Abstract

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Title: Role of PTEN's post-translational modifications in autophagy

Autophagy is a cellular phenomenon that simply eliminates 'unwanted' compounds. But beyond this apparent 'destruction', autophagy effectively plays the role of a recycling system. PTEN is a protein, primarily known for its tumour-suppressive actions but it is also involved in varied domains of cell metabolism. Like every other protein, post-translational modifications (PTM) modulate the various functions of PTEN. PTM are chemical modifications that occur on a protein after it has been synthesized and subsequently regulate the activity, localization, and stability of proteins. PTEN can be localized to the nucleus, cytoplasm or cell membrane which accordingly affects its functions and interactions. The domains that constitute PTEN are N-terminal PBD, phosphatase domain, C2 domain, C-terminal tail, and the PDZ-BD. The N-terminal PBD is the attachment site for PTEN's primary substrate PIP2 whereas PTEN's enzymatic nature is instilled in it by the phosphatase domain. The C2 domain assists in the association of this phosphatase domain with the plasma membrane where PTEN can express its phosphatase nature. The C-terminal tail is mainly involved in maintaining stability and function and the PDZ-BD is associated with additional signalling. PTEN exists in two conformations namely open and closed which serve as the gateway for the enzyme's active site. Numerous factors including PTM can modulate this alternating conformation shift. The PI3K/AKT intracellular signaling pathway plays a crucial role in regulating cellular events like autophagy. At the plasma membrane, AKT attaches itself to PIP3 and gets activated. PI3K assists in this conversion of PIP2 to PIP3 while PTEN dephosphorylates PIP3 back to PIP2. Hence, deleted or dysfunctional PTEN results in a dysregulated PI3K/AKT pathway. Two most important PTM that modulate PTEN function are phosphorylation and sumoylation. PTEN is phosphorylated at several locations, especially within the C-terminal tail, whereas the possible sumoylation sites are all located in the C2 domain.

We wanted to carry out this study on PTEN as it is frequently mutated or deleted in various types of cancer. Also, defective PTEN causes syndrome like PHTS which give rise to a propensity of developing cancers throughout life. Moreover, the paramount importance of a phenomenon like autophagy lies in the exhaustive role it displays in both physiology and pathophysiology. Taking both of these into account, we wanted to examine the effects of PTEN's phosphorylation and sumoylation on autophagy. We set off with finding out the alteration in autophagy patterns with respect to these PTM and the reasons behind it. We tried to further examine these alterations with respect to different causative processes of autophagy namely nutrient stress and ER stress. We finally tested our hypotheses on multiple cell lines, both PTEN null and PTEN positive cells, to reach a befitting consensus.

We found out that transfection of WT PTEN recovered autophagy in PTEN-null PC3 and U87MG cells. Sumoylation of PTEN was found to have boosted autophagy as observed from the reduced expression of autophagy related proteins in sumoylation deficient PTEN mutant transfected cells. Phosphorylation of PTEN curtailed autophagy as observed from the expression of autophagy in dephosphorylation-mimicking and phosphorylation-mimicking PTEN mutants transfected cells. This modulation by sumoylation and phosphorylation was also observed in PTEN positive normal (WI38) and cancer (A549, Hela) cells. The overall reason behind this modulation was that sumoylation of PTEN assisted whereas phosphorylation hindered plasma membrane localisation of PTEN. The observed effects of alteration in autophagy were similar when surfacing from both nutrient deprivation as well as ER stress, induced by EBSS and Tunicamycin treatment respectively.

WT PTEN transfection had triggered autophagy, and as the starvation period increased, there was a consistent rise in the number of active cells exhibiting autophagy. Because phosphatase deficient PTEN mutant couldn't fully recover autophagy, we could infer that it was catalytically inactive and PTEN's lipid phosphatase activity was deemed essential for the induction of autophagy. The C2 domain of PTEN is mostly responsible for its interaction with the plasma membrane as well as for the correct orientation of its

catalytic phosphatase domain. This is crucial from a functional standpoint because PTEN's enzymatic efficiency rely both on its penetration and orientation within the membrane. The location of cellular substrates inside the cell is determined by their sumoylation. PTEN was in fact directed to the cytoplasm by decreased sumoylation, as shown in the sumoylation deficient PTEN mutant transfected cells. PTEN's net positive charge on the C2 domain had supposed to have increased from sumoylation and it was subsequently drawn towards the negatively charged cell membrane, hence facilitating its association. As a result, sumoylation deficient mutant transfected cells showed prolonged AKT and mTOR phosphorylation both of which are deemed essential for subduing autophagy. On the other hand, the phosphorylation-mimicking and dephosphorylation-mimicking PTEN mutant transfected cells showed decreased and increased plasma membrane affinity respectively. Needless to say, the autophagy levels in the cells got changed accordingly. The C-terminal phosphorylation behaved as a molecular clamp on the C2 domain and consequently determined the aforementioned alteration in open and closed conformation of PTEN.
The parameters that we checked in our experiments for successful autophagy occurrence are namely MDC dye staining that preferentially accumulates in the autophagic vesicles; immunolabeling of LC3B which determines the formation of autophagosomes; western blot of cargo receptor protein p62 which binds to LC3B and provides perception about the autophagic flux; immunoblot of other autophagic marker proteins Beclin1, ATG5, ATG7, p-mTOR, p-Akt which are distinctly regulated during the whole process, and ultimately the ultrastructure of autophagic vesicles inside the cells using TEM. PTEN's sumoylation and phosphorylation status after autophagy was found out using a combination of both immunoprecipitation and western blot. Finally, PTEN's subcellular localisation was ascertained by immunolabeling followed by subsequent inspection under confocal microscopy.
PTEN's physiological functions have been repeatedly linked to its defective membrane recruitment. Here, we were able to show how autophagy and PTEN's post-translational modifications were related with respect to cell membrane association. In a nutshell, we had successfully rescued autophagy (stemming from both nutrient deprivation as well as ER stress) in PTEN null cells by transfecting WT PTEN. We had found out that the lipid phosphatase activity of PTEN was responsible for inducing autophagy via PI3K/Akt/mTOR axis. Most importantly we had observed (in both PTEN null and PTEN positive cells) that sumoylation of PTEN was aiding in autophagy while phosphorylation was curbing it. This was occurring due to the modulation of PTEN's cell membrane localisation by the PTM where PTEN's enzymatic activity controls autophagy.

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