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**PINK1 cleavage at position A103 by the mitochondrial protease PARL**

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**Objectives**

**Parkinson's disease** is a disorder of the brain that leads to shaking (tremors) and difficulty with walking, movement, and coordination. This paper focuses on probable causative mutations of an early onset Parkinson’s disease (PD) associated gene: PTEN-induced kinase 1 (PINK1). It gives a 63 kDa protein kinase that localizes in mitochondria. It plays versatile roles to provide protection against MPTP neurotoxins and overall wellbeing of the mitochondria. PINK1 protein is cleaved to produce a ∼53 kDa protein, ΔN-PINK1. If the full length (FL-PINK1) is not spliced properly it will accumulate in the mitochondria, and induce mitophagy i.e. removal of damaged mitochondria from the cell. There is an alternative cleavage product of FL-PINK1, namely ΔN2-PINK1. It is a ~45 kDa protein and more stable than ΔN-PINK1 because of its association with *Hsp* 90 chaperone.

Because of the neuroprotective properties of PINK1 and its intimacy with Parkinson’s disease phenotypes, characterization of this gene and an in depth study of its protein product is very important. Objectives of this research project were:

1. Determination of the cleavage site of PINK1.
2. Mutational analysis of the cleavage site residues.
3. Observation of the effect of PD associated mutations in PINK1.
4. Assessment of cellular consequences of impaired PINK1 cleavage.
5. Identification of the protease responsible for the cleavage of PINK1.

**Approach & Results**

1. **PINK1 Cleavage site determination:**

Recombinant HEK293T cells were cultured with the gene construct PINK1-3xHA. It was transiently expressed to obtain full-length and cleaved PINK1 (ΔN-PINK1) from lysates. To increase the amount of cleaved protein, the cells were treated with the proteasome inhibitor MG132 prior to lysis. 3x Hemagglutinin (HA) is a tag peptide used to detect PINK1 by immunoprecipitation using anti-HA-agarose beads. The product was isolated by SDS–PAGE run, Western blotted and stained by Coomassie Brilliant Blue. Then PINK1 53 kDa band was sequenced by *Edman N-terminal degradation*. 10 amino acid analysis confirmed the protein to be ΔN-PINK1 and revealed that the PINK1 cleavage site within the TM domain between residues A103 and F104.

1. **Mutational analysis of cleavage site residues:**

Mutational analysis of residues at and surrounding the cleavage site confirms the cleavage site. In position 104 when Phenyl alanine was changed to Aspartic acid then accumulation of both FL and ΔN-PINK1 increased. But when the Phenyl alanine was changed to Alanine, expression of FL PINK1 decreased but ΔN-PINK1 was still being amassed. Another important cleavage site was identified in the position 95. When the Proline residue was changed by an Alanine, splitting of PINK1 reduced substantially. As a result, concentration of FL PINK1 was building up with fewer ΔN-PINK1. The mutated proteins were cross checked for mitochondrial localization and positive result indicated that the transmembrane domain of PINK1 was left intact by these mutations.

1. **The effect of PD associated mutations:**

It was discovered that the cleavage site of PINK1 lies in between two PD associated mutations: C92F and Q115L. Another mutant variant is reported as R147H. Assessment of cleavage status showed that the production of the ΔN-PINK1 is unaffected by the presence of these PD mutations, which means these disease-associated mutations do not disarray the cleavage per se. But an accumulation of the FL-PINK1 protein compared with PINK1-wt specifies that they increase the ratio of FL to ΔN-PINK1 protein within cells.

1. **Cellular consequences of impaired PINK1 cleavage:**

♦ Transgenic Sh-SY5Ycells were produced with PINK-wt, PINK1-F104A and PINK1-P95A mutants in a pIRES-GFP vector. A cationic lipophilic dye, Tetra-Methyl-Rhodamine Methylester (TMRM) gives fluorescent intensity from within the mitochondria. It is proportional to the ΔѰm, mitochondrial membrane potential. The presence of Green Fluorescent Protein (GFP) marker helps to identify transfected cells. F104A mutants with enough ΔN-PINK1, had significantly higher basal ΔѰm than cells expressing PINK1-wt mutants. On the other hand, P95A mutants with predominant FL-PINK1 production had consistent lower level of ΔѰm than wild type PINK1 carriers.

♦ The same cells were assayed for Reactive Oxygen Species (ROS) production. Cytosolic hydroethidium (HEt) fluorescence and MitoSOX fluorescence were used for cytoplasm and mitochondria respectively. Expression of the PINK1-P95A i.e. full length uncut protein resulted in a significant increase in the basal cytosolic and mitochondrial ROS production compared with PINK1-wt or PINK1-F104A.

♦ Usually the mitochondria form an intricate interconnected network distributed evenly throughout the cell. But observation of PINK1-P95A and PINK1-C92F PD mutant cells by two different dyes - TMRM and DsRed-Mito confirms the alteration in mitochondrial distribution. They were aggregated in one part of the cell, and no clear network was visible.

♦ Utilizing the DsRed-Mito and pIRES-GFP constructs, this approach quantifies the degree of co-localization of the mitochondrial (DsRed-Mito) signal with the cytosolic (GFP) signal in the cells. This enables a measure of the relative volume of the cell occupied by the mitochondria and reflects the mitochondrial mass within a cell. In PINK1-P95A and PINK1-C92F PD mutant cells, a marked reduction was observed in co-localization compared to that of PINK1-wt or PINK1-F104A expressing cells.

♦  LC3 I-II cleavage is used as indicators of autophagy. There were no variation in basal and CCCP-induced levels of LC3 I-II cleavage in cells expressing vectors - PINK1-wt, PINK1-P95A and PINK1-F104A. It indicates that mitochondrial removal via mitophagy was not taking place, but by some other means.

1. **PARL is the protease responsible for the cleavage of PINK1:**

♦ Two mitochondrial proteases, high temperature requirement protein A2 (HtrA2) and presenilin-associated rhomboid-like protein (PARL) were probable candidates to cleave PINK1. HtrA2, the mitochondrial serine protease is known to interact with PINK1. But PARL was selected as a study in Drosophila suggested that Rhomboid-7, a PARL homologue is involved in dPINK1 processing. Mass spectrometry was used to identify protein-protein interaction between endogenous PINK1 and PARL in SH-SY5Y cells.

♦ Mouse embryonic fibroblasts (MEFs) were derived from PARL and HtrA2 knockout mice. PINK1 cleavage pattern demonstrated that in the absence of HtrA2, 53 kDa ΔN-PINK1 was generated but in the absence of PARL, PINK1 was aberrantly cleaved. PARL KO MEFs were co-transfected with PARL-wt, and successful splitting of PINK1 was observed. But PARL-S277G a mutant form, when inserted into the knockout cells failed to give functional ΔN-PINK1 products. This demonstrates the specificity of PINK1 cleavage by PARL.

♦ Proline is a conformationally restricted amino acid and is therefore predicted to induce a ‘kink’ in the α-helical structure of the PINK1 TM domain. PyMoL is used for structural modeling of the wild and P95A mutated TM domain shows that the P95A mutation removes this ‘kink’ and results in PINK1 displaying a conventional α-helix.

♦ PARL RNA interference (RNAi) in SH-SY5Y cells expressing PINK1-wt showed a significant increase in ROS production than those whose RNAi was scrambled and therefore PARL activity was smoothened.

**Conclusion**

PINK1 is cleaved between amino acids Ala-103 and Phe-104 to yield ΔNPINK1. A reduced ability to cleave PINK1, and the consequent accumulation of full-length protein, results in mitochondrial abnormalities similar to PINK1 knockout cells, including -

1. Disruption of even distribution of the mitochondrial network
2. Reduction in mitochondrial mass inside the cell independent of mitophagy activation
3. Lowering of Mitochondrial membrane potential, ΔѰm
4. Increase in generation of harmful Reactive Oxygen Species.
5. An increased ratio of FL- to ΔN-PINK1, expresses intermediate mitochondrial phenotype, which eventually contribute towards pathogenesis like swollen aggregates (Drosophila) or fragmented mitochondria (mammals)

PARL is the default protease required for production of ΔN-PINK1 from FL-PINK1 and subsequent conversion of it to ΔN2-PINK1. The cleavage of PINK1 by PARL is dependent upon structural recognition of a ‘kinked’ α-helix rather than a normal α helical structure.

**Future Research:**

The cleavage recognition site for PARL could not be elucidated from this research. This knowledge is critical to apprehend co-relation of non-related PD mutations with amassing of non-cleaved PINK1. This project also failed to assess whether naturally occurring N-terminal PD mutations C92F, Q115L or R147H cause accumulation of FL-PINK1 in patient tissue because of absence of certain materials.

Despite versatile works done on this topic, the researchers are open to criticism in their lack of focusing of ΔN2-PINK1, the next stable product of ΔN-PINK1 processing pathway. They limited their search in non-functionality of ΔN2-PINK1 in terms of protecting cells. If as a transient intermediate, ΔN-PINK1 imparts so many important functions, ΔN2-PINK1 the final stable product must have a bigger role to play in PD pathogenicity.

A promising finding from this research is the presence of an unknown mechanism of internal organelle processing apart from autophagy. There has to be a pathway by which the mitochondrial mass is decreasing without using LC3 I-II proteasome cleavage system.

Expression of more ΔN-PINK1 is beneficial as supported by the fact that an increased deposition of ΔN-PINK1 protein occurs in the surviving neurons of patients suffering from Parkinson’s disease. If PINK1 cleavage process at the endogenous level can be modulated, it can have future therapeutic applications.