In Search of the Identity of the XAP-1 Antigen: A Protein Localized to Cone Outer Segments

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PURPOSE. To determine the identity of the XAP-1 antigen. The XAP-1 antibody has been used by many investigators and is recognized as an index of photoreceptor outer segment maturity, yet its antigen remains unknown.

METHODS. Previous studies documented that the XAP-1 antigen is a photoreceptor membrane-associated protein. To enrich for this protein, the authors prepared outer segment preparations from mouse retinas. Crude membrane and cytoplasmic fractions from this preparation were then generated using ultracentrifugation. Proteins were solubilized using n-dodecyl β -D-maltoside and separated using SDS-PAGE. Aliquots of the crude membrane fraction were run on multiple lanes of a single gel, one lane of which was transferred to PVDF membrane and probed with the XAP-1 antibody. The remaining lanes were silver-stained. Very careful alignment of the Western blot with the silver-stained lanes indicated the presence of a single lightly stained band at the same position as the immunopositive band. nanoLC-ESI-MS/MS analysis was performed on the pooled protein bands. On determining the protein identity, confirmatory Western blot analysis and immunohistochemistry studies were performed.

RESULTS. Western blot analysis performed using the XAP-1 antibody indicated a single immunoreactive band at approximately 74 kDa in lysates from both total outer segment and crude membrane preparations. No immunoreactive band was present in the cytoplasmic lysate. MS analysis of pooled silver stained bands determined that the XAP-1 antigen is Grp78.

Western blot analysis and immunohistochemistry both support this identification.

CONCLUSIONS. Present evidence indicates that the XAP-1 antigen is Grp78, a protein that has been previously documented in the interphotoreceptor matrix surrounding cones. (*Invest Ophthalmol Vis Sci.* 2010;51:2736–2743) DOI:10.1167/iovs.09-4286

The mature vertebrate photoreceptor is a highly polarized and structurally unique photosensitive cell of the neural retina.^{1–4} The outer segments of these cells are composed of densely stacked, flattened membranous discs surrounded by a plasma membrane.^{5,6} Although the maintenance of proper outer segment structure is crucial for the survival and function of the photoreceptor cell, the mechanisms that regulate this complex physiologic process have not yet been fully elucidated.

The role of the retinal pigment epithelium (RPE) in the structural maintenance and daily outer segment membrane renewal process has been examined by many laboratories.^{7–11} The RPE is the nonneuronal tissue located adjacent to the retina, the apical processes of which surround photoreceptor outer segments.^{8,10} Previous work performed in our laboratory has shown that the RPE is important in intact retinas for the structural maintenance and membrane elaboration of photoreceptor outer segments in intact retinas^{12,13} and for the normal expression of some photoreceptor proteins, such as the *Xenopus* anti-photoreceptor-1 (XAP-1) antigen.¹⁴ Specifically, we demonstrated using RPE-supported *Xenopus laevis* tadpole retinas that photoreceptor cells with well-organized outer segment membranous discs express the XAP-1 antigen, which is localized to the area of the plasma membrane surrounding outer segments and the distal inner segments beyond the outer limiting membrane. In similarly maintained yet RPE-deprived retinas, nascent outer segments are elaborated as membranous whorls, and XAP-1 immunopositive labeling is undetectable.¹⁴ The addition of a neurosupportive agent to RPE-deprived retinas not only allows for proper outer segment membrane disc assembly,^{13,15} it supports XAP-1 antigen expression in a pattern similar that of to control retinas.¹⁴ The correlation between XAP-1 antigen expression and organized photoreceptor outer segments strongly suggests that the XAP-1 antigen may play an important role in the proper assembly and stability of photoreceptor outer segment membrane discs.¹⁴

Although the XAP-1 antibody has been used by multiple laboratories and is widely acknowledged to be a marker of photoreceptor maturity in both amphibian and mammalian retinas,^{16–18} the identity of the antigen to which it binds remains unknown. Because our previous studies indicated that the XAP-1 antigen might play an important role in photoreceptor outer segment assembly, it has become critical to characterize that protein. In this study, we have isolated the XAP-1

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antigen from a mouse photoreceptor outer segment-enriched preparation and subjected it to tryptic digestion and nanoelectrospray ionization quadrupole ion-trap mass spectrometric (nanoLC-MS/MS) analysis. The MS/MS data were used to search the Swiss-Prot protein database for the identity of the XAP-1 antigen. In addition to characterizing the protein by mass spectrometry and database searches, we also provide Western blot and immunohistochemistry data that further corroborate the protein characterization. Present evidence indicates that the XAP-1 antigen is Grp78, a protein previously localized to the interphotoreceptor matrix surrounding cones in porcine retinas.

MATERIALS AND METHODS

XAP-1 Antibody Production

The XAP-1 antibody is a monoclonal IgM antibody that was developed by Donald S. Sakaguchi and William A. Harris. The antibody was produced from mice immunized with stage 45 to 53 *X. laevis* tadpole optic nerves and retina homogenates.¹⁶ It is maintained at the Developmental Studies Hybridoma Bank at the University of Iowa, Department of Biological Sciences (Iowa City, IA).

Immunohistochemistry

Throughout this study, animals were handled in a manner consistent with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all studies were approved the Animal Care and Use review board of the University of Tennessee Health Science Center. Retinas from 1- to 3-month-old C57BL6/J mice or adult *X. laevis* frogs were obtained immediately after euthanatization and were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 6 hours. After thorough rinsing in phosphate buffer, retinas were embedded in low melting point agarose (Sigma, St. Louis, MO), and sections (~50- μ m thickness) were cut on a vibratome (VT1000S; Leica, Wetzlar, Germany). The XAP-1 monoclonal antibody (Developmental Studies Hybridoma Bank; 1:1 dilution) and rabbit anti-human Grp78 (Anaspec; 1:25 dilution) were used as the primary antibodies. High-temperature antigen retrieval using EDTA (pH 8.0) was performed on retinal sections before exposure to the anti-Grp78. To amplify the immunopositive signal, rabbit anti-mouse IgM (mu-chain specific; Pierce, Rockford, IL) and mouse anti-rabbit IgG antibodies (ImmunoPure; Thermo Scientific, Rockford, IL), respectively, were used as unconjugated secondary antibodies. The appropriate Alexa Fluor 488-conjugated tertiary antibodies (Invitrogen, Carlsbad, CA) were used to visualize the immunolabeling. Negative controls consisted of omission of primary antibodies. When appropriate, peanut agglutinin (PNA)-tagged with Alexa Fluor 594 and wheat germ agglutinin (WGA)-tagged with Alexa Fluor 633 were used to identify cone and rod photoreceptors, respectively. Iodide (TO-PRO III, 1:4000; Invitrogen) was used to label nuclei. Retinal sections were examined, and images were acquired using a confocal microscope (C1 Plus; Nikon, Tokyo, Japan).

Preparation of Samples for Western Blot Analyses

To prepare whole retinal samples, retinas from 1- to 3-month-old C57BL6/J mice were carefully dissected on ice immediately after euthanatization, rinsed in cold 0.1 M phosphate buffer, and incubated in phosphate buffer containing protease inhibitors (Sigma) and n-dodecyl β -D-maltoside (DDM; Pierce) at a final concentration of 1%. The retinas were incubated for 10 minutes, followed by two 5-minute periods of sonication in an ice water bath, and were centrifuged for 25 minutes (1000g, 4°C). The cleared supernatant was collected and used as the whole retinal lysate.

For the outer segment-enriched fraction, retinas in cold 0.1 M phosphate buffer containing protease inhibitors were briefly sonicated in an ice water bath for two 5-minute periods. Retina samples were then centrifuged for 25 minutes (1000g, 4°C). The supernatant was

collected, and proteins were solubilized in DDM (1% final concentration).

To isolate the crude membranes from the outer segment-enriched fraction, before protein solubilization, the samples were ultracentrifuged for 1 hour on an ultracentrifuge (Beckman Coulter, Hialeah, FL) at 200,000g using a fixed-angle rotor (type 70.1 Ti; Beckman Coulter). The supernatant containing the cytosolic fraction was separated from the pellet containing the enriched crude membrane fraction. Both fractions were individually resuspended in a small volume, and DDM was added to a final concentration of 1%. For all preparations, the samples were separated into aliquots, and the protein concentration was determined (BCA kit; Bio-Rad, Hercules, CA) and stored immediately at -80°C.

1D SDS-PAGE and Western Blot Analyses

Various protein samples (5–10 μ g) were diluted in an equal volume of sample buffer (NuPAGE LDS; Invitrogen) and were subjected to SDS-PAGE under reducing conditions. Gels (NuPAGE 4%–12% Bis-Tris; Invitrogen) were used for the electrophoretic separation of proteins at a constant voltage of 200 V. Prestained molecular weight marker (SeeBlue Plus2; Invitrogen) was used as a standard to determine the relative molecular mass of bands of interest. After electrophoresis, proteins were transblotted to nitrocellulose membrane (Nitrobind 0.45 μ M; Micron Separations, Westborough, MA) or polyvinylidene difluoride (PVDF; Hybond P; Amersham Pharmacia Biotech, Piscataway, NJ) at 30 V for 1 hour for subsequent Western blot analyses. After transfer, nonspecific binding sites were blocked with 5% blotto (Bio-Rad) for 1 hour at room temperature, followed by incubation in XAP-1 antibody (1:100, overnight at 4°C; Developmental Hybridoma Bank, Iowa City, IA). After rinsing, blots were incubated in unconjugated rabbit anti-mouse IgM antibody (mu-chain specific; Pierce; 1:1000 dilution for 1 hour at room temperature). After additional washes, the blots were incubated with goat anti-rabbit conjugated with horseradish peroxidase (HRP; Southern Biotechnology, Birmingham, AL; 1:10,000 for 30 minutes at room temperature) and were developed using enhanced chemiluminescence substrate (Supersignal WestFemto; Pierce). The blots were visualized using a Kodak imager (4000MM; Eastman Kodak, Rochester, NY).

nanoLC-MS/MS Analyses and Swiss-Prot Database Search

To prepare samples for mass spectrometric analysis, aliquots of the crude membrane fraction were run in parallel lanes on gels (NuPAGE 4%–12% Bis-Tris; Invitrogen) under reducing conditions. The gels were run for an extended period to allow for maximal separation in the 74-kDa relative molecular weight range. One lane was processed as a Western blot, as described. The remaining lanes of the gel were silver stained using a protocol that is compatible with mass spectrometry.¹⁷ To allow for proper alignment, a lane loaded with molecular weight markers was bisected; half was processed with the Western blot, and the other half was processed with the silver-stained lanes. A single band from each lane corresponding to the immunopositive band was excised and processed for in-gel protease digestion, as described previously.¹⁹ The nanoLC-MS/MS (LCQ^{Deca}; ThermoFinnigan, San Jose, CA) was used to obtain amino acid sequences from the tryptic peptides obtained from the bands corresponding to the XAP-1 antigen. The peptide MS and MS/MS spectra were acquired in the data-dependent mode to allow the mass spectrometer to automatically acquire a full-scan MS spectrum followed by five MS/MS scans of the most abundant ions from the MS scan. With the search engine (TurboSEQUEST; ThermoFinnigan), the nanoLC-MS/MS data obtained for the tryptic digest were used to automatically search the Swiss-Prot protein database to characterize the protein. Search criteria included the protein modifications cysteine carbamidomethylation and methionine oxidation. A subset of the Swiss-Prot database, created from Swiss-Prot version 12.2 and containing 32,027 protein entries, was searched. All peptide sequences with statistically significant scores were verified by

"blasting" them against the nonredundant GenBank CDS database. The Xcorr versus charge state cutoff scores for individual peptides were set at 5.0, 2.0, and 3.5 for single-, double-, and triple-charged peptide, respectively. All spectra contained expected fragment ion peaks, and their heights were at least fourfold above the average noise level.

Protein Depletion

Outer segment-enriched preparations were prepared from fresh retinas of 20 mice, as described. Protein estimation was carried out (DC Protein Assay kit; Bio-Rad) and the concentration was 0.8 $\mu\text{g}/\mu\text{L}$. To facilitate confirmation of the identity of the XAP-1 antigen as Grp78, the IgM heavy chain present in the outer segment preparation was immunodepleted. Our studies demonstrate that IgM or an IgM-like domain is present within the outer segment-enriched protein sample at a molecular mass very near that of the XAP-1 antigen and, therefore, could confound our confirmation studies. To remove the IgM heavy chain, 400 μg of the enriched OS preparation was incubated with 7 μg rabbit anti-mouse IgM antibody (mu-chain specific; Pierce) overnight at 4°C with constant rotation. The next day, 100 μL agarose beads (Protein A/G PLUS; Santa Cruz Biotechnology) were washed three times with lysis buffer (50 mM, Tris 8.0, 200 mM NaCl, 0.5% NP40, 1× proteinase inhibitors [Sigma]). The proteins bound by the rabbit anti-mouse IgM antibody were incubated with 30 μL washed protein A agarose beads at 4°C for 1 hour with constant rotation. The tube was spun at 1000g, for 3 minutes, and the supernatant containing outer segment proteins after one round of IgM deletion was carefully removed. A 50- μL aliquot was saved in -20°C for SDS-PAGE analysis. The beads were washed four to five times with lysis buffer. After a final wash, the buffer was removed, and the beads were washed with SDS sample buffer. A second IgM immunodepletion procedure was carried out to remove any residual IgM that might have remained in the outer segment-enriched preparation.

To provide additional evidence to confirm the identity of the XAP-1 antigen, IgM-depleted OS segment enriched fractions were subjected to two rounds of immunodepletion of Grp78. A protocol identical to that described was used with the exception that anti-Grp78 antibody (Anaspec, Fremont, CA) was substituted for anti-IgM heavy chain.

Multiplexed Western Blot Analysis of IgM-Depleted Outer Segment-Enriched Fractions

The following samples were subjected to SDS-PAGE under reducing conditions: enriched crude membrane fraction (5 μg), outer segment-enriched fraction (10 μg), enriched outer segment fraction after one round of IgM immunodepletion (10 μg), outer segment fraction enriched fraction after two rounds of IgM immunodepletion (10 μg), and outer segment-enriched fraction after two rounds each of IgM and Grp78 immunodepletion. Gels (NuPAGE 12% Bis-Tris; Invitrogen) were used for the electrophoretic separation of proteins at a constant voltage of 200 V using MOPS buffer. Prestained marker (SeeBlue Plus2, 2 μL ; Invitrogen) was used as standard to determine the relative molecular mass of the bands of interest.

After electrophoresis, proteins were transblotted to low-fluorescence PVDF membrane (Immobilon) at 30 V for 1.5 hours for subsequent Western blot analyses. The Western blot protocol for the detection of the proteins was conducted as described by the manufacturer (DyLight 680/800 Western blotting kit; Pierce) with some modifications. Briefly, after the transfer of proteins, the PVDF membrane was blocked for 3 hours at room temperature. The membrane was cut into two pieces, each with identical samples. Blot A was incubated in rabbit anti-human Grp78 (1:1000 dilution; Anaspec) overnight at 4°C. Blot B was also incubated with rabbit anti-human Grp78 along with the XAP-1 antibody (1:25 dilution). The blots were incubated in primary antibodies overnight at 4°C. After washing three times, both blots were incubated with both secondary antibodies (goat anti-mouse IgM Alexa fluor 680 [1:2000; Invitrogen] and goat anti-rabbit DyLight 800 [1:5000; Pierce]) for 2 hours at room temperature while they were protected from ambient

light. The multiplexed blot was imaged with an infrared imaging system (Odyssey; LI-COR Biosciences, Lincoln, NE).

RESULTS

Immunohistochemistry Using the XAP-1 Antibody

As shown by our immunohistochemical analyses, the XAP-1 antibody bound to the outer segments and the distal portion of the inner segments of mouse photoreceptors in a periodic pattern (Figs. 1A, 1C, 1D, 1F). In the outer retina, the labeling appeared to be restricted to the extracellular compartment near the plasma membrane. Colabeling of the retinal sections with PNA and WGA lectins that differentiate between cone and rod photoreceptors, respectively, indicated that in the mouse retina cone photoreceptors express the XAP-1 antigen. Rods show minimal to no immunolabeling (Figs. 1A, 1D). There was also weak and more diffuse immunopositive staining in the inner retinal layers (Fig. 1A). Images from the negative control (Fig. 1F, inset) illustrate that in the absence of the XAP-1 antibody, the immunopositive structures found in Figures 1A and 1D were also immunostained, though at a much lower intensity.

Western Blot Analysis

Western blot analysis was performed to determine the relative molecular mass of the XAP-1 antigen in the mouse retina and to estimate the relative amount of antigen in various retinal fractions (Fig. 2). For this analysis, the identical amount of protein (5 μg) was loaded in each lane. In most retinal samples (i.e., whole retina, enriched outer segment preparation, and crude membrane fractions of the outer segment-enriched preparation), a single immunopositive band was detected at approximately 74 kDa. However, no immunopositive band was found in the cytosolic fraction of the enriched outer segment preparation. A very light band was detected in the protein extract from the whole mouse retina. A moderately heavy band was detected in the sample derived from the outer segment-enriched fraction. The heaviest band was present in the crude membrane fraction derived from the outer segment-enriched sample. This result is expected given the immunolocalization pattern presented in Figure 1. No band was detected when the retinal protein sample was omitted. In preliminary studies (not shown), the intensity of the XAP-1-immunopositive band was visible but very weak when an anti-mouse HRP-conjugated secondary antibody was used. To overcome this limitation and amplify the immunopositive signal, we used an unlabeled secondary antibody (i.e., anti-IgM mu chain) followed by a tagged tertiary antibody. To our surprise, in controls in which the primary antibody was omitted yet the anti-IgM mu chain-specific secondary antibody was present, we detected a band at a slightly lower relative molecular mass than the XAP-1 antigen (Fig. 2). The relative density of the band was approximately 60% of that measured when the same protein sample was probed with the primary antibody before secondary and tertiary antibodies. These data demonstrate that the XAP-1 antigen and a protein recognized by anti-IgM mu chain are both expressed in the mouse retina and that they have nearly the same relative molecular mass.

nanoLC-MS/MS Analyses and Swiss-Prot Database Search

After separating maximally the proteins obtained from the crude membrane fraction of the outer segment-enriched preparation and carefully lining up the lane from the Western blot with the parallel silver-stained lanes, we selected the protein band that corresponded to the XAP-1 antigen.

FIGURE 1. Immunohistochemical localization of the XAP-1 antigen in the retina of the mouse. Sections showing the full retina (A–C) and higher magnification views of the outer retina (D–F). Retinas from C57BL6/J mice were immunostained using the XAP-1 antibody (A, D, green), PNA (B, E, red), and WGA (C, F, blue). (C, F) Composite images. Yellow (arrows) indicates areas of overlap of the XAP-1 antigen and PNA, indicating that in the outer retina, the XAP-1 antigen immunolabels cone photoreceptors. (F, inset) Negative control illustrating that, as with Western blot analysis, there is an IgM mu-chain or mu-chain-like protein in the retina that is localized to the area of the XAP-1 antigen. Scale bar, 10 μ m. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

Present within the area occupied by the immunopositive band (Fig. 3A) were three silver-stained bands (Figs. 3B, 3C). Based on the data of Figure 2, which illustrated that the lower portion of the immunopositive band was IgM mu chain or had an IgM-like domain, we carefully isolated from four individual lanes for mass spectrometric analysis the lightly stained uppermost band.

The chromatogram from the nanoLC-MS/MS analysis for the XAP-1 antigen band is shown in Figure 4A. Because of the low abundance of the XAP-1 antigen in the retina (Fig. 3), a small amount of protein was isolated and we obtained sequence data from a single peptide (i.e., ITPSYVAFTPEGER). The MS/MS spectrum of the peptide ion (doubly charged; precursor ion mass 784 Da for the [M+2H]²⁺ ion) is shown in Figure 4A. Based on the sequence of identified peptide, its probability score, the magnitude of the XCorr value, and the quality of the spectrum, the identity of the XAP-1 antigen was determined to be glucose-regulated protein precursor (Grp78). Illustrated in Figure 5 is the sequence of the full mouse Grp78 protein (black text) and the peptide match that was obtained by our MS/MS analysis (gray text).

Protein Depletion and Western Blot Confirmation of the Identity of the XAP-1 Antigen

By Western blot analysis, IgM or an IgM-like domain was demonstrated to be present in the mouse outer segment-enriched preparations at approximately the same relative molecular weight as the XAP-1 antigen; therefore, we removed it from the protein lysate. The purpose of this was to demonstrate that the immunopositive bands detected on probing with the XAP-1 and anti-Grp78 antibodies were perfectly superimposed. After one round of IgM depletion, a small amount of IgM remained in the outer segment-enriched protein lysate. After a second round of depletion, IgM was no longer detectable (Fig. 6A). In contrast, both XAP-1 antigen (Fig. 6B) and Grp78 (Fig. 6D) were retained in the protein sample after all IgM was removed.

FIGURE 2. Western blot analysis illustrating the presence of XAP-1-immunopositive bands in lysates obtained from various retinal preparations. XAP-1-immunopositive bands are found in lysates from the whole retina (retina), outer segment-enriched preparation (outer segment), and crude membrane fraction derived from the outer segment-enriched preparation. The intensity of the band is highest in the latter fraction and lightest in the former. No XAP-1-immunopositive bands are present in the cytosolic fraction. The negative control (right lane) indicates that an IgM mu chain or mu-chain-like protein is present in the outer segment-enriched preparation, though at a slightly lower relative molecular weight than the XAP-1 antigen.

FIGURE 3. Protein bands used for nano-LC MS/MS identification of the XAP-1 antigen. Proteins obtained from the crude membrane fraction of an outer segment-enriched preparation were electrophoretically separated. Western blot (A) and silver-stained proteins (B, C; enlargement of area indicated by *inset* in B). The uppermost band (B, C, *arrows*) from four lanes was isolated and prepared for LC-MS/MS analysis. The lower bands in B and C likely are the IgM mu chain or mu-chain-like protein that was illustrated in Figures 1 and 2.

Importantly, when the images from the multiplexed Western blot were merged, the XAP-1 antigen and Grp78 bands were superimposed (Fig. 6F).

To provide additional corroborative evidence of the identity of the XAP-1 antigen, we also removed Grp78 from the IgM immunodepleted sample and probed the remaining proteins with both the XAP-1 (Fig. 6B) and the Grp78 (Fig. 6D) antibodies. Neither antibody gave an immunopositive band, indicating that immunodepletion of Grp78 also removed XAP-1 antibody immunopositive proteins. Both immunodepletion studies provide corroborative evidence to support the identification of the XAP-1 antigen as Grp78.

Immunohistochemical Confirmation of the Identity of the XAP-1 Antigen

As additional verification of the identification of the XAP-1 antigen, we performed immunohistochemistry on mouse and *X. laevis* retinal sections using an anti-Grp78 antibody. In the mouse retina, cones were counterstained with PNA, and, in retinal sections from both mouse and frog, nuclei were stained with iodide (TO-PRO III; Invitrogen). After antigen retrieval, mouse cone photoreceptors were immunopositively labeled by anti-Grp78 in a pattern identical to that seen when sections were probed with the XAP-1 antibody (Fig. 7A). Because of the necessity to use heat and EDTA-based antigen retrieval before probing with anti-Grp78, in the mouse the morphology of cones, including their outer segments and interphotoreceptor matrices, was compromised. This procedure also prevented the possibility of performing double-labeling experiments using both the XAP-1 and the Grp78 antibodies, as the ability of the XAP-1 antibody to immunolabel the retina was abolished after antigen retrieval that was required for the Grp78 to recognize its antigen. Nonetheless, in the mouse, PNA labeling and Grp78-immunopositive labeling overlap (Fig. 7C). In addition to the labeling of cones of the mouse retina, the Grp78 antibody immunostained photoreceptor inner segments and the cytoplasm of cells in the inner nuclear and ganglion cell layers (Fig. 7A). In the outer retina of the frog, Grp78 antibody immunolabeling was not restricted to cones; rather, it outlined all photoreceptor outer segments and stained inner segments (Fig. 7D). Immunohistochemical staining shows that in the areas surrounding photoreceptor outer segments, the XAP-1 and Grp78 antibodies have identical labeling patterns within a species. Moreover, it is important to note that the labeling pattern we revealed around frog outer segments when using the Grp78 antibody (Fig. 7) was identical with that illustrated previously by us^{14,15} and other groups^{16,18} when using the XAP-1 antibody.

DISCUSSION

Since the first publication about it in 1992, the XAP-1 antibody has been used by retinal investigators as a marker of retinal maturity and photoreceptor cell type determination.^{16–18} Differing subsets of photoreceptors have been immunolabeled by the antibody, depending on the species studied. In *X. laevis*, both rods and cones were recognized,^{14,16,18} whereas in monkey and pig only cones were labeled.¹⁷ Previously, we documented that the ability of the antibody to bind to photoreceptors was correlated with proper outer segment formation,¹⁴ suggesting that the antigen may play a significant role in photoreceptor physiology. Despite the widespread use of the antibody, the antigen that is recognized by it has remained unknown.

Our earlier attempt to identify the XAP-1 antigen using *X. laevis* retinas proved inconclusive, with mass spectrometric characterization of protein spots from a two-dimensional Western blot yielding conflicting results with immunohistochemical studies.¹⁴ In the present study, we restructured our approach for the identification of the XAP-1 antigen to include the use of mouse retinal tissue (which has a higher expression level of the antigen than *Xenopus*; unpublished results, 2004), a native detergent for the generation of protein lysates (i.e., DDM), and protocols to enrich for sub-retinal preparations containing the XAP-1 antigen. We predict that these procedural modifications have facilitated the binding of the IgM antibody to the same antigen, whether it was retained within the tissue (i.e., during immunohistochemistry) or when extracted from its *in vivo* environment (i.e., SDS-PAGE and Western blot analysis). These

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215.1390	2	T	13	1453.6958	727.3515
312.1918	3	P	12	1352.6481	676.8277
399.2238	4	S	11	1255.5953	628.3013
562.2871	5	Y	10	1168.5633	584.7853
661.3556	6	V	9	1005.5000	503.2536
732.3927	7	A	8	906.4316	453.7194
879.4611	8	F	7	835.3945	418.2009
980.5088	9	T	6	688.3260	344.6667
1077.5615	10	P	5	587.2784	294.1428
1206.6041	11	E	4	490.2256	245.6164
1263.6256	12	G	3	361.1830	181.0951
1392.6682	13	E	2	304.1615	152.5844
---	14	R	1	175.1190	88.0631

FIGURE 4. LC-MS/MS results obtained from the protein band corresponding to the XAP-1 antigen. **(A)** The MS/MS spectrum of a peptide from the LC-MS/MS analysis. It contains product ions of the b- and y-series that matched peptide sequence ITPSYVAFTPEGER from Grp78 from *Mus musculus*. **(B)** The *m/z* ratios for theoretical product ions of the y- and b- series. Product ions that were observed in the MS/MS spectrum are shown in bold.

odifications proved critical for the success in identifying the antigen of the XAP-1 antibody as Grp78.

Grp78 (aka BiP or Hsp5) is a member of the Hsp70 family of heat shock proteins and plays a significant role in the unfolded protein response. Much of the research involving this protein has focused on its role in the folding and assembly of proteins in the endoplasmic reticulum.²⁰ Because of the KDEL sequence on its C terminus, it is expected that Grp78 would be found ubiquitously in the endoplasmic reticulum. However, in recent years, Grp78 has also been localized on the surfaces of various cells, in the culture medium in which cells have been grown, and in the sera of healthy patients.^{21–24} Although the mechanisms(s) by which Grp78 is secreted is not known with certainty, proposed possibilities include saturation of the KDEL receptor in the endoplasmic reticulum or association with lipophilic molecules that may mask the KDEL sequence, thus allowing Grp78 to exit the organelle.²¹ Hauck et al.²⁵ calculated the SecretomeP score of Grp78 and found that it was elevated (0.745), indicating that the probability is very high that

Grp78 exits the cell by nonclassical secretory pathways. Very recently a novel isoform of GRP78 was discovered that lacks both the N-terminal endoplasmic reticulum signal and the C-terminal KDEL endoplasmic reticulum retention sequences,²⁶ thus providing another mechanism by which this protein can be found in the areas outside the endoplasmic reticulum.

It has been demonstrated that Grp78 and other heat shock proteins have distinct functions in the ER compared with the extracellular compartment. Extracellularly, Grp78 protein activates the Ras/MAPK and PI3 kinase signaling pathways,²³ associates with T-cadherin and integrin β_3 , where it serves as a cell surface signaling receptor,²⁴ and regulates the secretion of anti-inflammatory cytokines such as interleukin 10.²² Grp78 has also been predicted to function as an intercellular signaling molecule.²²

In the endoplasmic reticulum of the retina, Grp78 has been declared as an indicator of stress from light-induced retinal degeneration,²⁷ in the *rd1* mouse,²⁸ and in retinal detachment.²⁹ When localizing the protein using immuno-

FIGURE 5. Protein sequence of *Mus musculus* Grp78. The peptide from our MS/MS analysis is shown in gray text and represents 2.14% coverage.

FIGURE 6. Depletion of IgM and Grp78 from the outer segment-enriched preparation. IgM and Grp78 were depleted from outer segment (OS) cell extracts. IgM and the XAP-1 antigen are shown in *red*, whereas Grp78 is shown in *green*. Overlap of the signals (E, F, *yellow*). A small amount of IgM was detected after one round of depletion, but none remained after a second round (A). Both the XAP-1 antigen (B) and Grp78 (C, D) remained in the sample after all IgM was removed. The XAP-1 antigen and Grp78 overlap at an identical relative molecular mass (F). Depletion of Grp78 (C, D) abolished the signal obtained with the XAP-1 antibody (B).

histochemistry in these studies, Grp78 was found throughout all layers of the retina, with the exception of the outer segments and the outer nuclear layer. Using mass spectrometric analysis, however, Hauck et al.²⁵ detected Grp78 in the interphotoreceptor matrix of porcine retinas. They also immunolocalized the protein to the extracellular compartment around cones, where they predicted that Grp78 exhibited neuroprotective properties.²⁵ Importantly, both our study and that of Hauck et al.²⁵ used high-temperature antigen retrieval, as recommended by the manufacturer of the antibody, to unmask the epitope required for antigen recognition by the Grp78 antibody in this area of the tissue. In the absence of antigen retrieval, we also failed to detect Grp78 in the area around cone outer segments in the mouse retina and all outer segments in frog retina, thus stressing the requirement of this procedure to allow for maximal

binding of the antibody to the retina, in particular the area around the distal portions of photoreceptors.

In conclusion, this study provides mass spectrometric, immunohistochemical, and Western blot evidence to support our assertion that the XAP-1 antigen is Grp78. The amino acid sequence used to generate the Grp78 antibody is known, though the epitope recognized by the XAP-1 antibody remains to be determined. Although both antibodies recognize an antigen surrounding outer segments of an identical relative molecular mass, the Grp78 antibody is unique in its ability to intensely stain the endoplasmic reticula of all retinal cells. These differences suggest that the Grp78 protein may have a modified three-dimensional structure because of varying posttranslational modifications or may be the result of alternative transcripts in the inner versus outer retinal compartments. It is possible that each antibody may preferentially label a unique variant.

FIGURE 7. Immunohistochemical localization of Grp78 in the retinas of mouse and frog. After antigen retrieval, retinas from C57BL/6/J mice were immunostained using anti-Grp78 (A, *green*), PNA (B, *red*), and iodide (C, *blue*). (C) Composite image. *Yellow* (arrows) indicates areas of overlap of the Grp78 and PNA, indicating that in the outer retina, Grp78 is localized to cone photoreceptors in a pattern identical with that of the XAP-1 antigen. (C, *inset*) Higher magnification image of the area within the *inset* in C. In the mouse, Grp78 is also found in all cell layers of the retina in a location corresponding to ER. (D) Examination of the frog retina revealed a different pattern. The peripheries of both rod and cone photoreceptor outer segments and the inner segments are immunolabeled in this species. Scale bar, 10 μ m. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

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