Proteomics and Phosphoproteomics Analysis of Human Lens Fiber Cell Membranes

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PURPOSE. The human lens fiber cell insoluble membrane fraction contains important membrane proteins, cytoskeletal proteins, and cytosolic proteins that are strongly associated with the membrane. The purpose of this study was to characterize the lens fiber cell membrane proteome and phosphoproteome from human lenses.

METHODS. HPLC-mass spectrometry-based multidimensional protein identification technology (MudPIT), without or with phosphopeptide enrichment, was applied to study the proteome and phosphoproteome of lens fiber cell membranes, respectively.

RESULTS. In total, 951 proteins were identified, including 379 integral membrane and membrane-associated proteins. Enriched gene categories and pathways based on the proteomic analysis include carbohydrate metabolism (glycolysis/gluconeogenesis, pentose phosphate pathway, pyruvate metabolism), proteasome, cell-cell signaling and communication (GTP binding, gap junction, focal adhesion), glutathione metabolism, and actin regulation. The combination of TiO₂ phosphopeptide enrichment and MudPIT analysis revealed 855 phosphorylation sites on 271 proteins, including 455 phosphorylation sites that have not been previously identified. PKA, PKC, CKII, p38MAPK, and RSK are predicted as the major kinases for phosphorylation on the sites identified in the human lens membrane fraction.

CONCLUSIONS. The results presented herein significantly expand the characterized proteome and phosphoproteome of the human lens fiber cell and provide a valuable reference for future research in studies of lens development and disease. (*Invest Ophthalmol Vis Sci.* 2013;54:1135-1143) DOI: 10.1167/iovs.12-11168

The ocular lens is a transparent tissue that focuses light onto the retina and protects the retina from ultraviolet damage. Anteriorly, the outermost layer of cells is a monolayer of epithelium while lens fiber cells form the bulk of the lens interior. Lens fiber cells contain high concentrations of important soluble proteins known as α -, β -, γ -crystallins;

however, membrane proteins are essential for maintenance of lens transparency and homeostasis. Membrane proteins are among the most important proteins within a cell and they mediate key biological cues for the cell to dynamically respond to environmental changes.¹ These proteins include important cell adhesion molecules, membrane-associated enzymes, membrane receptors and transporters, etc. Lens fiber cells are bounded by a plasma membrane and this membrane is the only membrane system for a majority of lens fiber cells since organelles are degraded during differentiation to mature lens fiber cells.² The vital roles of membrane proteins in the lens have been demonstrated previously by studying mutation and knockout of some major membrane proteins. For example, mutation in and knockout of the AQP0 gene,^{3,4} Lim2,^{5,6} or mutations in the Cx46 and Cx50 genes^{7,8} are cataractogenic.

Advances in MS-based proteomics have contributed greatly to the identification of low abundance proteins in complex mixtures.⁹ These technologies have been applied to better understand lens protein expression, posttranslational modifications, and protein aggregation.¹⁰⁻¹⁵ Proteomic analysis was also conducted to compare the proteomic difference between normal lens and cataract lens.^{15,16} Membrane proteins are normally underrepresented in the majority of proteome profiles due to their extreme hydrophobicity.^{17,18} In addition, crystallins and high abundance cytoskeletal proteins such as filensin and CP49 are strongly associated with the membrane protein fraction,¹⁹ resulting in the dilution of membrane protein signals. Recently, a membrane proteomics approach was utilized to produce descriptions of membrane signatures in the mouse lens fiber cells and 232 proteins were found enriched or unique in the lens membrane fraction.²⁰ A systematic study of human lens fiber cell membrane fraction has not been reported. With the advances in mass spectrometry and bioinformatics, further study of lens membrane fraction is expected to identify more membrane proteins as well as provide more information about proteins that are strongly associated with the membrane fraction.

Phosphorylation is one of the most important and well-studied posttranslational modifications.²¹ Recent advances in mass spectrometry and phosphopeptide enrichment allow the identification of in vivo phosphorylation sites with high accuracy.^{22,23} Phosphorylation of high abundance lens proteins have been studied previously,²⁴⁻²⁷ but these studies focused on single or a few proteins. Recent phosphoproteomic studies were reported for human lens²⁸ and porcine lens²⁹; however, less than 100 phosphorylation sites were identified and low-abundance membrane proteins were not the focus of these studies.

In this study, we performed a large-scale proteomic and phosphoproteomic analysis of the human lens membrane fraction using a multi-dimensional protein identification technology (MudPIT) in combination with a simple trifluoroethanol assisted in-solution digestion procedure.

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MATERIALS AND METHODS

Materials

Frozen human lenses were obtained from NDRI (Philadelphia, PA). Proteinase inhibitor (P8340) and phosphatase inhibitor (P5726) were purchased from Sigma-Aldrich (St. Louis, MO). Sequence-grade modified trypsin was obtained from Promega (Madison, WI) and sequence-grade pepsin was purchased from Princeton Separation (Adelphia, NJ). All other chemicals and HPLC grade solvents were purchased from Fisher (Fair Lawn, NJ).

Lens Membrane Protein Preparation

Three human lenses (aged 25, 37, and 58 years) were cut in half axially and decapsulated. For membrane preparations, a half lens was homogenized in buffer containing 25 mM Tris (pH8), 5 mM EDTA, 1 mM DTT, 150 mM NaCl, 1% (v/v) phosphatase inhibitor (Sigma-Aldrich) and 1% protease inhibitor (Sigma-Aldrich). After homogenization, the sample was centrifuged at 100,000g for 30 minutes and the supernatant was discarded. The pellets were washed twice with the above homogenizing buffer followed by three washes with homogenizing buffer containing 8 M urea. Centrifugation at 100,000g was performed to separate the supernatant and pellets for each wash. The remaining pellets were washed twice with water. The pellets were then washed with 0.1 M cold NaOH immediately followed by washing twice with water. Protein concentration was estimated by mixing a small aliquot of sample with equal volume of 5% SDS and measuring the absorption at λ 280 using a spectrophotometer (Nanodrop 2000 Spectrophotometer; Thermo Fisher Scientific, Wilmington, DE).

Enzyme Digestion

Membrane pellets obtained from a 25-year-old lens and a 37-year-old lens (2 mg of total protein each) were suspended in 20 μ L of 50% trifluoroethanol in 50 mM ammonium bicarbonate containing 10 mM DTT, respectively, and incubated at 56°C for 1 hour to reduce disulfide bonds. Reduced cysteines were alkylated by adding 2 μ L of 500 mM iodoacetamide and incubated at room temperature for 45 minutes. The sample was diluted 10 times by 50 mM ammonium bicarbonate buffer (pH 8.0). Sequence-grade modified trypsin (1 μ g; Promega) was added and the sample was incubated at 37°C for 18 hours. Likewise, membrane pellets (2 mg of protein) from a 58-year-old lens was suspended in 100 μ L of water containing 10 mM DTT at 56°C for 1 hour followed by alkylation at room temperature for 45 minutes by addition of 55 mM iodoacetamide. The excess reagents were removed by triplicate water washes of the pellets. The remaining pellets were suspended in 100 μ L 10 mM of HCl and 2 μ g of sequencing-grade pepsin (Princeton Separation) was added and the sample was incubated at 37°C for 6 hours. After digestion, the samples were centrifuged at 20,000g for 10 minutes, the supernatants were collected, and the remaining pellets were extracted with 100 μ L acetonitrile (ACN), 0.1% formic acid. The ACN extracts were dried by vacuum concentration and reconstituted in 20 μ L 5% ACN, 0.1% formic acid. The ACN extracts and the initially collected supernatants were pooled together and diluted 5 times by 0.1% formic acid and loaded onto a cartridge (SepPak C18; Waters, Milford, MA). The cartridge was washed with 0.1% formic acid and the bound peptides were eluted with 75% ACN, 0.1% formic acid. The sample was then dried by vacuum concentration and stored at -20°C until further analysis.

Phosphopeptide Enrichment

The desalted tryptic or peptic peptides from 2 mg total protein were reconstituted in 100 μ L of 2% ACN (0.05% HFBA) and added to 10 mg Titansphere beads (GL Sciences, Inc., Torrance, CA) that were prewashed with 80% ACN, 0.05% HFBA. Lactic acid was added to a final concentration of 150 mg/mL and the sample was incubated at RT

for 2 hours. The beads were then spun down at 20,000g and the supernatant was collected for analysis of nonphosphorylated peptides. The beads were washed twice with 100 μ L of 80% ACN, 0.05% HFBA followed by a single wash with 2% ACN, 0.05% HFBA. The phosphopeptides were eluted with 50 μ L 0.5 M ammonia followed by 50 μ L 5 M ammonia. The eluates were immediately dried by vacuum concentration and reconstituted in 0.1% formic acid. The two ammonia eluates were pooled for phosphoproteomic analysis.

LC-ESI/MS/MS

Five percent of the flowthrough from TiO₂ beads was used for proteomic analysis and 50% of the eluate from the TiO₂ beads was used for phosphoproteomic analysis. Peptides were loaded onto a custom packed biphasic C18/SCX trap column and analyzed by MudPIT as previously reported with a 13-step salt pulse (25 mM, 50 mM, 75 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 500 mM, 750 mM, 1 M, 1.5 M, and 2 M ammonium acetate) elution.³⁰ Phosphopeptide analysis was performed with a 11-step salt pulse elution (25 mM, 50 mM, 75 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 500 mM, 750 mM, 1 M ammonium acetate). After each salt pulse (5 μ L), peptides were eluted from the analytical column and electrosprayed into a mass spectrometer (LTQ Velos; ThermoScientific, San Jose, CA). The instrument was operated in a 7-step data dependent mode with one precursor scan event to identify the top six most abundant ions in each MS scan, which were then selected for fragmentation. Dynamic exclusion (repeat count 2, exclusion list size 300, and exclusion duration 60 seconds) was enabled to allow detection of less abundant ions.

Data Analysis

Tandem mass spectra were analyzed using a suite of custom-developed bioinformatics tools. For identification of proteins in the samples, all searches were configured to use a static modification of carbamidomethylation of cysteine and variable modification of oxidation of methionine residues. The protein database was a concatenated forward and reversed (decoy) Uniprot human database (version 155, December 31, 2009). For database searching of trypsin-digested samples, all MS/MS spectra were converted to mzXML files by ScanSifter, a tool under development at Vanderbilt University Medical Center and searched on a 2500-node Linux cluster supercomputer using a custom version of the TagRecon algorithm.³¹ Trypsin specificity was used with a maximum of two missed cleavage sites. For database searching of pepsin-digested samples, all MS/MS spectra were converted to DTA files by ScanSifter and searched on the same cluster by the SEQUEST algorithm³² using pepsin specificity of cleavage after FLWYAEQ. The combination of cross-correlation (Xcorr) and DeltaCN values was used for score optimization for proteomic analysis and results were filtered to a 5% peptide false discovery rate (FDR) with the requirement for a minimum of two unique peptides per reported protein using IDPicker.³³ To identify phosphorylated peptides, tryptic peptide analyses were searched by TagRecon and peptic peptide analyses were searched by SEQUEST as described above. Static modification of carbamidomethylation of cysteine and variable modification of oxidation of methionine and phosphorylation of Ser, Thr, and Tyr residues was set for searching phosphorylated peptides. Phosphorylated protein identification used the same criteria as above except DeltaCN was not used when reporting phosphorylated peptides by IDPicker. The protein level FDR was controlled to below 6% and all of the phosphopeptides that passed these criteria were manually verified. For Ser and Thr phosphorylation, a distinct neutral loss of phosphoric acid was required. The data were also reassembled by IDPicker to report phosphoproteins identified by a single phosphopeptide. This increased the FDR; however, the phosphopeptides that passed manual verification criteria are listed separately (see Supplementary Material and Supplementary Table S3c, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-11168/-DCSupplemental>).

RESULTS

Lens Membrane Proteome

A total of 933 protein groups were identified from trypsin-digested lens membrane fractions and 260 protein groups were identified from pepsin-digested lens membrane fraction based on at least two unique peptides. The final protein level FDR, 4% for tryptic peptide analyses and 2% for peptic peptide analyses, was calculated based on the number of decoy database hits to evaluate the search results. Among the 933 proteins detected in the trypsin-digested samples, 563 proteins (60%) were identified in both 25- and 37-year-old lens samples. Compared with the proteins detected by trypsin digestion, 18 proteins out of 260 proteins detected in the pepsin-digested sample were not detected in trypsin-digested samples. Combined tryptic and peptic analyses identified 951 proteins in the human lens membrane fraction. The complete list of proteins can be found in Supplemental Tables S1a and S1b (see Supplementary Material and Supplemental Tables S1a, S1b, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-11168/-DCSupplemental>). Even though fewer proteins were identified in the pepsin-digested sample, pepsin digestion is effective for membrane protein discovery as previously reported.¹³ For example, normalized spectral counts, a rough estimate of protein abundance, were 0.374 for the most abundant membrane protein AQP0 for peptic peptides compared with 0.000926 for AQP0 tryptic peptides. Moreover, the sequence coverage of AQP0 increased dramatically (from 9%–25% in the trypsin-digested samples to 84% in the pepsin-digested sample).

Predicted cellular localization of the detected proteins was analyzed by WebGestalt³⁴ and the result is shown in Figure 1. A total of 922 unique Entrez IDs were generated and 379 proteins (41%) were identified as membrane proteins, which includes integral membrane proteins and membrane-associated proteins. In addition, significant amounts of proteins were localized in macromolecular complex (285) and cytoskeleton (133) categories. Only 215 proteins (23%) were predicted to be localized in cytosol. The transmembrane helices were predicted by TMHMM,³⁵ a Hidden Markov Model for predicting transmembrane helices, and 178 proteins were predicted to have at least one transmembrane helix. The detailed results from TMHMM prediction are listed in Supplemental Table S2 (see Supplementary Material and Supplemental Table S2, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-11168/-DCSupplemental>).

Lens crystallin protein insolubilization with age has been well-documented in the lens, but remains poorly understood. Even though the lens membrane fractions have been washed by 8 M urea and 0.1 M NaOH, significant amounts of crystallins are still associated with the membrane as reported by Truscott et al.¹⁹ In the current work, α A-Crystallin and β B1-crystallin have the largest spectral counts. α B-, β A3-, β A4-, β B2-, β S-, γ C- and γ D-Crystallins also have high spectral counts (see Supplementary Material and Supplemental Tables S1a, S1b, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-11168/-DCSupplemental>). In addition, major lens cytoskeletal proteins are also among the proteins resistant to urea and alkali wash, including filensin, phakinin, and spectrin, indicating strong membrane-associated properties.

Overrepresented gene categories and pathways were estimated using Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatic tools³⁶ in which the whole human genome was used as the reference background. More than 50 terms of cellular function category and more than 100 terms of biological process category were overrepresented in human lens membrane fraction with a *P*

value less than 0.0001. Some top significantly enriched gene categories of cellular function and biological process are listed in Table 1. Overrepresented terms in the cellular function category indicate that structural proteins, proteins that are involved in signal transduction, and proteins involved in catalytic processes are enriched in the human lens membrane fraction. Enriched terms in biological process further support the result that proteins with structural, catalytic, and signal transduction roles are overrepresented. Enrichment analysis also indicated enrichment of proteins involved in translational elongation and ribosome pathway due to a series of detected ribosome proteins; however, the spectral counts for these proteins were low, suggesting low abundance. Since the membrane fraction was prepared from a half of the lens that contains both cortical and nucleus regions, these ribosome proteins are believed to originate from the superficial, nucleated fiber cells. Alternatively, they may be long-lived proteins present in more nuclear fiber cells. Ultracentrifugation with 100,000g was used to pellet the membrane fraction and under this speed, cell plasma membrane and organelles should both precipitate. Enriched terms in biological process also include glycolysis and carbohydrate metabolism. Ten KEGG pathways are enriched, which also include glycolysis/gluconeogenesis. Other enriched KEGG pathways include some important pathways in the lens such as proteasome, gap junction, and glutathione metabolism, etc. (Table 1).

Lens Phosphoproteome

Combining TiO₂ phosphopeptide enrichment and MudPIT analysis, we analyzed three human lens samples. In total, 803 unique phosphorylation sites on 264 proteins were identified in trypsin-digested samples. Among these identified phosphorylation sites, 773 sites were assigned unambiguously by TagRecon and confirmed by manual verification and 30 sites were ambiguous. A total of 536 phosphorylation sites were identified in the 25-year-old lens sample and 595 phosphorylation sites were identified in the 37-year-old lens sample. A total of 328 sites (41%) were detected in both lenses. In total, 106 phosphorylation sites in 49 proteins were detected in the pepsin-digested sample from the 58-year-old lens sample. Even though fewer phosphorylation sites were identified from the pepsin-digested sample, pepsin digestion resulted in identification of many new phosphorylation sites. Among the 106 phosphorylation sites identified in the pepsin-digested sample, 52 sites were not detected in the trypsin-digested sample. Moreover, several new phosphorylation sites adjacent to transmembrane domains in AQP0 were detected such as S8, S79, Y105/S106, and T199.

Combining analyses of trypsin- and pepsin-digested samples, a total of 855 phosphorylation sites were identified. Comparing our results with the large phosphorylation site databases (www.phospho.elm.eu.org, www.phosphosite.org, and www.uniprot.org; all in the public domain) and previous phosphorylation sites identified in lens proteins,^{25–29} 251 sites (29%) have been reported previously. This analysis results in 455 new phosphorylation sites identified in this study thereby extensively expanding the lens phosphoproteome. A detailed list of the phosphorylated peptides can be found in Supplemental Tables S3a and S3b (see Supplementary Material and Supplemental Tables S3a, S3b, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-11168/-DCSupplemental>). Proteins with at least five phosphorylation sites identified are listed in Table 2. In addition to phosphorylation sites reported above, phosphoprotein identification based on single peptides also gave another 14 phosphorylation sites (5 new phosphorylation sites). These sites are included in Supplemental Table 3c (see Supplementary Material and Supplemental Table 3c, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-11168/-DCSupplemental>).

FIGURE 1. Cellular localization of proteins detected in human lens fiber cell membrane fraction determined by WebGestalt. Among the proteins identified in the lens fiber cell membrane fraction, 922 unique Entrez IDs were generated and 379 proteins (41%) were identified as membrane proteins.

TABLE 1. Enriched Gene Categories and Pathways Estimated by DAVID Bioinformatic Tools³⁶

Terms		Number of Genes	P Value
Molecular function	Structural molecule activity	130	5.66E-39
	GTPase activity	58	6.53E-24
	Protein binding	599	2.62E-22
	Structural constituent of eye lens	17	1.68E-19
	GTP binding	71	2.26E-19
	Guanyl nucleotide binding	72	2.53E-19
	Catalytic activity	414	6.29E-18
	Nucleotide binding	209	7.14E-14
	Structural constituent of cytoskeleton	26	1.28E-13
Biological process	Pyrophosphatase activity	96	1.43E-13
	Translational elongation	43	9.63E-26
	Cellular carbohydrate catabolic process	38	1.12E-23
	Alcohol catabolic process	36	2.45E-22
	Glucose catabolic process	30	5.98E-21
	Glucose metabolic process	46	2.63E-20
	Glycolysis	16	4.27E-19
	Small GTPase mediated signal transduction	58	3.05E-15
	Cellular component assembly	110	4.35E-14
	Actin filament-based process	43	1.49E-10
	Cytoskeleton organization	62	1.55E-10
KEGG pathway	Ribosome	40	7.96E-20
	Glycolysis/Gluconeogenesis	29	4.61E-15
	Pentose phosphate pathway	14	2.27E-08
	Proteasome	17	8.07E-07
	Focal adhesion	39	1.83E-06
	Pyruvate metabolism	14	1.58E-05
	Tight junction	28	1.82E-05
	Gap junction	21	4.46E-05
	Glutathione metabolism	15	4.95E-05
	Regulation of actin cytoskeleton	36	1.03E-04

TABLE 2. Phosphorylation Sites* and Phosphoproteins with at Least Five Phosphorylation Sites

Accession Number	Protein Description	Phosphorylation Sites
B1ALY0	A-kinase anchor protein 2	<u>S187</u> , <u>S243</u> , <u>S244</u> , <u>S251</u> , <u>S260</u> , S350, S390, <u>T393</u>
O00192	Armadillo repeat protein deleted in velo-cardio-facial syndrome	<u>S184</u> , <u>S198</u> , <u>S200</u> , S203, S205, S267, <u>S335</u> , S602, S606, T642, <u>S813</u> , <u>S815</u> , <u>S851</u> , <u>T855</u> , S864, S871, T872, <u>S887</u> , S915
O43491	Band 4.1-like protein 2	S87, <u>T194</u> , <u>S231</u> , S386, <u>S402</u> , S529
O75781	Paralemm-1	S116, S124, T145, S152, S189, S245, S249, T250
P00352	Retinal dehydrogenase 1	<u>Y115</u> , <u>S116</u> , Y119, <u>T245</u> , T267, <u>S314</u> , S413, T424, Y426
P00558	Phosphoglycerate kinase 1	S153, S155, <u>S174</u> , S175, S203
P02489	Alpha-crystallin A chain	T13, S20, <u>S51</u> , <u>T43</u> , S45, <u>Y47</u> , <u>T55</u> , <u>S59</u> , <u>S62</u> , S66, S81, <u>T86</u> , <u>Y118</u> , S122, <u>S127</u> , <u>S130</u> , T148, T153, <u>S162</u> , <u>T168/S169</u> , S172, <u>S173</u>
P02511	Alpha-crystallin B chain	S19, S21, S43, S45, S53, S59, T63, <u>S66</u> , S76, <u>S85</u> , T132, <u>T134</u> , <u>S136</u> , <u>S138</u> , S139, <u>S153</u> , <u>T158</u>
P04075	Fructose-bisphosphate aldolase A	S36, T37, S39, S46, <u>T266</u> , S272, S276, <u>S281</u>
P04406	Glyceraldehyde-3-phosphate dehydrogenase	S151, T153, T154, S210, T211
P05023	Na/K-transporting ATPase subunit alpha-1	T226, S228, S452, S454, S484, S653, <u>S369</u> , <u>S375</u> , <u>S378</u> , <u>S380</u> , <u>S668</u>
P05813	Beta-crystallin A3	<u>S27</u> , <u>T34</u> , <u>Y36</u> , <u>S50</u> , T49, <u>S51</u> , <u>S80</u> , <u>S165</u> , S200, <u>T205/S206</u>
P07315	Gamma-crystallin C	<u>Y7</u> , <u>S16</u> , <u>T19</u> , <u>T20</u> , <u>T21</u> , <u>Y29</u> , <u>S31</u> , <u>S40</u> , Y66, <u>S73</u> , <u>S75</u> , <u>T85</u> , <u>S87</u> , <u>S106</u>
P07316	Gamma-crystallin B	<u>S16</u> , <u>Y17</u> , <u>T21</u> , <u>Y29</u> , <u>S31</u> , Y66, <u>S73</u> , <u>S75</u> , <u>S104</u> , <u>T107</u>
P07320	Gamma-crystallin D	Y7, <u>Y17</u> , <u>S20</u> , <u>Y29</u> , <u>S31</u> , <u>S40</u> , <u>S52</u> , S75, <u>T106</u> , <u>S110</u> , <u>Y154</u> , T160, <u>S174</u>
P08670	Vimentin	Y53, S73, S325, S419, S420
P09972	Fructose-bisphosphate aldolase C	S36, S39, S160, <u>T269</u> , <u>S272</u> , <u>S276</u>
P11166	Solute carrier family 2	T234, <u>T258</u> , S473, <u>S475</u> , T478
P11171	Protein 4.1	<u>S215</u> , <u>T378</u> , <u>S394</u> , <u>S446</u> , <u>S445</u> , <u>T494</u> , S521, S542, T559/S560
P13591	Neural cell adhesion molecule 1	<u>S514</u> , <u>S653</u> , <u>S664</u> , S780, S784
P14618	Pyruvate kinase isozymes M1/M2	S37, S57, S249, <u>T405/S406</u> , <u>S437</u>
P16152	Carbonyl reductase [NADPH] 1	S30, <u>S56</u> , <u>S149</u> , T162, <u>S191</u>
P17858	6-phosphofructokinase	<u>S134</u> , <u>T304</u> , S377, <u>S706</u> , <u>S720</u> , S775
P21333	Filamin-A	S968, S2180, <u>S2213</u> , T2336, S2327
P22914	Beta-crystallin S	T6, T9, Y11, T32, S35, <u>Y58</u> , <u>Y60</u> , <u>Y70</u> , S90, S94, <u>S105</u> , <u>T112</u> , <u>Y140</u> , <u>S172</u> , S167
P26232	Catenin alpha-2	<u>T264</u> , S262, S640, T653, S654, S905, <u>S939</u>
P30301	Aquaporin-0	<u>S8</u> , <u>S79</u> , <u>S106</u> , <u>T120</u> , T199, S229, S231, S235, S245, <u>T252</u> , <u>T260</u> , S641, S652, T654, S655, T658
P35221	Catenin alpha-1	<u>S824</u> , <u>S827</u> , <u>S2313/Y2314</u> , <u>T2315</u> , S2702
P35555	Fibrillin 1	<u>S87</u> , T91, S93, Y112, T118, S148, <u>T150</u> , S174, S175, <u>S204</u>
P43320	Beta-crystallin B2	<u>T120</u> , S128, <u>T146</u> , <u>S234</u> , S251, S254, S258, S259, S290, <u>T306</u> , <u>T374</u> , S403, S424, S428
P48165	Connexin 50	T147, <u>S149</u> , <u>S151</u> , <u>S181</u> , <u>S276</u> , <u>S394</u> , S415
P48637	Glutathione synthetase	<u>S8</u> , <u>T30</u> , S51, <u>S76</u> , T108, <u>S123</u> , <u>S181</u>
P53673	Beta-crystallin A4	S10, <u>T31</u> , S32, <u>S34</u> , <u>T37</u> , <u>T47</u> , S77, S81, S93, S97, S107, <u>S152</u> , T163, <u>S180</u> , S189, S190, <u>T192</u> , <u>Y198</u> , Y204, <u>T248</u>
P53674	Beta-crystallin B1	<u>S36</u> , <u>Y45</u> , <u>T55</u> , <u>S57</u> , S170
P55344	MP20	S199, T201, T202, T203, S239/Y240, <u>T303</u> , <u>T304</u> , S323/T324, <u>S338</u>
P60709	Actin, cytoplasmic 1	T31, T36, S40, S132, S164, S170, T196, S205, S219
P80723	Brain acid soluble protein 1	S257, <u>S312</u> , S1076, <u>S1166</u> , <u>S1966</u> , S2102, S2128, T2195, S2341, T2328, T2337/S2338, S2340/S2341
Q01082	Spectrin beta chain, brain 1	S31, <u>T565</u> , T828, <u>S2382</u> , S3760
Q01484	Ankyrin-2	S5, S233, <u>T327</u> , S339, S454, T460, T462, <u>S488</u> , T490, S607
Q12934	Filensin	<u>T12</u> , <u>S15</u> , S32, S33, S34, S35, <u>S38</u> , S81, S82, S91, <u>S156</u> , <u>S159</u> , <u>S208</u> , <u>S404</u> , S414
Q13515	Phakinin	T31, <u>S413</u> , S510, S512, S513
Q15124	Phosphoglucomutase-like protein 5	S720, <u>S794</u> , S1047, S1566, S1732, T3785, <u>S4406</u>
Q15149	Plectin-1	<u>S15</u> , <u>S138</u> , <u>S141</u> , T351, Y353, <u>S374</u> , <u>S377</u>
Q5T9C9	Phosphatidylinositol-4-phosphate 5-kinase-like protein 1	<u>T212</u> , <u>S311</u> , <u>S336</u> , <u>T345</u> , <u>S490</u>
Q6UWM7	Lactase-like protein	S23, S24, S46, S444, T1141
Q6ZSP3	highly similar to Potential phospholipid-transporting ATPase IB	<u>S155</u> , <u>S212</u> , <u>S228</u> , <u>S230</u> , S318, S358, <u>S367</u>
Q8IXS6	Paralemm-2	<u>S52</u> , <u>S373</u> , <u>S393</u> , <u>S561</u> , <u>S675</u> , S809, <u>T811</u> , <u>S940</u>
Q92823	Neuronal cell adhesion molecule	S7, <u>S51</u> , <u>S56</u> , <u>S58</u> , T111, S131, <u>S133</u> , <u>T230</u> , S381, <u>S424</u> , <u>S814/S816</u> , S900, S1065, S1082, S1401, <u>S1423</u> , <u>S1425</u> , S1439
Q9BXM0	Periaxin	S107, <u>S332</u> , <u>S407</u> , S546, S784
Q9H4G0	Band 4.1-like protein 1	S181, S182, S184, S860, S863
Q9NQC3	Reticulon-4	<u>S192</u> , <u>S215</u> , S234, S237, <u>T251</u> , S325, S330, <u>T334</u>
Q9ULX7	Carbonic anhydrase 14	S119, S121, <u>S131</u> , S132, <u>T249</u> , S347/T348, S353
Q9Y6H8	Connexin 46	

Underline indicates new phosphorylation sites.

* The full list of phosphorylation sites can be found in Supplemental Tables 3a-c (see Supplementary Material and Supplementary Tables 3a-c, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-11168/-DCSupplemental>).

www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-11168/-/DCSupplemental), but these sites were not included in further analysis of the data.

Phosphorylation was detected in 85 proteins that were not detected by the proteome study most likely due to the enrichment procedure. For example, multiple phosphorylation sites were detected on myosin-8, interferon-gamma receptor alpha chain, Ras-related protein Rab-2B, A-kinase anchor protein 2, and A-kinase anchor protein 9, etc. Other than crystallins, highly phosphorylated proteins detected in lens fiber cell membrane include transmembrane proteins such as Na/K-transporting ATPase subunit alpha-1, AQP0, MP20, connexin 50, and solute carrier family 2, as well as peripheral membrane proteins such as Armadillo repeat protein deleted in velo-cardio-facial syndrome, periaxin, paralemmin, and catenin, etc.

The distribution of identified phosphorylation sites on Ser, Thr, and Tyr is shown in Figure 2. A total of 826 unambiguous phosphorylation sites include 595 phosphoserine, 186 phosphothreonine, and 45 phosphotyrosine, giving a phosphoserine/phosphothreonine/phosphotyrosine ratio of 13:4:1. Compared with previous reports of 18:4:1 in human skeletal muscle,³⁷ 86:12:2 in HeLa cells,³⁸ and 55:12:2 in human embryonic stem cells,³⁹ the human lens has a higher level of phosphotyrosine and phosphothreonine residues.

Phosphorylation motifs were extracted using Motif-X algorithm⁴⁰ with the IPI human proteome as a background and the threshold for significance was set to $P < 10^{-6}$. Three overrepresentative motifs were identified and the result is shown in Figure 3. The three motifs included one proline-directed motif and two basophilic motifs. Potential kinases for identified phosphorylation sites were predicted by NetworKIN,⁴¹ and in total, 15 kinases for 475 phosphorylation sites were predicted. The detailed information is included in Supplemental Table 4 (see Supplementary Material and Supplementary Table 4, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-11168/-/DCSupplemental>). The number of distinct phosphorylation sites by predicted kinases is shown in Figure 4, demonstrating a major predicted role for PKA, PKC, CKII, p38MAPK, and RSK kinase families.

DISCUSSION

This study applied MudPIT to study the membrane proteome and phosphoproteome of human lens fiber cells. The results extensively expand previous results of lens proteomic^{10-12,20} and phosphoproteomic studies.^{28,29} Both our proteomic and phosphoproteomic results indicated that trypsin and pepsin digestion provided complementary information. Phosphorylation sites adjacent to transmembrane domains in AQP0 have been repeatedly detected in other lens samples (data not shown). This result raises a question of how many phosphorylation sites adjacent to transmembrane domains have been missed using the traditional trypsin digestion strategy. Unexpectedly, many new phosphorylation sites have been detected in highly abundant—and previously extensively studied—lens crystallins such as on the C-terminus of α A and α B-Crystallin, gamma crystallins, and some beta crystallins. Most newly identified crystallin phosphorylation sites were detected with low spectral counts indicative of low abundance. More careful quantitative studies are required to determine the stoichiometry of phosphorylation and any differences in phosphorylation status between soluble and membrane-associated crystallins.

It is well accepted that increasing amounts of crystallins are strongly associated with the membrane with age. High spectral counts and high sequence coverage for crystallins in this study

further support crystallin membrane-binding properties. Considering that crystallins and some cytoskeleton proteins in the lens aggregate to form large complexes that could pellet under ultracentrifugation, such complexes that are resistant to urea and alkali washes could contribute to the high spectral counts and sequence coverages observed in our studies.

Enrichment of gene categories and pathway analysis demonstrated overrepresented categories that play important roles in the lens. Enzymes involved in glycolysis/gluconeogenesis were significantly enriched in the lens membrane fraction, which is in agreement with the fact that a majority of lens fiber cells rely on anaerobic glycolysis to satisfy their energy requirements.⁴² Phosphorylation was detected on more than half of the enzymes (17 out of 29) involved in glycolysis/gluconeogenesis processes. Phosphorylation of a majority of glycolytic enzymes has been found in skeletal muscle³⁷ and the level of phosphorylation of some enzymes was age-dependent.³⁷ Further study is needed to characterize the roles and levels of phosphorylation on these enzymes in lens fiber cells of different ages. Proteins that are involved in cell-cell communication and signaling, including focal adhesion, gap junction, and GTP binding and hydrolysis, were—not unexpectedly—also enriched in the lens membrane fraction. Multiple phosphorylation sites were detected on important junction proteins such as connexins, catenins, and N-cadherin, indicating functions regulated by phosphorylation. Connexin 50 and connexin 46 are lens fiber cell connexins and both connexins are phosphorylated proteins.^{25-26,43} Compared with a previous report of 18 phosphorylation sites in bovine connexin 50 and 10 phosphorylation sites in bovine connexin 46,²⁶ 13 phosphorylation sites were detected in human connexin 50 and 7 phosphorylation sites were detected in human connexin 46. Phosphorylation regulates critical gap junction events including gating and connexin degradation^{44,45} (e.g., PKC γ activation decreases intercellular communication).⁴⁴ Activation of MAPK signaling significantly increased the coupling provided by connexin 50.⁴⁶ Based on kinases predicted by NetworKIN for the phosphorylation sites identified in this study, PKC is the predicted kinase for six phosphorylation sites in connexin 50 and MAPK is the predicted kinase for phosphorylation on S290 and T374 in connexin 50.

Glutathione plays a major role in the maintenance and regulation of the thiol-redox status in the lens and the human lens has high concentration of glutathione (up to 3 mM).⁴⁷ Consistent with the important function of glutathione in the lens, proteins involved in glutathione metabolism were also overrepresented in the human lens fiber cell membrane fraction. Informatics analysis also revealed the enrichment of proteins regulating the actin cytoskeleton, ribosome function, and proteasome activity. Actin has been identified as one of the major cytoskeletal proteins in the lens and is believed to play an important role during fiber cell differentiation and elongation.^{48,49} A series of proteasome proteins were detected in the lens membrane fraction even though these proteins belong to the cytoplasmic compartment. The ubiquitin proteasome pathway (UPP) has been shown to play a role in organelle degradation⁵⁰ and our results confirm the enrichment of proteasome pathway in lens fiber cells.

Overrepresented phosphorylation motifs predicated by Motif-X algorithm and NetworKIN analysis both supported the important role of AGC protein kinase and proline-directed kinase in lens fiber cells. The presence of some kinases in the lens fiber cells can be confirmed by our proteomic study. For example, both PKA catalytic and regulatory subunits (PRKACA, PRKACB, PRKARIA, PRKAR2A) as well as some MAPK family kinases (MAPK1 and MAPK3) were detected in the membrane fraction. A recent study⁵¹ showed a PKA tethering protein,

FIGURE 2. Phosphorylation site distribution on Ser, Thr, and Tyr residues. A total of 826 unambiguous phosphorylation sites include 595 phosphoserine, 186 phosphothreonine, and 45 phosphotyrosine giving a phosphoserine/phosphothreonine/phosphotyrosine ratio of 13:4:1.

AKAP2 (detected in this study), in complex with AQP0, suggesting a direct role of PKA-catalyzed phosphorylation in regulating AQP0. Inhibition of PKA activity leads to cortical cataracts.⁵²

In this paper, we applied MudPIT for lens fiber cell membrane fraction proteomic and phosphoproteomic analysis. Our results extensively expand the previous reported results in this field. The method used in this study was simple and

FIGURE 3. Overrepresented phosphorylation motifs predicted by Motif-X. Phosphorylation motifs were extracted using Motif-X algorithm with the IPI human proteome as a background and the threshold for significance was set to $P < 10^{-6}$. Three overrepresented motifs were identified including one proline-directed motif and two basophilic motifs.

FIGURE 4. Potential kinases for identified phosphorylation sites predicted by NetworKIN. Potential kinases for identified phosphorylation sites were predicted by NetworKIN and, in total, 15 kinases for 475 phosphorylation sites were predicted, suggesting a major role for PKA, PKC, CKII, p38MAPK, and RSK kinase families in the lens.

effective. It will be interesting and important to further study the spatial difference and age-dependence of lens fiber cell proteomics and phosphoproteomics corresponding to different stages of fiber cell differentiation. The results will provide valuable information to help to further understand lens development and aging.

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