



J Biol Chem. 2009 March 13; 284(11): 6946–6954.

PMCID: PMC2652289

doi: [10.1074/jbc.M805801200](https://doi.org/10.1074/jbc.M805801200)

## PHAPI/pp32 Suppresses Tumorigenesis by Stimulating Apoptosis<sup>\*</sup>

[Wei Pan](#),<sup>‡,1</sup> [Li S. da Graca](#),<sup>‡,1</sup> [Yufang Shao](#),<sup>‡</sup> [Qian Yin](#),<sup>§¶</sup> [Hao Wu](#),<sup>§¶</sup> and [Xuejun Jiang](#)<sup>‡§,2</sup>

<sup>‡</sup>Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 and the <sup>§</sup>Graduate School of Biomedical Sciences, and <sup>¶</sup>Department of Biochemistry, Weill Medical College of Cornell University, New York, New York 10021

<sup>1</sup>Both authors contributed equally to this work.

<sup>2</sup>To whom correspondence should be addressed: Cell Biology Program, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., Box 522, New York, NY 10021. Tel.: 212-639-6814; Fax: 212-717-3298; E-mail: [jiangx@mskcc.org](mailto:jiangx@mskcc.org).

Received July 29, 2008; Revised November 21, 2008

Copyright © 2009, The American Society for Biochemistry and Molecular Biology, Inc.

### Abstract

PHAPI/pp32 is a tumor suppressor whose expression is altered in various human cancers. Although PHAPI possesses multiple biochemical activities, the molecular basis for its tumor-suppressive function has remained obscure. Recently we identified PHAPI as an apoptotic enhancer that stimulates apoptosome-mediated caspase activation. In this study, we defined the structural requirement for its activity to stimulate caspase activation using a series of truncation mutants of PHAPI. Further, utilizing these mutants, we provide evidence to support the model that the apoptotic activity of PHAPI is required for its tumor-suppressive capability. Consistently, pp32R1, a close homolog of PHAPI and yet an oncoprotein, is not able to stimulate caspase activation. A highly discrete region between these two proteins localizes to an essential caspase activation motif of PHAPI. Additionally, PHAPI is predominantly a nuclear protein, and it can translocate to the cytoplasm during apoptosis. Disruption of the nuclear localization signal of PHAPI caused a modest decrease of its tumor-suppressive function, indicating that nuclear localization of PHAPI contributes to, but is not essential for, tumor suppression.

Apoptosis plays a crucial role in multiple physiological events, and its deregulation can lead to various human diseases, including cancer, neurodegeneration, and autoimmune disorders (1, 2). Apoptosis is executed by a family of proteases known as caspases (3, 4). In mammals, a major caspase activation pathway is the mitochondrial cytochrome *c*-mediated caspase activation pathway, also known as the intrinsic apoptotic pathway (5). In this pathway, a variety of apoptotic stimuli induce release of cytochrome *c* from mitochondria to the cytoplasm, a process controlled by the Bcl-2 family members (6–8). The released cytochrome *c* binds to Apaf-1, the essential mediator of the pathway, and triggers its nucleotide binding/exchanging activity (9, 10). Subsequently, the activated Apaf-1 protein forms a multimeric protein complex called the apoptosome, which in turn recruits and activates the initiator caspase, caspase-9. The active apoptosome-caspase-9 holoenzyme (9, 11) then cleaves and activates the effector caspases, including caspase-3 and caspase-7, and therefore causes apoptotic cell death.

The cytochrome *c*-mediated caspase activation pathway is under precise regulation. For example, in the upstream of mitochondria, there are both pro-apoptotic and anti-apoptotic Bcl-2 family proteins that dictate mitochondrial cytochrome *c* release (6–8). In the downstream of mitochondria, inhibitor of apoptosis proteins negatively regulate apoptosis by inhibiting caspase activity (12–15). The function of inhibitor of apoptosis proteins can be antagonized by Smac/Diablo (16, 17) and Omi/Htr2A (18, 19), which are also released from mitochondria during apoptosis. Further, nucleotide binding/exchanging of Apaf-1, a key biochemical event of the pathway, is also closely regulated. Recently, we found that the Apaf-1-dependent caspase activation can be enhanced by PHAPI (putative HLA-associated protein-I, also known as pp32) (20). Biochemically, PHAPI along with cellular apoptosis susceptibility and the heat shock protein HSP70, function as the nucleotide exchange factor for Apaf-1, a mechanism reminiscent of G protein regulation (21). Interestingly, PHAPI is predominantly a nuclear protein (22) and can translocate to the cytoplasm during apoptosis, a process involving the RNA-binding protein HuR (23). Therefore, subcellular translocation of PHAPI provides an additional regulatory mechanism for PHAPI-stimulated caspase activation.

In addition to its activity to promote apoptosis, PHAPI has been shown to possess other biochemical activities, including inhibition of protein phosphatase 2A (PP2A)<sup>3</sup> and histone acetyltransferase (HAT), both crucial regulators for various physiological processes (24, 25). PHAPI can also interact with Ataxin-1, a protein mutated in the neurodegenerative disease spinocerebellar ataxia type I (26), suggesting a possible role of PHAPI in this type of neurodegeneration.

The multiple biochemical activities of PHAPI suggest that this protein is involved in diverse biological events. To date, the most defined biological function of PHAPI is its role as a tumor suppressor. PHAPI expression appears to be greatly decreased during pancreatic cancer progression (27). In a study of human non-small cell lung cancer, PHAPI expression level correlated with improved outcome following chemotherapy (28). Functionally, overexpression of PHAPI can inhibit oncogene-induced tumorigenesis (22, 29, 30). Intriguingly, PHAPI has a close homolog known as pp32R1 (pp32-related-1), which is an oncoprotein instead of a tumor suppressor (29, 31). The expression of pp32R1 is switched on in certain human cancers with diminished PHAPI expression (31, 32).

The biochemical activity of PHAPI responsible for its tumor-suppressive function has not been defined. Although both PP2A and HAT have been implicated in cancer development, whether PHAPI is able to suppress tumorigenesis via inhibiting these two enzymes is not clear. On the other hand, the apoptosis-accelerating activity of PHAPI provides a plausible explanation for its tumor-suppressive role, which is consistent with recent studies showing that in human breast cancer and non-small cell lung cancer, PHAPI expression level correlates with the sensitivity of cancer cells to Apaf-1-mediated apoptosis (28, 33). To directly examine this possibility, we determined the structural requirement for the apoptotic activity of PHAPI and provided strong evidence to support the model that the apoptotic activity of PHAPI is required for its tumor-suppressive function.

## EXPERIMENTAL PROCEDURES

---

*Subcloning and Recombinant Protein Generation*—Full-length PHAPI was cloned into pET-28a(+) vector (Novagen) as described previously (20). The DNA fragments encoding for individual deletion mutants of PHAPI were generated by PCR and subsequently cloned into BamHI/SalI sites of pET-28a(+) (Novagen) for expression of recombinant proteins. The nuclear localization defective mutant of PHAPI (NLDM) was generated by site-directed mutagenesis (residues 236–239, KRKR mutated to

KLER). All plasmids were confirmed by DNA sequencing. Recombinant PHAPI and mutants were expressed as His<sub>6</sub>-tagged proteins in BL21(DE3) strain and purified using nitrilotriacetic acid-agarose (Qiagen) followed by Q-Sepharose chromatography. The purified proteins were dialyzed against Buffer A (20 mM HEPES, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol), and stored at -80 °C in small aliquots.

PCR cloning was performed to generate plasmids for mammalian expression of differentially tagged PHAPI, including pFLAG-CMV-PHAPI (using BglII/SalI sites), pCDNA3.1-hygro-HA-PHAPI (BamHI/SalI), pCMV5-myc-PHAPI (BglII/SalI), and pEGFP-C1-PHAPI (BglII/SalI). To generate pWZL-hygro-PHAPI and its mutants for retroviral infection, the coding sequences were cut out by BamHI/SalI from the corresponding pcDNA3.1-hygro-HA constructs and then inserted into pWZL-hygro through the same sites. All plasmids were confirmed by DNA sequencing.

*Assay for PHAPI-stimulated Caspase Activity*—The activity of PHAPI and its mutants to stimulate caspase activation was measured as described previously (20). This assay requires a Q-chromatographic fraction of HeLa cell extracts (HeLa S-100) that we named Q30. To prepare Q30, 10 ml of HeLa S-100 (~60 mg of total protein) was loaded onto a 1-ml HiTrap Q column (Amersham Biosciences) pre-equilibrated with Buffer A. After sample loading, the column was washed with 10 ml of Buffer A. Subsequently, the column was eluted with Buffer A containing 300 mM NaCl, and the eluted protein peak (Q30, ~4 ml) was collected. The Q30 fraction was stored at -80 °C in small aliquots.

The *in vitro* assay for the apoptotic function of PHAPI and its truncation mutants was conducted in a 20- $\mu$ l Buffer A system containing 3  $\mu$ l of Q30, 100 nM cytochrome *c*, 10  $\mu$ M dATP, 100  $\mu$ M DEVD-aminomethylcoumarin substrate (Calbiochem), and 500 nM recombinant PHAPI or its truncation mutants as indicated. The reaction mixtures were incubated at 30 °C using a Xfluo4 spectrometry reader (TECAN, Inc.), and generation of fluorescent signal (relative fluorescent units) as a result of cleavage of DEVD-aminomethylcoumarin by caspase-3, was measured automatically every 10 min at wavelengths of 360/465 nm (excitation/emission). Alternatively, caspase-3 activity can also be detected using the *in vitro* translated, [<sup>35</sup>S]methionine-labeled caspase-3 as the substrate in a 60-min reaction.

*Light Scattering Analysis*—Molar mass of purified Recombinant PHAPI was determined by static multiangle light scattering. Protein was injected onto a Superdex 200 HR 10/300 gel filtration column equilibrated in Buffer A. The chromatography system was coupled to a three-angle light scattering detector (mini-DAWN EOS) and refractive index detector (Optilab DSP, Wyatt Technology). The data were collected every 0.5 s at a flow rate of 0.25 ml/min. Data analysis was carried out using the ASTRA program (Wyatt Technology), yielding the molar mass and mass distribution (polydispersity) of the sample.

*Coimmunoprecipitation*—HeLa cells were grown in 60-mm plates and maintained in Dulbecco's modified Eagle's medium containing 10% of fetal bovine serum. As indicated, plasmids encoding Myc-tagged PHAPI, FLAG-tagged PHAPI, or vector controls (1  $\mu$ g each) were transiently transfected into HeLa cells with FuGENE 6 transfection reagent (Roche Applied Science). The cells were harvested 24 h after transfection. Cells were lysed by three cycles of freeze-and-thaw in Buffer L (20 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM  $\beta$ -mercaptoethanol, and 0.2% Nonidet P-40) containing protease inhibitor mixture (Roche Applied Science). Subsequently the cell extracts were subjected to immunoprecipitation using protein-A-agarose coupled with 9E10 anti-Myc antibody for overnight. The immunoprecipitates and corresponding inputs were analyzed by Western blot against Myc, FLAG, and histone antibodies as

indicated.

*PHAPI Translocation upon Stress Treatment*—HeLa cells were grown in 60-mm plates and maintained in Dulbecco's modified Eagle's medium containing 10% of fetal bovine serum. One microgram of plasmid encoding GFP-conjugated PHAPI wild-type (WT), GFP-conjugated PHAPI NLDM mutant, or GFP alone was transiently transfected into HeLa cells with FuGENE 6 transfection reagent (Roche Applied Science). After 24 h of transfection, the cellular localization of the GFP proteins was detected by fluorescent microscopy. To test PHAPI translocation upon stress treatment, the HeLa cells expressing GFP-PHAPI (WT) were treated either with 0.25  $\mu\text{M}$  staurosporine (Sigma) or 0.25  $\text{J}/\text{cm}^2$  of UV light using a UV cross-linker (Stratagene). Pictures were taken at the indicated time points.

*Soft Agar Colony Formation Assay*—Retroviruses were generated using pBABE-puro-Ras, pBABE-puro vector, pWZL-hygro-Myc, pWZL-hygro vector, pWZL-hygro-PHAPI, and its mutants as indicated. Primary mouse embryonic fibroblasts (cultured for three passages after isolation from mice) were plated in 10-cm plates ( $2 \times 10^5$ /plate). After overnight incubation, they were infected by three rounds of infection (4 h for each round) with indicated retroviruses. Forty-eight hours after infection, cells were allowed to grow for 3 days in medium containing 2  $\mu\text{g}/\text{ml}$  puromycin and 50  $\mu\text{g}/\text{ml}$  hygromycin B. After recovering from drug selection for 12 h in antibiotics-free medium, viral-infected cells were trypsinized and plated for soft agar colony-formation assay. For each assay,  $5 \times 10^5$  viral-infected cells were plated with soft agar medium to individual wells in 6-well plates. The plates were incubated for 2 weeks, and phase-contrast microscopic pictures were taken for each sample using a digital camera coupled to a microscope to show colony formation. Quantification and standard deviation were obtained from results of three independent experiments.

*Suspension Cell Culture and Measurement of Cellular Apoptosis*—One million of each infected mouse embryonic fibroblasts as used in the soft agar colony formation assay were suspended in 2 ml of suspension medium (Dulbecco's modified Eagle's medium containing 1% of bovine serum albumin and 0.5% of methyl cellulose), and then seeded into the Ultra Low Cluster 6-Well Plate (Corning Inc.) and incubated in a 37 °C incubator with 5%  $\text{CO}_2$ . Under this condition, cells are under suspension and anchorage-independent condition. Plates were periodically agitated to avoid cell aggregation. After 8 h of incubation, cells were harvested and lysed into Buffer A containing 140 mM additional KCl, 1% Nonidet P-40, and protease inhibitors. Caspase-3 activity in the cell extracts were measured to monitor suspension-triggered apoptosis in the cells expressing PHAPI or its individual mutants. The assay was conducted using 20  $\mu\text{g}$  of individual cell extracts and 100  $\mu\text{M}$  DEVD-aminomethylcoumarin substrate (Calbiochem) in 20- $\mu\text{l}$  Buffer A containing 140 mM additional KCl. The reaction mixtures were incubated at 30 °C using a Xfluo4 spectrometry reader (TECAN, Inc.), and generation of fluorescent signal (relative fluorescent units) as a result of cleavage of DEVD-aminomethylcoumarin by caspase-3, was measured automatically every 10 min at wave-lengths of 360/465 nm (excitation/emission).

## RESULTS

---

*Domain Analysis of PHAPI*—We previously identified PHAPI/pp32 as a regulatory component of the mitochondria-mediated caspase activation pathway (20). PHAPI functions to enhance the activity of the apoptosome complex to activate caspase-9. However, PHAPI does not have any recognizable motif that can directly link to apoptosis. Therefore, to further understand the role of PHAPI in apoptosis, we sought to determine the domain structures of PHAPI required for its activity to stimulate caspase activation.

PHAPI contains a short N-terminal fragment followed by four leucine-rich repeats (LRR1 to LRR4), and a short linker region (M region) connecting LRR4 to the highly negatively charged C terminus (Fig. 1A). We expressed and purified recombinant full-length PHAPI protein and a series of mutants with individual regions of the protein truncated (Fig. 1, A and B). Subsequently, we examined the activity of these recombinant proteins in stimulating Apaf-1/cytochrome *c*-mediated caspase activation. To do so, we used a chromatographic fraction (Q30) from HeLa cell cytosolic extracts and several other defined factors to reconstitute the caspase activation process (20). The Q30 fraction contains procaspase-9, procaspase-3, Apaf-1, and other required protein factors for the apoptotic activity of PHAPI (20, 21). Caspase activation was triggered by addition of 100 nM purified cytochrome *c* and 10  $\mu$ M nucleotide dATP and was measured by using cleavage of either a [<sup>35</sup>S]methionine-labeled procaspase-3 (Fig. 1C) or a fluorogenic peptide substrate of caspase-3 (Fig. 1D) as the readout.

Based on both functional assays (Fig. 1, C and D), we found that there are two regions of PHAPI protein essential for its apoptotic activity. One is the acidic C-terminal fragment, and the other is the short M region immediately flanking the C-terminal tail. LRR3 and LRR4 also appear to contribute to the apoptotic activity of PHAPI, because deletion of these two LRRs caused a modest but reproducible decrease of caspase activation measured by both assays (Fig. 1, C and D). Importantly, a truncated protein containing only the M region and the C-terminal tail of PHAPI, the two essential regions for the apoptotic activity of PHAPI, is sufficient to support the *in vitro* caspase stimulation activity of PHAPI (Fig. 1E).

*PHAPI but Not pp32R1 Can Stimulate Caspase Activation*—PHAPI has a close homologous protein, pp32R1 (Fig. 2A). Intriguingly, instead of being a tumor suppressor like PHAPI, pp32R1 is an oncogene (29, 31). We generated purified recombinant pp32R1 and examined its effect on Apaf-1-mediated caspase activation (Fig. 2B). Contrary to PHAPI, pp32R1 does not possess such apoptotic activity. This result, combined with the fact that PHAPI is a tumor suppressor, whereas pp32R1 is an oncoprotein, strongly suggests that the apoptotic activity and the tumor-suppressive capability of PHAPI have a causal relationship.

Mechanistically, why is pp32R1 not able to stimulate caspase activation albeit being highly homologous to PHAPI? We reason that such functional difference might be related to the most diverse region of these two proteins, correlating to residues 140–163 of PHAPI (Fig. 2A). These residues are within the M region of PHAPI, which is essential for its apoptotic activity. To test this possibility, we generated a mutant form of PHAPI recombinant protein in which residues 140–163 were swapped with the correspondent residues of pp32R1 (we named this mutant as PRP, Fig. 2C). As expected, the PRP mutant of PHAPI indeed showed a much decreased activity to enhance caspase activation (Fig. 2D).

Because pp32R1 is so similar to PHAPI but instead possesses oncogenic function, is it possible that pp32R1 promotes tumorigenesis by competing for the functional partner of PHAPI and thereby inhibiting its apoptotic activity? This turns out to not be the case, because in our *in vitro* caspase assay, neither pp32R1 nor the inactive PHAPI deletion mutants,  $\Delta$ M and  $\Delta$ C, can inhibit the activity of the full-length PHAPI to stimulate caspase activation (Fig. 3).

There is another potential mechanism that might explain the oncogenic function of pp32R1. It was reported that PHAPI is a homo-trimeric protein based on its behavior in gel-filtration chromatography (34). We also observed that both native PHAPI purified from HeLa cell extracts and recombinant PHAPI were resolved in early fractions of gel-filtration chromatography, suggesting a large complex formed by

homo-oligomerization (data not shown). If indeed PHAPI is a homo-trimer in cells and the homo-trimerization is required for its apoptotic activity, then one possibility for pp32R1 to abrogate this activity is to form inactive hetero-oligomers with PHAPI in cells. This potential activity would evade detection by our *in vitro* assay, because we used purified, recombinant PHAPI, which presumably was a stable homo-trimer before mixing with pp32R1.

However, gel-filtration chromatography alone is not sufficient to determine whether PHAPI is an oligomer or not, because, in addition to molecular weight, other parameters such as the shape of a protein will also affect its gel-filtration chromatographic behavior. Therefore, we performed additional experiments to examine this possibility. Firstly, we performed coimmunoprecipitation experiment to examine whether differentially tagged PHAPI can interact with each other when co-transfected into HeLa cells. As shown in [Fig. 4A](#), Myc-tagged PHAPI and FLAG-tagged PHAPI failed to interact with each other, whereas Myc-tagged PHAPI did co-precipitate endogenous histone, a previously reported interacting partner of PHAPI ([25](#)). This result argues against the possibility that PHAPI can form a homo-oligomer in cells. More conclusively, we measured the molecular mass of purified recombinant PHAPI by light scattering technique coupled with gel-filtration chromatography ([Fig. 4B](#)), the result (29.2 kDa) indicates that active PHAPI is a monomeric protein. Light scattering analysis also indicates that pp32R1 is a monomeric protein (data not shown). Therefore, our results suggest that pp32R1 exerts its oncogenic function through a mechanism not directly related to the apoptotic function of PHAPI.

*The Apoptotic Activity of PHAPI Contributes to Its Tumor-suppressive Function*—PHAPI has been shown to be a tumor suppressor, but the underlying molecular basis was not determined. Our previous finding that PHAPI can enhance Apaf-1-mediated caspase activation provided a potential mechanistic explanation ([20](#)). The fact that the closely related pp32R1 is an oncoprotein and has no effect on caspase activation ([Fig. 2](#)) further supports this model. In addition, it was reported recently that, in certain human cancers, expression levels of PHAPI correlated with sensitivity of cancer cells to apoptosis ([28](#), [33](#)). All these data are consistent with the possibility that the apoptotic activity of PHAPI is required for its tumor-suppressive function. To further test this hypothesis, we performed a soft agar colony formation assay to measure the tumor-suppressive function of a panel of PHAPI mutants with apoptotic activity defined in [Fig. 1](#). With such an assay, we can determine whether the apoptotic activity of PHAPI is correlated with its tumor-suppressive function.

In this assay, we utilized p53<sup>-/-</sup> mouse embryonic fibroblasts and induced tumorigenesis by introducing oncogenic Ras and Myc genes into the cells. The tumorigenic, anchorage-independent growth of the cells was measured based on their capability to form cell colonies in soft agar media. When full-length PHAPI or the individual truncation mutants are expressed in these cells, the tumor-suppressive function of these proteins to decrease soft agar colony formation can be detected.

As shown in [Fig. 5A](#), oncogenic Ras and Myc triggered potent cell transformation, and their oncogenic activity was dramatically suppressed by co-expression of the full-length PHAPI. Importantly, the truncation mutants of PHAPI that still possess intact apoptotic activity (such as  $\Delta$ LRR1 in [Fig. 5A](#), and data not shown) were also able to suppress Ras/Myc-induced tumorigenesis, whereas the two other mutants that do not have the apoptotic activity ( $\Delta$ M and  $\Delta$ C) completely lost the tumor-suppressive function. Therefore, the apoptotic activity of PHAPI correlates with its tumor-suppressive function.

To further examine whether the apoptotic activity of PHAPI indeed contributes to its tumor-suppressive function under this anchorage-independent growth condition, we measured the influence of PHAPI and

its mutants on apoptosis triggered by lack of anchorage in these Ras/Myc-expressed cells. To achieve anchorage independence, cells were grown under suspension condition. This condition mimics the soft agar growth condition but allows convenient cell harvesting and subsequent apoptosis measurement, which is rather difficult for soft agar condition. As shown in [Fig. 5B](#), 8 h after suspension growth, suspension-triggered apoptosis was monitored by measuring caspase-3 activity in individual cell extracts. Overexpression of wild-type PHAPI potently enhanced apoptosis induced by anchorage-independent condition, whereas  $\Delta M$  and  $\Delta C$ , two apoptosis-deficient mutants, failed to enhance apoptosis. Further,  $\Delta LRR1$ , which had a weaker tumor suppressive capability than the full-length PHAPI ([Fig. 5A](#)), possessed weaker cellular apoptosis activity as well ([Fig. 5B](#)). Based on this result, we conclude that the apoptotic activity of PHAPI contributes to its tumor-suppressive function under our assay condition. Because of the exquisite correlation between the tumor-suppressive function and the cellular apoptotic activity of PHAPI and its truncation mutants, it is highly possible that the apoptotic activity of PHAPI is the major causal reason for its tumor-suppressive function.

It is noteworthy that the cellular apoptotic activity of  $\Delta LRR1$  is weaker than that of the full-length PHAPI ([Fig. 5B](#)), although in the *in vitro* analysis they showed similar activity ([Fig. 1, C and D](#)). Therefore we propose that in cells there might be additional regulation of the apoptotic activity of PHAPI through its LRR1 domain.

*The Role of PHAPI Nuclear Localization in Apoptosis and Tumor Suppression*—Consistent with previous reports ([22](#), [23](#)), we also found that PHAPI is predominantly a nuclear protein when expressed in cells ([Fig. 6A](#)). This property of PHAPI immediately raised several questions. First, can PHAPI translocate to the cytoplasm to regulate apoptosome-mediated caspase activation, presumably a cytoplasmic event? Additionally, is the nuclear localization of PHAPI required for its tumor-suppressive function?

To examine whether nuclear export of PHAPI can be triggered in response of apoptosis, we expressed GFP-PHAPI in HeLa cells and then treated cells with multiple apoptotic stimuli. We found that both UV light and staurosporine induced significant nuclear export of GFP-PHAPI when compared with cells without treatment ([Fig. 6B](#)). This result is consistent with the recent report showing induction of PHAPI nuclear export by staurosporine ([23](#)). It should be noted that the UV/staurosporine-induced GFP-PHAPI nuclear export phenomenon was detected only in low percentage of the transfected cells. This might be due to several reasons. First, only ~20% of cells underwent apoptosis under these conditions (data not shown); second, many apoptotic cells shrank and floated. These cells contain very little space for the cytoplasm, and thus it is difficult to determine GFP-PHAPI nuclear export in these cells. Third, because PHAPI is a regulatory but not obligatory component of the mitochondrial caspase pathway, it is highly likely that its nuclear export is efficiently stimulated only by certain specific signals, which is an important question to address for understanding the role of PHAPI in apoptosis and tumor suppression.

To determine the role of the nuclear localization of PHAPI in its tumor-suppressive function, we generated a mutant form of PHAPI in which its putative nuclear localization signal on its C terminus was mutated (residues 236–239, KRKR, mutated to KLER). This NLDM possesses intact activity to stimulate caspase activation *in vitro* ([Fig. 7A](#)) but lost its nuclear localization ([Fig. 7B](#)). When we performed the soft agar colony formation analysis, we found that NLDM could still suppress Ras/Myc-induced tumorigenesis ([Fig. 7C](#)), indicating nuclear localization of PHAPI is not essential for its tumor-suppressive function. On the other hand, we found that NLDM reproducibly showed a weaker tumor-suppressive function than that of wild-type PHAPI ([Fig. 7C](#)), and it also induced cellular apoptosis to a

less extend than wild-type PHAPI (Fig. 7D), suggesting that nuclear localization also contributes to the cellular apoptotic activity and tumor-suppressive function of PHAPI. Because the NLDM mutant and the wild-type PHAPI showed the same level of activity in stimulating caspase activation *in vitro* (Fig. 7A), it is likely the nuclear localization of PHAPI engages additional mechanisms to enhance cellular apoptosis.

## DISCUSSION

---

PHAPI/pp32 has been shown to be a tumor suppressor based on both functional studies and human cancer pathological analyses. However, how PHAPI suppresses tumorigenesis at molecular level was not clear. Biochemically, PHAPI is able to regulate various reactions, including inhibition of PP2A and HAT, and stimulation of Apaf-1-mediated caspase activation, all of which might impact tumorigenesis. Some recent cancer biological studies support the possibility that the apoptotic activity of PHAPI is required for its tumor-suppressive function, because PHAPI expression in certain human cancers correlated with the sensitivity of cancer cells to apoptosis (28, 33). In this study, by utilizing multiple truncation mutants of PHAPI, we provided strong evidence to support the model that the apoptotic activity of PHAPI is required for its tumor-suppressive function. *In vitro* and cellular apoptotic activity of PHAPI and its mutants both correlate with their tumor-suppressive function. Importantly, PHAPI enhances cellular apoptosis induced by anchorage-independent growth condition under which we measured its tumor-suppressive function, indicating that, at least under this condition, the apoptotic activity of PHAPI contributes to tumor suppression.

Two regions of PHAPI, the highly acidic C-terminal domain and the M region connecting this C-terminal tail to the leucine-rich repeats, are essential for both the apoptotic activity and tumor-suppressive function of PHAPI (Figs. 1 and 5). Requirement of the M region is particularly interesting, because it is the major region that distinguishes PHAPI from its homolog pp32R1, and replacement of the M region of PHAPI with the correspondent peptide of pp32R1 abrogates the apoptotic activity of PHAPI (Fig. 2). Consistently, pp32R1 is not able to enhance caspase activation, although it also contains leucine-rich repeats and a long and acidic C terminus (Fig. 2). This result and the oncogenic nature of pp32R1 further support our conclusion that the apoptotic activity of PHAPI accounts for its tumor suppressive capability. To this end, the molecular basis underlying the oncogenic function of pp32R1 is not clear; pp32R1 cannot antagonize the apoptotic activity of PHAPI by any competitive mechanism (Fig. 3) or by interfering with the higher order structure of PHAPI, which surprisingly is a monomeric protein (Fig. 4). Further, contrary to PHAPI, pp32R1 is mainly a cytosolic protein (data not shown). Thus, it is more likely that pp32R1 promotes tumorigenesis in the cytoplasm.

The M region of PHAPI also distinguishes its apoptotic activity from some other reported biochemical activities of this tumor suppressor. It appears that PHAPI inhibits both PP2A and HAT enzymes via its highly negatively charged C terminus, because PHAPII/Set, another protein that inhibits both enzymes (35–37), has a completely different primary structure except that it also contains a long and highly acidic C terminus. Indeed, the highly acidic C termini of these two proteins can easily explain how they inhibit HAT activity: via a strong ionic interaction with histones, which are highly basic proteins, PHAPI and PHAPII/Set can mask histones from contact with HAT (25). Additionally, although PHAPII/Set can inhibit PP2A and HAT, it is not able to stimulate Apaf-1-dependent caspase activation (data not shown). Further, PHAPII/Set has been suggested to be oncogenic instead of tumor-suppressive (38, 39). In conclusion, our study not only demonstrates that the apoptotic activity of PHAPI is necessary for tumor suppression but also suggests that the biochemical activities of PHAPI to inhibit PP2A and HAT

probably play minor, if any, role in tumor suppression.

Importantly, PHAPI is predominantly a nuclear protein, and apoptotic stimulation can induce translocation of PHAPI to the cytoplasm, providing one more layer of regulatory mechanism for controlling apoptosis (Fig. 6) (23). Indeed, a PHAPI mutant (NLDM) that loses nuclear localization but retains intact activity to stimulate Apaf-1-dependent caspase activation is still functional to suppress tumorigenesis (Fig. 7), consistent with our hypothesis that the cytosolic apoptotic activity of PHAPI accounts for its tumor-suppressive function. Intriguingly, NLDM reproducibly showed a lower tumor-suppressive capability than the wild-type PHAPI (Fig. 7C), indicating that the nuclear localization of PHAPI might also contribute to tumor suppression. This nuclear tumor-suppressive function of PHAPI might be independent of apoptosis; alternatively, it might be due to the ability of PHAPI to shuttle other nuclear apoptotic proteins such as HuR to the cytoplasm, which represents an additional mechanism for PHAPI to accelerate apoptosis (23). In addition to the nuclear localization, our study also suggests that the LRR domains of PHAPI might also provide basis for additional regulation of the cellular apoptotic activity of PHAPI (Figs. 1D and 5B).

## Acknowledgments

---

We thank Dr. Ian Ganley and other members of the X. Jiang laboratory for critical reading and discussion.

## Notes

---

\*This work was supported, in whole or in part, by National Institutes of Health Grants R01 AI045937 (to H. W.) and R01 CA113890 (to X. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

## Footnotes

---

<sup>3</sup>The abbreviations used are: PP2A, protein phosphatase 2A; HAT, histone acetyltransferase; pp32R1, pp32-related-1; NLDM, nuclear localization defective mutant of PHAPI; CMV, cytomegalovirus; GFP, green fluorescent protein; WT, wild type; PRP, PHAPI recombinant protein with residues 140–163 swapped with the correspondent residues of pp32R1.

## References

---

1. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) Br. J. Cancer 26 239-257. [PMCID: PMC2008650] [PubMed: 4561027]
2. Horvitz, H. R. (1999) Cancer Res. 59 1701s-1706s. [PubMed: 10197583]
3. Thornberry, N. A., and Lazebnik, Y. (1998) Science 281 1312-1316. [PubMed: 9721091]
4. Budihardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. (1999) Annu. Rev. Cell Dev. Biol. 15 269-290. [PubMed: 10611963]
5. Jiang, X., and Wang, X. (2004) Annu. Rev. Biochem. 73 87-106. [PubMed: 15189137]
6. Chao, D. T., and Korsmeyer, S. J. (1998) Annu. Rev. Immunol. 16 395-419. [PubMed: 9597135]
7. Harris, M. H., and Thompson, C. B. (2000) Cell Death Differ. 7 1182-1191. [PubMed: 11175255]
8. Adams, J. M., and Cory, S. (2001) Trends Biochem. Sci. 26 61-66. [PubMed: 11165519]

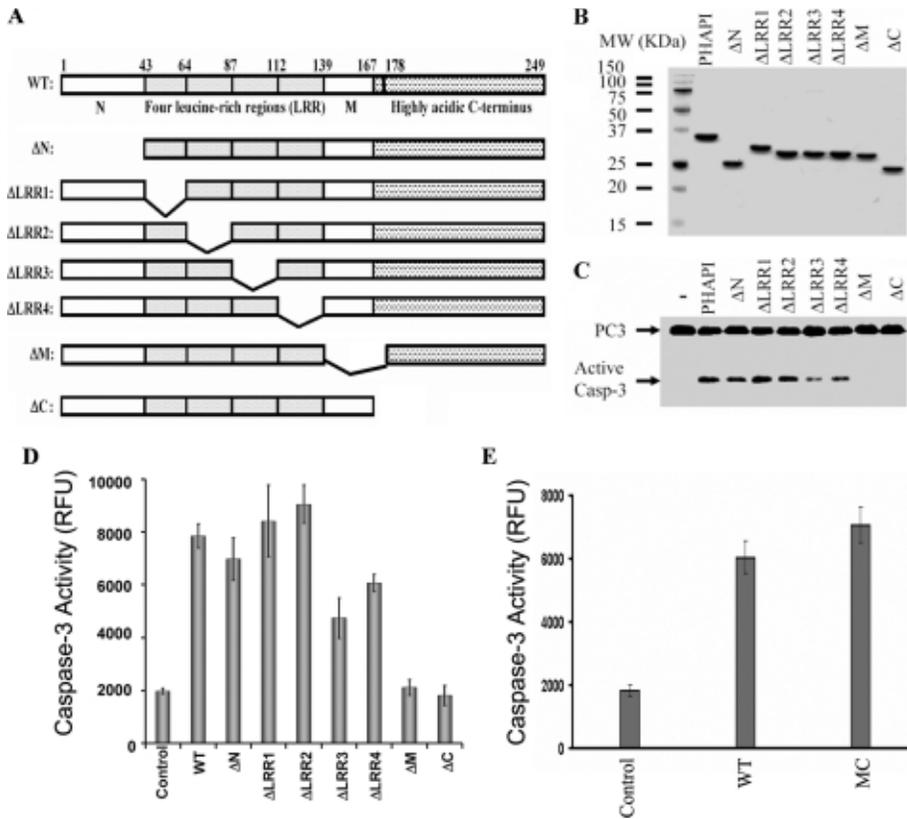
9. Jiang, X., and Wang, X. (2000) *J. Biol. Chem.* 275 31199-31203. [PubMed: 10940292]
10. Kim, H. E., Du, F., Fang, M., and Wang, X. (2005) *Proc. Natl. Acad. Sci. U. S. A.* 102 17545-17550. [PMCID: PMC1266161] [PubMed: 16251271]
11. Rodriguez, J., and Lazebnik, Y. (1999) *Genes Dev.* 13 3179-3184. [PMCID: PMC317200] [PubMed: 10617566]
12. Duckett, C. S., Nava, V. E., Gedrich, R. W., Clem, R. J., Van Dongen, J. L., Gilfillan, M. C., Shiels, H., Hardwick, J. M., and Thompson, C. B. (1996) *EMBO J.* 15 2685-2694. [PMCID: PMC450204] [PubMed: 8654366]
13. Uren, A. G., Pakusch, M., Hawkins, C. J., Puls, K. L., and Vaux, D. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93 4974-4978. [PMCID: PMC39390] [PubMed: 8643514]
14. Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) *Nature* 388 300-304. [PubMed: 9230442]
15. Deveraux, Q. L., and Reed, J. C. (1999) *Genes Dev.* 13 239-252. [PubMed: 9990849]
16. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000) *Cell* 102 33-42. [PubMed: 10929711]
17. Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2000) *Cell* 102 43-53. [PubMed: 10929712]
18. Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001) *Mol. Cell* 8 613-621. [PubMed: 11583623]
19. Verhagen, A. M., Silke, J., Ekert, P. G., Pakusch, M., Kaufmann, H., Connolly, L. M., Day, C. L., Tikoo, A., Burke, R., Wrobel, C., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2002) *J. Biol. Chem.* 277 445-454. [PubMed: 11604410]
20. Jiang, X., Kim, H. E., Shu, H., Zhao, Y., Zhang, H., Kofron, J., Donnelly, J., Burns, D., Ng, S. C., Rosenberg, S., and Wang, X. (2003) *Science* 299 223-226. [PubMed: 12522243]
21. Kim, H. E., Jiang, X., Du, F., and Wang, X. (2008) *Mol. Cell* 30 239-247. [PubMed: 18439902]
22. Chen, T. H., Brody, J. R., Romantsev, F. E., Yu, J. G., Kayler, A. E., Voneiff, E., Kuhajda, F. P., and Pasternack, G. R. (1996) *Mol. Biol. Cell* 7 2045-2056. [PMCID: PMC276049] [PubMed: 8970164]
23. Mazroui, R., Di Marco, S., Clair, E., von Roretz, C., Tenenbaum, S. A., Keene, J. D., Saleh, M., and Gallouzi, I. E. (2008) *J. Cell Biol.* 180 113-127. [PMCID: PMC2213623] [PubMed: 18180367]
24. Li, M., Makkinje, A., and Damuni, Z. (1996) *Biochemistry* 35 6998-7002. [PubMed: 8679524]
25. Seo, S. B., Macfarlan, T., McNamara, P., Hong, R., Mukai, Y., Heo, S., and Chakravarti, D. (2002) *J. Biol. Chem.* 277 14005-14010. [PubMed: 11830591]
26. Matilla, A., Koshy, B. T., Cummings, C. J., Isobe, T., Orr, H. T., and Zoghbi, H. Y. (1997) *Nature* 389 974-978. [PubMed: 9353121]
27. Brody, J. R., Witkiewicz, A., Williams, T. K., Kadkol, S. S., Cozzitorto, J., Durkan, B., Pasternack, G. R., and Yeo, C. J. (2007) *Mod. Pathol.* 20 1238-1244. [PubMed: 17906614]
28. Hoffarth, S., Zitzer, A., Wiewrodt, R., Hahnel, P. S., Beyers, V., Kreft, A., Biesterfeld, S., and Schuler, G. (2007) *Cell* 130 103-114. [PubMed: 17444444]

- M. (2008) *Cell Death Differ.* 15 161-170. [PubMed: 17962813]
29. Brody, J. R., Kadkol, S. S., Mahmoud, M. A., Rebel, J. M., and Pasternack, G. R. (1999) *J. Biol. Chem.* 274 20053-20055. [PubMed: 10400610]
30. Bai, J., Brody, J. R., Kadkol, S. S., and Pasternack, G. R. (2001) *Oncogene* 20 2153-2160. [PubMed: 11360199]
31. Kadkol, S. S., Brody, J. R., Pevsner, J., Bai, J., and Pasternack, G. R. (1999) *Nat. Med.* 5 275-279. [PubMed: 10086381]
32. Kadkol, S. S., El Naga, G. A., Brody, J. R., Bai, J., Gusev, Y., Dooley, W. C., and Pasternack, G. R. (2001) *Breast Cancer Res. Treat.* 68 65-73. [PubMed: 11678310]
33. Schafer, Z. T., Parrish, A. B., Wright, K. M., Margolis, S. S., Marks, J. R., Deshmukh, M., and Kornbluth, S. (2006) *Cancer Res.* 66 2210-2218. [PubMed: 16489023]
34. Ulitzur, N., Rancano, C., and Pfeffer, S. R. (1997) *J. Biol. Chem.* 272 30577-30582. [PubMed: 9374554]
35. Vaesen, M., Barnikol-Watanabe, S., Gotz, H., Awni, L. A., Cole, T., Zimmermann, B., Kratzin, H. D., and Hilschmann, N. (1994) *Biol. Chem. Hoppe Seyler* 375 113-126. [PubMed: 8192856]
36. Li, M., Makkinje, A., and Damuni, Z. (1996) *J. Biol. Chem.* 271 11059-11062. [PubMed: 8626647]
37. Seo, S. B., McNamara, P., Heo, S., Turner, A., Lane, W. S., and Chakravarti, D. (2001) *Cell* 104 119-130. [PubMed: 11163245]
38. Adler, H. T., Nallaseth, F. S., Walter, G., and Tkachuk, D. C. (1997) *J. Biol. Chem.* 272 28407-28414. [PubMed: 9353299]
39. Fukukawa, C., Shima, H., Tanuma, N., Okada, T., Kato, N., Adachi, Y., and Kikuchi, K. (2005) *Int. J. Oncol.* 26 751-756. [PubMed: 15703833]

## Figures and Tables

---

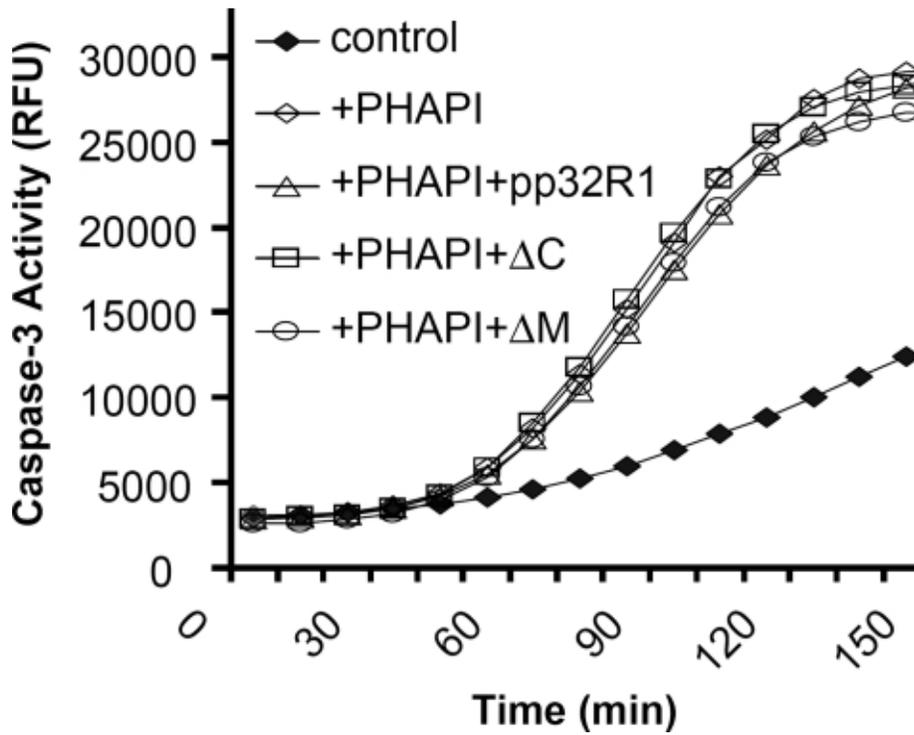
FIGURE 1.



**Determination of the domain structure required for the apoptotic activity of PHAPI.** *A*, schemes of PHAPI and its truncation mutants. Individual motifs and their corresponding amino acid positions are labeled for the full-length PHAPI. *B*, SDS-PAGE of purified recombinant PHAPI and its truncation mutants. 1  $\mu$ g of each protein was resolved by SDS-PAGE and stained with Coomassie Blue R250. *C* and *D*, analysis of the activity of PHAPI and its truncation mutants to stimulate Apaf-1-mediated caspase activation. The assay was performed as described under “Experimental Procedures” using 500 nM PHAPI or its truncation mutants as indicated. Either *in vitro* translated, [ $^{35}$ S]methionine-labeled caspase-3 (*C*) or fluorogenic peptide substrate of caspase-3 (*D*) was used as the substrate for the assay. The values of 90-min reaction are shown. *Error bars* represent standard error calculated from three independent experiments (same for other figures). *E*, analysis of the apoptotic activity of the PHAPI truncation mutant MC (amino acid 139–249, containing the M and C regions only). The values of 90-min reaction are shown.

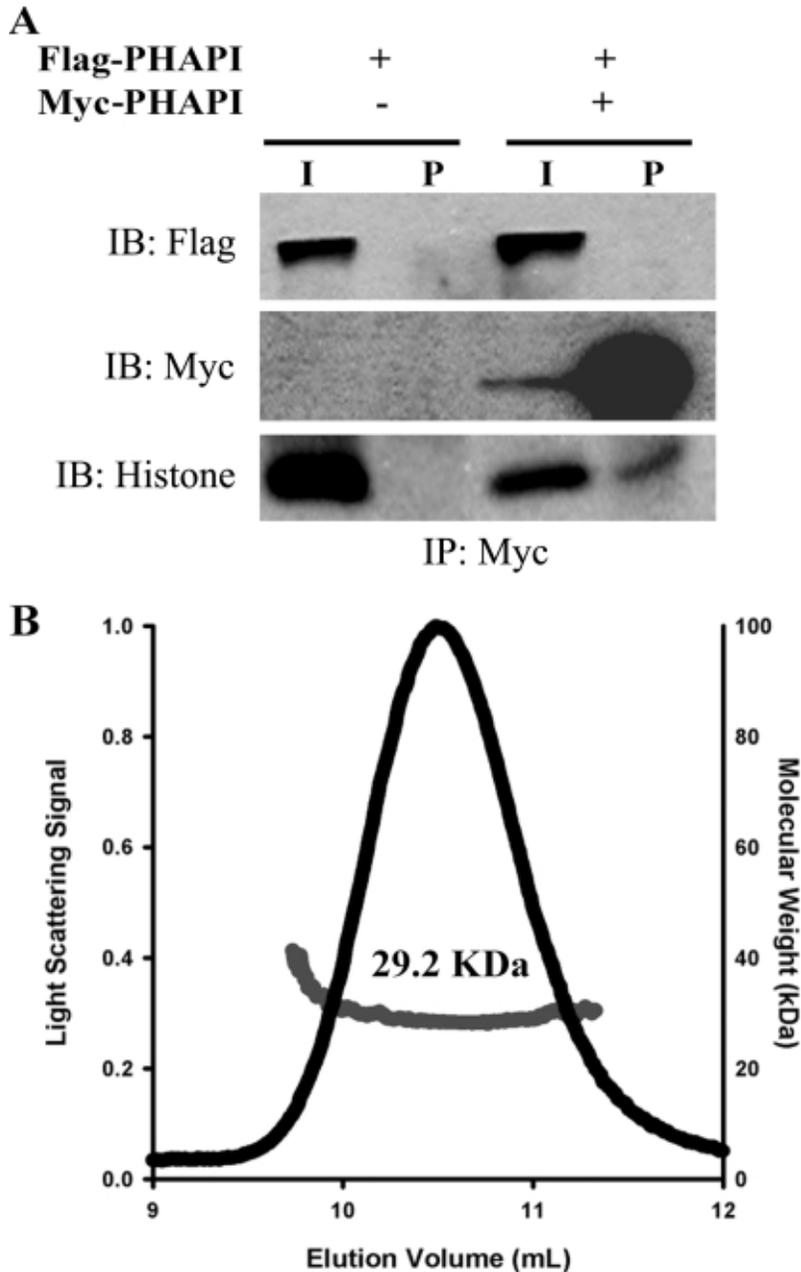
FIGURE 2.





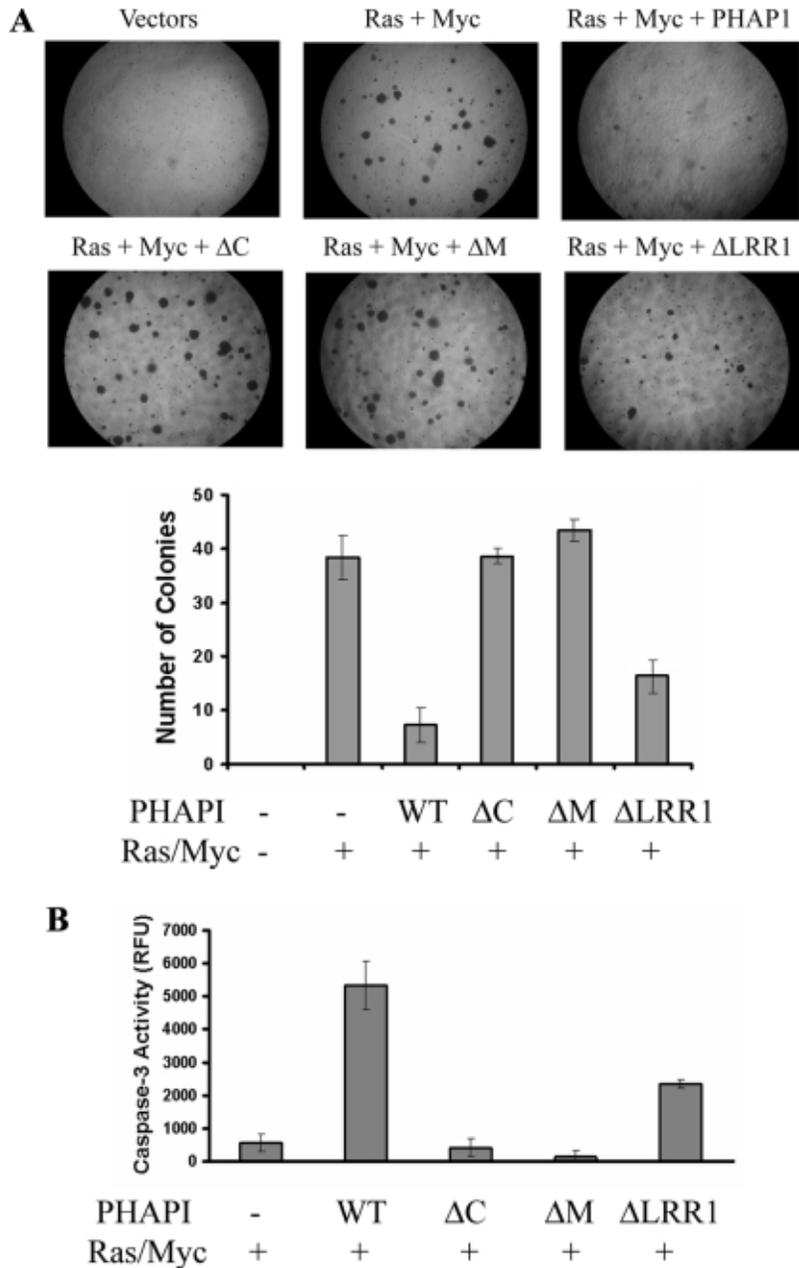
**pp32R1 does not inhibit the *in vitro* apoptotic activity of PHAPI.** The effect of pp32R1 and  $\Delta M$  or  $\Delta C$  mutant of PHAPI on the activity of PHAPI to stimulate Apaf-1-mediated caspase activation was examined. The assay was performed as described under “Experimental Procedures” using fluorogenic peptide substrate of caspase-3 and 500 nM PHAPI, pp32R1,  $\Delta M$ , or  $\Delta C$  as indicated.

FIGURE 4.



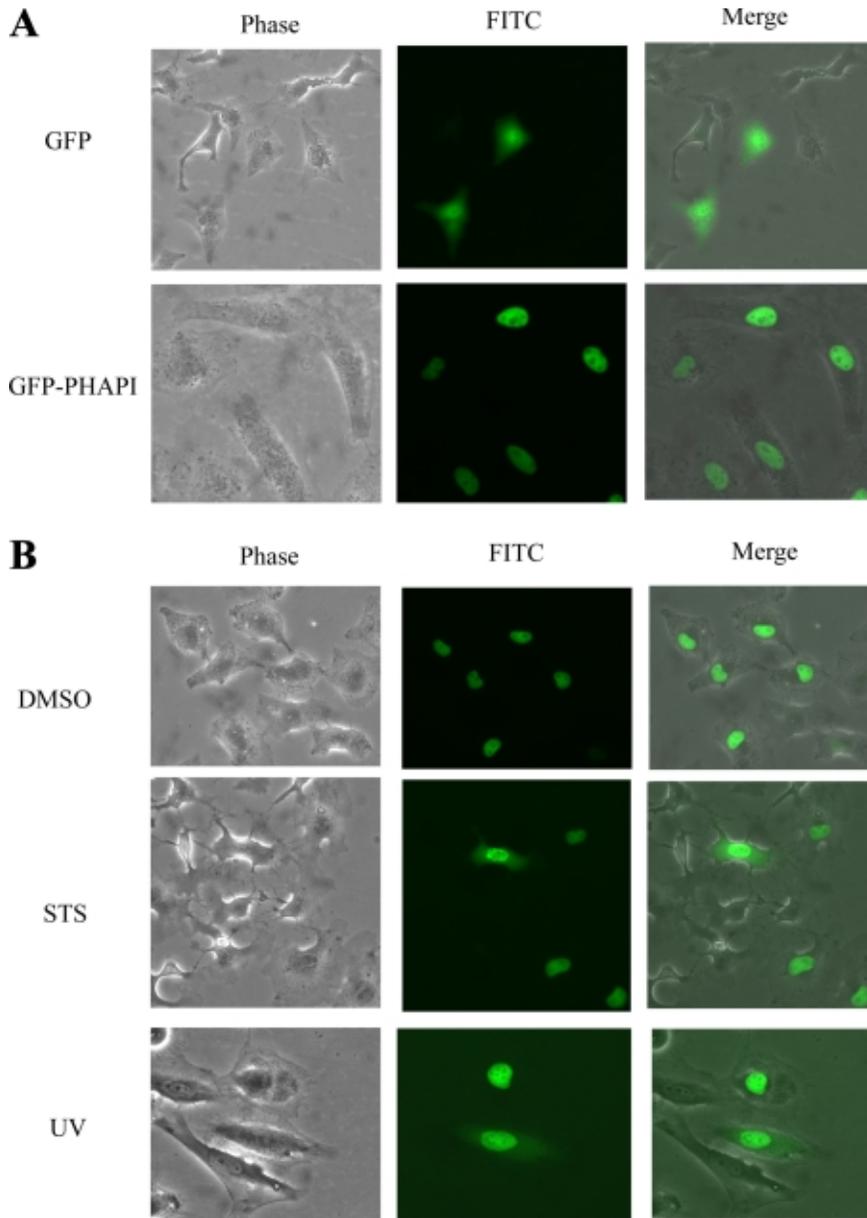
**PHAPI is not an oligomeric protein.** *A*, differentially tagged PHAPI did not interact with each other when expressed in HeLa cells. HeLa cells were transfected with plasmids encoding for FLAG-tagged PHAPI and/or Myc-tagged PHAPI as indicated. One day after transfection, cell extracts were prepared and coimmunoprecipitation using an anti-Myc antibody was performed as described under “Experimental Procedures.” Subsequently, the input extracts (*I*) and the immunoprecipitates (*P*) were subjected to Western blot analysis against anti-FLAG, anti-Myc, and anti-histone antibodies as indicated. *B*, light scattering analysis indicates PHAPI is a monomeric protein. Molar mass of purified Recombinant PHAPI was determined by static multiangle light scattering as described under “Experimental Procedures.” The measured molar mass of PHAPI is 29.2 kDa.

FIGURE 5.



**The apoptotic activity of PHAPI correlates with its tumor-suppressive function.** *A*, the tumor-suppressive function of PHAPI, and its truncation mutants, as indicated, was measured in a soft agar colony formation assay as inhibition of Ras/Myc-induced cell transformation. The assay was performed as described under “Experimental Procedures.” A set of representative pictures and the quantitative values with standard deviation from three independent experiments are shown. *B*, the effect of overexpression of PHAPI and its truncation mutants on apoptosis under anchorage-independent growth condition. The cells infected with the same set of genes as that in *panel A* were grown in suspension condition as described under “Experimental Procedures.” Eight hours later, cells were harvested, total cell lysates were prepared, and 20  $\mu$ g of extracts was subjected to caspase-3 activity to monitor cellular apoptosis triggered by lack of anchorage. The values of 120-min reaction are shown.

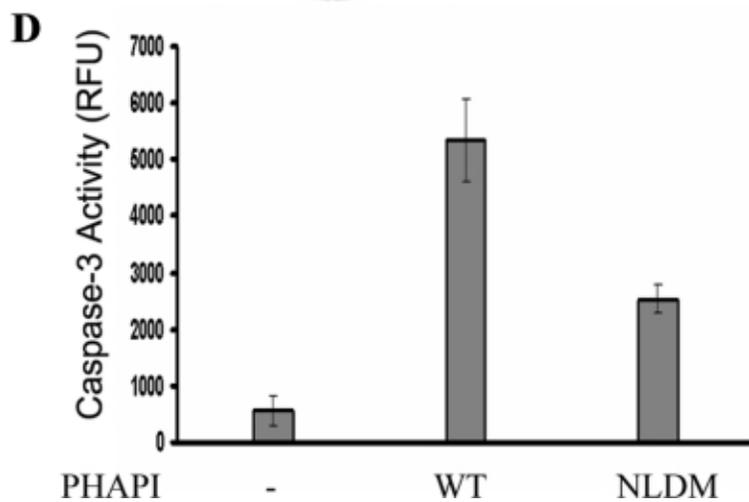
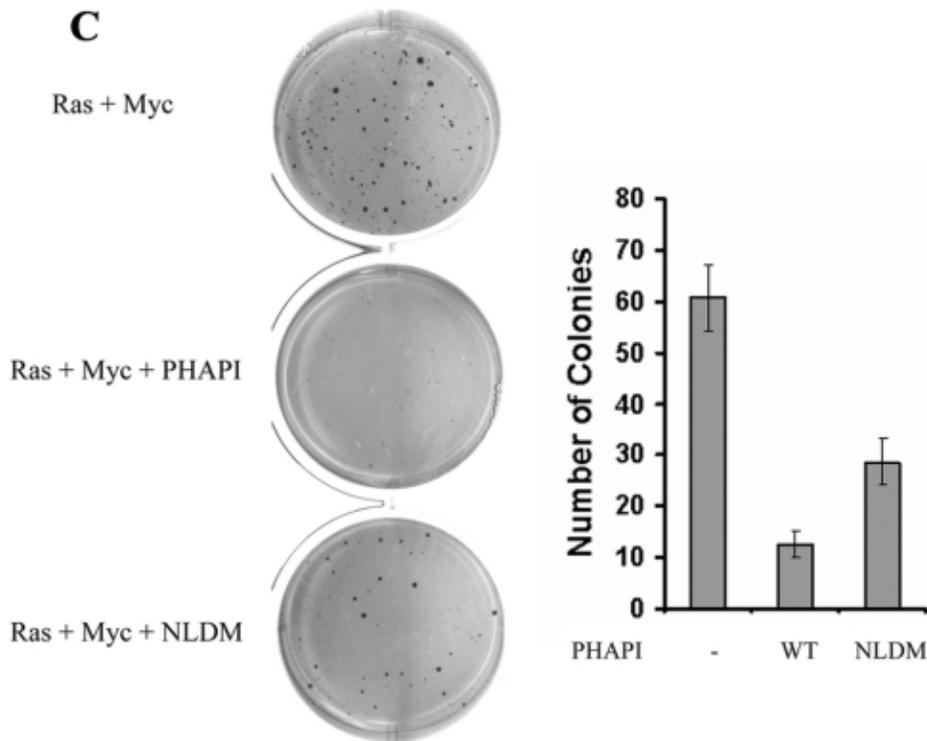
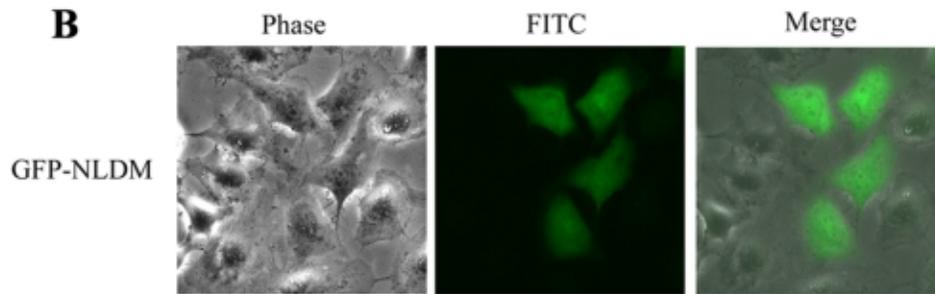
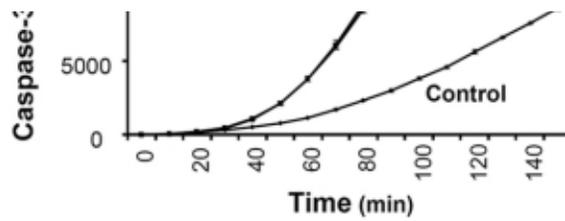
FIGURE 6.



**Apoptotic stimuli induce translocation of PHAPI from the nucleus to cytoplasm.** *A*, PHAPI is predominantly a nuclear protein. GFP alone or GFP-tagged PHAPI was expressed in HeLa cells, and their subcellular localization was detected by fluorescent microscopy. *B*, UV light and staurosporine can trigger translocation of PHAPI from the nucleus to cytoplasm. HeLa cells expressing GFP-PHAPI were treated with either UV light or staurosporine (*STS*) for the indicated times, and subsequently subcellular localization of GFP-PHAPI was detected by fluorescent microscopy.

FIGURE 7.





**A PHAPI mutant defective in nuclear localization possesses apoptotic and tumor-suppressive function.** *A*, the NLDM PHAPI has intact activity to stimulate Apaf-1-mediated caspase activation. The caspase assay was performed as described under “Experimental Procedures” using fluorogenic peptide substrate of caspase-3 and 500 nM PHAPI wild-type or NLDM mutant. *B*, the NLDM mutant lost the nuclear localization of wild-type PHAPI. Subcellular localization of GFP-NLDM expressed in HeLa cells was detected by fluorescent microscopy. *C*, the NLDM mutant possesses a modestly decreased tumor-suppressive function than wild-type PHAPI. The soft agar colony formation assay measuring the tumor-suppressive function of wild-type or NLDM PHAPI, as indicated, was performed as described under “Experimental Procedures.” A set of representative pictures and the quantitative values with standard deviation are shown. *D*, the NLDM mutant possesses a decreased cellular apoptotic activity than wild-type PHAPI. The cells infected with the same set of genes as that in *panel C* were grown in suspension condition as described under “Experimental Procedures.” Eight hours later, cells were harvested, total cell lysates were prepared, and 20  $\mu$ g of extracts was subjected to caspase-3 activity to monitor cellular apoptosis triggered by lack of anchorage. The values of 120-min reaction are shown.

---

Articles from The Journal of Biological Chemistry are provided here courtesy of **American Society for Biochemistry and Molecular Biology**