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Proteome Profile Changes in SH-SY5y Neuronal Cells after Treatment with Neurotrophic Factors

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ABSTRACT

Artemin, one of glial cell line-derived neurotrophic factors (GDNFs) supports sensory neuron. Although a role of artemin and GDNF as neurite outgrowth regulators in early neuron development has been suggested, the immediate effects of artemin and GDNF on neuronal cells have not been elucidated. Here, we investigated artemin and GDNF actions on the neuronal cell proteome. To identify immediate-early protein changes by artemin and GDNF in neuronal cells, we used a differential proteomics approach in SH-SY5y human neuronal cells treated with artemin or GDNF for 1 h. Eleven proteins that changed after both artemin and GDNF treatment were identified using two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization time-of-flight tandem mass spectroscopy. The calcium ion-binding chaperone calreticulin and calcium/calmodulin-binding nuclear matrix protein matrin 3 showed common quantitative differences after both artemin and GDNF treatment. Cytoskeletal proteins also showed quantitative profile differences, which are functionally relevant to cytoskeletal rearrangement leading to the neurite elongation in neurons. These protein changes were detected in neuronal cells without accompanying changes in mRNA levels. These results suggest that immediate changes induced by artemin and GDNF are related to cytoskeletal protein level changes without transcriptional regulation. J. Cell. Biochem. 112: 3845–3855, 2011. © 2011 Wiley Periodicals, Inc.

KEY WORDS: ARTEMIN; GDNF; PROTEOMICS; CYTOSKELETAL PROTEIN

Neurotrophic factors are a heterogeneous group of signaling molecules that regulate multiple aspects of the development and maintenance of the central and peripheral nervous systems. The glial cell line-derived neurotrophic factor (GDNF) family, which includes artemin [Kotzbauer et al., 1996; Milbrandt et al., 1998], persephin [Masure et al., 1999], and neurturin [Rosenblad et al., 2000], is a subfamily of the transforming growth factor β superfamily. GDNF family members play a crucial role in the development of peripheral autonomic and sensory neurons [Baloh et al., 2000; Airaksine and Saarma, 2002]. The GDNF family receptors comprise the GDNF receptor accessory proteins (GFR-α1, -α2, -α3, and -α4) that each binds preferentially to a different ligand as well as the tyrosine kinase receptor, Ret, which mediates signaling [Worby et al., 1998].

In recent studies on post-traumatic nerve regeneration and neuropathic pain, GDNF was found to reverse the post-traumatic changes in GFR-α1- and α2-expressing cells [Bennett et al., 2000; Boucher et al., 2000; Ramer et al., 2000; Schwab, 2000].

Artemin binds to GFR-α1 and α3 receptors and signals through Ret to support the survival of a subset of sensory neurons in the dorsal root ganglion (DRG) and the trigeminal ganglion [Baloh et al., 1998]. Artemin expression is observed in the adult brain and spinal cord [Baloh et al., 1998], although not at a very high level, suggesting that artemin may play a role in post-development.

A recent study showed that systemic artemin prevented and reversed neuropathic pain and normalized spinal nerve ligation-induced morphological changes [Gardell et al., 2003]. In addition, artemin and neurturin, as well as GDNF, are known to activate

Abbreviations: GDNF, glial cell line-derived neurotrophic factor; GFR, GDNF family receptor; DRG, dorsal root ganglion; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; 2D, two-dimensional; IPG, immobilized pH gradient; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LR, log ratio; MALDI-TOF MS, matrix-assisted laser-desorption ionization time-of-flight tandem mass spectroscopy; PBST, phosphate buffered saline-0.1% Tween 20; PBS, phosphate buffered saline; UCH-L1, ubiquitin carboxyl-terminal hydrolase L1; PDI, protein disulfide isomerase; GSH, glutathione; ER, endoplasmic reticulum.

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axonal growth. However, the mechanism by which artemin mediates the activation of axonal growth is unclear. To understand the molecular basis of GDNF-induced cell responses, it is important to identify the expression or function of proteins that are modified by both artemin and GDNF. Little is known about the early events in neuronal cells exposed to artemin or GDNF, since most studies have examined the physiological effects of artemin and GDNF. In the present study, we analyzed the molecular modifications induced by artemin in neuronal cells using a proteomics approach. This methodology provides important qualitative information on post-translational modifications to each protein as well as quantitative data on protein expression levels in response to a particular stimulus. The neuroblastoma cell line, SH-SY5y, is a derivative of undifferentiated peripheral neuroblasts that respond to GDNF and display the GDNF-responsive, GFRa-RET receptor [Baloh et al., 1998]. We observed artemin-induced neurite extension in SH-SY5y cell lines [Park and Hong, 2006]. Therefore, using the SH-SY5y cell line, we investigated the early events involved in artemin- and GDNF-induced cellular changes. To identify early events without transcriptional activation, we treated cells for 1 h with artemin or GDNF. This information is particularly important because it provides data on early cellular events, such as phosphorylation and protein degradation, involved in the signaling cascades triggered independently of protein synthesis.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENT

The human neuroblastoma cell line, SH-SY5y, was purchased from the Korean Cell Line Bank (Seoul, Korea) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). DMEM and FBS were purchased from Gibco-BRL (Rockville, MD), and artemin and GDNF were purchased from R&D systems (Minneapolis, MN). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO). The cells were incubated in a 5% CO_2-humidified incubator at 37°C, and treated with 200 ng/ml artemin or GDNF for 1 h.

PREPARATION OF PROTEIN EXTRACTS

To obtain total protein extracts, 5×10^7 cells were resuspended in a total volume of 500 μl buffer containing 8 M urea, 2 mM tributylphosphine, 4% (w/v) CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), 0.2% carrier ampholyte (pH 4–7, Bio-Rad, Hercules, CA), and a protease inhibitor cocktail (Sigma–Aldrich). Cells were disrupted by 10 strokes using a Model XL-2020 sonicator (Misonix, Farmingdale, NY). After 1-h incubation at room temperature, cell lysates were centrifuged at 16,000 *g* for 60 min at 13°C. The supernatant was collected, and protein concentrations were determined using the Bio-Rad detergent-compatible protein assay.

TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS AND GEL ANALYSIS

Proteins (1.5 mg) were hydrated overnight in 8 M urea, 4% CHAPS, 0.2% carrier ampholyte, and 0.0002% bromophenol blue at 4°C and applied to isoelectric focusing gel strips (immobilized pH gradient [IPG] strip: pH 4–7 linear, 17 cm in length; Bio-Rad). The gel strips were rehydrated overnight in 8 M urea, 4% CHAPS, 0.2% carrier ampholyte, and 0.0002% bromophenol blue at 20°C. Isoelectric focusing was initiated at 100 V for 2 h, 250 V for 2 h, and gradually increased to 10,000 V for 10 h. The focusing process was carried out for 10,000 V h and held at 500 V for 24 h. After isoelectric focusing, IPG strips were equilibrated with 0.05 M Tris-HCl (pH 8.8), 0.6 mM TATA-binding protein, 5% iodoacetamide, 6 M urea, 20% glycerol, and 2% sodium dodecyl sulfate (SDS) for 20 min. Strips were then transferred onto 12% SDS-polyacrylamide gels and sealed with 1% low-melting point agarose. SDS-polyacrylamide gel electrophoresis (PAGE) was run for 30 min at a constant current of 16 mA/gel, and then at 24 mA/gel at 15°C. After electrophoresis, the proteins were visualized by Coomassie Brilliant Blue G-250 staining of the gels. Images were digitalized using a Model GS-800 calibrated densitometer (Bio-Rad) and analyzed using PDQuest 2-D analysis software (Bio-Rad).

EVALUATION OF DIFFERENTIALLY REPRESENTED SPOTS

Quantitative differences were determined only when a matched spot was found to show statistically significant changes in tetraplicate gels. Matching spots in gels from the same sample were identified, and their intensities were measured using an Image Master 2-D system. Analysis was performed on approximately 200 different protein spots per sample. For each spot, the intensity value in a neurotrophic factor-treated gel was divided by its intensity value obtained in the control gel. The logs of these ratios (LRs) were then calculated. Means and median LR values were clustered around the zero value, which was expected if errors associated with the analysis were random and normally distributed. Spots showing a statistically significant differential expression ($P < 0.01$) between treatment case and control gel in duplicate experiments and common in both cases (GDNF and artemin treatment) were considered as different protein species.

IN-GEL ENZYMATIC DIGESTION AND MASS SPECTROMETRY

In-gel digestion was performed as previously described [Rosenfeld et al., 1992]. Spots were excised from the stained gel, destained with 0.1 M ammonium bicarbonate/50% acetonitrile (Sigma–Aldrich), and dried in a Speed Vac plus SC1 10 (Savant, Holbook, HY). The gel was rehydrated in a solution containing 1 M dithiothreitol and 0.1 M ammonium bicarbonate (pH 7.8) for 30 min at 56°C. After a subsequent incubation in a solution containing 1% iodoacetamide and 0.1 M ammonium bicarbonate (pH 7.8) for 30 min in the dark, the gel was washed with 0.1 M ammonium bicarbonate/50% acetonitrile and dried in the Speed Vac apparatus. The gel was rehydrated in a trypsin solution (Promega, Madison, WI). After incubation overnight at 37°C, peptides were sonicated for 30 min. Digested samples were removed and subjected to a desalting/concentration step on a μZipTipC18 column (Millipore) using acetonitrile as an eluent before matrix-assisted laser-desorption ionization time-of-flight tandem mass spectroscopy (MALDI-TOF MS) analysis. Peptide mixtures were loaded on the MALDI system using the dried-droplet technique and α-cyano-4-hydroxycinnamic acid (Sigma–Aldrich) as matrix, and were analyzed using a

Voyager-DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA). Internal mass calibration was performed using peptides derived from enzyme autoproteolysis. The Dataexplorer software package was used to identify spots from MS-Fit (Protein Prospector; http://prospector.ucsf.edu) and the Mascot server (Matrix Science, London, UK) by mass searching of human sequences. Candidates identified by peptide mapping analysis were evaluated further by comparing their calculated mass and isoelectric points using the experimental values obtained by 2D gel electrophoresis.

WESTERN BLOT ANALYSIS

For Western blot analysis, protein extracts were obtained from cells with or without artemin and analyzed by 10% SDS-PAGE. Then, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH). After transfer, membranes were incubated overnight with 10% (w/v) non-fat dry milk in phosphate buffered saline-0.1% Tween 20 (PBST) at 4°C and then incubated with the indicated antibody (Cell Signaling, Danvers, MA) for 3 h. After three washes with PBST, membranes were incubated with an anti-rabbit immunoglobulin coupled with peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). After 60 min of incubation at room temperature, the membranes were washed three times with PBST, and the blots were developed using an enhanced chemiluminescence kit (Amersham Biosciences, Buckinghamshire, UK). Normalization was performed using polyclonal anti-GAPDH antibody (Santa Cruz Biotechnology). Blots were quantified using a Gel Doc 2000 densitometer (Bio-Rad).

FLUORESCENCE DYE STAINING

The cultures were grown on coverslips and stained for F-actin using phalloidin-Alexa568 (Molecular Probes). Briefly, coverslips were washed with PBS, lightly fixed (in 4% paraformaldehyde for 5 min), and washed again. For F-actin staining, the fixed cells were permeabilized for 3 min at room temperature in PBS supplemented with 0.05% Triton X-100. After washing with PBS, the coverslips were treated with 5 U/ml phalloidin-Alexa568 and imaged by fluorescence microscopy using a WG filter set equipped with a Photometrics CoolSNAP camera (Roper Scientific, Duluth, GA).

IMMUNOCYTOCHEMISTRY

The coverslips were treated with 1% paraformaldehyde for 1 min. The cells were then washed with PBS, permeablized (1% Triton X-100 in PBS for 3 min), and washed again. After washing, the cells were treated successively with goat polyclonal anti-tropomyosin antibody (Santa Cruz Biotechnology) diluted in 1% bovine serum albumin and fluorescein isothiocyanate-conjugated anti-goat immunoglobulin G. The coverslips were mounted onto glass slides and imaged by fluorescence microscopy.

STATISTICAL ANALYSIS

Statistical analysis was performed using MINITAB. An asterisk (*) indicates $P < 0.01$ compared to control.

RESULTS

CHANGES IN SH-SY5Y PROTEOME PROFILE AFTER TREATMENT WITH ARTEMIN AND GDNF

Approximately 200 different spots focused in the 4–7 pH range were analyzed per sample. After exposing cells to either artemin or GDNF, five intensified spots, and six attenuated spots were common in both cases (Fig. 1). Each of these spots was excised, digested with trypsin, and analyzed by MALDI-TOF MS. Peptide mass fingerprint analysis and non-redundant sequence database matching allowed the unambiguous identification of all of the analyzed species. Figure 2 and Table I detail the nature of each identified spot, their 2D gel electrophoresis coordinates, and the relative sequence coverage.

Some proteins, including actin, actin γ propeptide, septin 2, matrin 3, laminin-binding protein, and tropomyosin, are cytoskeletal proteins. Several protein spots were members of the thiol-dependent interaction proteins, including calreticulin precursor, ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1), and protein disulfide isomerase-associated 3 precursor (PDI-associated 3 precursor). Additionally, metabolic enzymes such as glycosyl hydrolase family 31 and enolase 1 were down-regulated 0.3 ± 0.021 and 0.00001-fold, respectively, in both artemin- and GDNF-treated cases. The volume of each protein spot changed significantly ($P < 0.01$, Student's t-test; Table I). The spot intensities of septin 2, matrin, actin γ propeptide, PDI-associated 3 precursor, glycosyl hydrolase family 31, and enolase decreased. The spot intensities of calreticulin precursor, UCH-L1, laminin-binding protein, actin, and tropomyosin increased.

To further verify the 2D gel electrophoresis results, we performed Western blot analysis on total cell extracts from control cells and neurotrophic factor-treated cells lysed in radio-immunoprecipitation assay buffer. The blots were probed with antibodies to five selected proteins: matrin 3, UCH-L1, laminin-binding protein, enolase, PDI-associated 3 precursor, and GAPDH as loading control. Figure 3 shows the results of these experiments, confirming the measured expression level changes in the 2D gel electrophoresis data. From the real-time polymerase chain reaction data, mRNA levels did not show statistically significant differences between treatment and control cases (data not shown).

MODIFICATION OF TROPOMYOSIN AFTER EXPOSURE OF SH-SY5Y CELLS TO ARTEMIN AND GDNF

In the present study, tropomyosin was identified as a protein marker that is upregulated by the neurotrophic factors artemin and GDNF in SH-SY5y neuronal cells. The spot corresponding to tropomyosin was positioned at a pI value of approximately 5.0 (Spot No 1106; Fig. 4). Many of the isoforms are tissue- and filament-specific in their distribution. The tropomyosin identified in the present study was tropomyosin α-1 chain isoform 4. Its sequence was registered as NP_001018006.1 and identical to that of tropomyosin-3 α-1 chain isoform 4 for 268 amino acid residues. We observed the acidic shift of the tropomyosin spot detected at pI 4.9 of almost the same molecular weight that was due to phosphorylation by extracellular signal-regulated kinase. To identify whether the phosphorylation of tropomyosin is responsible for the observed

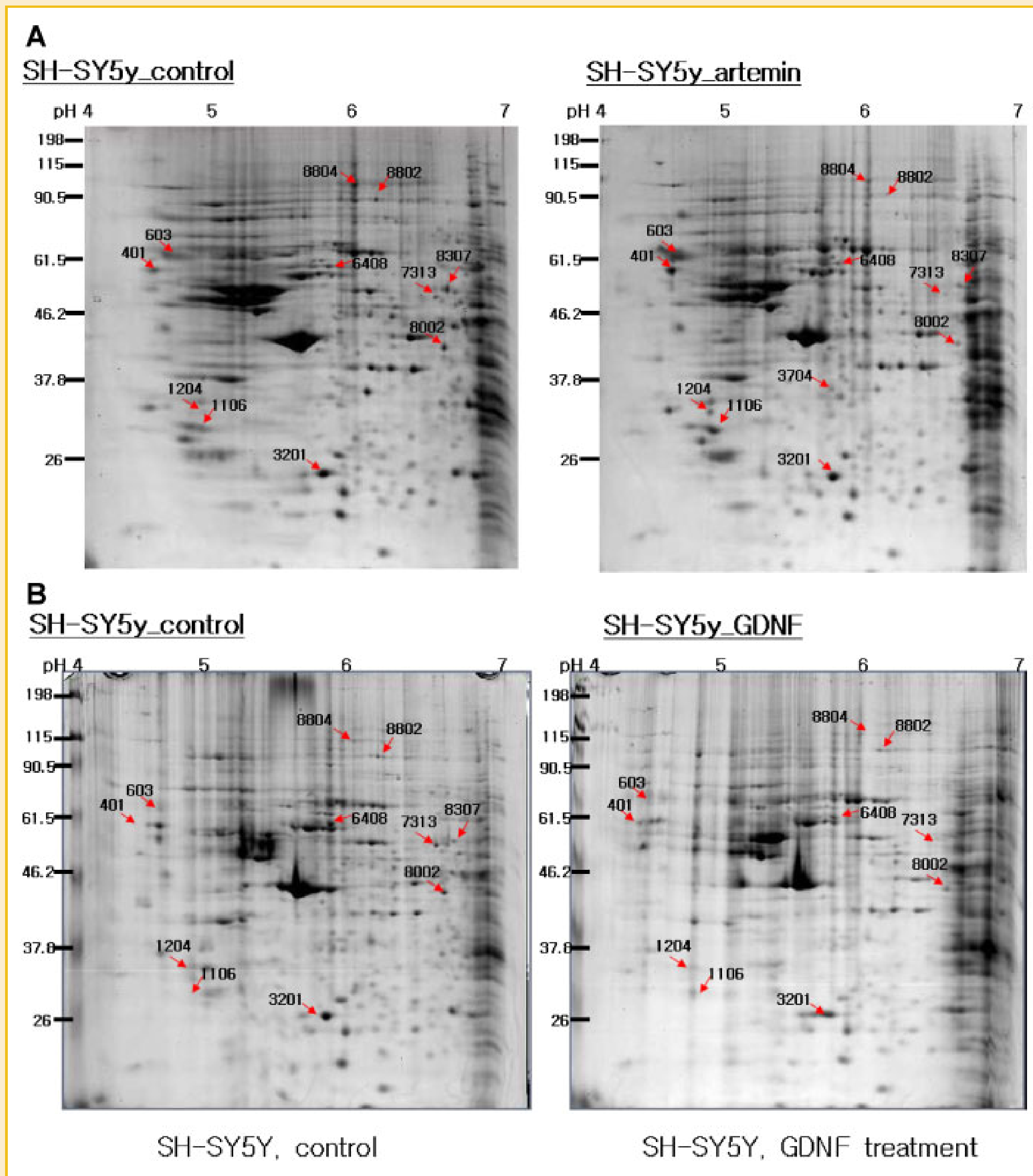

Fig. 1. 2D gel electropherograms of SH-SY5y cell protein patterns after treatment with artemin and GDNF. SH-SY5y cells were incubated with 200 ng/ml artemin or GDNF, incubated for 1 h, harvested, and lysed. Intracellular proteins were analyzed by 2D gel electrophoresis, and gels were stained with Coomassie Brilliant Blue G-250. A: Right panel: control cells; left panel: artemin-treated cells. B: Right panel: control cells; left panel: GDNF-treated cells. Vertical axes represent apparent molecular masses (kDa), and horizontal axes show pH values. Acquired images were repetitive. The data shown are representative of four separate experiments. Spot numbers are the same as those in Table I. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

acidic shift, the mitogen-activated protein kinase inhibitor PD98059 was added. As shown in Figure 4, the spot with a pI of 4.9 and a molecular weight of 27 kDa (pT) was weakened in cells treated with PD98059. The acidic shift of the spot with a pI 5.0 (T) to the pT spot was found to be weakened after treatment with neurotrophic factor. From the MALDI-TOF MS and peptide mass fingerprint analyses of the excised spots, these two spots were identified as the same tropomyosin isoform 4.

MODIFICATION OF UBIQUITIN CARBOXYL-TERMINAL HYDROLASE L1 AFTER EXPOSURE OF SH-SY5Y CELLS TO ARTEMIN AND GDNF

From our 2D gel electrophoresis data, we determined that the protein levels of UCH-L1, a neuronal de-ubiquitinating enzyme whose mutation has been linked to early onset familial Parkinson's disease, were upregulated in neurotrophic factor-treated SH-SY5y cells. Three human brain UCH-L1 isoforms, a full-length form and two amino-terminally truncated

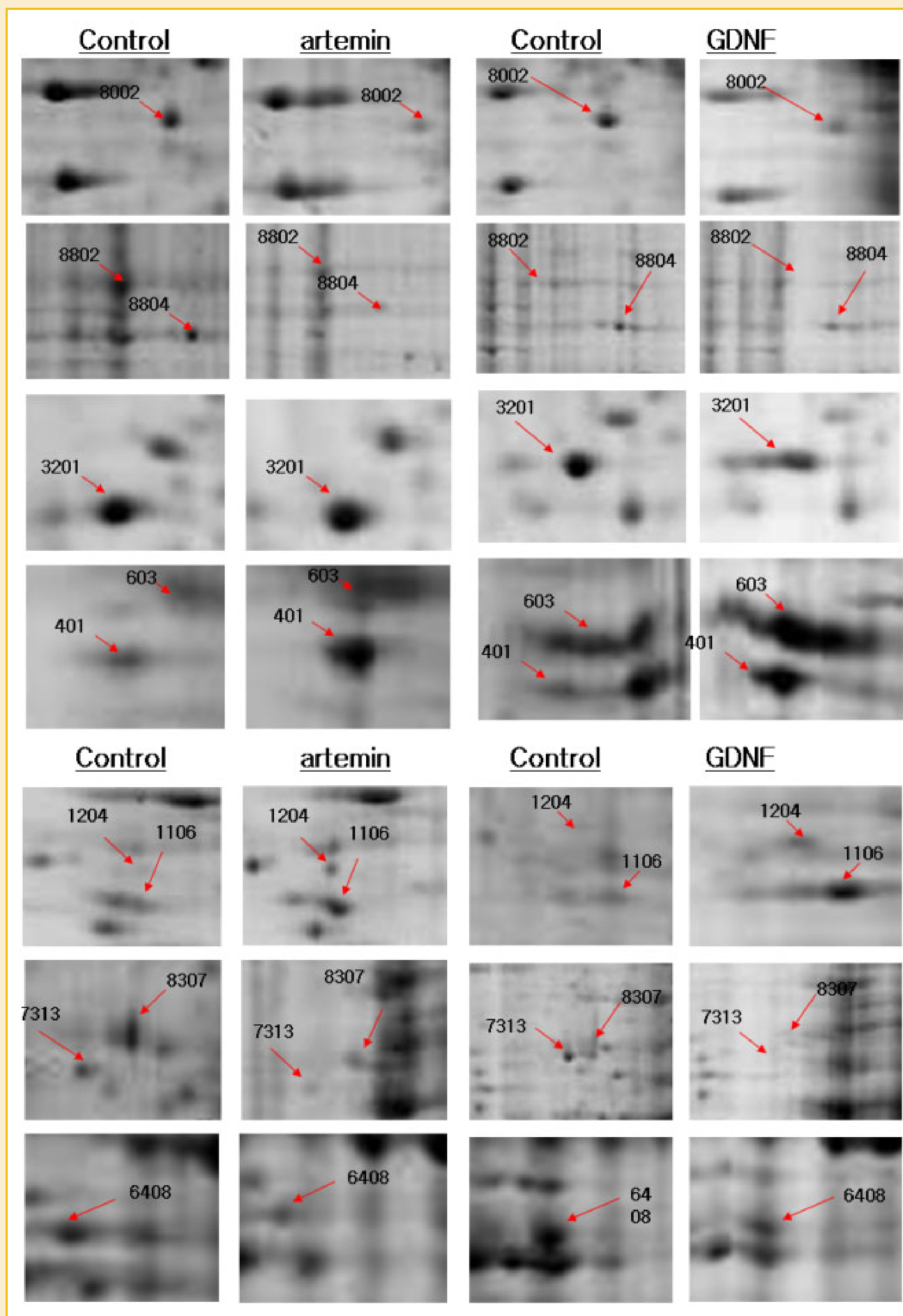

Fig. 2. Composition of 2D gel images of SH-SY5y cells exposed to artemin or GDNF. Regions comprising statistically significant protein spots were cropped from electropherograms shown in Figure 1. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

TABLE I. List of Spots/Proteins Sensitive to Neurotrphic Factor Treatment in SH-SY5y Cells Detected by Two-Dimensional Gel Electrophoresis and Identified by Peptide Mass Fingerprint Analysis

Spot number	Protein description	Theoretical Mr (kDa)	pI	Intensity variation	Accession number	Score/MALDI-TOF coverage (%)
8002	Septin2	36,824	6.85	Down-regulated (0.027 ± 0.003)	gi|62822169	143/59
8802	Matrin 3	95,078	5.87	Down-regulated (0.35 ± 0.0016)	gi|62750354	83/20
3201	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	25,151	5.33	Up-regulated (3.2 ± 0.13)	gi|21361091	77/53
401	Calreticulin precursor	48,283	42.9	Up-regulated (3.5 ± 0.09)	gi|4757900	224/46
8804	Glycosyl hydrolases family 31	107.158	5.71	Down-regulated (0.3 ± 0.02)	gi|577295	191/51
1204	Laminin-binding protein	32.833	4.78	Up-regulated (14932 ± 626)	gi|30583277	123/57
1106	Trpomyosin isoform	28.517	4.89	Up-regulated (14333 ± 374)	gi|854189	68/45
603	Actin	41.784	5.31	Up-regulated (3.82 ± 0.62)	gi|74191566	46/52
7313	Actin, gamma 1 propeptide	41.710	5.31	Down-regulated (0.0001 ± 0.000019)	Q71FK5 (swiiss-prot)	66/39
8307	Enolase 1	47.481	7.01	Down-regulated (0.00001 ± 0.0000021)	gi|13325287	196/52
6408	Protein disulfide isomerase-associated 3 precursor	56.747	5.98	Down-regulated (0.15 ± 0.008)	gi|21361657	166/61

Each protein change ratio has been represented as a mean of four individual values and standard deviations. The protein spots showing a statistically significant differential expression ($P < 0.01$) were selected.

forms, have been reported [Choi et al., 2004]. Our proteomics analyses reveal that the full-length UCH-L1 is altered by neurotrophic factors. To verify the proteomics identification of UCH-L1, Western blot analysis was performed. Immunodetection using anti-UCH-L1 antibody revealed the presence of a 27-kDa protein and a 17-kDa isoform as shown in Figure 5. Although the 27-kDa spot was attenuated in neurotrophic factor-treated cells, the 17-kDa isoform was increased in neurotrophic factor-treated

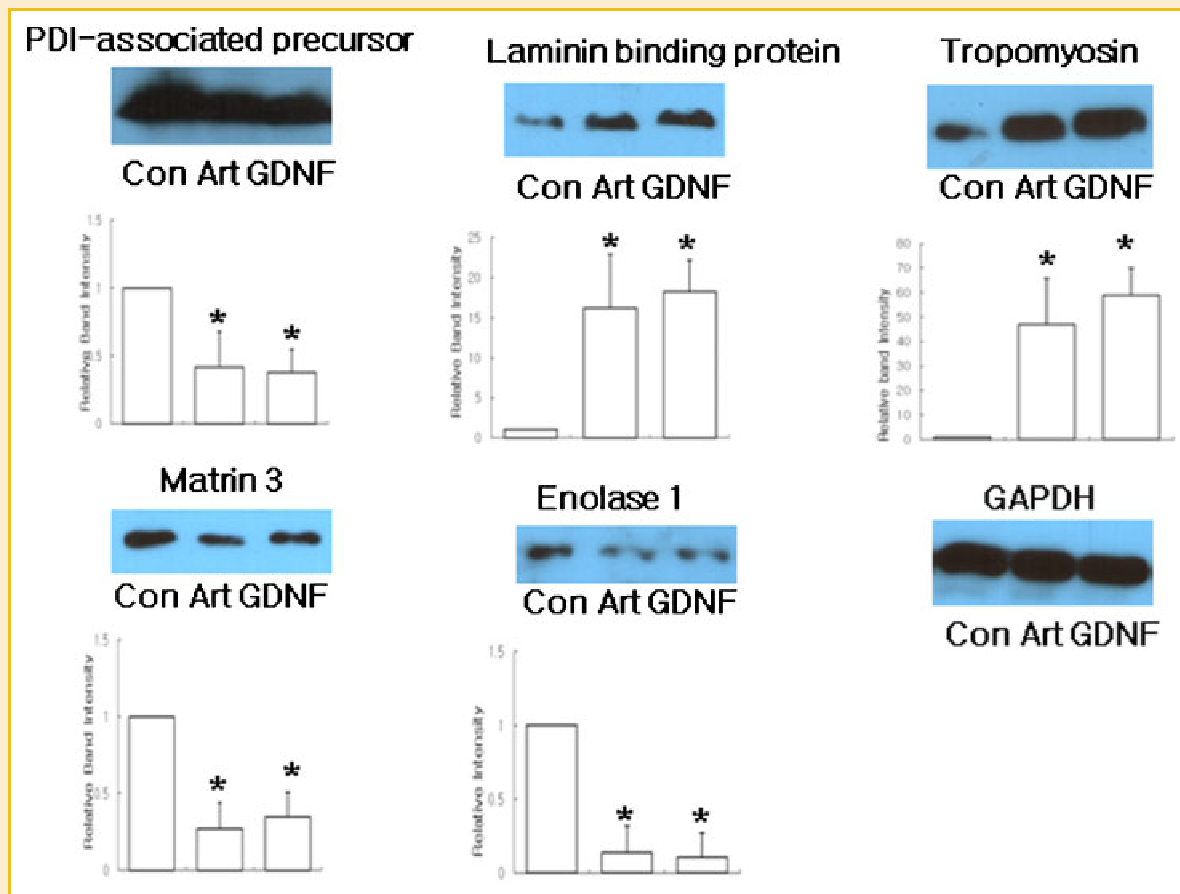

Fig. 3. Western blot analysis of PDI-associated 3 precursor, laminin-binding protein, matrin 3, tropomyosin and enolase 1 proteins. SH-SY5y cells were treated with 200 ng/ml artemin or GDNF for 1 h. Proteins were extracted, subjected to 10% SDS-PAGE, and transferred on nitrocellulose membranes. The levels of each group were normalized to GAPDH levels. Each data point represents a mean of three individual values and standard deviations. Quantitative analysis was performed using student's *t*-test, and results are expressed as activity relative to untreated control group. Asterisks indicate statistically significant differences between treatment and untreated control conditions ($^{*}P < 0.01$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

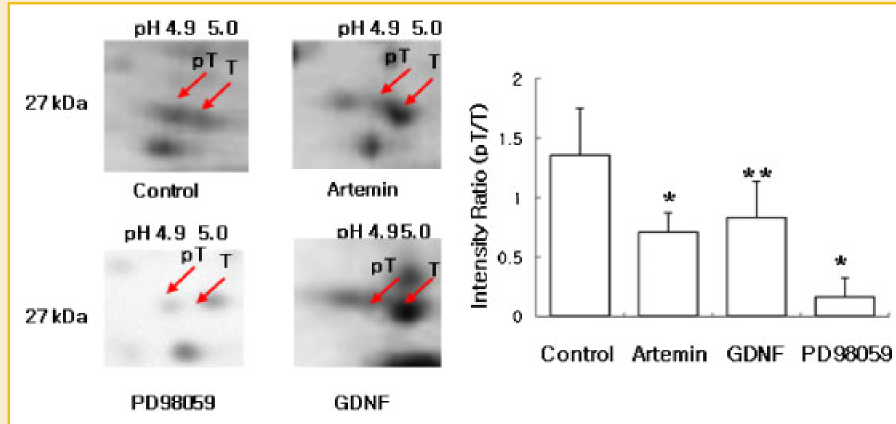

Fig. 4. Two-dimensional gel identification of tropomyosin and phosphorylated tropomyosin. SH-SY5y cells were treated with vehicle (dimethyl sulfoxide) or 5 mM PD98059 for 1 h. Proteins were extracted, analyzed by 2D gel electrophoresis, and stained with Coomassie Brilliant Blue G-250. Shifts of tropomyosin spots in the acidic direction are indicated by arrows. Acquired images were repetitive. The data shown are representative of four separate experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

cells, which suggests proteolytic processing or non-specific cleavage.

MODIFICATIONS OF PDI-ASSOCIATED PRECURSOR

Generally, the domains of chaperone proteins are assembled via disulfide bond, and the domains are interrelated with different sulfhydryl proteins. To determine whether the change in PDI-associated precursor levels is due to a change in disulfide bonds, we investigated the levels of PDI-associated precursor in the presence of glutathione (GSH), which interferes with disulfide bond formation. Accordingly, the purified protein was treated with GSH and separated by SDS-PAGE under non-reducing conditions, and the Western blots were probed with an anti-PDI-associated precursor antibody. Under these conditions, the dissociation of PDI-associated precursor, leading to a decrease in protein levels, was abrogated, indicating that intra-chain disulfide bonds between domains had not been reduced (Fig. 6).

MODIFICATION OF CYTOSKELETAL PROTEINS

Tropomyosins 1 and 4 are known to be associated with actin in vitro [Sen et al., 2001; Cooper, 2002]. The observation that several altered proteins are implicated in the organization of the cytoskeleton and cell-adhesion processes suggests that neurotrophic factors induce changes in cell cytoskeletal remodeling. To address this, we compared the actin cytoskeletal organization of neurotrophic factor-treated neuronal cells to control cells. In neurotrophic factor-treated cells, actin formed both long stress fibers with a striated pattern that crossed the cell body and bundles in the cell periphery. In contrast, in control cells, fewer fibers were formed and a diffuse staining pattern was observed in the central part of the cell. In addition, the fibers in control cells were more diffusely packed and more disorganized than those in neurotrophic factor-treated cells (Fig. 7).

To further confirm the observations about tropomyosin, we also performed immunocytochemistry on the bundles and cross-linked actin filaments that form stress fibers. Figure 8 shows that more

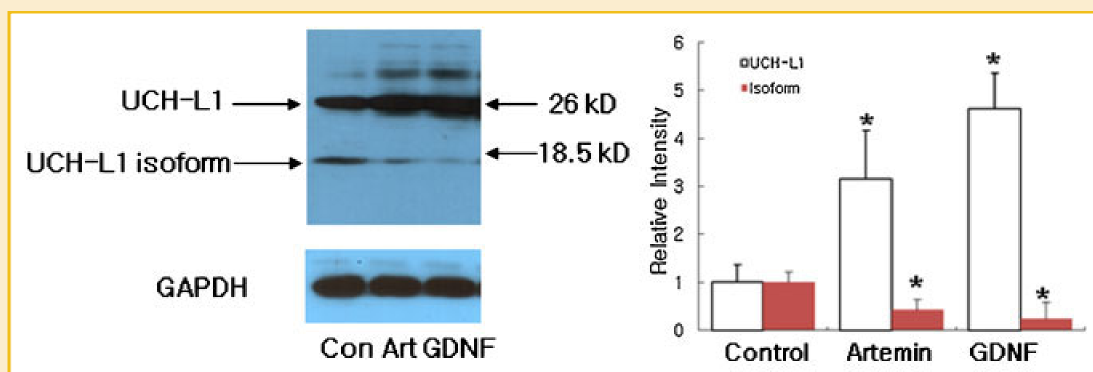

Fig. 5. Western blot analysis of the UCH-L1 protein. SH-SY5y cells were treated with 200 ng/ml artemin or GDNF for 1 h. Proteins were extracted, subjected to 10% SDS-PAGE, and transferred to nitrocellulose membranes. Data immunodetected with anti-UCH-L1 antibody are shown. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

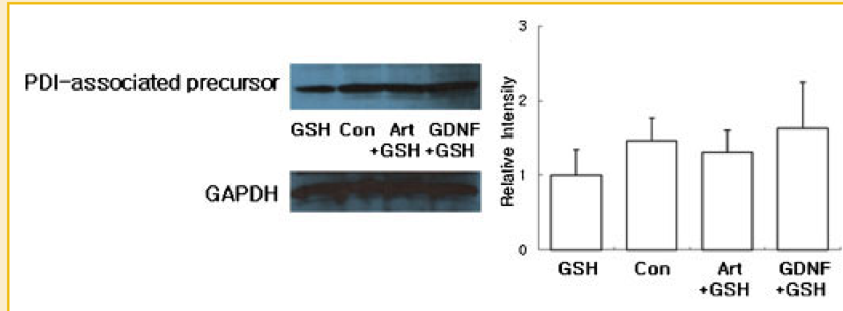

Fig. 6. Western blot analysis of PDI-associated 3 precursor protein. SH-SY5y cells were treated with 200 ng/ml artemin or GDNF with or without GSH for 1 h. Proteins were extracted, subjected to 10% native PAGE, and transferred to nitrocellulose membranes. Data immunodetected with anti-PDI-associated precursor antibody are shown. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

tropomyosin is detected in extracts of neurotrophic factor-treated cells than in control cells, further supporting the observation that actin fibers are more organized in neurotrophic factor-treated cells.

Overall, our data is consistent with data obtained using the 2D gel electrophoresis proteome, where the amount of actin increases and the amount of actin gamma propetide decreases in cells treated with neurotrophic factors.

DISCUSSION

Proteomics analysis identified a thiol/disulfide exchange catalyst as a marker altered by neurotrophic factors in SH-SY5y cells. PDI belongs to the superfamily of thiol/disulfide exchange catalysts that act as a protein-thiol oxidoreductase enzyme and shares sequence similarities with thioredoxin [Noiva, 1999]. PDI is composed of four

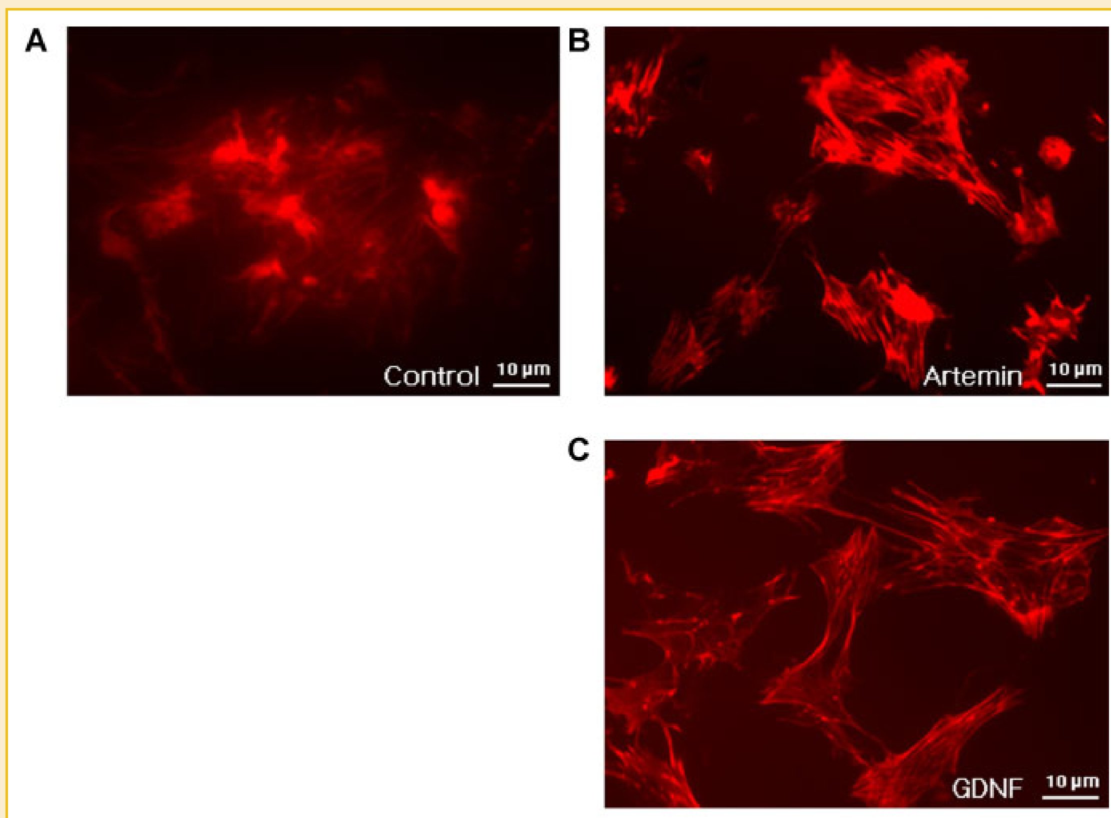

Fig. 7. Effects of artemin and GDNF on actin polymerization in SH-SY5y cells (A. control, B. Artemin, C. GDNF). Two hundred nanograms per milliliter artemin or GDNF were applied to the cultures for 3 h. After treatment, the cultures were stained for actin using phalloidin-Alexa568. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

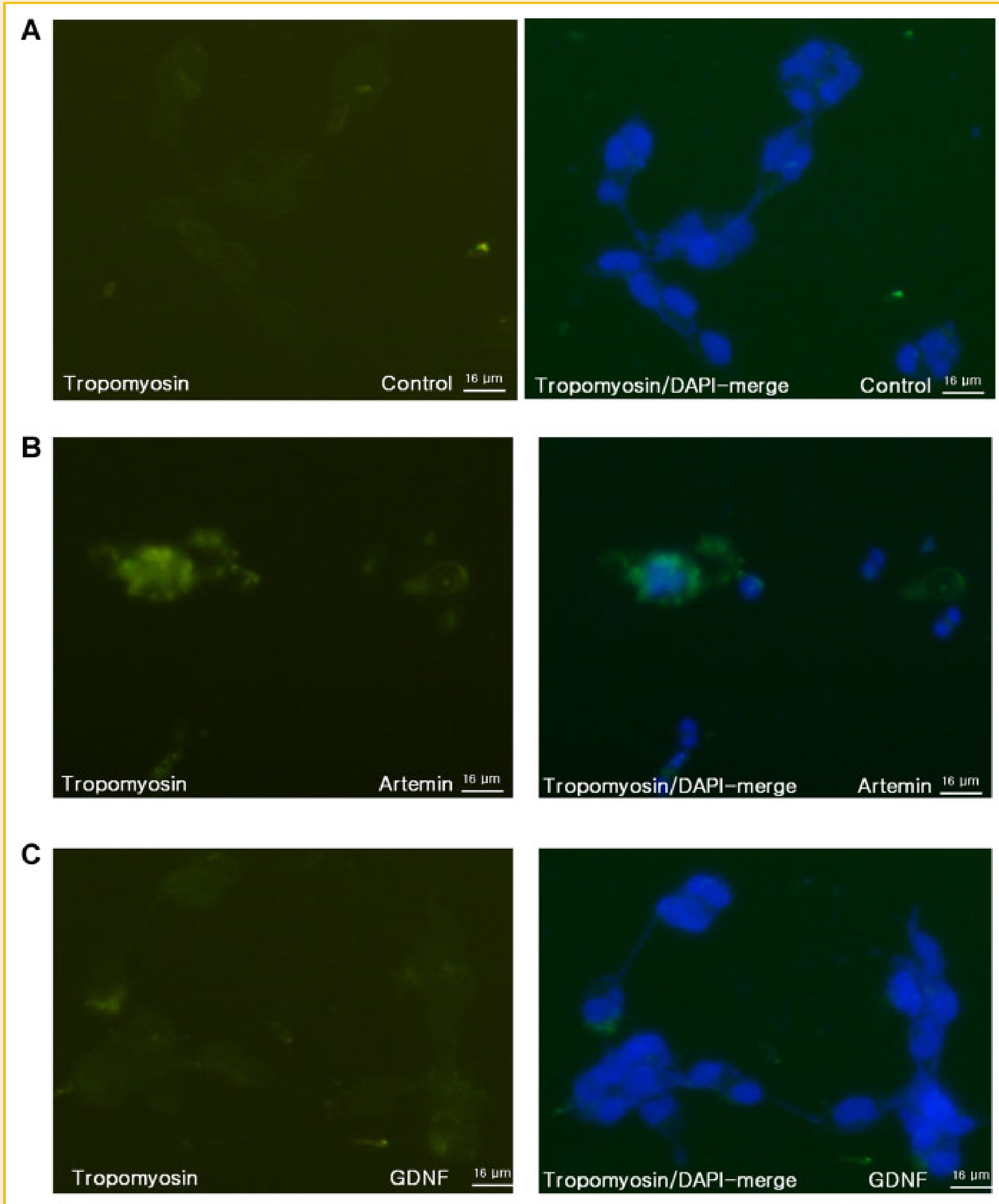

Fig. 8. Immunocytochemistry of tropomyosin in SH-SY5y cells. Artemin (200 ng/ml) or GDNF (200 ng/ml) were applied to the cultures for 3 h (A. control, B. Artemin, C. GDNF). After the treatment, the cultures were detected with anti-tropomyosin antibody and stained with DAPI (4′,6-diamidino-2-phenylindole). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

domains that have similarities with thioredoxin folds (a–b–b′–a′) [Kemmink et al., 1997]. In the present study, the spot corresponding to the PDI-associated precursor was positioned at a pI value of approximately 5.9, representing a mass of 59 kDa (Fig. 5). Its intensity was weaker in neurotrophic factor-treated cells than in control cells. The changes in PDI protein levels identified by MALDI-TOF were confirmed by Western blotting. PDI-associated precursor is a multi-domain chaperone and subunit of prolyl 4-hydroxylase (P4-H). P4-H consists of two distinct polypeptides, the catalytic a subunit and the b subunit, which is identical to the multifunctional enzyme, PDI [John and Bulleid, 1996]. PDI-associated precursor also plays a role in the formation of disulfide bonds within the major histocompatibility class I heavy chain [Lindquist et al., 2001].

PDI has two interrelated activities: the ability to catalyze the formation, reduction, and isomerization of disulfide bonds, and the ability to bind polypeptide chains [Freedman et al.,

1994]. This latter activity enables PDI to function as a molecular chaperone and to assist in the folding of polypeptides. It has been suggested that PDI forms more permanent associations with specific proteins, for example, as a subunit of P4-H and with microsomal triglyceride transfer protein [Pihlajaniemi et al., 1987; Wetterau et al., 1990]. The role of PDI as a molecular chaperone or a polypeptide-binding protein is mediated primarily through interactions between domains. It has been suggested that this binding is regulated by the redox state of PDI: its association requires the presence of GSH and its dissociation requires the presence of glutathione disulfide [Freedman et al., 1994]. It has been reported that wild-type PDI is a homodimer in the active state, and that this 26-kDa PDI is a disulfide-linked dimer [Noiva, 1999]. Actually, as shown in Figure 6, our data demonstrates that the binding between the PDI-associated precursor domains is dependent on the thiol buffer conditions and that the interactions between the PDI-associated precursor domains were blocked by and the levels of PDI-associated precursor were decreased by intracellular GSH redox states.

In our experiment, MALDI-TOF MS and quantitative analysis revealed that the protein spot with a sequence coverage range of 47% of calreticulin was a precursor that included the leader sequence, comprising amino acids 1–17. When we carried out Western blot analysis of cellular lysates obtained from SH-SY5y cells to further confirm the proteome result, treatment with 200 μg/ml artemin or GDNF for 1 h led to the upregulation of calreticulin ($P < 0.01$). Calreticulin is a chaperone that binds oligosaccharides on incompletely folded proteins and retains them in the endoplasmic reticulum (ER) [Spiro et al., 1996]. Like other chaperones, calreticulin prevents incompletely folded proteins from undergoing irreversible aggregation.

Calreticulin, when localized to the ER, has important functions in directing the proper conformations of proteins and glycoproteins, as well as the homeostatic control of cytosolic and ER calcium levels [Gold et al., 2010]. There is accumulating evidence for the diverse roles of calreticulin localized outside the ER, including localization to the outer cell surface of a variety of cell types, the cytosol, and the extracellular matrix [Gold et al., 2010]. Furthermore, recent studies showed that topical application of calreticulin has biological effects that enhance cutaneous wound healing in animal models [Gold et al., 2010]. The extracellular bioactivities of calreticulin have provided new insights into release of this protein from the ER to the cell surface. A role for calreticulin in cell migration might be directly related to its regulation on fibronectin and laminin compared to wild-type cells.

We performed Western blot analysis and immunocytochemistry for selected proteins that were influenced by GDNF and artemin in SH-SY5y cells. Since the cytoskeletal network plays an important role in cell shape and locomotion, which in turn are thought to be involved in growth control, such as division and neurite outgrowth, changes in the intermediate filaments and actin may contribute to the neuronal cell phenotype induced by neurotrophic factors. The changes in cellular protein modifications brought about by neurotrophic factors await further detailed analysis, and the precise manner by which actin cytoskeleton reorganization can be induced remains to be determined.

Our previous results indicated that 285 genes were differentially transcribed after a 3-h artemin treatment, including genes related to cell adhesion and actin polymerization [Park and Hong, 2006]. A series of genes involved in the regulation of actin dynamics, including coronin, Myr 5, Wiskott–Aldrich syndrome protein-interacting protein, cofilin, drebrin, and dynamin, were down-regulated by artemin, suggesting that these genes play previously undefined roles in the regulation of actin polymerization and synaptic vesicle movement. Artemin also down-regulated the expression of genes related to cell adhesion and matrix assembly, including biglycan, plectin, nestin, neuronatin, and the neuron-glia-CAM-related cell adhesion molecule, which is functionally important for neurite elongation in DRG neurons. Although in our previous study we showed that many of the genes that are known to interact with actin were transcriptionally regulated within 3 h, here we investigated how artemin was able to affect actin polymerization via protein modification without gene transcription in neuronal cells.

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