Differential Proteomic Analysis of the Mouse Retina

THE INDUCTION OF CRISTALLIN PROTEINS BY RETINAL DEGENERATION IN THE rd1 MOUSE*

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We have applied proteomic analysis to the degeneration of photoreceptors. In the rd1 mouse, a recessive mutation in the PDE6B gene leads to rapid loss of rods through apoptosis. By 5 wk postnatal, virtually all rod photoreceptors have degenerated, leaving one row of cones that degenerates secondarily. In order to assess comparative protein expression, proteins extracted from whole retina were resolved on a two-dimensional gel and identified by mass spectrometry combined with database screening. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry coupled to peptide mass fingerprinting was sufficient to identify most of the proteins, the remaining being identified with additional sequence information obtained by nano-electrospray ionization tandem mass spectrometry or liquid chromatography tandem mass spectrometry. The study revealed 212 spots, grouped into 109 different proteins. Differential analysis showed loss of proteins involved in the rod-specific photontransduction cascade, as well as induction of proteins from the crystallin family, in response to retinal degeneration. Identification of such pathways may contribute to new therapeutic approaches. Molecular & Cellular Proteomics 2:494–505, 2003.

The rd1 mouse carries a recessive mutation in the gene coding for the beta subunit of cGMP-phosphodiesterase (PDE6B) selectively expressed by rod photoreceptors (PR) and is a widely used model of inherited retinal degeneration (1). The defect in rod phosphodiesterase activity and consequent rise in intracellular cGMP concentration (2) lead to rapid loss of rods through apoptosis (3, 4). By 5 wk postnatal, virtually all rods have degenerated leaving one row of cones that undergo secondary degeneration (5). Experimental evidence suggests that secondary cone degeneration in this model results from loss of trophic support mediated by protein(s) secreted in the presence of rods (6, 7). Differential analysis would permit identification of proteins whose expression is lost following rod degeneration, and potentially those mediating cone viability. Analysis of gene expression in this model has already led to discovery of genes essential for photoreceptor function that are often found to be mutated and cause retinal degeneration in humans (8, 9). These analyses have measured the steady-state levels of mRNA using DNA chips (10, 11) and serial analysis of gene expression (12). It has been observed that mRNA levels are not directly correlated to protein expression (13). Because biological functions are performed by proteins, we conducted differential analysis of rd1 versus wild-type mouse retina at the protein level. In order to display retinal proteins, we separated retinal extracts by two-dimensional (2D) gel electrophoresis and identified stained protein spots by mass spectrometry followed by database searching. All the spots were first analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, the generated data being used to perform matching. This approach by peptide mass fingerprinting described in the early 90s permitted identification of >90% of the analyzed spots (14–17). For the remaining spots that could not be resolved by this approach, we used tandem mass spectrometry (MS/MS) with direct injection by nano-electrospray ionization (ESI)-MS/MS (18–20) or after an additional separation step, by nano-liquid chromatography (LC) MS/MS (21).

This study provides an analysis of the most abundant soluble proteins expressed in the adult mouse neural retina. In addition, comparison with rd1 retina after rods have degenerated demonstrates elevated expression of proteins of the crystallin family, indicating a possible endogenous mechanism of neuroprotection.

EXPERIMENTAL PROCEDURES

Animals—Care and handling of mice in these studies conformed to the Association for Research an Vision and Ophthalmology Resolu-
tion on the use of animals in research. C3H/HeN mice homozygous for the retinal degeneration 1 gene (rd1), and C57BL/6 normal sighted mice were obtained from Iffa-Credo Animal Suppliers (Lyon, France).

**Tissue Extraction and 2D Gel Electrophoresis**—All chemicals when not specified were obtained from Sigma (St. Louis, MO) and for dithiothreitol (DTT) and iodoacetamide were obtained from Fluka (Buchs, Switzerland).

Neural retinas were dissected in phosphate-buffered saline (PBS), and immediately transferred to extraction buffer: 50 mM Tris, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM DTT, protease inhibitors mixture (complete from Roche, Basel, Switzerland) for 45 min at 4 °C. After a few seconds of sonication, the concentration of each extract was determined by Bradford assay. Extracted protein (500 μg) was evaporated and suspended in rehydration buffer: 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 0.24% Triton X-100; 20 mM DTT, 20 mM spermine, 0.6%; Biolyte, pH range 3–10 (Bio-Rad, Hercules, CA). The samples were incubated 30 min at 20 °C and centrifuged 30 min at 15,000 rpm at 20 °C. Protein extracts were loaded strips of pH range 3–10 (Bio-Rad) during 15 h at 20 °C under 50 V in a PROTEAN isoelectric focusing cell (Bio-Rad). The isoelectric focusing was performed by steps of increased voltage: 1 ha t 150, 300, 600, and 1000 V followed by 15 h at 3000 V and finally 6 h at 500 V at 20 °C. The strips were incubated 30 min at 20 °C in electrophoresis buffer: 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 65 mM DTT and followed by 30 min in the same buffer containing 26 mM iodoacetamide. The second dimension was performed in a gradient gel (5–15% acrylamide) on a PROTEAN II (Bio-Rad) at 5 mA/gel for 3 h and 10 mA/gel overnight. The gels were stained by Coomassie brilliant blue (R 250; Sigma); spots were selected by visual inspection and gel slices were excised by scalpel.

**Western Blotting**—For Western blotting, 40 μg of protein from 2- and 5-wk-old C57BL/6 and C3H/He neural retina extracts were loaded on a 10% SDS-PAGE then transferred onto nitrocellulose membrane (Optitran; Schleicher & Schuell, Dassel, Germany). The nitrocellulose membrane was saturated overnight with 100 μl of 25 mM NH4HCO3 (Sigma) in PBS. The polyclonal anti-α-crystallin B (Chemicon, Temecula, CA) was incubated during 3 h at room temperature at a dilution of 1/5,000 in blocking solution then washed four times in PBS 0.1% Tween-20, then incubated 1 h at room temperature with goat anti-rabbit coupled to peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1/15,000 in blocking solution. The chemoluminescent reagents were added (ECL Plus; Amersham Biosciences, Buckingham, UK) and antibodies revealed by autoradiography on Biomax light film (Kodak, Rochester, NY). For standardization, a gel was loaded with the same extracts and probed with the monoclonal anti-α-tubulin (Sigma) at a dilution of 1/500 and revealed with an goat anti-mouse at 1/15,000 following the same procedure.

**In-gel Digestion Procedure**—Each gel slice was cut into small pieces with a scalpel, washed with 100 μl of 25 mM NH4HCO3 (Sigma), and then washed with 100 μl of acetoni tri le, as already described (22). After the washing step, gel pieces were completely

![Fig. 1.](image1)

**Fig. 1.** 2D gel electrophoresis separation of neural retina extracts after Coomassie staining. Left panel, wild-type mouse retina; right panel, rd1 mouse retina. Annotations according to Table I.

![Fig. 2.](image2)

**Fig. 2.** 2D gel electrophoresis separation of photoreceptors extracted from wild-type mouse retina isolated by vibratome sectioning.
**TABLE I**

List of identified proteins by MALDI-TOF MS and their annotations

| Spot no. | Protein Abbrev. | Accession no. | Molecular mass calculated (kDa) | Predicted function | Gene ontology |
|----------|----------------|---------------|--------------------------------|-------------------|---------------|
| A1       | Lumican        | LUM           | P51855                         | Extracellular Matrix | GO: 000578    |
| A2       | Splicing factor 3a1 | SF3          | NP_0080451                     | Splicing          | Cell Signalling |
| A5       | RAB-23 (open brain) | R23          | P35288                         | Transport         | GO: 0006868   |
| A6       | Hsp110         | h110          | Q61316                         | Chaperone         | GO: 0003773   |
| A8       | Tumor rejection Antigen | TRA        | P08113                         | Chaperone         | GO: 0003754   |
| A11      | Lamin B1       | LB1           | P14733                         | Cytoskeleton      | GO: 0005882   |
| A12      | Hnrnp          | Hnrp          | Q60577                         | Splicing          | GO: 0003529   |
| A14      | Vimentin       | Vim           | P20152                         | Cytoskeleton      | GO: 0005882   |
| A15      | Tubulin α-1    | Tbx           | P02551                         | Cytoskeleton      | GO: 0007018   |
| A17      | ATP synthase β | ATPβ          | P66480                         | Energetic metabolism | GO: 0006754 |
| A19      | Enolase 1      | Eno           | P17182                         | Glycolysis        | GO: 0006906   |
| A22      | β-actin        | βac           | P02570                         | Chaperone         | GO: 0005200   |
| A23      | Creatin kinase brain | Cki | Q04447                         | Glycolysis        | GO: 0004111   |
| A24-26   | Transducin β1  | Tβ1           | P4901                          | Vision            | GO: 0005718   |
| A24-26   | Transducin β2  | Tβ2           | P54312                         | Vision            | GO: 0005718   |
| A32      | PE-binding protein | Peb     | P70296                         | Splicing          | Cell Signalling |
| A33      | Histone h2b f  | H2b           | P10853                         | Energetic metabolism | GO: 0006754 |
| A35      | β-synuclein    | βSy           | Q16143                         | Synaptic transmission | GO: 0004356 |
| A37      | Cytochrome c oxidase | Cyc | P12787                         | Energetic metabolism | GO: 0006118   |
| A42      | Recoverin      | Rec           | P34057                         | Vision            | GO: 0007018   |
| A43      | 14-3-3 protein γ | 14γ       | P35215                         | Vision            | GO: 0005718   |
| A44      | Phosphoglycerate mutase | PgM | P18669                        | Energetic metabolism | GO: 0006754 |
| A47      | EF1α1          | E1α1          | P10126                         | Translation       | GO: 0003746   |
| A47      | EF1α2          | E1α2          | P27706                         | Translation       | GO: 0003746   |
| A49      | PTB-splicing factor | PTB        | P23746                         | Splicing          | GO: 0006371   |
| A55      | Glutamine synthase | Gls | P15105                        | Glutamate metabolism | GO: 0004356 |
| A62      | LDH-A          | LDH           | P61515                         | Chaperone         | GO: 0006457   |
| A67      | Cyclophilin A  | CyA           | P17742                         | Chaperone         | GO: 0006457   |
| A68      | Ubiquitin      | Ubn           | P02248                         | Protein degradation | GO: 0006700   |
| A69      | Vacular ATPase | vATP          | P50165                         | Energetic metabolism | GO: 0006754 |
| A70      | Calretinin     | CaR           | Q08331                         | Ca²⁺-binding      | GO: 0005509   |
| A71      | S-arrestin     | Arr           | P20443                         | Vision            | GO: 0007601   |
| A73      | Histone H2A.3  | H2a           | P20671                         | Translation       | GO: 0005718   |
| A74      | Chromatin assembly factor | CAF | Q13112                        | Translation       | GO: 0005634   |
| A76      | P 450 11B2     | P450          | P15539                         | Steroid hormone   | GO: 0006700   |
| A78      | Transaminase   | Trm           | P05202                         | Glycolysis        | GO: 0006520   |
| A79      | Hypothetical cytochrome c fam. | Act | P24752                        | Glycolysis        | GO: 0003985   |
| A80      | 60S protein ribosomal 4OS | 60S       | P40429                         | Translation       | GO: 0003735   |
| A81      | snRNP          | Snrp          | P43331                         | Splicing          | GO: 0006371   |
| A85      | T-box transcription factor | Tbx | P70325                        | Chaperone         | GO: 0003700   |
| A96      | Hsp 94         | Hsp           | P48722                         | Chaperone         | GO: 0006520   |
| A104     | Vesicular fusion P | P20 | P46460                        | Transport         | GO: 0008565   |
| A105     | Actin interacting protein | Act | Q08342                        | Cytoskeleton      | GO: 0005856   |
| A106     | Ulip 2         | Ulip          | O08553                         | Cell Signalling   | GO: 0005856   |
| A109     | Unc-18 homolog | Unc           | O08599                         | Transport         | GO: 0008565   |
| A113     | Ulip 4         | Ulip          | O35098                         | Cell Signalling   | GO: 0005856   |
| A116     | TACE           | TACE          | O08599                         | Cell Signalling   | GO: 0005856   |
| A117     | Dihydrolipoamide deshydrogenase | OADH | P03070                        | Energetic metabolism | GO: 0006096   |
| A134     | Fructose bisphosphate aldolase | FBP | P03024                        | Glycolysis        | GO: 0005856   |
| A138     | HnRNP A2       | HnRNP A2      | O08599                         | Splicing          | GO: 0003529   |
dried with a speed vacuum for the reduction-alkylation step as described. The supernatant was removed, and the washing procedure with 100 μl of NH4HCO3 and acetonitrile was repeated three times. Finally, gel pieces were again completely dried before trypsic digestion and swelled in a solution of trypsin (12.5 ng/μl; Promega, Madison, WI) in 25 mM NH4HCO3. The digestion was performed at 35 °C overnight, and the extraction step was performed with H2O/5% HCOOH. The mixture was sonicated 10 min and left a minimum of 1 h at room temperature, permitting passive elution of the peptides.

MALDI-TOF Mass Spectrometry—Mass measurements were carried out on a BIFLEX III™ MALDI-TOF mass spectrometer (Bruker, Bremen, Germany) equipped with the SCOUT™ high-resolution optics with X-Y multi-sample probe and griddles reflector. This instrument was used at a maximum accelerating potential of 19 kV and was operated in reflector mode. Ionization was accomplished with a 337-nm beam from a nitrogen laser with a repetition rate of 3 Hz. The output signal from the detector was digitized at a sampling rate of 2 GHz. A saturated solution of α-cyano-4-hydroxycinnamic acid in acetonitrile was used as matrix. A first layer of fine matrix crystals was

| Spot no. | Protein Description | Abb. | Coverage (%) | Accession no. | Molecular mass calculated (kDa) | Predicted function | Gene ontology |
|----------|---------------------|------|--------------|--------------|---------------------------------|-------------------|--------------|
| A139     | Mitochondrial malate deshydrogenase | Mit | 35 | P08249 | 35.6 | Glycolysis | GO : 0006099 |
| A140     | Capping β | Capping β | 25 | P47757 | 30.6 | Cytoskeleton | GO : 0005200 |
| A146     | UCP 2 | UCP 2 | 20 | P70406 | 33.4 | Energetic metabolism | GO : 00160689 |
| A150     | RAN | RAN | 34 | P17080 | 24.4 | Transport | GO : 0008565 |
| A151     | Triose P isomerase | Triose P isomerase | 21 | P99PG4 | 27.6 | Signal transduction | GO : 0007165 |
| A155     | Phospholipase A2 IIF | Phospholipase A2 IIF | 17 | Q61337 | 22.1 | Apoptosis | GO : 0006915 |
| A157     | Lactoylglutathione lyase | Lactoylglutathione lyase | 55 | P17751 | 26.6 | Signal transduction | GO : 0006906 |

**Table I—continued**

| Spot no. | Protein Description | Abb. | Coverage (%) | Accession no. | Molecular mass calculated (kDa) | Predicted function | Gene ontology |
|----------|---------------------|------|--------------|--------------|---------------------------------|-------------------|--------------|
| B1       | α crystallin A, major component | αCM | 86 | P02490 | 19.4 | Chaperone | GO : 0005198 |
| B2       | α crystallin A, minor component | αCm | 43 | P24622 | 22.5 | Chaperone | GO : 0005198 |
| B6       | β crystallin B3 | βC3 | 60 | Q9JJU9 | 24.3 | Chaperone | GO : 0005198 |
| B29      | Fus | Fus | 14 | P66959 | 52.7 | Splicing | GO : 0003723 |
| B31      | Carbonic anhydrase | CAII | 54 | P00920 | 29.0 | Glycolysis | GO : 0004089 |
| B33      | GDP dissociation Inhibitor 1 | GDI | 29 | P50926 | 36.6 | Transport | GO : 0008565 |
| B36      | α crystallin B chain | αCB | 21 | P29327 | 20.1 | Chaperone | GO : 0008565 |
| B39      | Peroxiredoxin 5 | Px5 | 40 | O08709 | 24.9 | Chaperone | GO : 0016209 |
| C6       | Tubulin β-5 chain | Tbβ | 37 | P05218 | 49.7 | Cytoskeleton | GO : 0005198 |
| C25      | Voltage-dependent anion channel 1 | VDC | 41 | Q60932 | 36.5 | Apoptosis | GO : 0008308 |
| C33      | ISGF-3G | ISG3 | 14 | Q61179 | 44.6 | Transcription | GO : 0003700 |
| C42      | Calreticulin | CNA | 48 | P14211 | 48.0 | Ca2⁺-binding | GO : 0005514 |
| C43      | Valosin containing Protein | MCP | 43 | Q01835 | 89.3 | Transport | GO : 0006810 |
| C45      | HspB6-1 α | αH86α | 45 | P07901 | 84.8 | Chaperone | GO : 0003773 |
| C46      | HspB6-1 β | βH86β | 28 | P11499 | 83.3 | Chaperone | GO : 0003773 |
| C48      | HsPa | αB | 55 | P20029 | 72.4 | Chaperone | GO : 0003773 |
| C49      | Hsp70.1 | h70 | 24 | P17879 | 70.0 | Chaperone | GO : 0003773 |
| C50      | Phosducin | Pho | 61 | Q9QW08 | 28.0 | Vision | GO : 0007601 |
| C51      | 14-3-3 protein ε | 14ε | 70 | P42655 | 29.2 | Vision | GO : 0005179 |
| C53      | HMG-1 | HMG | 51 | P07155 | 24.9 | Transcription | GO : 0006355 |
| C54      | K-ras | ras | 22 | P32883 | 21.7 | Signal transduction | GO : 0007264 |
| C56      | GAPDH | Gdh | 74 | P16858 | 35.8 | Glycolysis | GO : 0006906 |
| C58      | Acetyl glucosaminyl transferase | agt | 18 | P97402 | 45.5 | Extracellular matrix | GO : 0006906 |
| C63      | Cytoplasmic malate deshydrogenase | Mdh | 47 | P14152 | 36.5 | Glycolysis | GO : 0006906 |
| C65      | Glutamate oxaloacetate transaminase 1 | Got | 39 | P05201 | 46.2 | Glutamare metabolism | GO : 0006520 |
| C67      | Phosphoglycerate kinase | PhK | 56 | P09411 | 44.5 | Glycolysis | GO : 0006906 |
| C69      | Glutamate dehydrogenase | Gld | 10 | P26443 | 61.3 | Glutamate metabolism | GO : 0004353 |
| C70      | ATP synthase α | ATP | 31 | Q03265 | 59.8 | Energetic metabolism | GO : 0006754 |
obtained by spreading and fast evaporation of 0.5 μl of matrix solution. On this fine layer of crystals, a droplet of 0.5 μl of aqueous HCOOH (5%) solution was deposited. Afterward, 0.5 μl of sample solution was added and a second droplet of 0.2 μl of matrix saturated solution in 50% H₂O/50% acetonitrile was added. The preparation was dried under vacuum. Prior to deposit, samples were concentrated between 5- and 10-fold on zip-tip® (Millipore, Bedford, MA). The sample was washed once to three times by applying 1 μl of aqueous HCOOH (5%) solution on the target and then flushed after a few seconds. The calibration was performed in internal mode with four peptides, angiotensin, substance P, bombesin, and trypsin autolysis fragments with mono-isotopic (M+H)⁺ at m/z 1046.542, 1347.736, 1620.807, and 2211.107, respectively.

Nano-LC-MS/MS—Experiments were performed on extracted tryptic peptides from 2D gels. High-pressure LC separation was performed on a CapLC™ system (Waters, Milford, MA). The system was coupled via a nano-LC inlet to the quadrupole-TOF mass spectrometer (Micromass, Altrincham, UK) equipped with a nano-electrospray (Z-spray) source. Peptides were first desalted and then concentrated on a reverse phase precolumn of 300 μm ID (Microsorb, Santa Barbara, CA) by solvent C (H₂O/0.1% HCOOH) by solvent C (H₂O/0.1% HCOOH) delivered by an independent pump at a flow rate of 30 μl/min. The gradient was formed by the activation of two complementary pumps A and B, delivering respectively solvent A (H₂O/0.1% HCOOH) and solvent B (acetonitrile/0.1% HCOOH) at a flow rate of 3 μl/min. A split of 1/10 allowed the flow rate to be decreased to 300 nl/min. Peptides were eluted through a reverse-phase capillary column of 75 μm ID (PepMap, C18; LC Packings) with a linear gradient from 5 to 95% of solvent B over 60 min under the control of the MassLynx 3.5 software. The eluted peptides were analyzed by MS/MS with an automated MS-to-MS switching protocol when doubly or triply charged ions (precursor ions) were detected up to 10 counts/s over the m/z range 200 to 2000. In the MS experiment, precursor ions were measured in positive detection mode with a cone voltage of 30 V and selected by the quadrupole. The collision-induced dissociation (CID) MS/MS process was performed with collision energy between 20 and 50 eV in the collision cell and argon as the collision gas. The multi-mode analysis capacity of the quadrupole-TOF II permitted the analysis of co-eluted peptides with the ability to perform MS/MS experiments on eight different ions (eight different channels) in parallel. MS/MS spectra containing fragment ions in multiple charge states were deconvoluted using MaxEnt-3 software (Micromass) into single-charged and mono-isotopic ion spectra.

Database Search—Ions obtained from MALDI spectra were directly used for database searches using the software MS-Fit developed at the University of California, San Francisco MS facility and available on the internet to search against protein databases (Swiss-Prot and NCBI). Database searches were performed using the following values, protein molecular mass range of 10–200 kDa, trypsin digest with one missing cleavage allowed, cysteines modified by carbamidomethylation, possible oxidation of methionines, and mass tolerance of 50 ppm. The identification was based upon at least four matching peptides. The percentage of protein coverage (with peptides measured with 50 ppm mass tolerance) was taken into account for validation of protein identification.

Data resulted from LC-MS/MS were converted into a peak list containing all mono-isotopic peaks and submitted to the Swiss-Prot database via the Global Server 1.0 search engine (Micromass). The peptide mass was set to 0.5 Da and the MS/MS tolerance to 0.25 Da. The MS/MS analysis results were searched by BLAST after manual interpretation of spectra. Gene identities were extracted using specifically designed algorithms.

RESULTS

We performed 2D gel electrophoresis with whole-cell extracts of wild-type retina. The left panel of Fig. 1 illustrates the profile obtained with wild-type retina after coomassie staining. Coomassie blue was selected in preference to silver stain for reasons of compatibility with mass spectrometry, despite the better sensitivity of silver stain (23). The right panel of Fig. 1 shows that the profile obtained with 5-wk-old rd1 mouse retina is similar to that of wild type in many respects, with only a few spots missing from rodless retina and parallel expression of new proteins induced by rod degeneration. To directly visualize PR-specific proteins, gel analysis of the outer nuclear layer, which contains uniquely PR cell bodies, was performed (Fig. 2). This fraction was isolated by vibratome sectioning of flat-mounted retina from wild-type 5-wk-old mice (24). The protein spots were excised, washed, and digested...
Fig. 4. Nano-ESI MS/MS spectra obtained after isolation and fragmentation. a, ion (M+2H)^2+ at m/z 513.35; b, ion (M+2H)^2+ at m/z 488.32; c, ion (M+2H)^2+ at m/z 542.92. The interpreted amino acid sequences are depicted at the top of each spectrum. The spectra were treated by deconvolution before database searching.
with trypsin. The mass of the resulting peptides were measured using MALDI MS. In Table I, representing the list of proteins identified, the entries annotated An correspond to spots from analysis of wild-type total retina; the B series represents proteins identified from rd1 mouse total retina; and the C series is the list of proteins identified from the PR layer of wild-type retina. The relevance of these identifications is essentially based on the number of matching peptides combined with the high mass accuracy of the measure. Even if four matching peptides and 50 ppm mass tolerance for data base searches were required, the average peptide mass accuracy of identified proteins in Table I is 15 ppm (±5), and the number of matching peptides, expressed as % protein sequence coverage, always represents >4 peptides. These two criteria are reliable and allowed the unambiguous identification of the listed proteins (19).

Despite the limited resolution of 2D gel electrophoresis, through the use of MALDI-TOF MS it was possible to detect multiple proteins in the same spot. For multiple identifications, peptide masses assigned for the protein identified with the highest coverage were excluded, and the remaining list of masses was used to search the database (25). An example of the complexity of the mass spectrum obtained is shown in Fig. 3, where more than 100 peptides have been measured with mono-isotopic resolution. This analysis permitted the identification of four different proteins, phosphoglycerate mutase, phosphoglycerate kinase, creatine kinase, and pyruvate kinase. These proteins of very similar molecular mass from 44.5 to 57.9 kDa are not resolved on the gel, even if their isoelectric point show a variation over 1.5 pH units that should theoretically permit their separation. Post-translational modifications can induce variations in their isoelectric point and thus interfere with separation. These post-translational modifications also led to multiple spots for the same protein, for example glutamine synthase, lactate dehydrogenase, and pyruvate kinase (26). This phenomenon could also result from an incomplete isoelectric focusing (27).

In cases where MALDI-TOF analysis did not reach the criteria cited above, MS/MS was used to obtain sequence information and used in data mining. This additional procedure allowed unambiguous identification of proteins. In some cases, high abundance contaminant peptides (trypsin autolysis fragments, keratin contaminants) can suppress the detection of peptides of interest using the MALDI-MS approach (28). In order to detect these peptides, LC-MS/MS was performed. The chromatographic step (nano-LC) prior to MS analysis permitted fractionation of the peptide mixture prior to spectrometric analysis. Thus each peak characterized by its mass over charge ratio (m/z) is further analyzed by CID MS/MS (29). It is clear that MALDI and nano-ESI modes were very complementary, and the use of two different ionization processes contributed to detection of a wider range of peptides (30). In Fig. 4, three examples of MS/MS spectra are presented. Panels a and b represent MS/MS spectra obtained after CID experiments on doubly charged ions at m/z 513.35 and m/z 488.32, which both led to the identification of an unique protein, the elongation factor EF1α (Eef1). The interpretations of the spectra after deconvolution with MaxEnt 3 (MassLynx 3.5; Micromass) conducted on y series ions (classification according to Ref. 31) led to the interpretation of the amino acid sequences (GIGTV(PV)GR and PLQDVYK) shown in Fig. 4 and Table II. These peptides covered 6% of the protein sequence. With regard to the MS/MS spectrum in Fig. 4c, a unique sequence (SPI/LVVI/LSKGK) allowed unambiguous identification of the protein Ulip2. Independently of the search engine used to interrogate the database, use of either the interpreted sequence or the generated peak list led to unambiguous identification of Ulip2 protein. Table II gives the list of additional proteins identified using MS/MS and LC-MS/MS.

The identified proteins in Tables I and II have been annotated according to gene ontology, including their predicted functions (Fig. 5). Six categories were created: metabolism, expression, structure, neuronal functions, signaling, and defense, corresponding to major cellular and neuronal functions. The most diverse category was expression, with 34% identified proteins participating in transcription, splicing, translation, folding, transport, and protein degradation; then in descending order of abundance proteins with neuronal functions (20%), metabolism (19%), signaling (13%), defense (8%), and finally structural proteins (6%). Structural proteins represented the most abundant proteins: vimentin, tubulins (Tbα and β), and actin (Jac) (Fig. 1, left panel). This diversity was respected for proteins identified from photoreceptor cells (Fig. 2).

We next enlarged this study by comparing with data obtained from rodless rd1 mouse retina (Fig. 1, right panel). Proteins involved in PR function, such as β-transducin (Tβ1 and 2), recoverin (Rec), and 14.3.3 proteins (143/α/e), exhibit reduced levels in the rodless retina as expected (Fig. 1, left panel). After rod degeneration, a large increase in the content of the crystallin protein family was observed (αCM, m and αCb, and βC2 and 3 in Fig. 1, right panel). A low level of the α-crystallin A Major component (αCM) was also detected in the normal retina (Fig. 1, left panel). The induction of α-crystallin B (αCb) expression was examined using Western blotting (Fig. 6a). Expression was compared between wild-type and rd1 mice during and after rod degeneration, and only low levels of immunoreactivity were detected in wild-type retina at both ages (lanes 1 and 2) as compared with the internal standard (Fig. 6b).

This qualitative analysis led to the identification of 212 spots by MALDI-TOF MS, nano-ESI-MS/MS, and LC-MS/MS, representing 109 different proteins.

**DISCUSSION**

This is to our knowledge the first proteomic study of mouse retina, with unambiguous identification of 212 spots. Only 27
spots (13%) did not give any information. The vast majority of the 2D gel features were identified by peptide mass fingerprinting combined with MALDI-TOF MS analysis. Only a small number of silent spots were resolved by MS/MS and peptide sequencing. Concerning unidentified features, several reasons could be proposed. First, an ineffective tryptic digestion may result in a low yield of peptide extraction from the gel, and hence impaired mass spectrometric analysis. Second, the extensive post-translational modifications could generate unpredictable peptide masses, thus weakening the database search. Third, tryptic peptides outside the experimental mass range of 1000–4000 m/z will not be considered. Finally, the protein could be unknown and not yet included in the protein databases, and would thus require complete sequencing (Edman microsequencing or de novo MS/MS). The percentage coverage of identified proteins ranged from 6 to 86%. Several studies have demonstrated that validation of the identifications relies on several elements including random matches (32). In order to minimize false results, we took into account the correlation between the observed molecular mass of the protein (Figs. 1 and 2) and its identification (Table I).

The predicted locations of the proteins within cellular compartments represent the entire spectra, i.e. nuclear (37%), mitochondrial (25%), endoplasmic (15%), and membrane proteins (15%). Nevertheless, it should be noticed that rhodopsin was not found, while this 7-transmembrane receptor is very abundant in PRs. Out of five integral membrane proteins identified, three (TACE, acetylglucosaminyltransferase, and metalloendopeptidase) have only one transmembrane domain, and the other two (UCP2 and channel VdC) are multiple transmembrane domain proteins from mitochondrial membranes. The presence of the latter in our analysis may be a consequence of their specific subcellular location.

Fig. 5 is a schematic view of the diversity of functions required for normal retinal physiology. It is not surprising that proteins participating in expression are represented in abundance because the retina, and more specifically the PR, have the highest metabolic rate in the body, for example constant renewal of disks (33). Six splicing factors that most probably correlate with their abundance in retina were recognized. Even if these proteins mediate some basic biological process with no tissue specificity, the finding of disease-causing mutations in splicing factors demonstrates their importance in retinal function (34–36). Analysis of the retinal proteome provides a new approach in the search for genes involved in retinal dystrophies. One detected major protein, cyclophilin A, shares extensive homology to ninaA, a gene involved in photoreceptor degeneration in Drosophila (37, 38). The cyclophilin A gene maps to the RP9 locus, but no mutation mapping to that locus has been found in retinitis pigmentosa families, consistent with the recent identification of Pim-1

| Spots | Analysis | Precursor ion (charge) | Accession No. | Molecular mass calculated | Identified sequence | Identified protein | Predicted function | Gene ontology |
|-------|----------|-----------------------|---------------|---------------------------|---------------------|--------------------|-------------------|--------------|
| A45   | MS/MS    | 640.3 (2+)           | P0904929      | 24.9                      | GEPRKXXAK           | HMG-1              | Transcription     | GO:0006365   |
| A50   | MS/MS    | 611.3 (2+)           | H11001        | 57.9                      | VLTK                | PKTK               | Glycolysis        | GO:0006096   |
| C13   | MS/MS    | 564.4 (2+)           | H11001        | 42.0                      | FELR                | ATF-2              | Translation       | GO:0003746   |
| A77   | LC-MS/MS | 513.4 (2+)           | H11001        | 49.3                      | IGGIGTVPVGR         | Translation        | Cell signalling   | GO:0006740   |
| A83   | LC-MS/MS | 542.9 (2+)           | H11001        | 49.5                      | GSPLVVISQK          | EF-1               | Cell signalling   | GO:0006740   |
| B46   | LC-MS/MS | 898.0 (2+)           | H11001        | 49.5                      | VTVLENFOQK          | Tcp-10             | Immune response   | GO:0006365   |
| B47   | LC-MS/MS | 713.9 (2+)           | H11001        | 49.5                      | VTVLENFOQK          | Tcp-10             | Immune response   | GO:0006365   |

* Ion fragments are labeled according to the nomenclature of Roepstorff (31). y and b fragments correspond to rupture of the peptide bond with charge carried on the C and N terminus, respectively. The accompanying number corresponds to the position of the amino acid in the sequence.

2 S. Bhattacharya, personal communication.
**FIG. 5.** Schematic view of functions performed by the identified proteins. Italic letters correspond to annotation from gene ontology. The percentage of broader categories are indicated at the center of the graph.

**FIG. 6.** Western blotting analysis. Lanes 1 and 2, wild type; 3 and 4, rd1. Lanes 1 and 3, 15 days postnatal; 2 and 4, 35 days postnatal. a, α-crystallin B; b, α-tubulin.
gene mutations at that locus (39). Proteins involved in cell signaling were also identified (TACE, Ulip2, Ulip4, and Prp). The importance of tumor necrosis factor-α converting enzyme is highlighted by its specific inhibition by the TIMP3 gene product, involved in macular degeneration (40, 41). The proteins clustered in the category-annotated defense include apoptosis, redox control, and immune response (Fig. 5). The sustained protein synthesis in retinal tissues implies a large production of reactive oxygen products that are known to be neurotoxic (42, 43). Two peroxiredoxins (Px2 and Px5), enzymes involved in the reduction of these reactive products, were identified. Thioredoxin, a protein controlling redox potential, has been shown to prevent PR degeneration in vitro (44).

Using a differential approach, we found expression of crystallins from the small heat shock protein family (SHP) (45, 46) to be greatly increased after rod degeneration. A link between α-crystallin B induction and rod degeneration is suggested by our analysis by Western blotting (Fig. 5) and by previous studies (47). The level of α-crystallin B was found to be elevated at both ages examined, with a maximum at 15 days postnatal, the stage of maximal rod degeneration in the rd1 mouse (5). The mechanism of SHP regulation involves an increase in steady-state levels of their mRNA, as observed using micro-array analysis (data not shown). Up-regulation of α-crystallin B has been observed in many stress-inducing conditions, from oxidative stress to Alzheimer disease (46, 48). In addition, it is a specific constituent of drusen in patients suffering from aged-related macular degeneration (49). This observation has been extended by providing evidence that other members of the crystallin family are also highly up-regulated. The two proteins α-B and α-A constitute subunits of a multimeric complex that functions as a chaperone. α-crystallin B has been shown to block apoptosis induced by many stimuli (50). This activity is mediated by preventing the activation of the proenzyme caspase 3 (51) or alternatively by preventing the aggregation of the tau protein (52) with mutated proteins carrying a polyglutamine track (53). It is not known if the formation of a functional complex containing various SHP is necessary for inhibition of apoptosis. The presence of crystallin proteins after all rods have degenerated suggests that the protein is not produced exclusively by rods, but most likely by surrounding cells such as Müller glial cells in response to signals triggered by rod apoptosis (54). It is paradoxical that rod apoptosis induces the expression of an anti-apoptotic protein in surrounding cells. The increase in crystallin family protein content in cells that do not express the mutated protein (PDE6B) responsible for the disease might constitute a general mechanism, because it has been observed in many pathological conditions (46). This pathway might provide alternative targets for the development of neuroprotective therapies in this model.

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