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Research Article

Lactacystin-induced apoptosis of cultured mouse cortical neurons is associated with accumulation of PTEN in the detergent-resistant membrane fraction

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Abstract. The tumor suppressor function of PTEN is attributed to its phospholipid phosphatase activity that dephosphorylates the plasma membrane phosphatidylinositol-(3,4,5)-triphosphate [PtdIns(3,4,5)P_3]. Implicit in this notion is that PTEN needs to be targeted to the plasma membrane to dephosphorylate PtdIns(3,4,5)P_3. However, the recruitment of PTEN to the plasma membrane is not fully understood. Here, we demonstrate PTEN accumulation in the detergent-insoluble fraction of neuronal cells in response to treatment by the proteasome inhibitor lactacystin. First, lactacystin induces apoptosis and the activation of caspase-3 in cultured cortical neurons. Second, PTEN undergoes proteolysis to form a truncated 50-kDa form that lacks parts of its C-terminal tail. Third, the truncated PTEN is stably associated with the detergent-insoluble fraction in which the plasma membrane marker protein flotillin-1 resides. Taken together, our results suggest that truncation and accumulation of PTEN to the detergent-insoluble membrane fraction are two events associated with the apoptotic signals of the proteasome inhibitor in cortical neurons.

Key words. Tumor suppressor; neuronal death; proteasome; caspase-3.

PTEN is a novel phospholipid and protein phosphatase. Its tumor suppressor function is attributed to its phospholipid phosphatase activity that specifically dephosphorylates the plasma membrane phospholipid second messenger phosphatidylinositol-(3,4,5)-triphosphate [PtdIns (3,4,5)P_3], a product of phosphoinositide-3 kinase (PI3-kinase). Mutation of the PTEN gene is associated with many cancer types such as gliomas and endometrial cancer [1, 2]. Furthermore, mammalian cells transfected with PTEN are more sensitive to the induction of apoptosis [3, 4], suggesting that its interplay with PI3-kinase is an important regulatory step in the cellular cascades related to apoptosis.

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The PTEN structure can be divided into a phosphatase domain in the N-terminal portion, a C2 domain and a C-terminal tail. The phosphatase domain contains the active site responsible for catalyzing the dephosphorylation reaction. Mutations abolishing PTEN phosphatase activity are frequently found in cancer. Both phosphatase and C2 domains contain phospholipid-binding motifs that are essential for targeting PTEN to the plasma membrane and for activation of PTEN [2, 5]. The 50-residue C-terminal tail contains the physiological phosphorylation sites Ser-380, Thr-382 and Thr-383 and the ITKV motif at the C-terminus that mediates binding of PTEN to PDZ domain-containing cellular proteins [1, 6]. In most mammalian cells, PTEN is mainly localized in the cytosol, with little or no association with the plasma membrane [7]. In the PC12 cell line, a significant portion of PTEN is also localized in the nucleus [8]. Since PtdIns(3,4,5)P_3 is located on the plasma membrane, PTEN needs to translocate from the cytosol and/or the nucleus to the plasma membrane before it can dephosphorylate the phospholipid substrate. However, the mechanism of PTEN recruitment to the plasma membrane is still poorly understood. Data presented in a recent report by Das et al. [5] suggest that dephosphorylation of Ser-380, Thr-382 and Thr-383 in the C-terminal tail enhances PTEN targeting to the plasma membrane and nucleus. Nevertheless, the physiological and pathological conditions that induce PTEN targeting to the plasma membrane have not been identified. Inhibition of the proteasome has been postulated to be responsible for the pathogenesis of Alzheimer's disease [9–11]. Inhibition of proteasome function by lactacystin treatment induces apoptosis of cultured cortical neurons and cerebellar granule cells via stimulation of mitochondrial cytochrome c release and activation of a caspase-3-like protease activity [12, 13]. Here, we report that neuronal apoptosis and activation of caspase-3 induced by lactacystin treatment are associated with conversion of PTEN to a truncated form that lacks parts of the C-terminal tail, and accumulation of both PTEN and its truncated fragment in the detergent-insoluble membrane fraction. These data suggest the association of PTEN with the plasma membrane in a pathological condition.

Materials and methods

Primary cortical neurons

Cultures of mouse neocortical neurons (gestational days 15–16) were prepared from cortices microdissected from the brains of fetuses and subjected to trypsin digestion and mechanical trituration [14]. The dissociated cells were harvested by centrifugation and resuspended in Neurobasal (NB) medium with 2.5% B-27 supplement, 0.25% GlutaMAX-I supplement (all from GIBCO), 10% fetal calf serum and 1% penicillin and streptomycin. Cells were seeded to a density of 2×10^5 cells/cm^2 culture area previously coated with 100 µg/ml poly-D-lysine. The cultures were maintained in a humidified 5% CO_2 incubator at 37 °C. After 24 h in vitro, the culture medium was replaced with serum-free NB medium with 2.5% B-27 supplement, 0.25% GlutaMAX-I supplement and 1% penicillin and streptomycin. Immunocytochemical staining of the cultures for microtubule-associated protein 2 and glia fibrillary acidic protein indicated > 95% of the cells were neurons with minimal contamination by glia [14].

DNA staining

Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). Fixed cells were then incubated with Hoechst 33285 at a final concentration of 2 µg/ml. Stained nuclei were observed and analyzed under a fluorescence microscope (Leica DM IRB).

MTT assay

MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] was dissolved in RPMI medium 1640 (GIBCO) at a stock concentration of 5 mg/ml [15]. MTT solution (30 µl) was added to each well of the 24-well plate containing cells in 300 µl culture medium and the plate was incubated at 37 °C for 30 min. The culture medium was then removed by aspiration. An aliquot of 200 µl DMSO was added to dissolve the formazan formed in each well and the absorbance of the solution at the wavelength of 570 nm was read using a TECAN plate reader.

Fluorogenic peptide substrate assay for proteasome activity

Fluorogenic peptide substrates including substrate II (Z-Leu-Leu-Glu-AMC), substrate III (Suc-Leu-Leu-Val-Tyr-AMC) and substrate VI (Z-Ala-Arg-Arg-AMC) were used to assay for the postglutamyl, chymotrypsin-like and trypsin-like peptidase activities of the neuronal cell proteasome, respectively. Briefly, cells were lysed in ice-cold homogenization buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 1 mM DTT, 5 mM ATP, 20% glycerol, 0.04% Nonidet P-40). Protein concentration was determined and equal amounts of protein (5 µg) were incubated with 50 µM of substrate II, substrate III, or substrate VI in assay buffer (50 mM Tris and 0.5 mM DTT) prior to protease activity measurement using a TECAN plate reader with an excitation wavelength of 360 nm and emission wavelength of 465 nm. All fluorogenic substrates were purchased from Calbiochem.

Caspase-3 activity measurement

Cells were lysed in buffer containing 10 mM HEPES (pH 7.4), 2 mM EDTA, 5 mM DTT and 0.1% (v/v) NP40. The lysates were subjected to centrifugation and the supernatants were used for enzymatic assays. Reactions were carried out in lysis buffer containing 10 µg of protein and

50 μM acetyl-Asp-Glu-Val-Asp-aminotrifluoromethylcoumarin (Alexis Biochemicals). Fluorescent AFC formation was measured at an excitation wavelength of 400 nm and emission wavelength of 505 nm using a plate reader (Molecular Devices Spectra Max GeminiXS).

Preparation of crude cell lysate for Western blotting

For whole-cell lysate preparation, neurons were lysed in 5× sample buffer (0.5 M Tris, pH 6.8, 10% SDS, 20% glycerol, 0.05% bromophenol blue, 20% β-mercaptoethanol). When RIPA buffer (10 mM Tris HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) was used for extraction, cells were lysed in RIPA buffer and the insoluble materials were pelleted by centrifugation at 14,000 rpm for 10 min. Pellets were redissolved in 5× sample buffer. Equal volumes of the whole-cell lysate and the pellet obtained from extraction using RIPA buffer were loaded onto SDS-PAGE gels. An aliquot of the supernatant containing an equal amount of proteins was used for Western blot analysis. After SDS-PAGE, proteins were electrotransferred to PVDF membranes and probed with antibodies. A PTEN polyclonal antibody was raised in immunized rabbits using five synthetic peptides from different regions of PTEN. The peptides were K^{352}ANRYFSDNFKVKLYF^{367}, T^{2}AIIKEIVSRNKRRYQED^{19}, T^{232}RREDKFMYFEF^{243}, E^{388}NEPFDEDQHTQITKV^{403} and K^{260}QNKMLKKDKMF^{271}. Two other PTEN antibodies which recognized the N-terminal of PTEN [anti-PTEN (N-19)] from Santa Cruz (sc-6818) and C-terminal of PTEN [anti-PTEN (26H9)] from Cell Signaling Technology (No. 9556) were obtained commercially. Anti-β-tubulin (ATN01) was purchased from Cytoskeleton Inc.

Microscopy

For confocal microscopy, cells were fixed with 4% paraformaldehyde in PBS. Fixed cells were treated with 100 mM NH_4Cl and with 0.2% Triton X-100 and then blocked with 10% goat serum in PBS, immunolabeled with primary antibodies and with Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes). Images were obtained using laser scanning confocal microscope (Carl Zeiss LSM510). For immunocytochemistry, fixed cells were quenched in 1% H_2O_2 and non-specific binding was subsequently blocked with 10% normal goat serum and 0.1% Triton X-100 in Tris-buffered saline (TBS) for 1 h at 4 °C. Cells were incubated with polyclonal antibody to PTEN (1:2000) overnight at 4 °C. Secondary antibody was incubated at room temperature for 3 h in solution with 2% normal goat serum and 0.1% Triton X-100 in TBS. Detection of immunoreactive cells was carried out using 3,4-diaminobenzidine (DAB) substrate solution (0.5 mg/ml DAB and 0.01% H_2O_2 in TBS). Immunoreactive cells were visualized under bright-field microscopy. For transmission electron microscopy, cells were fixed in 3% glutaraldehyde and 2% paraformaldehyde. After osmication in 2% osmium tetroxide, samples were dehydrated in an ascending series of ethanol and embedded in araldite. Ultra-thin sections (70 nm) were cut and mounted on formvar-coated copper grids. Grids were stained with uranyl acetate and lead citrate before viewing in a Philips BioTwin CM 120 transmission electron microscope.

Preparation of plasma membrane fractions using Percoll gradient centrifugation

All steps were carried out at 4 °C. Each well was washed once with 3 ml buffer A (0.32 M sucrose, 1 mM EDTA, 20 mM Tris, pH 7.8) containing a protease inhibitor cocktail (Complete mini, Roche) and phosphatase inhibitors (25 mM sodium fluoride, 2 mM sodium orthovanadate and 10 mM sodium pyrophosphate) and the cells were scraped off and collected in 3 ml buffer A. The cells were pelleted by centrifugation for 5 min at 1000 g. Cells were resuspended in 0.5 ml buffer A, placed in a Dounce homogenizer and homogenized using a tight-fitting plunger for 20 strokes. The suspension was transferred to a 1.5-ml Eppendorf tube and centrifuged at 1000 g for 10 min. The post nuclear supernatant (PNS) was stored on ice and the pellet from each tube was resuspended in 0.5 ml buffer A, homogenized and centrifuged again. The PNSs were combined and layered on top of 30% Percoll in buffer A and centrifuged at 84,000 g for 30 min in a Beckman SW 41 Ti rotor. This method was modified from Smart et al. [16]. The separation of subcellular components was tested and confirmed by performing Western blotting using markers such as anti-histone H1 (Santa Cruz, sc-8030), anti-oxphos Complex IV subunit II (Molecular Probes, A6404), and anti-GRP78 (N-20) (Santa Cruz, sc-1050) antibodies. Plasma membrane fractions were determined using c-src anti-serum, anti-flotillin-1 (BD Transduction Laboratories, 610820) and anti-caveolin-1 (Santa Cruz, sc-894). Cytosolic fractions were determined by measuring lactate dehydrogenase (LDH) using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Protein concentrations of fractions were determined using a BCA protein assay kit (Pierce).

Results and discussion

Lactacystin treatment suppresses proteasome activity and induces apoptosis of mouse cortical neurons

Previously, Qiu et al. [12] reported that treatment of rat cortical neurons with proteasome inhibitor induced apoptosis. To investigate further how proteasome inhibitor induces neuronal cell death, we examined the morphological and biochemical changes of mouse cortical neurons induced by treatment with the proteasome inhibitor lactacystin. As shown in figure 1, treatment of the cultured

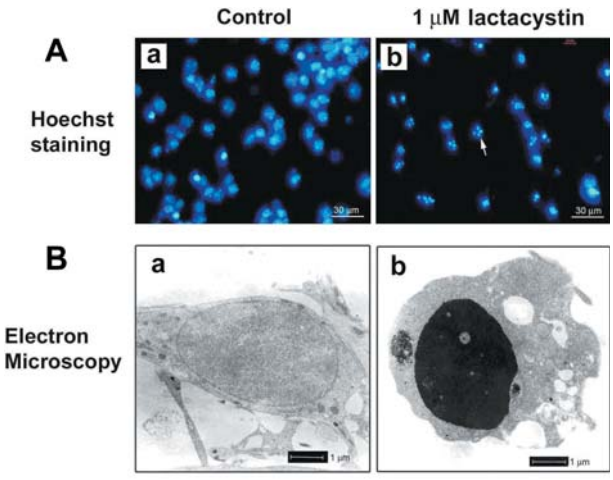

Figure 1. Morphological changes of cultured cortical neuronal cells induced by lactacystin treatment. (*A*) Staining of apoptotic nuclei using Hoechst 33285. Control and treated (1 μM lactacystin for 48 h) cells were stained and viewed under the fluorescence microscope (Carl Zeiss LSM510). White arrow indicates the apoptotic nuclei in treated cells. (*B*) Transmission electron micrographs of cultured cortical neurons. Control and lactacystin-treated (1 μM lactacystin for 48 h) cells showing the normal (a) and condensed nuclei (b).

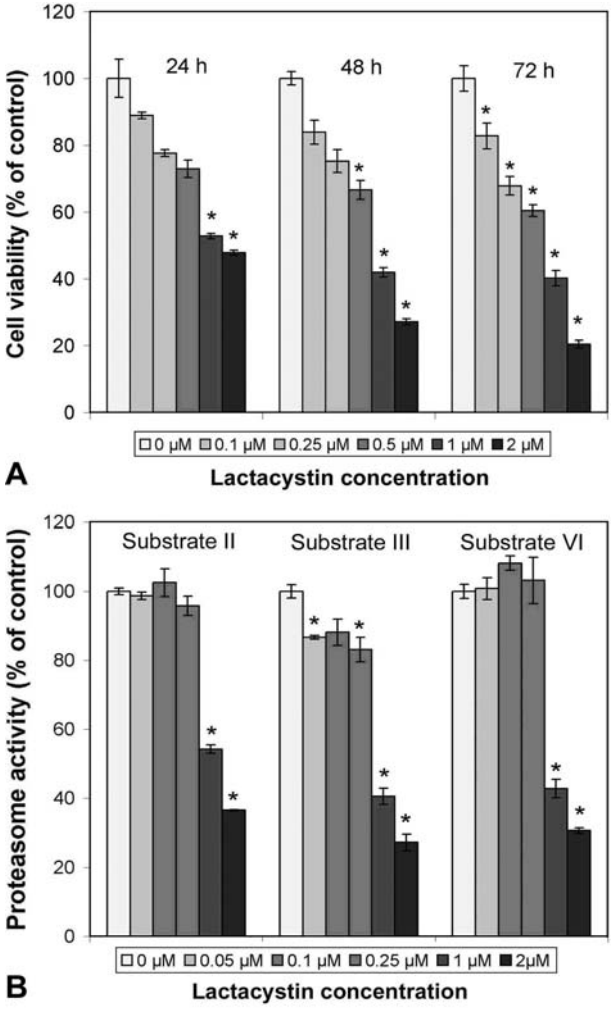

Figure 2. The effects of lactacystin treatment on viability and proteasome activity of cultured cortical neurons. (*A*) Cell viability of lactacystin-treated (1 μM lactacystin for 48 h) cultured cortical neurons was determined using the MTT assay (as described in materials and methods). Cell injury was found to be concentration and time dependent. Values are the mean ± SE of at least three independent samples and *$p < 0.05$ (ANOVA with Tukey's test) compared with the corresponding control. (*B*) Proteasome activity measurement of cultured cortical neurons treated with lactacystin (0, 0.05, 0.1, 0.25, 1 and 2 μM for 48 h) using fluorogenic peptide substrate. Five micrograms of total protein was incubated with substrate II, substrate III or substrate VI for the measurement of postglutamyl, chymotrypsin-like or the trypsin-like peptidase activity, respectively, and the fluorescent products were detected using a TECAN plate reader. Values are the mean ± SE of at least three independent samples and *$p < 0.05$ (ANOVA with Tukey's test) compared with the corresponding control.

neuronal cells with 1 μM lactacystin for 48 h induced apoptosis-associated phenotypic changes including cell shrinkage, DNA condensation and chromatin fragmentation. Counting of the condensed and fragmented nuclei revealed that about 70% of the treated cells underwent apoptosis 48 h after the treatment (data not shown). The MTT assay confirmed that the viability of the treated cells decreased in a dose- and time-dependent manner (fig. 2A). Decrease in cell viability was concomitant with the dose-dependent decrease in proteasome activities – the postglutamyl peptidase activity, chymotrypsin-like peptidase activity and the trypsin-like peptidase activity of the proteasome in the treated cells (1 μM for 48 h) were decreased to 54.3 ± 1.2%, 40.6 ± 2.4 % and 42.8 ± 2.7%, respectively, of the level in untreated cells (fig. 2B). To ascertain if the caspase-3 signaling pathway is involved in the lactacystin-induced neuronal cell death, we examined the level of the active caspase-3 generated from proteolysis of the procaspase-3, and the caspase-3 activity level in cultured neurons treated with different doses of lactacystin. As shown in figure 3, Western blotting of the whole-cell lysate extracted from lactacystin-treated neurons revealed the presence of a cleaved form of caspase-3. The appearance of the cleaved form of caspase-3 was accompanied by a dose-dependent activation of caspase-3 activity in the lactacystin-treated neurons. Taken together, our results demonstrate that the treatment of lactacystin at 1 μM for 48 h could efficiently inhibit proteasome activity, activate caspase-3 activity and induce apoptosis of cultured neuronal cells.

Changes of PTEN level in cultured neuronal cells during development

PTEN has been implicated in enhancing apoptosis of neurons induced by several pathological conditions [4]. Since the regulatory properties of PTEN are not fully understood, the exact role played by PTEN in enhancing neuronal apoptosis is not known. As a part of our ongoing study of the molecular mechanism of lactacystin-in-

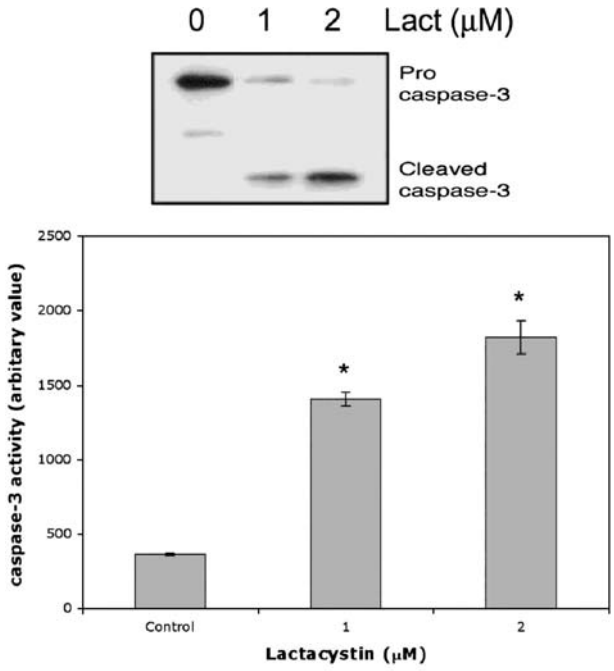

Figure 3. Detection of active caspase-3 in cultured cortical neurons treated with lactacystin. Western blot showing the presence of a cleaved form of caspase-3 and the increase in caspase-3 activity in lactacystin-treated (0, 1 and 2 μM for 48 h) cells. Statistically significant (*$p < 0.01$) over control cells determined by one-way ANOVA with Tukey's test.

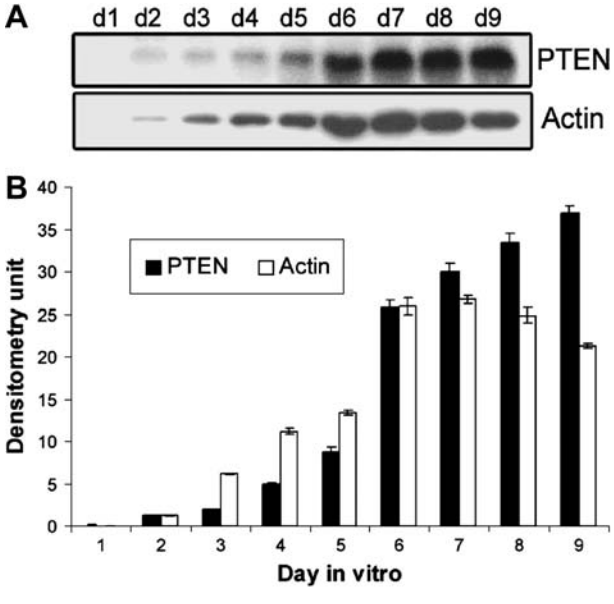

Figure 4. Western blot analysis of PTEN expression in the cultured mouse primary cortical neurons. (*A*) PTEN expression in cultured mouse cortical neurons from day 1 to day 9 in vitro. Equal amount of protein (5 μg) were loaded and run on SDS-PAGE, transferred to PVDF membrane and immunolabeled with anti-PTEN and anti-actin. Since the expression of actin increased from day 1 to 6 before reaching a plateau level, it is used only as an internal control for cultures from day 6 onward. (*B*) Graph represents the densitometric intensities of the bands in *A*.

duced neuronal cell death, we examined whether lactacystin treatment induces biochemical changes in PTEN. As a background study, we examied the time-dependent changes in PTEN expression level in cultured primary cortical neurons. Figure 4 shows that PTEN expression can be detected as early as day 2 in the in vitro culture, and the expression level increases in a time-dependent fashion to the maximum level on day 6–9 in culture. Our observation is reminiscent of the report by Luukko et al. [17] of the high level of PTEN expression in embryonic mouse brain. Since the PTEN expression level of the cultured neurons at day 5 is relatively high and reaches the maximum level at day 7 in culture, and that the neuronal cells require treatment with lactacystin for 48 h before they exhibit prominent apoptotic phenotypes (fig. 1), we used neurons at day 5 to day 7 in culture for our investigation of the effect of lactacystin on PTEN expression level and subcellular localization.

Lactacystin treatment enhances the conversion of PTEN to a 50-kDa truncated fragment and accumulation of both forms of PTEN in the detergent-insoluble membrane fraction

Western blot analysis of the whole-cell lysate of neurons in the untreated cells (lactacystin concentration = 0; fig. 5) revealed two forms of PTEN – a 55-kDa form and a 50-kDa truncated form. PTEN in untreated cells exists predominantly as the 55-kDa form. Treatment with increasing concentrations of lactacystin decreased the total expression level of PTEN (fig. 5). Intriguingly, the treatment also caused the conversion of a significant amount of the 55-kDa species to the truncated 50-kDa species. After lysis of the neuronal cells with RIPA buffer, PTEN levels in the soluble and the insoluble fractions were determined by Western blotting. As shown in figure 6A, only the 55-kDa species appeared in the soluble fraction while both the 55-kDa and the 50-kDa species were present in the insoluble fraction (fig. 6B). Since RIPA buffer contains detergents (NP40, deoxycholate and SDS), the soluble portion represents the cytosolic fraction and the detergent-soluble membrane fraction of the neuronal cells, while the insoluble portion represents the detergent-resistant plasma membrane. To establish the identity of the 50-kDa species, we reprobed the blots with two other PTEN antibodies: the PTEN N-19 and PTEN 26H9 that target the N-terminal segment and the C-terminal segment, respectively. The PTEN 26H9 antibody failed to recognize the 50-kDa truncated PTEN (fig. 6B), indicating that it lacks parts of the C-terminal tail. Taken together, the data in figure 6 demonstrate that lactacystin treatment of neuronal cells suppresses PTEN expression, induces proteolysis of PTEN to the truncated 50-kDa form of PTEN that is preferentially targeted to the detergent-resistant fraction.

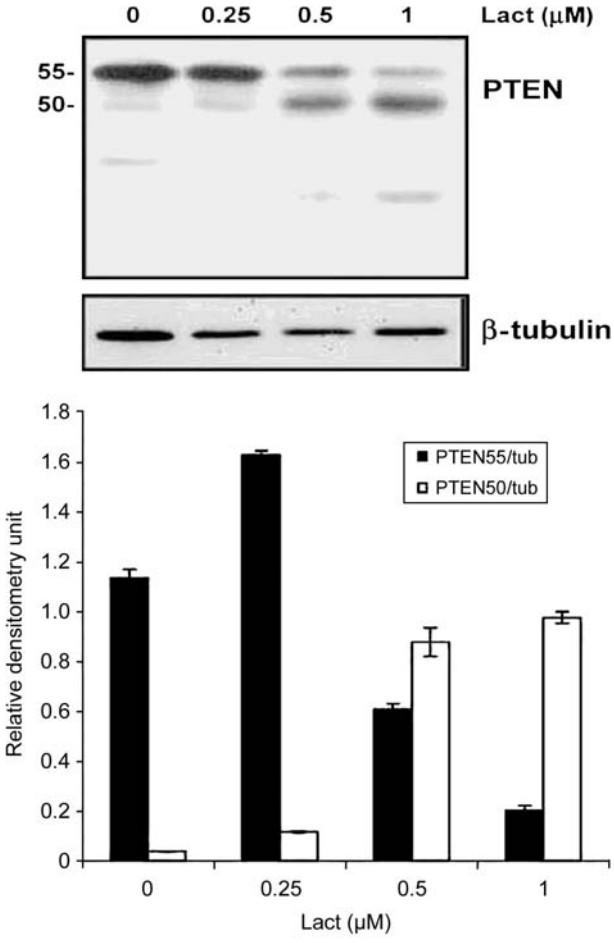

Figure 5. Western blots and graphic representation of the relative amounts of the 55-kDa and 50-kDa PTEN species found in the whole cell lysate of cortical neurons treated with increasing concentrations of lactacystin. Mouse cortical neurons were treated with increasing concentrations of lactacystin (0, 0.25, 0.5 and 1 μM) for 48 h and proteins were harvested using 5× sample loading buffer. Proteins were resolved in a 10% gel SDS-PAGE and electrotransferred to PVDF membrane. The blot was later immunolabeled with PTEN polyclonal antibody raised from five peptides chemically synthesized according to five regions of PTEN protein. β-Tubulin was the internal control for equal loading of protein. The graph represents the densitometric intensities of the 50- and 55-kDa PTEN species found with different lactacystin concentrations.

Recently, Torres et al. [18] reported that PTEN is cleaved by caspase-3 at several target sites, located in unstructured regions within the C terminus of the molecule. In our study, caspase-3 was activated upon treatment with lactacystin (fig. 3). This makes caspase-3 the most likely candidate for cleaving PTEN to its 50-kDa truncated form during lactacystin-induced apoptosis.

Lactacystin treatment enhances accumulation of a PTEN detergent-insoluble fraction that copurified with plasma membrane protein markers

As PtdIns(3,4,5)P_3 is localized on plasma membrane, the detergent-insoluble fraction in which PTEN and its truncated form accumulate upon lactacystin treatment are likely parts of the plasma membrane. To examine this notion, we performed immunocytochemistry to investigate how lactacystin treatment affects localization of PTEN in cultured cortical neurons. Figure 7A shows that PTEN in the untreated neurons is localized predominantly in the cytosol and nucleus. The image, however, could not reveal if PTEN is associated with the plasma membrane. Since flotillin-1 is expressed exclusively in specific microdomains of the plasma membrane such as the lipid raft, we therefore compared its localization with that of PTEN. As shown in figure 7B, the localization of flotillin-1 has little, if any, overlap with that of PTEN in the untreated cells. In contrast, the significant overlap of the confocal images of PTEN and flotillin-1 in the lactacystin-treated neurons suggests that PTEN is colocalized with flotillin-1 in the lactacystin-treated cells.

To investigate further the subcellular distribution of PTEN in lactacystin-treated and untreated neuronal cells, the post-nuclear supernatants of cell lysates from both the treated and untreated cells were subject to Percoll gradient centrifugation. Analysis of the centrifugation fractions with various protein markers revealed: (i) the plasma membrane lipid raft markers caveolin-1 and flotillin-1 resided predominantly in fractions 4–6 (fig. 8A), corresponding to the low-density membrane fraction; (ii) besides residing in fractions 4–6, the plasma membrane-bound protein tyrosine kinase c-Src was also present in fractions 21–22, which correspond to the high-density membrane fraction (data not shown), and (iii) the catalytic activity of the cytosolic protein marker LDH peaked at fraction 2 (fig. 8B), suggesting that fractions 1–3 were derived from the cytosolic compartment of the neuronal cells. In the untreated cells, PTEN was present in the cytosol (fractions 1–3), the low-density membrane fraction (fractions 4–6) and the high-density membrane fraction (fractions 21–22). However, in the lactacystin-treated cells, PTEN and its truncated fragment were almost absent in the cytosol and accumulated in both the low-density and high-density membrane fractions. In summary, the data shown in figures 7 and 8 lend further support to the conclusion drawn from the biochemical data (fig. 6) that lactacystin treatment induces accumulation of PTEN and its truncated fragment in the membrane fraction.

Colocalization of anti-PTEN and anti-flotillin-1 immunoreactivities in the lactacystin-treated cells (fig. 7) and comigration of PTEN and its truncated fragment with the plasma membrane markers in Percoll density gradient centrifugation (fig. 8) suggest that the detergent-insoluble fractions in which both forms of PTEN accumulate (fig. 6) are parts of the plasma membrane. However, unequivocal proof that both forms of PTEN are targeted to the plasma membrane such as the lipid raft upon lactacystin treatment entails demonstration of a correlative de-

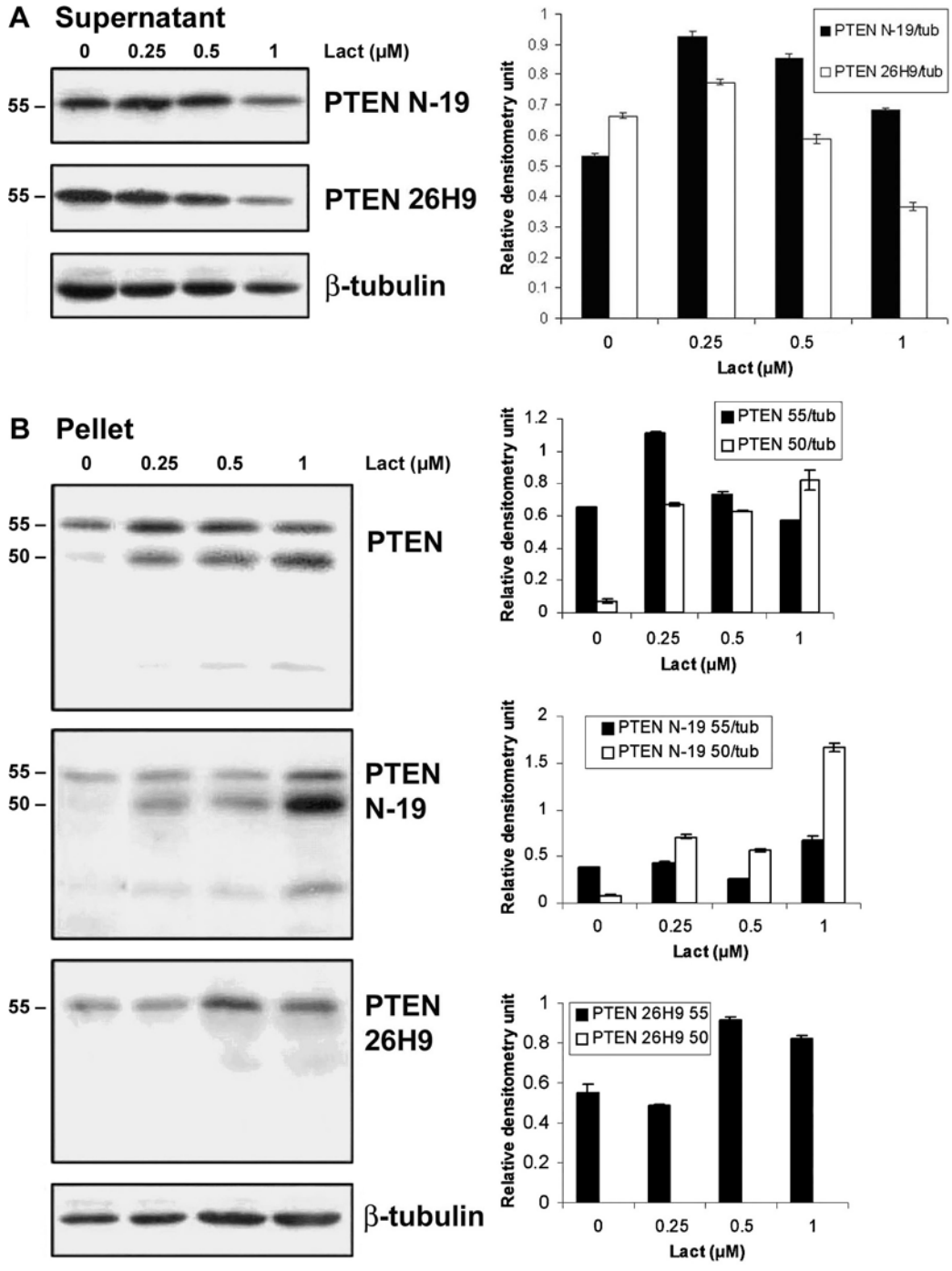

Figure 6. Distribution of the 55-kDa PTEN and the 50-kDa PTEN species in the soluble and insoluble fractions of cortical neurons treated with lactacystin. (*A*) The 55-kDa PTEN species in the detergent-soluble fraction of the lactacystin-treated cells. (*B*) The 55-kDa and 50-kDa species of PTEN in the detergent-insoluble fraction of the lactacystin-treated (1 μM for 48 h) cells. Relative amounts of the 55-kDa and 50-kDa species of PTEN were quantitated by densitometric scan of the immunoreactive bands in the Western blots and are expressed in densitometry units.

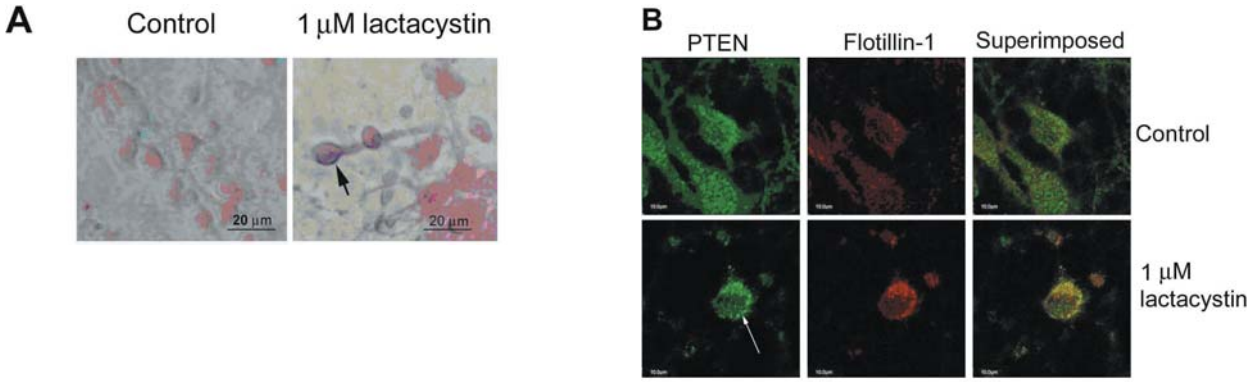

Figure 7. Effects of lactacystin treatment on PTEN subcellular localization in cultured neuronal cells. (*A*) Immunocytochemistry showing PTEN localized at the plasma membrane during neuronal apoptosis (arrow). (*B*) Immunofluorescence showing PTEN colocalized with flotillin-1 during neuronal apoptosis. Cortical neurons were treated with 1 μM lactacystin for 48 h for the induction of neuronal apoptosis.

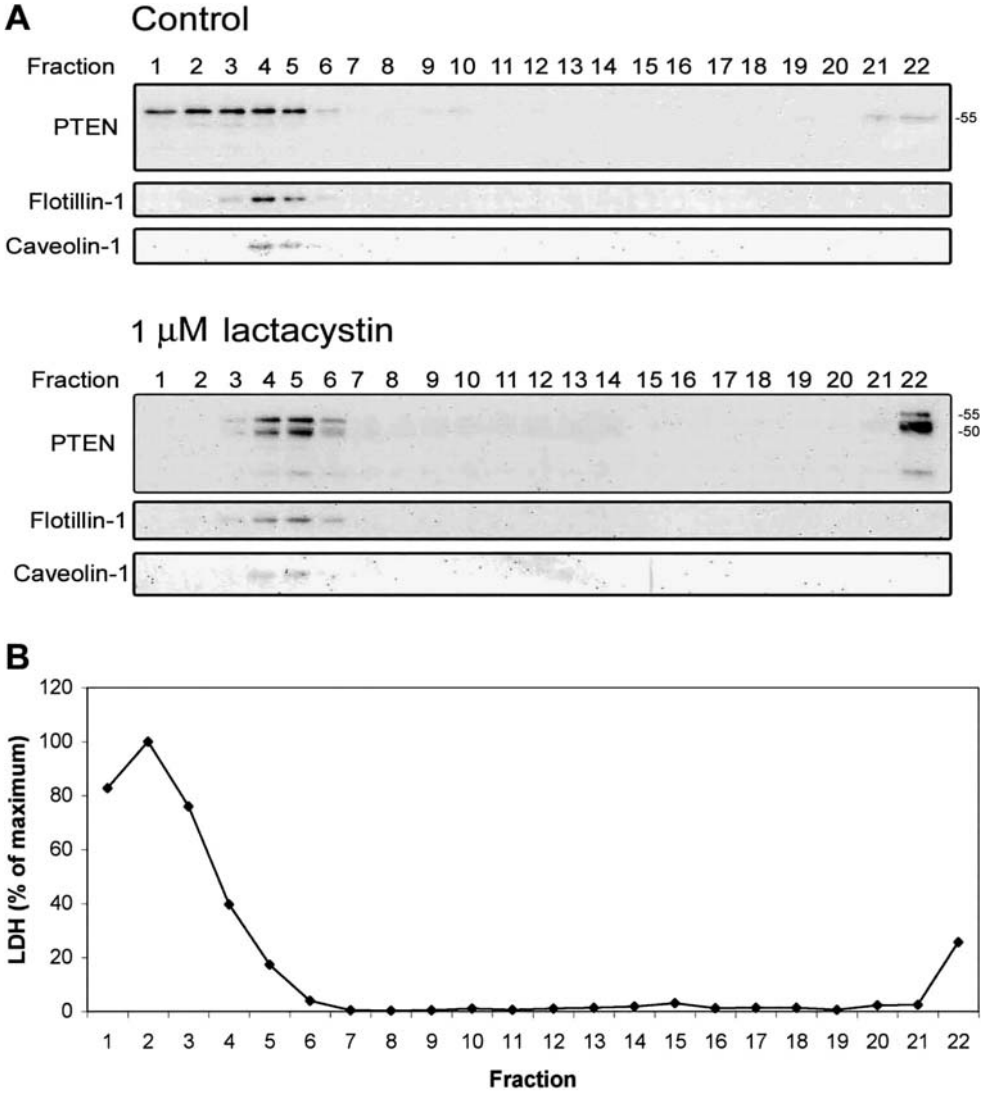

Figure 8. Lactacystin-induced changes in PTEN distribution in the various subcellular compartments of neuronal cells separated by Percoll gradient centrifugation. (*A*) The plasma membranes of cortical neurons treated and untreated with 1 μM lactacystin for 48 h were isolated by Percoll gradient centrifugation. Fractions (from top to bottom of the gradient) were collected and immunoblotted for PTEN, flotillin-1 and caveolin-1 (plasma membrane lipid raft markers). (*B*) LDH enzyme activity measurement of fractions from the cells treated with 1 μM lactacystin for 48 h. Graph shows the majority of LDH was in fraction 2, which corresponds to the cytosolic fraction.

crease in plasma membrane PtdIns(3,4,5)P_3 level and/or binding of PTEN and its fragments to protein complexes that are found exclusively in the plasma membrane.

Implications of the appearance of the 50-kDa truncated PTEN in neuronal cells induced by lactacystin treatment

Previously, Das et al. [5] demonstrated that deletion of the segment corresponding to residues 354–403 or prevention of PTEN C-terminal tail phosphorylation by replacing Ser-380, Thr-382 and Thr-383 with alanine targets the 'phosphatase-dead' [C124A] PTEN mutant to the plasma membrane. Their results suggest that the C-terminal tail, upon phosphorylation, prevents PTEN from targeting to the plasma membrane. This notion is reminiscent of our finding that the C-terminally truncated 50-kDa PTEN species preferentially accumulates in the detergent-insoluble membrane fraction in the lactacystin-treated neuronal cells. The truncated PTEN species is possibly an activated form of PTEN, which upon dephosphorylation of PtdIns(3,4,5)P_3 in the plasma membrane, suppresses cell growth and promotes apoptosis by antagonizing the PI3-kinase/PKB signaling pathway.

Despite of our findings, we still do not know the exact role played by PTEN in mediating the apoptotic effects of lactacystin. Future investigation should focus on (i) identifying the proteases responsible for converting the full-length PTEN to the truncated PTEN, (ii) defining the segments deleted from PTEN to generate the 50-kDa truncated form in neurons, (iii) ascertaining how the deletion facilitates targeting of PTEN to the plasma membrane and (iv) ascertaining if the truncated PTEN is enzymatically active.

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