Suppression of Cancer Cell Migration and Invasion by Protein Phosphatase 2A through Dephosphorylation of \( \mu \)- and m-Calpains*

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The \( \mu \)- and m-calpains are major members of the calpain family that play an essential role in regulating cell motility. We have recently discovered that nicotine-activated protein kinase C \( \tau \) enhances calpain phosphorylation in association with enhanced calpain activity and accelerated migration and invasion of human lung cancer cells. Here we found that specific disruption of protein phosphatase 2A (PP2A) activity by expression of SV40 small tumor antigen up-regulates phosphorylation of \( \mu \)- and m-calpains whereas C2-ceramide, a potent PP2A activator, reduces nicotine-induced calpain phosphorylation, suggesting that PP2A may function as a physiological calpain phosphatase. PP2A co-localizes and interacts with \( \mu \)- and m-calpains. Purified, active PP2A directly dephosphorylates \( \mu \)- and m-calpains in vitro. Overexpression of the PP2A catalytic subunit (PP2A/C) suppresses nicotine-stimulated phosphorylation of \( \mu \)- and m-calpains, which is associated with inhibition of calpain activity, wound healing, cell migration, and invasion. By contrast, depletion of PP2A/C by RNA interference enhances calpain phosphorylation, calpain activity, cell migration, and invasion. Importantly, C2-ceramide-induced suppression of calpain phosphorylation results in decreased secretion of \( \mu \)- and m-calpains from lung cancer cells into culture medium, which may have potential clinical relevance in controlling metastasis of lung cancer. These findings reveal a novel role for PP2A as a physiological calpain phosphatase that not only directly dephosphorylates but also inactivates \( \mu \)- and m-calpains, leading to suppression of migration and invasion of human lung cancer cells.

The process of tumor cell invasion and metastasis is conventionally understood as the migration of individual cells that detach from the primary tumor, enter lymphatic vessels or the bloodstream, and seed in distant organs (1). Cancer cells disseminate from the primary tumor either as individual cells, using amoeboid- or mesenchymal-type movement, or as cell sheets, stands, and clusters using collective migration. Cancer cell migration is typically regulated by integrins, matrix-degrading enzymes (i.e. metalloproteases, calpains, etc.), cell-cell adhesion molecules, and cell-cell communication (1). Tumor invasion is a complex process that involves cellular migration and interaction with the microenvironment at an ectopic site (2). The extracellular matrix forms a dense molecular meshwork so that cells may fail to penetrate without proteolytic degradation of the matrix molecules (3). Thus, the proteolytic ability in cells is critical in the processes of cell migration and invasion. Proteolytic enzymes, such as metalloproteases or cysteine proteases (i.e. calpains), dissolve tiny holes in the sheath-like covering (basement membrane) surrounding the blood vessels to allow cancer cells to invade (4–5). Once the tumor cells enter the stroma, they can easily gain access to lymphatic and blood vessels for further dissemination (6).

Understanding the mechanisms that regulate migration and invasion of tumor cells is important for development of novel therapies to control metastasis. Mounting evidence indicates that the calpain family of proteases plays an essential role in regulating cell migration and invasion, which potentially contribute to the metastasis of a variety of cancers, including lung cancer (7–9). The mammalian calpain superfamily consists of 16 known genes. Fourteen of these genes encode proteins that contain cysteine protease domains; the other two genes encode smaller regulatory proteins that associate with some of the catalytic subunits to form heterodimeric proteases (9). However, \( \mu \)- and m-calpains are the major species in the calpain family that are ubiquitously and constitutively expressed in mammalian cells. A distinguishing feature of calpain activity is its ability to confer limited cleavage of protein substrates into stable fragments rather than complete proteolytic digestion (10). Thus, calpain-mediated proteolysis represents a major pathway of post-translational modification that influences various aspects of cell physiology, including apoptosis, proliferation, migration, and invasion (10–11). Calpain cleavage of focal adhesion components, including focal adhesion kinase, paxillin, talin, and possibly others, promotes the disassembly of these complexes, which leads to reduced cell adhesion and increased cell motility (12–13).

Recent reports indicate that, in addition to \( \text{Ca}^{2+} \) binding, phosphorylation of calpain can also enhance its activity to accelerate cell migration and invasion (7–8, 14). Epidermal growth factor, nicotine, and nitrosamine 4-(methylthio)pyridine-1−(3-pyridyl)-1-butane have been found to induce
calpain phosphorylation through activation of MAPKs
with phosphate-free RPMI 1640 medium and metabolically labeled with [32P]orthophosphoric acid for 90 min. After treatment with nicotine or C2-ceramide, cells were washed with ice-cold 1× PBS and lysed in detergent buffer. μ- or m-Calpain was immunoprecipitated using a μ- or m-calpain antibody, respectively. The samples were subjected to 10% SDS-PAGE, transferred to a nitrocellulose membrane, and exposed to Kodak X-Omat film at −80 °C. Phosphorylation of μ- or m-calpain was determined by autoradiography. The same filter was then probed by Western blot analysis using a μ- or m-calpain antibody, respectively, and developed using an ECL kit from Amersham Biosciences as described previously (7–8).

Dephosphorylation of μ- or m-Calpain in Vitro—H460 cells were metabolically labeled with [32P]orthophosphoric acid and treated with nicotine for 60 min. 32P-labeled μ- or m-calpain was immunoprecipitated using an agarose-conjugated μ- or m-calpain antibody, respectively. The beads were washed three times in detergent buffer and resuspended in 60 μl of phosphate assay buffer containing 50 mM Tris-HCl, pH 7.0, 20 mM β-mercaptoethanol, 2 mM MnCl2, 0.1% bovine serum albumin. Purified PP2A (10 ng) was added, and the samples were incubated at 30 °C for 10 min. The reaction was terminated by the addition of 2× SDS sample buffer. The sample was boiled for 5 min before loading onto SDS-PAGE. Calpain phosphorylation was determined by autoradiography.

Detection of Calpain Activity in Living Cells—Cells were plated at 50–80% confluence in a glass chamber and incubated with 0.5% FBS medium for 24 h. The cells were then treated with nicotine or C2-ceramide in the presence of t-Boc-LM-CMAC (30 μM) for 30–60 min. Samples were then observed under a fluorescent microscope (excitation 329 nm, emission 409 nm) as described (7–8).

Calpain Zymography with FITC-Casein—Calpain zymography using FITC-casein was performed as described (22–23). Briefly, cells were washed with 1× PBS and lysed in the zymography lysis buffer containing 50 mM Hepes, pH 7.6, 150 mM NaCl, 10% (v/v) glycerol, 5 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 0.1% (v/v) Triton X-100, 10 μg/ml leupeptin, and 10 mM 2-mercaptoethanol. FITC-casein (0.05%, w/v) was mixed with 10% acrylamide solution (74:1 of acrylamide and bisacrylamide). The gel was incubated for at least 1 h at 4 °C for complete polymerization. 15 μg of cell lysate containing endogenous μ- and m-calpains in 20 μl of sample buffer (20% glycerol, 0.1 M Tris-HCl, pH 6.8, 10 mM EDTA, 10 mM 2-mercaptoethanol, and 0.02% bromphenol blue) was loaded into each gel well and subjected to electrophoresis under non-denaturing conditions in a buffer containing 25 mM Tris-HCl, 125 mM glycine, 1 mM EDTA, pH 8.0, and 10 mM 2-mercaptoethanol. After electrophoresis, the gel was incubated in a buffer containing 50 mM Tris-HCl, pH 7.0, 5 mM CaCl2, and 10 mM 2-mercaptoethanol overnight at room temperature. Because μ- or m-calpain-mediated cleavage of FITC-casein can result in the disappearance of fluorescence, this could produce dark zones in a bright background. The region of m-calpain band in the gel could be distinguished from μ-calpain because of its higher mobility (23). Photographs were taken under (ultraviolet) UV light.

Cell Migration and Invasion Assay—Cells were treated with nicotine or C2-ceramide as indicated. Cell migration was

EXPERIMENTAL PROCEDURES

Materials—Anti-μ-calpain, m-calpain, PP2A/C, fluorescein isothiocyanate (FITC)-conjugated anti-goat, and rhodamine-conjugated anti-rabbit IgG antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Nicotine was purchased from Sigma. C2-ceramide and purified PP2A were obtained from Calbiochem. Synthetic calpain substrate t-butoxycarbonyl-Leu-Met-chloromethylaminocoumarin (t-Boc-LM-CMAC) was obtained from Molecular Probes (Eugene, OR). The QCMTM chemotaxis 24-well colorimetric cell migration and cell invasion assay kits were purchased from Chemicon International, Inc. (Temecula, CA). Anti-small t antigen antibody was purchased from Research Diagnostics Inc. (Concord, MA). The HA-tagged PP2A/C/pCDNA3 construct was generously provided by Dr. Brain Law (University of Florida). Wild-type small t antigen cDNA in pCMV5 was kindly provided by Dr. Marc Mumby (University of Texas Southwestern Medical Center, Dallas, TX). All reagents used were obtained from commercial sources unless otherwise stated.

Cell Lines and Cell Culture—H69, H82, H157, H460, H1299, and A549 human lung cancer cells were obtained from ATCC (Manassas, VA). A549 cells were maintained in F-12K medium with 10% fetal bovine serum and 4 mM L-glutamine. H69, H82, H157, H460, and H1299 cells were maintained in RPMI 1640 with 10% fetal bovine serum.

Metabolic Labeling, Immunoprecipitation, and Western Blot Analysis—Cells were cultured with 0.5% fetal bovine serum (FBS) medium overnight. Cells were then washed three times

2 The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; SCLC, small cell lung cancer; t-Boc-LM-CMAC, t-butoxycarbonyl-Leu-Met-chloromethylaminocoumarin; PP2A/C, protein phosphatase 2A catalytic subunit; small t, SV40 small tumor antigen; siRNA, small interfering RNA; RNAi, RNA interference; PBS, phosphate-buffered saline; FBS, fetal bovine serum; HA, hemagglutinin; FITC, fluorescein isothiocyanate.
assessed using a QCM™ 24-well colorimetric cell migration assay kit (Chemicon) following the manufacturer’s instructions. This new technique does not require cell labeling, scraping, washing, or counting. Cells that migrated through the polycarbonate membrane were incubated with "Cell Stain Solution" and then subsequently extracted and detected on a standard microplate at 560 nm. Cell invasion was assessed using the Chemicon cell invasion assay kit. This assay was performed in an invasion chamber, which is a 24-well tissue plate with 12 cell culture inserts. The inserts contain an 8-μm pore size polycarbonate membrane over which a thin layer of ECMatrix™ is dried. The extracellular matrix layer occludes the membrane pores, blocking non-invasive cells from migrating through. Invasion cells migrate through the extracellular matrix layer and cling to the bottom of the polycarbonate membrane. The insert membrane with invaded cells on the bottom was placed in the wells with cell stain/dissociation solution after incubation and reincubation for 30 min at 37 °C. Absorbance was measured with a microplate reader at 560 nm. Each experiment was repeated three times, and data represent the mean ± S.D. of three determinations.

**Monolayer Wound Healing Assay—**Cells were seeded into a six-well tissue culture dish and allowed to grow to 90% confluency in complete medium. Cell monolayers were wounded by a plastic tip (1 mm) that touched the plate as described (8). Wounded monolayers were then washed four times with medium to remove cell debris and incubated in 0.5% FBS medium in the absence or presence of nicotine (0.2 μM) or inhibitor for various times up to 24 h. Cells were monitored under a microscope equipped with a camera (Deiss).

**Immunofluorescence—**Cells were seeded and cultured on a Lab-Tek® chamber slide (Nalge Nunc International) overnight at 37 °C with 5% CO2. Cells were washed with 1X PBS, fixed with cold methanol and acetone, and blocked with 10% goat and rabbit serum. Cells were incubated with goat against human μ- or m-calpain and rabbit against human PP2A/C primary antibodies for 60 min. After washing, samples were incubated with FITC-conjugated anti-goat and rhodamine-conjugated anti-rabbit secondary antibodies for 60 min. Cells were washed with 1X PBS and observed under a fluorescent microscope (Zeiss). Pictures were taken and colored with the same exposure setting for each experiment. To determine subcellular regions of protein co-localization, individual red- and green-stained images derived from the same field were merged using Openlab 3.1.5 software from Improvision, Inc. (Lexington, MA). Areas of protein co-localization appear yellow.

**Knock Down of PP2A Catalytic Subunit by RNA Interference (RNAi)—**Human PP2A/C siRNA (Santa Cruz Biotechnology) was transfected into H460 cells by using Lipofectamine™ 2000 according to the manufacturer’s instructions. A control siRNA (non-homologous to any known gene sequence) was employed as a negative control. The levels of PP2A/C expression were determined by Western blot using a PP2A/C antibody. Three independent experiments were conducted for specific silencing of the targeted PP2A/C gene.

**Collection of Vesicles from Culture Medium—**Vesicles in culture medium were collected as described previously (7). Briefly, H460 cells were cultured in RPMI 1640 medium with 0.1% FBS and treated with nicotine in the presence or absence of increasing concentrations of C2-ceramide for 24 h. After treatment, samples were centrifuged at 500 × g for 10 min to pellet the cells. The supernatant was further centrifuged at 21,000 × g for 10 min to pellet the cell debris. To pellet the medium vesicles, the resulting supernatant was centrifuged at 100,000 × g for 60 min in an ultracentrifuge (Optima XL-80K Ultracentrifuge with a SW 28 rotor; Beckman Coulter). The pellets were washed with ice-cold PBS and then resuspended in 100 μl of buffer A (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 5 mM 2-mercaptoethanol). Samples were mixed with 2× SDS sample buffer, boiled for 5 min, and then subjected to 10% gradient SDS-PAGE. Levels of μ- and m-calpain in the vesicles from cell culture medium were analyzed by Western blotting.

**RESULTS**

**Disruption of PP2A Activity by Expression of Small T Antigen**

**Results in Phosphorylation of μ- and m-Calpains in Association with Up-regulation of Calpain Activity, Acceleration of Wound Healing, Migration, and Invasion of Human Lung Cancer Cells—**

Our previous findings and those of others have demonstrated that phosphorylation of μ- and m-calpains through activation of MAPKs ERK1/2 and/or protein kinase C t positively regulates calpain activity and cell motility (7, 8, 14). Because both μ- and m-calpains are extensively co-expressed with serine/threonine protein phosphatase 2A (PP2A) in both small cell lung cancer (SCLC) cells (i.e. H69 and H82) and non-small cell lung cancer cells (i.e. H157, H460, A549, and H1299) (Fig. 1A), PP2A may act as a physiological phosphatase to regulate calpain function. It is well known that the SV40 small tumor antigen (small t) can interact with the 36-kDa catalytic C and the 63-kDa A subunits of PP2A, which can specifically disrupt PP2A activity (24). To test whether inhibition of PP2A activity affects calpain phosphorylation, the pCMV5/small t antigen construct was transfected into H460 cells. After transfection, cells were metabolically labeled with [32P]orthophosphoric acid. Intriguingly, expression of small t antigen significantly enhances phosphorylation of both μ- and m-calpains (Fig. 1B and C). To test whether expression of small t antigen affects the proteolytic activity of calpains, t-Boc-LM-CMAC, a synthetic calpain substrate, was used for measurement of calpain activity in living cells as described previously (7). Cleavage of t-Boc-LM-CMAC by active calpains can induce retention of the chloromethyl-nocoumarin portion of the molecule in the cells and can result in increased fluorescence (7). The pCMV5/small t antigen construct was transfected into H460 cells. After 48 h, cells were incubated with t-Boc-LM-CMAC for 60 min. Results indicate that expression of small t antigen enhances calpain activity (Fig. 1D). To further test the effect of small t antigen on activity for individual μ- or m-calpain, calpain zymography using FITC-casein was employed as described under “Experimental Procedures.” This technique can analyze calpain activity after electrophoresis in gels. Samples are run under nondenaturing conditions on acrylamide gels in which FITC-casein has been copolymerized. After electrophoresis, the gels are incubated overnight in a buffer containing a reducing agent. During this time, FITC-casein in the active μ- or m-calpain
band is digested to fragments sufficiently small to diffuse out of the gel, and \( \mu \)- or m-calpain itself is also extensively degraded. The gels are viewed and photographed with UV illumination. The \( \mu \)- or m-calpain activity produces dark (nonfluorescent) zones in a bright background. Results reveal that expression of small t antigen enhances activities of both \( \mu \)- and m-calpains (Fig. 1E), suggesting that phosphorylation may activate both \( \mu \)- and m-calpains. To test the effect of small t antigen on cell motility, a wound healing assay was employed as described under “Experimental Procedures.” Compared with vector control cells, wound repair is significantly accelerated in cells expressing small t antigen (Fig. 1F). Consistently, expression of small t antigen significantly enhances both migration and invasion of human lung cancer cells (Fig. 1G). These findings strongly suggest that PP2A may function as a physiological calpain phosphatase to induce calpain dephosphorylation in lung cancer cell motility signaling. Other lung cancer cell lines (i.e. H69, H82, H1299, and A549) were also tested, and similar results were obtained (data not shown). These results support the notion that calpain phosphorylation may be a dynamic process that is regulated by calpain kinases (i.e. ERK1/2 and protein kinase C) (7–8, 14) and phosphatases (i.e. PP2A). Thus, protein kinase-mediated constitutive phosphorylation of \( \mu \)- and m-calpains may occur in untreated cells because specific inhibition of PP2A activity by small t antigen up-regulates calpain phosphorylation (Fig. 1).

Ceramide Inhibits Nicotine-induced Calpain Phosphorylation in Association with Suppression of Calpain Activity, Wound Healing, Migration, and Invasion of Human Lung Cancer Cells—Because ceramide is a potent pharmacological activator of PP2A (25–26), it is possible that ceramide may induce calpain dephosphorylation through activation of PP2A. To test this, H460 cells were metabolically labeled with \( ^{32} \)Porthophosphoric acid and treated with nicotine in the absence or presence of increasing concentrations of C2-ceramide. Results clearly indicate that C2-ceramide suppresses nicotine-induced phosphorylation of
PP2A Dephosphorylates Calpain

μ- and m-calpains in a dose-dependent manner (Fig. 2A). Importantly, treatment of cells with C2-ceramide blocks nicotine-stimulated calpain activity, wound healing, and cell migration and invasion (Fig. 2, B–E). These findings suggest that ceramide-activated PP2A may reduce the calpain phosphorylation level via dephosphorylation.

PP2A Co-localizes and Interacts with μ- and m-Calpains—To assess a potential direct role for PP2A as a physiological calpain phosphatase, subcellular distribution of PP2A/C and calpain was examined by immunofluorescent staining. A goat antibody against human μ- or m-calpain, rabbit polyclonal PP2A/C antibody, and FITC-conjugated anti-goat (green) or Rhodamine-conjugated anti-rabbit (red) secondary antibodies were used so that cells could be simultaneously stained without cross-reaction. As shown in Fig. 3A, μ- or m-calpain is primarily co-localized with PP2A/C in the cytoplasm of H460 cells. To test whether ceramide-activated PP2A enhances an association between PP2A and calpain, H460 cells were treated with increasing concentrations of C2-ceramide for 60 min. A co-immunoprecipitation experiment was carried out using an agarose-conjugated μ- or m-calpain antibody. The μ- or m-calpain-associated PP2A/C (i.e. bound PP2A/C) was analyzed by Western blotting using a PP2A/C antibody. Results reveal that treatment of cells with C2-ceramide promotes PP2A/C to associate with μ- or m-calpain in a dose-dependent manner (Fig. 3B). Thus, PP2A/C may potentially function as a physiological phosphatase for both μ- and m-calpains to regulate their activities in human lung cancer cells.

PP2A Dephosphorylates and Inactivates μ- and m-Calpains in Association with Inhibition of Wound Healing, Migration, and Invasion of Human Lung Cancer Cells—PP2A is the most abundant known serine/threonine-specific protein phosphatase expressed in mammalian cells (15). To test whether PP2A can directly dephosphorylate calpain, H460 cells were metabolically labeled with [32P]orthophosphoric acid and treated with nicotine for 60 min. Phosphorylated μ- or m-calpain was immunoprecipitated using an agarose-conjugated μ- or m-calpain antibody, respectively. The 32P-labeled μ- or m-calpain was used as substrate and incubated with purified, active PP2A at 30 °C for 10 min. Results show that PP2A efficiently removes the 32P-labeled phosphate from μ- or m-calpain, suggesting that PP2A can directly dephosphorylate μ- or m-calpain in vitro (Fig. 4A). To further test whether PP2A can dephosphorylate calpains in vivo, a HA-tagged PP2A/C/pC DNA3 con-
PP2A Dephosphorylates Calpain

**A**

![Images of PP2A/C and m-Calpain localization](Image)

**B**

![Images of co-immunoprecipitation experiment](Image)

**FIGURE 3.** PP2A co-localizes and interacts with m- and m-calpains. A, H460 cells were fixed with methanol and acetone. After washing with 1× PBS, cells were incubated with goat against human m-calpain and rabbit against human PP2A/C antibodies. FITC-conjugated anti-goat and rhodamine-conjugated anti-rabbit secondary antibodies were used to visualize rabbit against human PP2A/C antibodies. FITC-conjugated anti-goat and rhodamine-conjugated anti-rabbit secondary antibodies were used to visualize m-calspain (green) and PP2A/C (red) localization patterns under a fluorescent microscope. Red- and green-stained images were merged using Openlab 3.1.5 software. Areas of co-localization appear yellow. B, H460 cells were treated with increasing concentrations of C2-ceramide for 120 min. A co-immunoprecipitation experiment was performed using agarose-conjugated m- or m-calpain, respectively. The m- or m-calpain-associated PP2A/C and total calpain were analyzed by Western blot.

Depletion of PP2A/C by RNAi Enhances Calpain Phosphorylation and Activity, Which Results in Accelerated Wound Healing, Migration, and Invasion of Human Lung Cancer Cells—Our data strongly suggest that PP2A functions as a physiological calpain phosphatase to dephosphorylate calpain, which may lead to decreased calpain activity. To test whether PP2A is essential for calpain dephosphorylation, H460 cells were transfected with PP2A/C siRNA as described under “Experimental Procedures.” Results show that the PP2A/C siRNA efficiently and specifically reduces PP2A/C expression in H460 cells whereas control siRNA has no effect (Fig. 5A). Importantly, specific disruption of PP2A/C expression by RNAi not only results in phosphorylation of both m- and m-calpains but also further enhances nicotine-stimulated calpain phosphorylation in association with increased calpain activity, accelerated wound healing, cell migration, and invasion (Fig. 5). These findings suggest that PP2A may be essential for dephosphorylation of calpains to negatively regulate motility of human lung cancer cells.

**TREATMENT OF CELLS WITH C2-CERAMIDE SUPPRESSES NICOTINE-STIMULATED SECRETION OF m- AND m-CALPAINS FROM HUMAN LUNG CANCER CELLS—**Calpain has been reported to be a positive regulator of cell migration and invasion because it localizes to focal adhesions and cleaves many focal adhesion-related proteins, including integrin receptors, focal adhesion kinase, and talin (27). Therefore, secretion of calpains from inside of cells into extracellular matrix is critical for the effect of calpain on cell motility. We have previously discovered that nicotine or nitrosamine 4-(methylisothio- samin)-1-(3-pyridyl)-1-butanone-induced calpain phosphorylation promotes secretion of m- and m-calpains from lung cancer cells into culture medium through a nonclassical pathway involving vesicles (7–8). To test whether C2-ceramide-induced dephosphorylation of calpains affects their secretion, H460 cells were treated with nicotine (50 nM) in the absence or presence of increasing concentrations of C2-ceramide for 24 h. The medium vesicles were collected as described under “Experimental Procedures.” Western blotting analysis indicates that C2-ceramide inhibits nicotine-induced nonclassical secretion of both m- and m-calpains in a dose-dependent manner (Fig. 6).

**DISCUSSION**

Calpain activity is required for de-adhesion of the cell body and rear to enable productive locomotion of adherent cells during wound repair and tumor invasion (28). Either inhibition of calpain activity by a pharmacological agent or genetic disruption of calpain expression by gene silencing resulted in decreased cell migration and/or invasion (7, 29–30), indicating that calpains are required components in the cellular machinery that governs cell motility. Because calpain activity is significantly elevated in various tumor cells when compared with nonmalignant cells (31–32) and antisense-mediated suppression of m-calpain restricts invasion of carcinoma cells in vivo (2), this strongly suggests that elevated calpain activity in tumor cells may potentially facilitate tumor metastasis in a mechanism...
by accelerating migration and invasion. Thus, identifying the mechanisms leading to calpain being activated or inactivated may be of benefit in developing novel therapeutic strategies for treatment of metastatic tumors.

Although calpain activity is regulated by multiple mechanisms, the best studied mechanism is activation by calcium because calpains contain calcium binding EF-hand motifs in domains IV and VI. However, structural data suggest that conformational changes caused by calcium occupancy of the EF hands alone are insufficient to align the active site catalytic residues properly (9). In addition, the levels of calcium required to activate calpains in vitro do not exist within living cells. Therefore, other regulatory mechanism(s) including phosphorylation may play a critical role in regulating calpain activity.

Our previous findings and those of others discovered that MAPKs ERK1/2 and protein kinase C function as physiological calpain kinases that can directly phosphorylate and activate calpains (7, 8, 14), suggesting that protein kinase(s)-mediated calpain phosphorylation may be another important mechanism for calpain activation. However, it is currently unclear whether calpain dephosphorylation mediated by a protein phosphatase plays a role in regulating calpain activity and cell motility. PP2A is a key protein serine/threonine phosphatase that is responsible for 30–50% of the total cellular serine/threonine dephosphorylation activity. PP2A can negatively regulate cell motility because inhibition of PP2A activity with okadaic acid stimulates migration of various tumor cells (19–20). Evidence reported here demonstrates that PP2A functions as a calpain phosphatase because specific disruption of PP2A activity by expression of small t antigen enhances phosphorylation of both μ- and m-calpains in association with increased calpain activity and cell motility (Fig. 1). Furthermore, PP2A not only co-localizes and interacts with both μ- and m-calpains but also directly dephosphorylates μ- and m-calpains in vitro (Figs. 3 and 4), indicating its potential direct role as a calpain phosphatase.

Importantly, overexpression of PP2A/C results in calpain dephosphorylation, decreased calpain activity, and reduced cell migration and invasion (Fig. 4). By contrast, specific knockdown of PP2A with small interfering RNA (siRNA) results in calpain phosphorylation and increased calpain activity (Fig. 2). These findings suggest that PP2A plays a critical role in regulating calpain activity and cell motility. Therefore, therapeutic strategies targeting PP2A may have potential therapeutic applications in the treatment of metastatic tumors.

### References

down of PP2A/C expression by RNAi increases calpain phosphorylation in association with increased calpain activity and accelerated cell motility (Fig. 5). These findings provide genetic evidence that PP2A is a physiological calpain phosphatase in vivo. Thus, PP2A suppression of cell migration and invasion may occur in a novel mechanism by directly dephosphorylating and inactivating the major calpain species, the \( \mu \)- and m-calpains.

The sphingolipid ceramide functions as a powerful second signal effector molecule that regulates diverse cellular processes, including apoptosis, cell cycle, cell senescence, cell motility, and cellular differentiation (33). It is clear that ceramide can stimulate PP2A activity by binding to its catalytic domain (25). Here we found that the PP2A activator C2-ceramide not only promotes PP2A/C to associate with \( \mu \)- or m-calpain but also potently induces calpain dephosphorylation (Figs. 2A and 3B), suggesting that \( \mu \)- and m-calpains may function as downstream targets in the ceramide-induced suppression of cell motility (Fig. 2).

We have previously demonstrated that nicotine or nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced calpain phosphorylation promotes secretion of active \( \mu \)- and m-calpains from lung cancer cells through a nonclassical pathway involving vesicles, which may have potential to cleave substrates in the extracellular matrix (7–8). Because C2-ceramide potently blocks nicotine-stimulated calpain secretion (Fig. 6), this indicates that C2-ceramide-induced dephosphorylation of \( \mu \)- and m-calpains by activating PP2A impedes calpain secretion, which may contribute to retardation of cell motility.

In summary, our findings have identified PP2A as a novel physiological calpain phosphatase that can directly dephosphorylate the major calpain species, \( \mu \)- and m-calpains, in the context of cigarette smoke exposure in lung cancer cells.
ceramide production may have clinical relevance for the treatment and invasion of human lung cancer cells. Thus, therapeutic suppression of calpain secretion, leading to reduced migration and invasion of human lung cancer cells. Therefore, therapeutic activation of PP2A results in decreased calpain activity and suppression of calpain secretion, leading to reduced migration and invasion of human lung cancer cells. Thus, therapeutic activation of PP2A to dephosphorylate calpain by enhancing ceramide production may have clinical relevance for the treatment of cancer metastasis.

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