The tumor suppressor SHIP1 colocalizes in nucleolar cavities with p53 and components of PML nuclear bodies

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Abbreviations: DFC, dense fibrillar component; DIC, Differential interference contrast; EGFP, enhanced green fluorescent protein; FC, fibrillar center; GC, granular component; LMB, leptomycin B; NES, nuclear export signal; PBMC, Peripheral Blood Mononuclear Cell; PtdIns(3, 4, 5)P3, phosphatidylinositol-(3, 4, 5)-trisphosphate; PtdIns(3, 4)P2, phosphatidylinositol-(3, 4)-bisphosphate; PML, Promyelocytic Leukemia; RNA pol, RNA polymerase; SHIP1, src homology 2 domain-containing inositol phosphatase 1; UPP, ubiquitin-proteasome pathway.

Introduction

The src homology 2 domain-containing inositol phosphatase isoform 1 (SHIP1) possesses important functions as negative regulator of proliferation in haematopoietic cells.1 This regulatory function of SHIP1 is mediated by its inositol 5-phosphatase activity catalyzing dephosphorylation of phosphoinositides (PI) and inositol phosphates (IP) at the D5 position of the inositol ring.2 Regulation of phosphoinositide- and inositol phosphate-mediated cellular processes by SHIP1 i.e. conversion of PtdIns(3,4,5)P3 to PtdIns(3,4)P2 at the plasma membrane, SHIP1 modifies PI3-kinase mediated signaling. We have recently demonstrated that SHIP1 is a nucleo-cytoplasmic shuttling protein and SHIP1 nuclear puncta partially colocalize with FLASH, a component of nuclear bodies. In this study, we demonstrate that endogenous SHIP1 localizes to intranucleolar regions of both normal and leukemic haematopoietic cells. In addition, we report that ectopically expressed SHIP1 accumulates in nucleolar cavities and colocalizes with the tumor suppressor protein p53 and components of PML nuclear bodies (e.g. SP100, SUMO-1 and CK2). Moreover, SHIP1 also colocalizes in nucleolar cavities with components of the ubiquitin-proteasome pathway. By using confocal microscopy data, we generated 3D-models revealing the enormous extent of the SHIP1 aggresomes in the nucleolus. Furthermore, treatment of cells with the proteasome inhibitor MG132 causes an enlargement of nucleolar SHIP1 containing structures. Unexpectedly, this accumulation can be partially prevented by treatment with the inhibitor of nuclear protein export Leptomycin B. In recent years, several proteins aggregating in nucleolar cavities were shown to be key factors of neurodegenerative diseases and cancerogenesis. Our findings support current relevance of nuclear localized SHIP1.

Keywords: aggresome, cancer, nucleus, leptomycin B, MG132, nucleolar cavities, PML bodies, p53, SHIP1, ubiquitin proteasome pathway.

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Nucleus
been shown that accompanying deletion of PTEN and SHIP1 causes development of lethal B cell lymphomas in mice. These data strongly point to a tumor suppressor function of SHIP1 in haematopoietic cells.

Despite of its predominant localization in the cytoplasm, SHIP1 exhibits active nucleo-cytoplasmic shuttling and enzymatically active SHIP1 is localized in nuclear puncta of leukemia cells. The non-membrane-enclosed nucleoli mainly consisting of RNA and proteins are called ”ribosome factories of the cell”. Nucleoli are well organized structures and can be divided into the sub-compartments fibrillar centers (FCs), dense fibrillar components (DFCs) and granular components (GCs). In recent years, localization of multiple proteins to intranucleolar regions distinct from the ribosome production compartments was observed. These regions were termed as nucleolar vacuoles, aggresomes or cavities. Several proteins shown to aggregate in these cavities are components of the ubiquitin-proteasome pathway. Furthermore, we demonstrate that the proteasome inhibitor MG132 induces strong aggregation of SHIP1 in nucleolar cavities.

Material and Methods

Cell culture and transient gene expression

The factor independent leukemia cell line TF-1-IIING1-8 was derived from the GM-CSF dependent erythroleukemia cell line TF-1. TF-1-IIING1-8 cells were cultured in a humidified atmosphere at 37°C in the presence of 5% CO2 using RPMI 1640 medium (Invitrogen, Darmstadt, Germany) supplemented with 10% (v/v) fetal calf serum without GM-CSF. H1299 cells (non-small cell lung cancer) were cultured in a humidified atmosphere at 37°C in the presence of 5% CO2. Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 10% (v/v) FBS, 4 mM L-glutamine, 4.5 g/l glucose and 1% (v/v) penicillin/streptomycin was used. Leukocytes were separated from blood samples of healthy donors using Ficoll gradient by using Lymphoprep®. Leukocytes were separated from blood samples of healthy donors using Ficoll gradient by using Lymphoprep®. The blood samples were layered on top of 3 ml S3 containing 0.88 M sucrose, 0.5 mM MgCl2 and × Protease inhibitor and centrifuged at 1420g and 4°C for 5 min. After centrifugation of the cells at 219xg and 4°C for 5 min the pellet was resuspended in 3 ml S1 containing 0.25 M sucrose, 10 mM MgCl2 and 1x Protease inhibitor by pipetting up and down. The solution was layered on top of 3 ml of S2 (0.35 M sucrose, 0.5 mM MgCl2 and 1× Protease inhibitor) and centrifuged at 1420g and 4°C for 5 min. The resulting pellet was resuspended in 3 mL S2 and continuously sonicated for 5 seconds on ice (Sonopuls GM 70; Power: MS72/D, Bandelin, Berlin, Germany). The sonicated solution was layered on top of 3 ml S3 containing 0.88 M sucrose, 0.5 mM MgCl2 and × Protease inhibitor and centrifuged at 3000xg and 4°C for 10 min. For additional purification of the isolated nucleoli, the pellet was resuspended in 0.5 mL S2 and centrifuged at 219xg and 4°C for 5 min. This purified nucleoli pellet was resuspended in 100 μL MPER buffer (Mammalian Protein extraction reagent, Thermo scientific, Braunschweig, Germany) and stored at −80°C until Western Blot analysis.

Fluorescence microscopy

Fixation of cells with paraformaldehyde and examination of the intracellular protein localization by fluorescence microscopy were performed as described. For confocal microscopy, cells were washed with PBS, fixed with 3% (v/v) paraformaldehyde/ PBS (10 min, 37°C) and permeabilized with 0.3% (v/v) Triton X-100/PBS (4 min, RT). Subsequently, cells were blocked in PBS supplemented with 1% (w/v) BSA for 30 min at room temperature and incubated with mouse anti-SHIP1 antibody P1C1 (1:500, Santa Cruz, Dallas, USA) for 16 hours at 4°C and, subsequently, with a goat anti-mouse IgG antibody labeled with Alexa Fluor 568 (Life Technologies, Darmstadt, Germany) for 1 hour at RT. Further antibodies used are mouse anti-UBF (F-9, sc-13125, Santa Cruz, Dallas, USA), mouse anti-Pol I, II, III RPB6 (B6-1, sc-21751, Santa Cruz, Dallas, USA), rabbit anti-Fibrillarin (ab5821, Abcam, Cambridge, UK), mouse anti-Fibrillarin (ab5821, Abcam, Cambridge, UK), mouse anti-Fibrillarin (ab5821, Abcam, Cambridge, UK).
anti-Fibrillarin (ab18380, Abcam, Cambridge, UK), rabbit anti-20s proteasome α/β subunits (Z03711, Biotrend, Destin, USA), mouse anti-Ubiquitin (P4D1, #3936, Cell Signaling, Denvers, USA), mouse anti-p53 (sc-126, Santa Cruz, Dallas, USA), rabbit anti-Akt (pan) (11E7, Cell Signaling, Denvers, USA), goat anti-rabbit IgG Alexa Fluor 488 (Life Technologies, Darmstadt, Germany), goat anti-mouse IgG Alexa Fluor 568 (Life Technologies, Darmstadt, Germany). Normal mouse IgG (sc-2025; Santa Cruz, Dallas, USA) was used as negative control for immunohistochemistry. Cells were grown on chamber slides -μ-Slide 4 well; iBiTreat (Ibidi, Munich, Germany). Images were taken with a Leica TCS SP5 equipped with 63x HCX PL APO CS Oil objective, NA: 1.40-0.60 (iris), WD (mm): 0.1, DIC Obj Prism: E. 3D analysis was performed by Imaris software (Bitplane, Belfast, United Kingdom).

Construction of plasmids

Full-length cDNA of human p53 (IMAGE ID: 3544714) was obtained from Source BioScience LifeSciences (Nottingham, UK). The coding sequence was amplified by PCR techniques using the following primer pair (5'-ATAGATCTATGGAGGAGCCGGAGT-CAGAT-3', 5'-TATCTAGATCATCTGAGTCAGGCCCCTTC-3'). Thereby, BglII and XbaI restriction sites were introduced at the 5'- and 3'-end, respectively. Subsequently, these sites were used for cloning the fragment into the expression vector pEGFP-C1 (Clontech, La Jolla, CA, USA) prepared from Dam/-Dcm- E.coli strain SCS110. Creation of a vector expressing an EGFP fusion protein of human SHIP1 is described elsewhere. A vector expressing a DsRed2 fusion protein of human CK2α was generated as described elsewhere. H2B-EGFP expression vector was created by Geoffrey M. Wahl and provided (no. 11,680) by addgene (Cambridge, USA). Mouse Ubc9-EGFP and EGFP-hADAR expression vectors were kindly provided by Ali Bazebbahi and Maria Carmo-Fonseca, respectively. Vectors expressing GFP-SUMO-1 and EGFP-S100 were kindly gifts from Giannino Del Sal and Hans Will. The pcDNA4/TO-based plasmid encoding an EGFP fusion protein of human AF1033 was a kind gift from Juan J. Arredondo. The pcDNA4/TO-based plasmid encoding an EGFP fusion protein of human SHIP1 is described elsewhere. A vector expressing a DsRed2 fusion protein of human CK2α was generated as described elsewhere. H2B-EGFP expression vector was created by Geoffrey M. Wahl and provided (no. 11,680) by addgene (Cambridge, USA). Mouse Ubc9-EGFP and EGFP-hADAR expression vectors were kindly provided by Ali Bazebbahi and Maria Carmo-Fonseca, respectively. Vectors expressing GFP-SUMO-1 and EGFP-S100 were kindly gifts from Giannino Del Sal and Hans Will. The pcDNA4/TO-based plasmid encoding an EGFP fusion protein of human AF1033 was a kind gift from Juan J. Arredondo.

Expression of the fusion protein was induced by addition of tetracycline for 24 h at final concentration of 1 μg/ml. LeGO-iG2-Puro+ vector was kindly provided by Kristoffer Riecken and Boris Fehse. Human SHIP1 cDNA was sub-cloned into the LeGO-iG2-Puro+ vector using a BamHI/BamHI strategy.

Quantification of nuclear/cytoplasmic distribution

Image analysis using ImageJ software (Rasband, W., NIH, Washington, DC, USA) was performed as previously described. A minimum of 50 cells was randomly selected and examined after fixation in each experiment. For the determination of the nuclear/cytoplasmic ratio, fluorescence intensities of 3 rectangular regions of interest in both the nucleus and the cytoplasm of each cell were averaged to calculate the ratio of nuclear over cytoplasmatic fluorescence intensity (n/c ratio). For statistical evaluation the program SigmaStat 3.10 (Systat Software Inc., Erkrath, Germany) was used. Student’s t-test was performed and a value of p < 0.05 was considered statistically significant.

Results

Nucleolar localization of endogenous SHIP1 in haematopoietic cells

First, we examined the distribution of endogenous SHIP1 in the haematopoietic cell line TF-1-IIING1-8 (Fig. 1A, B; Figs. S1A and S1B) and in PBMC from a healthy donor (Fig. 1C).

In untreated TF-1-IIING1-8 cells endogenous SHIP1 was partly found in the nucleoplasm in shape of nuclear puncta, but rarely found within the nucleoli (Fig. 1A and Fig. S1A). However, after MG132 treatment, endogenous SHIP1 was found within the nucleoli of both normal and leukemic haematopoietic cells marked by immunostaining of fibrillarin. In addition, we observed SHIP1-positive dots in the nucleoli of TF-1-IIING1-8 cells using DIC microscopy (Fig. 1B3 and S1B3). Since endogenous SHIP1 seems to accumulate in the nucleolus in response to the exposure to the proteasome inhibitor MG132, we were interested in the localization of the involved proteasomes. It was shown before that in particular PML nuclear body-associated proteins PML, SP100 and SUMO-1 redistribute to the nucleolus upon inhibition of proteasome-dependent protein degradation. Proteasomes are large multi-subunit complexes localized both in the nucleus and cytosol. They selectively degrade intracellular proteins and are responsible for the degradation of many proteins involved in cellular processes such as apoptosis, differentiation, proliferation and cell cycling.

The 26S proteasome in eukaryotes consists of one 20S and 2 19S subunits. Therefore, we analyzed the distribution of the endogenous 20S proteasome core subunit by immunofluorescence in untreated TF-1-IIING1-8 cells. Thereby, we detected that haematopoietic cells possess a nucleolar cavity that contains the endogenous 20S proteasome core (Fig. 1D).

Nucleolar localization of ectopically expressed SHIP1 in a non-haematopoietic cell-line

The small nucleoli (compared to the size of their nuclei) and presumably the very small nucleolar cavities of haematopoietic cells seriously hamper the identification of nucleolar substructures. Size and percentage of cavities strongly seem to depend on cell line and of course of concentrations and incubation times of proteasome inhibitor.

Therefore, we performed additional investigations using a non-haematopoietic cell line more suitable for such studies (H1299; human epithelial lung carcinoma cell-line). We investigated the localization of the endogenous 20S proteasome core in untreated H1299 cells. These cells possess easily detectable nucleolar cavities (Fig. 2A1). As expected, these nucleolar regions were detected in DIC microscopy (Fig. 2A2).

In untreated H1299 cells, ectopically expressed SHIP1 was observed mainly in the cytoplasm. In a small proportion of the cells (15% ± 5.7%; mean ± SD) one or a few distinct SHIP1-positive spots were detected within the nucleolus (Fig. 2B1 and 2B2). In this case almost every nucleolus of the cell possess SHIP1, however detection of SHIP1 depends on size of the cavity and, in case of small cavities, of the selected z-stack. After
Figure 1. Endogenous SHIP1 is enriched in the nucleolus of haematopoietic cells after proteasome inhibition. Endogenous SHIP1 is stained in untreated (A) and MG132 (8h) treated (B) TF-1-IIING1-8 cells (red). Nucleoli are marked by endogenous Fibrillarin (green). In addition nucleoli can be identified in DIC images as regions of lower refractive index. Endogenous SHIP1 is also stained in haematopoietic cells of healthy donors (C, red). Furthermore staining of the endogenous 20s proteasome is shown in untreated TF-1-IIING1-8 cells (D, green). Nucleolar cavities are marked by white arrows.
treatment with 20 μM MG132 for 8 hours, SHIP1 was localized to distinct intranucleolar foci (Fig. 2C1) in 31% ± 12.7% (mean ± SD) of the treated cells. In this case the SHIP1-positive spots were significantly (p < 0.05) larger (2.21 ± 1.43 μm) than in untreated cells (0.94 ± 0.50 μm). In average, there can be found 3.43 SHIP1 spots in 3.43 nucleoli per cell in untreated cells. Whereas 5.57 SHIP1 spots can be found in 3.5 nucleoli per cell in MG132 treated cells. It is conceivable that the many spots in untreated cells fuse to one large aggregate in MG132 treated cells.

After 16 hours of MG132 treatment the viability of the cells was mainly affected (data not shown). Furthermore, the SHIP1-containing nucleolar regions became more irregularly shaped and took up a predominant portion of the nucleolus. Such irregularly shaped SHIP1-containing nucleolar spots were also observed in some cases after 8 hours of MG132 treatment (Fig. 2C2).

Notably, Moran and co-workers have reported that puromycin N-acetyl-transferase (PAC) expressing-vectors can unexpectedly cause a response to misfolded proteins associated with the formation of nuclear aggresome-like structures in human cell lines. PAC expression promotes formation of nuclear inclusions by PML, SUMO-1, HSP70 and 20S proteasome. However, these inclusions are found outside of the nucleoli. In contrast, aggresome-like structures detected by us were located within the nucleoli. Furthermore, nucleolar SHIP1-positive spots were also found after expression of SHIP1 fused to EGFP using non PAC expressing vectors. In addition, nucleolar staining of endogenous 20S proteasome spots was also observed in parental TF-1-IIING1-8 (Fig. 1D) and H1299 cells (Fig. 2).

**Figure 2.** Ectopically expressed SHIP1 localizes to nucleolar cavities of H1299 cells before and after proteasome inhibition and is markedly reduced after LMB treatment. (I) The distribution of the endogenous 20S proteasome is shown in untreated H1299 cells (A1; green). The nucleolus can be identified in DIC image as regions of lower refractive index (A2). Ectopically expressed SHIP1 is shown in untreated (B) and MG132 (8h) treated (C) H1299 cells. Nucleolar cavities are marked by red and white arrows. (II) H1299 cells were treated with 20 nM Leptomycin B for 5 hours. Subsequently staining of ectopically expressed SHIP1 was carried out (B1). Cells were also treated with Methanol as control (A1). Nucleoli are marked by red arrows.
co-localize with endogenous Upstream binding factor (UBF) (Fig. S2I A) in the fibrillar center (FC) and endogenous Fibrillarin (Fig. S2I B) in the dense fibrillar component (DFC) nor with endogenous human RPB6, a shared subunit of polymerase I, II and III (Fig. S2I C). In all cases EGFP-SHIP1 granules were detected within the nucleolus of untreated H1299 cells, but did not co-localize with one of these markers.

In addition, ectopically expressed SHIP1 never co-localized with transiently expressed Histone 2B (H2B-EGFP) (Fig. S2II A) and the RNA editing adenosine deaminase ADAR2 (EGFP-hADAR2) (Fig. S2II B) in the nucleolar cavities of H1299 cells. Notably, both proteins fused to EGFP were localized to the nucleolus, but excluded from the nucleolar cavities.

To get a more detailed view of the structure of the subnucleolar SHIP1 containing component, we generated 3D-models based on confocal microscopy data of EGFP-SHIP1 expressed in H1299 cells. In these models SHIP1 presents itself within the nucleolar cavity as ovoid and spans partly almost the entire vertical z-stack of the cell (Fig. 3) [Please note the magnification of the images and the different angles showing the enormous extent (~2.3 μm in xy and ~4.7 μm in z; structures are bigger than the microscope PSF: 200 nm in xy and 500 nm in z) of the SHIP1 aggresomes in the nucleolar cavities]. Here, endogenous UBF (Fig. 3, A1 and A2) is stained in red and the nucleolar EGFP-SHIP1 aggresomes are surrounded by this marker protein.

Figure 3. 3D analysis of nucleolar EGFP-SHIP1-aggresomes. Visualization was carried out by Imaris Software. To see the nucleolar region of interest, the models were different cut. Representation of EGFP-SHIP1 (green) and staining of endogenous UBF (red) in an untreated H1299 cell is shown from different angles. Magnification of the nucleolar region in (A2-A5) is shown, EGFP-SHIP1 (green) and UBF (red) are seen. Nucleolar aggresomes are marked by white arrows. Scale bar is included in μm in red.
SHIP1 partially co-localize with the tumor suppressor p53 and other proteins known to be important factors in tumorigenesis in the nucleolus.

Localization of p53 within nucleolar cavities has been intensively investigated. To examine the potential colocalization of SHIP1 and p53 in the nucleolus, we detected ectopically expressed SHIP1 in untreated EGFP/p53-expressing cells by fluorescence microscopy. Indeed, ectopically expressed SHIP1 and transiently expressed EGFP/p53 co-localized in nucleolar cavities of untreated H1299 cells (Fig. 4A) in about 82.5% of cases. After 20 μM MG132 treatment for 8 hours and 3 μM for 16 hours, protein accumulation of SHIP1 and p53 in nucleolar cavities increased and colocalization can be observed (Fig. 4B). However, after 16 hours treatment in some cells SHIP1 and p53 are co-localized in the nucleolar cavity, but also localized to different regions within the nucleolus (Fig. 4C).

As recently shown by Boyd and coworkers, the nuclear export inhibitor leptomycin B (LMB) stabilizes high levels of p53 and causes a noticeable reduction in p53 ubiquitination by MDM2, particularly in the nucleolar fraction. To further investigate the influence of LMB on the nucleolar localization of SHIP1, H1299 cells were treated with 20 nM Leptomycin B for 5 hours. By covalently modifying exportin 1, LMB completely abolishes classical nuclear export processes in eukaryotic cells. Interestingly, the nucleolar localization of ectopically expressed SHIP1 was noticeably reduced after LMB treatment (Fig. 2II B1) compared to untreated cells (Fig. 2II A1).

A similar effect can be seen for H1299 cells, which are transiently transfected with an EGFP-SHIP1-triple-NLS construct.
In this case only control cells show a clear localization pattern of SHIP1 in the nucleolus (S4 C1), whereas the LMB treated cells show an obvious accumulation of SHIP1 in the nucleus attached to a marked reduction in nucleolar localization (S4 D1). Interestingly, the EGFP-SHIP1-triple-NLS mutant does not localize to nucleolar cavities, but is located in other parts of the nucleolus. In addition to that MG132 treatment of the cells (S4 B1) does not seem to have an influence on SHIP1 localization compared to the control (S4 A1). It should be noted that also EGFP alone fused to a triple NLS sequence shows clear nucleolar accumulation (data not shown).

Furthermore, we investigated the colocalization of ectopically expressed SHIP1 in H1299 cells with other proteins known to be important factors in tumorigenesis and are mainly shown before to localize to nucleolar cavities. Indeed, colocalization of SHIP1 with interferon stimulated antigen SP100 fused to EGFP (Fig. S3I A), serine/threonine protein kinase CK2α fused to DsRed2 (Fig. S3I B), leukemia-associated protein AF10 fused to EGFP (Fig. S3I C), the endogenous 20s proteasome core (Fig. S3II A), endogenous Ubiquitin (Fig. S3II B) and SUMO-1 fused to GFP (Fig. S3II C) was observed in nucleolar cavities.

Analysis of endogenous SHIP1 in isolated nucleoli from TF-1-IIING1-8 cells

We further analyzed the presence of nucleolar SHIP1 in TF-1-IIING1-8 cells. Protein extracts were proposed from purified nucleoli of TF-1-IIING1-8 cells expressing endogenous SHIP1 protein. Western blot analysis showed SHIP1 protein in total cell lysate as well as in the nucleoli fraction. In this case Fibrillarin served as nucleolus marker for checking the success of nucleoli purification. Enriched levels of Fibrillarin protein are seen in the nucleoli fraction, compared with that in total cell lysate. Furthermore Calnexin was used as cytosolic/endoplasmic reticulum marker to check the purity of the nucleoli fraction.

Only negligible amounts of total AKT and Calnexin were detected in the nucleoli fraction. Further, marked p53 protein levels in total cell lysate as well as in the nucleoli fraction were observed.

Discussion

In this study, we show for the first time that endogenous SHIP1 localizes to intranucleolar regions of both normal and leukemic haematopoietic cells. However, endogenous SHIP1 becomes first visible in immunostaining upon treatment with the proteasome inhibitor MG132. The accumulation of endogenous SHIP1 in the nucleoli of MG132 treated cells indicates that SHIP1 may target to the nucleoli under normal conditions as seen in Western blot analysis of purified nucleoli of TF-1-IIING1-8 cells. So it is possible that the nucleolus may have a function in the regulation of proteasomal protein degradation.

In addition to that, ectopically expressed SHIP1 colocalizes with p53 and several other key factors of oncogenesis in nucleolar cavities. Interestingly, size and frequency of SHIP1 containing nucleolar cavities increased after treatment with the proteasome inhibitor MG132. Treatment with MG132 also caused a significant accumulation of SHIP1 in the cell nucleus. This effect was not only seen in H1299 cells stably expressing SHIP1, but also in H1299 cells transiently expressing SHIP1 fused to EGFP. These findings point to a degradation of polyubiquitinated SHIP1 in the nucleus by the ubiquitin-proteasome pathway. Possibly, aggregation of ubiquitinated SHIP1 in the nucleolus and its subsequent degradation are mechanisms preventing its tumor suppressor function and may play a functional role in tumorigenesis. As recently shown, the tyrosine phosphorylation of SHIP1 by the oncoprotein BCR-ABL induces its polyubiquitination directly or indirectly via Src family kinases and, subsequently, promotes its proteasomal degradation. Furthermore, Interleukin-4 also leads to tyrosine phosphorylation and thus to proteasomal degradation of SHIP1 in bone marrow-derived macrophages.

Figure 5. Analysis of endogenous SHIP1 levels of isolated nucleoli from TF-1-IIING1-8 cells. Western blot analysis of isolated nucleoli from TF-1-IIING1-8 showing marked levels of endogenous SHIP1 (second lane). Endogenous SHIP1 can also be found in total cell lysate (TCL) (first lane). Endogenous Fibrillarin and endogenous Calnexin serve as nucleolus and cytosolic/endoplasmic reticulum markers, respectively. Endogenous total AKT and endogenous p53 levels are also examined in TCL and the nucleolus fraction.
Aggregation of polyubiquitinated proteins often results from insufficient protein degradation by the ubiquitin-proteasome system. In this context the nucleolus may represent a key stress response organelle and proteotoxic stress may lead to aggregation of key proteins of oncogenesis, including cancer-related transcription factors and cell cycle regulators (e.g. p53 and cyclin D) as well as proteins involved in neurodegenerative diseases (e.g., ataxin-1, Malin). It was also demonstrated that CK2-mediated PML phosphorylation plays an important role in causing PML polyubiquitination and degradation upon cellular stress and carcinogenesis. In addition, it was recently shown that CK2 acts as a crucial protein in the formation and clearance of aggresomes by phosphorylation of HDAC6 in response to misfolded protein stress.

Proteasomes partially associate with PML nuclear bodies and PML bodies in the nucleus may function as proteolytic centers of the cell, since they are enriched in components of the proteasome system. Impaired proteolysis in the cell causes further accumulation of ubiquitinated proteins at PML bodies, thereby, forming aggresomes. These aggresomes are able to impair the function of the proteasome system and, thereby, to promote apoptosis. SP100 and SUMO-1 are well-known representatives of the PML nuclear body and have been shown to localize to nucleolar cavi- ties. So our data suggest a similar role of SHIP1 in nucleolar cavi- ties as it deserves for SP100 and SUMO-1. Moreover it is also conceivable that SHIP1 may play a functional role in PML bodies, which has to be checked in future studies. PML nuclear body-associated protein TTRAP also localizes in nucleolar cavities upon proteasome inhibition.

In addition to the tumor suppressor p53, it was recently shown by Li and co-workers that the tumor suppressor and member of the PI3K-Akt signaling pathway PTEN also localizes to the nucleolus and that nucleolar PTEN plays a role in regu- lating nucleolar homeostasis and maintaining nucleolar morphol- ogy. SHIP1 is like PTEN an inositol phosphatase with tumor suppressor function and may have similar effects in the nucleolus like PTEN.

Furthermore, we demonstrate that nucleolar targeting of SHIP1 can be reduced by treatment of the cells with LMB. The Exportin 1-specific inhibition of nuclear export reduced the nucleolar localization of SHIP1, thereby, suggesting that LMB prevents SHIP1 from being ubiquitylated. The LMB effect on the nucleolar targeting of SHIP1 is highly interesting, since a chimeric EGFP fusion protein of SHIP1 containing 3 additional NLS sequences and being significantly enriched in nucleoli is removed from the nucleoli after LMB treatment. Possibly, in LMB treated cells an ubiquitin ligation factor fails to reach SHIP1. Notably, the E3 ubiquitin ligase MDM2 accumulates in nuclear bodies after treatment with LMB. Furthermore, an important role of CRM1 (the target of LMB) in the regulation of intranuclear transport between the Cajal bodies and the nucleolus has been reported.

Summing up, our results demonstrate that endogenous SHIP1 localizes to nucleolar regions. After treatment with the proteasome inhibitor MG132, size and frequency of SHIP1-contain- ing nucleolar regions significantly increased. As recently shown, several proteins involved in key processes of oncogenesis aggregate in nucleolar cavities. For example, p53 as well as components of the PML nuclear bodies and the ubiquitin pro- teasome pathway can be found there. Nevertheless, the exact func- tion of nucleolar SHIP1 has to be analyzed in further experiments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.

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