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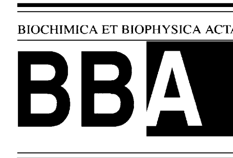

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Methionine-rich repeat proteins: a family of membrane-associated proteins which contain unusual repeat regions

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Abstract

We report the protein isolation, cloning and characterization of members of an unusual protein family, which comprise the most abundant proteins present in the squid eye. The proteins in this family have a range of molecular weights from 32 to 36 kDa. Electron microscopy and detergent solubilization demonstrate that these proteins are tightly associated with membrane structures where they may form tetramers. Despite this, these proteins have no stretches of hydrophobic residues that could form typical transmembrane domains. They share an unusual protein sequence rich in methionine, and contain multiple repeating motifs. We have therefore named these proteins Methionine-Rich Repeat Proteins (MRRPs). The use of structure prediction algorithms suggest very little recognized secondary structure elements. At the time of cloning no sequence or structural homologues have been found in any database. We have isolated three closely related cDNA clones from the MRRP family. Coupled in vitro transcription/translation of the MRRP clones shows that they encode proteins with molecular masses similar to components of native MRRPs. Immunoblot analysis of these proteins reveals that they are also present in squid brain, optic lobe, and heart, and also indicate that MRRP-like protein motifs may also exist in mammalian tissues. We propose that MRRPs define a family of important proteins that have an unusual mode of attachment or insertion into cell membranes and are found in evolutionarily diverse organisms.
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Keywords: Squid; *Loligo forbesi*; *Loligo pealei*; Eye; Membrane protein

1. Introduction

The squid eye has been extensively studied because the photoreceptors are typically invertebrate being a particularly advantageous model for the IP_3 signaling pathway, while the eye structure is optically like a vertebrate eye [1]. Squid photoreceptors employ a classical IP_3/Ca^{2+} pathway that is an important central process in most cell types. Consequently, the squid visual system uses many of the same signaling components as mammals. The squid visual system is also suitable for the examination of structural proteins as the eye shape optics are very similar to that in vertebrates and since parallels exist in the recruitment of some eye structural proteins in squid and vertebrates. For example, in both species, lens structural proteins have evolved from other proteins with distinct but different functions, an example being glutathione S transferase in squid [2]. This type of molecular recruitment also occurs in corneal epithelium where the major cornea proteins in mammals are class 3 aldehyde dehydrogenase and enolase, while in the chicken, cornea cyclophilin is abundant [3]. These data suggest that eye structural proteins can be recruited from

Abbreviations: DDAPS, *N*-Dodecyl-*N*,*N*-dimethyl-3-ammonio-propane sulfonate; HEK, human embryonic kidney; IP_3, inositol 1,4,5-triphosphate; LDAO, *N*,*N*-Dimethyl dodecylamine-*N*-oxide; PMSF, phenylmethylsulfonyl fluoride; RACE, rapid amplification of cDNA ends

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proteins that are present in different tissues and other species during evolution.

The largest eyes, relative to body size, in the animal kingdom are found in the squid [4]. Consequently, large amounts of protein can be isolated and this facilitates the isolation and direct sequencing of many of the protein components of the eye. Rhodopsin, G protein subunits, phospholipase Cβ and the squid transient receptor potential (sTRP) channel have been purified and cloned from the squid *Loligo forbesi* [5–9]. The squid visual system is therefore ideal for the cloning and characterization of both known and novel proteins.

Since polypeptides with molecular masses in the range of 32–36 kDa appear to be the most abundant proteins in squid eye, it is reasonable to hypothesize that they must have an important function. In this study, we describe the cloning, molecular, and biochemical characterization of this novel protein family.

2. Experimental

2.1. Materials

Adult specimens of *L. forbesi* were obtained from the Marine Biological Association, Plymouth, UK. Adult specimens of *Loligo pealei* were obtained from Marine Biological Laboratories, Woods Hole, MA, USA. Eyes and other tissues were dissected from freshly killed animals, frozen in liquid nitrogen and stored at −70 °C or in liquid nitrogen until use. For protein sequencing and all nucleic acid analysis, the *L. forbesi* species of squid was used. For all other protein characterization experiments, including injection of MRRP into rabbits for the generation of polyclonal antisera, the *L. pealei* species of squid was used. Both a λgt10 *L. forbesi* squid eye cup cDNA [10] and an *E. coli*-based *L. forbesi* squid eye cup cDNA library in pBluescript™ Stratagene [8] were used to obtain cDNA clones of the components of the MRRP family. Oligonucleotide primers were synthesized by the University of Leeds facility or by Sigma-GENOSYS. Boehringer Mannheim High Fidelity Taq polymerase was used with a RACE Kit purchased from Clontech. Polymerase chain reaction (PCR) reactions were performed in a Perkin-Elmer Cetus thermocycler.

2.2. MRRP isolation for protein sequencing and antisera production

The eyes were dissected from freshly caught squid and the lens and aqueous humor were removed. The remaining eye cups were quickly frozen in liquid nitrogen and stored frozen until use. Frozen eyes cups were used for protein preparation as previously described [11] with some modifications. Briefly, frozen eye cups were defrosted and shaken in saline buffer and then homogenized before layering either on a 36% sucrose cushion or on a sucrose density gradient 40/60% before centrifugation. The resulting pellet was removed and washed by being resuspended in 50 mM Tris–HCl, pH 7.4, and centrifuged in a bench top microcentrifuge at 13,000×*g*, 4 °C, for 10 min. In some cases, to solubilize MRRP, the sucrose or collagenase pellet was removed and resuspended in 50 mM Tris pH 7.4 plus 2% detergent *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-propane sulfonate (DDAPS) or *N*,*N*-dimethyl dodecylamine-*N*-oxide (LDAO). The MRRP pellet was subjected to electrophoresis on a 10%, 12%, or 15% SDS-PAGE and the band corresponding to the MRRP protein was excised and the protein purified from the gel slice according to standard protocols [12]. For protein sequencing, the eluted protein was dialyzed against 10 mM Tris–HCl, pH 7.0, 1 mM PMSF at 4 °C for 24 h. The purified MRRP protein was proteolytically cleaved with either trypsin or V8 (*S. aureus*) endoproteinase. The peptides generated were separated by SDS-PAGE and electroblotted on polyvinylidene difluoride (PVDF) membrane as described in Ref. [12]. The protein sequence was inspected and regions were selected for the design of degenerate primers.

2.3. mRNA isolation and RT-PCR

Poly (A+) enriched RNA was isolated as previously described from *L. forbesi* squid eye cups [8]. First-strand cDNA was reverse transcribed using poly (A+)-enriched RNA from squid eye cup by priming with oligo (dT) [13]. Several degenerate primer combinations were used in the PCR with 1 μl of the first-strand cDNA reaction as template and 1 μM of each primer, 0.16 mM dNTPs, and 1.5 mM $MgCl_2$ at 95 °C 45 s, 51 °C 1 min, 72 °C 1 min for 30 cycles. The primer pair (CCGGAATTCAAYTAYCARATG-GAYATG), that has a 5′ *Eco*RI site incorporated, and (CCCCAIGGRTCRTTRTA) generated a PCR product that was then blunt ended with Klenow fragment, cut with *Eco*RI, subcloned into pBluescript™ (Stratagene) and sequenced using M13-based primers.

2.4. cDNA library screening and 5′ RACE

Initially, a λgt10 *L. forbesi* squid eye cDNA library [6] was screened using a digoxygenin-labeled MRRP subcloned PCR product (DIG screening kit Boehringer-Mannheim) as a probe. The positive λ clone was subcloned into pBluescript™ Stratagene and sequenced using M13 based primers. The λ clone is truncated at the 5′ end so RACE (Clontech kit) was used to obtain the 5′ end of the cDNA using primers designed to this λ clone. First-strand cDNA was generated by priming squid eye cup poly (A+) enriched RNA with random hexamers [13,14]. Adapters were ligated to the ends of first-strand DNA as per the Clontech kit instructions. Two gene-specific primers (ATTCGTGTGCT-GAGAGAACGGGTTGACGGGTTG and TATATCCATC-CAGCGTCCCTGCATATCC) were used in combination

with each of two adapter primers (Clontech Kit). Expand™ High Fidelity Taq Polymerase (Boehringer Mannheim) was used with the following reaction conditions: 10 pmol of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 5 μl of a 1:50 dilution of the first-strand adapter ligated cDNA reaction at 95 °C 5 min, 95 °C 45 s, 70 °C 1 min, and 68 °C 1 min for 30 cycles. The PCR products were subcloned into PCR2.1TA (Invitrogen). A RACE clone was identified and used as a probe in a pooled library screen [15] of an *E. coli*-based squid eye cDNA library in pBluescript™ (Stratagene) [8], using a combined PCR and Southern blotting approach [16]. Briefly, the RACE cDNA was random primed with α ^{32}P dCTP, 10 mCi/ml (ICI) using the oligo labeling kit from Amersham Biosciences according to the manufacturer's instructions. The labeled probe was separated from unincorporated nucleotide using Nuctrap™ columns Stratagene. PCR was performed on plasmid DNA extracted from the total pooled *E. coli* library using a cDNA-specific primer (ATTCGTGTGCTGAGAGAACGGGTTG) in combination with both M13R and M13F primers. The PCR reactions were subjected to electrophoresis on a 1.5% agarose gel, the gel was transferred [16] to nitrocellulose (Scheicher & Schuell) and hybridized to the RACE probe under high stringency conditions. This continued in turn until a single mixed culture of positive clones was obtained. To isolate single colonies, colony lifts [16] were performed on plated cultures by transferring to nitrocellulose filters to which ^{32}P labeled RACE probe was hybridized. Two full-length MRRP clones were isolated as a result of this screen. DNA sequencing was performed using DNA sequencer 377A (Applied Biosystems) at both the University of Leeds and the University of Durham DNA sequencing facilities.

The open reading frame from conceptually translated protein sequence was used to search both protein, OWL ([17] and SWISSPROT), and nucleic acid databases including expressed sequence tags (ESTs) using the Genetics Computer Group (GCG) Wisconsin package suite of programs (BLAST, FASTA, and TFASTA [18,19]. To look at the regions of the MRRP cDNA that have the highest probability of being protein-encoding regions, a codon preference table for *L. forbesi* squid downloaded from the internet [20] was transferred into the GCG program Codon Preference and the program Codon Frequency. A PROSITE search [21] was performed using the combined MRRP protein sequence of the 5′ RACE and λ clone to look at sequence motifs in the MRRP protein sequence. The program TopPred II [22] was used to analyze protein sequence hydrophobicity and to look at possible membrane topology. All other protein sequence analysis programs used were run on the Expert Protein Analysis System (ExPASy) proteomics server [23].

2.5. Northern blotting analysis

Total RNA was isolated as previously described [8] and poly (A+)-enriched RNA was isolated by loading the total squid eye cup RNA on to an oligo(dT)-cellulose column. Electrophoresis, Northern transfer, and hybridization were performed according to methods in Current Protocols in Molecular Biology [24]. Briefly, 30 μg of denatured poly (A+)-enriched RNA was electrophoresed on a 1.2% agarose formaldehyde gel and transferred to a nitrocelluose filter (Scheicher & Schuell) for hybridization. The MRRP race cDNA was random primed with α^{32}P dCTP (ICI) 10 mCi/ml at 10^5 cpm/ml using a random primer labeling kit from Amersham Biosciences according to the manufacturer's instructions. The labeled probe was hybridized to the filter and exposed to X ray film [16] for 2 days at −70 °C with an intensifying screen.

2.6. Polyclonal antisera generation, SDS-PAGE, and immunoblotting

Polyclonal antisera to gel purified MRRP was generated in rabbits, through the University of Leeds Biomedical service, as described in Ref. [25]. Approximately 200–300 μg of gel-purified MRRP from both sucrose cushion and eye white preparations was mixed with Freund's complete adjuvant (Sigma) and then injected subcutaneously into each rabbit. This was followed by three boost injections with Freund's incomplete adjuvant (Sigma) at 4-week intervals with test bleeds at 10 days following each boost. Dot blot analysis was performed using the test bleed sera titrated from 1:1000 to 1:10,000 on 0.5 μg of purified MRRP to determine the best dilution to use in the immunoblots. SDS-PAGE was performed according to standard procedures [26].

Tissues were dissected from freshly killed animals, and stored at −70 °C until use. HEK cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 30 μg/ml penicillin/streptomycin to confluence in a 75-cm^2 flask. The HEK cells were pelleted and used to make a protein lysate. For the immunoblots, 0.5 μg of purified MRRP, tissue and cell protein lysates prepared by incubation in Laemmli sample buffer [26] plus β-mercaptoethanol on ice, Sigma 7SDS, and prestained markers were subjected to electrophoresis on a 10%, 12%, or 15% gel. The proteins from the gel were transferred onto FLUOROTRANS™ PVDF membrane using a semidry blotter according to standard protocols [27]. For the immunoblots, all washes and incubations were performed at room temperature. The membrane was blocked in TBS/Tween 20 (0.2%) plus 5% nonfat dry milk. Washes were carried out in TBS/Tween 20 and the PVDF membrane was incubated with antisera at 1:1000 overnight. The membrane was washed again in TBS/Tween 20 and then incubated with secondary antibody (goat anti-rabbit, Sigma) IgE at 1:2000 for 1–2 h. After washing, the membrane was incubated with Exavidin Peroxidase at 1:1000 for 1–2 h. The membrane was washed, developed with 0.1 mg/ml 3,3′ diaminobenzidine, 0.03% H_2O_2, and enhanced with 0.008% $NiSO_4$.

2.7. Coupled in vitro transcription and translation

In vitro transcription and translation were performed according to the manufacturer's instructions in a TNT™ coupled reticulocyte lysate system (Promega). Reactions were carried out for 90 min at 30 °C in a final volume of 25 μl using T3 RNA polymerase, 0.9 μg of template DNA and 2 μl of ^{35}S-methionine (10 mCi/ml), ICN. Some reactions were supplemented by the addition of pancreatic microsomal membranes as per manufacturer's instructions.

To isolate the microsomal membranes, a 5-μl sample of the completed translation was retained for analysis by SDS-PAGE, and the remaining 20 μl was mixed with 80 μl Tris, 100 mM NaCl, pH 7.9. After centrifugation at 110,000×g, 1 h, 4 °C, the microsomal pellet was resuspended in the same buffer and the centrifugation step was repeated to wash the membranes. The resulting pellet was resuspended and electrophoretically resolved on SDS-PAGE along with the supernatants. The gels were dried and exposed to X-ray film at room temperature for 2–4 days.

2.8. Immunolabeling of in vitro translated MRRP in microsomal membranes with 5 nm colloidal gold anti-rabbit antibodies

Microsomal membranes were isolated from a non-radioactive coupled in vitro transcription/translation reaction as described in the "Coupled in vitro transcription and translation" section. The pellet was washed with 100 μl 50 mM Tris pH 7.4, centrifuged at 100,000×g, and resuspended in 200 μl PBS pH 7.4. The resuspended pellets were divided evenly into two tubes, washed in 200 μl PBS, and resuspended in 100 μl PBS–BSA buffer (20 mM phosphate, 150 mM NaCl pH 7.4 plus 0.5% BSA, 0.1% gelatin and 0.05% Tween 20). The resuspended membranes were incubated in the PBS–BSA buffer in a blocking step for 30 min with gentle agitation at room temperature. The MRRP antiserum was added at a final dilution of 1:100 and incubated overnight with gentle agitation at 4 °C. The samples were then centrifuged at 100,000×g, resuspended in 100 μl PBS–BSA buffer for 5 min, and centrifuged. This wash step was repeated, and the pellet resuspended in 100 μl PBS–BSA buffer. Colloidal gold 5-nm conjugated–anti-rabbit antibody (Nanoprobes, Inc.) was added to a final dilution of 1:50 and incubated for 2 h with gentle agitation at room temperature. The immunolabeled membranes were centrifuged at 100,000×g, washed twice in PBS–BSA buffer, and the final pellet was resuspended in 30 μl PBS. The samples were applied to carbon coated copper EM grid, stained, and viewed under EM as described in the "Electron microscopy" section.

2.9. Collagenase treatment

Frozen eye cups were defrosted at room temperature for 15 min. The white tissue that surrounds the lens and extends between the optic nerve and pigmented retina was gently scraped away from the black retina. 50 mg of dissected eye white tissue was resuspended in 0.5 ml of 50 mM Tris buffer, pH 7.4 plus 0.1 mg/ml of collagenase (Sigma) and 20 mM $CaCl_2$ and incubated overnight at 37 °C. The sample was then microcentrifuged at 5000×g for 10 min and the resulting pellet was washed three times and resuspended in 1 ml of 50 mM Tris pH 7.4 buffer (method adapted from Ref. [28]). For electron microscopy studies, samples of the preparations were taken from this step and partially delipidated by incubation with LDAO (0.112% final concentration, protein concentration of 3.8 mg/ml) for 2 h at room temperature. The samples were then microcentrifuged at 13,000×g, 4 °C for 30 min. The pellet was washed three times and resuspended in 0.5 ml 50 mM Tris–HCl pH 7.4.

2.10. Electron microscopy

Solutions of MRRP-containing membranes, from the above-described collagenase treatment, prior to and after detergent treatment with LDAO (see above) were prepared for electron microscopy by negative staining according to Valentine et al. [29] using an aqueous solution of uranyl acetate (4% w/v). Specimens were observed in a Philips CM10 or Jeol JEM-1200EX transmission electron microscope operated at accelerating voltages of 100 or 80 kV, respectively. Electron micrographs were recorded on Agfa Scientia 23 D 56 electron image sheet film at calibrated magnifications. For the determination of the lattice parameters of crystalline arrays, digitized data were analyzed using the PC-based electron crystallographic software package CRISP [30].

3. Results

3.1. Isolation of MRRPs, their protein properties, and MRRP immunoblots

The MRRP protein family appear to be the most abundant proteins present in squid eye. MRRPs were separated away from other eye proteins in a protein homogenate of total eye cup tissue (minus the lens and aqueous humor) using a sucrose gradient or sucrose cushion (Fig. 1A) or by direct isolation using collagenase from eye white tissue (Fig. 6A). MRRPs were not soluble in any solution without detergent present. They could not be solubilized by all detergents, but were soluble in N-dodecyl-N,N-dimethyl-3-ammonio-propane sulfonate (DDAPS) or N,N-dimethyl dodecylamine-N-oxide (LDAO). At best, MRRPs were only partially soluble in 100% formic acid. When run on SDS-PAGE purified MRRPs migrate at molecular weights of 32–36 kDa (Fig. 1A). The brackets in Fig. 1 indicate the protein bands corresponding to the 32–36 kDa MRRPs in Fig. 1. This is the region that was gel purified for both protein sequencing and

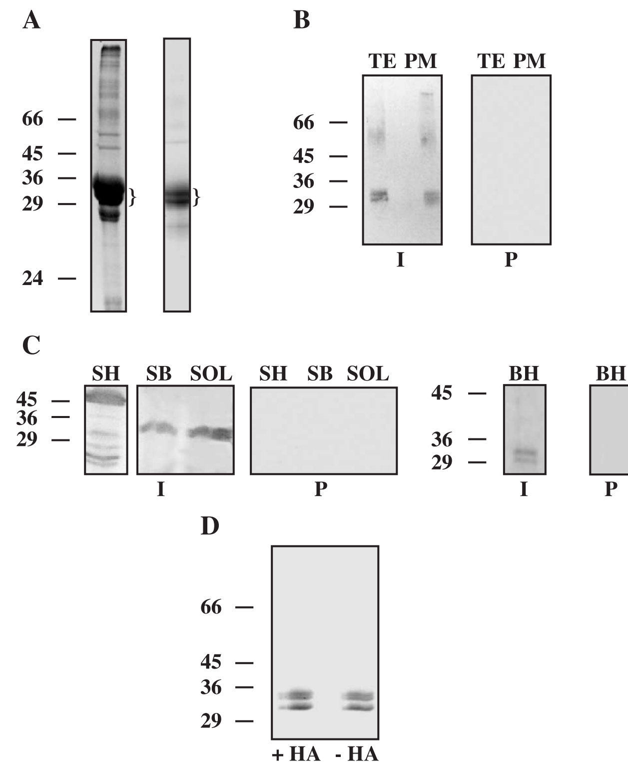

Fig. 1. Biochemical characterization of native MRRPs. (A) MRRP pellet isolated from a sucrose cushion was electrophoresed on a 12% SDS-PAGE gel and Coomassie blue stained. In the right panel, MRRPs loaded on the gel at a lower concentration can be seen as a doublet (bracket). (B) MRRP antisera recognize MRRP bands of 32–36 kDa on immunoblots of total eye cup lysate and purified MRRP. Immunoblot of silver-stain detectable amounts of total eye lysate (TE) and MRRP-purified sucrose cushion pellet (PM). A doublet of MRRP bands from 32–36 kDa are recognized by the MRRP polyclonal antisera in both total eye lysate (TE) and MRRP sucrose pellet (PM) in panel I that are not detected by the preimmune sera (panel P). (C) Polyclonal antisera identify MRRP-like protein bands in immunoblots of different tissues. Squid tissue lysates (SH-heart, SB-brain, and SOL-optic lobe). The MRRP band of 32 kDa is seen in squid brain and optic lobe whereas in addition to this band squid heart has smaller bands and a prominent band of 45 kDa in panel I that are not detected by the preimmune antisera (panel P). A characteristic MRRP-like doublet of 32–36 kDa is detected in bovine heart (BH—panel I) that was not detected by MRRP preimmune antisera (panel P). (D) MRRP protein migration appears unaffected by hydroxylamine treatment. Sucrose cushion purified MRRP was incubated in the presence (+HA) and the absence (−HA) of hydroxylamine and subjected to electrophoresis. There is no apparent shift in molecular weight indicating that MRRP is not extensively fatty acid acylated.

for the rabbit injections in the generation of MRRP polyclonal antisera. MRRPs subjected to SDS-PAGE at a lower concentration can been seen to run as a doublet in the right panel of Fig. 1A.

To study the tissue expression of MRRPs, we generated polyclonal antisera in rabbits to purified MRRPs. On immunoblots of squid total eye homogenate and MRRPs isolated from a sucrose cushion pellet, the antisera detect a doublet of 32–36 kDa that is indistinguishable from native MRRP electrophoretically resolved on SDS-PAGE (Fig. 1B). The fact that the lanes containing total eye lysate (TE) and the purified sucrose pellet (PM) look the same and that these bands are not detected with preimmune sera demonstrate the specificity and low background of the MRRP antisera. In squid heart, brain and optic lobe lysates, MRRP antisera also recognized bands of 32–36 (Fig. 1C SH, SB, SOL—panel I). In addition, a prominent band of approximately 45 kDa could be seen in squid heart. A 45-kDa band was also detected in bovine optic nerve (data not shown). These bands were not detected by preimmune sera (SH, SB, SOL—panel P). In bovine heart, a doublet of 32–36 kDa similar to the doublet detected in squid eye was seen (BH—panel I). Immunoreactive bands were not detected with the preimmune serum (BH—panel P) demonstrating the specificity of the MRRP antisera. To test whether MRRP is palmitylated on cysteine residues, the purified protein was incubated with 1 M hydroxylamine [31] and run along with untreated protein on 15% PAGE. There was no apparent shift in the hydroxylamine-treated sample, indicating that MRRP is not extensively palmitylated (Fig. 1D), although we cannot rule out palmitylation on a few cysteine residues.

3.2. Cloning and characterization of three cDNAs encoding MRRPs

Peptide sequence information from the polypeptide fragments produced by limited proteolysis with V8 (*S. aureus*) endopeptidase together with the alignment of conceptually translated protein sequences from the MRRP cDNAs is shown in Fig. 3. Degenerate primers designed to the polypeptide fragment sequences were used for PCR

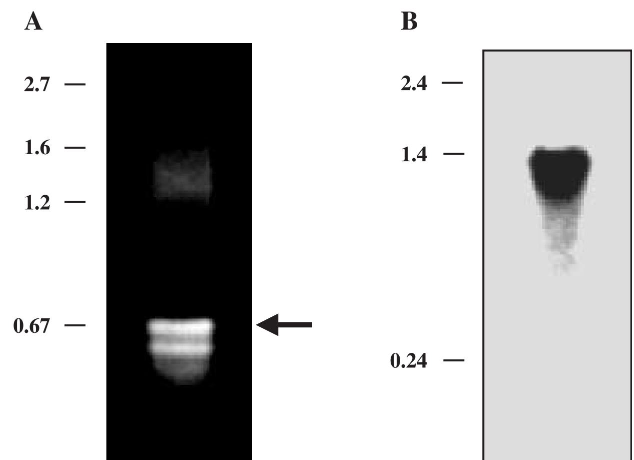

Fig. 2. 5′ RACE and Northern blot analysis of MRRP poly (A+) enriched RNA. (A) 5′ MRRP RACE. The PCR products obtained from 5′ RACE using a cDNA-specific antisense primer (see "Experimental" section) were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide to visualize the bands. Two PCR bands were consistently seen in all 5′ RACE PCR products including a larger band of approximately 0.65 kb (including 5′ RACE adapter sequence) which was subcloned. (B) MRRP Northern blot, 30 μg of squid eye cup poly (A+) enriched RNA was fractionated on a 1.2% agarose formaldehyde denaturing gel, transferred to nitrocellulose, and probed under high-stringency conditions using random-primed α ^{32}P labeled 0.65 kb MRRP RACE cDNA. A major band at 1.4 kb is observed.

amplification of *L. forbesi* eye cDNA. The most prevalent PCR product of 0.38 kb was subcloned into pBluescript and used to screen a λgt10 *L. forbesi* eye cDNA library. From this screen, a 5′ truncated 0.89-kb clone was isolated, subcloned into pBluescript, and sequenced. To obtain the 5′ coding region for this clone, 5′ RACE was performed on squid eye cDNA. Two PCR products were generated with both cDNA-specific primers (see "Experimental" section). These two PCR products were repeatedly obtained even using nested combinations of primers (Fig. 2A) which both hybridize to MRRP cDNA probe, indicating these are specific products (results not shown). The larger band of 0.65 kb (including the 5′ adapter primer sequence) was obtained using the cDNA-specific primer (ATTCGTG-TGCTGAGAGAACGGGTTG)/5′ adapter primer pair. This 0.65-kb product (RACE clone, Fig. 2A, arrow) was isolated and subcloned into PCR2.1TA. The RACE clone was sequenced and 0.61 kb (minus the 5′ RACE adapter) of the nucleotide sequence appeared to contain the full 5′ coding region for MRRP overlapping with the λ clone by 0.11 kb. The combined clones correspond to a cDNA of 1.3 kb with a polyadenylation signal (AATAAA) at nucleotide number base 1256 (DNA sequence not shown) and predicted to give an open reading frame of 264 amino acids with a calculated molecular mass of 32.8 kDa. This combined set of partial cDNAs is called MRRP1 (Fig. 3).

The 0.65 kb RACE subclone was used to screen a *E. coli*-based *L. forbesi* eye cup cDNA library in pBluescript. From this screen, two full-length cDNA clones of 1.4 kb were isolated. The codon frequency data from using the GCG program Codon Frequency indicated that the open reading frame chosen has the highest codon frequency and that the region beginning with the first methionine residue encountered is predicted to be a coding region. This first methionine was chosen as the initiating methionine as this would give the correct predicted molecular mass for the MRRPs based on the size of both the native and in vitro translated proteins for the MRRP2 and MRRP3 clones

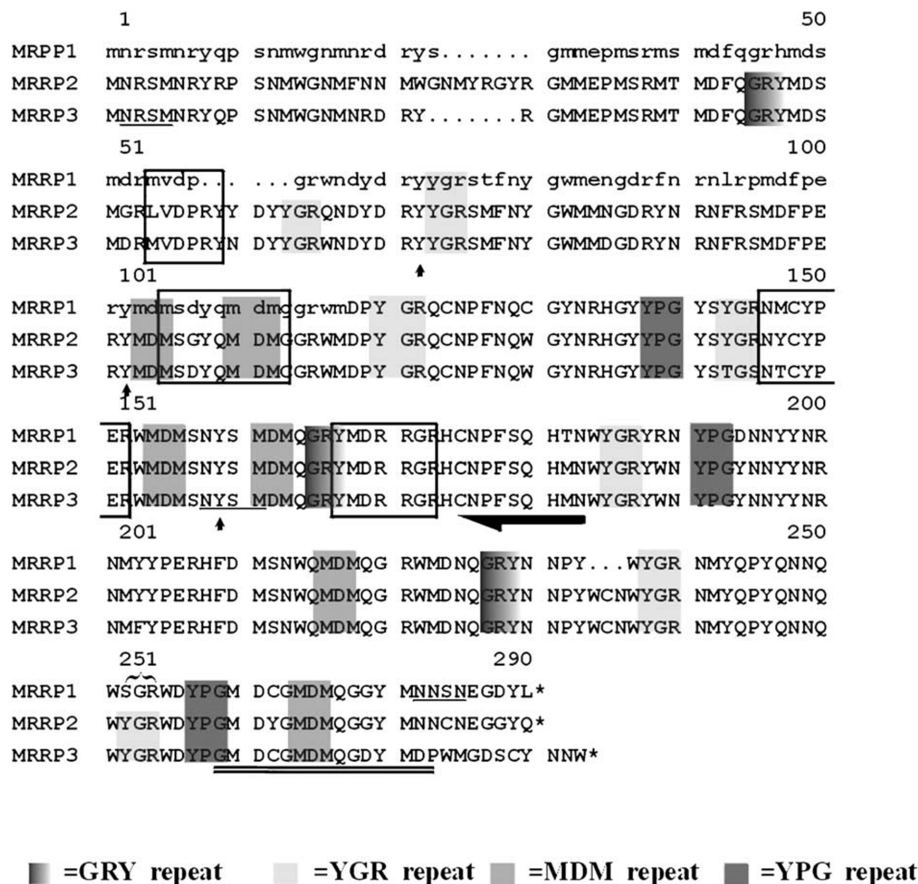

Fig. 3. Alignment of the predicted amino acid sequence for MRRP clones. The MRRP1 amino acid sequence was derived by combining the sequence of the 5′ RACE (lower case) and λ clone (uppercase). Both MRRP2 and MRRP3 amino acid sequences were derived from full-length cDNA clones. The MRRP1 protein consists of 264 amino acids with a predicted molecular weight of 32.8 kDa. MRRP2 has 280 amino acids and a molecular weight of 35.3 kDa whereas MRRP3 has 276 amino acids and a molecular weight of 34.5 kDa. Regions corresponding to the peptide sequences from the protein sequencing of gel-purified MRRP are boxed. Protein repeat regions are shaded. The potential N-glycosylation (single underline), potential tyrosine kinase (arrow heads), and a potential protein kinase C phosphorylation site (SGR-bracket) as determined by a PROSITE search are also shown. The region to which the primer used for 5′ RACE was designed is underlined with an arrow. These sequence data and the corresponding nucleotide sequences are available from EMBL/GenBank/DDBJ under accession number AJ316568.

(Figs. 1 and 5A), but did not contain a eukaryotic ribosome binding site [32]. The 0.65-kb RACE clone was used as a probe in Northern blot analysis on *L. forbesi* eye cup mRNA. The results show that under high-stringency conditions, a transcript of 1.4 kb, which corresponds with the size of full-length MRRP cDNA clones, was detected (Fig. 2B). All of the clones have an open reading frame of 264–280 amino acids with an in frame stop codon at the end of the amino acid sequences confirmed by DNA sequencing. The alignment of the conceptually translated protein sequences of these clones is shown in Fig. 3. The proteins are rich in methionine with this residue having the highest percentage in the proteins (13.3–13.8%). Tyrosine and asparagine are found to occur at the next highest percentages (10–13%). The sequence is also abundant in arginine, glycine, and cysteine residues (9–12%). There are also many repeating microdomains throughout the protein sequence, some of which differ in the three protein sequences (Fig. 3). There were no transmembrane domains for the MRRP clones predicted by the program TopPred II (see Fig. 4). A PROSITE search indicated three potential N-glycosylation sites (Fig. 3), although these may not be glycosylated in vivo since the in vitro translation of MRRP clones plus and minus membranes demonstrates there is no apparent shift in molecular weight (Fig. 5A). In addition, a protein kinase C and three tyrosine kinase phosphorylation sites are also predicted (Fig. 3). None of the three MRRP sequences were predicted to have a signal peptide, suggesting that the protein(s) is intracellular.

The MRRP1 amino acid sequence was used for programs found on web servers. The Expert Protein Analysis System (ExPASy) server (http://expasy.cbr.nrc.ca/ [23]) and its web

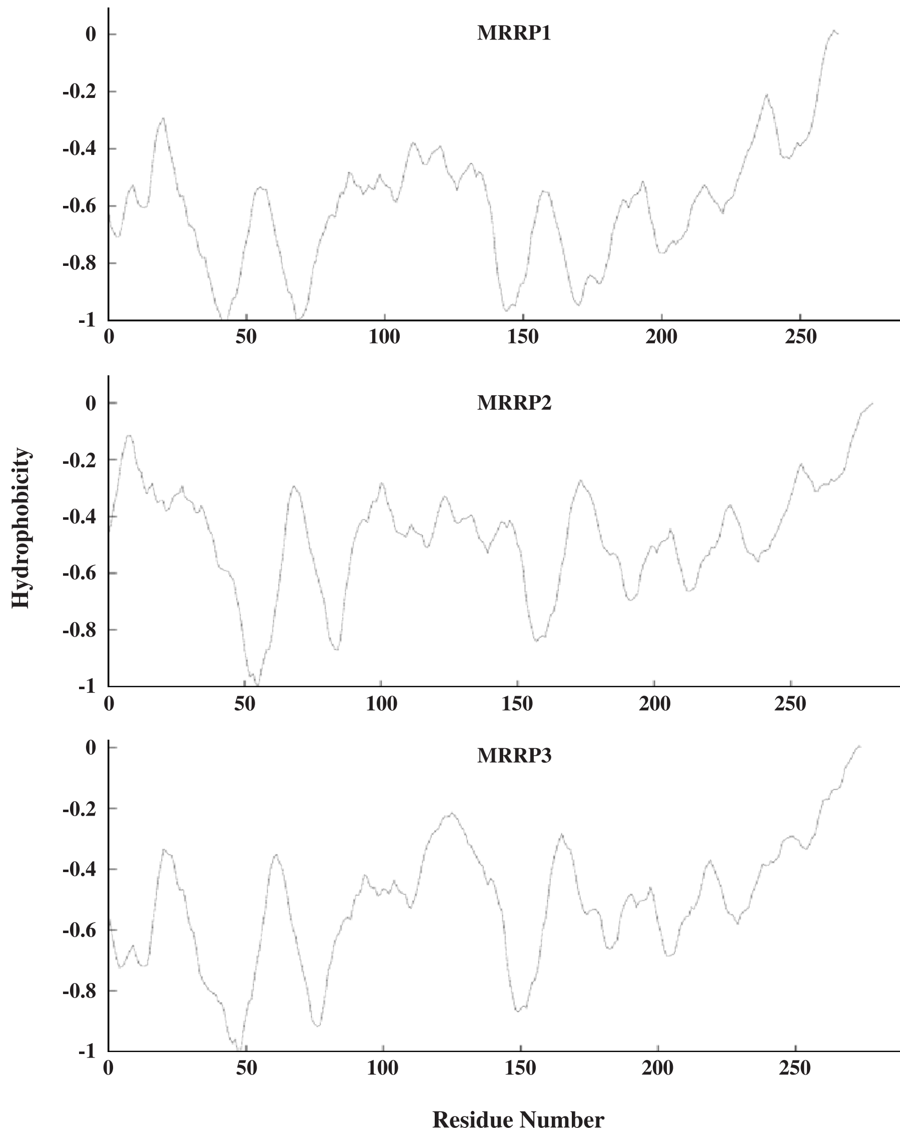

Fig. 4. Hydrophobicity analysis of the conceptually translated amino acid sequences for MRRP clones 1–3. The hydophobicity profiles (hydrophobicity vs. sequence residue number) were generated by the program TopPred II. No typical transmembrane domains are predicted for any of the MRRP clones corresponding amino acid sequence.

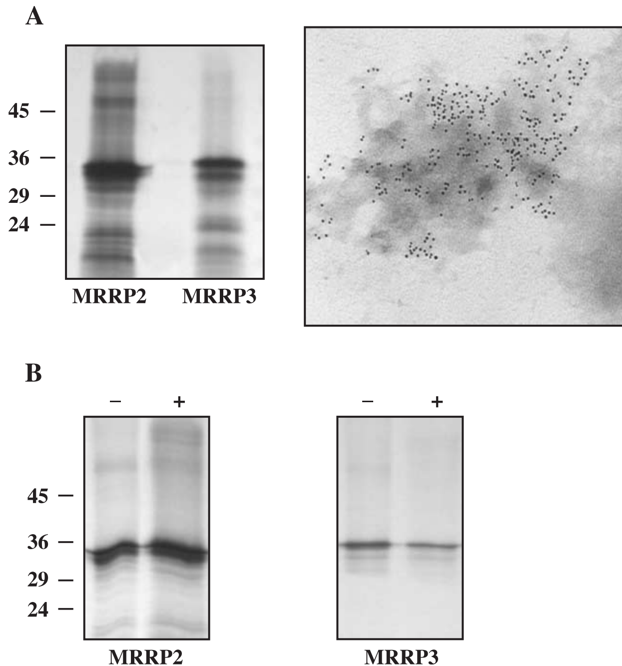

Fig. 5. Coupled in vitro transcription/translation of MRRP2 and MRRP3 cDNA clones and electron micrograph of immunolabeled in vitro translated MRRP. (A) Left panel—0.9 μg of the clones in pBluescript™ were used in the coupled reactions using T3 RNA polymerase and ^{35}S-methionine in the presence of membranes as described below. Right panel—electron micrograph of in vitro translated MRRP2 containing membranes stained with MRRP antisera and visualized by 5 nm colloidal gold secondary antibody labeling. This micrograph demonstrates that MRRP antisera recognize in vitro translated MRRP. (B) 0.9 μg of the clones in pBluescript™ were used in the coupled reactions using T3 RNA polymerase and ^{35}S-methionine in both the absence (−) and presence (+) of microsomal membranes. The reaction total lysate from the reactions without membranes and isolated microsomal membranes from reactions performed in the presence of membranes were electrophoresed on a 10% SDS-PAGE gel. The dried-down gels were exposed to X-ray film for 2–4 days and the films were developed. There is a major protein band at 35–36 kb and a minor band at 34 kDa for the MRRP3 clone whereas the MRRP2 clone yields four bands ranging in molecular weight from 32–34 kDa. In the presence of microsomal membranes (+), there is no major shift in the sizes of these in vitro translation protein bands.

links was used to look at possible homologs based on amino acid composition and also to make structure predictions. Various homologs of crystallins and sodium channels were detected several times using different programs. The Structure Assignment With Text description (SAWTED) server (http://www.bmm.icnet.uk/~sawted) was used to detect homologs based on structure predictions and fold recognition programs.

In Fig. 4, the hydrophobicity profiles for MRRP clones 1, 2, and 3 are shown. The DNA sequence of the clones were conceptually translated using the program Translate in the GCG software package. The sequences were then copied into the program TopPred II, which generated the hydrophobicity profiles using a GES index. All the values for the hydrophobicity are below zero indicating that there are no predicted α-helical transmembrane domains for any of the three amino acid sequences.

3.3. In vitro translation of MRRP

In order to verify that the MRRP cDNAs encode proteins of the correct size, coupled in vitro transcription/translation of the two full-length clones MRRP2 and MRRP3 were performed. The results demonstrate they encode proteins of 32–36 kDa, which are the same size as the isolated MRRP protein (Fig. 5A). The MRRP2 in vitro translated product comprises four bands that migrate at a lower molecular weight than MRRP3 at about 32–34 kDa. MRRP2 is predicted to encode 280 amino acids and a molecular weight of 35.3 kDa. The MRRP3 in vitro translated product migrated at 34–36 kDa, which matches well with the cDNA predicted to encode a protein of 276 amino acids with a predicted molecular mass of 34.6 kDa. To examine whether MRRP in vitro translated products associate with membranes, the cDNA were also translated in the presence of microsomal membranes (Fig. 5B, left panel). The results show that the in vitro translated products pelleted with the membranes and that the protein bands are the same apparent size as the products translated in the absence of membrane. The MRRP therefore appear not to be glycosylated in this system. To confirm that MRRP2 and MRRP3 clones encode MRRP proteins that are components of native MRRPs (i.e., the clones encode proteins that are recognized by MRRP antisera which detect native MRRPs), immunolabeling of isolated microsomal membranes containing MRRP in vitro translated products was performed. In Fig. 5A (right panel), an electron micrograph shows isolated in vitro translated MRRP2 containing microsomal membranes that were first incubated with MRRP antisera and then labeled with colloidal gold secondary antibody. This appears to be a patch of a microsomal membrane, as opposed to a vesicle, that is densely labeled with the colloidal gold secondary antibody. Immunolabeling of isolated microsomal membranes containing MRRP3 in vitro translated products gave similar results as MRRP2 where densely labeled areas of immunoreactivity could be seen in the electron micrographs (data not shown). These results demonstrate that MRRP antiserum recognizes the in vitro translated products for both the MRRP2 and MRRP3 clones. The proteins encoded by these clones make up part of the family of immunoreactive native proteins in squid tissues recognized by MRRP antisera.

3.4. Electron microscopy studies of native MRRP

3.4.1. Native MRPPs

Immunoblot analysis of protein isolated from an eye white tissue collagenase preparation (Fig. 6A) demonstrates that the major protein in eye white of 32–36 kDa (arrow) is recognized by MRRP antisera. The collagenase preparation contains mainly native MRRPs in the membrane compartment. These membranes have been tested and found to contain phospholipids that are of the typical types found in cell plasma membranes. We estimate that MRRPs are approximately 90% of the total protein in this preparation.

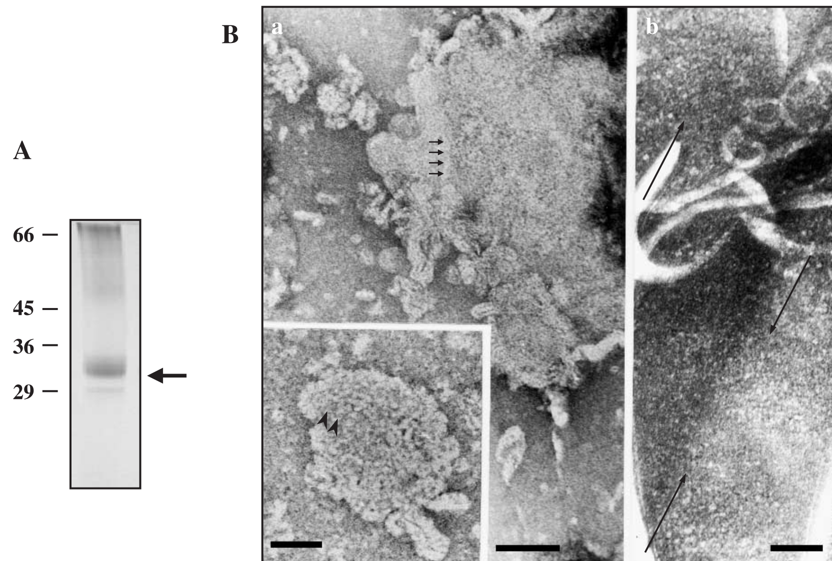

Fig. 6. Collagenase preparation of squid eye white and electron micrographs of native MRRP containing membranes. (A) Coomassie blue stained gel of MRRP collagenase preparation demonstrates that MRRP is present in eye white and makes up at least an estimated 90% of the total eye white proteins. This is the preparation used to obtain membranes containing native MRRPs for electron microscopy studies. (B) Electron micrographs of negatively stained MRRP-containing membranes before (left panel) and after (right panel) detergent treatment with 0.112% LDAO. In (a), small arrows point at an area of the membrane with irregular substructures. At higher magnification (inset), single stain-excluding particles can be clearly discerned (arrowheads). In (b), distinct lattices as revealed after partial delipidation are highlighted by large arrows (see text for further details). The scale bars correspond to 100 nm in inset of left panel a and in right panel b and 50 nm in left panel a.

Consequently, this was the protein material used for the native MRRPs electron microscopy studies described in the experimental procedures. Electron microscopic observation of MRRP-containing membranes revealed vesicles of various sizes (Fig. 6B, left panel a). While some areas appeared relatively smooth, the majority of the membrane surfaces exhibited a marbled appearance, and at higher magnification (left panel a, inset) stain-excluding particles (arrowheads) can be clearly discerned. These particles densely populated the membrane surface suggesting that MRRP molecules are in close contact. When MRRP-containing membranes were incubated in the presence of 0.112% LDAO, MRRP assembled into crystalline arrays (Fig. 6B, right panel b). Fourier analysis of the periodicity of these arrays revealed a 7.2 nm repeat (±0.2 nm) along two principal axes defining the unit cell as a=b=7.2 nm. The included angle was determined to be 90°. These unit cell parameters, together with the fact that MRRP was not solubilized by the detergent treatment, suggest a p4 plane group composed of one tetrameric MRRP molecule. The presence of tetramers is also in agreement with measurements performed on membranes showing single particles in the absence of detergent (Fig. 6B, panel a and inset). The fact that the regular arrays were obtained by partial delipidation favors the idea that MRRPs are tightly associated with the membrane. As the detergent extracts some of the lipid, MRRP molecules can move closer together, facilitating their assembly into ordered arrays. In contrast to this, upon complete detergent-solubilization of MRRP-containing membranes, MRRP undergoes spontaneous association into large amorphous aggregates. From this, in conjunction with the a–b dimensions and the included angle, it appears that the only compatible plane group is p4 implying there are four asymmetric units per unit cell, i.e., MRRP forms tetramers. The presence of tetramers also agrees with measurements performed on membranes in the absence of detergent with particles that are not assembled into a lattice (Fig. 6B, left panel a and N. Evans, unpublished results).

4. Discussion

In this study, we have identified and cloned the cDNAs for three related proteins, which are the most abundant proteins present in squid eye. As these proteins are highly enriched in methionine residues and have many repeating motifs rich in methionine, tyrosine, asparagine, arginine, glycine, and cysteine, we have named these proteins methionine-rich repeat proteins (MRRPs). They have very unusual protein sequences, which has been confirmed in a recent report [43]. A Propsearch, which searches for proteins with similar amino acid compositions, identified many different crystallin protein types as matches. Methionine-rich proteins have been classed as seed, insect storage, synaptic vesicle-related, signal recognition particle proteins or proteins that contain RNA-binding domains [33–37]. However, none of the methionine-rich regions of these proteins are homologous to the MRRP repeat motifs. It is possible that the MRRPs methionine residues are targets for oxidation. The oxidative stress repair enzyme methionine sulfoxide reductase is highly expressed in pigment epithelial

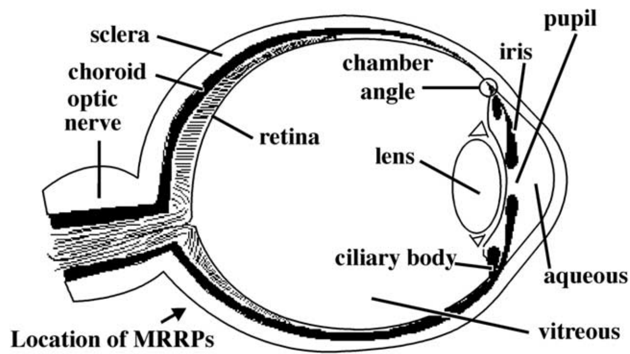

Fig. 7. MRRPs appear to be localized to the eye tissue analogous to the sclera in the mammalian eye. Diagram of mammalian eye anatomy labeling various eye tissues. The arrow points to the area corresponding to squid eye white tissue where MRRPs are abundantly expressed. This area is the mammalian sclera. The eye white tissue in squid may have the same function as the sclera. This figure was adapted from an eye anatomy figure at http://www.richmondeye.com/index.html.

cells of the retina [38] and may provide oxidative defense for MRRPs in the squid eye as well. The nature of the repeating domains in MRRPs is unclear and we have no defined function of this protein family based on this study. However, the extreme abundance of MRRPs in squid eye suggests the possibility that they may be structural proteins.

The detergent-induced array formation also argues for the tight binding of MRRP to the membrane. With transmembrane proteins, detergent-induced crystallization within the immediate native lipid environment is well documented [39,40]. In the present case, an analogous phenomenon is observed since the assembly proceeds with the protein still associated with membranes. In the EM studies, MRRPs appear to protrude from the membrane densely populating the lipid surface. The MRRPs density is so high that upon partial delipidation ordered arrays are formed. The lattice parameters argue for a tetramer, but we cannot rule out that (in the absence of detergent) MRRPs also exists in other oligomeric states. It is conceivable that the different MRRP types form heteromultimers conferring different functions.

Porins are bacterial outer membrane proteins that are mainly expressed at high levels and behave as integral membrane proteins. Unlike other integral membrane proteins, porins do not contain α-helices but instead insert into membranes by folding into antiparallel β-pleated strands in the form of a closed barrel [41]. MRRPs, like porins, have a very tight association with membranes, behave like integral membrane proteins, and do not contain typical transmembrane domains. These biochemical characteristics of MRRPs and the protein secondary structure prediction of many β-turns, some β-pleated strands and coil-coil domains (data not shown) can be explained by a β-barrel type structure that can insert into membranes where polar and charged residues are somehow shielded from the hydrophobic cell membrane. In general, porins are present as homotrimers in the outer membranes of bacteria and function as diffusion pores [41]. MRRPs are located in the squid eye white tissue that could correspond to the sclera in the mammalian eye (Fig. 7). The sclera does not contain blood vessels and therefore relies on diffusion of oxygen and nutrients from adjacent tissues or fluid [42]. MRRPs could perhaps function as general diffusion pores, like the general porins, to allow the diffusion of small molecules. We cannot rule out, however, other functions, particularly of a structural nature, for MRRP-like proteins. If MRRP-like domains are universally present in mammalian tissues, genetic recruitment of the MRRP gene may have occurred in evolution as in squid lens and cornea proteins [3].

In summary, we have isolated clones encoding unusual proteins, which we have named Methionine-Rich Repeat Proteins (MRRPs) because they are rich in the amino acid methionine and have many repeating micro-domains. Immunoblot analysis of both squid and bovine tissues using antisera generated to isolated MRRPs detects protein species of MRRPs that are 32–36 kDa. Electron microscopy studies in conjunction with Fourier transform analysis suggest that MRRP associates with a cell membrane fraction, and is able to form ordered arrays of tetrameric MRRP. The fact that proteins with MRRP-like motifs may exist in evolutionary diverse organisms strongly suggests that MRRP defines an important family of membrane-associated proteins, which may have an unusual nature of attachment to cell membranes. The MRRP family may perform a structural or transport role.

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References

[1] D.L. Gilbert, W.J. Adelman Jr., J.M. Arnold, Squid as Experimental Animals, Plenum Press, New York, 1990.
[2] S.I. Tomarev, R.D. Zinovieva, Squid major lens polypeptides are homologous to glutathione S-transferases subunits, Nature 336 (1988) 86–88.
[3] R.A. Cuthbertson, S.I. Tomarev, J. Piatigorsky, Taxon-specific recruitment of enzymes as major soluble-proteins in the corneal epithelium of 3 mammals, chicken, and squid, Proc. Natl. Acad. Sci. U. S. A 89 (1992) 4004–4008.
[4] C.F.E. Roper, K. Boss, The giant squid, J. Sci. Am. 246 (1982) 82–89.
[5] M.D. Hall, M.A. Hoon, N.J.P. Ryba, J.D.D. Pottinger, J.N. Keen, H.R. Saibil, J.B.C. Findlay, Molecular-cloning and primary structure of squid (*Loligo forbesi*) rhodopsin, a phospholipase C-directed G-protein-linked receptor, Biochem. J. 274 (1991) 35–40.
[6] N.J.P. Ryba, J.D.D. Pottinger, J.N. Keen, J.B.C. Findlay, Sequence of the beta-subunit of the phosphatidylinositol-specific phospholipase C-directed GTP-binding protein from squid (*Loligo forbesi*) photoreceptors, Biochem. J. 273 (1991) 225–228.

[7] J.S. Lott, N.J.P. Ryba, J.D.D. Pottinger, J.N. Keen, A. Carne, J.B.C. Findlay, The gamma-subunit of the principal G-protein from squid (*Loligo forbesi*) photoreceptors contains a novel N-terminal sequence, FEBS Lett. 312 (1992) 241–244.

[8] A. Carne, R.A. McGregor, J. Bhatia, A. Sivaprasadarao, J.N. Keen, A. Davies, J.B.C. Findlay, A beta-subclass phosphatidylinositol-specific phospholipase-C from squid (*Loligo forbesi*) photoreceptors exhibiting a truncated C-terminus, FEBS Lett. 372 (1995) 243–248.

[9] P.D. Monk, A. Carne, S.-H. Liu, J.W. Ford, J.N. Keen, J.B.C. Findlay, Isolation, cloning, and characterisation of a trp homologue from squid (*Loligo forbesi*) photoreceptor membranes, J. Neurochem. 67 (1996) 2227–2235.

[10] N.J.P. Ryba, M.D. Hall, J.B.C. Findlay, R. Tirindelli, A method for efficient and rapid cloning of cDNA into Lambda-GT10 that facilitates directional sub-cloning into plasmid vectors, Nucleic Acids Res. 19 (1991) 1953.

[11] H.R. Saibil, M. Michel-Vilaz, Squid rhodopsin and GTP-binding protein crossreact with vertebrate photoreceptor enzymes, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 5111–5115.

[12] J.B.C. Findlay, M.J. Geisow (Eds.), Protein Sequencing: A Practical Approach, IRL Press, Oxford, 1989.

[13] U. Gubler, B.J. Hoffman, A simple and very efficient method for generating cDNA libraries, Gene 25 (1983) 263–269.

[14] R.J. Harvey, M.G. Darlison, Random-primed cDNA synthesis facilitates the isolation of multiple 5′-cDNA ends by RACE, Nucleic Acids Res. 19 (1991) 4002.

[15] L. Alphey, PCR-based method for isolation of full-length clones and splice variants from cDNA libraries, BioTechniques 22 (1997) 481–486.

[16] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

[17] D. Akrigg, T.K. Attwood, A.J. Bleasby, J.B.C. Findlay, A.C.T. North, N.A. Maughan, D.J. Parry-Smith, D.N. Perkins, J.C. Wootton, Serpent—an information-storage and analysis resource for protein sequences, CABIOS 8 (1992) 295–296.

[18] J. Devereux, P. Haeberli, O. Smithies, A comprehensive set of sequence—analysis programs for the vax, Nucleic Acids Res. 12 (1984) 387–395.

[19] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (1990) 403–410.

[20] Y. Nakamura, T. Gojobora, T. Ikemura, Codon usage tabulated from the international DNA sequence databases, Nucleic Acids Res. 26 (1998) 334.

[21] A. Bairoch, P. Bucher, K. Hofmann, The PROSITE database, its status in 1997, Nucleic Acids Res. 25 (1997) 217–221.

[22] M.G. Claros, G. von Heijne, Toppred-II—an improved software for membrane–protein structure predictions, CABIOS 10 (1994) 685–686.

[23] R.D. Appel, A. Bairoch, D.F. Hochstrasser, A new-generation of information-retrieval tools for biologists—the example of the expasy www server, Trends Biochem. Sci. 19 (1994) 258–260.

[24] F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl, Current Protocols in Molecular Biology, John Wiley and Sons, New York, 1987.

[25] E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988.

[26] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[27] M.G. Pluskal, M.B. Przekop, M.R. Kavontian, C. Vecoli, D.A. Hicks, Immobilon pvdf transfer membrane—a new membrane substrate for western blotting of proteins, BioTechniques 4 (1986) 272–283.

[28] S.Y. Ali, S.W. Sajdera, H.C. Anderson, Isolation and characterization of calcifying matrix vesicles from epiphyseal cartilage, Proc. Natl. Acad. Sci. U. S. A. 67 (1970) 1513–1520.

[29] R.C. Valentine, B.M. Shapiro, E.R. Stadtman, Regulation of glutamine synthetase. XII. Electron microscopy of the enzyme from *Escherichia coli*, Biochemistry 7 (1968) 2143–2152.

[30] S. Hovmöller, CRISP: crystallographic image processing on a personal computer, Ultramiscroscopy 41 (1992) 121–135.

[31] A.I. Magee, A.H. Koyama, C. Malfer, D. Wen, M.J. Schlesinger, Release of fatty-acids from virus glycoproteins by hydroxylamine, Biochim. Biophys. Acta 798 (1984) 156–166.

[32] M. Kozak, Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes, Cell 44 (1986) 283–292.

[33] A.A. Kortt, J.B. Caldwell, G.G. Lilley, T.J.V. Higgins, Amino-acid and cDNA sequences of a methionine-rich 2S protein from sunflower seed (*Helianthus annuus* L), Eur. J. Biochem. 195 (1991) 329–334.

[34] L.M. Corpuz, H. Choi, S. Muthukrishnan, K.J. Kramer, Sequences of 2 cDNAs and expression of the genes encoding methionine-rich storage proteins of *Manduca sexta*, Insect Biochem. 21 (1991) 265–276.

[35] D.T. Hess, T.M. Slater, M.C. Wilson, J.H.P. Skene, The 25 KDA synaptosomal-associated protein snap-25 is the major methionine-rich polypeptide in rapid axonal-transport a major substrate for palmitoylation in adult CNS, J. Neurosci. 12 (1992) 4634–4641.

[36] D. Zopf, H.D. Bernstein, A.E. Johnson, P. Walter, The methionine-rich domain of the 54 KD protein subunit of the signal recognition particle contains an RNA-binding site and can be cross-linked to a signal sequence, EMBO J. 9 (1990) 4511–4517.

[37] K.V. Datar, G. Dreyfuss, M.S. Swanson, The human HNRNP M proteins—identification of a methionine arginine-rich repeat motif in ribonucleoproteins, Nucleic Acids Res. 21 (1993) 439–446.

[38] J. Moskovitz, N.A. Jenkins, D.J. Gilbert, N.G. Copeland, F. Jursky, H. Weissbach, N. Brot, Chromosomal localization of the mammalian peptide-methionine sulfoxide reductase gene and its differential expression in various tissues, Proc. Natl. Acad. Sci. U. S. A. 93 (8) (1996) 3205–3208 (APR 16).

[39] A. Holzenburg, F.H. Wilson, M.E. Finbow, R.C. Ford, Structural investigations of membrane–proteins—the versatility of electron-microscopy, Biochem. Soc. Trans. 20 (1992) 591–597.

[40] B.K. Jap, M. Zulauf, T. Scheybani, A. Hefti, W. Baumeister, U. Aebi, A. Engel, 2D crystallization—from art to science, Ultramicroscopy 46 (1992) 45–84.

[41] R. Koebnik, K.P. Locher, P. Van Gelder, Structure and function of bacterial outer membrane proteins: barrels in a nutshell, Mol. Microbiol. 37 (2) (2000) 239–253.

[42] G.J. Tortora, S. Reynolds Grabowski, Principles of Anatomy and Physiology, 7th ed., HarperCollins, New York, 1993.

[43] W.J. Crookes, L.L. Ding, Q.L. Huang, J.R. Kimbell, J. Horwitz, M.J. McFall-Ngai, Reflectins: the unusual proteins of squid reflective tissues, Science 303 (5655) (2004) 235–238.