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Phosphoproteomic Analysis of AML14.3D10 Cell Line as a Model System of Eosinophilia

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Eosinophils act as effectors in the inflammatory reactions of allergic diseases including atopic dermatitis. Atopic dermatitis patients and others with allergic disorders suffer from eosinophilia, an accumulation of eosinophils due to increased survival or decreased apoptosis of eosinophils. In this study, a differential phosphoproteome analysis of AML14.3D10 eosinophil cell line after treatment with IL-5 or dexamethasone was conducted in an effort to identify the phosphoproteins involved in the proliferation or apoptosis of eosinophils. Proteins were separated by 2-DE and alterations in phosphoproteins were then detected by Pro-Q Diamond staining. The significant quantitative changes were shown in nineteen phosphoproteins including retinoblastoma binding protein 7, MTHSP75, and lymphocyte cytosolic protein 1. In addition, seven phosphoproteins including galactokinase I, and proapolipoprotein, were appeared after treatment with IL-5 or dexamethasone. Especially, the phospho-APOE protein was down-regulated in IL-5 treated AML14.3D10, while the more heavily phosphorylated APOE form was induced after dexamethasone treatment. These phosphoproteome data for the AML14.3D10 cell line may provide clues to understand the mechanism of eosinophilia as well as allergic disorders including atopic dermatitis.

Keywords: AML14.3D10, Atopic dermatitis, Eosinophilia, Phosphoproteome

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease that affects children (up to 20%) and adult (1-3%) worldwide. The manifestations of AD result from a complex interplay of susceptibility genes, environmental factors, pharmacological abnormalities, skin barrier defects, and immunological phenomena (Abramovits, 2005). AD skin lesions are characterized by increased IgE-bearing Langerhans cells, inflammatory dendritic cells, macrophages, eosinophils, and activated T lymphocytes. In particular, eosinophil numbers as well as eosinophil granule protein levels in peripheral blood are elevated in most AD patients and appear to correlate with disease activity (Trautmann *et al*., 2001; Simon *et al*., 2004). In addition to the increased production of eosinophils, inhibition of eosinophil apoptosis by IL-5 appears to play an important role at sites of allergic inflammation (Trautmann *et al*., 2000; Domae *et al*., 2003). In practice, purified blood eosinophils from AD patients that were cultured *ex vivo* had a reduced death kinetics compared with normal eosinophils. However, expression patterns of proteins associated with AD in eosinophils are not fully clarified (Kilty *et al*., 1999; Ogawa *et al*., 2004; Yoon *et al*., 2005; An *et al*., 2007).

Proteomic analysis involves using a combination of techniques, including two-dimensional electrophoresis (2-DE), image analysis, and mass spectrometry (MS). Proteins in a complex sample mixture can be separated by 2-DE to produce a high resolution 2-DE map in which stained proteins are visualized as spots of various sizes and intensities depending on the amount of protein in the sample. A computer-assisted comparison of the spot patterns in different 2-DE gels is then performed to assess changes in protein levels that are related to the disease or the process of interest. Differentially expressed protein spots selected and isolated from the 2-DE proteome maps can then be identified by mass spectrometry analysis. The proteomic approaches coupled with genomics have been applied to analyze many diseases and to probe for therapeutics (Park *et al*., 2004; Yoon *et al*.,

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2005; Park *et al*., 2006). Among various post-translational modifications of proteins, phosphorylation is involved directly or indirectly in a variety of important cellular events (Raggiaschi *et al*., 2005; Kang *et al.*, 2007). Thus, analysis of the entire phosphorylated proteins in a cell, so-called phosphoproteome, is an attractive subject.

The AML14.3D10 human myeloid leukemic cell line has been used as a standard cell line model for the study of eosinophil (Baumann and Paul, 1998). In this study, a phosphoproteomic analysis of the human eosinophil cell line AML14.3D10 was performed in an effort to identify the proteins involved in the survival or death of an eosinophil. Using phosphoprotein-specific dye after 2-DE of AML14.3D10 cell lysates treated with IL-5 (as a survival factor) or dexamethason (as a pro-apoptotic agent), several candidate phosphoproteins that are potentially involved in the survival or the death of eosinophils were identified.

Materials and Methods

Cell Lines, cell culture, and reagents. AML 14.3D10 was maintained in RPMI 1640 medium containing 10% v/v FBS (Gibco) and 1% v/v Antibiotic-Antimycotic (Gibco). It was then incubated for 24 h. For the experiments, cells were incubated for 24 h in fresh medium, followed by treatment with IL-5 (50 ng/ml) or 10 μM dexamethasone for 24 h at 37°C in a 5% CO_2 incubator.

IL-5 or dexamethasone treatment. Approximately 1×10^6 AML14.3D10 cells were resuspended in 20 ml of fresh complete medium and incubated for 24 h. They were then stimulated with IL-5 (final 50 ng/ml) or dexamethasone (final 10 μM) for 24 h at 37°C in a 5% CO_2 incubator. Following this, cells were collected by immediate centrifugation.

Sample preparation for 2-DE. AML14.3D10 cells were washed with PBS supplemented with 2 mM sodium orthovanadate (Na_3VO_4). The supernatant was removed and then resuspended with PBS. After being transferred into a new tube, it was centrifuged at 2,000 rpm for 5 min and the supernatant was removed. Following this, the sample was resuspended with 200 μl lysis buffer and sonicated two times followed by centrifuging at 13,000 *g* for 20 min at 4°C. The supernatant was then removed. The protein concentration was determined by a Bradford assay. To precipitate the protein, four volumes of 100% acetone were added to the protein. It was stored at −20°C for 2 h and then centrifuged at 13,000 *g* for 30 min at 4°C, and the supernatant was removed. To remove the remaining salts, the pellet was washed for 10 min with 80% acetone and centrifuged at 13,000 *g* for 10 min. It was then vacuum-dried and stored at −80°C. The resulting pellet was resuspended in a rehydration buffer [7 M urea, 4% w/v CHAPS, 0.5% w/v IPG buffer (pH 4-7 NL), 2% w/v DTT and a trace of bromophenol blue].

2-DE. IEF was performed with an IPGphor™ unit (Amersham Biosciences, Uppsala, Sweden) using precast 18 cm pH 4-7 nonlinear IPG gel strips (Amersham Biosciences). Equal amounts (1 mg) of total proteins were mixed with rehydration solution. IEF was then performed at 300 V for 1 h, 3,500 V for 1.5 h and 3,500 V for 18 h. The current was limited to 20 mA per gel strip. After IEF separation, gel strips were equilibrated for 20 min in a reducing buffer of 65 mM DTT, 6 M urea, 2% SDS, and 30% glycerol in 1.5 M Tris-HCl (pH 8.8), followed by another 20 min in an alkylating buffer of 135 mM iodoacetamide, 6 M urea, 2% SDS, and 30% glycerol in 1.5 M Tris-HCl (pH 8.8). The equilibrated strips were placed onto a SDS-PAGE apparatus and sealed with 0.5% agarose in a Laemmli running buffer. Discontinuous SDS-PAGE was performed in a Protean 2D slab plate (Bio-Rad) using 12% polyacrylamide gels. Electrophoresis was performed at 16°C at a maximum current of 20 mA per gel.

Pro-Q staining. Fluorescent staining of 2-DE gels using Pro-Q Diamond phosphoprotein gel stain kit (Molecular Probes, Eugene, OR) was performed according to the manufacturer's guidelines. Briefly, the gels were fixed in 50% methanol, 10% acetic acid overnight, washed with three changes of deionized water for 15 min per wash, followed by incubation in Pro-Q Diamond phosphoprotein gel stain for 180 min. They were then destained with three changes of 20% acetonitrile in 5 mM sodium acetate (pH 4.0) for 1 h. This was done three times. Useful images could be obtained at 3 h after staining, employing three successive destaining trials. Images were acquired using a Typhoon™ 9400 imager (Amersham Biosciences) with an excitation of 532 nm and an emission of 580 nm to determine the phosphostain reacting proteins.

Image analysis. Gels were analyzed using Phoretix™ 2D Expression software (Nonlinear Dynamics) after being scanned using a transmissive flatbed scanner (UMAX) calibrated at an AGFA 25 × 125 mm scale. The protein spot volume was evaluated based on the lowest boundary mode of background selection. The spot volumes were normalized based on the total spot volume for each gel. Protein spot intensity was defined as the normalized spot volume which is the ratio of the single spot volume to the total spots volumes on a 2-DE gel. The computer analysis allowed automatic detection and quantification of protein spots, as well as matching between control gels and gels from treated samples. The significance of differences of protein spots were evaluated by student's t-test, with $p < 0.05$ taken as indicating significance.

Protein identification. Spots were excised as 1-2 mm^2 slices, destained with freshly prepared 100 mM ammonium bicarbonate (ABC)/100% acetonitrile (ACN) followed by 100% ACN for 5 min two times and dried in a Speed Vac Plus SC110A (Savant, Holbrook) vacuum concentrator. The dried gel pieces were rehydrated with 20 ml of 10 ng/μl trypsin solution, and digestion was allowed to continue at 37°C for 16 h. The in-gel tryptic peptides were extracted with 70% ACN and 0.1% trifluoroacetic acid (TFA) for 30 min three times. The extracted solutions were mixed and then dried in a vacuum concentrator. The resulting peptides were solubilized with 0.1% v/v TFA for MS analysis. Mass spectra were acquired via Axima CFR+ matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS, Shimadzu Biotech, Japan). The obtained mass spectra were analyzed using a MASCOT PMF search (http://www.matrixscience.com). For protein identification, we performed database searching against

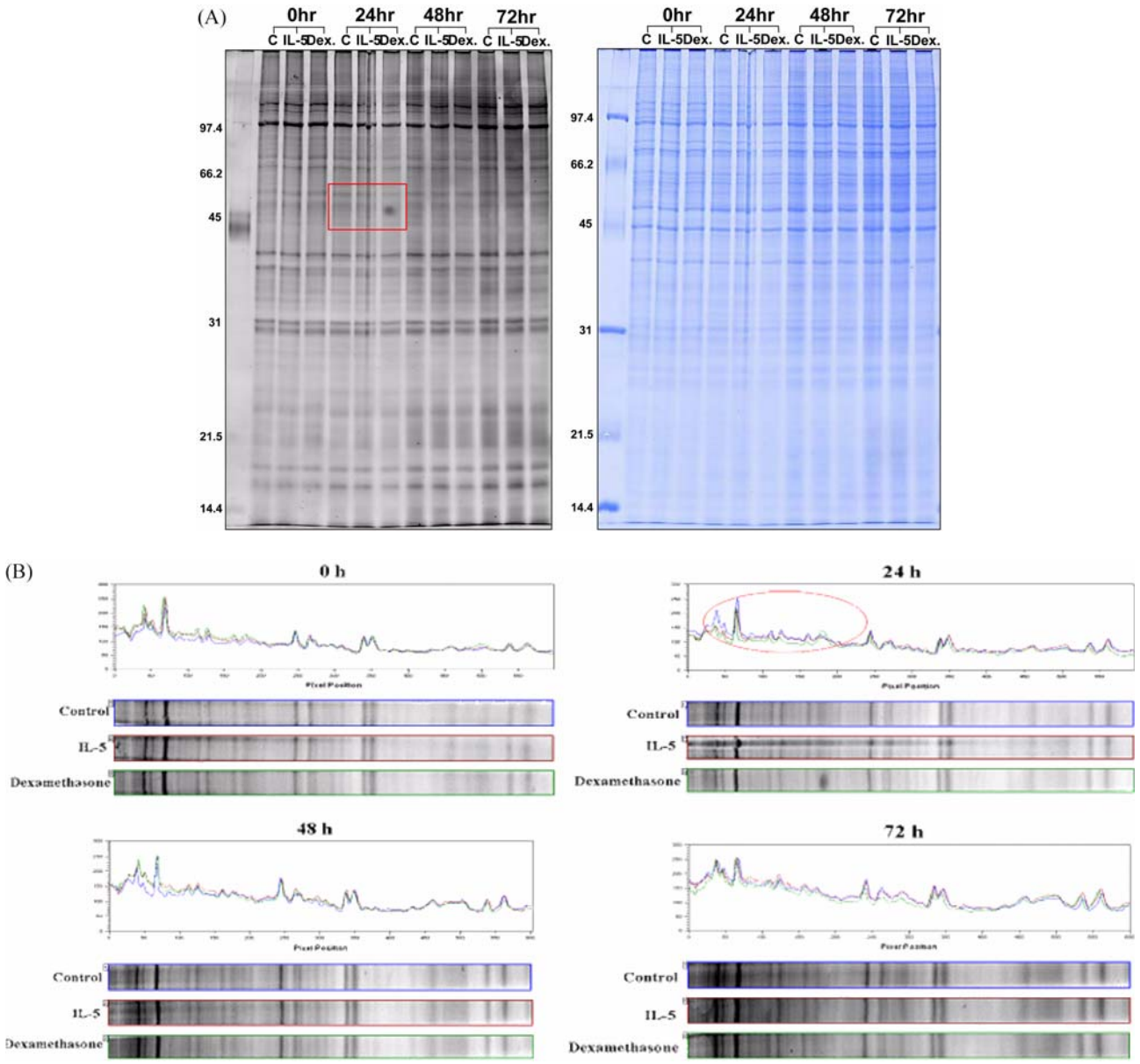

Fig. 1. Screening of the total phosphoproteins expression pattern after treatment with IL-5 or dexamethasone. (A) Time-course 1-DE images of phosphoproteins of eosinophils after treatment with IL-5 or dexamethasone. The total proteins were separated by 12% SDS-PADE, and then stained with Pro-Q Diamond (left panel) and Coomassie Brilliant Blue (right panel). (B) Densitometric analysis of the expression pattern of the phosphoproteins. The regions showing slightly different expression pattern were indicated (by either box or circle).

the NCBInr database using the MASCOT program and the p*I* acquired from our image analysis. We used a protein molecular mass range of 6-200 kDa and a mass tolerance of 100 ppm for the internal calibration. The proteins that failed by peptide mass finger printing were tried again by LC-MS/MS (Q-TOF Premier, Micromass).

Results and Discussion

Screening of total phosphoprotein expression pattern. To establish suitable conditions for the phosphoproteome change, the changes in the phosphoprotein expression pattern in AML14.3D10 eosinophil cell line induced by treatment with IL-5 or dexamethasone were investigated using 1-DE (Fig. 1A). The total phosphoproteins between the untreated control AML cell line and the IL-5 or dexamethasone treated AML cell line showed a similar expression pattern. Compared with the control, however, slight differences in the phosphoprotein expression pattern after the treatment with IL-5 or dexamethasone were detected at 24 h (Fig. 1B). Therefore, a proteome analysis of phosphoproteins treated with IL-5 or dexamethasone for 24 h was carried out.

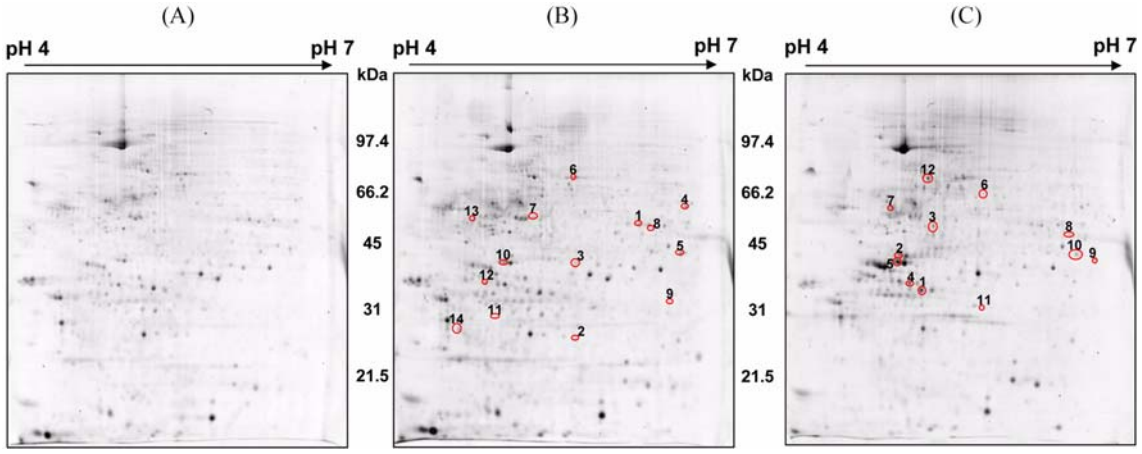

Fig. 2. Representative 2-DE gel images of IL-5 or dexamethasone treated AML14.3D10. The circles mark spots that show significant changes in expression levels. The indicated spot numbers in Table 1 and 2 correspond to the spots shown in Fig. 2. 2-DE was performed with 1 mg of AML14.3D10 cell extract proteins after treatment with IL-5 (B) or dexamethasone (C), using pH 4-7NL immobilized pH gradients for IEF and 12% gels for SDS-PAGE.

Phosphoproteome analysis of the AML14.3D10 cell line after the IL-5 treatment. The proteins of AML14.3D10 cells after treatment with IL-5 or dexamethasone were separated by 2-DE. Alterations in phosphoproteins were then detected by Pro-Q Diamond staining. Among approximately 1,000 protein spots stained by coomassie brilliant blue, approximately 244, 247, and 223 different phosphoprotein spots were detected in the untreated control, IL-5 treated, and dexamethasone treated samples, respectively. Protein spot changes greater than 1.5 fold in magnitude in three or more replicated gels were selected for protein identification. In the gels that were treated with IL-5 for 24 h, a total of 14 reproducible protein changes were observed (Fig. 2 and 3, Table 1). Compared with the control, six phosphoproteins were up-regulated, five phosphoproteins were down-regulated, and three phosphoproteins were appeared when treated with IL-5.

A eukaryotic translation elongation factor 1γ (eEF1γ) was appeared in the IL-5 treated sample. The Ser-386 of eEF1γ is known to be phosphorylated. This site was identified at Cell Signaling Technology (CST) using PhosphoScan®, CST's MS/MS platform for phosphorylation site discovery. Sites were variously discovered in leukemia, lymphoma and carcinoma model cell lines and tumors. Additionally, it has been reported that eEF1γ was phosphorylated by cdc2 kinase during meiotic maturation of *Xenopus* oocytes (Belle *et al*., 1990). However, it is unclear whether phospho-eEF1γ is involved in atopic dermatitis.

The phospho-mortalin (p-MTHSP75) spot was decreased after the IL-5 treatment. Mortalin is a chaperone protein associated with cell survival, stress response, intracellular trafficking, control of cell proliferation, mitochondrial biogenesis, and cell fate determination (Wadhwa *et al*., 2002). Mortalin is not induced by heat and is associated with cell aging and immortalization. It was known that mortalin is phosphorylated in a Ca^{2+}-dependent manner. However, the exact role of phosphorylation in the cell is unclear (Osorio *et al*., 2006). Among the proteins interacting with mortalin, AIF and Apaf-1 have critical roles in apoptosis. Therefore, the phosphorylation of mortalin may be involved in proliferation/apoptosis of eosinophil.

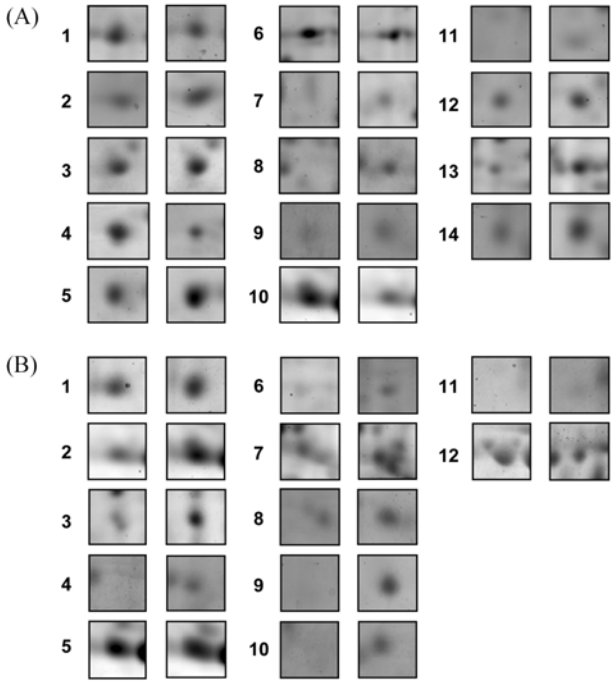

Fig. 3. Enlarged areas of silver stained 2DE gels of extracts from control and AML14.3D10 cells treated with IL-5 (A) or dexamethasone (B). 2-D gel images were analyzed with the phoretix™ 2D Expression software (Nonlinear Dynamics). The indicated spot numbers correspond to the spots shown in Fig. 2.

The phosphatidylinositol transfer protein (PITP) family is a

Table 1. Fourteen differentially expressed proteins in IL-5 treated AML14.3D10 cell line

Spot No.	ID	Seq. cov. (%)	Matched peptides	PI	Mass (kDa)	Expectation value	Mascot (score)	NCBI	Fold change†
1	angiotensinogen	5	2	5.78	53407	0.00063	81	gi|532198	-2.1
2	Chain C, Crystal Structure Of Lipid-Free Human Apolipoprotein A-I	72	24	5.27	28061	1.2E-98	998	gi|90108666	-1.9
3	apolipoprotein E	41	13	5.81	36242	2.0E-66	676	gi|178853	-2.0
4	hemopexin precursor	4	2	6.57	52254	9.9E-06	99	gi|386789	+2.9
5	translation initiation factor eIF3 p40 subunit; eIF3p40	13	4	6.38	40075	9.9E-25	259	gi|3986482	+1.9
6	MTHSP75	29	21	5.97	74019	3.9E-114	1152	gi|292059	-1.9
7	unnamed protein product	18	8	5.63	48311	3.9E-22	232	gi|22761285	Induction
8	Eukaryotic translation elongation factor 1γ	6	3	6.25	50457	9.9E-04	79	gi|15530265	Induction
9	PITPNB protein [Homo sapiens]	41	11	5.89	31903	3.1E-37	384	gi|21594294	+1.6
10	suppressor of G2 allele of SKP1	17	5	5.11	38066	2.5E-13	145	gi|5730041	-2.2
11	Proapolipoprotein	8	2	5.45	28944	0.005	73	gi|178775	Induction
12	RNA polymerase subunit	40	8	4.74	31728	6.3E-38	390	gi|2920711	+1.7
13	hypothetical protein LOC84306 [Homo sapiens]	5	2	4.71	40304	0.0013	79	gi|14150141	+1.9
14	Chain B, 14-3-3 Protein Theta (Human) Complexed To Peptide	28	11	5.17	29408	7.9E-45	460	gi|71042777	+2.2

†The values are the means from three independent determinations.

family of transfer proteins that can bind and exchange one molecule of phosphatidylinositol (PI) or phosphatidylcholine (PC) and facilitate the transfer of these lipids between different membrane compartments. In mammals, three soluble isoforms of PITP (a, b and RdgBb) have been identified. PITPNB was originally purified as a sphingomyelin (SM) transfer protein using an assay that monitored the transfer of pyrenylacyl-labelled SM from donor to acceptor vesicles (Westermann *et al*., 1995). The ability to transfer SM was unique to PITPB. The Ser-261 of PITPNB is constitutively phosphorylated but has no effect on phospholipids transfer activity (Tiel *et al*., 2002). Thus far, the involvement of PITPNB in atopic dermatitis has not been reported. However, the data on PITPNB up-regulation after treatment with IL-5 suggest the possibility of an interconnection between regulation of SM metabolism and atopic dermatitis by phosphorylation of PITPNB.

Phosphoproteome analysis of the AML14.3D10 cell line after a dexamethasone treatment. When dexamethasone was treated as a pro-apoptotic agent, a total of 223 phosphoprotein spots were detected. Spots showing significant changed expression levels after treatment with dexamethasone compared with those from the control were are shown in Fig. 2. Among them, seven phosphoproteins were up-regulated, one phosphoprotein spot was down-regulated and four phosphoproteins were appeared (Fig. 3). The identified proteins showing differentially expressed patterns are listed in Table 2.

Lymphocyte cytosolic protein 1 (L-plastin), a malignant transformation-associated protein, is a member of family of actin filament cross-linkers. Fig. 4A showed matched peptide fragments and a representative MS/MS spectrum of L-plastin. In addition, MASCOT search results from MS/MS data suggest the possibility that Ser-5 of L-plastin might be phosphorylated although statistical significance is not achieved (since the ion score is low) (Fig. 4B). Recently, Friederich group reported the phosphorylation of Ser-5 in L-plastin (Janji *et al*., 2006). Ser-5 phosphorylation promotes its targeting to the actin cytoskeleton and increases its filament bundling activity in cells and *in vitro* (Wang and Brown, 1999). Phospho-L-plastin was decreased when dexamethasone was treated. However, it is unclear how phosphorylation of L-plastin is involved in atopic dermatitis.

Phospho-apolipoprotein E (p-APOE) was induced by dexamethasone, whereas decreased by IL-5 treatment. Thus, p-APOE can be used as an indicator for proliferation or apoptosis of eosinophils. APOE is known to mediate the

Table 2. Twelve differentially expressed proteins in dexamethasone treated AML14.3D10 cell line

Spot No.	ID	Seq. cov. (%)	Matched peptides	PI	Mass (kDa)	Expectation value	Mascot (score)	NCBI	Fold change†
1	Alternative splicing factor (ASF)	36	11	5.61	32321	0.0063	72	gi|179073	+2.9
2	hypothetical protein LOC345651	8	4	5.39	42318	7.9E-09	132	gi|63055057	+3.1
3	placental protein 17b1; PP17b1	22	9	5.37	47350	3.9E-49	502	gi|4206374	+2.8
4	apolipoprotein E	24	7	5.65	36302	6.3E-42	430	gi|178849	Induction
5	WD-40 repeat protein	19	5	4.93	38814	1.5E-18	196	gi|4519417	+1.7
6	unnamed protein product	17	15	5.17	59720	3.9E-53	543	gi|28317	+2.4
7	retinoblastoma binding protein 7	13	9	4.89	48132	6.2E-24	250	gi|4506439	+1.8
8	26S proteasome subunit 9	14	5	6.08	47646	1.5E-05	98	gi|2150046	+2.5
9	Mitochondrial ribosomal protein s22	33	12	7.7	41425	3.1E-38	393	gi|14424546	Induction
10	galactokinase 1	17	7	6.04	42702	3.9E-22	232	gi|4503895	Induction
11	60S acidic ribosomal protein PO [Homo sapiens]	41	13	7.68	27602	9.9E-34	349	gi|5815233	Induction
12	lymphocyte cytosolic protein 1 (L-plastin) [Homo sapiens]	57	38	5.29	70814	6.3E-154	1522	gi|8217500	-2.1

†The values are the means from three independent determinations.

binding, internalization, and catabolism of lipoprotein particles. There are three common APOE alleles of the APOE gene. The three APOE alleles differentially affect an individual's risk for developing Alzheimer's disease (Hoe *et al*., 2005; Raftery *et al*., 2005). Processes mediated by the APOE isoforms that may influence the progression of Alzheimer's disease include cholesterol transport and synapse formation, modification of neurite outgrowth and synaptic plasticity, destabilization of microtubules and β-amyloid clearance (Nathan *et al*., 2002; Beffert *et al*., 2004). Roles for APOE in repairing damage and promoting cell survival are also suggested by APOE up-regulation in conjunction with glial activation in neuronal injuries (Poirier *et al*., 1991). Recently, it was reported that there is nuclear and cytosolic pools of APOE in the human fibroblast. In particular, nuclear APOE protein levels were up-regulated during apoptosis. In addition, through *in vivo* studies using radio-labeling and immunoprecipitation, it was revealed that APOE could be phosphorylated (Quinn *et al*., 2004). In the present study, the induced APOE spot was detected at a more acidic position compared to that corresponding to its p*I* value on the 2-DE gel when dexamethasone was treated. This result suggests the possibility that a hyperphosphorylated nuclear APOE form is induced in response to apoptotic stimuli. Thus far, it is unclear how APOE affects atopic dermatitis. From the data in this study it is possible to postulate that phosphorylation of APOE may be involved in the survival or death of eosinophils and furthermore in the progression of atopic dermatitis.

Galactokinase is a major enzyme for galactose metabolism. Phosphogalactokinase I was appeared after the treatment with dexamethasone. Galactokinase was known to be up-regulated by hypoxia-inducible factor I (HIF-1) under hypoxia condition (Greijer *et al*., 2005). There is no report about involvement of galactokinase with atopic dermatitis, yet survival of cells under apoptotic conditions seems to be increased by up-regulation of pathways leading to more energy. Therefore, galactokinase I is up-regulated by dexamethasone treatment to increase cellular energy level. However, it is unclear how phosphorylation of galactokinae I has influence on its enzymatic activity.

In conclusion, the phosphoproteomic analysis of AML14.3D10, the eosinophil model cell line, provides an understanding of the molecular mechanisms of eosinophilia as well as clues for biomarkers for the diagnosis of and new drug development for atopic dermatitis.

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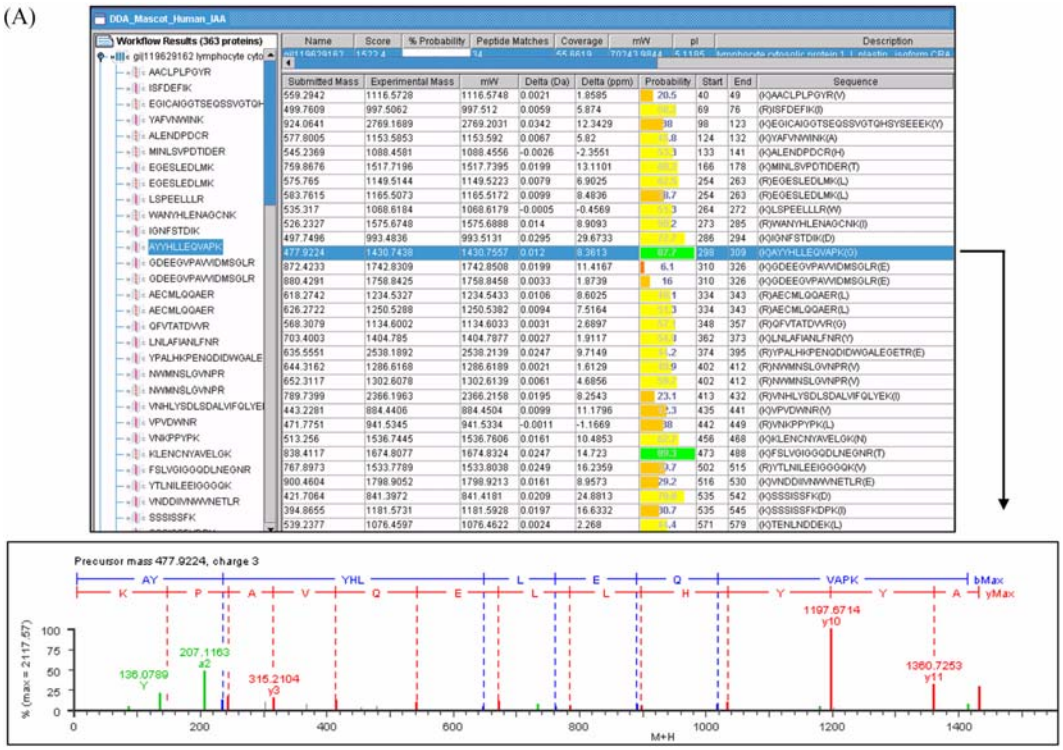

#	b	b^{++}	b^0	b^{0++}	Seq.	y	y^{++}	y^*	y^{*++}	y^0	y^{0++}	#
1	58.0287	29.5180			G							12
2	127.0502	64.0287	109.0396	55.0235	S	1323.5555	662.2814	1306.5289	653.7681	1305.5449	653.2761	11
3	226.1186	113.5629	208.1080	104.5577	V	1254.5340	627.7707	1237.5075	619.2574	1236.5235	618.7654	10
4	313.1506	157.0790	295.1401	148.0737	S	1155.4656	578.2365	1138.4391	569.7232	1137.4551	569.2312	9
5	428.1776	214.5924	410.1670	205.5871	D	1068.4336	534.7204	1051.4071	526.2072	1050.4230	525.7152	8
6	557.2202	279.1137	539.2096	270.1084	E	953.4067	477.2070	936.3801	468.6937	935.3961	468.2017	7
7	686.2628	343.6350	668.2522	334.6297	E	824.3641	412.6857	807.3375	404.1724	806.3535	403.6804	6
8	817.3032	409.1553	799.2927	400.1500	M	695.3215	348.1644	678.2949	339.6511	677.3109	339.1591	5
9	964.3386	482.6730	946.3281	473.6677	M	564.2810	282.6441	547.2545	274.1309	546.2704	273.6389	4
10	1093.3812	547.1942	1075.3707	538.1890	E	417.2456	209.1264	400.2191	200.6132	399.2350	200.1212	3
11	1206.4653	603.7363	1188.4547	594.7310	L	288.2030	144.6051	271.1765	136.0919			2
12					R	175.1190	88.0631	158.0924	79.5498			1

Fig. 4. A representative LC-MS/MS spectrum and database query results. (A) Matched peptide fragment and error between experimental mass and theoretical mass. A representative spectrum of a peptide fragment ion is shown. (B) MS/MS spectrum of [4]GSVSDEEMMELR[15]. The mascot search result suggests the possibility that Ser-5 of lymphocyte cytosolic protein (L-plastin) could be phosphorylated.

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