

The Human Eosinophil Proteome. Changes Induced by Birch Pollen Allergy

Charlotte Woschnagg,[†] Jens Forsberg,^{‡,⊥} Åke Engström,[‡] Federico Odreman,[§] Per Venge,[†] and Rodolfo C. Garcia^{*,†,§}

Department of Medical Sciences, Clinical Chemistry, Uppsala University, 75185 Uppsala, Sweden, Department of Medical Biochemistry and Microbiology, Uppsala University, P.O. Box 582, 75123 Uppsala, Sweden, and Leukocyte Biology and Proteomics Groups, International Centre for Genetic Engineering and Biotechnology, Area Science Park, Padriciano 99, 34012 Trieste, Italy

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Proteins from human eosinophils were separated bidimensionally and identified by mass spectrometry (336 spots/bands, 98 different proteins). Of these, 24.7% belonged to the cytoskeleton/migration group. Highly basic proteins (11.3%) were concentrated in the granule-containing cell fraction. We detected novel hyperacidic forms of cofilin-1, profilin-1 and adenylyl cyclase-associated protein, and hyperbasic forms of eosinophil-derived neurotoxin/eosinophil protein X and major basic protein homologue. We also found evidence of the triglycosylation of the heavy chain of eosinophil peroxidase. In addition, through comparative 2D image analysis, spot quantification and MS, it was found that hsc70, actin-capping protein and hyperacidic forms of eosinophil peroxidase heavy chain are overexpressed in cells from birch pollen allergic subjects, at the peak of a season. The link between these findings and an increased cellular antigen-presenting capacity and motility are discussed.

Keywords: eosinophil fractions • basic proteins • cytoskeletal proteins • birch pollen allergy • hsc70

Introduction

Eosinophils are multifunctional cells involved in numerous inflammatory phenomena such as parasitic infections and allergic diseases.^{1,2} They are recruited to inflammatory sites by virtue of the action of cytokines and chemokines,³ where they participate in the modulation of immune responses through the release of mediators,⁴ pro-inflammatory cytokines⁴ and toxic proteins⁵ from their cytoplasmic granules. The main characteristics of these cells are, in brief, their ability to migrate, transmigrate, encircle target particles, their high susceptibility to activation and their cytotoxic and immune-modulating capacity.

Proteomic analysis allows the comprehensive description of the protein composition of cells and physiological fluids and its changes in disease conditions or upon treatment with drugs.⁶ Under conditions allowing a good resolution, the combination of isoelectrofocusing and polyacrylamide electrophoresis (2-DE) results in the visualization of a large number of the different proteins contained in a certain tissue, cell type or physiological fluid. Even though there is a limit to the

number of spots that can be separated bidimensionally, this is compensated by advantages such as the possibility of establishing both qualitative and quantitative differences in the protein patterns corresponding to different physiological or pathological situations, and of detecting proteins post-translationally modified.

The identification of the proteins and protein forms present in eosinophils is a necessary step toward the analysis of differences in their expression during inflammatory situations. This would contribute to the knowledge of activation mechanisms in general and could lead to defining new markers of eosinophil activation. No study of the proteins present in eosinophils has been reported to date. Work by Levi Schaeffer et al.⁷ has established differences in the bidimensional patterns of eosinophil protein expression under the effect of tumor necrosis factor- α , granulocyte macrophage colony stimulating factor and a human mast cell line extract. Their isoelectrofocusing (IEF) covered the range 3.5–8.5, and no proteins were identified presumably because the amount of material available was insufficient, and this was the reason to increase detection sensitivity by using metabolically labeled eosinophils. In addition, a comparative proteomic analysis of eosinophils from the peripheral blood of subjects with atopic dermatitis has shown an increase in the expression of 51 proteins when compared with healthy controls.⁸ These proteins cover a variety of functions such as signaling, regulation of metabolism, apoptosis and inflammation.

During allergy, changes are likely to occur all along from eosinophil maturation at the bone marrow through priming

* To whom correspondence should be addressed. Dr. Rodolfo C. Garcia, Leukocyte Biology Group, International Centre for Genetic Engineering and Biotechnology, Area Science Park, Padriciano 99, 34012 Trieste, Italy. Tel.: +39-040-3757371. Fax: +39-040-226555. E-mail: garcia@icgeb.org.

[†] Department of Medical Sciences, Clinical Chemistry, Uppsala University.

[‡] Department of Medical Biochemistry and Microbiology, Uppsala University.

[⊥] Present address: Strategic Research Center for Stem Cell Biology and Cell Therapy, Klinikgatan, Lund University, 22184 Lund, Sweden.

[§] International Centre for Genetic Engineering and Biotechnology.

and degranulation at peripheral blood level followed by recruitment to tissues, activation and, finally, apoptosis.⁹ These events will inevitably be reflected in qualitative and quantitative changes in cellular proteins. A number of eosinophil functions have been reported to be altered at peripheral blood level in inflammatory conditions. During pollen allergy and/or asthma, migratory responses are increased,¹⁰ there is a greater propensity to secrete granule proteins,^{11,12} piecemeal degranulation takes place,¹³ adhesion to ICAM-1/VCAM-1 is primed,¹⁴ the expression of CD9 and CD11b is increased,¹⁵ and oxidative metabolism is affected.^{16,17} Therefore, studies of the protein composition of peripheral blood eosinophils and the changes elicited during inflammatory disorders such as allergy are likely to provide relevant information about alterations in course before transmigration and tissue localization as well as those in cells returning to the bloodstream from the lymphatic system.

The present study reports in the first place the identification of a number of proteins present in eosinophils and their subcellular fractions, among which we have detected some protein forms yet undescribed. Second, we performed a comparison of the proteomes of eosinophils from control and birch pollen allergic individuals which showed some significant differences. Some of the differentially expressed proteins were identified, and the possible functional implications are discussed.

Materials and Methods

Reagents. Immobilized pH gradient strips (pI 3–11, non-linear), DeStreak IEF rehydration solution and IPG solutions were purchased from GE Healthcare (Uppsala, Sweden). IEF strips pI 9–12, Zoom 2D protein solubilizer, Zoom carrier ampholytes 9–11 and Coomassie Brilliant Blue G250 stain were from Invitrogen (Carlsbad, CA). Dithiothreitol (DTT) was obtained from USB (Cleveland, OH). Sequencing-grade trypsin was purchased from Promega (Madison, WI). Bradford protein assay reagent was purchased from Bio-Rad (Hercules, CA). Percoll was from Pharmacia (Uppsala, Sweden). Both MACS system and anti-CD16 beads were from Miltenyi Biotec (Bergisch-Gladbach, Germany). Protease inhibitors (Complete) were from Roche Diagnostics (Mannheim, Germany). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Material Collection and Processing. 1. Subjects. Blood was drawn from an asymptomatic individual on different occasions within a period of 3 years, with informed consent. Using cells from the same donor avoids variability due to different polymorphisms in different individuals.

For the allergy studies, 5 birch pollen allergic, nonmedicated subjects with seasonal symptoms of rhinitis (allergics: A₁–A₅) and 5 healthy, nonallergic blood donors (control group: C₁–C₅) were bled at the peak of the Swedish birch pollen season. The allergic subjects presented elevated blood eosinophil counts ($340 \times 10^6/\text{L}$, range 200–500) compared with the nonallergic controls ($180 \times 10^6/\text{L}$, range 100–300) during the period of pollen exposure, indicating an ongoing inflammatory process.

Ethical approval was obtained from Uppsala University, Ethics committee, Faculty of Medicine, with the number 98293, dated September 8, 1998.

2. Cells and Cell Extracts. Eosinophils were isolated from peripheral venous blood using the magnetic cell separation system (MACS), as previously described.¹⁸ The mean cell purity was 99.5% ($n = 4$, range 98.4–100) in the case of eosinophils from the asymptomatic subject used for the eosinophil pro-

teome studies, and 99% ($n = 10$, range 98–100) in the case of the allergy studies.

Cells were resuspended with 6% sucrose in 10 mM PIPES (pH 7.2) containing protease inhibitors and processed in either of two ways: (i) treated with NP-40 (1% (w/v) final concentration), constituting what is referred to as “whole cell extracts”; or (ii) disrupted by sonication (15 pulses of 3 s at 20% amplitude in a sonicator from Chemical Instruments AB, Lidingö, Sweden). Disrupted cells were centrifuged at 800g for 3 min, at 4 °C, and the turbid supernatant (cytoplasmic fraction) was layered on a 1 mL cushion of 9% sucrose and subjected to ultracentrifugation at 200 000g for 1 h, in a 50.4Ti Beckman rotor, at 4 °C. The liquid above the cushion constitutes the cytosolic fraction. The pellet (membrane-bound organelles + plasma membranes) was resuspended with 9% sucrose containing protease inhibitors, after which 1% (w/v) NP-40 (final concentration) was added.

For the allergy comparative study, purified eosinophils (av. 7×10^6 , range 3×10^6 to 11.5×10^6) were washed with 9% sucrose in 10 mM PIPES (pH 7.2) and the salt-free cell pellets were directly solubilized in DeStreak solution (pI range 3–11) as described below for TCA precipitates.

Protein Isolation. Protein concentrations were determined by the method of Bradford. Proteins were precipitated with 10% (w/v) trichloroacetic acid (TCA), left overnight at 4 °C and centrifuged at 10 000g for 15 min. In the case of membrane fractions, 1% (w/v) NP-40 was added before the TCA. The protein pellets were washed 3 times with 600 μL of cold ethanol/ether (1:1, v/v), centrifuging each time at 10 000g for 10 min. The wet pellets were centrifuged for additional 5 min to remove the remainder of the ethanol/ether. Pellets were air-dried for approximately 5 min and solubilized for 4–12 h at room temperature (RT) with DeStreak IEF rehydration solution (260 μL) containing 1% (v/v) IPG 3–11 solution, in the case of pI 3–11 IEF separations; or with Zoom Protein solubilizer 1 containing 10 mM DTT and 1% (v/v) Zoom carrier ampholytes 9–11, for 2 h, at RT, in the case of pI 9–12 IEFs.

In all cases, samples solubilized in DeStreak solution (either TCA precipitates or cells directly solubilized) were stored at –80 °C. Before use, they were sonicated at 10% amplitude for 5 s and centrifuged at 10 000g for 3 min. The clear supernatants were used for the bidimensional separations (2-D = IEF + SDS-PAGE), and the small pellets (IEF rehydration solution-insoluble material) were kept and analyzed by monodimensional SDS-PAGE, as described below.

Protein Separations. 1. Monodimensional: Samples (DeStreak-insoluble material) were suspended in sample buffer [2.5 mM Tris, 2% (w/v) SDS, 10% (v/v) glycerol and 10 mM dithioerythritol] and heated at 60 °C for 30 min. They were run on 12% SDS-polyacrylamide gels.

2. Bidimensional: 2.1. pI 3–11. Samples (clear solutions) to be subjected to IEF contained 200–500 μg of protein in the case of the proteome studies of whole cells and cell fractions, or were from salt-free cell pellets containing an average number of 7×10^6 eosinophils (range 3×10^6 to 11.5×10^6) in the case of the allergy comparative study.

IEF separations were carried out on 13 cm long pH-gradient strips (3–11 NL). Samples (240 μL) were loaded by in-gel rehydration at RT, for 14 h. IEF was carried out on an EttanIPGphor IEF unit (Amersham Biosciences), at 20 °C, using the following program: 50 V for 4 h, 0–1000 V (gradient) for 3 h and 8000 V up to 32 000–40 000 Vh. Each focused strip was equilibrated first with 2.5 mL of a solution containing 6 M

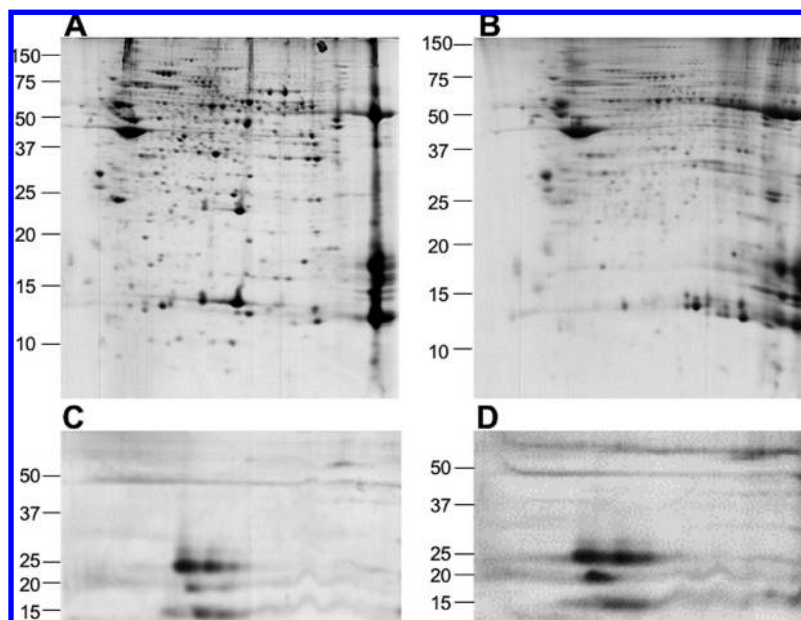


Figure 1. Bidimensional protein maps of eosinophil whole cell extract (A and C) and membranous fraction (B and D). (A and B) first dimension, IEF p/ 3–11; second dimension, SDS-PAGE (14% gels), (C and D) first dimension, IEF p/ 9–12; second dimension, SDS-PAGE (4–12% gradient gels), Molecular mass markers are indicated on the left-hand side of each picture, in kilodaltons (kDa). The p/ 3–11 separations illustrated (A and B) are 2 out of a total of 6 (see Supporting Information Figure 1).

urea, 30% glycerol, 50 mM Tris-HCl (pH 8.8), 2% (w/v) SDS, 10 mg/mL DTT and bromophenol blue (tracking dye), for 10 min, at RT. After removing this solution, a second 10 min equilibration was performed with 2.5 mL of a solution containing the same components except for DTT that was replaced by iodoacetamide (25 mg/mL). Proteins were then separated electrophoretically in the second dimension by placing the strips horizontally on 12 or 14% SDS-polyacrylamide gels (200 × 200 × 1.5 mm) that were run overnight at 12 mA per gel. Standard protein markers (Precision Plus Dual Color, BioRad) covering the range 10–250 kDa were run alongside each strip.

2.2. pI9–12. Clear samples containing 200 μ g of protein in 200 μ L of Zoom protein solubilizer 1 plus ampholytes pH 9–11 were used to reswell 7 cm long IEF strips for 18 h, at RT. The strips were focused at 2000 V for a total of 14 000 Vh and treated successively with DTT- and iodoacetamide-containing equilibration solutions, as described above. The second-dimension SDS-PAGE was performed in 4–12% precast gels (Nu PAGE, Invitrogen).

Protein Visualization and Calculation of Parameters. Coomassie staining (Bi- and monodimensional separations shown in Figure 1 and Figures 1–3 of Supporting Information): gels were fixed for 1 h in 10% (v/v) ethanol–7% (v/v) acetic acid and stained with Colloidal G-250 Coomassie Blue stain solution (Colloidal Blue Staining Kit, Invitrogen) for 1 day in order to achieve maximum intensities and minimize the possible influence of differences in dye-protein affinities. Excess dye was washed off with distilled water.

Silver staining (gels corresponding to the allergy studies, Figure 5) was performed according to a standard, improved sensitivity protocol.¹⁹

All gels were scanned at 300 dpi in a GS-800 Calibrated Densitometer (BioRad, Hercules, CA).

Isoelectric point values were calculated according to a 3–11 pH curve provided by the supplier of the nonlinear IEF strips [http://www.amershambiosciences.com] or considering a linear

curve in the case of the 9–12 IEF strips from Invitrogen. Experimental, apparent molecular masses of the proteins were calculated from the semilogarithmic curves of molecular mass ($\log_{10}$) versus migration distance corresponding to each of the gels being analyzed. In the case of gels presenting distorted areas, the calculation of molecular masses within those areas may deviate from the correct values by up to 20%.

Image Analysis. The scanned silver stained gel images corresponding to the allergy studies were sent to the Ludesi Analysis Center (Lund, Sweden, <http://www.ludesi.com>) for professional image analysis using dedicated software. Spot detection, segmentation and matching followed a strict protocol to ensure a high level of correctness. Gels were matched using all-to-all spot matching, avoiding the bias caused by the use of a reference gel. The integrated intensity of each of the spots was measured, background corrected and normalized. Normalization removes systematic gel intensity differences originating, for example, from variations in staining, scanning time and protein loading by mathematically minimizing the median expression difference between matched spots. This allows a satisfactory quantification and comparison of different gels. Differential expression of proteins was defined on the basis of >1.5-fold change between group averages and $p < 0.05$. Differences involving at least 4 out of the 5 samples of each group were considered as meaningful.

Protein Identification by Mass Spectrometry. Protein spots from Coomassie stained gels were excised, washed two times with 50 mM ammonium bicarbonate/50% acetonitrile and once with 100% acetonitrile and allowed to dry. In the case of silver stained gels, spot plugs were treated with 15 mM potassium ferricyanide/50 mM sodium thiosulfate, then washed as described above. The dried gel plugs were then subjected to overnight in-gel digestion at 37 °C, using 10 μ L of 20 μ g/mL sequencing grade modified trypsin (Promega, Madison, WI) in 20 mM ammonium bicarbonate per gel plug. Peptides were extracted with 50% acetonitrile/0.1% TFA and

dried under nitrogen gas in a TurboVap 96 (Zymark Corporation, Hopkinton, MA) or in a speedvac centrifuge. In-gel digestions and extractions were carried out in 96 well polypropylene microplates (Grainer Bio-One, Frickenhausen, Germany) using the Ettan Digester (GE Healthcare, Uppsala, Sweden).

Extracted protein digests were dissolved in 5 μ L of 10% acetonitrile/0.1% TFA and subsequently analyzed by MALDI-TOF and, in some cases, also by MALDI-TOF/TOF mass spectrometry. Mass spectra (MS and MS/MS) were recorded on an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Samples were prepared as "dried droplets" on polished steel MALDI target plates (Bruker Daltonics, Bremen, Germany) using α -cyano-4-hydroxy-*trans*-cinnamic acid as matrix.

Monoisotopic peaks were selected and mass spectra were internally calibrated using trypsin autolysis products, amino acids 108–115 (MH^+ : 842.51), 98–107 (MH^+ : 1045.56) and 58–77 (MH^+ : 2211.10). Masses of known contaminants were removed and the identities of the proteins were determined by searching the peptide mass fingerprint data against theoretical trypsin digests of human proteins available at the Mass Spectrometry Protein Database, using the Mascot search engine.²⁰ Peptide mass tolerance was set to 0.05 Da, allowing a maximum of one missing cleavage. Carbamidomethylations on cysteine residues were set as fixed modifications and oxidation of methionine residues was considered as a variable modification. A protein was accepted as identified if its probability-based Mowse score was ≥ 60 ($p < 0.05$). Protein digests that returned either no significant protein matches in the PMF searches, protein matches with low sequence coverage or protein matches with a low number of matching peptides were further analyzed using MS/MS in order to obtain or confirm identities. MS/MS data were combined with those from the corresponding MS spectrum using the software BioTools (Bruker Daltonics, Bremen, Germany). Mascot MS/MS ion searches were then carried out using the combined data. Parameters were set as described above, with the addition of the MS/MS tolerance, which was set to 0.7 Da.

Results

Separation of Proteins from Whole Eosinophil Extracts and Cytosolic and Membrane Fractions. We performed a series of 2-DE separations (IEF/SDS-PAGE) of whole eosinophils as well as of cytosolic and membrane fractions, the latter including all membranous and membrane-bound material of the cell. The IEF separations covered the pI range 3–11, and the second dimension was carried out in 12 or 14% gels that were run for variable lengths of time in order to improve the resolution of different sections of the protein distribution maps. Figure 1 illustrates two out of a total of six pI 3–11 bidimensional separations (A and B), as well two pI 9–12 separations (C and D), from which spots were cut out for protein identification. The 2-D protein distributions show an abundance of highly basic proteins in membrane-bound eosinophil fractions and whole cell extracts (Figure 1A,B). These basic proteins can be seen vertically distributed along the right-hand side of the gels.

In order to obtain a better separation of protein forms of pI close or >11 , we also performed separations using IEF strips spanning from pI 9 to 12, followed by a second dimension on gradient gels (Figure 1C,D).

A small proportion of the proteins precipitated with TCA from whole eosinophil extracts or from membrane fractions, but not from cytosolic fractions, were observed not to be completely solubilized for IEF by the urea/thiourea-containing DeStreak solution even after 12 h at RT. Centrifugation at 10 000g revealed a pellet of insoluble material, which was heated with a denaturing solution containing SDS and DTT and run on monodimensional gels in order to establish its protein composition. This DeStreak-insoluble material was found to contain eosinophil lysophospholipase, eosinophil peroxidase, eosinophil granule major basic protein, actin and histones (Supplemental Figure 3).

Identification of Proteins. We were able to identify proteins in 330 spots excised from eight 2-D gels (whole cell extracts, cytosolic and membrane fractions) and 6 bands from a 1-D gel of urea/thiourea-insoluble material. Out of the 330 spots from 2-D gels, 307 corresponded to pI 3–11 and 23 to pI 9–12 IEF separations. According to the position of each spot on the gels, they were assigned an apparent molecular weight (M_r) and a pI , which were used as additional criteria to establish protein identities (Table 1). Bands from monodimensional gels could only be assigned a molecular weight. As an example of the MS analysis we performed, Figure 2 illustrates a section of the peptide mass spectrum of an acidic form of cofilin-1 (spot 66, Table 1). It shows 7 out of the 10 peaks corresponding to the matching peptides found, and a description of identification parameters and coverage is also provided.

The total of the spots and bands identified were accounted for by 98 different proteins, since a number of them were found in more than a subcellular fraction, were repeated in different 2-D separations, were present in single separations as several isoforms of different pI , or were protein fragments.

A graphical representation of experimental versus theoretical isoelectric points shows a sizable proportion of protein spots of pI both substantially lower and higher than the theoretical value (Figure 3A). Table 2 lists the proteins for which forms with pI s differing considerably from theoretical values have been observed. We have excluded spots showing an important pI variation accompanied by a reduction in molecular size, because in those cases pI variations are likely to be due to the missing terminal fragments. We also excluded spots placed in an area of horizontal or vertical streaking, which could have led to artifactual positioning. The greater positive pI deviations, from +0.4 to +1.4 pH units, corresponded to forms of EPX/EDN and MBP2. Negative shifts from –0.2 to –2.3 pH units were observed for forms of coronin-1A, adenyl cyclase-associated protein 1 (CAP1), cofilin-1, profilin-1 and EPO heavy chain.

Regarding molecular masses, experimental and theoretical values were in agreement, within the margins of experimental/calculation errors (Figure 3B). Values lower than the theoretical one represent protein fragments most likely originated by proteolysis. Higher values, instead, are related to post-translational modifications of the protein backbone, as is the case for integrin- $M\alpha$ (CD11b), which is known to be extensively glycosylated.²¹ In some cases, higher values may be calculation artifacts due to an uneven gel run, with this effect being relatively greater in the high molecular weight region of gels. In others, they are due to an anomalous migration on SDS-PAGE due to intrinsic characteristics of the molecule, as is the case for PTB-associated splicing factor²² and moesin.²³

Table 1. Proteins Identified in Human Eosinophils^a

spot nr	protein	accession number	theor. M_r /pI	exp. M_r /pI	sequence coverage (%)	matched peptides	MS MS	MOWSE score
16	Actin, cytoplasmic 2	P63261	40.6/5.55	45.0/5.20	46	18		161
328	Actin-binding protein p57	P31146	50.9/6.25	58.6/5.70	29	17		160
205	Actin-capping protein, α 1	P52907	32.9/5.45	40.3/5.31	55	12		140
84	Actin-capping protein, β	P47756	31.4/5.36	29.4/5.43	44	15		141
243	Actin-interacting protein 1	O75083	66.2/6.17	75.9/6.08	26	16		152
261	Actin-like protein 3	P61158	47.7/5.61	49.2/5.45	42	19		164
206	Actin-related protein 2/3 complex subunit 2	O15144	34.4/6.84	36.7/6.51	41	13		174
217	Adenylyl cyclase-assoc. protein 1 (CAP1)	Q01518	51.8/8.12	66.8/7.67	35	16		80
218		Q01518	51.8/8.12	66.8/7.20	29	11		74
220		Q01518	51.8/8.12	66.8/6.45	32	12		95
270	Alpha-actinin 1	P12814	103.5/5.22	96.6/5.73	15	12		81
9	Alpha-enolase	P06733	47.4/6.99	50.1/6.66	33	14		151
81	Annexin I	P04083	38.7/6.57	35.5/6.13	68	25		204
85	Annexin III	P12429	36.4/5.63	31.3/5.41	54	21		186
86	Annexin III	P12429	36.4/5.63	31.3/5.28	39	14		131
83	Annexin IV	P09525	35.9/5.84	31.6/5.50	59	21		159
318	Annexin VI	P08133	76.0/5.42	78.2/5.25	30	19		173
331	Annexin XI	Q6ICSO	54.7/6.91	56.0/6.20	32	16		199
291	Bactericidal/permeability-increasing protein	Q8IW58	54.1/9.50	51.9/7.03	27	11		78
141	Calgranulin A	P05109	10.9/6.51	9.6/6.50	58	8		110
62	Calgranulin B	P06702	12.8/5.55	12.9/5.52	95	13		98
58	Calgranulin C	P80511	10.4/5.82	9.8/5.55	47	6		92
207	Calponin-2	Q99439	34.0/6.92	40.9/6.40	23	7		70
252	Calreticulin	Q53G71	47.1/4.30	56.9/4.13	29	15		130
127	Carbonate dehydratase	P00915	28.4/6.90	26.3/6.32	59	11		86
336	Catalase	P04040	59.8/6.95	61.0/6.45	40	17		218
78	Cathepsin G	P08311	25.8/11.51	25.1/10.47	44	9		74
327	Citrate synthase	O75390	52.0/8.13	45.0/6.87	3		1	
68	Cofilin-1	P23528	18.7/8.26	17.8/5.92	60	10		
66		P23528	18.7/8.26	17.7/6.97	59	10		118
65		P23528	18.7/8.26	17.5/8.08	65	12	2	
221	Coronin-1A	P31146	51.6/6.25	66.8/6.31	20	10		73
222		P31146	51.6/6.25	66.8/6.15	24	12		118
224		P31146	51.6/6.25	66.8/5.87	29	15		153
134	Cyclophilin A	P62937	18.0/7.82	15.1/8.02	25	4		78
337	EH domain-containing protein 1	Q2M3Q4	60.6/6.35	63.5/6.04	40	19		195
93	Elastase inhibitor	P30740	42.8/5.90	43.0/5.70	40	17		149
172	Eosinophil cationic protein (ECP)	P12724	15.7/10.72	19.4/10.50	35	7		86
162		P12724	15.7/10.72	20.8/10.16	41	8		72
166	Eosinophil-derived neurotoxin (EPX/EDN)	P10153	15.5/9.20	22.9/10.60	17		2	
165		P10153	15.5/9.20	22.9/10.50	17		2	
164		P10153	15.5/9.20	22.9/10.40	17		2	
171		P10153	15.5/9.20	22.9/10.30	8		3	
163		P10153	15.5/9.20	22.9/10.20	17		2	
193		P10153	15.5/9.20	19.5/9.67	35		3	
96	Eosinophil granule major basic protein (MBP)	P13727	13.8/10.80	12.6/n.a.	11	1	1	
120	Eosinophil lysophospholipase	Q05315	16.5/6.83	12.3/6.52	64	13		105
169	Eosinophil major basic protein homologue, C-lectin	Q9Y2Y8	13.4/9.15	15.8/10.50	9		1	
168		Q9Y2Y8	13.4/9.15	15.8/10.30	9		1	
180		Q9Y2Y8	13.4/9.15	14.1/9.74	93	9	1	
25	Eosinophil peroxidase (EPO), heavy chain	P11678	53.4/10.79	55.6/10.97	16	13		108
27		P11678	53.4/10.79	54.7/10.65	23	21		118
213		P11678	53.4/10.79	56.9/9.37	48	21		144
104	Eosinophil peroxidase (EPO), light chain	P11678	12.7/10.70	14.9/10.22	42	19		91
117	Eukaryotic translation elongation factor 1 α 1	Q6IPN6	50.4/9.10	45.3/9.42	21	11	1	
170	Eukaryotic translation elongation factor 1 α 1 var.	Q53HM9	50.4/8.98	51.9/11.36	15	7	1	
344	Fructose-bisphosphate aldolase A	P04075	39.7/8.39	40.0/8.25	29	11		85
322	GDP dissociation inhibitor 2	Q6IAT1	51.1/6.11	51.8/5.67	56	20		212
312	Gelsolin	P06396	86.0/5.77	100.0/5.48	18	12		110
294	Glucose-6-phosphate isomerase	P06744	63.2/8.44	55.5/8.35	31	17		108
330	Glucose-6-phosphate-1-dehydrogenase	P11413	59.6/6.44	58.6/6.14	36	20		218
290	Glutathione reductase-homologue	P00390	56.3/8.74	51.9/7.47	27	10		79
71	Glutathione S-transferase	P09211	23.4/5.43	23.3/5.37	60	8		124
128	Glyceraldehyde-3-phosphate dehydrogenase	P04406	36.1/8.58	34.1/8.45	48	17	1	
308	Glycogen phosphorylase	P06737	97.4/6.74	109.6/6.15	32	24		253

Table 1. Continued

spot nr	protein	accession number	theor. M_r /pI	exp. M_r /pI	sequence coverage (%)	matched peptides	MS MS	MOWSE score
231	GRP 78	P11021	72.3/5.01	89.1/5.01	40	23		219
319	Heat shock cognate protein, 70 kDa	P11142	71.1/5.37	78.2/5.17	39	24		210
209	Heterogeneous nuclear ribonucleoprotein B1	P22626	37.5/8.97	40.7/8.27	37	11		106
247	Heterogeneous ribonuclear particle protein L	P14866	60.7/6.65	75.0/6.40	22	11		132
195	High mobility group box protein 1	Q5T7C4	18.5/9.72	30.9/9.42	37	6		85
44	Histone H2A	Q99878	13.8/10.88	12.6/10.72	33	4	1	
190	Histone H2B type 1	P62807	13.7/10.32	16.2/10.27	48	8		138
162	Histone H2B type 2E	Q16778	13.9/10.14	20.8/10.16	64	12		103
46	Histone H3	P84243	15.3/11.27	14.5/10.76	31	7		81
41	Histone H4	P62805	11.4/11.36	9.0/10.76	57	9		105
262	HSP70-1	P08107	70.3/5.48	65.8/5.30	43	30		254
250	Integrin, α M	P11215	128.4/6.88	281.8/6.26	26	26		256
249		P11215	128.4/6.88	281.8/6.07	29	31		308
300	Lactoferrin	P02788	78.2/8.50	79.4/8.87	54	31		262
208	Lasp-1 protein	Q14847	30.2/6.11	42.2/6.20	32	8	1	
268	Leukotriene A-4 hydrolase	P09960	69.7/5.80	65.8/5.66	43	26		222
88	L-lactate dehydrogenase B chain	P07195	36.8/5.72	35.0/5.48	33	10		106
262	L-plastin	P13796	70.3/5.20	65.8/5.30	30	13		82
263		P13796	70.3/5.20	65.8/5.11	42	23		171
254	Lymphocyte-specific protein 1	P33241	37.4/4.69	48.8/4.48	23	7		64
59	Lysozyme	P61626	14.7/9.28	13.6/10.40	42	8	1	
349	Macrophage capping protein	P40121	38.8/5.88	41.4/5.54	8	2	2	
265	Matrix metalloproteinase-9	P14780	78.4/5.69	84.5/4.60	31	22		144
239	Moesin	P26038	67.8/6.09	92.3/6.20	28	18		159
235		P26038	67.8/6.09	92.3/5.76	34	23		188
69	NGAL/HNL	P80188	22.6/9.02	21.7/8.67	68	17		160
342	p47-phox	P14598	44.9/9.12	47.2/9.15	51	20		184
124	Peroxiredoxin-2	P32119	22.0/5.66	21.2/5.45	43	10		113
10	6-Phosphogluconate dehydrogenase	P52209	53.5/6.88	49.0/6.68	21	11		100
143	Phosphoglycerate kinase 1	P00558	44.9/8.30	40.7/8.25	41	13		160
75	Phosphoglycerate mutase 1	P18669	28.8/6.75	27.7/6.35	45	12		102
60	Profilin-1	P07737	15.1/8.47	13.5/8.47	71	10	2	
61		P07737	15.1/8.47	13.5/7.10	52	8		84
201	Proteasome activator 28 α -subunit	Q06323	28.9/5.78	33.7/5.52	56	15		179
203	Proteasome subunit, α , 1	Q53YE8	29.9/6.15	35.1/5.95	38	9		125
197	Proteasome subunit, α , 2	P25787	25.9/7.12	29.2/6.42	3		1	
225	Protein disulfide isomerase	P30101	56.8/5.61	65.8/5.56	32	17		128
251	Protein disulfide isomerase	P30101	56.8/5.61	65.8/4.93	38	22		237
215	PTB-associated splicing factor	P23246	76.2/9.45	118.9/9.42	25	19		109
295	Pyruvate kinase	P14618	58.3/7.95	58.5/8.08	37	17		133
72	Rho-GDP-dissociation inhibitor 2	P52566	23.0/5.10	24.0/5.06	43	10		80
314	Serum albumin	Q56G89	67.7/5.63	72.8/5.50	41	24		331
90	TALDO1 protein	Q8WV32	36.5/5.80	37.2/5.61	32	12		131
296	Transketolase	P29401	67.9/7.58	64.9/7.47	35	23		219
212	Translation elongation factor 1	Q6IPN6	50.1/9.10	55.3/9.00	3	1		98
350	Tropomyosin-3	P06753	32.8/4.68	31.8/4.82	54	21		224
258	Tubulin beta chain	P07437	50.2/4.75	51.3/4.92	44	22		190
290	UTP-glucose-1-phosphate uridylyltransferase 2	Q16851	56.9/8.15	51.9/7.47	20	11		78
118	Vimentin.	P08670	53.6/5.06	52.7/5.06	61	31		258
305	Vinculin	P18206	117.2/5.83	141.9/5.67	33	30		281
73	14-3-3 protein ζ/δ	P63104	27.7/4.73	26.6/4.72	53	17		130

^a The complete list of spot/band identifications and the gels from which spots were excised (Figure 1–3 in Supporting Information), are specified in Table 1 (Supporting Information). Protein fragments have been excluded from the table above.

Function analysis (Figure 4) shows that the proteins identified are distributed among the following groups: cytoskeleton/migration (24.7%), metabolism (17.6%), granule secretory components (11.3%), gene regulation (10.3%), protein synthesis/modification/degradation (7.2%), membrane fusion/trafficking (6.2%), chaperones and protein folding (5.2%), redox (4.1%), bactericidal mechanisms (4.1%), signaling (3.1%), calcium-binding (3.1%) and receptors (1.0%).

Among the proteins found, a few are known to be neutrophil components: lactoferrin, lysozyme, human neutrophil lipocalin/neutrophil gelatinase-associated lipocalin (HNL/NGAL) and bactericidal/permeability increasing protein (Table 1). The source of these proteins could be the small amount of neutrophils (mean 0.5%, range 0–1.6) that contaminate eosinophil preparations. Another plausible explanation would be the uptake by eosinophils, at plasma level, of proteins secreted by

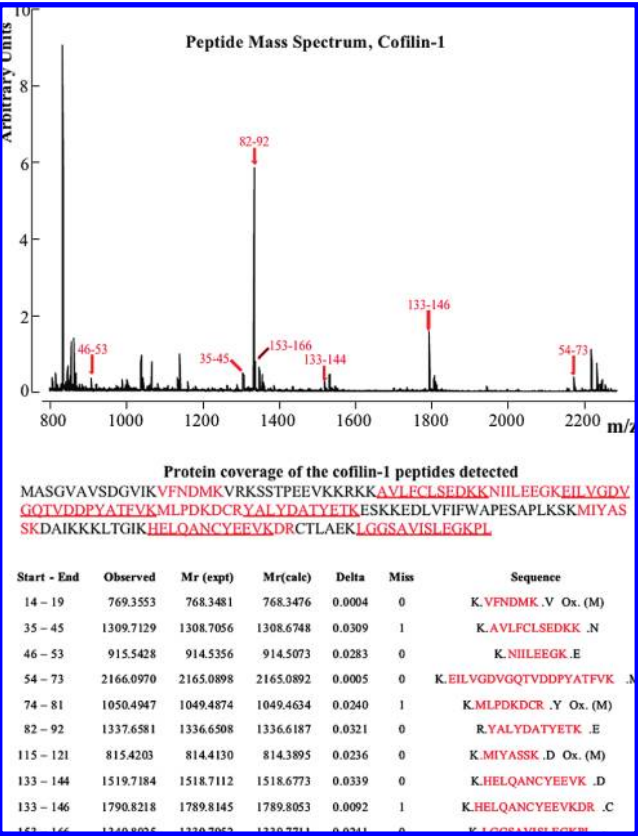


Figure 2. Cofilin-1: mass spectrum of tryptic digest, sequence coverage and parameters. The peptide mass spectrum of the tryptic digest of spot no. 66 is shown. This spot corresponds to a hyperacidic form of cofilin-1 with $pI = 6.97$ against a theoretical value of 8.26 (see Table 1). The Y-axis arbitrary unit values are intensity $\times 10^4$, according to BioTools software. The arrows indicate the m/z peaks of 7 of the peptides that matched cofilin, out of a total of 10. The numbers above each arrow show the sequence stretch covered by each peptide. The 10 matching peptides are indicated in red and red underlined on the protein sequence, and are listed on the table below the protein sequence. Other Mascot Search results were Score, 118; Number of mass values searched, 40; Sequence coverage, 59%.

neutrophils. This would be similar to what is known to occur conversely, namely, the uptake of the eosinophil proteins ECP and EPO by neutrophils.^{24,25} It cannot be excluded either that some classically neutrophil proteins might also be synthesized by eosinophils.

Comparative Analysis of Eosinophils from Asymptomatic and Birch Pollen Allergic Subjects. We performed bidimensional separations of proteins from eosinophils obtained from two groups of individuals: asymptomatic (control group, $n = 5$) and birch pollen-sensitive (allergy group, $n = 5$), at the time of maximum airborne birch pollen during Spring 2004. The gels were analyzed commercially for group differences, according to position and intensity (spot volume). Differences between both groups were found for a total of 16 spots, out of a total number of spots of 417 (average, range 261–590, $n = 10$). Considering as meaningful those changes observed in at least 4 out of the 5 subjects of each group, and where differences in mean spot intensities between groups was $\geq 50\%$, the number of relevant spots was reduced to 12. These 12 spots are indicated on the template gel shown in Figure 5. They were accurately excised employing the tridimensional profiles pro-

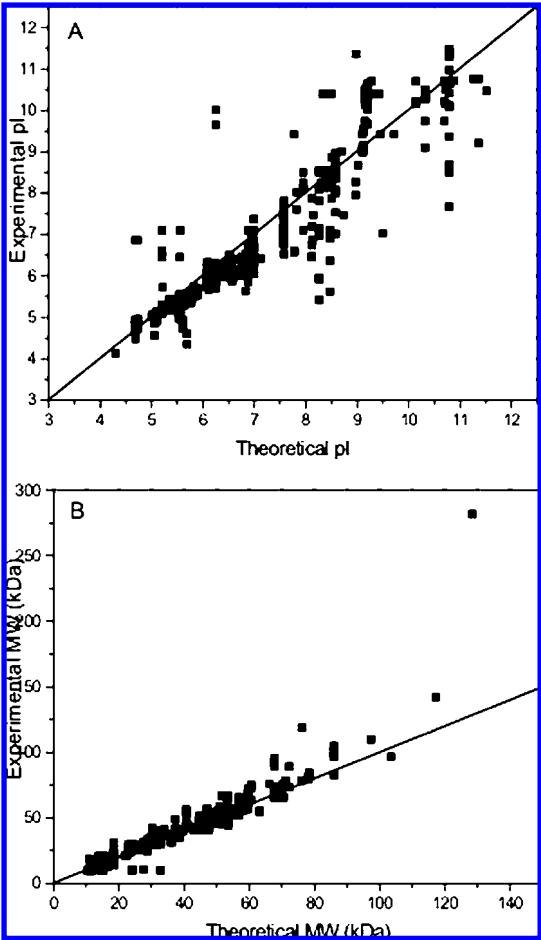


Figure 3. Experimental versus theoretical pI s (A) and M_r (B). All the values of experimental and theoretical pI s and M_r s used to build the graphs are found in Table 1 (Supporting Information), and include the values shown in Table 1.

Table 2. Protein Forms of pI Largely Apart from the Theoretical Value

protein ^b	theor. pI	negative pI shifts Δ^a	positive pI shifts Δ^a
EPX/EDN	9.20		+0.5 to +1.4
EPO heavy chain	10.79	−0.7 to −1.4	
MBP2	9.15		+0.6 to +1.3
Cofilin-1	8.26	−0.4 to −2.3	
Profilin-1	8.47	−1.4 to −1.6	
CAP1	8.12	−0.9 to −1.7	
Coronin-1A	6.25	−0.2 to −0.4	

^a pI shifts = experimental minus theoretical pI value (pH units).
^b EDN/EPX, eosinophil-derived neurotoxin/eosinophil protein X; EPO, eosinophil peroxidase; MBP2, eosinophil major basic protein homolog; CAP1, adenyl cyclase-associated protein 1. Spot localizations: EDN/EPX, Supporting Information Figure 1B2 (spot 193), Supporting Information Figure 2A (spots 163-6 and 171), Supporting Information Figure 2B (spots 159-61); EPO heavy chain, Supporting Information Figure 1B2 (spots 213-4); MBP, Supporting Information Figure 2A (spots 168-9 and 173), Fig. 2 Suppl. B (spots 174-6); Cofilin-1, Supporting Information Figure 1A1 (spot 136), Supporting Information Figure 1A2 (spots 66 and 68), Supporting Information Figure 1B2 (spot 194), Supporting Information Figure 1C (spot 142); Profilin-1, Supporting Information Figure 1A2 (spot 61), Supporting Information Figure 1B2 (spot 188); CAP1, Supporting Information Figure 1A3 (spot 332), Supporting Information Figure 1B2 (spots 218-20); Coronin-1A, Supporting Information Figure 1A2 (spot 276), Supporting Information Figure 1B2 (spots 222-4).

vided by the Ludesi imaging analysis. MS identification was possible for 4 of the spots (nos. 2, 7, 9 and 12), with the amount of material in the rest being insufficient. The identity of the

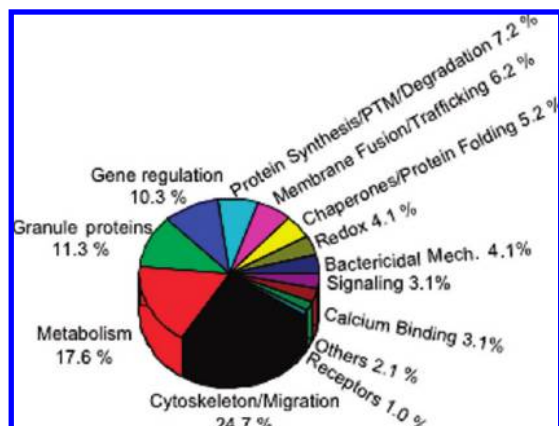


Figure 4. Distribution of eosinophil proteins according to their biological function. Functions were assigned on the basis of Swiss-Prot annotations and scientific literature.

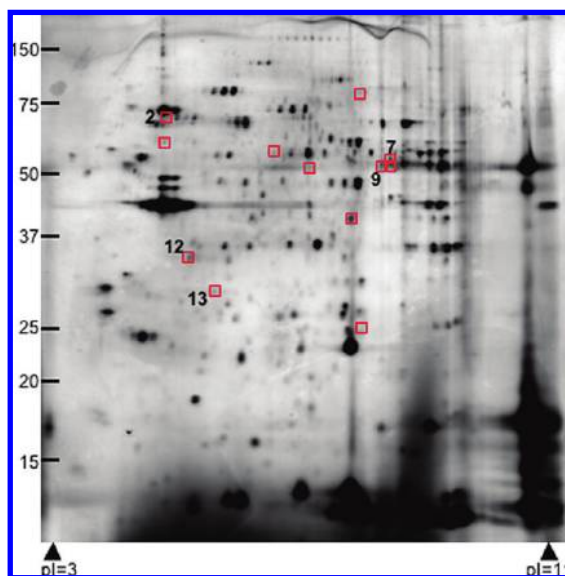


Figure 5. Eosinophil proteins differentially expressed during birch pollen allergy. Silver stained gel corresponding to one of the allergic subjects (A_3) used as a template to show the position of the spots, indicated by red squares, for which differential expression was detected.

protein corresponding to a fifth spot (no. 13) was tentatively inferred by accurate comparison of the 2-D protein maps corresponding to the gels from allergic subjects with the gels A2 and C2 shown in Supporting Information Figure 1, for which the MS identification of a protein in exactly the same position had been successful. This inference was supported by the fact that the putative protein corresponding to spot no. 13 was the β subunit of the same protein whose $\alpha 1$ subunit had also been found increased (spot no. 12). Spot intensity comparisons (allergy vs control) corresponding to the spot nos. 2, 7, 9, 12 and 13 mentioned above are shown in Figure 6. They illustrate the significance of the group differences observed for each protein.

Identification gave the following results (Table 3): spot no. 2, 70 kDa heat shock cognate protein (hsc70); spot no. 12, actin-capping protein (CAP1) $\alpha 1$ chain; spot no. 13, CAP1 β chain; and spot nos. 7 and 9, two hyperacidic isoforms of the eosinophil peroxidase heavy chain. The pI and M_r of hsc70 and the $\alpha 1$ and β subunits of CAP1 were close to their theoretical

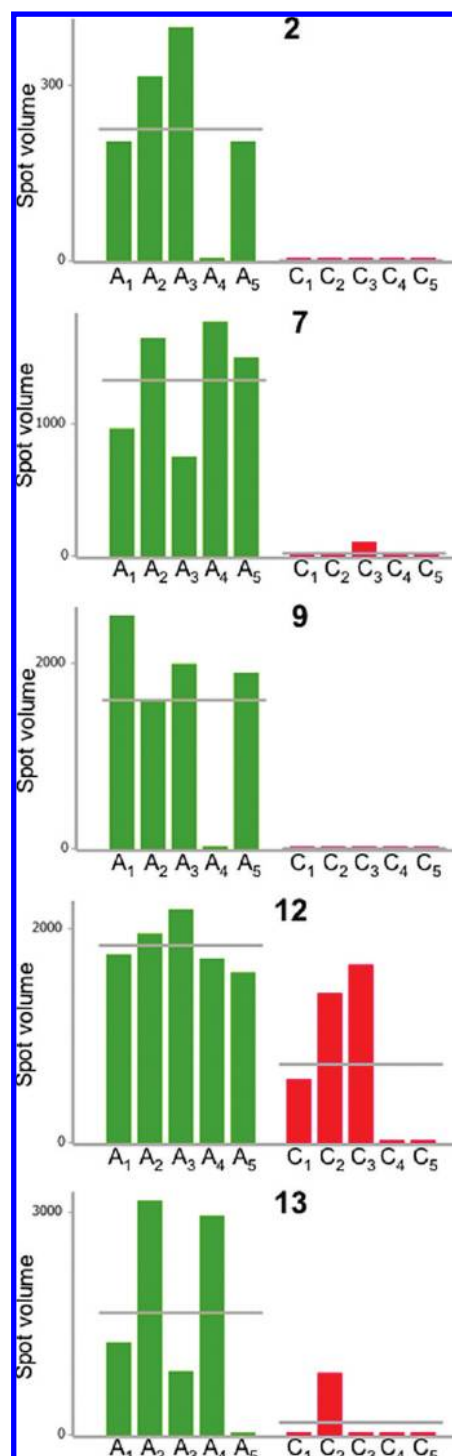


Figure 6. Quantification of the eosinophil proteins identified that were differentially expressed during allergy. Bar graphs of spot volumes, according to the report by the Ludesi Analysis Center (<http://www.ludesi.com>), A_1-A_5 , Birch pollen allergic subjects, C_1-C_5 , Control (asymptomatic) subjects.

values, while the pIs of the peroxidase heavy chain were >3 units lower than their basic theoretical value, without a significant change in M_r (Table 3). The proteins hsc70 and actin-capping protein, found increased in the eosinophils from birch pollen allergic subjects, are also on the list of eosinophil proteins in Table 1. Instead, isoforms of eosinophil peroxidase heavy chain as hyperacidic as the ones found during allergy had not been detected during the eosinophil proteome studies

Table 3. Proteins Overexpressed in Eosinophils from Birch Pollen-Sensitive Subjects^a

spot no. (Figure 4)	Protein identity	Swiss-Prot accession number	M_r/pI (theor.)	M_r/pI (exp.)	sequence coverage	matching peptides	score	fold increase ^c
2	hsc70	P11142	71.1/5.37	69.2/5.19	22%	10	103	∞
7	EPO heavy chain	P11678	53.4/10.79	53.7/7.48	5%	3	65	61.5
9	EPO heavy chain	P11678	53.4/10.79	50.7/7.30	5%	3	91	∞
12	Actin-capping, α 1 subunit	P52907	32.9/5.40	32.9/5.30	46%	8	101	2.5
13	Actin-capping, β subunit ^b	P47756	31.4/5.36	28.5/5.44	—	—	—	9.8

^a The experimental values of pI/M_r were calculated on the gel shown in Figure 5. ^b Protein identity tentatively ascribed by accurate comparison of the 2-D gels corresponding to the allergic subjects with those shown in Figure 1 (Suppl), A2 and B2, for which MS protein identification had been performed. ^c Fold increase ∞ means that the spot was present only in the 2-D gels corresponding to allergic subjects and not those of the control subjects.

(Table 1) nor were observed in the control group of the allergy study. It could not be established whether the light chain of EPO was more expressed in allergic subjects, as the heavy chain is, due to the very high background of silver staining in the low molecular mass area of the gels analyzed.

Discussion

The present work reports for the first time the identification of a number of human eosinophil proteins separated bidimensionally. Additionally, it describes the differential expression of proteins in eosinophils from subjects allergic to birch pollen compared with those from asymptomatic individuals, analyzed simultaneously at the peak of a pollen season.

Protein Composition of Eosinophils. We have identified the proteins present in 336 spots/bands obtained upon bidimensional (IEF/SDS-PAGE) and monodimensional (SDS-PAGE) separations of whole cells and of cytosolic and membrane subcellular fractions. These spots/bands corresponded to a total of 98 different proteins, since many of them were present as several spots of different pI s and/or as protein fragments. The pI of some of the protein forms detected here deviate significantly from their theoretical value (Table 2), and we discuss possible causes.

The bidimensional separations performed show an abundance of basic proteins in whole cell extracts and membrane fractions as opposed to the cytosol fraction. This is not surprising, since eosinophil granules are contained in both whole cell extracts and membrane fractions, and are rich in highly basic proteins such as eosinophil cationic protein (ECP), EPX/EDN, EPO and MBP.²⁶ Altogether, 11.3% of the proteins detected are from secretory compartments, and most of them are basic.

Three isoforms of EPX/EDN of pI between 9.67 and 10.60 (theoretical $pI = 9.20$) were observed on pH 3–11 IEF separations, whereas six forms were seen between pI 10.0 and 10.6 when the pH range was narrowed to 9–12. EPX/EDN variants within the pI range 8.5–10.5 have only been reported in human urine.²⁷ The hyperbasic forms of EPX/EDN observed here ($\Delta pI = +0.5$ to $+1.4$) could be originated, at least in part, by the methyl-esterification of aspartic acid residues that are abundant in the carboxy-terminal portion of the molecule. Methylations are compatible with some of the peptide peaks obtained after tryptic digestion, but it is not possible to know whether they are physiological or a consequence of the presence of methanol during the staining procedure. Glycosylation of EDN/EPX has also been reported,²⁸ but precise information about its nature is lacking.

ECP forms of two different pI s (10.50 and 10.16, $\Delta pI = 0.34$) were detected on pI 9–12 separations. These forms could correspond to the two molecules arising from the known

polymorphism of ECP, Arg97Thr.²⁹ The theoretical ΔpI between these two variants is 0.25 pH units (10.72 for Arg97 and 10.47 for Thr97 ECP, according to <http://www.expasy.org>). Since ECP is known to be glycosylated,^{30–32} pI variations could also result directly or indirectly from glycosylation differences. ECP phosphorylation has not been reported.

Apart from forms of the expected pI , we detected EPO heavy chains of pI s up to 1.4 units below the theoretical value of 10.79. We cannot rule out horizontal streaking artifacts due to the relative abundance of this protein in eosinophils, combined with its characteristic surface adherence. Nonetheless, the fact that forms of EPO heavy chain even more acidic, of pI 7.30 and 7.48, were detected in the eosinophils from birch pollen allergic subjects (Table 3) but not in those from the control group is in favor of the real existence of these molecules. We observed the presence of serially glycosylated residues among the tryptic peptides from one of the hyperacidic heavy EPO forms (Figure 7A). This supports the idea of EPO being a glycoprotein, which up to now was only hypothetical and based only on the presence of 4 putative N-glycosylation sites (<http://www.cbs.dtu.dk/services/NetNGlyc/>).³³ It can be speculated that modifications conferring negative charges such as sialylation might be occurring on carbohydrate side chains, which could explain the large negative pI shift. Other possible modifications are poly-ADP-ribosylation or polyglutamylation. The first is typically a nuclear protein modification and the second is not likely since polyglutamylation appears to require glutamic acid-rich stretches³⁴ and EPO heavy chain is poor in glutamic acid.

Three to four isoforms of eosinophil major basic protein homologue (MBP2) were observed, with pI s ranging from 9.7 to 10.5 against a theoretical value of 9.2. Such basic forms have not yet been reported in the literature. Methylated peptides were detected among the tryptic peptides; therefore, methyl-esterification could be contributing to the pI shift, as in the case of EPX/EDN. Nevertheless, methylation can be also a result of the gel staining procedure.

Cytoskeleton/migration proteins are abundantly represented in the eosinophil proteome, constituting 25% of the total. This reflects the importance of cell motility and adhesion and granule exocytosis among eosinophil functions, all of which involve actin polymerization-depolymerization. Cytoskeletal remodeling is modulated by a variety of actin-monomer-binding proteins.

Cofilin-1 was detected as isoforms of molecular mass close to the theoretical value but with pI s ranging from 5.92 to 8.55 against a theoretical pI of 8.26. This protein has actin-depolymerizing and severing activity³⁵ that is abolished by phosphorylation at Ser3.^{36,37} In neutrophils, cofilin undergoes dephosphorylation upon cell stimulation.³⁸ The detection of

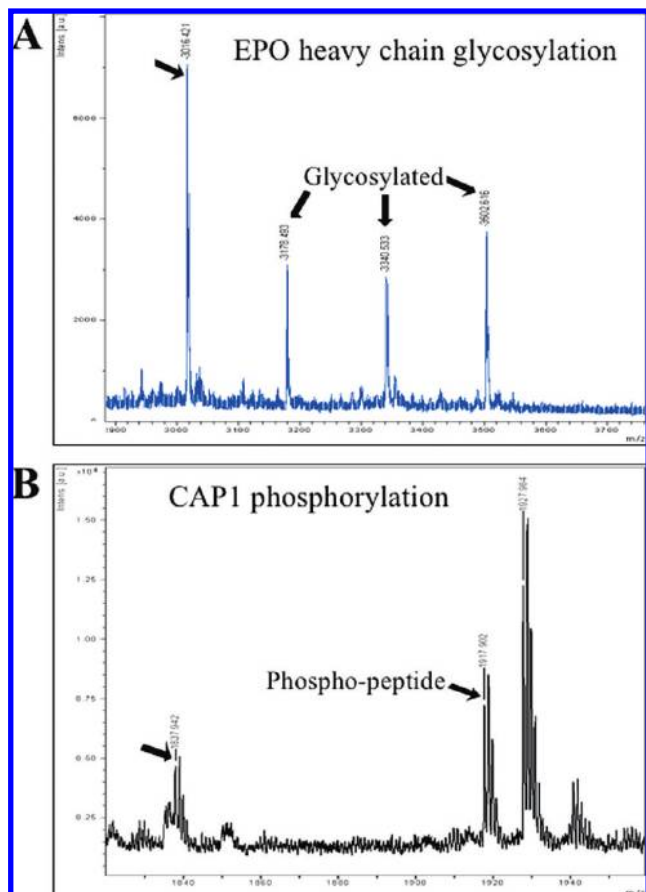


Figure 7. Mass spectra showing peptides that are evidence of EPO heavy chain glycosylation (A) and CAP1 phosphorylation (B). (A) The arrows indicate peaks of unglycosylated ($m/z = 3016.421$) and glycosylated ($m/z = 3178.493$, 3340.533 and 3502.616) peptides obtained from a tryptic digest of spot 213 (EPO heavy chain, see Table 1 and Figure 1B2 in Supporting Information). (B) MS of unphosphorylated ($m/z = 1837.942$) and phosphorylated ($m/z = 1917.902$) peptides corresponding to spot 220 (adenylyl cyclase-associated protein 1/CAP1, see Table 1 and Figure 1B2 in Supporting Information).

hyperacidic forms, with pI s 0.4–2.3 units below the theoretical value, suggests that cofilin-1 is partially inactive in resting eosinophils. Phosphorylation at more than one site or/and other modification/s such as lysine acetylation³⁹ and/or methylation would be necessary to explain the pI reductions observed. N-substitutions are possible on 25 lysine residues representing 15% of the polypeptide length. A highly acidic form of cofilin ($pI < 7$) has also been reported in human epidermis.⁴⁰

Moesin, an actin filament-binding protein, was detected as 6 isoforms within a pI range of 0.6 pH units around the theoretical value. This protein has been shown to be spatiotemporally controlled by reversible phosphorylation in neutrophils.⁴¹ Cell stimulation triggers dephosphorylation at Thr558 and this is associated to uropod retraction and rear release of adherent cells, that is, to motility. The number of forms we detected, though, cannot be explained by just one but by multiple phosphorylation events and/or by other modifications.

In addition to a form with the expected pI , we observed hyperacidic species of profilin-1, another actin-monomer-binding protein,³⁵ with pI s lower than the theoretical by 1.4–1.6 units. Phosphorylation of a serine residue at the carboxy-

terminal end by rat brain protein kinases C has been reported,⁴² but this alone would not explain such a ΔpI . Lysine acetylation, which would reduce its pI , is also known to occur.³⁹ The absence of glutamic acid-rich regions within the sequence makes polyglutamylation unlikely.

Three/four isoforms of adenylyl cyclase-associated protein 1 (CAP1) with pI s lower than the theoretical value by up to 1.7 pH units were observed, apart from one with a pI close to the theoretical one. Such acidic forms have not been reported to date. CAP1 binds actin monomers and favors a rapid actin dynamics in conjunction with cofilin.⁴³ We have detected a tryptic digest molecule compatible with the monophosphorylation of the peptide 295–312 of CAP1 (Figure 7B), in agreement with recent reports about the phosphorylation of S308 in platelets⁴⁴ and S310 in cancer cells.⁴⁵ Nevertheless, hyperacidic CAP1 forms should be the result of multiple phosphorylations, and/or of the addition of negative charges, for example, by glutamylation. Lysine acylation³⁹ is also possible, considering that the lysine content of CAP1 is almost 10%.

Isoforms of gelsolin, an actin-severing protein, were found within a narrow pI range close to the theoretical value. The tyrosine kinase c-Src has been shown to modify gelsolin mainly at Tyr438, as well as at several other minor sites.⁴⁶ Interaction of the tyrosine kinase PYK2 with gelsolin has been reported to result in actin filament uncapping at barbed ends in osteoclasts,⁴⁷ and hence in an increase in actin polymerization.

Coronin-1A is a protein exclusively expressed in leukocytes that accumulates at sites of rearrangement of the F-actin cytoskeleton and participates in the integration of plasma membrane signaling and cytoskeleton modulation.⁴⁸ It has been reported to participate in the assembly of the NADPH oxidase system of neutrophils.⁴⁹ We have detected acidic isoforms of coronin-1A compatible with multiple phosphorylations, in agreement with published work.⁴⁸

The fact that among the eosinophil protein components we found several endoplasmic reticulum components, namely, protein disulfide isomerase, calreticulin, grp78 and hsp70, is consistent with the capacity of these cells to process proteins.

We identified calgranulins A, B and C in eosinophils. These proteins, abundant in neutrophils, are associated to the interaction between phagocytes and endothelium⁵⁰ and to transmigration.⁵¹

Changes Elicited by Birch Pollen Allergy. The comparison of the proteomes of eosinophils from asymptomatic individuals with those from birch pollen allergic subjects, both isolated at a seasonal peak of airborne pollen, resulted in the detection of some significant changes. The changes consisted mostly (>90%) of increases in protein levels during allergy. We were able to assign an identity to the proteins in five of the spots with increased intensity: hsc70, two chains of the actin-capping protein (CapZ) and two eosinophil peroxidase heavy chain hyperacidic forms (Table 3). These proteins are not among those found differentially expressed in eosinophils from atopic dermatitis patients.⁸

The detection of novel peroxidase heavy chain forms in eosinophils during allergy is an intriguing question. The fact that the pI of these forms is >3 units lower than the theoretical value, without a change in M_r , is indicative of a post-translational modification introducing pluri-negative charges (e.g., by extensive sialylation or by polyglutamylation) or/and neutralizing a large number of side chain positive charges, for example, by lysine acylation³⁹ or oxidation⁵² or arginine deimination (citrullination).⁵³ A hypothetical sialylation has

been discussed above in this section (Protein Composition of Eosinophils) and is supported by our data showing glycosylated peptide peaks among the acidic forms of EPO heavy chain (Figure 7A). Glycosylated residues could constitute a frame on which sialylation is possible, although no sialylated peptide was detected. Polyglutamylation and acylation seem unlikely explanations due to the low glutamic acid and lysine content, respectively, of the EPO heavy chain. Protein oxidation or deimination, instead, would be compatible with an ongoing inflammatory situation like allergy, where reactive oxygen species are being generated. The lack of detection of protein forms with *pI*s intermediate between the theoretical, 10.79, and 7.39/7.48 (hyperacidic forms) favors the hypothesis of either a block modification or many simultaneous events that introduce a large number of negative charges or, alternatively, cancel positive ones. Because the light chain of EPO was undetectable in the gels due to a high silver staining background in the low molecular mass area, it was not possible to know whether the up-regulation of acidic EPO heavy chain forms is accompanied by a variation in the levels of the light chain.

Hsc70 is a constitutively expressed member of the Hsp70 class of heat shock proteins. These proteins are classically involved in protein folding and unfolding, possess peptide-binding capacity⁵⁴ and have also emerged as implicated in innate and adaptive immune systems.^{55,56} As a chaperone, hsc70 binds a number of proteins and mediates their correct folding and unfolding.⁵⁷ It is present in the cytosol, from where it has been reported to transport proteins to lysosomes for degradation. The lysosomal localization of a subset of hsc70^{58,59} results in its co-localization with MHC-II molecules. Published work demonstrates that overexpression of hsc70 in macrophages enhances the MHCII-mediated presentation of an exogenous protein, the data being consistent with a role for hsc70 in binding and protecting peptides from degradation and/or facilitating the kinetics of peptide transfer to MHC-II molecules.^{60,61} Besides their secretory function, eosinophils also act as antigen-presenting cells.^{62–64} Our finding of up-regulated levels of hsc70 in eosinophils from birch pollen allergic individuals at a seasonal allergy peak is consistent with a hypothetically increased antigen-presentation function, which would be helped by hsc70. This process starts in the lungs during allergen challenge, from where eosinophils migrate to the local lymph nodes and can present antigens.^{62,65} A proportion of antigen presentation-competent eosinophils could subsequently drain to the circulating blood, and be the hypothetical source of the significant increase in eosinophil hsc70 content observed by us. In that case, we would be actually detecting an average increment, implying that the hsc70 increase could be higher in the fraction of peripheral blood eosinophils of lymph node origin.

CapZ is an α/β heterodimeric, nonsevering actin-binding protein that selectively binds to the positive ends of actin filaments inhibiting polymerization and resulting in their being shorter.⁶⁶ CapZ from polymorphonuclear leukocytes is an abundant cytoplasmic protein that shares properties with that from *Dictyostelium*.⁶⁷ It is considered the major barbed-end terminator during cell motility, and it plays a major role in lamellipodial protrusion and motility.^{68,69} It has also been reported to augment the oriented movement of *Dictyostelium*.⁷⁰ Since we have found increased levels of both hsc70 and CapZ in eosinophils during allergy, it is worth noting that hsc70 has been reported to be a positive modulator of CapZ activity^{71,72} and therefore of cell motility. Hence, our results suggest that

blood eosinophils from allergic individuals, or at least a fraction of them, show clear signs of an increased potential to respond to chemoattractants.

The present study provides novel information on changes observed in human eosinophils during birch pollen allergy, at a comparative proteomics level. Being the first report on this subject, validation by ELISA or Western blot analysis will be needed using eosinophils from additional allergic individuals isolated during different pollen seasons.

Conclusions

The MS analysis of 336 spots/bands of eosinophil proteins from mono- and mostly bidimensional separations (IEF/SDS-PAGE) resulted in the identification of 98 different proteins. Among them, a number of novel hyperacidic and hyperbasic forms were present within the secretory granule and cytoskeleton/migration function groups. Protein forms with an experimental *pI* considerably lower than the theoretical value were detected for cofilin-1 ($\Delta pI \leq 2.3$), profilin-1 ($\Delta pI \leq 1.6$) and CAP1 ($\Delta pI \leq 1.7$). Forms with a *pI* substantially higher than expected were observed for EDN/EPX ($\Delta pI \leq 1.4$) and MBP2 ($\Delta pI \leq 1.3$). We also identified the triglycosylation of the heavy chain of EPO, within a protein form with an experimental *pI* 0.5 units below the theoretical value. Questions therefore rise about the origin and role of these yet undescribed protein forms.

Additionally, the differential expression of proteins in eosinophils from subjects allergic to birch pollen compared with those from asymptomatic individuals is described, after bidimensional analysis of cells obtained at the peak of a pollen season. Upon further MS identification, overexpression of 70 kDa heat shock cognate protein (hsc70), actin-capping protein (CapZ) and two novel, hyperacidic forms ($\Delta pI \leq 3.5$) of the heavy chain of eosinophil peroxidase were detected in cells from the allergy group. The induction of hsc70 is consistent with a protected/facilitated peptide transfer to MHC-II molecules and an up-regulated antigen-presentation function in eosinophils from allergic individuals. The induction of CapZ could be linked to hsc70 since the latter is also known to be a positive modulator of CapZ, which plays a major role in lamellipodial protrusion and cell motility.

In summary, eosinophils present in the circulation of birch pollen allergic individuals have been found to show signs of an increased potentiality for antigen-presentation and motility.

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Supporting Information Available: Supplemental Table 1 containing the list of proteins successfully identified in the spots/bands excised from the gels shown in Figure 1, with complete information about all spots processed (accession number, theoretical and experimental molecular mass and *pI*, MS-related information, gel from which the spot was cut out). Table 1 within the manuscript main body is an edited protein list containing all proteins but where protein fragments and some isoforms have been omitted. Supplemental Figure 1 shows all of the IEF 3–11 bidimensional separations from which spots were excised and then subjected to MS identification. Supplemental Figure 2 shows two IEF 9–12 bidimensional

separations from which spots were excised and subjected to MS identification. Supplemental Figure 3 shows a monodimensional SDS-PAGE of DeStreak-insoluble material, from which bands were excised and subjected to MS identification. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) Rothenberg, M. E.; Hogan, S. P. The eosinophil. *Annu. Rev. Immunol.* **2006**, *24*, 147–174.
- (2) Gleich, G. J. Mechanisms of eosinophil-associated inflammation. *J. Allergy Clin. Immunol.* **2000**, *105* (4), 651–663.
- (3) Lampinen, M.; Carlson, M.; Hakansson, L. D.; Venge, P. Cytokine-regulated accumulation of eosinophils in inflammatory disease. *Allergy* **2004**, *59* (8), 793–805.
- (4) Adamko, D. J.; Odemuyiwa, S. O.; Vethanayagam, D.; Moqbel, R. The rise of the phoenix: the expanding role of the eosinophil in health and disease. *Allergy* **2005**, *60* (1), 13–22.
- (5) Carlson, M.; Peterson, C.; Venge, P. The influence of IL-3, IL-5, and GM-CSF on normal human eosinophil and neutrophil C3b-induced degranulation. *Allergy* **1993**, *48* (6), 437–442.
- (6) Anderson, N. L.; Anderson, N. G. Proteome and proteomics: new technologies, new concepts, and new words. *Electrophoresis* **1998**, *19* (11), 1853–1861.
- (7) Levi-Schaffer, F.; Temkin, V.; Simon, H. U.; Kettman, J. R.; Frey, J. R.; Lefkowitz, I. Proteomic analysis of human eosinophil activation mediated by mast cells, granulocyte macrophage colony stimulating factor and tumor necrosis factor alpha. *Proteomics* **2002**, *2* (11), 1616–1626.
- (8) Yoon, S. W.; Kim, T. Y.; Sung, M. H.; Kim, C. J.; Poo, H. Comparative proteomic analysis of peripheral blood eosinophils from healthy donors and atopic dermatitis patients with eosinophilia. *Proteomics* **2005**, *5* (7), 1987–1995.
- (9) Adamko, D.; Lacy, P.; Moqbel, R. Eosinophil function in allergic inflammation: from bone marrow to tissue response. *Curr. Allergy Asthma Rep.* **2004**, *4* (2), 149–158.
- (10) Hakansson, L.; Carlson, M.; Stalenheim, G.; Venge, P. Migratory responses of eosinophil and neutrophil granulocytes from patients with asthma. *J. Allergy Clin. Immunol.* **1990**, *85* (4), 743–750.
- (11) Carlson, M.; Hakansson, L.; Peterson, C.; Stalenheim, G.; Venge, P. Secretion of granule proteins from eosinophils and neutrophils is increased in asthma. *J. Allergy Clin. Immunol.* **1991**, *87* (1 Pt. 1), 27–33.
- (12) Carlson, M.; Hakansson, L.; Kampe, M.; Stalenheim, G.; Peterson, C.; Venge, P. Degranulation of eosinophils from pollen-atopic patients with asthma is increased during pollen season. *J. Allergy Clin. Immunol.* **1992**, *89* (1 Pt. 1), 131–139.
- (13) Karawajczyk, M.; Seveus, L.; Garcia, R.; Bjornsson, E.; Peterson, C. G.; Roomans, G. M.; Venge, P. Piecemeal degranulation of peripheral blood eosinophils: a study of allergic subjects during and out of the pollen season. *Am. J. Respir. Cell Mol. Biol.* **2000**, *23* (4), 521–529.
- (14) Hakansson, L.; Heinrich, C.; Rak, S.; Venge, P. Priming of eosinophil adhesion in patients with birch pollen allergy during pollen season: effect of immunotherapy. *J. Allergy Clin. Immunol.* **1997**, *99* (4), 551–562.
- (15) Fernvik, E.; Gronneberg, R.; Lundahl, J.; Hed, J.; Andersson, O.; Johansson, S. G.; Hallden, G. The degree of natural allergen exposure modifies eosinophil activity markers in the circulation of patients with mild asthma. *Allergy* **1996**, *51* (10), 697–705.
- (16) Woschnagg, C.; Garcia, R.; Rak, S.; Venge, P. IL-5 priming of the PMA-induced oxidative metabolism of human eosinophils from allergic and normal subjects during a pollen season. *Clin. Exp. Allergy* **2001**, *31* (4), 555–564.
- (17) Woschnagg, C.; Rak, S.; Venge, P. Oxygen radical production by blood eosinophils is reduced during birch pollen season in allergic patients. *Clin. Exp. Allergy* **1996**, *26* (9), 1064–1072.
- (18) Woschnagg, C.; Venge, P.; Garcia, R. C. The effect of IL-5 treatment on the stimulation-induced phosphorylation of proteins in blood eosinophils. *Cytokine* **2004**, *28* (3), 137–148.
- (19) Mortz, E.; Krogh, T. N.; Vorum, H.; Gorg, A. Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis. *Proteomics* **2001**, *1* (11), 1359–1363.
- (20) Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **1999**, *20* (18), 3551–3567.
- (21) Gbarah, A.; Gahmberg, C. G.; Ofek, I.; Jacobi, U.; Sharon, N. Identification of the leukocyte adhesion molecules CD11 and CD18 as receptors for type 1-fimbriated (mannose-specific) *Escherichia coli*. *Infect. Immun.* **1991**, *59* (12), 4524–4530.
- (22) Urban, R. J.; Bodenburger, Y.; Kurosky, A.; Wood, T. G.; Gasic, S. Polypyrimidine tract-binding protein-associated splicing factor is a negative regulator of transcriptional activity of the porcine p450scs insulin-like growth factor response element. *Mol. Endocrinol.* **2000**, *14* (6), 774–782.
- (23) Lankes, W. T.; Furthmayr, H. Moesin: a member of the protein 4.1-talin-ezrin family of proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88* (19), 8297–8301.
- (24) Bystrom, J.; Garcia, R. C.; Hakansson, L.; Karawajczyk, M.; Moberg, L.; Soukka, J.; Venge, P. Eosinophil cationic protein is stored in, but not produced by, peripheral blood neutrophils. *Clin. Exp. Allergy* **2002**, *32* (7), 1082–1091.
- (25) Zabucchi, G.; Menegazzi, R.; Soranzo, M. R.; Patriarca, P. Uptake of human eosinophil peroxidase by human neutrophils. *Am. J. Pathol.* **1986**, *124* (3), 510–518.
- (26) Ackerman, S. J.; Loegering, D. A.; Venge, P.; Olsson, I.; Harley, J. B.; Fauci, A. S.; Gleich, G. J. Distinctive cationic proteins of the human eosinophil granule: major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin. *J. Immunol.* **1983**, *131* (6), 2977–2982.
- (27) Thongboonkerd, V.; Semangoen, T.; Chutipongtanate, S. Enrichment of the basic/cationic urinary proteome using ion exchange chromatography and batch adsorption. *J. Proteome Res.* **2007**, *6* (3), 1209–1214.
- (28) Tiffany, H. L.; Li, F.; Rosenberg, H. F. Hyperglycosylation of eosinophil ribonucleases in a promyelocytic leukemia cell line and in differentiated peripheral blood progenitor cells. *J. Leukocyte Biol.* **1995**, *58* (1), 49–54.
- (29) Trulsson, A.; Bystrom, J.; Engstrom, A.; Larsson, R.; Venge, P. The functional heterogeneity of eosinophil cationic protein is determined by a gene polymorphism and post-translational modifications. *Clin. Exp. Allergy* **2007**, *37* (2), 208–218.
- (30) Rosenberg, H. F.; Tiffany, H. L. Characterization of the eosinophil granule proteins recognized by the activation-specific antibody EG2. *J. Leukocyte Biol.* **1994**, *56* (4), 502–506.
- (31) Boix, E.; Carreras, E.; Nikolovski, Z.; Cuchillo, C. M.; Nogues, M. V. Identification and characterization of human eosinophil cationic protein by an epitope-specific antibody. *J. Leukocyte Biol.* **2001**, *69* (6), 1027–1035.
- (32) Eriksson, J.; Woschnagg, C.; Fernvik, E.; Venge, P. A SELDI-TOF MS study of the genetic and post-translational molecular heterogeneity of eosinophil cationic protein. *J. Leukocyte Biol.* **2007**, *82* (6), 1491–1500.
- (33) Thomsen, A. R.; Sottrup-Jensen, L.; Gleich, G. J.; Oxvig, C. The status of half-cystine residues and locations of N-glycosylated asparagine residues in human eosinophil peroxidase. *Arch. Biochem. Biophys.* **2000**, *379* (1), 147–152.
- (34) van Dijk, J.; Miro, J.; Strub, J. M.; Lacroix, B.; van Dorsselaer, A.; Edde, B.; Janke, C. Polyglutamylation is a post-translational modification with a broad range of substrates. *J. Biol. Chem.* **2008**, *283* (7), 3915–3922.
- (35) Paavilainen, V. O.; Bertling, E.; Falck, S.; Lappalainen, P. Regulation of cytoskeletal dynamics by actin-monomer-binding proteins. *Trends Cell Biol.* **2004**, *14* (7), 386–394.
- (36) Arber, S.; Barbayannis, F. A.; Hanser, H.; Schneider, C.; Stanyon, C. A.; Bernard, O.; Caroni, P. Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* **1998**, *393* (6687), 805–809.
- (37) Niwa, R.; Nagata-Ohashi, K.; Takeichi, M.; Mizuno, K.; Uemura, T. Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. *Cell* **2002**, *108* (2), 233–246.
- (38) Boldt, K.; Rist, W.; Weiss, S. M.; Weith, A.; Lenter, M. C. FPRL-1 induces modifications of migration-associated proteins in human neutrophils. *Proteomics* **2006**, *6* (17), 4790–4799.
- (39) Kim, S. C.; Sprung, R.; Chen, Y.; Xu, Y.; Ball, H.; Pei, J.; Cheng, T.; Kho, Y.; Xiao, H.; Xiao, L.; Grishin, N. V.; White, M.; Yang, X. J.; Zhao, Y. Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. *Mol. Cell* **2006**, *23* (4), 607–618.
- (40) Hensbergen, P.; Alewijnse, A.; Kempenaar, J.; van der Schors, R. C.; Balog, C. A.; Deelder, A.; Beumer, G.; Ponc, M.; Tensen, C. P. Proteomic profiling identifies an UV-induced activation of cofilin-1 and destrin in human epidermis. *J. Invest. Dermatol.* **2005**, *124* (4), 818–824.
- (41) Yoshinaga-Ohara, N.; Takahashi, A.; Uchiyama, T.; Sasada, M. Spatiotemporal regulation of moesin phosphorylation and rear release by Rho and serine/threonine phosphatase during neutrophil migration. *Exp. Cell Res.* **2002**, *278* (1), 112–122.

- (42) Singh, S. S.; Chauhan, A.; Murakami, N.; Styles, J.; Elzinga, M.; Chauhan, V. P. Phosphoinositide-dependent in vitro phosphorylation of profilin by protein kinase C. Phospholipid specificity and localization of the phosphorylation site. *Recept. Signal Transduct.* **1996**, *6* (2), 77–86.
- (43) Bertling, E.; Hotulainen, P.; Mattila, P. K.; Matilainen, T.; Salminen, M.; Lappalainen, P. Cyclase-associated protein 1 (CAP1) promotes cofilin-induced actin dynamics in mammalian nonmuscle cells. *Mol. Biol. Cell* **2004**, *15* (5), 2324–2334.
- (44) Zahedi, R. P.; Lewandrowski, U.; Wiesner, J.; Wortelkamp, S.; Moebius, J.; Schutz, C.; Walter, U.; Gambaryan, S.; Sickmann, A. Phosphoproteome of resting human platelets. *J. Proteome Res.* **2008**, *7* (2), 526–534.
- (45) Imami, K.; Sugiyama, N.; Kyono, Y.; Tomita, M.; Ishihama, Y. Automated phosphoproteome analysis for cultured cancer cells by two-dimensional nanoLC-MS using a calcined titania/C18 biphasic column. *Anal. Sci.* **2008**, *24* (1), 161–166.
- (46) De Corte, V.; Demol, H.; Goethals, M.; Van Damme, J.; Gettemans, J.; Vandekerckhove, J. Identification of Tyr438 as the major in vitro c-Src phosphorylation site in human gelsolin: a mass spectrometric approach. *Protein Sci.* **1999**, *8* (1), 234–241.
- (47) Wang, Q.; Xie, Y.; Du, Q. S.; Wu, X. J.; Feng, X.; Mei, L.; McDonald, J. M.; Xiong, W. C. Regulation of the formation of osteoclastic actin rings by proline-rich tyrosine kinase 2 interacting with gelsolin. *J. Cell Biol.* **2003**, *160* (4), 565–575.
- (48) Gatfield, J.; Albrecht, I.; Zanolari, B.; Steinmetz, M. O.; Pieters, J. Association of the leukocyte plasma membrane with the actin cytoskeleton through coiled coil-mediated trimeric coronin 1 molecules. *Mol. Biol. Cell* **2005**, *16* (6), 2786–2798.
- (49) Grogan, A.; Reeves, E.; Keep, N.; Wientjes, F.; Totty, N. F.; Burlingame, A. L.; Hsuan, J. J.; Segal, A. W. Cytosolic phox proteins interact with and regulate the assembly of coronin in neutrophils. *J. Cell Sci.* **1997**, *110* (Pt. 24), 3071–3081.
- (50) Frosch, M.; Streyl, A.; Vogl, T.; Wulffraat, N. M.; Kuis, W.; Sunderkotter, C.; Harms, E.; Sorg, C.; Roth, J. Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. *Arthritis Rheum.* **2000**, *43* (3), 628–637.
- (51) Eue, I.; Pietz, B.; Storck, J.; Klempt, M.; Sorg, C. Transendothelial migration of 27E10+ human monocytes. *Int. Immunol.* **2000**, *12* (11), 1593–1604.
- (52) Hazell, L. J.; van den Berg, J. J.; Stocker, R. Oxidation of low-density lipoprotein by hypochlorite causes aggregation that is mediated by modification of lysine residues rather than lipid oxidation. *Biochem. J.* **1994**, *302* (Pt. 1), 297–304.
- (53) Gyorgy, B.; Toth, E.; Tarcsa, E.; Falus, A.; Buzas, E. I. Citrullination: a posttranslational modification in health and disease. *Int. J. Biochem. Cell Biol.* **2006**, *38* (10), 1662–1677.
- (54) Haug, M.; Schepp, C. P.; Kalbacher, H.; Dannecker, G. E.; Holzer, U. 70-kDa heat shock proteins: specific interactions with HLA-DR molecules and their peptide fragments. *Eur. J. Immunol.* **2007**, *37* (4), 1053–1063.
- (55) Javid, B.; MacAry, P. A.; Lehner, P. J. Structure and function: heat shock proteins and adaptive immunity. *J. Immunol.* **2007**, *179* (4), 2035–2040.
- (56) Basu, S.; Srivastava, P. K. Heat shock proteins: the fountainhead of innate and adaptive immune responses. *Cell Stress Chaperones* **2000**, *5* (5), 443–451.
- (57) Bercovich, B.; Stancovski, I.; Mayer, A.; Blumenfeld, N.; Laszlo, A.; Schwartz, A. L.; Ciechanover, A. Ubiquitin-dependent degradation of certain protein substrates in vitro requires the molecular chaperone Hsc70. *J. Biol. Chem.* **1997**, *272* (14), 9002–9010.
- (58) Terlecky, S. R.; Chiang, H. L.; Olson, T. S.; Dice, J. F. Protein and peptide binding and stimulation of in vitro lysosomal proteolysis by the 73-kDa heat shock cognate protein. *J. Biol. Chem.* **1992**, *267* (13), 9202–9209.
- (59) Agarraberes, F. A.; Terlecky, S. R.; Dice, J. F. An intralysosomal hsp70 is required for a selective pathway of lysosomal protein degradation. *J. Cell Biol.* **1997**, *137* (4), 825–834.
- (60) Panjwani, N.; Akbari, O.; Garcia, S.; Brazil, M.; Stockinger, B. The HSC73 molecular chaperone: involvement in MHC class II antigen presentation. *J. Immunol.* **1999**, *163* (4), 1936–1942.
- (61) Li, P.; Gregg, J. L.; Wang, N.; Zhou, D.; O'Donnell, P.; Blum, J. S.; Crotzer, V. L. Compartmentalization of class II antigen presentation: contribution of cytoplasmic and endosomal processing. *Immunol. Rev.* **2005**, *207*, 206–217.
- (62) Shi, H. Z.; Humbles, A.; Gerard, C.; Jin, Z.; Weller, P. F. Lymph node trafficking and antigen presentation by endobronchial eosinophils. *J. Clin. Invest.* **2000**, *105* (7), 945–953.
- (63) Tamura, N.; Ishii, N.; Nakazawa, M.; Nagoya, M.; Yoshinari, M.; Amano, T.; Nakazima, H.; Minami, M. Requirement of CD80 and CD86 molecules for antigen presentation by eosinophils. *Scand. J. Immunol.* **1996**, *44* (3), 229–238.
- (64) Akuthota, P.; Wang, H. B.; Spencer, L. A.; Weller, P. F. Immunoregulatory roles of eosinophils: a new look at a familiar cell. *Clin. Exp. Allergy* **2008**, *38* (8), 1254–1263.
- (65) Duez, C.; Dakhama, A.; Tomkinson, A.; Marquillies, P.; Balhorn, A.; Tonnel, A. B.; Bratton, D. L.; Gelfand, E. W. Migration and accumulation of eosinophils toward regional lymph nodes after airway allergen challenge. *J. Allergy Clin. Immunol.* **2004**, *114* (4), 820–825.
- (66) Borisy, G. G.; Svitkina, T. M. Actin machinery: pushing the envelope. *Curr. Opin. Cell Biol.* **2000**, *12* (1), 104–112.
- (67) Maun, N. A.; Speicher, D. W.; DiNubile, M. J.; Southwick, F. S. Purification and properties of a Ca(2+)-independent barbed-end actin filament capping protein, CapZ, from human polymorphonuclear leukocytes. *Biochemistry* **1996**, *35* (11), 3518–3524.
- (68) Mejillano, M. R.; Kojima, S.; Applewhite, D. A.; Gertler, F. B.; Svitkina, T. M.; Borisy, G. G. Lamellipodial versus filopodial mode of the actin nanomachinery: pivotal role of the filament barbed end. *Cell* **2004**, *118* (3), 363–373.
- (69) Lai, F. P.; Szczodrak, M.; Block, J.; Faix, J.; Breitsprecher, D.; Mannherz, H. G.; Stradal, T. E.; Dunn, G. A.; Small, J. V.; Rottner, K. Arp2/3 complex interactions and actin network turnover in lamellipodia. *EMBO J.* **2008**, *27* (7), 982–992.
- (70) Hug, C.; Jay, P. Y.; Reddy, I.; McNally, J. G.; Bridgman, P. C.; Elson, E. L.; Cooper, J. A. Capping protein levels influence actin assembly and cell motility in dictyostelium. *Cell* **1995**, *81* (4), 591–600.
- (71) Haus, U.; Trommler, P.; Fisher, P. R.; Hartmann, H.; Lottspeich, F.; Noegel, A. A.; Schleicher, M. The heat shock cognate protein from Dictyostelium affects actin polymerization through interaction with the actin-binding protein cap32/34. *EMBO J.* **1993**, *12* (10), 3763–3771.
- (72) Tardieux, I.; Baines, I.; Mossakowska, M.; Ward, G. E. Actin-binding proteins of invasive malaria parasites and the regulation of actin polymerization by a complex of 32/34-kDa proteins associated with heat shock protein 70 kDa. *Mol. Biochem. Parasitol.* **1998**, *93* (2), 295–308.

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