Analysis of Cyclin-Dependent Kinase 2-Regulated Phosphorylation of Stathmin in Etoposide-Induced Apoptotic HeLa Cells by Two-Dimensional Electrophoresis and Mass Spectrometry

Yong-ung Kim, Kyo-Tan Koo, Joon-Seok Choi, Ying-Hua Jin, Hyungshin Yim, You-Take Oh, and Seung Ki Lee*

College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, San 56–1, Sillim-dong, Gwanak-gu, Seoul 151–742, Republic of Korea

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The candidate proteins that are involved in the cyclin-dependent kinase 2 (cdk2) signaling pathway were analyzed by comparing different proteins between dominant negative cdk2 overexpressed and control HeLa cells using two-dimensional electrophoresis (2-DE) and mass spectrometry (MS). The 2-DE and MS indicated that stathmin and its monophosphorylated form were induced in etoposide-treated HeLa cells compared to untreated cells and this effect was inhibited by overexpression of dominant negative mutant form of cdk2. Further analysis showed that serine-25 (Ser-25), which comprises the conserved target motif for phosphorylation by mitogen-activated protein kinase (MAPK), was the major phosphorylation site of monophosphorylated form of stathmin. These findings indicate that etoposide-induced expression and phosphorylation at Ser-25 of stathmin might be mediated by activation of the MAPK signaling pathway, which is mediated by the cdk2 activation during the onset of the anticancer agent induced apoptotic events in the cancer cells.

Key words —— stathmin, cyclin-dependent kinase 2, cancer cell, etoposide, proteomic analysis

INTRODUCTION

Stathmin is a highly conserved 19 kDa cytosolic phosphoprotein that has been proposed to be a relay molecule in integrating diverse intracellular signaling pathways, and level of expression and state of phosphorylation of the protein are modulated in reponse to integrated cellular factors and the pattern of forms of the protein present in cell depend on the state of cell reflecting proliferation, differentiation and functions.[1)] Stathmin has also been revealed to promote depolymerization of microtubules by tight complex formation of one stathmin with two tubulin heterodimers,[2)] and this microtubule-destabilizing activity is inhibited by its increasing phosphorylation.[3)] The phosphorylation state of stathmin varies during the cell cycle, and its phosphorylation during mitosis seems important for the progression of the cell cycle.[4)] Stathmin exists as two isoforms, α and minor β with different isoelectric points and molecular weights, encoded by a single cellular mRNA,[5)] and furthermore, both isoforms can be phosphorylated up to four serine residues, serine-16 (Ser-16), -25, -38, and -63.[6)] It has been reported that Ser-16 is a site for cAMP-dependent protein kinase (PKA), protein kinase C (PKC) and calmodulin-dependent protein kinases (CaM),[6–8)] Ser-25 and Ser-38 are major sites for both cell-cycle-regulated cyclin dependent kinases (cdks) and mitogen-activated protein kinase (MAPK),[6,8–10)] and Ser-63 is a site for PKA.[6)] Cdk2, associated with cyclin E and cyclin A during the G1 and S phase, respectively, has been implicated in control of G1 and S phase events in the cell cycle.[11)] So far, cdk2 has been reported to phosphorylate several protein substrates including transcription factors such as E2F family members, the tumor suppressor retinoblastoma protein (pRB) and related proteins, and other regulators of the cell cycle.[11)] Thus, cdk2 will likely control many of the extrinsic and/or intrinsic signaling pathways involved in the regulation of pro-

*To whom correspondence should be addressed: College of Pharmacy, Seoul National University, San 56–1, Sillim-dong, Gwanak-gu, Seoul 151–742, Republic of Korea. Tel.: +82-2-880-7879; Fax: +82-2-872-1795; E-mail: sklcrs@plaza.snu.ac.kr

liferative or antiproliferative processes. Dominant negative mutant form of cdk2 have been shown to suppress several apoptosis induced by tumor necrosis factor α (TNF-α), staurosporine, a pan-specific protein kinase inhibitor, and growth factor deprivation in human cells like HeLa cells and endothelial cells.[12–14)]

We and others have reported recently that essential activation of cdk2 is involved in a variety of apoptosis induced by anticancer agents including etoposide, paclitaxel, and doxorubicin in several human tumor cells and overexpression of dominant negative mutant form of cdk2 suppressed these apoptosis.[15,16)] Now, the proteomic studies with two-dimensional electrophoresis (2-DE) and MS have been demonstrated to be valuable for the analysis of real states of cellular protein subsets characteristic of various biological events.[17,18)] In the present study, we overexpress the dominant negative mutant form of cdk2 and induced apoptosis by treatment with etoposide in HeLa cells, and examine the proteins that are likely to be involved in the cdk2 signaling pathway in apoptotic cells by comparing the level of expression and state of phosphorylation of proteins between dominant negative cdk2 overexpressed and mock-transfected control HeLa cells using 2-DE and MS. We show that etoposide treatment increases the cellular amount of the nonphosphorylated isoform and its monophosphorylated form of stathmin, and overexpression of dominant negative mutant form of cdk2 inhibits this effect. We determine that Ser-25 is the major site phosphorylated on the monophosphorylated form of stathmin. We discuss the specific phosphorylation at Ser-25 of stathmin in the cdk2 signaling pathway to correlate the role of essential activation of cdk2 with the onset of apoptotic events in the anticancer agent treated cancer cells.

MATERIALS AND METHODS

Cell Line, Plasmids and Chemicals —— Human cervical carcinoma cell line, HeLa cells were from American Type Culture Collection. Dulbecco's modified Eagle medium (DMEM), calf serum and trypsin-EDTA solution were purchased from Invitrogen (Grand Island, NY, U.S.A.). Wild type and dominant negative cdk2 expressing plasmids under control of the CMV promoter (pCMVcdk2, pCMVcdk2-dn) were a generous gift from Dr. E. Mark (Harvard Medical School, Boston, MA, U.S.A.). Other chemicals were of the best grade commercially available.

Cell Culture, Transfection and Treatment —— HeLa cells were grown as a monolayer at 37°C and 5% CO_2 in a culture vessel in DMEM supplemented with Antimycotic-Antibiotic (Invitrogen; 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) and 5% calf serum (Invitrogen). The cells, in which the average initial cell population was 5×10^5 cells, were seeded and allowed to adhere to the bottom of 100 mm disposable plastic petri dishes and incubated for 24 hr in a CO_2 incubator. The cells (2×10^5) were transiently transfected with 10 μg of either pCMV or pCMVcdk2-dn essentially according to the transfection method with calcium phosphate.[18)] Twenty-four hours after transfection, the cells were induced to undergo apoptosis by treatment with 30 μg/ml etoposide for the indicated time period of 0, 6, and 12 hr.

Sample Preparation ——After incubation, the cells were pelleted and washed with PBS (pH 7.4). The cells were then lysed in 200 μl of lysis buffer containing 8 M urea, 4% (w/v) CHAPS, 40 mM Tris, 2 mM tributylphosphine, and 2% (v/v) carrier ampholytes pH 3–10 (Bio-Rad, Hercules, CA, U.S.A.). The cell lysate was centrifuged at 20000 × *g* for 10 min and the supernatant was stored at –80°C until use. The total protein concentration in the supernatant was determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, U.S.A.) on the basis of the method as described.[19)]

2-DE —— The separations were carried out with a slight modification of the method as described.[20)] For the first dimension separation, cell lysate containing 250 μg of proteins were loaded onto pH 3–10 isofocusing gels of linear IPG strips (Bio-Rad). IEF was performed on the Protean cell (Bio-Rad) at room temperature at 100000 Vh using the voltage gradient recommended by the manufacturer. For the second dimension separation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the Hoefer SE 600 apparatus (Amersham Pharmacia Biotech, Uppsala, Sweden) using 12% polyacrylamide gels at room temperature at a constant current of 50 mA per plate. After 2-DE, the gel were silver-stained using a silver staining kit (Sigma-Aldrich, St. Louis, MO, U.S.A.) for detection of the protein spots.

In-Gel Digestion —— In-gel digestion was carried out with a slight modification of the method as described.[21)] After gel electrophoresis, silver stained protein spots were cut out into 1 mm^3 size of gel

pieces. The gel pieces were destained for 20 min at room temperature in the dark with 200 μl of freshly prepared silver destaining solution (30 mM potassium ferricyanide, 100 mM sodium thiosulfate). Then, the gel pieces were washed for 15 min at room temperature with 3–4 times with 200 μl of Milli-Q grade water until the gel pieces are clear and washed for 20 min at room temperature over 3 times with 100 μl of 50% acetonitrile (ACN), 0.2 M ammonium bicarbonate solution. After destaining and washing, the gel pieces were dehydrated for 10 min at room temperature in 200 μl of 100% ACN. Trypsin (modified trypsin, sequence grade, Promega, Madison, WI, U.S.A.) was dissolved in 1 mM HCl at a concentration of 0.06 μg/μl and stored –20°C in aliquots, and immediately prior to the experiment, trypsin solution at a concentration of 0.003 μg/μl was prepared by diluting it 20-fold with 0.2 M ammonium bicarbonate (pH 8.5). Subsequently, the gel pieces were kept for 20 hr at 30°C in 65 μl of 0.2 M ammonium bicarbonate (pH 8.5) containing 0.195 μg of trypsin. The generated tryptic peptides were extracted from gel pieces to the supernatant by treatment for 30 min at room temperature with 50 μl of 50% ACN, 0.3% TFA solution. The collected supernatant was concentrated at 50°C by evaporation of the organic phase.

Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) —— The sample peptide concentrate was dissolved in 10 μl of 0.3%TFA solution. The resin in ZipTip$_{\mu\text{-C18}}$ (Millipore, Bedford, MA, U.S.A.) was swollen 2 times with applying 10 μl of 50% ACN and was equilibrated 2 times with applying 10 μl of 0.3% TFA solution. The sample peptides were adsorbed on resin with applying 8 times of 10 μl of the sample peptide solution on it. The adsorbed sample peptides were washed and desalted with applying 2 times of 10 μl of 0.3% TFA solution. Matrix solution [*ca*. 5 mg/ml α-cyano-4-hydroxycinnamic acid (α-CHCA)] was prepared fresh by 2-fold diluting clear supernatant of 10 mg of α-CHCA dissolved at room temperature in 1 ml of 50% ACN, 0.3% TFA solution with the same solution. The adsorbed sample peptides were extracted with applying 5 times of 1–2 μl of matrix solution, and finally spotted on the MS sample plate. The sample peptides mounted on the MS sample plate were air dried for about 30 min, and ionization process was started automatically when the instrument reached the specified high vacuum. MALDI-TOF mass spectra were obtained in delayed extraction reflection mode using angiotensin I (M_r 1296.69) and adrenocorticotropic hormone (ACTH; clip 18–39: M_r 2465.20) as external standards on a Voyager DETM STR Biospectrometry Workstation (Applied Biosystems, U.S.A.). MALDI-TOF mass spectra of protein spot were analyzed by searching for their peptide mass fingerprints in a non-redundant database NCBI (NCBI, NIH, Bethesda, MD, U.S.A.) using the search program MS-FIT (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm). Database searches were performed with the mass tolerance of 50 ppm for masses of tryptic peptides and the individually adjusted minimum number of peptides required for a match depending on the extent of background contributions.

Electrospray Ionization Quadrupole Time of Flight Tandem Mass Spectrometry (ESI Q-TOF MS/MS) —— The sample peptide concentrate was dissolved in 30 μl of 0.3%TFA solution and resultant sample was concentrated and desalted using ZipTip$_{\mu\text{-C18}}$. The recovered extracts are eluted into borosilicate glass capillary spraying needle by the addition of 3 μl of 50% ACN, 0.3% formic acid solution. The ionization process of the sample peptides was started in an atmospheric pressure ionization source at 40°C and an applied capillary potential of 1.2 kV. ESI Q-TOF tandem mass spectra were obtained on a Q-TOF Ultima Global Mass Spectrometer (Micromass U.K., Manchester, U.K.). ESI Q-TOF tandem mass spectra of peptide from tryptic peptides of protein spot was analyzed by searching in a non-redundant database NCBI using the Mascot computer program (http://www.matrixscience.com/search_form_select.html).

RESULTS

2-DE Detection of Proteins

The proteins that are likely to be involved in the cdk2 signaling pathway in cells were detected by comparing different protein spots in 2-DE gels between dominant negative cdk2 overexpressed and mock-transfected control HeLa cells. The analysis of proteins is concentrated on a series of three spots representing a subset of proteins in which each of these seems likely to be different only in its phosphorylation state. Two protein spots in this subset are slightly downregulated in dominant negative cdk2 overexpressed samples compared to mock-transfected controls (Fig. 1). Since the reproducibility of 2-DE gel data seems to be sufficient as the

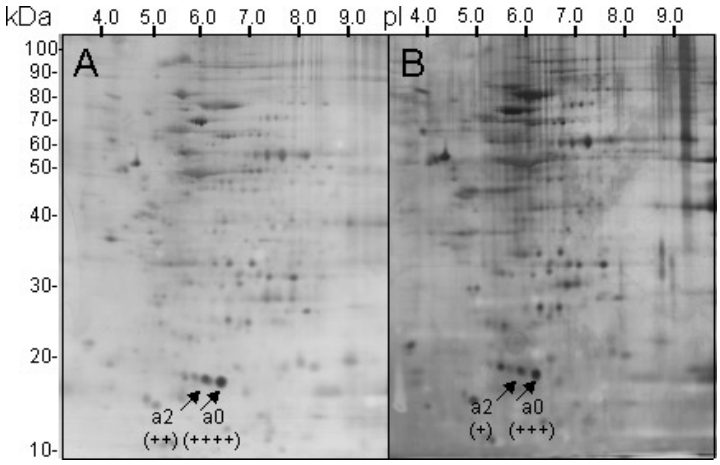

Fig. 1. 2-DE Analysis of the Two Different Proteins in Cellular Proteins of HeLa Cells between Dominant Negative cdk2 Overexpressed and Mock-Transfected Control HeLa Cells

2-DE patterns of silver-stained protein spots with marked positions of the two most different spots of samples of 250 μg proteins from cells after transfection (A) with pCMV, (B) with pCMVcdk2-dn. The intensity scale (++++, +++, ++, +) of protein spots corresponds qualitatively to four apparent intensities.

total protein spots were closely matched in gels from the independently prepared sets of HeLa cell samples, different protein spots in two sets of 2-DE gels between pCMVcdk2-dn transfected and pCMV transfected cells represent candidate proteins that are involved in the cdk2 signaling pathway. Thus, this model that is coupled with dominant negative mutant form of cdk2 is a suitable approach for the detection of proteins that are involved in the cdk2 signaling pathway.

2-DE Analysis of Proteins

Then, proteins that are associated with etoposide-induced apoptosis in the cdk2 signaling pathway in these candidate proteins that are involved in the cdk2 signaling pathway were examined by comparing different protein spots in 2-DE gels of etoposide-treated HeLa cells between pCMVcdk2-dn transfected and pCMV transfected control cells. The amounts of spot a0 and a2 in a subset of apparent M_r 19 kDa and apparent pI 6.2–5.6 were significantly increased in the 12 hr etoposide-treated HeLa cells compared to the 0 hr untreated cells in the mock-transfected controls. On the contrary, the amounts of these two spots are significantly downregulated in dominant negative cdk2 overexpressed HeLa cells compared to mock-transfected control HeLa cells in the 12 hr etoposide-treated samples (Fig. 2). The essential activation of cdk2 for the progression of apoptosis was previously observed in about 12 hr of 30 μg/ml etoposide treatment in HeLa cells (data not shown). These findings indicate that these proteins of two different spots are involved in the cdk2 signaling pathway in the progression of etoposide-induced apoptotic events in HeLa cells.

MS Identification of Proteins

Spot a0 and a2 were excised from a 2-DE gel, in-gel digested with trypsin, and analyzed by means of MS. The apparent M_r determined by 2-DE for the protein corresponding to spot a0 is 19 kDa, in good agreement with the calculated molecular weight of 17303 for human stathmin, which contains a high proportion of charged amino acids (47%). The prominent peak at *m*/*z* 1388.75 in MALDI-TOF MS of tryptic peptides of spot a0 most likely corresponded to the peptide 15–27 in the stathmin sequence. The peak at *m*/*z* 1387.75 in ESI Q-TOF MS/MS of spot a0 was sequenced to the peptide 15–27 in the stathmin sequence. Together with the results of 2-DE analysis, spot a0 and a2 were unequivocally identified by peptide mass profiling by MALDI-TOF MS and ESI Q-TOF MS/MS as the nonphosphorylated isoform and its monophosphorylated form of stathmin, respectively (Table 1). These findings indicate that expression of the nonphosphorylated isoform of stathmin is upregulated and its monophosphorylated form is increased in response to cdk2 activation in etoposide-induced apoptotic HeLa cells.

MS Analysis of Phosphorylation Sites in Phosphorylated Stathmin

The phosphorylation of the respective peptides

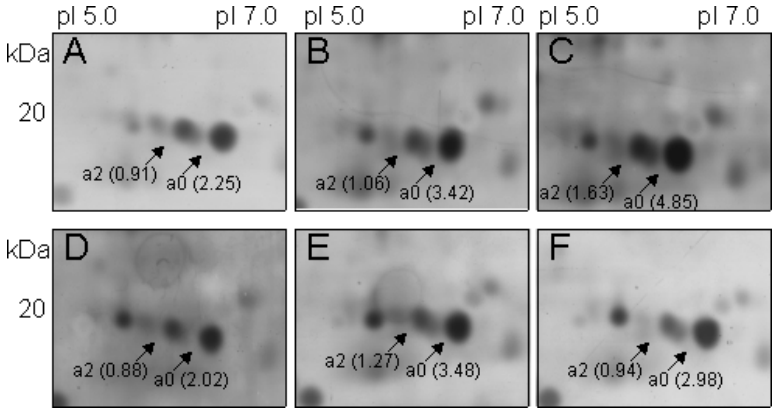

Fig. 2. 2-DE Analysis of the Two Different Proteins of Etoposide-Treated HeLa Cells between Dominant Negative cdk2 Overexpressed and Mock-Transfected Control HeLa Cells

2-DE patterns of protein spots from cells incubated (A) at 37°C for 0 hr, (B) at 37°C for 6 hr, (C) at 37°C for 12 hr in the presence of 30 μg/ml etoposide after transfection with pCMV, (D) at 37°C for 0 hr, (E) at 37°C for 6 hr, (F) at 37°C for 12 hr in the presence of 30 μg/ml etoposide after transfection with pCMVcdk2-dn. The values of spot relative volumes (%) in the parentheses for two proteins were obtained by Phoretix 2D software, and significant differences in the values of spot relative volumes of two proteins in the 12 hr etoposide-treated dominant negative cdk2 overexpressed samples compared to the corresponding mock-transfected controls were observed from two independent sets of experiments.

Table 1. Identification of Two Different Proteins of Etoposide-Treated HeLa Cells Affected by Overexpression of Dominant Negative cdk2

Spot No.	Identification method	Experimental pI/molecular mass (Da)	From database pI/molecular mass (Da)	Protein name	NCBI No.
a0	Mass fingerprinting (MALDI-TOF MS) MS sequencing (ESI Q-TOF MS/MS)	6.2/19250	5.8/17303	stathmin 1	5031851
a2	Mass fingerprinting (MALDI-TOF MS) MS sequencing (ESI Q-TOF MS/MS)	6.0/18750	5.8/17383	stathmin 1[a)]	5031851

a) monophosphorylated.

were estimated on the basis of the presence of the relevant molecular mass at the *m*/*z* value 80, corresponding to the mass region of the *m*/*z* of the PO_3^- ion group, below the molecular mass of the $[M + H]^+$ ion of the respective phosphorylated peptides. The phosphorylated peptide identified in MALDI-TOF MS analysis was submitted to ESI Q-TOF MS/MS analysis and the site phosphorylated on the monophosphorylated form of stathmin was determined. The peak at *m*/*z* 1468.72 in MALDI-TOF MS of tryptic peptides of spot a2 most likely corresponded to the monophosphorylated form of peptide 15–27 in the stathmin sequence. The sequence identified in ESI Q-TOF MS/MS of the peak at *m*/*z* 1467.71 of spot a2 corresponded to the peptide 15–27 in the stathmin sequence and Ser-25 was found to be the major phosphorylation site of monophosphorylated form of stathmin (Fig. 3). The confirmation of the major phosphorylation at this site was obtained from the analysis of the phosphorylation of monophosphorylated form of stathmin, which was identical with Ser-25, from each of prepared gels of the experimental set. Further analysis of monophosphorylated form of stathmin by MS indicated that the monophosphorylated form was a mixture of protein phosphorylation at either Ser-25 and Ser-38 although the majority of the protein is the form phosphorylated at Ser-25 (data not shown). These findings indicate that Ser-25 is identified as the site of phosphorylation in the monophosphorylated stathmin.

DISCUSSION

It is generally accepted that many cellular apoptotic events during death appear to be parallel with mitotic cell cycle events during proliferation in appropriately restricted temporal and spatial subcellular compartments.[22)] Thus, the closely related

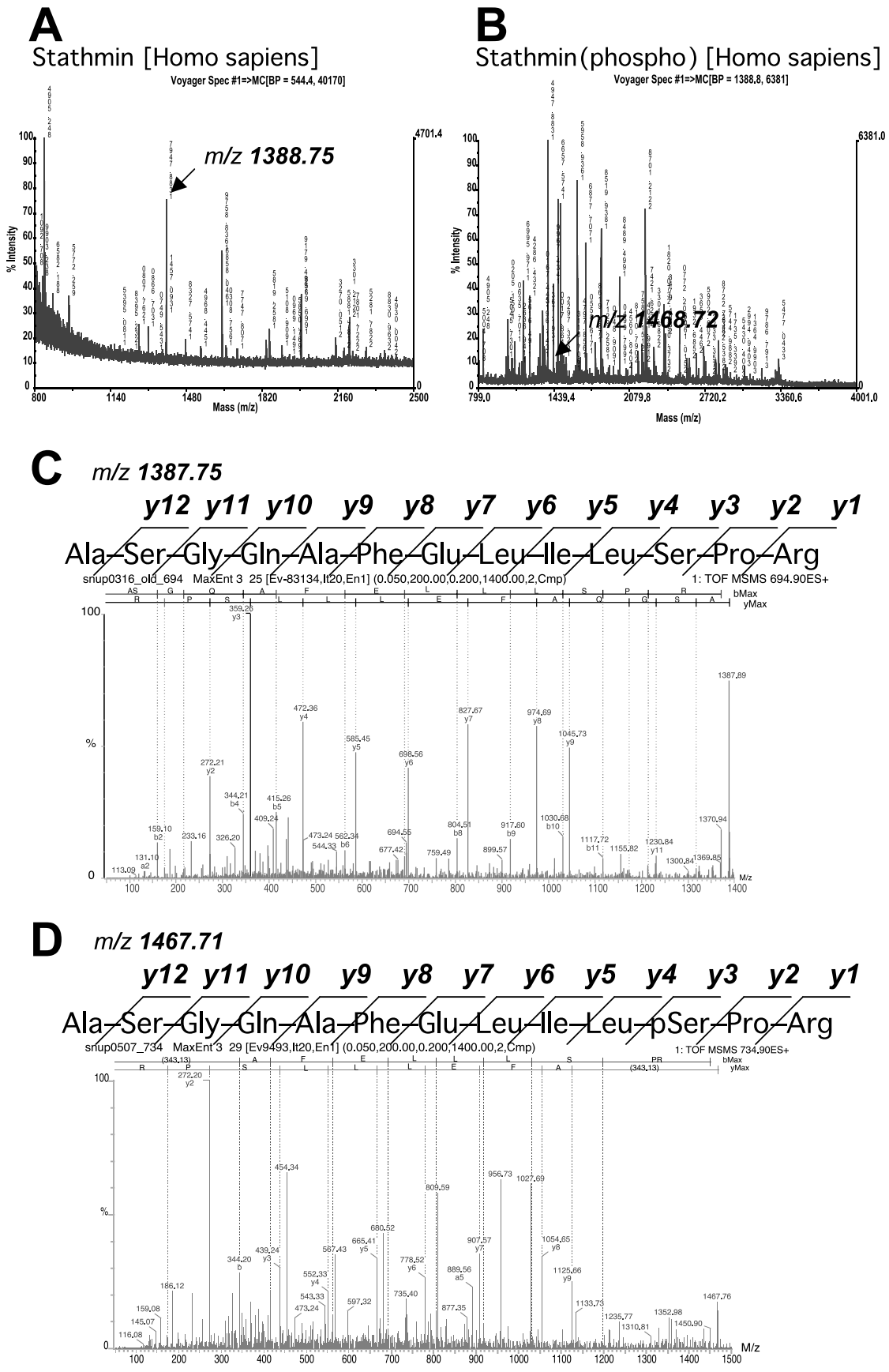

Fig. 3. MS Analysis of Two Different Proteins by MALDI-TOF MS and ESI Q-TOF MS/MS

MALDI-TOF mass spectra of tryptic peptides of (A) spot a0, (B) spot a2. In a parallel experiment, ESI Q-TOF tandem mass spectra of the peptide and the corresponding phosphorylated peptide from tryptic peptides of (C) spot a0, (D) spot a2, respectively.

pathways seem to be involved evolutionarily in fundamental molecular framework of both mitosis and apoptosis, and it appears that several proteins and molecules are involved in the control of both mitotic and apoptotic signaling pathways. We previously reported that the essential activation of cdk2 is involved in the progression of etoposide-induced apoptosis in HeLa cells.[16] We examined the proteins that are likely to be involved in the cdk2 signaling pathway in apoptotic cells by comparing the level of expression and state of phosphorylation of proteins between dominant negative cdk2 overexpressed and mock-transfected control HeLa cells by means of 2-DE and MS. Indeed, 2-DE and MS analysis showed that etoposide treatment increases the cellular amount of the nonphosphorylated isoform and its monophosphorylated form of stathmin, while overexpression of dominant negative mutant form of cdk2 inhibits this effect. Further MS analysis showed that Ser-25 was the major phosphorylation site of the monophosphorylated form of stathmin. Stathmin is expressed in all tissues at moderately high levels, and its expression is increased in cancer cell lines such as HeLa cells. Since stathmin is known to play an important role in the progression of mitosis, these observations indicate possible links between apoptosis and cell cycle regulation most likely in anticancer agent induced apoptotic cancer cells.

An *in vitro* phosphorylation study showed that both Ser-25 and Ser-38 of stathmin were phosphorylated by either cdc2 or cdk2.[8] However, it has been reported that stathmin is phosphorylated primarily at Ser-25 and to a lesser extent at Ser-38 during heat shock or chemical stress in HeLa cells,[10] and in contrast, this protein is phosphorylated mostly at Ser-38 and to a lesser extent at Ser-25 during heat shock, which lead the cells to apoptosis, in a human T lymphoblastic cell line, JURKAT cells.[23,24] Although there is some overlap in the sequence specificity of phosphorylation sites of stathmin, sequence specificity of the substrate sites at Ser-25 and Ser-38 correspond to the major target motifs in which serine/threonine residues followed by proline, for phosphorylation by proline-directed kinases involving cdks and MAPK.[6,8–10] Thus, cdks and/or MAPK activation is likely to be implicated in these phosphorylations. Although cdks phosphorylate serine or threonine residues with the canonical consensus sequence S/TPXK/R in studies using a number of different peptide substrates, it has generally been assumed that cdk2 and cdc2 have similar but distinct substrate sequence specificities.[25] With regard to this idea, the substrate sequence at Ser-25 of stathmin, SPRS, seems to form phosphorylation site exclusively for cdc2, whereas the substrate sequence at Ser-38 of this protein, SPPK, seems to form phosphorylation site for cdk2 as well as cdc2.[25] Thus, Ser-25 in stathmin, at which the etoposide-induced phosphorylation was inhibited by the presence of dominant negative mutant form of cdk2, might comprise the conserved target motif for phosphorylation by protein kinases other than cdk2. Further, phosphorylation at Ser-25 of stathmin may be due to activation of the MAPK signaling pathway and its phosphorylation may be mediated by MAPK.[26] Consistent with this idea, it has been reported that the MAPK signaling pathway can be significantly activated by some conditions, which include anticancer agent treatment such as etoposide treatment in HeLa cells,[27] while this pathway is constitutively inactivated in JURKAT cells.[28] Collectively, these observations suggest that upregulated expression and increased phosphorylation at Ser-25 of stathmin might be mediated by the MAPK signaling pathway, which is mediated by the cdk2 activation during the onset of etoposide-induced apoptotic events in HeLa cells. Further, the present approach by means of 2-DE and MS coupled with dominant negative mutant form of cdk2 has proven to be suitable for the analysis of real states of cellular protein subsets involved in the cdk2 signaling pathway during a specific cellular state such as apoptosis.

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