The radioactively phosphorylated histone H1 was subject to acid hydrolysis. The hydrolysate was mixed with the phosphoamino acid standards, spotted onto a TLC plate before electrophoresis. Ninhydrin-stained phosphoamino acid standards on the TLC plate (left). Autoradiogram of the TLC plate (right).

Figure 4. PD-associated G386A and G409V mutations reduce PINK1 kinase activity. (A) Mutation of Gly-386 and Gly-409 is expected to cause reduction in the PINK1 kinase activity: Gly-386 and Gly-409 of PINK1 reside in the activation loop, which governs both the activity and substrate recognition of many protein kinases. On the basis of the sequence alignment between PKA and PINK1, Gly-386 and Gly-409 of PINK1 falls within the conserved subdomains VII (blue) and VIII (pale green) of PKA crystal structure (PDB ID: 1ATP). Gly-186 of PKA (equivalent to Gly-386 of PINK1) is hydrogen bonded to Asp-184 of PKA (equivalent to Asp-384 of PINK1) to stabilize its electrostatic interaction with Mg2+-ATP. Gly-200 of PKA (equivalent to Gly-409 of PINK1) is hydrogen bonded to the backbone of the protein substrate analog, which dictates substrate recognition and binding. These interactions are important for bringing γ-phosphate of ATP and the target hydroxyl group in the substrate [represented by the methyl group (magenta) of the substrate analog] together in close proximity for the phosphorylation reaction. Therefore, mutations in Gly-386 and Gly-409 of PINK1 are likely to disrupt the ATP and substrate binding, respectively. Green-dotted lines indicate hydrogen bonds. The image was generated with the Swiss pdb viewer. (B) Purified PINK1(KD) (9.2 pmol), KD(G386A) (20 pmol) and KD(G409V) (35 pmol) were incubated with 15 mg histone H1 in the presence of kinase assay buffer and 5 mM [γ-32P]ATP. The extent of histone H1 phosphorylation over time is shown by autoradiograms. (C) The protein bands corresponding to histone H1 were excised and the extent of phosphorylation was quantified and expressed in picomoles of 32P incorporated in histone H1 per micromole of recombinant PINK1 protein and the data were plotted over time. The specific kinase activity of PINK1(KD), KD(G386A) and KD(G409V) was determined from the data presented to be 25, 1.2 and 3.4 pmol of 32P incorporated per minute per micromole of recombinant PINK1 protein, respectively.
The C-terminal tail governs the kinase activity of PINK1

The PD-associated W437X mutation is a non-sense mutation, which causes truncation of the C-terminal tail and a portion of the kinase domain (residues 437–511) of PINK1. Another PD-associated mutation D525fsX562 is a frame-shift mutation starting at codon 525. Theoretically, this mutation would give rise to a PINK1 mutant lacking most parts of the C-terminal tail. These mutations are expected to cause PD by reducing PINK1 kinase activity. To investigate the validity of this notion and decipher the functional role of the C-terminal tail, we generated PINK1(KD + T) and compared its kinase activity with PINK1(KD).

Figure 5 shows that the specific kinase activity of PINK1(KD + T) is 6-fold higher than that of PINK1(KD), revealing for the first time that the C-terminal tail contains one or more regulatory motifs capable of enhancing PINK1 kinase activity. Our result also supports the notion that PD-associated truncating mutations can cause PD by reducing PINK1 kinase activity.

The C-terminal tail influences PINK1’s selectivity of phosphorylation sites in histone H1 but not casein

The C-terminal tail of PKA is responsible for recruiting protein substrates and positioning them in the catalytic cleft (32). To examine the possible role of the C-terminal tail of PINK1 in regulating PINK1 substrate recognition, we performed tryptic phosphopeptide mapping of casein and histone H1 phosphorylated by PINK1(KD) and PINK1(KD + T). For most protein kinases, phosphorylation of substrates is governed by the structural features in residues surrounding the phosphorylation site of the protein substrates. Given that casein and histone H1 have 23 and 17 potential phosphorylation sites, respectively, it is expected that only some of these sites are preferentially phosphorylated by PINK1. This notion is supported by the data shown in Figure 6. The tryptic phosphopeptide maps of casein phosphorylated by the two PINK1 proteins are almost identical (Fig. 6A and B), indicating that PINK1(KD) and PINK1(KD + T) displayed similar patterns of preference for phosphorylation sites in casein. In contrast, histone H1 phosphorylated by the two PINK1 proteins gave distinct tryptic phosphopeptide maps (Fig. 6C and D). The tryptic phosphopeptide map (Fig. 6E) of a mixture of histone phosphorylated by PINK1(KD) and that phosphorylated by PINK1(KD + T) reveals that the two PINK1 proteins phosphorylated common (Sites a–i) and distinct (Sites j–n) sites in histone H1 (depicted schematically in Fig. 6F and G). Close inspection reveals that PINK1(KD) phosphorylated nine sites in histone H1 with Sites b, d, f and g being the preferred

Figure 5. The effect of C-terminal tail on PINK1 kinase activity. Purified PINK1[KD] (9.2 pmol) and PINK1[KD + T] (6.9 pmol) were incubated with 15 µg histone H1 in the presence of kinase assay buffer and 5 mM [γ-32P]ATP. (A) The extent of histone H1 phosphorylation over time is shown by autoradiograms. (B) The protein bands corresponding to histone H1 were excised and the extent of phosphorylation was quantified and expressed in picomoles of 32P incorporated in histone H1 per micromole of recombinant PINK1 protein and the data were plotted over time. The specific kinase activity of PINK1(KD) and PINK1(KD + T) were calculated to be 25 and 140 pmoles 32PO4 incorporated per minute per micromole of recombinant PINK1 protein, respectively.

Figure 6. The C-terminal tail influences PINK1’s selectivity of phosphorylation sites in histone H1 but not casein. (A) Tryptic phosphopeptide map of casein phosphorylated by PINK1(KD). (B) Tryptic phosphopeptide map of casein phosphorylated by PINK1[KD + T]. (C) Tryptic phosphopeptide map of histone H1 phosphorylated by PINK1(KD). (D) Tryptic phosphopeptide map of histone H1 phosphorylated by PINK1(KD + T). (E) A mixture of the samples analyzed in (C) and (D). (F) Schematic of the phosphopeptide maps shown in (C). (G) Schematic of the phosphopeptide maps shown in (D). O: Origin. The arrows in (G) indicate the phosphopeptide fragments that were preferentially phosphorylated by PINK1(KD + T).
sites of phosphorylation (Fig. 6D and G). In addition to these nine sites, PINK1(KD + T) phosphorylated five other sites (Sites j–n) in histone H1. Thus, it is clear from the data that the C-terminal tail can influence the phosphorylation site selectivity of the PINK1 kinase domain. Although histone H1 is also phosphorylated by PKA, the tryptic phosphopeptide map of histone H1 phosphorylated by PKA is very different from those of the two PINK1 constructs (Supplementary Material, Fig. S3). Taken together, the data suggest that (i) PINK1 recognizes specific structural determinants surrounding the target phosphorylation sites in its substrates as substrate selectivity determinants and (ii) the C-terminal tail of PINK1 participates in determining the substrate selectivity of PINK1.

**DISCUSSION**

With the baculovirus-infected insect cell expression system, we were able to generate intact recombinant PINK1 constructs for investigation into the enzymatic properties of PINK1. These PINK1 proteins are catalytically active; they preferentially phosphorylate histone H1 with specific enzymatic activities ranging from 25–140 pmol PO$_4$ incorporated/min/μmol of enzyme. Although histone H1 is not the physiological substrate of PINK1, it allowed us to develop an assay method to investigate the catalytic properties, the impact of PD-associated mutations on PINK1 kinase activity as well as the regulatory properties of PINK1. Indeed, with this assay method we found that (i) PINK1 is a protein serine/threonine kinase—it phosphorylates histone H1 exclusively at serine and threonine residues, (ii) PINK1 kinase activity is significantly reduced by two PD-associated mutations mapped to the kinase domain, supporting the hypothesis that loss-of-function mutations of *PINK1* induce PD by abrogating PINK1 kinase activity (3), (iii) the C-terminal tail contains regulatory motifs that enhance PINK1 kinase activity, partly by assisting the kinase domain in substrate recognition. It is reported that PINK1 can protect neuronal cells from undergoing apoptosis through an unknown mechanism when the cells are treated with neurotoxins (11,15). One of the avenues to decipher this mechanism is to identify the physiological substrates of PINK1. The availability of active recombinant PINK1 proteins permits the search for PINK1 substrates.

**Catalytic properties of PINK1**

It is clear from the results shown in Figure 6 that the subset of serine and threonine residues targeted by PINK1 in histone H1 is distinct from those phosphorylated by PKA. Most protein kinases recognize the specific features in the primary structures around the phosphorylation sites in protein substrates as substrate selectivity determinants. For PKA, the optimal phosphorylation site sequence is R/K-R/K-x-S/T-Hb, where x represents any amino acid and Hb represents a hydrophobic residue (reviewed in 39). The results shown in Figure 6 and Supplementary Material, Figure S3 suggest that the optimal phosphorylation site sequence of PINK1 is different from that of PKA. The availability of active recombinant PINK1 proteins allows future studies employing the peptide library approach to define the optimal phosphorylation target sequence of PINK1 (reviewed in 40). Knowledge of the optimal phosphorylation sequence of PINK1 will facilitate the search for PINK1 protein substrates because mitochondrial proteins with sequences similar to that of the PINK1 optimal phosphorylation sequence are potential physiological substrates of PINK1.

The results shown in Figure 6 indicate that the C-terminal tail of PINK1 can influence phosphorylation site selectivity in substrates. It is possible that the C-terminal tail contains functional motif(s) that bind to specific sequences in a protein substrate and in turn direct the kinase domain to phosphorylate a specific site in the substrate. In light of this, it would be worthwhile in future investigation to use recombinant C-terminal tail of PINK1 as the ‘bait’ to search mitochondrial extract of neuronal cells for potential PINK1 protein substrates that bind to the C-terminal tail.

**Regulatory properties of PINK1**

Similar to many protein kinases, the kinase activity of PINK1 is likely governed by configuration of the activation segment in the kinase domain. Indeed, mutations of Gly-386 and Gly-409 in this segment induce significant reduction of PINK1 kinase activity (Fig. 4). As shown in Figure 4A, Gly-386 corresponds to the conserved DFG motif that forms the Mg$^{++}$-chelating loop of protein kinases. It is therefore conceivable that mutation of Gly-386 to alanine can disrupt the structural integrity of the Mg$^{++}$-chelating loop and consequently induces significant reduction in PINK1 kinase activity (Fig. 4). Relevant to this, the homologous Gly (Gly-2019) in the Mg$^{++}$-chelating loop of another PD-related protein kinase LRRK2 was found mutated to serine in many PD patients (reviewed in 35). Biochemical analysis revealed that, in contrast to the inactivating effect of G386A mutation, the G2019S mutation induces a significant increase in kinase activity of LRRK2, suggesting that this missense mutation of PINK1 causes PD by activating LRRK2. What is the structural basis for the opposing effects arising from substitution of the conserved Gly in the Mg$^{++}$-chelating loop in PINK1 and LRRK2? Determination of the three-dimensional structures of PINK1 and LRRK2 kinase domain will reveal the answers to this question.

In many protein kinases, configuration of the activation segment is regulated by phosphorylation of a conserved serine/threonine or tyrosine residue in the activation segment. Phosphorylation of this residue in most cases activates the enzyme. For example, the active conformations of PKA and c-Src kinase are stabilized by phosphorylation of Thr-197 and Tyr-416, respectively, in the activation segment (reviewed in 31,41,42). Using the recombinant PINK1 construct spanning the segment corresponding to residues 112–496, Beilina et al. (9) and Silvestri et al. (8) detected autophosphorylation of the recombinant kinase *in vitro*. In contrast to their findings, we failed to detect autophosphorylation of PINK1(KD) and PINK1(KD + T). Thus, the autophosphorylation site of PINK1 is unlikely to be located in the activation segment and other parts of the kinase domain. The PINK1 construct used by these groups of researchers contains the segment corresponding to residues 112–147,
which is not included in both PINK1 constructs used in our study. It is possible that the autophosphorylation site resides in this segment. The other explanation for the discrepancy of our results and those reported by Beilina et al. (9) and Silvestri et al. (8) relates to the intracellular compartment in which recombinant PINK1(KD) and PINK1(KD + T) were expressed. It is noteworthy that owing to the lack of a mitochondrial targeting motif, these two purified recombinant PINK1 proteins were expressed and folded in the cytosol of S9 cells (Supplementary Material, Fig. S1). Similar to other mitochondrial proteins encoded by nuclear genes, native PINK1 is likely synthesized in the cytosol and then imported into the mitochondria in an unfolded configuration. Presumably, once the unfolded PINK1 protein enters the mitochondria, it is processed and folded to the functional configuration with the assistance of chaperones (43). It is possible that PINK1 acquires the ability to undergo autophosphorylation only when it is folded in the functional configuration in the mitochondria. Alternatively, PINK1 can undergo autophosphorylation only when it binds to a regulatory protein in mitochondria. In view of this, the enzymatic properties such as the ability to undergo autophosphorylation and kinetics of phosphorylation of histone H1 should be compared between recombinant PINK1(KD + T) used in the current studies and endogenous PINK1 purified from the mitochondria of mammalian cells.

**PINK1 interplays with Parkin and superoxide dismutase 1 to maintain survival of neurons in Drosophila**

In addition to PINK1, the Parkin gene encoding an E3 ubiquitin ligase is also a recessive PD-causative gene. Thus, in addition to mitochondrial dysfunction, an impaired ubiquitin–proteasome system can also cause PD. Using Drosophila as a model system, three groups of researchers recently reported that PINK1 and Parkin act in a common signalling pathway to maintain mitochondrial integrity (12–14,44). Their studies revealed that Parkin operates downstream of PINK1 in this pathway (12–14). In addition, Wang et al. (44) demonstrated that expression of human superoxide dismutase 1 (SOD1) suppressed degeneration of neurons in Drosophila induced by Drosophila PINK1 inactivation. Their results suggest that PINK1 maintains survival of neuronal cells by protecting the cells from undergoing oxidative stress. Hence, there are two important outstanding questions: (i) How might PINK1, which is located in mitochondria, regulate the activity of Parkin in the cytosol? (ii) How might PINK1 protect neurons from undergoing oxidative stress? Identifying the physiological substrates of PINK1 will provide the answers to these questions. The availability of active recombinant PINK1 constructs will permit a search for the protein substrates, which upon phosphorylation, mediate regulation of Parkin by PINK1.

The four groups of researchers mentioned above also demonstrated that deletion of the Drosophila PINK1 (dPINK1) gene induced pathogenic phenotypes including mitochondrial dysfunction, shortened lifespan and dopaminergic neuron degeneration (12–14,44). These phenotypes can be rescued by expressing wild-type human PINK1 in the dPINK1-deficient Drosophila (14), indicating that the role of PINK1 in maintaining mitochondrial integrity and preventing degeneration of dopaminergic neurons is conserved from Drosophila to man. However, upon deletion of the C-terminal 72-residue segment, the ability of human PINK1 to rescue these phenotypes in dPINK1-deficient Drosophila is lost. On the basis of these results, Yang et al. (14) suggested that the C-terminal tail may affect the following enzymatic properties of PINK1: (i) kinase activity, (ii) the ability to bind to its substrates, (iii) the ability to bind to other cofactors (14). The data shown in Figures 5 and 6 give further credence to their prediction of the role of the C-terminal tail in governing PINK1 kinase activity and binding of PINK1 to substrates. Our future investigation will include examining whether the C-terminal tail mediates PINK1 binding to some unknown regulatory cofactors in vivo.

**How do heterozygous C-terminal truncation mutations of PINK1 contribute to PD?**

Intriguingly, heterozygous PINK1 mutations can also give rise to PD (5). For example, the Q456X non-sense mutation has been reported as a heterozygous PD-associated mutation (5). Truncated PINK1 resulting from this mutation lacks the C-terminal tail and a small portion of the kinase domain. On the basis of our data presented in Figure 5, it is expected that this truncated form of PINK1 exhibits very low or no kinase activity. Since Q456X is observed in PD patients as a heterozygous mutation, it is possible that both the wild-type PINK1 encoded by the normal allele, and the truncated PINK1 mutant encoded by the mutant allele are co-expressed in patients carrying this mutation. Bonifati et al. (5) suggested that the truncated PINK1 mutant may act as a dominant negative mutant, which interferes with the activation and/or function of wild-type PINK1 encoded by the normal allele. Future investigation into the role of the C-terminal tail in regulating PINK1 function may provide the answer to this outstanding question.

In summary, our study has laid the groundwork for elucidating the molecular basis of regulation and neuroprotective function of PINK1, both are areas critical to understanding the mechanism of PD pathogenesis.

**MATERIALS AND METHODS**

**Reagents**

Bovine histone H1 and dephosphorylated casein were from Sigma-Aldrich. Poly(Glu,Tyr), a random copolymer of glutamate and tyrosine (molar ratio of Glu: Tyr = 4:1) was purchased from Sigma-Aldrich. It is an efficient substrate for most known protein tyrosine kinases (38). Bacfectin®, pBacPAK9 vector and Bsu36I-digested BacPAK6 viral DNA were from BD Biosciences. QuickChange® II site-directed mutagenesis kit was from Stratagene. The human PINK1 cDNA clone was from OriGene Technology. The anti-PINK1 antibody raised against the segment corresponding to residues 258–274 of PINK1 was from Imgenex Corporation. The anti-Flag-tag antibody, the anti-Flag-tag antibody agarose and the catalytic subunit of PKA were from Sigma-Aldrich.
Generation of pBacPAK9-recombinant PINK1 transfer vectors

Full length PINK1 gene was retrieved from the human PINK1 cDNA clone by restriction digestion with EcoRI and XbaI. Truncated PINK1 constructs encoding PINK1(121–581), PINK1(148–581) [also referred to PINK1(KD + T)] and PINK1(148–515) [also referred to PINK1(KD)] were amplified by PCR from the human PINK1 cDNA clone. PCR reactions were used to introduce the restriction sites as well as the sequences encoding (i) the poly-His tag at the N-terminal end of PINK1(121–581) and the C-terminal end of PINK1(148–515), and (ii) a FLAG sequence (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) at the N-terminal end of both PINK1(148–581) and PINK(148–515). The PCR products were digested with EcoRI and XbaI endonucleases and then ligated to pre-digested pBacPAK9 vector to generate the following plasmids: pBacPAK9-PINK1, pBacPAK9-PINK1 (121–581), pBacPAK9-PINK1(148–581) and pBacPAK9-PINK1(148–515). Authenticity of the plasmids containing the cDNA encoding full length and the truncated PINK1 constructs was confirmed by DNA sequencing.

Generation of pBacPAK9-PINK1(148–515)[G386A] and pBacPAK9-PINK1(148–515)[G409V] plasmids by site-directed mutagenesis

The glycine residues at positions 386 (G386) and 409 (G409) of PINK1 were mutated to alanine (G386A) and valine (G409V) residues, respectively, in the PINK1(148–515) construct, by using the QuickChange® II site-directed mutagenesis kit according to manufacturer’s instructions. Authenticity of the resultant plasmids, pBacPAK9-PINK1(148–515)[G386A] and pBacPAK9-PINK1(148–515) [G409V] was confirmed by DNA sequencing.

Generation of recombinant PINK1 baculovirus expression vector

To generate the recombinant PINK1 baculovirus expression vector, 1 × 10^6 Sf9 cells were co-transfected with respective PINK1-pBacPAK9 plasmid, Bsu361-digested BacPAK6 viral DNA and Bacfectin®, according to the manufacturer’s instructions. The cell lysates were analyzed by immunoblotting with anti-PINK1 antibody and anti-Flag mouse monoclonal antibodies to confirm the production of recombinant PINK1 in the infected Sf9 cells.

Purification of recombinant PINK1 proteins

Sf9 cells (1 l and at cell densities ranging 0.6–0.9 × 10^6 cells/ml) were cultured in Grace’s medium (Invitrogen®) in the presence of 7% ‘Cosmic’ calf serum (Hyclone) before they were infected with the recombinant PINK1 baculovirus at a multiplicity of infection ≥1. At 50 h after infection, the cells were harvested for protein purification by centrifugation at 1000g for 5 min. The cell pellet was washed with serum-free Grace’s medium (Invitrogen®) and centrifuged at 1000g for 5 min again. Subsequent protein purification procedures were carried out at 4°C. The cell pellet was homogenized in Lysis Buffer [50 mM Tris–HCl, pH 7, 15% glycerol, 0.1 mg/ml soybean trypsin inhibitor, 0.2 mg/ml benzamidine–HCl, 0.1 mg/ml phenylmethylsulfonylfluoride (PMSF)] and centrifuged at 100 000g for 30 min. The supernatant was collected for subsequent protein purification.

Supernatants derived from cell lysates containing recombinant full-length PINK1, PINK1(121–581) and PINK1(148–581) [also called PINK(KD + T)] constructs, were subjected to DEAE anion exchange column chromatography. These supernatants were diluted 1:1 with DEAE Buffer (25 mM Hepes, pH 7.0, 15% glycerol, 0.2 mg/ml benzamidine–HCl, 0.1 mg/ml PMSF, 1 mM dithiothreitol) and loaded onto a DEAE Sepharose column (80 ml bed volume) pre-equilibrated with DEAE buffer. The proteins were eluted with a 0–1 M NaCl gradient in DEAE buffer. The elution profiles of recombinant PINK1 proteins were monitored by immunoblotting with anti-PINK1 antibody.

PINK1(148–515) [also called PINK1(KD)] and its PD-associated mutants contain a 6-histidine tag to allow purification by affinity chromatography onto a column with nickel–nitritriacetic acid (nickel–NTA) agarose (QIAGEN®). Supernatants derived from cell lysates containing these recombinant PINK1 proteins were incubated in 5 ml of nickel–NTA agarose gel for 2 h. The nickel–NTA agarose gel was washed with 10 ml of wash buffer (25 mM Hepes, pH 7.0, 15% glycerol, 0.2 mg/ml benzamidine–HCl, 0.1 mg/ml PMSF, 1 mM NaCl). The proteins were eluted with 10 ml of Elution Buffer (wash buffer as above containing 300 mM imidazole, at pH 7.5). The elution profiles of recombinant PINK1 proteins were monitored by anti-Flag western blotting.

Flag-tagged recombinant PINK1 proteins were immunoprecipitated with anti-Flag antibody agarose (Sigma-Aldrich). The immunoprecipitate was sequentially washed with 2 × 1 ml of DEAE Buffer with 1 M NaCl and 2 × 1 ml DEAE Buffer. All subsequent experiments were performed with immunoprecipitated recombinant PINK1 proteins.

Quantitation of Flag-tagged PINK1 proteins purified by anti-Flag antibody agarose

All Flag-tagged PINK1 proteins including PINK(KD + T), PINK(KD), KD(G386A) and KD(G409V) were affinity purified by immunoprecipitation with anti-Flag antibody agarose prior to enzymatic analysis. The Flag-Hck(222–503), containing the kinase domain and the C-terminal regulatory domain of the Hck tyrosine kinase were generated and purified as described previously (45). It was used as the Flag-protein standard for quantitation of the PINK1 proteins in the immunoprecipitates. Briefly, aliquots of the immunoprecipitates and known amounts (4–40 pmol) of the Flag-protein standard were analyzed by anti-Flag western blotting.

The anti-Flag immunoreactivities of the PINK1 proteins and the protein standard were quantitated and expressed as densitometry units. The values from the Flag-protein standard were used to generate the standard curve. From the curve and the immunoreactivities of the PINK1 proteins, their amounts were determined. Supplementary Material, Figure S1 illustrates how we determined the amounts of the immunoprecipitated PINK1 proteins with this method.

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Phosphorylation of histone H1, casein and poly(Glu,Tyr) copolymer by recombinant PINK1 proteins

Phosphorylation of exogenous substrates (1 μg each of histone H1, dephosphorylated casein or poly(Glu,Tyr) copolymer) was performed at 30° C for 1 h in a final volume of 25 μl containing the Assay Buffer (20 mM Tris–HCl, pH 7.5, 10 mM MgCl₂, 1 mM MnCl₂ and 50 μM Na₃VO₄), 10 μM [γ-32P]ATP and 10 μl (bed volume) of recombinant PINK1 proteins bound to the anti-Flag antibody agarose. The amounts of PINK1 proteins used in the experiment were quantitated by anti-Flag western blotting to be 4.9 pmol, 11 pmol and 19 pmol for PINK(KD), KD(G386A) and KD(G409V), respectively. The reaction was stopped with the addition of 5× SDS–PAGE sample buffer and resolved by 10% Tris–Glycine SDS–PAGE. The SDS–PAGE gel was dried and exposed to a phosphoimager plate for subsequent quantitative analysis.

Time course of phosphorylation of histone H1 by recombinant PINK1 proteins

Histone H1 (15 μg) was phosphorylated at 24° C for 56 min by the resuspended immunoprecipitated recombinant enzymes (6.9–35 pmol PINK1 proteins in 30 μl of anti-Flag antibody agarose) in a final volume of 75 μl of the assay buffer with 5 μM [γ-32P]ATP. Aliquots (5 μl) of the reaction were taken out and mixed with 5× SDS–PAGE sample buffer at 8 minute intervals. The proteins were resolved by SDS–PAGE. The SDS-gel was dried and exposed to a phosphoimager. 32P-labelled histone H1 bands were excised and the associated radioactivity was determined by scintillation counting.

The amounts of recombinant PINK1 proteins present in the above reactions were quantified by comparing their immunoreactivity with anti-Flag antibody and that of a Flag-tagged protein standard of known concentration.

Phosphoamino acid analysis

Histone H1 and casein were phosphorylated by immunoprecipitated Flag-tagged recombinant PINK1 as mentioned earlier. After SDS–PAGE, proteins in the gel were transferred onto polyvinylidene fluoride membrane (Millipore). The 32P-labelled histone H1 was detected by autoradiography and the corresponding portion of the membrane was excised. The 32P-labelled histone H1 was hydrolyzed by 6 M HCl at 110°C for 90 min. After repeated freeze-drying to remove HCl, the hydrolysate was mixed with phosphoamino acid standards [1 μg each of phosphotyrosine, phosphoserine and phosphothreonine (Sigma-Aldrich)] and spotted onto a cellulose thin-layer chromatography (TLC) plate (Macherey-Nagel). The amino acid mixture was separated by thin-layer electrophoresis (TLE) in TLE buffer [5% (v/v) acetic acid, 0.5% (v/v) pyridine in H₂O] at 500 V for 80 min, as described previously (46). The phosphoamino acids were detected by ninhydrin spray [0.1% (w/v) in acetone], followed by heating of the TLC plate. The 32P-labelled phosphoamino acids were detected by phosphoimager.

Phosphopeptide mapping of radioactively phosphorylated casein and histone H1

PINK1(KD) (~100 pmol) and PINK1(KD + T) (~40 pmol) affinity-purified by anti-Flag antibody agarose (bed volume = 10 μl) were used to phosphorylated histone H1 or casein (15 μg each) at 30° C for 30 min in a final volume of 40 μl of the assay buffer with [γ-32P]ATP. Since casein is a much poorer substrate of the PINK1 (Fig. 3A), ATP of a lower concentration (1 μM) and high specific radioactivity (~145 000 cpm/pmol) was used in the reaction. For histone H1 phosphorylation, the ATP concentration and specific radioactivity used for the reaction were 10 μM and ~36 000 cpm/pmol, respectively. The reactions were stopped by the addition of SDS sample buffer. Following SDS–PAGE, proteins in the gel were transferred onto a nitrocellulose membrane. The 32P-labelled casein and histone H1 were detected by autoradiography and the corresponding portions of the membrane were excised and digested with 1 mg/ml trypsin in the presence of 5% (v/v) CH₃CN in 100 mM NH₄HCO₃. In the presence of 5% CH₃CN, the tryptic fragments including the 32P-labelled peptides were detached from the membrane. The resulting solution was freeze-dried and the tryptic peptide fragments were separated by TLE in the first dimension and TLC in the second dimension as described previously (33). Aliquots containing ~2000 cpm of the phosphopeptide fragments each were spotted on the TLC plates for analysis. For the mixture of histone H1 phosphorylated by PINK1(KD) and that phosphorylated by PINK19(KD + T) (Fig. 6E), an aliquot containing ~1000 cpm of phosphopeptide fragments was taken from each sample. The two aliquots were mixed together prior to spotting onto the TLC plate. To maximize the space on the TLC plate (24 × 24 cm) available for the separation of the positively charged tryptic phosphopeptide fragments, the samples were spotted 16 cm from the cathode end [labelled as (−) in Fig. 6] and 8 cm from the anode end [labelled as (+) in Fig. 6].

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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