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Nuclear pool of phosphatidylinositol 4 phosphate 5 kinase 1α is modified by polySUMO-2 during apoptosis

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ABSTRACT

Phosphatidylinositol 4 phosphate 5 kinase 1α (PIP5K) is mainly localized in the cytosol and plasma membrane. Studies have also indicated its prominent association with nuclear speckles. The exact nature of this nuclear pool of PIP5K is not clear. Using biochemical and microscopic techniques, we have demonstrated that the nuclear pool of PIP5K is modified by SUMO-1 in HEK-293 cells stably expressing PIP5K. Moreover, this SUMOylated pool of PIP5K increased during apoptosis. PolySUMO-2 chain conjugated PIP5K was detected by pull-down experiment using affinity-tagged RNF4, a polySUMO-2 binding protein, during late apoptosis.

1. Introduction

Phosphorylation at positions 3–5 of the inositol ring in phosphatidylinositol generates several phosphoinositide species that serve as potent signal transducers in multiple cellular events [1]. One of the important phosphoinositide modifying enzymes, PIP5K adds a phosphate, specifically to the 5th position on the inositol ring having its fourth position previously phosphorylated generating Phosphoinositide 4,5 bisphosphate (PIP2). PIP5K is a 61 kDa protein which migrates at about 68 kDa in SDS PAGE [2]. This enzyme has been shown to be involved in numerous cellular events like cell migration, membrane ruffling etc. Studies from several laboratories demonstrated nuclear localization of PIP5K and implicated a range of functions for it [3,4]. However, none of the above studies gave any indication on the nature of posttranslational modification (PTM) of PIP5K.

The nuclear pool of several cytosolic proteins like the Golgi protein p115 [5] and Actin [6] have been shown to be modified by SUMOylation. The modification involves covalent attachment of small ubiquitin like modifier, SUMO to the ε-amino group in the side chain of lysine in the polypeptide substrate, which results in isopeptide bond formation. SUMO is known to have four paralogues designated SUMO-1 to SUMO-4 [7]. The most intensely studied form, the SUMO-1, is an 11 kDa protein [8]. SUMO-2 and -3 differ from each other by only three N-terminal residues and thus is often referred to by SUMO-2/3. They share approximately 50% protein sequence similarity with SUMO-1 [9]. SUMOylation is a reversible and highly dynamic process [7]. SUMO is conjugated to and de-conjugated from target proteins in a series of steps that involve enzymes corresponding to those of the ubiquitin-pathway. SUMOylation has been linked to pathways as diverse as intracellular trafficking, cell cycle, DNA repair, and RNA metabolism [10].

In the present study, we have used biochemical and microscopic techniques to characterize a differential SUMOylation pattern of PIP5K in normal and apoptotic HEK-293 cells. We have shown that nuclear PIP5K is modified by SUMO-1 in normal cells. Furthermore, during apoptosis the amount of this SUMO-1 conjugated PIP5K is enhanced and PIP5K modified by distinct poly SUMO chains constituted of SUMO-1 and SUMO-2 is also observed.

2. Materials and methods

2.1. Materials

The following antibodies were used: anti-FLAG (SIGMA, F3165), anti-SUMO-1 (SIGMA, S8070), anti-SUMO-2 (SIGMA, S9571), β-actin (CST, 4970), β-tubulin (CST, 2128), anti-Nucleophosmin (ABCAM, FC82291), anti-PARP (CST, 9532), anti-GFP (ABCAM, ab6556). Anti mouse and anti rabbit secondary antibodies conjugated with AlexaFluor 488 (A11001 and A11008 respectively) and 568 (A11004 and A11011 respectively) were purchased from Life Technologies. Secondary antibodies conjugated with Alkaline Phosphatase (A3562 (mouse) and A3687 (rabbit)) were from SIGMA. Inverted Eclipse Ti-U microscope was purchased from Nikon, Japan.

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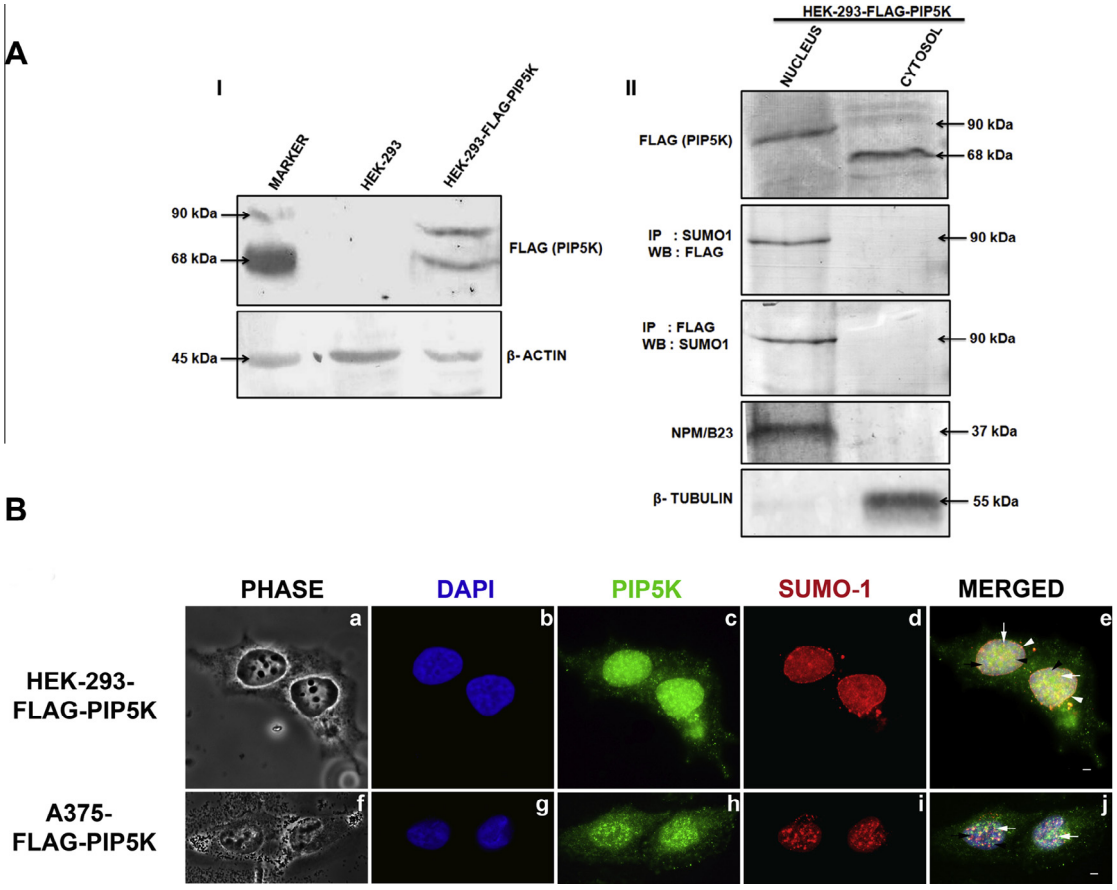

Fig. 1. Nuclear pool of PIP5K is modified by SUMO-1. (A) I: 100 μg whole cell lysate from HEK-293 and HEK-293-FLAG-PIP5K cells were resolved in 10% SDS–PAGE and probed with anti-FLAG and anti β-actin antibodies as indicated. II: Nuclear and cytosolic lysates (100 μg each) from HEK-293-FLAG-PIP5K were run on SDS–PAGE, and probed with anti-FLAG (first panel), anti-nucleophosmin (NPM/B23; nuclear marker) (fourth panel) and β-Tubulin (cytosolic marker) (fifth panel) antibodies. Nuclear and cytosolic lysates from HEK-293-FLAG-PIP5K cells were separately immunoprecipitated with anti-SUMO-1 (second panel) and anti-FLAG (third panel) and were immuno probed with anti-FLAG (second panel) and anti-SUMO-1 (third panel) antibodies respectively. (B): HEK-293-FLAG-PIP5K cells (a–e) and A375-FLAG-PIP5K cells (f–j) were double stained with anti FLAG (green) and anti SUMO-1 antibodies (red). Merged panel (e, j) also include DAPI stained (blue) nuclear images. White arrows in the merged panels (e and j) indicate the yellow/orange colored zones in the nuclei (e and j) and in the nuclear membrane rim (panel e; white arrowhead). Black arrowhead and black arrows represent distinct anti-FLAG & anti-SUMO-1 immunoreactive zones respectively. Scale bar represents 2 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2. Cell culture, transfection and stable lines

HEK-293 and A375 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2. Cells were transfected using Qiagen Effectene transfection reagent according to manufacturer's protocol. Stable cell lines were made by selecting the cells using 500 μg/ml G418 for a week and then maintained at 50 μg/ml G418 concentration.

2.3. Plasmids and constructs

Dr. J. Kunz from BMC, Houston, Texas kindly donated us the constructs for kinase dead mutant of PIP5K (PIP5KD) and human GFP-PIP5K1α. PIP5K was subcloned from GFP-PIP5K1α into pCMV-Tag2B to generate FLAG-PIP5K1α. FLAG-RNF4 was a kind gift from Prof. J. Palvimo, Institute of Biomedicine, University of Eastern Finland.

2.4. Cellular extracts, immuno-precipitaion and Western blot

Cells were harvested by centrifugation at 2500*g* and washed in cold PBS. The cells were then re-suspended in three volumes of Buffer A (15 mM NaCl, 2 mM $MgCl_2$, 20 mM Tris pH 7.5, 1 mM EDTA and 1× Protease Inhibitor (PI) cocktail, Sigma) and was left on ice for 10 min, lysed in a Dounce Homogenisor, checked for proper lysis using trypan blue and centrifuged at 4500*g*. The supernatant was re-centrifuged and collected as the crude cytoplasmic fraction. The pelleted nuclei was resuspended in Buffer A, re-centrifuged and finally re-suspended in ice cold PBS having 1 mM PMSF and 1× PI cocktail. The nuclear fraction was then clarified by brief sonication. All fractions were boiled in 1% SDS for 5 min and finally diluted at 1:10 with PBS for SUMOylation assay. Immunoprecipiatation and immunoblotting were done as described previously [11,12]. Alkaline phosphatase tagged secondary antibodies and NBT/BCIP system was used to detect proteins on the membrane.

2.5. Immunofluorescence and microscopy

Immunofluorescence was done as described previously [13]. AlexaFluor 488 and AlexaFluor 568 conjugated anti-mouse and anti-rabbit secondary antibodies were used for immuno-detection following incubation with appropriate primary antibodies. Mounted cells were imaged using NIKON Inverted Research Microscope ECLIPSE TI-U. Images were processed using NIS-Elements and Adobe Photoshop CS5.

2.6. MTT assay

HEK-293-FLAG-PIP5K and untransfected HEK-293 cells grown in a 96-well (5000 cells in each well) plates were treated with 50 μg/ml etoposide for the indicated duration of time. Three wells were used for each time point. One set of untreated cells were kept as control. After the treatment, the media from each well was replaced by 100 μl fresh media followed by 10 μl of MTT reagent (CCK-8, Sigma). Following which, the readings were collected at an interval of one 1 h up to 4 h in a microplate reader (BioRad; Model No. iMark, USA) according to the manufacturer's protocol. The average readings of one through 4 h for the sets were calculated. The quantitation of the viable cells in control and etoposide treated cells (in triplicate) are represented in a tabular format.

3. Results and discussion

3.1. Nuclear PIP5K is modified by SUMO-1 in vivo

PIP5K is a soluble enzyme and has been shown to be localized both in the cytosol and nucleus [14]. While cytosolic PIP5K has been implicated in several cellular events like endocytosis, membrane ruffling and migration; the nuclear pool has been shown to be involved in pre-mRNA splicing events [4]. Very little has been documented about the PTM(s) of the nuclear PIP5K. Western-blot analysis of the lysate from HEK-293 cell line stably expressing FLAG-PIP5K with anti-FLAG antibody revealed two distinct bands; one around 90 kDa and another at 68 kDa, the latter corresponds to the reported molecular weight of PIP5K (Fig. 1A; I). Analyzing the nuclear and cytosolic lysates from these cells, we observed that the 90 kDa species was exclusively found in the nuclear fraction while the cytosolic fraction retained the 68 kDa band (Fig. 1A; II). The increment of about 22 kDa as seen in these blots can only be explained as covalent attachment of any other macromolecule to PIP5K.

Ubiquitination and SUMOylation could possibly be the modifications that might result in such a shift in mobility in SDS PAGE. In our case, we did not find any evidence for ubiquitination of PIP5K (data not shown). Analysis of human PIP5K protein sequence by the SUMOplot™ (http://www.abgent.com/sumoplot) displays six possible sites for SUMOylation, two of which (K33 and K244) have a probability score higher than 0.80. The increase in 22 kDa can aptly be explained by the addition of two SUMO-1 moieties. Therefore, we wanted to investigate if the nuclear form of PIP5K is modified by SUMO-1. We analyzed the nuclear and cytoplasmic fractions from HEK-293-FLAG-PIP5K cells by immuno-precipitation followed by immuno-blot using anti-SUMO-1 and anti FLAG antibodies respectively and vice versa. The presence of the 90 kDa band in the nuclear fraction and the absence of any band in the cytoplasmic fraction clearly indicated that the nuclear pool

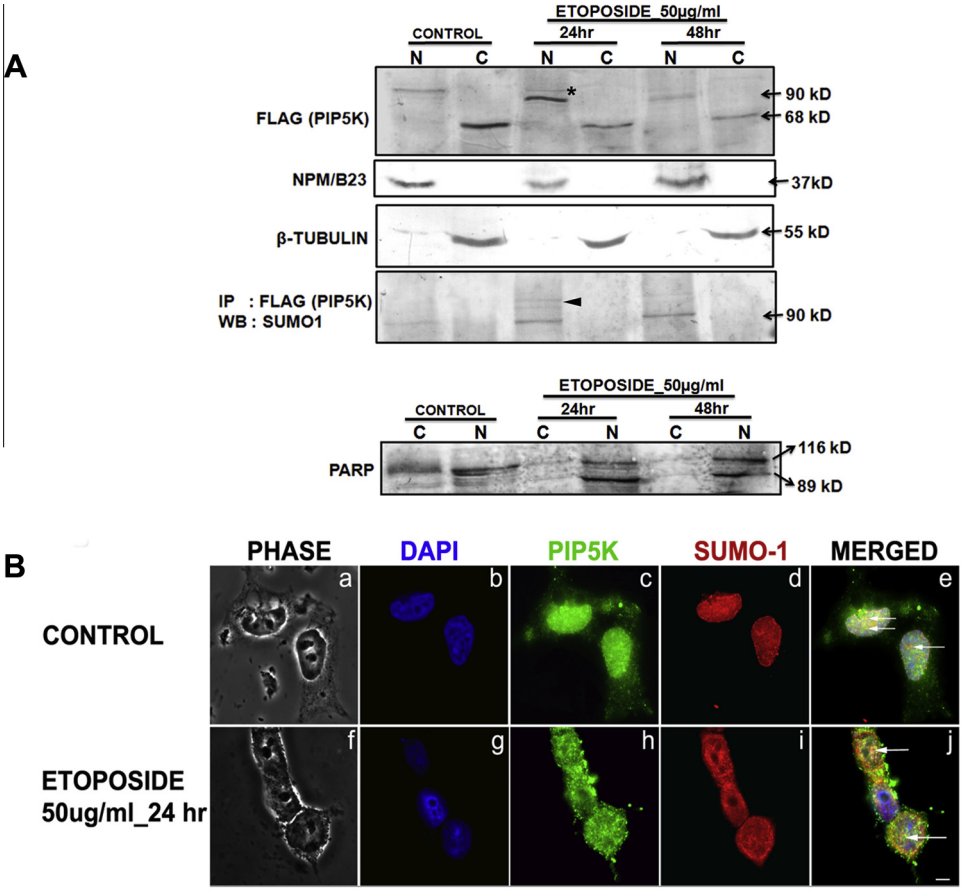

Fig. 2. Enhancement of SUMOylated PIP5K in the nuclei during apoptosis. (A) Nuclear (N) and cytosolic (C) lysates from control and apoptotic (24 h and 48 h) HEK-293-FLAG-PIP5K cells were probed for PIP5K (FLAG), NPM/B23 and β- Tubulin as indicated. (*) in the figure indicates appearance of higher molecular weight species in 24 h apoptotic nuclear fraction. Anti-FLAG immunoprecipitates from nuclear and cytosolic lysates were immunoblotted to probe with anti-SUMO-1 antibody. Arrowhead indicates higher molecular weight forms of PIP5K during 24 h apoptosis. Nuclear and cytosolic lysates were probed with anti-PARP antibody as indicated. (B) Untreated control (a–e) and 50 μg/ml of etoposide treated HEK-293-FLAG-PIP5K cells (f–j) were double stained with anti FLAG (PIP5K) (green; c and h) and anti SUMO-1 (red; d and i) antibodies. Merged panels (e and j) also contain DAPI stained nucleus in blue (b and g). Arrows indicate co-localized PIP5K and SUMO-1. The extent of co-localization in panel e is much less compared to that in panel 'j' indicating higher SUMOylated PIP5K in apoptotic nuclei. Scale bar represents 2 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of PIP5K is modified by SUMO-1 (Fig. 1A; II). To further investigate the SUMOylation of PIP5K, HEK-293-FLAG-PIP5K cells were immuno-stained using anti-FLAG and anti-SUMO-1 antibodies. The co-localization of PIP5K and SUMO-1 in the nucleus strengthens the fact that was derived from the immunoblot experiment above (Fig. 1B; arrows). SUMO conjugates being mostly nuclear [15], very little or no co-localization was seen within the cytoplasm. Discrete nuclear regions immunoreactive to anti-FLAG (Fig. 1B; e and j; black arrowhead) and anti-SUMO-1(Fig. 1B; e and j; black arrow) were also seen. Similar results were obtained using A375 melanoma cells stably expressing FLAG-PIP5K (Fig. 1B). It is notable that SUMO-1 and SUMOylated PIP5K are also localized along the nuclear membrane (Fig. 1B panel e; white arrowheads). Thus, it can be concluded that the majority of the nuclear pool of PIP5K is modified by SUMO-1.

3.2. Enhanced SUMOylation of nuclear PIP5K during apoptosis

Various cytosolic proteins are known to undergo nuclear migration during apoptosis [16,17]. The sub-cellular distribution pattern of PIP5K during apoptosis still remains unclear. To explore the localization of PIP5K during apoptosis, we induced apoptosis in HEK-293-FLAG-PIP5K cells by 50 μM etoposide for indicated times. Cleaved PARP fragment at 89 kDa in the apoptotic nuclear fraction of the treated cells confirmed apoptosis (Fig. 2A). Apoptotic cytosol showed a gradual decrease in the amount of PIP5K which was evident by diminished 68 kDa anti-FLAG immunoreactive band (Fig. 2A) which complied with results from other groups who showed cleavage of cytosolic PIP5K by activated caspase 3 during apoptosis [18]. Consequently, there was an enhanced nuclear localization of SUMOylated PIP5K during 24 h apoptosis (Fig. 2A). In addition to the 90 kDa species, higher molecular weight bands were also seen in the 24 h apoptotic nuclear extract (Fig. 2A; asterisk). Considering the number of SUMOylation sites as predicted by SUMOplot™, the presence of the higher molecular weight bands was indicative of PIP5K being modified by polySUMOylation, i.e., assembly of polymeric chains of SUMO-1. Previous report shows that certain proteins like human topoisomease I undergo polySUMOylation [19]. To confirm the presence of polySUMO-1-PIP5K, anti-FLAG immunoprecipitaes from control and apoptotic lysates were subjected to Western blot with anti-SUMO-1 antibody. The result showed the presence of higher molecular size bands in the 24 h apoptotic nuclear fraction (Fig. 2A; arrowhead) indicating the presence polySUMO-1-PIP5K. Consistent with the biochemical data, immunofluorescence analysis of apoptotic HEK-293-FLAG-PIP5K cells showed a higher degree of co-localization of PIP5K and SUMO-1 in the nucleus (Fig. 2B; panel e; arrows) as compared with the control cells (Fig. 2B, panel j; arrows).

It can be argued that higher protein load and/or increased catalytic activity due to over-expression of PIP5K might result in a forced nuclear translocation and subsequent SUMOylation of

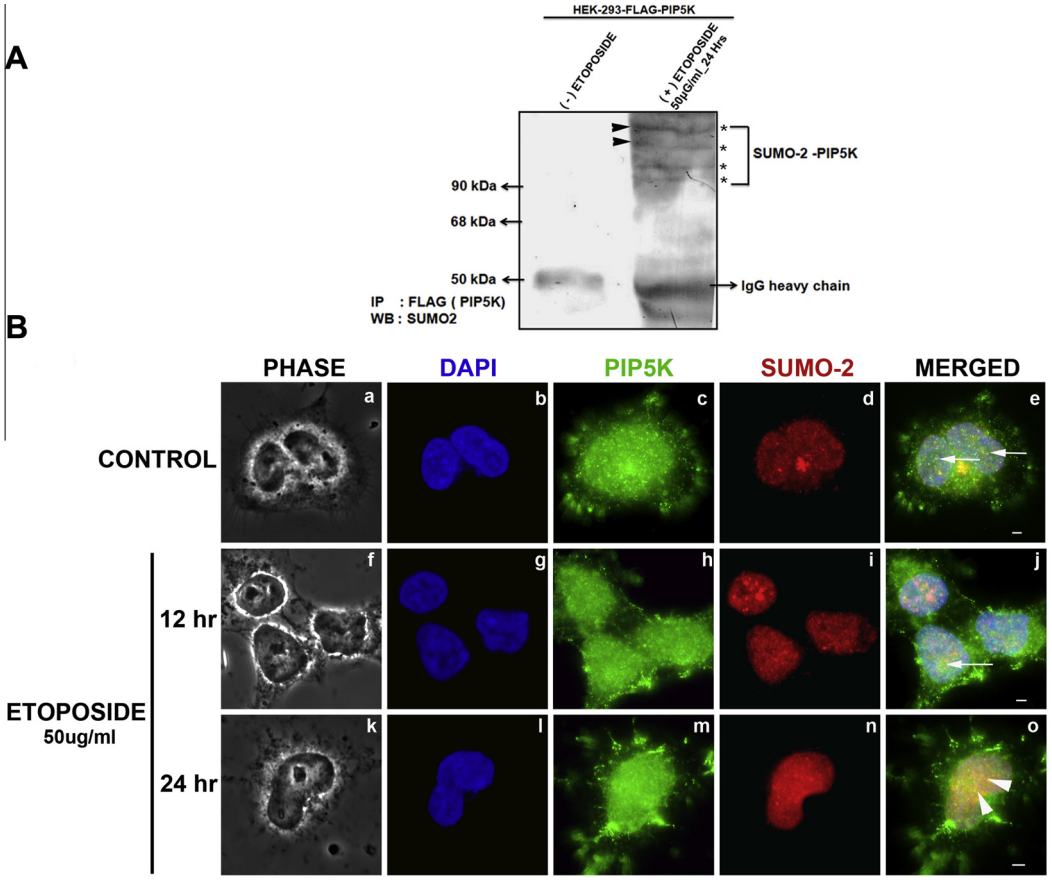

Fig. 3. PIP5K is modified by SUMO-2 mainly during apoptosis. (A) Untreated control and etoposide treated whole cell lysates from HEK-293-FLAG-PIP5K were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-SUMO-2 antibody. SUMO-2 immunoreactive bands were present in apoptotic fraction (asterisk). The arrowheads in the treated fraction indicate higher molecular weight species of SUMO-2 modified PIP5K. Arrow at 50 kDa in both the lanes indicates the IgG heavy chains used. (B) Control (a–e), 12 h (f–j) and 24 h etoposide treated (k–o) HEK-293-FLAG-PIPK cells were double stained for PIP5K (green) and SUMO-2 (red). Nuclei (b, g and l) have been DAPI stained and shown in blue. Arrows in panel e and j indicate little or no co-localization of PIP5K and SUMO-2. In contrast, PIP5K and SUMO-2 are highly co-localized in the 24 h apoptotic nuclei (panel j; arrowheads). Scale bar represents 2 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

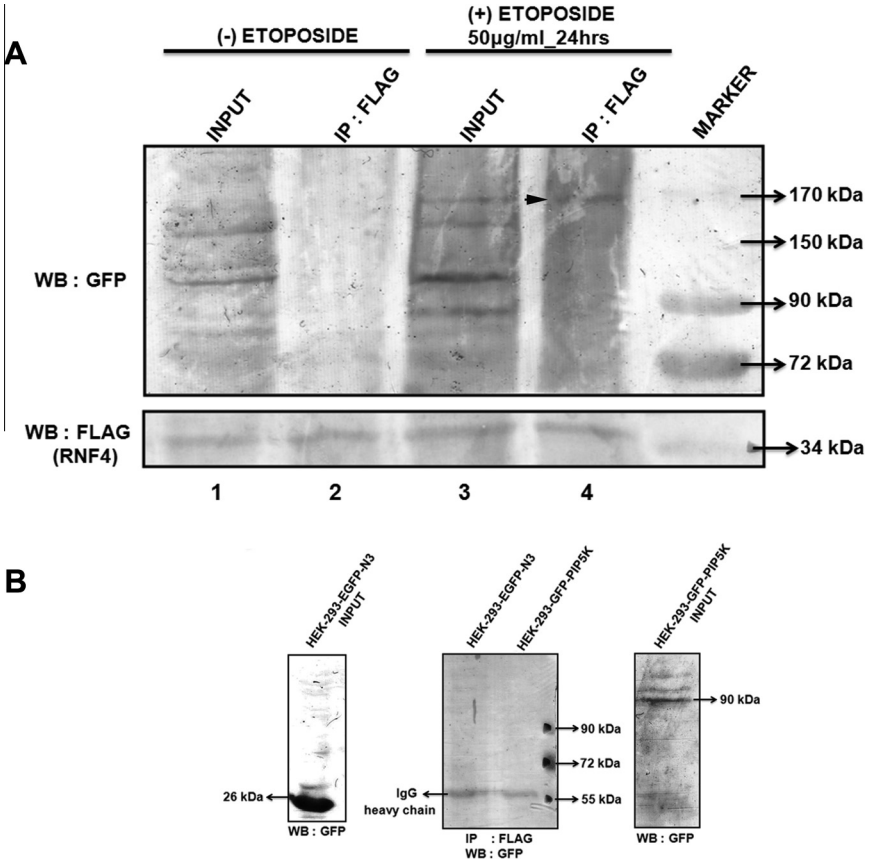

Fig. 4. PIP5K is also modified by polySUMO-2 chains. (A) GFP-PIP5K was transiently transfected to HEK-293 cells stably expressing FLAG-RNF4 following which they were treated with etoposide to induce apoptosis. Lysates of untreated control (lanes 1 and 2) and treated cells (lanes 3 and 4) were immunoprecipitated using anti-FLAG antibody and resolved in 7.5% SDS–PAGE (lanes 2 and 4). The gel was immunoblotted with anti-GFP and anti-FLAG antibodies to detect GFP-PIP5K (upper panel) and RNF4 (lower panel) respectively. Input samples for immunoprecipitation were analyzed in the same SDS–PAGE (lanes 1 and 3). Arrow head in lane 4 indicates anti-FLAG immunoreactive higher molecular weight species of GFP-PIP5K (upper panel). The lower panel represents RNF4 in the input lysates (lanes 1 and 3) and immunoprecipitates (lanes 2 and 4). (B) Left panel: Cell lysate of HEK-293 cells transiently expressing GFP that was immuno-blotted with anti-GFP antibody showing GFP at 26 kDa. The middle panel shows anti-FLAG immuno-precipitates from HEK-293-EGFP-N3 and HEK-293-GFP-PIP5K cells probed with anti-GFP antibody. The heavy chain of the antibody band has been marked at around 55 kDa region. The right panel represents Western-blot of HEK-293-GFP-PIP5K whole cell lysate using anti-GFP antibody detecting GFP-PIP5K at 90 kDa.

PIP5K. To verify, we have stably over-expressed a FLAG tagged PIP5KD (KD; kinase dead) in HEK-293 cells. Immunofluorescence analysis of these cells showed that PIP5KD was highly secluded from the nucleus both in control (Fig. S1, panel c; arrowheads) and apoptotic cells (Fig. S1, panel h; arrowheads). The merged panels (Fig. S1; panel e and j; arrows) also showed that the SUMO-1 specks did not co-localize with PIP5KD in the nuclei. This result ruled out the possibility that higher protein load had caused the accumulation of the SUMOylated form(s) in the nucleus.

3.3. PIP5K is modified by SUMO-2, mainly during apoptosis

While some substrates are preferentially modified by one SUMO isoform, there are others that can be modified by both SUMO-1 and SUMO-2/3 [20]. It has been documented that various cellular stresses cause increase of global SUMO-2/3 conjugation [21–23]. To see whether PIP5K was modified by SUMO-2 during apoptosis, we subjected anti-FLAG immuno-precipitates from lysates of control and apoptotic HEK-293-FLAG-PIP5K cells to Western blot with anti-SUMO-2 antibody. PIP5K was appreciably modified by SUMO-2 as seen from SUMO-2 immunoreactive bands present only in the apoptotic fraction (Fig. 3A; asterisk). Among these, the bands of much higher molecular size are indicative of PIP5K modified by polySUMO-2 chains (Fig. 3A; arrowheads). Immuno-fluorescence analysis revealed very meager co-localization of PIP5K and SUMO-2 both in untreated and 12 h apoptotic cells (Fig. 3B; panels e and j, arrows). On the other hand, the merged panel of 24 h apoptotic cells showed substantial co-localization of PIP5K and SUMO-2 in the nucleus (Fig. 3B: panel o; arrowhead). It is to be noted that almost 90% of HEK-293-FLAG-PIP5K cells were viable after 12 h of etoposide treatment as contrary to around 77% for untransfected HEK-293 cells. This percentages of viable cells were about 50 for both the cell types at the end of 24 h treatment (Supplementary Table 1). This result shows that modification of PIP5K by SUMO-2 is related to the extent of apoptotic cell death.

3.4. PIP5K is modified by polySUMO-2 only during apoptosis

Recent studies showed that polySUMO-2 modified proteins were efficiently detected by pull-down experiments using affinity tagged RNF4, a specific E3 ubiquitin ligase which is known to bind preferentially to polySUMO-2 rather than SUMO-2 monomers [24,25]. We, therefore, adopted a similar strategy to investigate whether the higher order SUMO-2 immunoreactive bands as seen in Fig. 3A were polySUMO-2 conjugated PIP5K. To do that we created a stable HEK-293 cell line expressing FLAG-RNF4. We transiently transfected this cell line with GFP-PIP5K and subsequently induced apoptosis with 50 µg/ml etoposide. Anti-FLAG immunoprecipitates from untreated (Fig. 4A; lane 2) and apoptotic (Fig. 4A; lane 4) cells were subjected to Western blot with anti-GFP (Fig. 4A; upper panel) and anti-FLAG (Fig. 4A; lower panel) antibodies. The GFP immunoreactive species of PIP5K (at around 170 kDa as opposed to the native size of 90 kDa) was only seen in the immunoprecipitate from apoptotic lysates (Fig. 4A;

upper panel, lane 4) whereas it was almost undetectable in the untreated cell lysate (Fig. 4A; upper panel, lane 2). This typically confirmed the formation of polySUMO-2 conjugated PIP5K only during apoptosis. As control, we have subjected anti-FLAG immunoprecipitates obtained from HEK-293 cells expressing either EGFP-N3 or GFP-PIP5K to Western blot using an anti-GFP antibody which yielded no immune-reactive species (Fig. 4B; middle panel). This shows that the anti FLAG antibody did not non-specifically pull down GFP or GFP tagged proteins.

Taken together, the present study shows that the majority of the nuclear PIP5K is modified by SUMO-1. Induction of apoptosis not only results in an enhancement of SUMO-1 modified PIP5K but also gives rise to its polySUMO-1 conjugated forms in the nucleus. Moreover, our results demonstrate that only apoptotic nuclei contains SUMO-2 and polySUMO-2 modified PIP5K; the latter being confirmed by its binding with RNF4, a specific polySUMO-2 binding protein. Although SUMO-2 modification of proteins was shown to occur during different types of stress including apoptosis, our study demonstrates the conjugation of polySUMO-2 to a protein during apoptosis. This study shows that SUMO-2 conjugated PIP5K is clearly visible in cells after 24 h of etoposide treatment but only marginally detectable in the treated cells after 12 h (Fig. 3B). In addition to this, polySUMO-2 conjugated PIP5K was found in the cells treated for 24 h (Fig. 4A; upper panel, lane 4). Interestingly, results from MTT assay reveals that cells stably expressing PIP5K are resistant to apoptotic death for up to 12 h; while the extent of viable cells after 24 h is highly comparable to that of the untransfected counterparts (Supplementary Table 1). Reports from other laboratories have shown that the degradation of PML is induced by its modification with polySUMO-2 and its subsequent interaction with RNF4 [25]. This result in conjunction with ours indicates a correlation between polySUMO-2 conjugation of PIP5K and its probable degradation by RNF4, a polySUMO2 specific E3 ubiquitin ligase during mid to late apoptosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.058.

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