Proteomic analysis of SP600125-controlled TrkA-dependent targets in SK-N-MC neuroblastoma cells: Inhibition of TrkA activity by SP600125

Eun Joo Jung1, Hyung Chul Park2, Ky Hyun Chung2 and Choong Won Kim1

1 Department of Biochemistry, Institute of Health Sciences, Gyeongsang National University School of Medicine, Jinju, South Korea
2 Department of Urology, Gyeongsang National University School of Medicine, Jinju, South Korea

The c-Jun N-terminal kinase (JNK) is well known to play an important role in cell death signaling of the p75 neurotrophin receptor. However, little has been studied about a role of JNK in the signaling pathways of the tropomyosin-related kinase A (TrkA) neurotrophin receptor. In this study, we investigated JNK inhibitor SP600125-controlled TrkA-dependent targets by proteomic analysis to better understand an involvement of JNK in TrkA-mediated signaling pathways. PDQuest image analysis and protein identification results showed that hnRNP C1/C2, α- tubulin, β-tubulin homolog, actin homolog, and eIF-5A-1 protein spots were upregulated by ectopic expression of TrkA, whereas α-enolase, peroxiredoxin-6, PROS-27, HSP70, PPI-gamma, and PDH E1-alpha were downregulated by TrkA, and these TrkA-dependent upregulation and downregulation were significantly suppressed by SP600125. Notably, TrkA largely affected certain PTM(s) but not total protein amounts of the SP600125-controlled TrkA-dependent targets. Moreover, SP600125 strongly suppressed TrkA-mediated tyrosine phosphorylation signaling pathways as well as JNK signaling, indicating that SP600125 could function as a TrkA inhibitor. Taken together, our results suggest that TrkA could play an important role in the cytoskeleton, cell death, cellular processing, and glucose metabolism through activation or inactivation of the SP600125-controlled TrkA-dependent targets.

Keywords:
Cell biology / JNK / Proteomic analysis / PTM / SP600125-controlled / TrkA-dependent targets

1 Introduction

Neurotrophins (NTs) such as nerve growth factor (NGF), brain-derived neurotrophic factor, NT-3, and NT-4 are a family of closely related proteins that regulate cell survival, death, maintenance, and development in both the central and peripheral nervous systems [1, 2]. The effects of NTs are exhibited through activation of two distinct receptor types: the tropomyosin-related kinase (Trk) subfamily (TrkA, TrkB, and TrkC) and the p75 neurotrophin receptor (p75NTR) that belongs to the tumor necrosis factor receptor superfamily [3]. NGF activates various intracellular signaling pathways through activation of the TrkA and p75NTR receptors, involved in the regulation of both cell survival and death in neuronal and non-neuronal cell types.

The transmembrane TrkA receptor possesses an intrinsic tyrosine kinase activity in the intracellular domain. NGF...
binding to TrkA leads to receptor dimerization and enhances TrkA enzyme activity [4]. Thus, the activated TrkA phosphorylates Src homology 2-containing protein (Shc), and this adaptor protein induces an intermediate protein connection with Grb-2, Sos, and Raf, activating the extracellular signal-regulated protein kinase (ERK) pathway, which is one of the mitogen-activated protein kinase signaling pathways [4]. This NGF-mediated TrkA-dependent ERK signaling enhances transcription of proteins involved in cell proliferation and survival [4]. The activated TrkA also phosphorylates phosphatidylinositol 3-kinase and stimulates phosphatidylinositol 3-kinase-Akt signaling pathway, involved in the regulation of cell proliferation and survival [4, 5]. In contrast, other mitogen-activated protein kinase members such as c-Jun N-terminal kinase (JNK) and p38 have been known to be activated by TrkA-dependent phospholipase C-γ pathway, suggesting a role of TrkA in the cell death signaling [4]. At present, little has been studied about a role of JNK in the TrkA-dependent signaling pathways, whereas JNK has been well known to play a critical role in the p75NTR-dependent cell death signaling. In addition, p75NTR can function as a cell survival factor through activation of the nuclear factor-kappaB transcription factor [6].

To reveal TrkA-dependent cellular effects, ectopic expression of TrkA was widely used in various cell types. The effects of ectopic TrkA seemed to be different according to the cell type and protein expression level. It has been known that ectopic expression of TrkA plays an important role in neuronal responses in an NGF-dependent or NGF-independent manner [7]. TrkA overexpression caused activation of its tyrosine kinase activity and promoted cell growth, migration, and invasion in breast cancer cells [8]. In contrast, ectopic expression of TrkA inhibited angiogenesis and tumor growth in neuroblastoma cells [9, 10] and induced apoptosis in a p53-dependent mechanism [11]. NGF-mediated TrkA-dependent apoptosis was also associated with a Ras/Raf signaling pathway in medulloblastoma cells [12]. Moreover, TrkA-dependent apoptotic cell death was strongly related to the production of γ-H2AX, a phosphorylated form of histone H2AX at serine-139, in the absence of cellular stimuli such as DNA damage-inducing reagents in U2OS osteosarcoma cells, and this phenomenon was significantly suppressed by JNK inhibitor SP600125, suggesting a critical role of JNK in the TrkA-dependent cell death signaling [13, 14]. The TrkA-dependent cell death was promoted by ectopic expression of H2AX via upregulation of TrkA tyrosine-490 phosphorylation [15], whereas it was inhibited by ectopic expression of caveolin-1 via downregulation of tyrosine-490 phosphorylation [16], indicating that tyrosine-490 phosphorylation of TrkA is critically required for its cell death function. Recently, it was demonstrated that ectopic TrkA and TrkC, but not TrkB, caused death of mouse embryonic stem cells [17], further supporting a role of TrkA in cell death signaling.

Studies on the TrkA-dependent cellular effects have been somewhat accomplished using TrkA inhibitor GW441756 [18]. We have previously reported that the induction of TrkA tyrosine-490 phosphorylation by TrkA overexpression was significantly inhibited by GW441756, resulting in the downregulation of its cellular effects such as γ-H2AX production [13], Bak cleavage [16], and inhibition of colony formation by cancer cells [19]. In addition, we recently identified TrkA-dependent targets associated with tyrosine phosphorylation signaling pathways using the effects of GW441756 in SK-N-MC neuroblastoma cells [20]. Moreover, we demonstrated that TrkA overexpression caused tyrosine phosphorylation of JNK as well as ERK, indicating an involvement of JNK in the TrkA-dependent signaling pathways [20]. To better understand this, here we identified SP600125-controlled TrkA-dependent targets by proteomic analysis in TrkA-inducible SK-N-MC cells. This research could highly contribute on a new signal transduction and mechanism in various TrkA-dependent signaling pathways through activation or inactivation of the SP600125-controlled TrkA-dependent targets.

2 Materials and Methods

2.1 Reagents

DMEM, tetracycline (Tet)-screened FBS, and penicillin/streptomycin were from Gibco-BRL. Tet was from Duchefa. Blasticidin and zeocin were from Invitrogen. Super signal west pico stable peroxide solution was from Pierce. SP600125 was from Calbiochem. Protran nitrocellulose membrane (BA83) was from Whatman. GW441756, trichloroacetic acid, Bradford reagent, CBB G, and CHCA were from Sigma-Aldrich. Acrylamide/bis-acrylamide 37.5:1 solution (40%), urea, Tween-20, ammonium sulfate, and DMSO were from Amresco. IPG strip (pH 4–7) 7 cm and 17 cm were from Bio-Rad. IPG buffer (pH 4–7) was from Amersham Biosciences. Recombinant DNase I (RNase-free) was from Takara. Sequencing grade modified trypsin was from Promega. AS601245 was from (Enzo Life Sciences). Antibodies used in the study were: β-actin (Sigma-Aldrich), hnRNP C1/C2 (4F4, Santa Cruz), α-tubulin (B-5–1–2, Sigma-Aldrich), PP1-gamma (C-19, Santa Cruz), α-enolase (L-27, Santa Cruz), peroxiredoxin-6 (D-9, Santa Cruz), TrkA (763, Santa Cruz), phospho-TrkA (E-6, Santa Cruz), acetylated α-tubulin (6–11B-1, Santa Cruz), HSP70 (K-20, Santa Cruz), phospho-tyrosine (PY20, Transduction Laboratories), phospho-Y204-ERK1/2 (Bioworld Technology), ERK1 (Transduction Laboratories), phospho-T183/Y185-JNK1/2/3 (Bioworld Technology), NK1 (D-6, Santa Cruz), phospho-S63-c-Jun II (Cell Signaling Technology), actylated-lysine (Cell Signaling Technology), goat anti-rabbit HRP conjugate (Bio-Rad), and goat anti-mouse HRP conjugate (Enzo Life Sciences).

2.2 Cell culture

TrkA-inducible cells by the Tet-On system were previously established in SK-N-MC cells [19]. The cells were maintained...
with medium A (DMEM, Tet-screened 10% FBS, 1% penicillin/streptomycin) containing 1.25 μg/mL blasticidin and 25 μg/mL zeocin in a humidified 5% CO₂ incubator at 37°C. Ectopic expression of TrkA was induced by adding of 2 μg/mL Tet for 12 h in the medium A and then treated with DMSO as a control solvent, SP600125, GW441756, or AS601245 with the indicated amounts and times.

### 2.3 Sample preparation, 2DE and Coomassie Blue stain

Samples were prepared for proteomic analysis, separated by 2DE and stained with Coomassie Blue staining solution as described previously [20]. Briefly, whole cells were washed with PBS, lysed in 2DE lysis buffer, treated with DNase I, and then precipitated with trichloroacetic acid. After washing with acetone, the pellet was dissolved in rehydration solution followed by protein quantification using Bradford reagent. IPG strips (pH 4–7, 17 cm) were rehydrated for 14 h at 20°C in the IEF cell with the protein samples (500 μg each) containing 0.5% IPG buffer (pH 4–7) and performed by 1D IEF. The strips were reduced for 15 min in equilibration buffer (50 mM Tris (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, and trace amount of bromophenol blue) containing 10 mg/mL DTT, and then alkylated for 15 min in the same buffer containing 25 mg/mL iodoacetamide. 2DE of the strips was performed on 8.5–14% sucrose gradient polyacrylamide gels, which were prepared using the light 8.5% SDS-PAGE solution and the heavy 14% SDS-PAGE solution containing 15% sucrose. The gels were fixed in fixing solution and stained with Coomassie Blue staining solution. After destaining with H₂O, gel images were obtained using a UMAX scanner (PowerLook 2100XL).

### 2.4 Image analysis and statistical significance

Quantitative analysis of Coomassie Blue stained images was carried out using PDQuest software (Bio-Rad) according to the manufacturer’s instructions. Quantity of total about 1500 protein spots was normalized by valid spot intensity. SP600125-controlled TrkA-dependent targets were determined by the inhibitory effects of SP600125 on more than twofold upregulated or downregulated TrkA-dependent protein spots. In addition, certain protein spots with a significant SP600125 effect were also determined as SP600125-controlled TrkA-dependent targets, although they were less than twofold regulated by TrkA. The statistical significance of image analysis was determined by the Student’s t-test (statistical level of *p < 0.05 is significant).

### 2.5 MALDI-TOF MS and database searching

SP600125-controlled TrkA-dependent protein spots were excised from Coomassie Blue stained 2DE gels and performed with in-gel digestion using modified porcine trypsin as described [20]. The tryptic digested peptides were mixed with saturated matrix solution (15 mg CHCA, 15 mg NC membrane, 75% acetone, 25% 2-propanol) and calibrants (bradykinin and neurotensin). And then, the mixed tryptic peptides were immediately spotted onto MALDI-TOF sample target plate. Mass measurement of tryptic peptides was performed with MALDI-TOF MS (Voyager-DE-STR; Applied Biosystems), and mass spectra were acquired for the mass range of 800–3500 Da and calibrated with bradykinin m/z (904.4681) and neurotensin m/z (1672.9175) as standard peaks. The proteins were identified by PMF on the basis of the Swiss-Prot and NCBInr database using the search program MASCOT (http://www.matrixscience.com), allowing peptide mass tolerance of 1.2 Da and one missed cleavage. Significance of the searched data was judged from the scores more than 56 (p < 0.05) and at least seven matched peptide masses.

### 2.6 MALDI-TOF/TOF MS/MS and database searching

After in-gel digestion of 2DE gel spots, peptide mass spectra were obtained by MALDI-TOF/TOF MS/MS (ABI 4800 plus; Applied Biosystems) as described previously [20, 21].
Search for protein identity was carried out using the NCBI-nr database by ProteinPilot v.3.0 software (with MASCOT as the database search engine). The parameters for searching were peptide and fragment ion mass tolerance of 50 ppm, one missed trypsin cleavage, carbamidomethylation of cysteine, oxidation of methionine and monoisotopic. Significance of the identified proteins was based on the number of matching peptide masses and comparison of experimental and theoretical properties of the proteins, in addition to database searched protein scores; greater than 84% was considered as a statistically significant ($p < 0.05$).

### 2.7 Confirmation of the identified proteins

To confirm the results identified by MALDI-TOF MS and MS/MS analysis, SP600125-controlled TrkA-dependent protein spots were excised from Coomassie Blue stained 2DE gels followed by 10% SDS-PAGE. In addition, protein samples (5 µg each) prepared for 2DE were separated by 10% SDS-PAGE. After 1DE, the proteins were transferred to NC membrane and analyzed by Western blot using the super signal ECL detection system [20].

### 2.8 2DE/Western blot analysis

IPG strips (pH 4–7, 7 cm) were rehydrated at 20°C in the IEF cell with protein samples (10 µg each) containing 0.5% IPG buffer (pH 4–7) followed by 1D IEF in a maximum current of 50 µA/IPG strip; at 250 V for 15 min, at 4000 V for 2 h, and then at 4000 V for 30 000 V-h. The strips were reduced for 15 min in equilibration buffer containing 10 mg/mL DTT, and then alkylated for 15 min in the same buffer containing 25 mg/mL iodoacetamide. 2DE of the strips was performed on 10% SDS polyacrylamide gels, and then separated protein spots were transferred to NC membrane and analyzed by Western blot [20].

### 3 Results

#### 3.1 2DE and image analysis of SP600125 effects on TrkA-dependent targets

SP600125, which is one of the most extensively studied ATP-competitive JNK inhibitors, has been known as a useful tool for studying JNK-mediated signal transduction [22–24]. To reveal an involvement of JNK in the TrkA-dependent signaling pathways, the JNK inhibitor SP600125-controlled TrkA-dependent targets were investigated by 2DE and Coomassie Blue stain in TrkA-inducible SK-N-MC cells. The representative 2DE images were selected from five independent experiments (Fig. 1), and PDQuest image analysis was performed with two sets of well-separated 2DE images. Thus, 16 protein spots were determined as major SP600125-controlled TrkA-dependent targets; ten protein spots were upregulated by TrkA, whereas six protein spots were downregulated by TrkA, and these TrkA-dependent upregulation and downregulation were significantly suppressed by SP600125 treatment.

#### 3.2 Identification of SP600125-controlled TrkA-dependent protein spots

We performed MALDI-TOF MS and MALDI-TOF/TOF MS/MS analysis to identify the sixteen SP600125-controlled TrkA-dependent protein spots. The ten upregulated protein spots were identified as heterogeneous nuclear ribonucleoproteins C1/C2 (hnRNP C1/C2; SSP 3209, 3212, 3350, 3352), α-tubulin (SSP 4415, 4506, 4535), β-tubulin homolog (SSP 2430), actin homolog (SSP 5329), and eukaryotic translation initiation factor 5A isoform B (eIF-5A-1; SSP 6017) with a statistical significance ($p < 0.05$; Tables 1 and 2). In addition, the six downregulated protein spots were identified as serine/threonine-protein phosphatase PP1-gamma catalytic subunit (PP1-gamma; SSP 7211), pyruvate dehydrogenase E1-alpha subunit (PDH E1-alpha; SSP 7237), α-enolase (SSP 7420), peroxiredoxin-6 (SSP 8101), proteasome subunit alpha type-6 (PROS-27; SSP 8106), and HSP 70 (HSP70; SSP 2620) with a statistical significance ($p < 0.05$; Tables 1 and 2). 2DE profile of the 16 SP600125-controlled TrkA-dependent protein spots has shown in Fig. 2.
Figure 3. Quantitative analysis of SP600125 inhibitory effects on TrkA-dependent protein spots. Protein spots of (A) four hnRNP C1/C2, (B) three α-tubulin, and (C) β-tubulin homolog, actin homolog and elf-5A-1 were upregulated by ectopic expression of TrkA, whereas protein spots of (D) α-enolase, peroxiredoxin-6 and PROS-27 and (E) HSP70, PP1-gamma and PDH E1-alpha were downregulated by TrkA. Both TrkA-dependent upregulation and downregulation were significantly suppressed by SP600125 (compare protein spots indicated by arrows in left four panels). Relative intensities of the protein spots determined by PDQuest software are shown in right panels. Each bar represents the mean ± SD for two independent experiments, and data significance was evaluated with a Student’s t-test, *p < 0.05.
Table 1. Identification of SP600125-controlled TrkA-dependent protein spots by MALDI-TOF MS analysis.

| Spot no. | Protein identification by MALDI-TOF MS | Tryptic fragment coverage/matches | Mascot probability score/expect (p) | UniProtKB entry/database | Protein mass (Da) | pI |
|----------|--------------------------------------|----------------------------------|---------------------------------|--------------------------|------------------|----|
| 3209     | Heterogeneous nuclear ribonucleoproteins C1/C2 (hnRNP C1/C2) | 20%/7 fragment | 73/0.00095 | HNRPC/Swiss-Prot | 33 650 | 4.95 |
| 3212     | hnRNP C1/C2                          | 26%/8 fragment | 87/3.9e-05 | HNRPC/Swiss-Prot | 33 650 | 4.95 |
| 3350     | hnRNP C1/C2                          | 23%/8 fragment | 86/0.0014 | HNRPC/Swiss-Prot | 33 650 | 4.95 |
| 3352     | hnRNP C1/C2                          | 22%/8 fragment | 87/3.9e-05 | HNRPC/Swiss-Prot | 33 650 | 4.95 |
| 4415     | Tubulin alpha-1C chain /Tubulin alpha-1B chain (α-Tubulin) | 20%/7 fragment | 83/0.00011 | TBA1C/Swiss-Prot | 49 863 | 4.96 |
| 4506     | Tubulin alpha-1C chain /Tubulin alpha-1B chain (α-Tubulin) | 22%/7 fragment | 85/5.8e-05 | TBA1C/Swiss-Prot | 49 863 | 4.96 |
| 4535     | Tubulin alpha-1B chain /Tubulin alpha-1A chain (α-Tubulin) | 29%/10 fragment | 111/1.6e-07 | TBA1B/Swiss-Prot | 50 120 | 4.94 |
| 7211     | Serine/threonine-protein phosphatase PP1-gamma catalytic subunit (PP1-gamma) | 24%/8 fragment | 102/3.4e-05 | PP1G/Swiss-Prot | 36 960 | 6.12 |
| 7237     | Pyruvate dehydrogenase E1-alpha subunit (PDH E1-alpha) | 24%/11 fragment | 122/1.4e-05 | E0TKV7/NCBInr | 41 828 | 5.66 |
| 7420     | Alpha-enolase (α-Enolase)            | 24%/10 fragment | 106/1.4e-05 | ENOA/Swiss-Prot | 47 139 | 7.01 |
| 8101     | Peroxiredoxin-6                      | 24%/7 fragment | 90/0.0006 | PRD6X/Swiss-Prot | 25 019 | 6.00 |
| 8106     | Proteasome subunit alpha type-6 (PROS-27) | 32%/8 fragment | 104/2.1e-05 | PSA6/Swiss-Prot | 27 382 | 6.34 |

3.3 Quantitative inhibition effects of SP600125 on TrkA-dependent protein spots

Relative intensities of the SP600125-controlled TrkA-dependent protein spots on Coomassie Blue stained 2DE gels were measured by PDQuest software. Four spots of hnRNP C1/C2 (SSP 3209, 3212, 3350, 3352) and three spots of α-tubulin (SSP 4415, 4506, 4535) were more than twofold upregulated by TrkA, which was inhibited by SP600125 (Fig. 3A and B). In contrast, α-enolase (SSP 7420), peroxiredoxin-6 (SSP 8101), and PROS-27 (SSP 8106) protein spots were significantly downregulated by TrkA, which was inhibited by SP600125 (Fig. 3C). In addition, HSP70 (SSP 2620), PP1-gamma (SSP 7211), and PDH E1-alpha (SSP 7237) protein spots were downregulated by TrkA, which was inhibited by SP600125 (Fig. 3D). In addition, HSP70 (SSP 2620), PP1-gamma (SSP 7211), and PDH E1-alpha (SSP 7237) protein spots were downregulated by TrkA, which was inhibited by SP600125 (Fig. 3D).

Table 2. Identification of SP600125-controlled TrkA-dependent protein spots by MALDI-TOF/TOF MS/MS analysis.

| Spot no. | Protein identification by MALDI-TOF/TOF MS/MS | Tryptic fragment coverage/matches | Mascot probability score/expect (p) | UniProtKB entry/database | Protein mass (Da) | pI |
|----------|-----------------------------------------------|----------------------------------|---------------------------------|--------------------------|------------------|----|
| 2430     | Highly similar to Tubulin beta-7 chain (β-Tubulin homolog) | 35%/24 fragment | 215/3.1e-15 | B7ZAF0/NCBInr | 46 936 | 4.83 |
| 2620     | Chaperone protein DnaK ; Heat shock protein 70 (HSP70) | 36%/28 fragment | 447/3.0e-03 | E0TLC1/NCBInr | 65 004 | 5.04 |
| 5329     | Highly similar to Actin (Actin homolog)        | 72%/25 fragment | 345/3.1e-02 | B4DW52/NCBInr | 38 950 | 5.19 |
| 6017     | Eukaryotic translation initiation factor 5A isoform B (eIF-5A-1) | 60%/9 fragment | 306/2.5e-02 | IF5A1/NCBInr | 17 049 | 5.08 |
3.4 Confirmation of SP600125-controlled TrkA-dependent protein spots

To prove the identification results analyzed by MALDI-TOF MS and MALDI-TOF/TOF MS/MS, SP600125-controlled TrkA-dependent protein spots and β-actin spot as a negative control were excised from Coomassie Blue stained 2DE gels and analyzed by 1DE/Western blot. Except for SSP 8101 spot, which was very weakly recognized by peroxiredoxin-6 antibody for some reason and thus visualized by long-time exposure, most of the identified protein spots were strongly recognized by the indicated antibodies as expected: SSP 3209, 3212, 3350, and 3352 spots by hnRNP C1/C2 antibody; SSP 4415, 4506, and 4535 spots by α-tubulin antibody; SSP 7211 spot by PP1-gamma antibody; SSP 7420 spot by α-enolase antibody (Fig. 4).

Next, the samples prepared for 2DE were analyzed by 1DE/Western blot to prove whether the identified proteins are regulated by TrkA and SP600125. As shown in Fig. 5A, ectopic expression of TrkA induced its tyrosine-490 phosphorylation in TrkA-inducible SK-N-MC cells, and both TrkA protein amount and tyrosine-490 phosphorylation level seemed to have no effect by SP600125 (first and second panels). In addition, ectopic expression of TrkA did not influence on acetylation of α-tubulin and protein amounts of α-tubulin, hnRNP C1/C2, PP1-gamma, and full length HSP70 (Fig. 5A, third to seventh panels). However, a certain protein of lower molecular weight than full length HSP70 was detected by HSP70 antibody in a control condition containing 0.1% DMSO, and it was disappeared by ectopic expression of TrkA (Fig. 5A, seventh panel; indicated by an arrow and asterisk). Moreover, the lower protein that could be a cleaved HSP70 or a certain HSP70 homolog was detected as a couple of protein spots by 2DE/Western blot analysis using 17 cm IPG strip (pH 4–7) in a normally proliferating TrkA-inducible SK-N-MC cells (Fig. 5B, indicated by an arrow and asterisk). These results suggest that TrkA could be involved in downregulation of full length HSP70 cleavage or a certain HSP70 homolog.

Again, to prove whether the identified protein spots are regulated by TrkA and SP600125, the samples were analyzed by 2DE/Western blot using 7 cm IPG strip (pH 4–7). Interestingly, we found that most of the identified protein spots were modified in a control condition by an unknown mechanism (Fig. 6, arrow areas in left panels). The modified hnRNP C1/C2 protein spots (SSP 3352, 3350, 3212, 3209) and
Proteomics 2014, 14, 202–215

3.5 Investigation of GW441756 effects on SP600125-controlled TrkA-dependent protein spots

Recently, hnRNP C1/C2, α-tubulin, α-enolase, peroxiredoxin-6, and PROS-27 were identified as major GW441756-controlled TrkA-dependent targets in SK-N-MC neuroblastoma cells by us [20]. Thus, here we investigated the effects of GW441756 on the SP600125-controlled novel TrkA-dependent protein spots. The results revealed that unknown TrkA-dependent β-tubulin homolog, actin homolog, eIF-5A-1, PP1-gamma, and PDH E1-alpha protein spots were also controlled by GW441756, but not as much as the major GW441756-controlled TrkA-dependent protein spots (data not shown). However, SSP 3350, one of the SP600125-controlled hnRNP C1/C2 protein spots, was not controlled by GW441756 on the TrkA-dependent upregulation, whereas other hnRNP C1/C2 protein spots SSP 3352, 3212, and 3209 had significant inhibitory effects by GW441756 (Fig. 8A). Like hnRNP C1/C2 protein spot SSP 3350, TrkA-dependent downregulation of HSP70 protein spot SSP 2620 was not controlled by GW441756 (Fig. 8B).

3.6 Inhibition of TrkA activity by SP600125

Intriguingly, we showed here that most of the SP600125-controlled TrkA-dependent targets were similarly regulated by TrkA inhibitor GW441756 although there was somewhat difference in 2DE image (Fig. 8). To further investigate this, TrkA was induced with Tet for 12 h in SK-N-MC-TrkA cells and then treated with GW441756, SP600125, and other JNK inhibitor AS601245 for 8 h. After removing media thoroughly, the cells were immediately extracted with SDS sample buffer.
to exclude a potential dephosphorylation and other modifications followed by 10% SDS-PAGE and Western blot analysis. Surprisingly, TrkA-mediated tyrosine phosphorylation processes including ERK phosphorylation were gradually suppressed in a dose dependent manner by both GW441756 and SP600125, indicating that SP600125 could function as a TrkA inhibitor similarly to GW441756 (Fig. 9A, second and third panels). As expected, activation of TrkA-dependent JNK signaling including c-Jun phosphorylation was significantly suppressed by SP600125 but not by GW441756 at least at these cellular circumstances (Fig. 9A, fifth and seventh panels). In addition, TrkA-dependent JNK activation was inhibited by AS601245, but TrkA-mediated tyrosine phosphorylation processes were not suppressed by AS601245 (Fig. 9B, second and third panels). Again, our results demonstrated here that TrkA did not largely affect protein amount of full length HSP70 and α-tubulin, however, a cleaved form of HSP70 or a certain HSP70 homolog indicated by an arrow and asterisk was significantly inhibited by TrkA, in consistent with the results of Fig. 5A (Fig. 9A, eighth and ninth panels).

Interestingly, we found that a cleaved form of α-tubulin or a certain α-tubulin homolog was significantly upregulated by TrkA, and it was blocked by SP600125 (Fig. 10A, indicated by an arrow and asterisk). Moreover, ectopic expression of TrkA altered acetylation levels of cellular proteins as shown in Fig. 10B (especially in areas indicated by arrows). Taken together, our results strongly suggest that TrkA could be involved in various PTM signaling pathways such as acetylation and cleavage of cellular proteins as well as tyrosine phosphorylation.

4 Discussion

To better understand an involvement of JNK in TrkA-mediated signaling pathways, we identified JNK inhibitor
Figure 9. Effects of GW441756, SP600125, and AS601245 on TrkA-mediated cellular processes. SK-N-MC-TrkA cells were cultured in the absence or presence of Tet for 12 h and then untreated (lanes 1–2 in A) or treated with the indicated amounts of GW441756 (GW; lanes 3–8 in A), DMSO as a control solvent (DM; lanes 9–10 in A, lanes 1–2 in B), SP600125 (SP; lanes 11–16 in A), or AS601245 (AS; lanes 3–8 in B) for 8 h. After removing media, the cells were immediately extracted with SDS sample buffer followed by Western blot analysis using the indicated antibodies.
TrkA dependent targets consisted of α-tubulin, β-tubulin homolog, actin homolog, peroxiredoxin-6, hnRNP C1/C2, eIF-5A-1, PROS-27, PPI-gamma, α-enolase and PDH E-alpha, whereas GW441756-uncontrolled TrkA dependent targets included hnRNP C1/C2 and HSP70 (Fig. 11). Since activation of TrkA-dependent JNK signaling was also significantly suppressed by SP600125 (Fig. 9A), our results strongly suggest an involvement of JNK in various TrkA-mediated signaling pathways.

Neuronal microtubule cytoskeleton was regulated by tubulin PTM, and many neurodegenerative disorders were related with altered microtubule-based transport [25]. Moreover, JNK was activated by moscatilin-induced tubulin depolymerization in human colorectal cancer cells [26]. JNK activation and tubulin depolymerization were involved in anticancer drug-induced cell cycle arrest and apoptosis in human breast cancer cells [27] and prostate cancer cells [28]. In addition, JNK activation induced actin cytoskeleton changes [29] and played an important role in the regulation of actin stability and migration in neurons and cancer cells [30]. Both α-tubulin and β-tubulin existed in numerous isotypic forms encoded by different genes and underwent various PTMs such as acetylation, phosphorylation, deetyrosylation, polyglutamylation, and polyglycylation [31]. Thus, our results suggest that TrkA could play an important role in the cytoskeleton through regulation of α-tubulin, β-tubulin homolog, and actin homolog.

Peroxiredoxins and HSP70 have been well known to play an important role as a cell survival factor. Peroxiredoxins belong to a family of multifunctional antioxidant thioredoxin-dependent peroxidases and play an important role in cellular protection against oxidative stress [32]. Six different peroxiredoxin isozymes showed distinct distribution profiles in different brain regions and different cell types, and peroxiredoxin-6 was expressed in glial cells but not in neurons [33]. Peroxiredoxin-6 was predominantly localized in the cytoplasm, and the intracellular location was changed to the mitochondria after ischemia-reperfusion in mice, protecting cells against mitochondrial dysfunction [34]. Moreover, peroxiredoxins suppressed JNK activation stimulated by tumor necrosis factor-α [35] and radiation [36], indicating a cell survival role of peroxiredoxins in various signaling pathways. HSP70 is a major member of the HSP family and protects cells against cellular stresses. HSP70 overexpression inhibited aminoglycoside-induced hair cell death [37], whereas reduced expression of HSP70 was associated with triptolide-mediated cell death of neuroblastoma [38]. The chaperone function of HSP70 was required for protecting cells against stress-induced apoptosis [39]. HSP70 prevented activation of JNK [40] and c-Jun [41], leading to inhibition of apoptotic cell death. Thus, our results suggest that TrkA could play an important role in the regulation of cell death through an interaction with peroxiredoxin-6 and HSP70.

hnRNP C1/C2 belongs to the family of nucleic acid binding proteins that are involved in the regulation of pre-mRNA processing and mature mRNA exporting out of the nucleus.
(NRF-2011-0003111).

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2011-003111).

The authors have declared no conflict of interest.

5 References

[1] Roux, P. P., Barker, P. A., Neurotrophin signaling through the p75 neurotrophin receptor. Prog. Neurobiol. 2002, 67, 203–233.

[2] Skaper, S. D., The neurotrophin family of neurotrophic factors: an overview. Methods Mol. Biol. 2012, 846, 1–12.

[3] Skaper, S. D., The biology of neurotrophins, signalling pathways, and functional peptide mimetics of neurotrophins and their receptors. CNS Neurol. Disord. Drug Targets 2008, 7, 46–62.

[4] Arrighi, N., Bodei, S., Zani, D., Simeone, C. et al., Nerve growth factor signaling in prostate health and disease. Growth Factors 2010, 28, 191–201.

[5] Sarkar, D., Reid, A. H., Yap, T. A., de Bono, J. S., Targeting the PI3K/AKT pathway for the treatment of prostate cancer. Clin. Cancer Res. 2009, 15, 4799–4805.

[6] Freund-Michel, V., Frossard, N., The nerve growth factor and its receptors in airway inflammatory diseases. Pharmacol. Ther. 2008, 117, 52–76.

[7] Giannakopoulou, D., Daquin-Nerrière, V., Mitsacos, A., Kouvelas, E. D. et al., Ectopic expression of TrkA in the adult rat basal ganglia induces both nerve growth factor-dependent and -independent neuronal responses. J. Neurosci. Res. 2012, 90, 1507–1521.

[8] Lagadec, C., Meignan, S., Adriaenssens, E., Foveau, B. et al., TrkA overexpression enhances growth and metastasis of breast cancer cells. Oncogene 2009, 28, 1960–1970.

[9] Eggert, A., Grotzer, M. A., Ikegaki, N., Liu, X. G. et al., Expression of the neurotrophin receptor TrkA down-regulates expression and function of angiogenic stimulators in SH-SY5Y neuroblastoma cells. Cancer Res. 2002, 62, 1802–1808.

[10] Eggert, A., Grotzer, M. A., Ikegaki, N., Liu, X. G. et al., Expression of neurotrophin receptor TrkA inhibits angiogenesis in neuroblastoma. Med. Pediatr. Oncol. 2000, 35, 569–572.

[11] Lavoie, J. F., LeSauteur, L., Kohn, J., Wong, J. et al., TrkA induces apoptosis of neuroblastoma cells and does so via a p53-dependent mechanism. J. Biol. Chem. 2005, 280, 29199–29207.

[12] Chou, T. T., Trojanowski, J. Q., Lee, V. M., A novel apoptotic pathway induced by nerve growth factor-mediated TrkA activation in medulloblastoma. J. Biol. Chem. 2000, 275, 565–570.

[13] Jung, E. J., Kim, C. W., Kim, D. R., Cytosolic accumulation of γ-H2AX is associated with tropomyosin-related kinase A-induced cell death in U2OS cells. Exp. Mol. Med. 2008, 40, 276–285.

[14] Jung, E. J., Kim, D. R., Control of TrkA-induced cell death by JNK activation and differential expression of TrkA upon DNA damage. Mol. Cells 2010, 30, 121–125.

[15] Jung, E. J., Kim, D. R., Ectopic expression of H2AX protein promotes TrkA-induced cell death via modulation of TrkA tyrosine-490 phosphorylation and JNK activity upon DNA damage. Biochem. Biophys. Res. Commun. 2011, 404, 841–847.

[16] Jung, E. J., Kim, C. W., Caveolin-1 inhibits TrkA-induced cell death by influencing on TrkA modification associated with tyrosine-490 phosphorylation. Biochem. Biophys. Res. Commun. 2010, 402, 736–741.

[17] Nikoletopoulou, V., Lickert, H., Frade, J. M., Rencurel, C. et al., Neurotrophin receptors TrkA and TrkC cause neuronal death whereas TrkB does not. Nature 2010, 467, 59–63.
[18] Vera, C., Tapia, V., Kohan, K., Gabler, F. et al., Nerve growth factor induces the expression of chaperone protein calreticulin in human epithelial ovarian cells. *Horm. Metab. Res.* 2012, 44, 639–643.

[19] Jung, E. J., Kim, D. R., Apoptotic cell death in TrkA-overexpressing cells: kinetic regulation of ERK phosphorylation and caspase-7 activation. *Mol. Cells* 2008, 26, 12–17.

[20] Jung, E. J., Lee, S. Y., Kim, C. W., Proteomic analysis of novel targets associated with TrkA-mediated tyrosine phosphorylation signaling pathways in SK-N-MC neuroblastoma cells. *Proteomics* 2013, 13, 355–367.

[21] Kwon, Y. S., Ryu, C. M., Lee, S., Park, H. B. et al., Proteome analysis of *Arabidopsis* seedlings exposed to bacterial volatiles. *Planta* 2010, 232, 1355–1370.

[22] Bennett, B. L., Sasaki, D. T., Murray, B. W., O’Leary, E. C. et al., SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl. Acad. Sci. U.S.A.* 2001, 98, 13681–13686.

[23] Weston, C. R., Davis, R. J., The JNK signal transduction pathway. *Curr. Opin. Genet. Dev.* 2002, 12, 14–21.

[24] Davies, C., Tournier, C., Exploring the function of the JNK (c-Jun N-terminal kinase) signalling pathway in physiological and pathological processes to design novel therapeutic strategies. *Biochem. Soc. Trans.* 2012, 40, 85–89.

[25] Chalkiadaki, G., Nikitovic, D., Katonis, P., Berdiaki, A. et al., c-Jun N-terminal kinase phosphorylation and caspase-3 activation in drug-resistant human melanoma cells. *Int. J. Cancer* 2009, 178, 235–244.

[26] Janke, C., Kneussel, M., Tubulin post-translational modifications: encoding functions on the neuronal microtubule cytoskeleton. *Trends Neurosci.* 2010, 33, 362–372.

[27] Chen, T. H., Pan, S. L., Guh, J. H., Liao, C. H. et al., Moscatilin induces apoptosis in human colorectal cancer cells: a crucial role of c-Jun NH2-terminal protein kinase activation caused by tubulin depolymerization and DNA damage. *Clin. Cancer Res.* 2008, 14, 4250–4258.

[28] Chen, J., Gu, H. Y., Lu, N., Yang, Y. et al., Microtubule depolymerization and phosphorylation of c-Jun N-terminal kinase-1 and p38 were involved in gamogbic acid induced cell cycle arrest and apoptosis in human breast carcinoma MCF-7 cells. *Life Sci.* 2008, 83, 103–109.

[29] Chang, W. L., Chang, C. S., Chiang, P. C., Ho, Y. F. et al., 2-Phenyl-5-(pyrrolidin-1-yl)-1-(3,4,5-trimethoxybenzyl)-1H-benzimidazole, a benzimidazole derivative, inhibits growth of human prostate cancer cells by affecting tubulin and c-Jun N-terminal kinase. *Br. J. Pharmacol.* 2010, 160, 1677–1689.

[30] Chalkiadaki, G., Nikitovic, D., Katonis, P., Berdiaki, A. et al., Low molecular weight heparin inhibits melanoma cell adhesion and migration through a PKCa/JNK signaling pathway inducing actin cytoskeleton changes. *Cancer Lett.* 2011, 312, 235–244.

[31] Ludueña, M., Multiple forms of tubulin: different gene products and coherent modifications. *Int. Rev. Cytol.* 1998, 178, 207–275.

[32] Immenschuh, S., Baumgart-Vogt, E., Peroxiredoxins, oxidative stress, and cell proliferation. *Antioxid. Redox. Signal.* 2005, 7, 768–777.

[33] Hattori, F., Okawa, S., Peroxiredoxins in the central nervous system. *Subcell. Biochem.* 2007, 44, 357–374.

[34] Eismann, T., Huber, N., Shin, T., Kuboki, S. et al., Peroxiredoxin-6 protects against mitochondrial dysfunction and liver injury during ischemia-reperfusion in mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2009, 296, G266–G274.

[35] Kang, S. W., Chang, T. S., Lee, T. H., Kim, E. S. et al., Cytosolic peroxiredoxin attenuates the activation of JNK and p38 but potentiates that of ERK in HeLa cells stimulated with tumor necrosis factor-α. *J. Biol. Chem.* 2004, 279, 2535–2543.

[36] Kim, Y. J., Lee, W. S., Ip, C., Chae, H. Z. et al., Prx1 suppresses radiation-induced c-Jun NH2-terminal kinase signaling in lung cancer cells through interaction with the glutathione S-transferase P1c-Jun NH2-terminal kinase complex. *Cancer Res.* 2006, 66, 7136–7142.

[37] Taleb, M., Brandon, C. S., Lee, F. S., Harris, K. C. et al., Hsp70 inhibits aminoglycoside-induced hearing loss and cochlear hair cell death. *Cell Stress Chaperones* 2009, 14, 427–437.

[38] Antonoff, M. B., Chugh, R., Skube, S. J., Dudeja, V. et al., Role of Hsp70 in triptolide-mediated cell death of neuroblastoma. *J. Surg. Res.* 2010, 163, 72–78.

[39] Mosser, D. D., Caron, A. W., Bourget, L., Meriin, A. B. et al., The chaperone function of hsp70 is required for protection against stress-induced apoptosis. *Mol. Cell. Biol.* 2000, 20, 7146–7159.

[40] Gabai, V. L., Meriin, A. B., Mosser, D. D., Caron, A. W. et al., Hsp70 prevents activation of stress kinases. A novel pathway of cellular thermotolerance. *J. Biol. Chem.* 1997, 272, 18033–18037.

[41] Bienemann, A. S., Lee, Y. B., Howarth, J., Uney, J. B., Hsp70 suppresses apoptosis in sympathetic neurons by preventing the activation of c-Jun. *J. Neurochem.* 2008, 104, 271–278.

[42] Dreyfuss, G., Kim, V. N., Kataoka, N., Messenger-RNA-binding proteins and the messages they carry. *Nat. Rev. Mol. Cell Biol.* 2002, 3, 195–205.

[43] Weighardt, F., Biamonti, G., Riva, S., The roles of heterogeneous nuclear ribonucleoproteins (hnRNP) in RNA metabolism. *Bioessays* 1996, 18, 747–756.

[44] Stone, J. R., Collins, T., Rapid phosphorylation of heterogeneous nuclear ribonucleoprotein C1/C2 in response to physiologic levels of hydrogen peroxide in human endothelial cells. *J. Biol. Chem.* 2002, 277, 15621–15628.

[45] Stone, J. R., Maki, J. L., Collins, T., Basal and hydrogen peroxide stimulated sites of phosphorylation in heterogeneous nuclear ribonucleoprotein C1/C2. *Biochemistry* 2003, 42, 1301–1308.

[46] Sun, Z., Cheng, Z., Taylor, C. A., McConkey, B. J. et al., Apoptosis induction by elf5A1 involves activation of the intrinsic mitochondrial pathway. *J. Cell. Physiol.* 2010, 223, 798–809.

[47] Li, A. L., Li, H. Y., Jin, B. F., Ye, Q. N. et al., A novel elf5A complex functions as a regulator of p53 and p53-dependent apoptosis. *J. Biol. Chem.* 2004, 279, 49251–49258.

[48] Tanahashi, H., Kito, K., Ito, T., Yoshioka, K., MafB protein stability is regulated by the JNK and ubiquitin-proteasome pathways. *Arch. Biochem. Biophys.* 2010, 494, 94–100.
[49] Sánchez-Pérez, T., Ortiz-Ferrón, G., López-Rivas, A., Mitotic arrest and JNK-induced proteasomal degradation of FLIP and Mcl-1 are key events in the sensitization of breast tumor cells to TRAIL by antimicrotubule agents. *Cell Death Differ.* 2010, 17, 883–894.

[50] Zhu, D. Y., Cui, R., Zhang, Y. Y., Li, H. et al., Involvement of ubiquitin-proteasome system in icariin-induced cardiomyocyte differentiation of embryonic stem cells using two-dimensional gel electrophoresis. *J. Cell. Biochem.* 2011, 112, 3343–3353.

[51] Sogawa, K., Masaki, T., Miyachi, A., Sugita, A. et al., Enhanced expression of PP1 gamma 1, a catalytic subunit isoform of protein phosphatase type 1, in invasive ductal carcinoma of the breast. *Cancer Lett.* 1997, 112, 263–268.

[52] Monick, M. M., Powers, L. S., Gross, T. J., Flaherty, D. M. et al., Active ERK contributes to protein translation by preventing JNK-dependent inhibition of protein phosphatase 1. *J. Immunol.* 2006, 177, 1636–1645.

[53] Sogawa, K., Yamada, T., Muramatsu, Y., Sumida, T. et al., Decrease of nuclear protein phosphatase 1 activity and induction of mitotic arrest and apoptosis by a marine microalgal polysaccharide in human myeloid leukemia U937 cells. *Res. Commun. Mol. Pathol. Pharmacol.* 1998, 99, 267–282.

[54] Capello, M., Ferri-Borgogno, S., Cappello, P., Novelli, F., α-enolase: a promising therapeutic and diagnostic tumor target. *FEBS J.* 2011, 278, 1064–1074.

[55] Butterfield, D. A., Lange, M. L., Multifunctional roles of enolase in Alzheimer’s disease brain: beyond altered glucose metabolism. *J. Neurochem.* 2009, 111, 915–933.

[56] Zhou, W., Capello, M., Fredolini, C., Piemonti, L. et al., Mass spectrometry analysis of the post-translational modifications of α-enolase from pancreatic ductal adenocarcinoma cells. *J. Proteome Res.* 2010, 9, 2929–2936.

[57] Tomaino, B., Cappello, P., Capello, M., Fredolini, C. et al., Circulating autoantibodies to phosphorylated α-enolase are a hallmark of pancreatic cancer. *J. Proteome Res.* 2011, 10, 105–112.

[58] Schöder, H., Knight, R. J., Kofoed, K. F., Schelbert, H. R. et al., Regulation of pyruvate dehydrogenase activity and glucose metabolism in post-ischaemic myocardium. *Biochim. Biophys. Acta.* 1998, 1406, 62–72.

[59] Wada, N., Matsuishi, T., Nonaka, M., Naito, E. et al., Pyruvate dehydrogenase E1α subunit deficiency in a female patient: evidence of antenatal origin of brain damage and possible etiology of infantile spasms. *Brain Dev.* 2004, 26, 57–60.

[60] Pilegaard, H., Birk, J. B., Sacchetti, M., Mourtzakis, M. et al., PDH-E1α dephosphorylation and activation in human skeletal muscle during exercise. Effect of intralipid infusion. *Diabetes* 2006, 55, 3020–3027.