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Light-Induced Phosphorylation of Crystallins in the Retinal Pigment Epithelium

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Abstract

Protein phosphorylations have essential regulatory roles in visual signaling. Previously, we found that phosphorylation of several proteins in the retina and the retinal pigment epithelium (RPE) is involved in anti-apoptotic signaling under oxidative stress conditions, including light exposure. In this study, we used a phosphoprotein enrichment strategy to evaluate the light-induced phosphoproteome of primary bovine RPE cells. Phosphoprotein-enriched extracts from bovine RPE cells exposed to light or dark conditions for 1 hour were separated by 2D SDS-PAGE. Serine and tyrosine phosphorylation were visualized by 2D phospho western blotting and specific phosphorylation sites were analyzed by tandem mass spectrometry. Light induced a marked increase in tyrosine phosphorylation of beta crystallin A3 and A4. The most abundant light-induced up-regulated phosphoproteins were crystallins of 15–25-kDa, including beta crystallin S and zeta crystallin. Phosphorylation of beta crystallin suggests an anti-apoptotic chaperone function in the RPE. Other chaperones, cytoskeletal proteins, and proteins involved in energy balance were expressed at higher levels in the dark. A detailed analysis of RPE phosphoproteins provides a molecular basis for understanding light-induced signal transduction and anti-apoptosis mechanisms. Our data indicates that phosphorylation of crystallins likely represents an important mechanism for RPE shielding from physiological and pathophysiological light-induced oxidative injury.

Keywords

phosphoproteome; retinal pigment epithelium; crystalline; 2D electrophoresis; mass spectrometry

Introduction

Phosphorylation of specific amino acids is an important modulator of protein function that regulates protein subcellular localization, protein–protein interactions, conformational

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changes, and signal transduction [1]. The change in charge that accompanies phosphorylation creates a switch mechanism, allowing reversible phosphorylation to modify intracellular signaling in response to specific microenvironmental conditions and thereby act as a basic survival tool. Phosphorylation is mediated by the action of protein kinases, which add phosphate to serine, threonine and tyrosine residues at a relative ratio of 1000 to 100 to 1 [2]. This differential modification reflects the distinct pKa values of these amino acids, and the distinct properties of their respective specific Ser/Thr kinases and Tyr kinases. Equally important in the dynamic regulation of protein phosphorylation is the activity of specific protein phosphatases. Phosphorylation may also interact with other post-translational modifications such as ubiquitination through positive or negative feedback mechanisms, a crosstalk that adds an additional dimension to the combinatorial possibilities of this switch mechanism [3].

The retinal pigment epithelium (RPE) is a cellular monolayer that provides crucial support to the retina, and transports oxygen, nutrients, and vitamin A. RPE is essential to the visual cycle responsible for maintaining sight, which is in turn mediated by the continuous regeneration of 11-*cis*-retinaldehyde as a rhodopsin chromophore. Loss of RPE cells is thought to be an early event in retinal degeneration [6]. Many critical reactions involved in RPE responses to environmental changes, such as oxidative stress, light exposure and changes in nutrients, especially those involving a rapid response, are mediated by phosphorylation events rather than by changes at the transcriptional level [7,8]. For example, we have demonstrated that oxidative stress, such as bright light exposure, increases the phosphorylation of Janus kinase 2 (Jak2), which is the downstream regulator of erythropoietin previously [9].

Typically, phosphoproteins have been studied using chemical and affinity-based methods [4,5]. However, the rapid and dynamic nature of the underlying changes and the low abundance of phosphoproteins reflecting their substoichiometry present challenges to the quantitative study of the phosphoproteome. Thus, the isolation of the phosphoproteome or phosphopeptidome represents a potentially advanced step in this analysis. In this study, we introduced a system-wide, unbiased, and high-throughput approach to investigate global phosphoproteomes of light- and dark-exposed RPE using a phosphoproteome enrichment method. This detailed analysis of the phosphoproteome provides a basis for understanding the molecular mechanisms of phosphorylation-dependent signaling in the RPE.

Materials and Methods

Preparation of RPE proteins

Fresh bovine eyes were obtained from a local abattoir (Brown Packing Company, Gaffney, SC) immediately after excision from the animal. The post-mortem stability, procedures for preparing RPE cells and general proteomic techniques have been described in detail previously [11–15]. Briefly, bovine eyes were opened 5 mm posterior to the limbus, and the vitreous and retina were removed. After washing with a phosphate-buffered saline (PBS), eye-cups were incubated in 0.25% trypsin in Dulbecco's minimum essential medium (DMEM; Gibco, Grand Island, NY) for 60 minutes at 37°C. RPE cells were collected under a dissecting microscope using a Pasteur pipette. After adding the culture medium (DMEM/

F12) containing 10% fetal bovine serum (FBS), cells were centrifuged and resuspended in a culture medium, and plated into 6-well plates (Nunc). Second passage cells were used for experiments. Cells were incubated under dark or light (700 lux) conditions in a humidified chamber at room temperature for 1 hour. RPE cells were incubated in lysis buffer consisting of 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 1 mM NaF, 1 mg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride for 1 hour on ice. The cell debris was discarded by centrifuging at 15115 × g for 15 minutes at 4°C, and the supernatant was obtained and stored at -80°C for later use.

Phosphoprotein and phosphopeptide enrichment

Phosphoproteins were collected by charge-based spin-column chromatography using a phosphoprotein enrichment kit (Pierce). The protein concentration was determined by a Bradford protein assay (Biorad), and adjusted to a final concentration of 0.5 mg/mL with a binding/wash buffer containing 0.25% CHAPS, as suggested by the manufacturer. Columns were loaded with 2 mg proteins, and incubated, washed and eluted, as described by the manufacturer. Eluted proteins were applied to a desalting column (Thermo scientific) prior to 2D electrophoresis. Phosphopeptide enrichment was performed as described previously [10]. Briefly, trypsin-digested peptides were enriched using a phosphopeptide isolation kit (Pierce). The peptides were mixed with a binding buffer (10% acetic acid, 50 µL), and were added to a SwellGel Disc (Pierce, Gallium III resin). The peptide-resin mixture, kept at a pH below 3.5, was incubated for 8 minutes at room temperature and gently swirled every minute to maintain the resin in suspension. After incubation, the peptide-resin mixture was centrifuged at 760 × g for 1 minute. The collected resin was washed twice with 50 µL washing buffer 1 (0.1% acetic acid), and twice with 50 µL washing buffer 2 (0.1% acetic acid, 10% acetonitrile). Phosphopeptides were incubated for 5 minutes and then eluted in 50 µL elution buffer (100 mM ammonium bicarbonate, pH 9.0). Eluted phosphopeptides were analyzed by Bruker tandem time-of-flight (TOF-TOF) mass spectrometry at the Mass Spectrometry Facility at the University of South Carolina.

Alkaline phosphatase treatment of the phosphoproteome

The phosphorylation status of isolated phosphoproteins was confirmed *in vitro* by dephosphorylation of the phosphoproteome. Enriched RPE phosphoproteins were treated with alkaline phosphatase (calf intestinal, Promega Corporation, MI) in a reaction buffer consisting of 1 mM Tris-HCl (pH 8.0) and 10 mM MgCl₂ and incubated at 37°C for 60 minutes. Dephosphorylated proteins (50 µg) were analyzed by two-dimensional (2D) SDS-PAGE and compared to control samples that were not treated with phosphatase. Proteins were visualized by Coomassie blue staining and analyzed by TOF-TOF mass spectrometry.

2D gel electrophoresis and western blotting

Isoelectric focusing was performed on an IEF system (GE Healthcare) using 7- and 11-cm IEF strips with a pH gradient of 3–10 (Biorad) at a total of 12360 Vh (11 cm strip) or 6860 Vh (7 cm strip). Enriched phosphoproteins (100 µg) or total RPE proteins were mixed with a 2X rehydration buffer (7 M Urea, 2 M Thiourea, 2% pH 3–10 Pharmalyte, 100 mM DTT, 200 µl 4% CHAPS) and actively rehydrated. Alkaline phosphatase-treated samples (50 µg)

were loaded onto 7-cm strips. Strips were equilibrated for 15 minutes in a SDS equilibration buffer (50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS), followed by reduction and alkylation (2% DTT, 2.5% iodoacetamide) for 15 minutes. Strips were loaded on 8%–16% gradient precast SDS-PAGE gels (Bio-Rad). Electrophoresis was run in duplicate, one for Coomassie/silver staining and one for 2D western blotting. For 2D western blotting, proteins were transferred onto a PVDF membrane and the membrane was blocked with 1% BSA in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature. The membrane was incubated overnight at 4°C with an anti-phospho-serine (Santa Cruz biotechnology, Santa Cruz, CA) or anti-phospho-tyrosine (Santa Cruz biotechnology, Santa Cruz, CA) primary antibodies, diluted 1:1000. The membrane was then washed with TBST and incubated with a horseradish peroxidase-conjugated anti-mouse secondary antibody (Bio-Rad), diluted 1:20000. Immunoreactive protein spots were visualized using an enhanced chemiluminescence (ECL) system (GE Healthcare, Piscataway, NJ). Films or gels were scanned and protein spots were analyzed densitometrically using ImageJ [11] and TopSpot software. Gels were silver stained following Dunn's mass-compatible silver staining method [12]. To visualize phosphoproteins, 2D gels were stained by Giovanni Candiano's blue silver method [13] or Pro-Q Diamond phosphoprotein staining kit (Invitrogen). Using actin and tubulin, included as positive landmark spots, silver-stained images were aligned with 2D western blots, and target gel spots were excised, digested, and analyzed by mass spectrometry.

Mass spectrometry analysis

For protein digestion, gel pieces were washed twice with 50% acetonitrile (MeCN) containing 25 mM ammonium bicarbonate, followed by 100% MeCN, and then dried. Gel pieces were rehydrated in a digestion buffer (25 mM ammonium bicarbonate containing 20 ng/ μ L trypsin [Promega Corporation, MI] and 20% MeCN) by incubating for 1 hour at 4°C, and then digested by incubating overnight at 37°C. After incubation, the digestion buffer was collected and peptides in gel pieces were further extracted using 25 mM ammonium bicarbonate and 50% MeCN. Peptides were combined and concentrated to a final volume of 10 μ L using a Speed Vac. Peptides were purified and desalted using a C₁₈ zip-tip (Millipore Corporation, MA), and concentrated to 3 μ L. Purified concentrated peptides were mixed with alpha-cyano 5-hydroxy cinnamic acid (12 mg/mL) as an organic matrix in 70% MeCN with 0.1% trifluoroacetic acid (TFA), loaded onto a Bruker anchorchip, and crystallized. The mass spectrometer was operated at an accelerating voltage of 19 kV in the reflectron mode, with an m/z range of 600 to 3500. Spectra were acquired from an average of 200 laser shots. Monoisotopic peptide mass was obtained and internally calibrated with peptides from trypsin autoprolysis (m/z 842.509 and 2211.105). Typical mass accuracy was within a tolerance of 100 ppm.

For tandem mass analysis, LIFT-MS/MS spectra were obtained by metastable ions generated by laser-induced decomposition of selected precursor ions. This was accomplished by inserting a LIFT cell in the ion flight path in the first TOF region. Parent and fragment ions were re-accelerated and traveled with different velocities according to their masses, and were focused on the detector after passing through the reflectron. LIFT-TOF/TOF spectra were acquired with an Ultraflex III TOF/TOF mass spectrometer using Flexcontrol 3.0

software (Bruker Daltonics, Billerica, MA). Ionization was accomplished using a Smart beam Nd:YAG laser. Peptide ions were accelerated at 25 kV. In LIFT TOF/TOF mode, precursor ions were accelerated to 8 kV and selected in a timed ion gate. In the LIFT-cell, fragments were further accelerated to 19 kV. Reflector potential was 29 kV. MS/MS spectra were analyzed using the MASCOT protein-searching engine (Matrix Science, London, UK), based on NCBI (<http://www.ncbi.nlm.nih.gov/>) and Expasy (<http://expasy.org/>) database.

Results

To investigate the RPE phosphoproteome, we used 2D electrophoresis to separate proteins from primary bovine RPE cells incubated under light or dark conditions for 1 hour. RPE proteins induced by light were visualized using mass spectrometry-compatible silver staining [9] (Fig 1A). Serine- and tyrosine-specific phosphorylation was visualized by 2D-western-blot analysis using anti-phosphoserine and anti-phosphotyrosine antibodies, respectively (Fig 1B, 1C). MALDI-TOF mass spectrometry analysis revealed that a large proportion of the proteins up-regulated first and foremost by light in the RPE belong to the beta crystallin family (Table 1). Tyrosine phosphorylation of beta crystallin A3 and A4 (#1–8 in Table 1) was markedly increased in the light, as shown by phospho-Tyr-specific western blotting, whereas serine phosphorylation of beta crystallin B2 (#10 in Table 1) was decreased in the light. Notably, opposite effects on serine and tyrosine phosphorylation may yield positive or negative regulation with the same stimulus, as has been shown in insulin signaling [14]. Enzymes associated with phosphorylation or energy metabolism, including Ser/Thr protein kinase 4 and ATP synthase, were downregulated in the light.

To overcome the challenge of detecting low-abundance phosphoproteins (10–50% of total proteins) in the RPE, we used metal-based (Ga^{+3}) affinity chromatography to isolate phosphorylated proteins. With this method, phosphoproteins were concentrated using a spin column containing a proprietary metal/buffer matrix that extracts 10–25% of total proteins (based on charge), with negligible nonspecific binding. Phosphoprotein-enriched protein samples were separated by 2D PAGE and proteins were visualized by silver staining (Fig 2). Light-induced proteins were abundant in 15–25 kDa ranges, whereas dark-induced proteins were observed in 40–60 kDa. Protein spots exhibiting a change in phosphorylation status were further analyzed by TOF-TOF mass spectrometry. Major phosphoproteins with a molecular weight of 15–25 kDa were identified as crystallin family (Table 2). Two proteins identified as beta crystallin S and zeta crystallin are new candidates for phosphorylation modification. Fold changes in phosphorylation, sequence coverage, and the number of peptides identified by tandem mass spectrometry are shown in Table 2. Other chaperones, cytoskeletal proteins, and proteins associated with energy metabolism were expressed at 1.5- to 6-fold up-regulated in the dark (Table 3). Tandem mass spectrometry revealed the specific phosphorylation sites in crystallin alpha-A and alpha-B (Fig. 3). Phosphorylations at serine residues were next to proline or near proline. The results of the phosphoproteome enrichment experiment were confirmed using an affinity-based phosphopeptide-enrichment strategy. In this experiments, in-solution trypsin digested peptides were concentrated using a metal based column and analyzed by TOF-TOF mass analysis.

To confirm reversible phosphorylation reactions of enriched phosphoproteins, we tested dephosphorylation reactions of target proteins. RPE phosphoproteins were treated with alkaline phosphatase (AP). Phosphoproteins from light- or dark-exposed RPE cells, treated with (+AP) or without (–AP) alkaline phosphatase, were separated by 2D PAGE and visualized by Coomassie staining (Fig. 4). A comparison of +AP and –AP (control) conditions showed that alkaline phosphatase treatment reduced or eliminated phosphoprotein spots partially. In particular, alkaline phosphatase reactions demonstrated that the target proteins, beta crystallin B1 and B2, glutamine synthetase, and tubulin alpha1B and beta2, were specifically dephosphorylated. However, major phosphorylated spots in light were still remained after phosphatase treatment implying that crystalline family may require their-specific phosphatase for dephosphorylation reaction.

Discussion

Phosphorylation is a key reversible post-translational modification involved in controlling the localization, interaction, and activity of specific proteins [1]. Under conditions of moderate oxidative stress, many proteins exert a cytoprotective effect through changes in their expression level and/or as a result of post-translational modifications [7,8]. Previously, we demonstrated that phosphorylation of Jak2 is involved in neuroprotective signaling in an oxygen imbalance model (1% hypoxic, 50% hyperoxic, H₂O₂, bright light, and constant light) in RPE and retinal cell cultures [9]. In the current study, we investigated global phosphoproteome changes in the RPE after short-term light exposure to provide a basis for understanding the early molecular signatures of light-induced signal transduction. Phosphoproteins from primary bovine RPE cells were enriched using a Ga³⁺/TiO₂-based column purification procedure and were visualized by silver staining or phospho-staining after separation by 2D SDS-PAGE.

In a parallel experiment, whole phosphopeptides, from in-solution trypsin digestion and phosphopeptide enrichment, were analyzed by TOF-TOF mass analysis to identify specific phosphorylation sites. The sequences surrounding pSer, pThr and pTyr were categorized based on functional domain context. To confirm the target-specific phosphorylation changes, we treated the entire phosphoproteome with gamma-phosphatase to remove phosphate groups. Using this 2D phospho-specific western-blot (pSer and pTyr) and mass spectrometry (TOF-TOF) approach, we identified approximately 60 phosphoproteins. A partial list of phosphoproteins includes members of the crystallin family, heat-shock proteins, retinoid-binding proteins, kinases and serotransferases.

Light exposure or oxidative stress can trigger apoptosis of the photoreceptor and RPE. For homeostatic responses, stress conditions might expect up-regulated neuroprotective and/or anti-apoptotic proteins. Among the major light-induced up-regulated proteins and phosphoproteins in the bovine RPE cells were members of the crystallin family, including β - and γ -crystallin. Once thought to be confined to the lens, crystallin proteins are now known to be expressed in various extralenticular tissues [15–17]. The physiological roles of crystallins in the lens, as well as their modifications, intrinsic changes, and mobility on 2D gels have been investigated [18–22].

The ability of α -crystallins to act as molecular chaperones to prevent the non-specific aggregation of denatured proteins and to suppress mitochondrial oxidative stress-mediated apoptosis of photoreceptor cells have been well established [23,24]. However, much less is known about the cellular functions of β/γ -crystallins [15]. Recently, the locations and functions of crystallins in the retina have been described [15,25]. α A- and α B-crystallin are located in the ganglion cell layer and photoreceptor layer, whereas β -crystallin is detected in all nuclear layers of the retina [15]. The functional role of crystallins as chaperones, anti-apoptotic proteins or signal transducers in the retina have also been described [15,25–27]. Phosphorylated α B-crystallin directly interacts with Bax and caspase-3 to suppress their pro-apoptotic action, and thus exerts a cytoprotective effect in the retina [27]. Light-induced up-regulation of α A-crystallin and increased phosphorylation of α A-crystallin in the aged retina have been reported [26]. The specific function of each crystallin, particularly β -crystallin in the RPE, is still largely unknown, although levels of α B-crystallin are increased after heat shock and oxidative stress [28], and α B-crystallin immunoreactivity has been found in both rod outer segments and the RPE after light exposure [25]. Phosphorylation-dependent signaling might be different between tissues, and this might reflect the microenvironmental diversity among different tissues, including differences in stress stimuli. Changes in the global phosphoproteome may be indicative of an early signaling event under oxidative stress conditions in the RPE. Increased phosphorylation of zeta crystallin, β -crystallin A3, A4, and S, in particular, might suggest a stress-response role for these proteins in the RPE. Accordingly, crystallin phosphorylation might provide an anti-apoptotic signal to attenuate a light-induced degenerative pathway. In this context, stress-induced up- or down-regulation of crystallins is known to occur in various diseases or age-related conditions [29,30].

Cytoskeletal proteins in the RPE, including actin and tubulin, were down-regulated in light. The functional relationship between heat-shock proteins and cytoskeletal proteins, such as intermediate filaments, vimentin and actin, is well characterized [15,31]. N-terminal phosphorylation of heat-shock proteins is correlated with stabilization of cytoskeletal elements [31].

Oxidative stress-mediated protein phosphorylation protects RPE cells from apoptosis and thus enhances RPE cell survival [8]. Constant light-induced retinal/RPE degeneration has been suggested as a major risk factor in age-related macular degeneration (AMD) [32,33]. However, light exposure may also initiate protective signaling in the RPE via crystallin phosphorylation. The global phosphoproteome of the light-exposed RPE characterized here will help to identify key elements of phosphorylation signaling that follow light-induced oxidative stress and serve as a framework for understanding retinal degeneration at the molecular level under a variety of stress conditions.

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Fig. 1. Analysis of light-associated changes in protein tyrosine/serine phosphorylation by 2D gel electrophoresis

Primary bovine RPE cells were incubated under light or dark conditions for 1 hour at room temperature. The retinal pigment epithelium proteins were isolated and analyzed by 2D electrophoresis and western blotting using anti-pSer and anti-pTyr antibodies. (A) Light-treated RPE cells. Proteins were separated by 2D-electrophoresis and visualized by silver staining. Phosphorylated proteins were visualized by pSer (B) or pTyr (C) western blot. (D) Dark-induced proteins were visualized by silver staining, pSer (E), and pTyr (F) western blot, respectively.

Fig. 2. Analysis of the phospho-enriched RPE proteome

Total RPE proteins were enriched by affinity purification using a Ga^{3+} column. The phosphoproteome of light-induced (A) and dark-incubated control (B) RPE cells are shown. Proteins analyzed by mass spectrometry are shown in numbers. Identified proteins are listed in Table 2 and 3.

Fig. 3. MALDI-TOF-TOF mass analysis of phosphoprotein-enriched RPE proteome
Phosphoproteins were analyzed by tandem mass spectrometry to identify phosphorylated amino acid residues. Mass spectra analyzed by MALDI-TOF-TOF shows amino acid sequence and phosphorylated serine residues. The peptide sequence and parental protein names are shown and phosphate groups on serine within the parent peptide sequence are denoted by pS. X axis represents mass to charge ratio (m/z) and Y axis shows relative ion intensity.

Fig. 4. Confirmation of phosphoproteins in the RPE by alkaline phosphatase treatment

The phosphoproteome of light-treated (A, B) and dark-incubated (C, D) RPE cells were split into two aliquots and incubated with alkaline phosphatase (+ AP) or without alkaline phosphatase (– AP). The samples were separated by 2D gel electrophoresis and proteins were visualized by Coomassie blue staining. In alkaline phosphatase-treated samples, phosphorylated protein spots were down-regulated or disappeared entirely. However, major phosphorylated spots were still remaining implying crystalline family might be resistant to non-specific phosphatase reaction.

Fig. 5.

Table 1

Light- or dark-induced Proteins in RPE cells.

Spot no.	Light-induced up-regulated proteins	Accession no.	Seq. Cov. (%) ^a	MW (Dalton) ^b	pI ^b
1	Beta A4 crystallin	P11842	49.5	23861	5.8
2	Beta A4 crystallin	P11842	27.6	23861	5.8
3	Beta A4 crystallin	P11842	27.6	23861	5.8
4	Beta A4 crystallin	P11842	27.6	23861	5.8
5	Beta A3 crystallin	Q6DTZ7	33	25568	6
6	Beta A3 crystallin	P11843	25.1	25131	6
7	Beta crystallin S	P06504	37.1	20928	6.4
8	Beta A3 crystallin	Q6DTZ7	25	25568	6
10	Beta-crystallin B2	P02522	64.4	23298	6.4
21	Beta A3 crystallin	Q6DTZ7	20	25568	6
22	Retinal pigment epithelium-specific 65 kDa protein	Q28175	19.9	60945	6
23	serum albumin precursor	P02769	30.1	69294	5.8

Spot no.	Light-induced down-regulated proteins	Accession no.	Seq. Cov. (%)	MW (Dalton)	pI
12	Actin, cytoplasmic 1	P60712	45.1	41737	5.3
14	Serine/threonine-protein kinase 4	Q5E9L6	12.7	55588	4.9
18	ATP synthase subunit beta, mitochondrial precursor	P00829	37.5	56284	5.1
28	Serotransferrin precursor	Q29443	8.5	77754	6.8
30	Glutamyl aminopeptidase	Q32LQ0	19	109802	5.1
32	Septin-6	Q3SZN0	18.3	48775	6.4
33	Interphotoreceptor matrix proteoglycan 1 precursor	Q9GMS5	14	89379	5.2
35	Glutamine synthetase	P15103	11.8	42032	6.4
36	Protein KTI12 homolog	Q14815	32.2	38783	6.4

^aThe coverage of matched peptide sequences relative to the complete amino acid sequence of the protein

^bTheoretical molecular weight and isoelectric point

Table 2

Light-induced phosphoproteins in RPE cells.^b

Spot no.	Protein	Accession No.	Score ^a	Seq. Cov. (%)	MW (Dalton)	pI	No. of identified peptides	ms/ms	Known PO ₄ modification	Relative intensity (light/dark ratio)
CHAPERONE										
P1	Alpha-crystallin chain A	gi 117373	73	40	19835	5.78	3		Phosphoprotein	#
P2	Alpha-crystallin chain A	gi 117373	84	34	19835	5.78	3		Phosphoprotein	#
P3	Alpha-crystallin chain A	gi 27805855	70	55	19835	5.78	3		Phosphoprotein	12.6
P4	Beta-crystallin B2	gi 27807379	77	32	23397	6.45	3		Phosphoprotein	16.3
P5	Beta-crystallin S	gi 27805845	73	55	21125	6.38	1		Unknown	32.0
p6	Alpha-crystallin chain B	gi 27805849	84	53	20024	6.76	4		Phosphoprotein	5.4
p7	Beta-crystallin A3	gi 117403	78	52	25571	5.98	2		Phosphoprotein	#
8	Beta-crystallin B2	gi 157834682	78	45	20816	5.98	1		Phosphoprotein	#
9	Alpha-crystallin chain B	gi 27805849	70	40	20024	6.76	2		Phosphoprotein	#
10	Beta-crystallin A3	gi 117403	61	20	25571	5.98	2		Phosphoprotein	#
11	Beta-crystallin A3	gi 117403	71	52	25571	5.98	1		Phosphoprotein	4.9
12	Beta-crystallin A3	gi 117403	71	47	25571	5.98	2		Phosphoprotein	#
13	Beta-crystallin B2	gi 27807379	152	58	23397	6.45	2		Phosphoprotein	#
14	Beta-crystallin B2	gi 27807379	81	33	23397	6.45	3		Phosphoprotein	#
15	Beta-crystallin B4	gi 50262254	56	34	23729	5.78	3		Phosphoprotein	#
16	Beta-crystallin B4	gi 27806951	42	34	24130	5.78	1		Phosphoprotein	#
17	Beta-crystallin B4	gi 27806951	64	31	24130	5.78	2		Phosphoprotein	#
18	Alpha-crystallin chain B	gi 27805849	53	28	20024	6.76	1		Phosphoprotein	#
19	Alpha-crystallin chain A	gi 117357	56	39	20010	5.44	3		Phosphoprotein	#
20	Alpha-crystallin chain A	gi 27805855	52	27	19835	5.78	3		Phosphoprotein	#
21	Beta-crystallin B3	gi 27806955	58	33	24296	6.39	3		Phosphoprotein	#
22	Beta-crystallin B2	gi 27807379	41	22	20816	5.98	2		Phosphoprotein	#
23	Alpha-crystallin chain B	gi 27805849	61	42	20024	6.76	2		Phosphoprotein	#
24	Beta-crystallin S	gi 3212411	38	35	10412	6.29	2		Unknown	#
25	Beta-crystallin A3	gi 117403	51	28	25571	5.98	2		Phosphoprotein	#
61	Beta-crystallin B1	gi 27806961	85	32	28354	7.66	2		Phosphoprotein	#

Spot no.	Protein	Accession No.	Score ^a	Seq. Cov. (%)	MW (Dalton)	pI	No. of identified peptides	Known PO ₄ modification	Relative intensity (light/dark ratio)
62	Beta-crystallin B1	gi 27806961	102	32	28354	7.66	3	Phosphoprotein	#
63	Beta-crystallin B1	gi 27806961	58	18	28354	7.66	3	Phosphoprotein	#
70	Heat shock protein beta-1	gi 85542053	121	45	22436	5.98	2	Phosphoprotein	#

^aProbability-based MASCOT MOWSE score is $-10 \times \text{Log}(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 55 are considered significant ($p < 0.05$).

^bPhosphoproteins from light- and dark-exposed RPE cells were enriched using metal-based affinity chromatography and analyzed as described in Materials and Methods. Crystallins were significantly enriched in light-treated PRE cells as a result confirmed by western blotting. Transferrin was used as an internal control.

[#]No matching spot was found in the dark.

Table 3

Dark-induced phosphoproteins in RPE cells.

Spot no.	Protein	Accession No.	Score	Seq. Cov. (%)	MW (Dalton)	pI	No. of identified ms/ms peptides	Known PO ₄ modification	Relative intensity (dark/light ratio)
CHAPERONE									
33	Heat shock protein 70	gi 157832116	86	39	42543	6.64	2	Phosphoprotein	2.7
50	Heat shock 90kD protein 1, alpha	gi 60592792	80	15	85077	4.93	2	Phosphoprotein	1.4
RETINOID METABOLISM									
26	Retinaldehyde-binding protein, CRALBP	gi 234606	113	37	36579	4.99	3	Unknown	1.5
28	Guanine nucleotide-binding protein, beta-1 subunit	gi 1942392	71	39	38151	5.60	2	Unknown	1.7
48	Interphotoreceptor retinoid-binding protein	gi 27806445	90	12	140181	5.14	3	Unknown	1.1
CYTOSKELETON									
27	Actin, cytoplasmic 1(Beta-actin)	gi 116242946	43	36	42064	5.22	2	Phosphoprotein	1.3
29	Tubulin, beta, 2	gi 77736271	72	33	50255	4.79	1	Phosphoprotein	2.7
32	Tubulin, alpha 1B	gi 168804008	91	34	50804	4.94	3	Phosphoprotein	2.5
ENERGY METABOLISM									
35	Pyruvate kinase	gi 73587283	38	19	61427	8.62	2	Phosphoprotein	1.7
36	Creatine kinase, mitochondrial 1	gi 27805877	48	18	47266	8.60		Phosphoprotein	2.1
37	Creatine kinase, mitochondrial 1	gi 27805877	57	20	47266	8.60	2	Phosphoprotein	2.2
44	Alpha enolase	gi 4927286	85	27	47589	6.44	3	Phosphoprotein	2.8
45	Glutamine synthetase	gi 95006991	80	28	42632	6.38	1	Phosphoprotein	5.9
51	Creatine kinase B-type	gi 125292	42	13	42960	5.47	1	Phosphoprotein	3.8
52	Creatine kinase B-type	gi 125292	95	32	42960	5.47		Phosphoprotein	4.3
65	Aconitase 2, mitochondrial	gi 27806769	56	11	86045	8.08	1	Unknown	1.4
IRON TRANSPORT									
38	Serotransferrin	gi 2501351	42	12	79870	6.75	3	Phosphoprotein	1.0

Spot no.	Protein	Accession No.	Score	Seq. Cov. (%)	MW (Dalton)	pI	No. of identified ms/ms peptides	Known PO ₄ modification	Relative intensity (dark/light ratio)
39	Serotransferrin	gi 2501351	65	15	79870	6.75	3	Phosphoprotein	1.2
40	Serotransferrin	gi 2501351	46	15	79870	6.75		Phosphoprotein	1.1
ZYMOGEN									
57	Plasminogen	gi 27806815	50	8	93894	7.68	1	Phosphoprotein	1.2