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Phosphoproteome Profiling for Cold Temperature Perception

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ABSTRACT

Temperature sensation initiates from the activation of cellular receptors when the cell is exposed to a decrease in temperature. Here, we applied a phosphoproteome profiling approach to the human lung epithelial cell line BEAS-2B to elucidate cellular cold-responsive processes. The primary aim of this study was to determine which intracellular changes of phosphorylation are accompanied by cold sensation. Eighteen protein spots that exhibited differentially phosphorylated changes in cells were identified. Most of the proteins that were phosphorylated after 5 or 10 min were returned to control levels after 30 or 60 min. Identified proteins were mainly RNA-related (i.e., they were involved in RNA binding and splicing). Temperature (18 and 10°C) stimuli showed homologies that were detected for time course changes in phosphoproteome. The data indicated a time-shift between two temperatures. The phosphorylation of putative cold responsive markers, such as ribosomal protein large P0 and heterochromatin-associated proteins 1, were verified by Western blotting in cells transfected with TRPM8 or TRPA1. J. Cell. Biochem. 112: 633–642, 2011.

KEY WORDS: PHOSPHOPROTEOME; CELLULAR COLD-RESPONSIVE PROFILING; RNA-BINDING PROTEIN

Recognition of temperature is an important element of sensory perception and allows humans to evaluate both their external environment and internal status. At the molecular level, cold is detected by transient receptor potential melastatin 8 (TRPM8) or TRPA1, a nonselective cation channel expressed on a subset of peripheral afferent fibers. It is known that thermo-sensitive afferents express ion channels of the transient receptor potential (TRP) family that respond at distinct temperature thresholds, thus establishing a molecular basis for thermo-sensation. Much is known about those channels mediating the perception of noxious heat; however, those proposed to be involved in cold sensation, TRPM8 and TRPA1 channel proteins, have only recently been addressed. While TRPM8 almost certainly performs a critical role in cold signaling, its part in nociception is still under investigation. TRPA1 is activated by the pungent ingredients in mustard and cinnamon, but it also has been postulated to mediate our perception of noxious cold temperatures. However, a number of conflicting reports have suggested that the role of this channel in cold sensation needs to be confirmed [McKemy, 2005]. In particular, the molecular and biochemical pathways inside cells that regulate cold-induced responses are largely unknown. In general, cold exposure induces adaptive thermogenesis, which elevates energy expenditure in mammals via mitochondrial uncoupling [Wijers et al., 2008]. The respiratory epithelium is constantly exposed to the external environment, and prolonged inhalation of cold air is detrimental to human airways [Sabnis et al., 2008]. Here, we hypothesized that TRPM8 and TRPA1 could function as a proximal sensor and mediator of compensatory events in the cooling of respiratory tract cells. Phosphorylation events are involved in integrating and transmitting information initiated by all the chemical and physical stimuli (e.g., ligand receptor interactions, environmental stresses) and, ultimately, in translating these stimuli into cellular responses. The activation of thermo-sensitive receptors could be coupled with changes in phosphorylation of representative determinants leading to gating, adaptive thermogenesis, and compensatory events due to cooling. Therefore, we hypothesized that cold-perceptive phosphorylation might lead to ER calcium gating, mitochondrial uncoupling, adaptive thermogenesis, and acclimatization to cold.

Post-translational modifications (PTMs) of proteins are considered one of the major determinants categorizing the organism's complexity [Venter et al., 2001]. To date, at least 200 different PTMs have been identified, of which only a few are reversible and important for the regulation of biological processes. One of the most studied PTMs is protein phosphorylation because it is vital for a large number of protein functions that are important to cellular processes spanning from signal transduction, cell differentiation,

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and development to cell-cycle control and metabolism [Delom and Chevet, 2006]. A primary role of phosphorylation is to act as a switch to turn "on" or "off" a protein activity or a cellular pathway in an acute and reversible manner [Hunter, 1995]. Furthermore, it is estimated that one of every three proteins is phosphorylated at some point in its life cycle [Zolnierowicz and Bollen, 1999]. Today, it is well known that almost all processes regulated by protein phosphorylation are reversible and controlled by the combined action of two different classes of enzymes, namely protein kinases and phosphatases. These kinases and phosphatases constitute approximately 2% of the human genome [Venter et al., 2001; Manning et al., 2002; Alonso et al., 2004]. Analysis of the entire cellular phosphoprotein panel, the so-called phosphoproteome, has been an attractive study subject since the discovery of phosphorylation as a key regulatory mechanism of cell life. However, despite a growing knowledge of many phosphorylation consensus sequences, this PTM usually cannot be predicted accurately from the translated gene sequence alone. Thus, characterization of protein modification by phosphorylation is one of the major investigations that needs to be accomplished in the post-genomic era. Understanding of the regulatory role played by phosphorylation begins with the discovery and identification of phosphoproteins. Long-lasting cellular responses via changes in gene expression, protein translation, and enzyme activity can be analyzed by differential gene expression alone or complemented with proteome analysis. However, differential gene expression is insufficient for investigating immediate early responses integrating multiple physiological inputs into highly complex and dynamic phosphorylation events that are not captured in these analyses. In light of the importance of short-term activational changes, analysis of the phosphoproteome may be necessary to fully understand temperature sensing receptor signaling. In this study, we explored the hypothesis that activation of thermo-sensitive receptors could be coupled with changes in phosphorylation of representative determinants leading to gating, adaptive thermogenesis, and compensatory events due to cooling.

We identified expression of TRPM8 and TRPA1 in the human bronchial epithelial cell line BEAS-2B and used this cell line to obtain a phosphoproteome profile. Since changes in protein phosphorylation are immediately process driven by various types of stimulation, the influence of time should not be neglected, but the kinetic study of protein phosphorylation changes has not been exploited yet. Here, we compared different temperatures (10 and 18°C) and analyzed data derived from a time course phosphorylation profiling experiment, which consisted of a study on the effects of cold temperature on BEAS-2B cells.

MATERIALS AND METHODS

CELL CULTURE

Human bronchial epithelial (BEAS-2B) cells (CRL-9609) were purchased from American Type Culture Collection (Rockville, MD) and cultured in a bronchial epithelial cell growth medium bullet kit (BEGM kit) supplemented with a gentamycin–amphotericin B mix (Clonetics/Lonza Corp., Basel, Switzerland). Culture flasks (Corning, Corning, NY) for the cells were pre-coated at 37°C with BEGM fortified with collagen (30 μg/ml), fibronectin (10 μg/ml), and BSA (10 μg/ml). Cells were maintained at 37°C in an air-ventilated and humidified incubator with 5% carbon dioxide. The HEK293 (human embryonic kidney) cells were cultured in minimum essential medium (MEM: Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. The cell line was purchased from Korean Cell Line Bank (Seoul, Korea). The cells were incubated in a 5% CO_2 humidified incubator at 37°C.

PROTEIN SAMPLE PREPARATION

Cultured cell pellets were washed twice with ice-cold PBS and homogenized directly by motor-driven homogenizer (PowerGen125, Fisher Scientific, Waltham, MA) in a sample lysis solution composed of 7 M urea, 2 M thiourea containing 4% (w/v) 3-[(3-cholamidopropy)dimethyammonio]-1-propanesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT), 2% (v/v) pharmalyte, and 1 mM benzamidine. Proteins were extracted for 1 h at room temperature with vortexing. After centrifugation at 15,000*g* for 1 h at 15°C, the insoluble material was discarded, and the soluble fraction was used for two-dimensional gel electrophoresis. The protein content of the final extracts was estimated using the bicinchonic acid (BCA) kit according to the manufacturer's protocol (Bio-Rad, Hercules, CA).

PREPARATION OF PHOSPHOPROTEIN ENRICHED EXTRACTS

To obtain phosphoprotein extracts, PhosPro™ Phosphoprotein enrichment kit (Genomine, Pohang, Korea) was used. In brief, the total protein solution prepared was diluted with a dilution solution (Genomine) to adjust to a final concentration of 0.67 mg/ml. To make a phosphoprotein-specific complex, 240 μl of solution A (Genomine) was added and rapidly and vigorously mixed by vortexing for a few seconds before incubation for 15 min with inverting or gentle vortexing. After subsequent addition of 360 μl of solution B (Genomine) and brief mixing, the sample solution was incubated for 15 min with gentle vortexing for the settling of the aggregated materials. After approximately 4 ml of upper clear solution was discarded, the remaining aggregate suspension was transferred to a new tube and centrifuged at 12,000 rpm for 5 min to allow precipitation of the phosphoprotein complex. The supernatant was discarded, and the aggregate in the hard pellet was dissolved in 0.7 ml dissolving solution (Genomine). Next, 750 μl of delipidation solution (methanol/chloroform, 600:150) was added with vortexing and was centrifuged at 12,000 rpm for 10 min. The middle phase protein disk was recovered without the lower or upper phase solution. Next, the protein disk was washed twice with sufficient (~1 ml) methanol. The protein pellet was completely dried in air or an oven and dissolved in a solution for isoelectric focusing (IEF).

ISOELECTRIC FOCUSING AND TWO-DIMENSIONAL ELECTROPHORESIS

Protein (200 μg) was diluted to a final volume of 80 μl in a sample buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 100 mM DTT, and 0.5% carrier ampholyte (pH 4–7, Bio-Rad) and loaded on a 17 cm long gel with a pH 4–7 gradient. A rehydrated, immobilized pH gradient immobiline polyacrylamide gel (IPG) strip

was positioned gel side down on the strip tray and covered with mineral oil. The voltage was sequentially increased from 150 to 3,500 V over 3 h to allow entry of the sample followed by maintenance at 3,500 V, with focusing complete after 96 kV-h. Prior to conducting the second dimension, strips were incubated for 10 min in equilibration buffer (50 mM Tris–Cl, pH 6.8 containing 6 M urea, 2% SDS, and 30% glycerol), first with 1% DTT and secondly with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS–PAGE gels (20 cm × 24 cm, 10–16%). SDS–PAGE was performed using a Hoefer DALT 2D system (Amersham Biosciences, Buckinghamshire, UK), following manufacturer's instruction. The gels were run at 20°C for 1,700 V-h. After two-dimensional electrophoresis (2-DE), the gels were stirred in fixing solution [40% (v/v) ethanol and 10% (v/v) acetic acid in distilled water] for 1 h and stirred in a rehydration solution [5% (v/v) ethanol and 5% (v/v) acetic acid in distilled water] three times for 30 min. The proteins were visualized by a ProQ diamond phosphoprotein gel stain (Invitrogen, Carlsbad, CA) of the gels for 1 h and washed with ProQ diamond phosphoprotein destaining solution (Invitrogen) for 30 min. The images were digitized using image photographing equipment and exposed to a Cy3 emission filter for 20 s and analyzed by PDQuest 2D analysis software (Bio-Rad).

2-DE GEL IMAGE ANALYSIS

Electrophoretically separated proteins were visualized by Coomassie brilliant blue G-250 staining of the gels. Enriched phosphorylated proteins could be detected by staining with the fluorescent dye ProQ Diamond. Quantitative differences were determined only when a matched spot displayed the same degree of down- or up-regulation in duplicate experiments. Matching spots in gels from the same sample were identified, and their intensities were measured using an Image Master 2D system. Analysis was performed on approximately 30 different phosphoprotein spots per sample. For each spot, the intensity value obtained in the low temperature was divided by that obtained in the control gel. The logs of these ratios (LR; the means and median values clustered around the 0 value) were calculated. LR was expected if errors associated with the analysis were random and normally distributed. Spots showing an expression fivefold fewer or greater than the control were considered to represent a statistically significant, differentially expressed protein species.

IN-GEL ENZYMATIC DIGESTION AND MASS SPECTROMETRY

Spots were excised from the stained gel, destained with 50 mM ammonium bicarbonate in 40% acetonitrile, and dried with a Speed Vac plus SC1 10 (Savant, Holbook, HY). The excised spot was rehydrated in 10 ng/μl trypsin in 50 mM ammonium bicarbonate. After the rehydrated spot was placed on ice for 45 min and treated with 50 mM ammonium bicarbonate (10 μl), it was incubated at 37°C for 12 h.

MATRIX-ASSISTED LASER-DESORPTION IONIZATION TIME-OF-FLIGHT TANDEM MASS SPECTROSCOPY (MALDI-TOF-MS/MS)

Digested samples were removed and subjected to a desalting/concentration step on an mZipTipC18 column (Millipore, Billerica, MA) using acetonitrile as an eluent before MALDI-TOF-MS/MS analysis. Peptide mixtures were loaded on the MALDI system using the dried-droplet technique and α-cyano-4-hydroxycinnamic acid (Sigma, Saint Louis, MS) as the matrix, and they were analyzed using a 4700 Reflector spec #1 mass spectrometer (Applied Biosystems, Framingham, MA). Internal mass calibration was performed using peptides derived from enzyme autoproteolysis. The Data Explorer software package (Applied Biosystems) was used to identify spots from the ProFound database by mass searching all sequences.

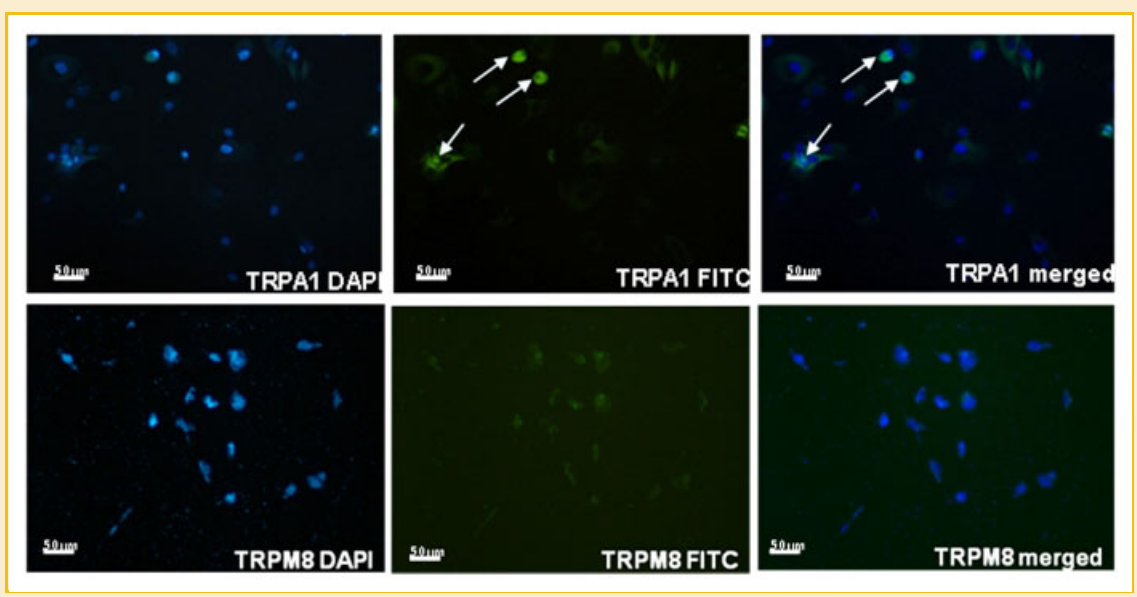

Fig. 1. Immunostaining of TRPM8 and TRPA1 in BEAS-2B cells. TRPM8 and TRPA1 proteins were detected by primary rabbit anti-TRPM8 or rabbit anti-TRPA1 antibody and FITC-conjugated anti-rabbit IgG (1:500). The cells were counterstained with DAPI. TRPM8 and TRPA1 proteins were observed on the cell bodies. The images were taken at a magnification of 400×.

Candidates identified by peptide mapping analysis were evaluated further by comparing their calculated masses and isoelectric points using the experimental values obtained by 2-DE.

IMMUNOCYTOCHEMISTRY FOR TRPM8 AND TRPA1

The cells were fixed with pre-chilled 1% paraformaldehyde for 1 min at room temperature and incubated with rabbit anti-TRPM8 or rabbit anti-TRPA1 antibody (diluted in 1% BSA, 1:100) (Abcam, Cambridge, UK) overnight at 4°C. After the primary antibody treatment, the cells were incubated with FITC-conjugated anti-rabbit IgG (dilute in 1% BSA, 1:500) (Santa Cruz Biotechnology, Delaware, CA) for 2 h at room temperature. Next, cells were counterstained using 4,6-diamino-2-phenylondole (DAPI, Sigma). The cover-slips were mounted on a slide glass, and the fluorescence

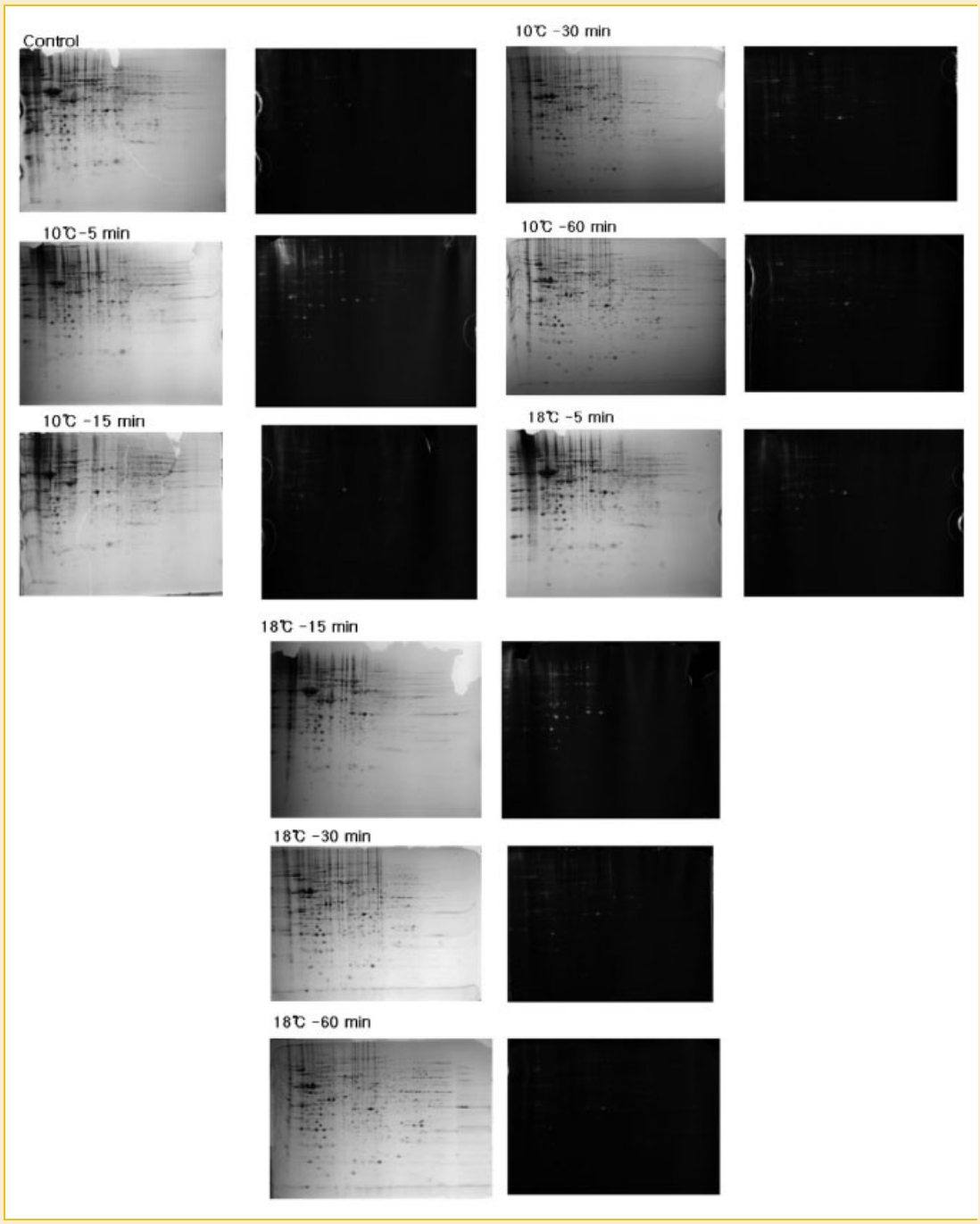

Fig. 2. BEAS-2B cell phosphoprotein patterns after treatment at 10 and 18°C by 2D gel electrophoresis. BEAS-2B cells were incubated at 10 or 18°C for 5, 15, 30, or 60 min; harvested; and lysed. Intracellular enriched phosphoproteins were analyzed by 2D gel electrophoresis, and gels were stained with Coomassie Brilliant Blue G-250 and washed with ProQ diamond phosphoprotein destaining solution. Right panel: Coomassie Brilliant Blue staining; left panel: ProQ diamond. Vertical axes represent apparent molecular mass (kDa), and horizontal axes represent pH values. Acquired images were repetitive. The data shown are representative of three separate experiments.

was detected using a Nikon fluorescence microscope (Nikon, Tokyo, Japan). The images were taken with a Nikon Eclipse TE2000-U camera (Nikon).

TRANSFECTION AND IMMUNOPRECIPITATION

Transient transfection was performed using Lipofectamine Plus reagent (Invitrogen). Briefly, 1×10^4 HEK293 cells distributed in the medium were dispensed into wells of a 96-well plate. After 24 h, cells were transfected with 0.10 μg of TRPM8 or TRPA1 pcDNA3 constructs. TRPM8 and TRPA1 pcDNA3 plasmids were provided by Dr. David Julius (University of California, San Francisco, CA). Cells were incubated for another 24 h, trypsinized, and the whole cell protein was obtained by lysing the cells on ice for 20 min in 700 μl of lysis buffer (0.05 M Tris–HCl, pH 7.4, 0.15 M NaCl, 1% Noniodet P-40 (NP-40), 0.5 M PMSF, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate). The lysates were sonicated for 20 s, centrifuged at 15,000*g* for 10 min, and the supernatant was saved. Protein concentration was determined using a BCA kit according to the manufacturer's protocol (Bio-Rad). Cell lysates (150 μg) were incubated with 2 μl primary antibody (anti-heterochromatin-associated proteins 1 antibody: Cell Signaling (Beverly, MA) or anti-ribosomal protein large P0 antibody: Santa Cruz Biotechnology) overnight at 4°C. Next, 15 μl Protein A/G Plus (Gendepot, Barker, TX) was added, and the complex was incubated for 4 h at 4°C. The pellet was washed three times with lysis buffer. The immunoprecipitated complexes were released with 2× sample buffer for Western blot analysis using a phospho-specific antibody.

WESTERN BLOT ANALYSIS

To determine the phosphorylation levels of heterochromatin-associated proteins 1 and ribosomal protein large P0, immunoprecipitated proteins were analyzed by 12% SDS–PAGE. The separated proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). After transfer, the membrane was saturated by incubation overnight at 4°C with 5% (w/v) nonfat dry milk in Tris-buffered saline (TBS)−0.1% Tween-20 (TBST), and it was incubated with anti-phospho serine/threonine/tyrosine antibody (Abcam) for 3 h. After washing with TBST, the membrane was incubated with an anti-rabbit immunoglobulin coupled with peroxidase (Abcam). After 60 min of incubation at room temperature, the membrane was washed three times with TBST, and the blots were developed using enhanced chemiluminescence (ECL1; Amersham Biosciences). Normalization was performed using polyclonal GAPDH antibody (Santa Cruz Biotechnology). Blots were quantified using a Gel Doc 2000 densitometer (Bio-Rad).

QUANTITATIVE ANALYSIS

Blots were quantified using a Gel Doc 2000 densitometer (Bio-Rad). In all experiments, statistical significance was calculated for the data from three or four independent experiments using one-way ANOVA or Student's *t*-test (Sigma Plot, LaJolla, CA). The error bars represent the standard deviations of the mean.

RESULTS

DISTRIBUTION OF TRPM8 AND TRPA1 RECEPTORS IN BEAS-2B CELLS

TRPM8 and TRPA1 receptors play a role in low temperature perception. To investigate the existence of TRPM8 and TRPA1, cultured BEAS-2B cells were stained with goat anti-TRPM8 or -TRPA1 antibody and FITC-conjugated IgG. DAPI was used to detect the cell nucleus. As shown in Figure 1, both TRPM8 and TRPA1 proteins were detected, and the distribution coincided with the cell body.

PHOSPHOPROTEIN ISOLATION AND TWO-DIMENSIONAL GEL SEPARATION

The resulting enriched phosphorylated protein was analyzed with two-dimensional gel electrophoresis (Fig. 2). To investigate the alterations associated with low temperature sensing on a molecular

TABLE I. List of Phosphorylated Spots/Proteins Sensitive to Temperature Treatment in BEAS-2B Cells Detected by Two-Dimensional Gel Electrophoresis and Identified by Peptide Mass Fingerprint Analysis

Spot between	Protein description	Measured Mr (kDa)/PI	MALDI-TOF coverage (%)
2110	Splicing factor, arginine/serine-rich 7 (SFRS7), splicing factor 9G8	27.6/11.9	40
3002	Proteasome 26S ATPase subunit4 isoform2	43.6/5.1	29
3006	Chain B, crystal structure of the human Y14 Magoh complex	12.2/4.3	49
3008	Heterochromatin-associated proteins 1 (HP1α)	22.3/5.9	31
3103	Splicing factor, arginine/serine-rich 1 (SFRS1)	22.6/7.9	51
3208	Chain A, solution structure of alpha subunit of human elf2	36/5.9	47
3205	Splicing factor, arginine/serine-rich 10 (SFRS 10), transformer-2-beta isoform 3	22/10.3	43
6106	Ribosomal protein, large, P0 (RPLP0)	34.4/5.4	49
7010	Splicing factor, arginine/serine-rich 3 (SFRS3)	19.5/11.8	36
2113	α-Tubulin	50.8/4.9	41
2202	hnRNP C (C1/C2)	32.4/5.9	34
2006	Proteasome (prosome, macropain) subunit, alpha type, 3	28.7/5.2	28
2101	Eukaryotic translation elongation factor 1 delta isoform 2	31.2/4.9	28
3409	Splicing factor SF3a60	59.1/5.3	37
5203	Calponin 3	36.6/5.7	28
5512	Ras-GAP SH3 domain-binding protein (G3BP)	52.2/5.4	25
6708	Matrin 3	95.1/5.9	28
7506	CTP synthase	67.4/6.0	33
8510	Progerin	69.5/6.2	29

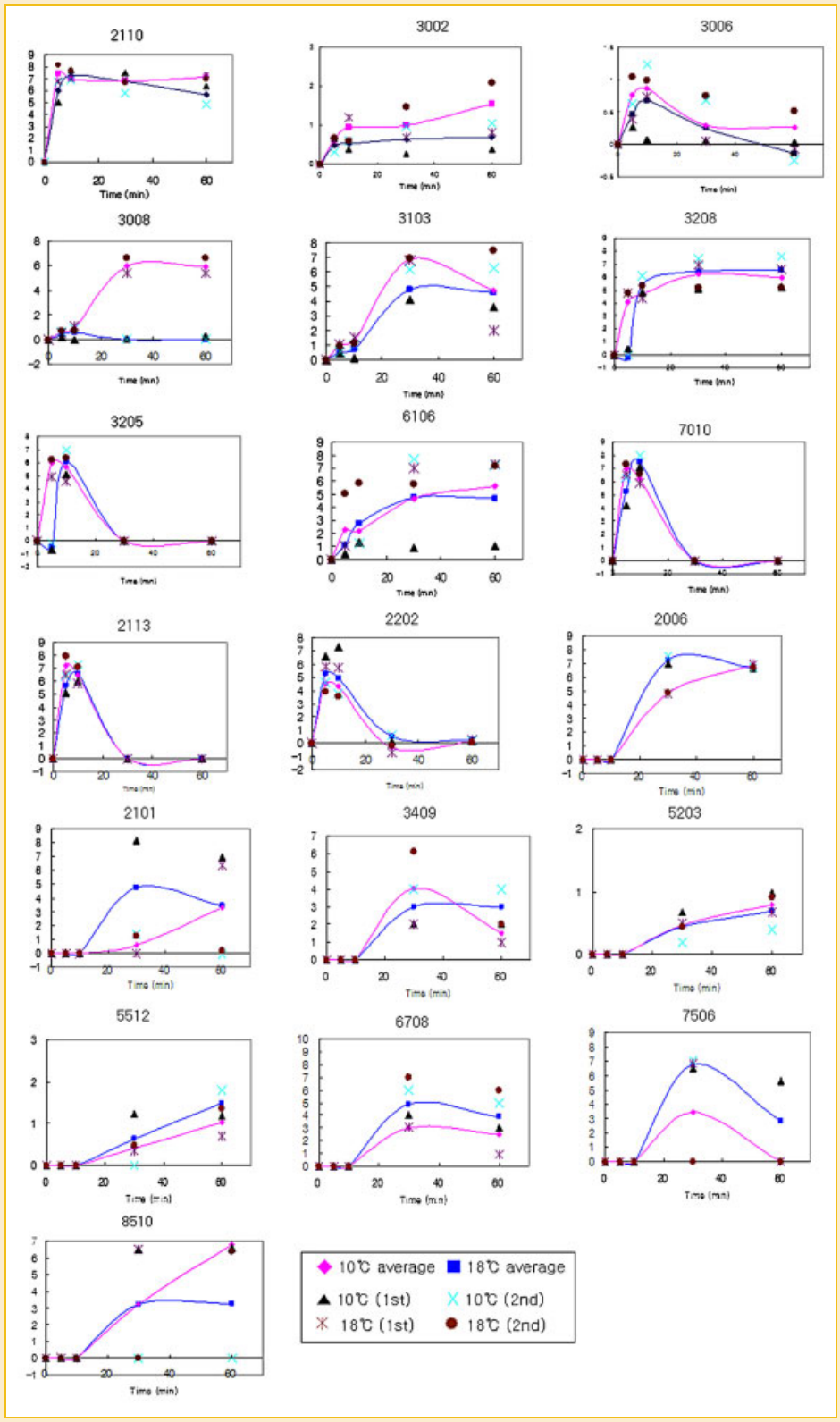

Fig. 3. Typical kinetics of proteins regulated in response to low temperature treatment of BEAS-2B cells. The kinetics of phosphorylation change over time is depicted for selected proteins (Table I). Each (▲) or (X) represents single values from each gel (10°C-treated). Each (●) or (✳) represents single values from each gel (18°C-treated). Lines connect the average standardized abundance values (pink, 10°C-treated; blue, 18°C-treated). The *Y*-axis represents the log ratio of treatment per control.

basis, phosphoproteome analysis for the proteins in the BEAS-2B cells was performed by 2D gel electrophoresis and MALDI-TOF MS/MS after treatment at 10 or 18°C for 5, 15, 30, or 60 min. The phosphoprotein spot stained with ProQ Diamond was analyzed. More than 20 phosphoprotein spots were visualized on the 2-DE gel, and the differences in spot intensities between control and cold-treated cells were compared and analyzed for each gel. Nineteen protein spots whose expression differed between control and cold treatment were apparent. These proteins were identified, as shown in Table I.

TIME-COURSE ANALYSIS OF PROTEIN PHOSPHORYLATION FROM LOW TEMPERATURE-RESPONSIVE BEAS-2B CELLS

Our experimental design was formalized with a time series, in which replicated proteomes for each time-point are technical replicates, with no special relationship between each other. We also used unequally spaced sampling intervals (5 min between the first two time-points, 30 min until the 1 h time-point) and two replicates at each time-point (Fig. 3). Some similarities in phosphorylation behavior existed for splicing factor arginine/serine-rich 7 (spot number 2110), proteasome 26S ATPase subunit4 isoform 2 (3002), and Eif 2 chain A (3208). The line shows the trend estimation of the data related to time points, exhibiting an increase until 10 min followed by a saturated trend, which can be statistically distinguished from random behavior.

The second group [chain B: crystal structure of the human Y14 Magoh complex (3006), heterochromatin-associated proteins 1 (HP1α) (3008−10°C), transformer-2-beta isoform 3 (3205), splicing factor arginine/serine-rich 3 (7010), α-tubulin (2113), and hnRNP C (C1/C2) (2002)] increased in phosphorylation between 5 and 10 min and decreased back to their minimum levels between 30 and 60 min of treatment.

For HP1α (3008−18°C), SFRS1 protein (3103), and ribosomal protein large P0 (RPLP0, 6106), the phosphorylation level continued to increase and reached their maximum level between 30 and 60 min .

Finally, there is a group of proteins whose phosphorylation was up-regulated between 2- and 1,000-fold of control after 30 min of exposure: proteasome subunit alpha type 3 (2006), eukaryotic translation elongation factor 1 delta isoform 2 (2101), splicing factor SF3a60 (3409), calponin 3 (5203), Ras-GAP SH3 domain-binding protein (G3BP, 5512), matrin 3 (6708), CTP synthase (7506), matrin 3 (6708), and progerin (8510).

Most of the proteins that were phosphorylated after 5 or 10 min returned to control levels after 30 or 60 min. Physiological functions of these proteins can be associated with RNA binding and splicing.

TIME PROFILING USING IMMUNOPRECIPITATON

To confirm the phosphorylation profile changes induced by low temperature, HEK293 cells were transfected with the TRPM8 or TRPA1 receptor gene and incubated at low temperature for various lengths of time. Next, the proteins were immunoprecipitated using anti-HP1α or anti-RPLP0 antibodies and detected with anti-

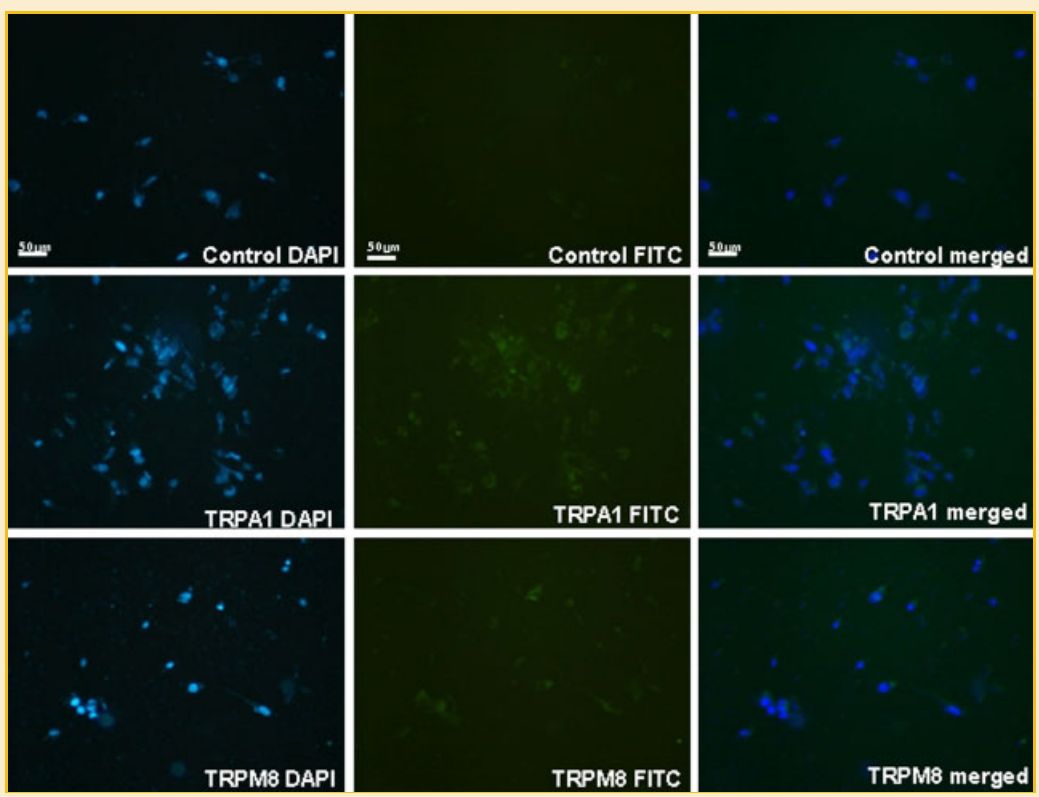

Fig. 4. Immunostaining of TRPM8 and TRPA1 in HEK293 cells transfected with TRPM8 and TRPA1 genes. HEK293 cells were transfected with TRPM8 or TRPA1 pcDNA3 constructs. TRPM8 and TRPA1 proteins were detected by primary rabbit anti-TRPM8 or rabbit anti-TRPA1 antibody and FITC-conjugated anti-rabbit IgG (1:500). The cells were counterstained using DAPI. TRPM8 and TRPA1 proteins were observed on the cell bodies. The images were taken at a magnification of 400×.

phospho serine/threonine/tyrosine antibody. As shown in Figure 4, the expression of TRPM8 and TRPA1 in HEK293 cells was observed. The phosphorylation of HP1α protein of TRPA1-transfected cells increased from 2 min after the treatment at 10°C until 10 min, and it was restored quickly, as shown in Figure 5. Phosphorylation of the HP1α protein of TRPM8-transfected cells increased from 2 min after treatment at 18°C. This behavior was consistent with the proteome profiling results. The phosphorylation of RPLP0 of TRPA1-transfected cells increased from 15 min after treatment at 10°C and increased from 2 min after treatment at 18°C. This behavior also was consistent with the proteome profiling results.

DISCUSSION

We presented the analysis performed on a time series of phosphoproteome data from the bronchial epithelial cell line BEAS-2B treated at 10 and 18°C. This study provides the first quantitative investigation of the thermo-sensitive phosphoproteome and its dynamic changes in response to low temperature. The time-dependent phosphorylation is rhythmic, and maximum values are observed within 5 or 10 min at 18 or at 10°C. Moreover, the peak (at the time of half increase) is phase advanced at 18°C compared to 10°C (proteasome 26S ATPase subunit4 isoform2, 2.8 ± 1.01 min; chain B crystal structure of the human Y14 Magoh complex, 3.8 ± 1.69 min; SFRS1 protein, 6.8 ± 2.11 min; chain A, solution structure of alpha subunit of human Eif2, 3.1 ± 1.15 min; transformer-2-beta isoform 3, 3.4 ± 1.33 min; RPLP0, 2.7 ± 1.05; α-tubulin, 2.6 ± 1.14). The duration of the peak obtained at 18°C is not different from that obtained at 10°C (30 min two-way ANOVA, $P > 0.05$). Taken together, these results permit us to conclude (1) that the TRPA1- and TRPM8-mediated intracellular signaling shows the same time profile pattern which is tightly regulated by the duration, and (2) that the phase delay (peak time shift) of phosphorylation is observed between temperatures. Here, we suggest a novel concept, the phosphorylation capacity of cells, which indicates the amount of

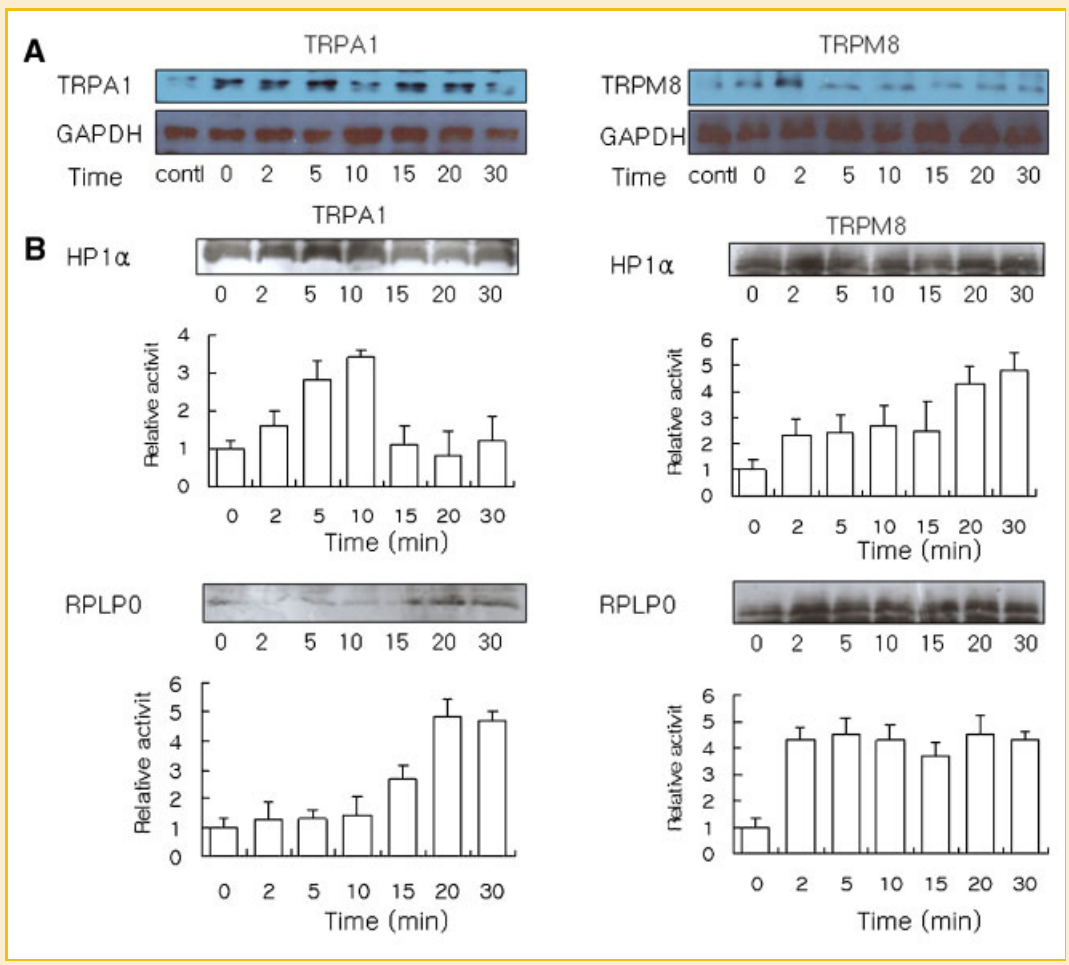

Fig. 5. A: Western blot of TRPM8 and TRPA1 in HEK293 cells transfected with TRPM8 and TRPA1 genes. HEK293 cells were transfected with TRPM8 or TRPA1 pcDNA3 constructs. Cells were incubated for the indicated times at 10°C (TRPA1-transfected cells) or 18°C(TRPM8-transfected cells), and whole cell protein was obtained by lysing the cells. TRPM8 and TRPA1 proteins were detected by Western blot analysis (Contl: extract of cells without transfection). B: Immunoprecipitation of HP1α and RPLP0 in HEK293 cells transfected with TRPM8 and TRPA1. Cell lysates were incubated with 2 μl primary antibody overnight at 4°C. Next, 15 μl Protein A/G Plus was added, and the complex was incubated for 4 h at 4°C. The pellet was washed three times with lysis buffer. The immunoprecipitated complexes were released and applied to Western blot analysis using phospho-specific antibody.

phosphorylation change induced by temperature, and it appears to act in a manner similar to that of heat capacity. These data suggest that temperature capacity in biological systems can be translated to phosphorylation capacity.

RNA-binding proteins, such as arginine/serine rich splicing factors and ribosomal protein, were transiently phosphorylated in BEAS-2B cells in response to cold temperature stimulus. The SR protein family is comprised of a number of phylogenetically conserved and structurally related proteins with a characteristic domain rich in arginine and serine residues known as the RS domain. They play significant roles in constitutive pre-mRNA splicing and also are important regulators of alternative splicing. In addition, they participate in post-splicing activities, such as mRNA nuclear export, nonsense-mediated mRNA decay, and mRNA translation. These wide-ranging roles of SR proteins highlight their importance as pivotal regulators of mRNA metabolism [Long and Caceres, 2009].

A dynamic cycle of phosphorylation and dephosphorylation is required for pre-mRNA splicing [Mermoud et al., 1994]; this cycle is related, at least in part, to the phosphorylation status of SR proteins. Several protein kinase families have been shown to phosphorylate the RS domain of SR proteins including the SR protein kinase (SRPK) family [Gui et al., 1994; Wang et al., 1998; Ngo et al., 2008], the Clk/Sty family of dual specificity kinases [Colwill et al., 1996], and topoisomerase I [Rossi et al., 1996]. RS domain dephosphorylation also plays an important role in sorting SR proteins in the nucleus, where shuttling SR proteins and nonshuttling SR proteins are recycled via different pathways [Lin et al., 2005]. Importantly, alternative splicing is extensively regulated by signal transduction pathways, whereby signaling cascades can link the splicing machinery to the exterior environment [Lynch, 2007]. For instance, the SR protein SRp38 is dephosphorylated upon heat shock by the phosphatase PP1 and becomes a potent splicing repressor [Shin et al., 2004; Shi and Manley, 2007]. Arginine/serine rich splicing factor 7 (also known as splicing factor 9G8) promotes the nucleoplasmic export of mRNA [Huang and Steitz, 2001]. Magoh are associated with mRNA in the same position after export to the cytoplasm, and they require translation of the mRNA for removal [Lau et al., 2003]. The heterogeneous nuclear RNP (hnRNP) family is comprised of a structurally diverse group of RNA-binding proteins with roles in many aspects of RNA biogenesis including pre-mRNA splicing [Martinez-Contreras et al., 2007]. Among the proteins that were differentially phosphorylated by cold temperature, splicing factor arginine/serine-rich proteins (SFRS1, 3, 7, 10), splicing factor SF3a60, hnRNP, Magoh, and RPLP0 are RNA splicing or RNA-binding proteins. Altogether, low temperature stimulation from the exterior environment seems to be linked to the splicing machinery through phosphorylation signaling. Among the proteins that were differentially phosphorylated by cold temperature, calponin, matrin 3, progerin, and tubulin are known as scaffold proteins. The F-actin-binding protein calponin functions as a scaffold [Appel and Margan, 2010]. Matrin 3, an abundant protein of the internal nuclear matrix has been associated with components of the nuclear matrix [Tubo et al., 1987]. Progerin, the mutant of prelamin A is a structural component of the nucleoskeleton [Fong et al., 2009].

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