Heterologous expression in *Escherichia coli* of native and mutant forms of the major intrinsic protein of rat eye lens (MIP26)

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The complete cDNA of rat eye lens major intrinsic protein (MIP26) was sequenced using the dideoxy chain termination method. The sequence displayed 89% nucleotide identity and 95% identity at the amino acid level with bovine MIP26 [Gorin, Yancey, Cline, Revel and Horwitz (1984) Cell **39**, 49–54]. Both native and mutant cDNAs coding for rat MIP26 were amplified by PCR and subcloned into the pPOW expression vector for expression in *Escherichia coli*. A membrane signal peptide (PelB) was used for secretion of MIP26 into the cytoplasmic membrane. A hydrophilic octapeptide tail (FLAG) was fused to either the N- or C-terminus of MIP26 to aid monoclonal antibody-mediated identification and purification. Heterologously expressed MIP26 was identified by using a monoclonal antibody corresponding to the FLAG peptide located at the termini of MIP26. Immunofluorescently labelled monoclonal antibody was used to determine the localization of MIP26 in the cytoplasmic membrane. The majority of the protein was integrated into cell plasma membrane. MIP26 was extracted with *n*-octyl β-D-glucopyranoside and then purified on an affinity gel column. Rat MIP26 cDNA contains an -Asn-Gly- sequence at the C-terminus, which has been shown in other proteins to be particularly susceptible to spontaneous deamidation [Takemoto and Emmons (1991) Curr. Eye Res. **10**, 863–869]. We therefore modified the MIP26 molecule using a site-directed mutagenesis method to generate a mutant MIP26 at the appropriate asparagine residue ($Asn^{244} \rightarrow Asp$) near the C-terminus. The mutation was confirmed by DNA sequencing. The mutant MIP26 protein was also expressed in *E. coli* and incorporated predominantly into the cytoplasmic membrane.

INTRODUCTION

The eye lens is a transparent avascular organ which focuses light on to the retina. It is predominantly composed of elongated non-dividing fibre cells surrounded by plasma membranes containing intercellular junctions. Lens fibre cells communicate directly with each other by exchanging ions and metabolites. Such communicating gap junctions are essential for normal cell function. It has been suggested that this intercellular transmembrane pathway maintains the transparency of the lens (Peracchia et al., 1985). Defects in such structures could be associated with the development of cataract, the major cause of blindness world wide (Harding and Crabbe, 1984; Harding, 1991).

The major intrinsic protein of the lens fibre cell membrane is a polypeptide with an apparent molecular mass of 26 kDa referred to as MIP26 or MP26 (Broekhuyse et al., 1976; Benedetti et al., 1981; Takemoto and Emmons, 1991). It is a member of the MIP family of transmembrane proteins (Chepilinsky, 1994) MIP26 represents more than 50% of the total membrane protein of the mature bovine eye lens (Paul and Goodenough, 1983). The predicted structure for MIP26 is that it traverses the lipid bilayer six times with both N- and C-termini on the cytoplasmic side of the membrane (Gorin et al., 1984).

The C-terminal arm of MIP26 is known as the channel gate, and the gating activity is regulated by Ca^{2+}-activated calmodulin (Peracchia and Girsch, 1985; Swamy and Abraham, 1992). When these channels open, they allow Ca^{2+} ions to move down their electrochemical gradient across the plasma membrane into the cytoplasm, leading to a significant increase in cytosolic free Ca^{2+}. The increased Ca^{2+} then activates the Ca^{2+}-binding regulatory protein calmodulin. Calmodulin, with its bound Ca^{2+}, can then bind to MIP26 causing the channels to close rapidly to control the level of Ca^{2+} in the fibre cell cytoplasm.

Abnormally high Ca^{2+} levels in the lens are characteristic of many cataracts such as selenite cataract (David and Shearer, 1984) and cataract in diabetics (Hightower and McCready, 1989).

During normal aging, MIP26 is known to undergo post-translational cleavage and loss of small fragments ($\approx$ 4–10 kDa) from the C-terminal arm. The conversion of MIP26 into small fragments progresses with age and is essentially complete by the age of 23 (Harding, 1991); it may contribute to selenite cataract (David and Shearer, 1984; Takemoto and Emmons, 1991).

Spontaneous non enzymic deamidation of asparagine residues is a common modification in many tissues during aging when the residue is present on the N-terminal side of glycine (Harding and Crabbe, 1984; Geiger and Clarke, 1987; Takemoto and Emmons, 1991). It has been shown that, during aging of the bovine lens, the asparagine residue at position 246, which is immediately N-terminal of a glycine residue, is completely deamidated *in vivo* (Takemoto and Emmons, 1991). As the MIP26 molecule from rat lens contains this sequence at residues 244 and 245, we modified the asparagine residue (uncharged polar R group) to an aspartic acid residue (negatively charged polar R group) by using a novel method for site-directed mutagenesis.

MATERIALS AND METHODS

Bacterial strains and media

Two strains of *Escherichia coli* were used as plasmid hosts: *E. coli* DH5α (F^-, rec^-, $meth^-$) from Bethesda Research Laboratories

Abbreviation used: MIP26, major intrinsic protein of eye lens fibre (molecular mass 26 kDa).

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(Gibco–BRL, Paisley, U.K.) and *E. coli* TOPP 2 [*F'*, *proAB*, *lacqZΔM15*, Tn*10*, (*tet*^{1})] from Stratagene (Cambridge, U.K.).

E. coli strain DH5α was used as the host for recombinant plasmids and grown in Luria broth/agar (Sambrook et al., 1989). Recombinant MIP26 cDNA was expressed in *E. coli* strain TOPP 2 cultured in Superbroth (Hatt et al., 1992).

DNA and enzymes

The complete rat MIP26 cDNA was obtained as an insert in the plasmid pUC18 kindly donated by Dr. A. Shiels, formerly of the University of East Anglia, Norwich, U.K. Plasmid pPOW was a gift from Dr. B. E. Power of the CSIRO Division of Biomolecular Engineering, Victoria, Australia.

Restriction endonucleases and T4 DNA ligase were purchased from Gibco–BRL. *Taq* polymerase was from Perkin–Elmer Cetus (Perkin–Elmer Corp., Warrington, Cheshire, U.K.) and used according to the supplier's instructions. Oligonucleotide primers for PCR and sequencing were synthesized by Genosys Biotechnologies (Cambridge, U.K.). The FLAG expression system was purchased from International Biotechnologies (New Haven, CT, U.S.A.).

DNA sequencing

Recombinant plasmid DNA was sequenced by the dideoxy chain termination method using a Sequenase Kit (USB, Cleveland, OH, U.S.A.), modified by using T7 DNA polymerase (Tabor and Richardson, 1987).

PCR

PCR was used to amplify MIP26 cDNA from cloned plasmid pUC18 in an *Eco*RI cutting site. The oligonucleotides were used as primers for PCR with *Stu*I–*Eco*RI restriction sites for identification of the PCR product, and to insert it into the *Msc*I–*Eco*RI-cleaved pPOW expression vector.

A FLAG marker expression system, based on fusion of a 1 kDa hydrophilic octapeptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) to either the N- or C-terminus of MIP was used, allowing immunological detection, localization, and purification of the expressed protein (Hopp et al., 1988). The primer was constructed to incorporate an *Stu*I-recognition site, and the rat MIP26 N-terminal coding region in primer 1 (5′ end) was: 5′-nonsense DNA overhang for restriction endonuclease binding (12 bases), *Stu*I restriction endonuclease site (six bases), MIP26 cDNA-binding sequence (17 bases), i.e. 5′-ACATGAACTTGCAGG-CCTGGGAACTTCGGTCTGCC-3′ (35-mer). The reverse primer 2 (3′ end) was: 5′-nonsense DNA overhang for restriction endonuclease binding (nine bases), *Eco*RI restriction endonuclease site (six bases), stop codon (three bases), FLAG octapeptide and rat MIP26 C-termini coding region (15 bases), i.e. 5′-CTTCGAACAGAATTCTTACTTGTCATCGTCGTC-CTTGTAGTCCAGGGCCTGAGTCTT-3′ (57-mer).

The PCR protocol was based on the method of Saiki et al., (1988). Amplification reaction mixtures containing primers, template, dNTPs and buffer were heated to 95 °C for 5 min before the addition of *Taq* polymerase and $MgCl_2$ to introduce nicks, to facilitate amplification of the template. We typically employed 28 cycles of amplification by denaturing DNA at 94 °C for 1 min, annealing primers at 50 °C for 1.5 min, and extending primers at 72 °C for 1.5 min.

After the last of 28 cycles, the samples were left at 72 °C for an additional 10 min for a final primer extension.

We have found that the efficiency of inverse PCR is enhanced by amplification of linear rather than circular DNA. For this, the circularized DNA was treated with a restriction enzyme before PCR. The PCR product was purified away from the low-melting-point agarose gel using Magic PCR Preps (Promega, Southampton, Hants., U.K.) which eliminated nucleotides, excess primers, primer dimers and short fragments.

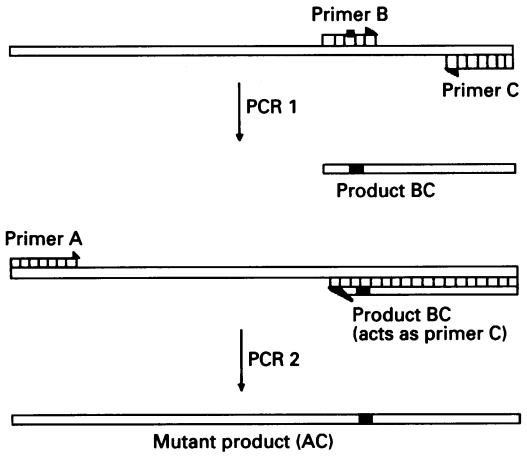

Figure 1 Mutagenesis by overlap extension

In our modification of the method, the mutant product (BC) of the first PCR cycle was made by 'mispriming' with primers B and C. The mutant BC product was used to act as primer C on the template in PCR 2 to complete the mutant MIP26 cDNA fragment. An arrowhead (→ or ←) indicates the 5′- to -3′ direction of each strand of the primers and PCR products. Changes to the original sequence, represented by the black rectangle, were made by 'mispriming' with primers B and C.

Site-directed mutagenesis of rat MIP26

Site-directed mutagenesis using a novel modification of 'mutation by overlap extension' was carried out on the 3′ end of the MIP26 gene, replacing Asn^{244} with aspartic acid. The effect of such a substitution on MIP26 structure was investigated. The mutagenesis was performed using PCR by performing a two-step reaction with sets of primers that overlap in the region of the mutant (Horton and Pease, 1991). The mutagenic primer had the sequence 5′-GACTCCAATGGACAG-3′. This sequence corresponds to nucleotides 724–738 of the cDNA except for nucleotide 730 where A replaced G (underlined in the sequence above). This base change converts the codon for Asn at position 244 in the MIP26 into the codon for Asp.

The mutated fragment (63 nucleotides) of the first reaction of overlap extension was recovered from low-melting-point agarose gels by using the Mermaid kit which is suitable for purifying fragments of less than 200 bases (obtained from Stratech Scientific, Luton, Beds., U.K.). The gel-purified mutated fragment was then used as primer C (3′ end) in the second reaction to complete the mutant whole fragment of MIP26 cDNA (Figure 1). Instead of primer C we used the mutated fragment from the first reaction of PCR (by including primer B and C) in the second reaction to complete the whole mutant product. This simple method produces a high mutagenic efficiency (100%) when the mutation is placed inside the product molecule, at a distance of less than 100 bases from either end (Dilsiz and Crabbe, 1994).

Subcloning of rat MIP26 cDNA and preparation of plasmids

After PCR amplification and subsequent purification, the fragments were cloned directly into a pGEM-T vector (Promega). The plasmid was prepared by cutting with *Eco*RV and adding a

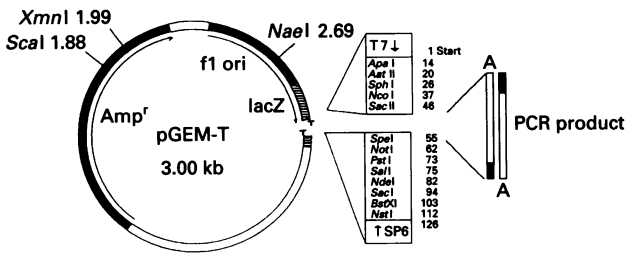

Figure 2 pGEM-T vector for direct cloning of PCR-amplified nucleic acid

The vector was constructed by cutting with *Eco*RV and adding a 3′-terminal thymidine (T) to both ends. The insert with the 3′-terminal adenine extension (A) is added to the amplification products generated by thermostable DNA polymerase. This method (TA cloning) was used to simplify the direct cloning of PCR product.

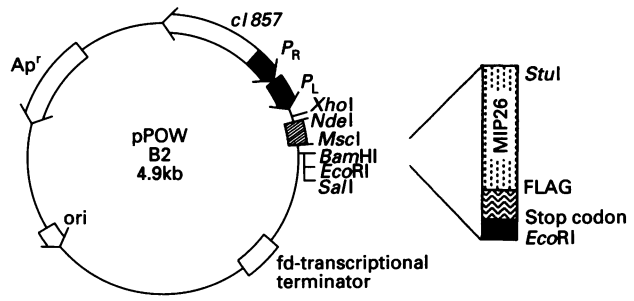

Figure 3 *E. coli* expression vector pPOW

The pPOW system was constructed with λ left and right promoters in tandem, the temperature-sensitive repressor gene *cI857* and the pelB signal sequence for protein secretion (Schauder et al., 1987; Power et al., 1992).

3′-terminal thymidine to both ends. These single 3′-thymidine overhangs at the insertion site greatly improve the efficiency of direct ligation of PCR fragments into the plasmid (Figure 2).

The ligation mixtures were incubated at 22 °C for 6 h, and then subjected to heat inactivation at 65 °C for 15 min, phenol/chloroform extraction and ethanol precipitation. Diluted ligation mixture (1:1) was used to transform *E. coli* strain DH5α and then incubated on ice for 45 min. The cells were subjected to heat-shock treatment at 42 °C for 45 s, and 0.8 ml of Luria broth was added. The cells were then incubated at 37 °C for 1 h with shaking. They were finally spread on to Luria agar plates containing 50 μg/ml ampicillin, 4 mM isopropylthiogalactoside and 0.05% X-gal indicator and incubated at 37 °C overnight.

Recombinants were selected by blue/white colour colony selection on the plates. Individual white colonies were picked and grown in Luria broth/ampicillin. Plasmid DNA was prepared and digested with *Stu*I and *Eco*RI to reveal the presence of MIP26 cDNA. The insert in this plasmid was resequenced to ensure no misincorporation during PCR. The *Stu*I and *Eco*RI restricted gene encoding MIP26 from recombinant pGEM-T was gel purified using the 'Magic-PCR prep' method (Promega), and then ligated to the *Msc*I and *Eco*RI-cut expression vector pPOW (Figure 3) to express the protein. In this vector, expression is under the control of the λ P_RP_L promoters and is heat-inducible in the presence of the temperature-sensitive repressor *cI857*.

The ligation mixture was transformed into *E. coli* strain DH5α. The cells were spread on to Luria agar/ampicillin plates and incubated at 37 °C overnight. Plasmid DNA was prepared from transformant *E. coli* strain DH5α by an affinity-chromatography-based method using the Magic mini-preps kit (Promega). Recombinant plasmids were then identified by digestion with enzymes. Positively orientated recombinants were sequenced using dideoxy-nucleotide-chain-termination sequencing (Tabor and Richardson, 1987) in order to verify the authenticity of the native and mutant sequences.

Expression and preparation of MIP26

E. coli TOPP 2 cells were transformed by the standard *E. coli* transformation procedure modified from Hanahan (1985) used for propagation of plasmid for protein production. Transformants were grown at 30 °C in Superbroth containing thiamine and biotin to an A_{600} of 4.0. MIP26 expression was induced by increasing the temperature to 42 °C for 15 min and then incubating the culture at 37 °C for 4 h.

Cells were collected by centrifugation at 3000 *g* for 5 min at 4 °C and resuspended in one-tenth of the original volume in lysis buffer (50 mM Tris/HCl, pH 7.5, 20% sucrose) at 0 °C. EDTA was then added to a final concentration of 1 mM, and cells were incubated at 0 °C for 30 min. Lysozyme was added (at a final concentration of 100 μg/ml) and the mixture incubated at room temperature for 20 min. Cells were collected by centrifugation at 12000 *g* for 10 min at 4 °C to form spheroplasts. Periplasmic proteins were removed by osmotic shock in one-tenth of the original volume in cold deionized water (0 °C, 10 min), followed by centrifugation at 12000 *g* for 5 min at 4 °C. The pellet was resuspended in lysis buffer (as above) and transferred to a French press at 4 °C. The cell suspension was disrupted by three passages through the press at 82.8 MPa.

The cell suspension was centrifuged at 20000 *g* for 30 min at 4 °C and the supernatant containing soluble protein was transferred to a new tube. The pellet containing inclusion bodies and membrane-associated proteins was washed with ice-cold deionized water and centrifuged at 20000 *g* for 30 min at 4 °C.

For sucrose-gradient centrifugation, the pellet was resuspended in one-tenth of the original volume buffer (25 mM Tris/HCl buffer, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride, 2 μg/ml leupeptin and 60% sucrose). Density-gradient centrifugation was carried out as described by Tadayyon et al. (1994), using a Beckman TL-100 ultracentrifuge (Beckman Instruments). Fractions were analysed for the presence of MIP26 by SDS/PAGE and Western blotting.

The pellet from membrane fractions was resuspended in one-tenth of the original volume of 25 mM Tris/HCl buffer, pH 7.5, prepared as above, to which 2.5% (w/v) octyl β-D-glucopyranoside was added. The solution was left on ice for 30 min. The samples were then centrifuged at 12000 *g* for 5 min at 4 °C and the supernatant containing solubilized membrane proteins was analysed by SDS/PAGE.

SDS/PAGE

Proteins of the bacterial cell membrane fraction were separated on denaturing SDS/polyacrylamide gels using a Bio-Rad Miniprotein II dual slab cell minigel apparatus; a discontinuous gel system was used (Laemmli, 1970).

As MIP26 is aggregated by boiling (Wong et al., 1978), the samples were denatured in SDS solubilization buffer at 37 °C, then resolved (30 min) on a 12.5% polyacrylamide gel. This was followed by staining with 0.1% of Coomassie Blue R-250 in 40% methanol/10% acetic acid for 1 h and then destaining in 40% methanol/10% acetic acid overnight.

Immunoblot analysis

Immunoblot analysis was used to detect MIP26 in cytoplasmic membrane proteins extracted from *E. coli*. After electrophoresis, the proteins on the gel were electrophoretically transferred to nitrocellulose membranes (Pharmacia, Milton Keynes, Beds., U.K.) at 200 mA for 1.5 h by the method of Towbin et al. (1979). The nitrocellulose membranes were blocked in blocking solution [3% BSA in Tris-buffered saline (TBS)] for 20 min at room temperature. The membranes were rinsed (3 × 5 min washes in TBS) and incubated with 10 μg/ml primary anti-(FLAG M2) monoclonal antibody in TBS for 1 h at 37 °C (the antibody is specific to the FLAG octapeptide at the C-terminus of the MIP26 molecule). The membranes were further washed (3 × 5 min with TBS on a shaker) and incubated with goat anti-mouse IgG–horseradish peroxidase-conjugated secondary antibody (Promega) for 2 h at 37 °C. Membranes were again washed as above in TBS and immunoreactive proteins visualized by staining with 4-chloronaphthol (Promega). Blots were finally rinsed with distilled water and dried.

Purification of MIP26 from *E. coli* cytoplasmic membrane

MIP26 was purified from crude *E. coli* cytoplasmic membrane fractions by affinity chromatography using an anti-(FLAG M2) affinity gel system. Anti-(FLAG M2) affinity gel was packed in a column and equilibrated with PBS, pH 7.4. The column was run at 1.0 ml/min at room temperature. Adsorbed MIP26 protein was eluted with 10 ml of 0.1 M glycine, pH 3.0, and the column re-equilibrated with 20 μl of 1 M Tris base at pH 8.0.

Anti-(FLAG M2) affinity gel consists of the anti-(FLAG M2) IgG monoclonal antibody coupled to an agarose matrix via a hydrazide linkage to its carbohydrate group located at the Fc region of the antibody.

Immunofluorescent localization of MIP26 in *E. coli*

The spheroplast form of *E. coli* was suspended in 0.1 ml of 0.05% glutaraldehyde/2% paraformaldehyde/0.1 M PBS, and 1% BSA was added to prevent non-specific binding. Anti-(FLAG M2) antibody was added and the mixture incubated for 1 h at room temperature. This was followed by incubation with a second antibody fluorescein isothiocyanate conjugate for 2 h at room temperature. The sections were examined for epi-fluorescence using a Zeiss microscope with an excitation wavelength of 380–490 nm. Photomicrographs were recorded on Kodak T-max film.

Expression and purification of mutant MIP26

Mutant MIP26 cDNA was inserted into the *Msc*I–*Eco*RI sites of the pPOW expression vector. *E. coli* TOPP 2 strain was transformed with pPOW vector containing mutant MIP26 cDNA. Conditions of cell growth, induction of expression and purification of mutant protein were essentially as described for native MIP26.

RESULTS

Sequence analysis of native and mutant rat MIP26 cDNA

The complete rat MIP26 cDNA containing 783 bases (coding potential 261 amino acids, calculated molecular mass 26 kDa) was sequenced in order to identify and characterize the gap junction protein, MIP26, of the eye lens (Figure 4). It was identical with the sequence reported by Kent and Shiels (1990).

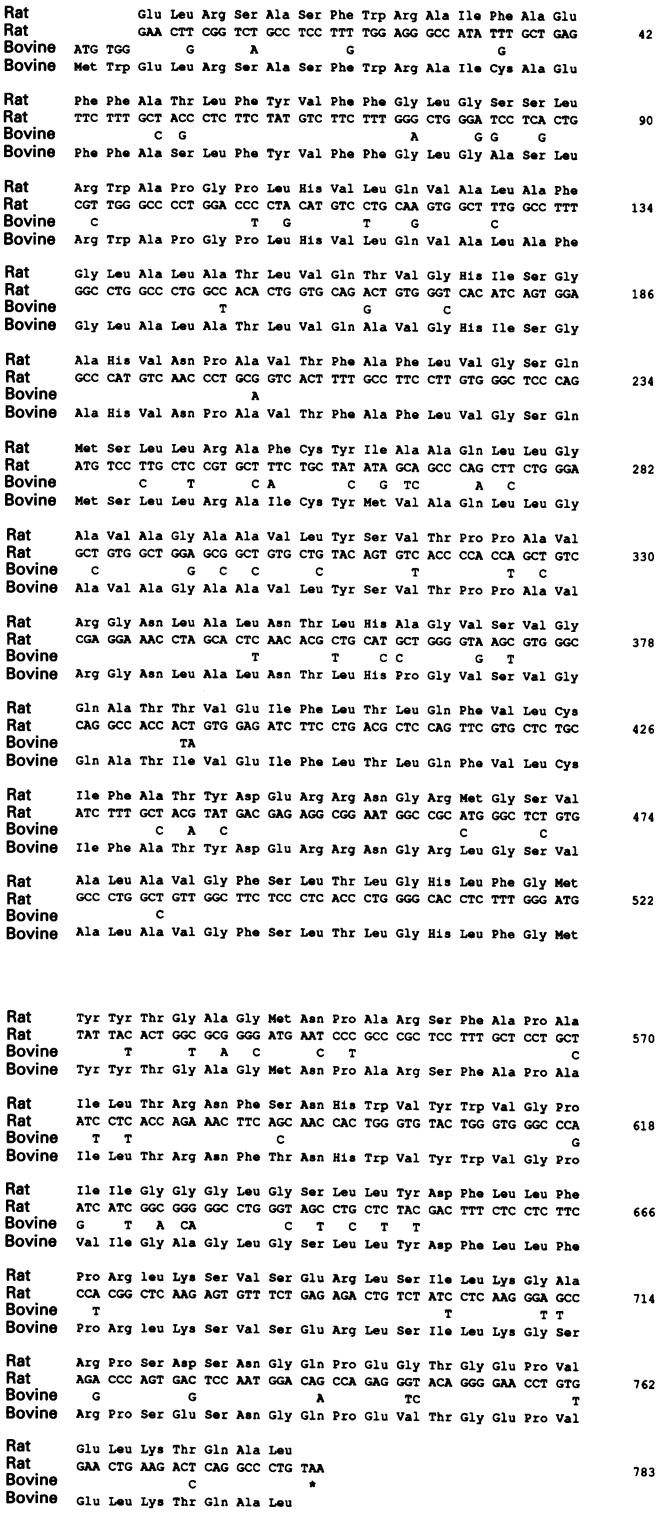

Figure 4 Comparison of nucleotide and amino acid sequences of rat MIP26 with those of bovine MIP26

The cDNA of rat MIP26 has an open reading frame of 783 bases (261 residues), whereas bovine MIP26 has 789 bases (263 residues).

It displayed 89% nucleotide identity (Gorin et al., 1984) and 95% identity at the amino acid level with bovine MIP26 (Figure 4).

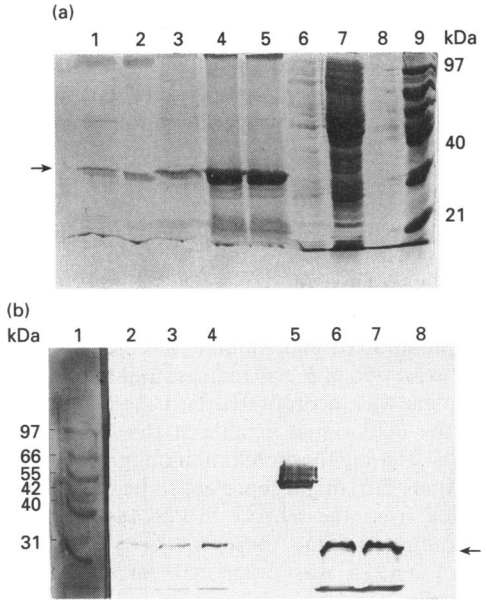

Figure 5 SDS/PAGE and Western-blot analysis of samples of MIP26 expressed in *E. coli*

(**a**) SDS/polyacrylamide gel: lanes 1–5, membrane fractions from *E. coli* after transformation with pPOW-MIP26 (the arrow indicates MIP26); lanes 6–8, membrane fractions from pPOW-transformed *E. coli* (negative control); lane 9, marker proteins. (**b**) Western blot. Proteins in unstained gels were transblotted on to nitrocellulose membrane and then detected with Anti-(FLAG M2) monoclonal antibody and anti-mouse IgG–horseradish peroxidase conjugate as described in the Materials and methods section. Lane 1, marker proteins; lanes 2–4, 6 and 7, fractions from the membrane; lane 8, negative control of the membrane fraction from *E. coli*; lane 5, positive control of bacterial alkaline phosphatase containing the FLAG peptide. The arrow indicates MIP26.

Rat MIP26 cDNA was amplified by PCR from cloned plasmid pUC18 using a pair of primers. Site-directed mutagenesis using overlap extension was carried out in two steps. The PCR products corresponding to native and mutant MIP26 cDNA were then ligated into the PCR cloning vector pGEM-T. Recombinants containing MIP26 were identified using *Stu*I and *Eco*RI. *Stu*I and *Eco*RI cleavage of transformants demonstrated that approximately 95% of white colonies contained recombinant plasmids. Several PCR clones were sequenced using PCR oligonucleotides to check the mutated region of MIP26 cDNA. All clones contained the mutated nucleotide G at position 730 and DNA sequences of mutant MIP26 showed that there were no unexpected mutations.

Expression of MIP26 in *E. coli* cells

The purified fragment of MIP26 cDNA was cloned into the *Msc*I and *Eco*RI multicloning site in the expression vector pPOW and transformed in *E. coli*. Transformants were screened for the presence of the MIP26 cDNA by using the restriction enzymes *Sal*I and *Bam*HI; transformants with inserts of the desired size (720 bp) in positive orientation were found in 50% of the colonies tested.

Recombinant clones were then sequenced using double-stranded sequencing and the forward and reverse sequencing primers that had identity with the vector.

The plasmid POW containing the secretion signal PelB allows secretion of MIP26 fusion protein into the cytoplasmic membrane of *E. coli*. When the protein has been exported through the membrane, the secretion signal is cleaved from the mature protein.

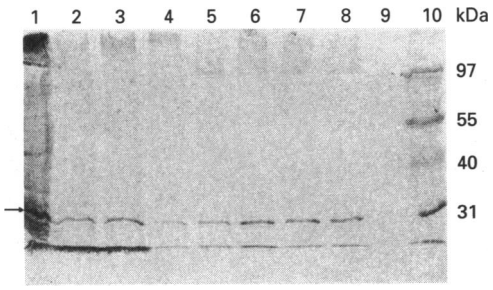

Figure 6 Purification of MIP26 from membrane fractions

Lane 1, octyl β-D-glucopyranoside-solubilized extract of *E. coli* membranes; lanes 2–8, MIP26 protein (→) eluted from the immunoaffinity column using glycine/HCl (pH 3.0); lane 9, eluted negative control of membrane fraction from *E. coli*; lane 10, marker proteins.

The MIP26 gene was designed to express a hydrophilic octapeptide (FLAG) fused to the C-terminus to aid antibody-mediated identification and purification. Optimum synthesis was achieved in *E. coli* TOPP 2 cells using fortified SB medium, growth conditions and heat induction as described in the Materials and methods section.

Cells were treated with EDTA/lysosyme to permeabilize the outer membrane. Such treatment causes release of the soluble periplasmic proteins, including any MIP26 protein resulting from cleavage of the fusion protein. Warming the MIP26 sample in loading buffer containing SDS for electrophoresis was found to result in aggregation of MIP26. For this reason, samples were solubilized at 4 °C before SDS/PAGE analysis. Analysis of fractions after sucrose-density-gradient centrifugation showed that MIP26 was confined to the membrane fraction; unlike the heterologous expression of γ_E-crystallin (Goode and Crabbe, 1994a) there was no evidence of its presence in inclusion bodies or other fractions. The cell fractions were then separated on SDS/PAGE mini-gels and stained with Coomassie Blue R-250 (Figure 5a). Immunoblot analysis of the eluted MIP26 revealed essentially a single band of the expected mobility, i.e. monomeric MIP26. Immunoblots using anti-(FLAG) monoclonal antibody show that MIP26 was present in plasma membranes isolated from *E. coli* (Figure 5b). These data demonstrate that the product was indeed MIP26, and that no extraneous proteins were present. SDS/PAGE analysis of plasma membranes isolated from *E. coli* demonstrated almost exclusively an MIP26 protein band with an apparent molecular mass of 26 kDa. Heterologously expressed MIP26 was purified to homogenity using an immunoaffinity purification system (Figure 6). The N-terminal sequence of the protein was determined: Glu-Leu-Arg-Ser-Ala-Ser-Phe-Trp. Estimation of yield by absorbance (Dunn, 1989) and by bicinchoninic acid assay (Pierce Inc.) (Smith et al., 1985) gave a value of 10 mg of MIP per litre of induced cell culture. The level of expression, based on total protein extracted from *E. coli* membranes, was estimated to be about 34% of total membrane protein.

To determine the location of MIP26 in the host cell *E. coli*, indirect immunofluorescence experiments were performed using Anti-FLAG antibody to detect FLAG peptide fused to either the N- or C-terminus of MIP26 after the removal of the cell wall.

None of the normal *E. coli* spheroplasts with control vectors showed any fluorescence with the anti-FLAG antibody. About 25% of *E. coli* spheroplasts with the pPOW vector containing

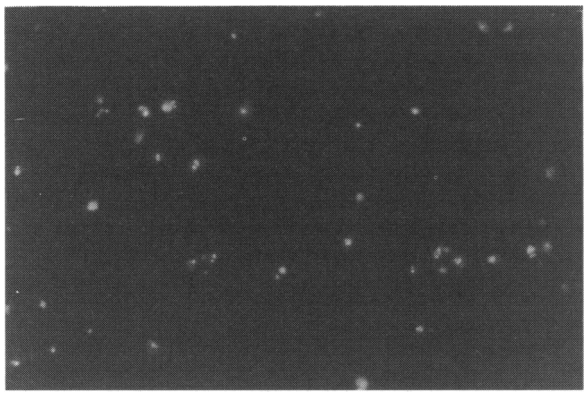

Figure 7 Immunofluorescence of N-terminal FLAG–MIP26 fusion protein in *E. coli* cells

Spheroplasts were probed with Anti-(FLAG M2) monoclonal antibody and second antibody–fluorescein isothiocyanate conjugate and then observed under the fluorescence microscope. Some fluorescent spheroplasts were visible when the FLAG peptide was fused to the N-terminus of MIP26 expressed in *E. coli* TOPP 2 cells but no spheroplast immunofluorescence was observed when the FLAG peptide was fused to the C-terminus of MIP26, indicating that the C-terminus of MIP26 was always located on the cytoplasmic side of the membrane after expression in *E. coli* cells.

MIP26 and FLAG marker peptide fused at the N-terminus of MIP26 showed immunofluorescence (Figure 7). We observed no spheroplast immunofluorescence when the FLAG peptide was fused to the C-terminus of MIP26, indicating that it is always located on the cytoplasmic side of the membrane after expression in *E. coli* cells. These data suggest that most of the heterologously expressed protein had been incorporated into the cytoplasmic membrane of *E. coli* in the correct orientation.

The mutant protein was also synthesized efficiently and located in the membrane of *E. coli*. Most of the purified mutant MIP26 ran as a single sharp band on the SDS/PAGE. The results of the mutagenesis experiments revealed no clear differences between the protein yield of native and mutant MIP26. No differences were detected either in the size or level of synthesis of the native and mutant proteins.

DISCUSSION

The sequence of the rat MIP26 reported here [the last 353 bases were sequenced and published by Shiels et al. (1988)] differs from the bovine sequence reported by Gorin et al. (1984) at 16 amino acid residues. This indicates a strong identity (95%) between the nucleotide sequences of rat and bovine MIP26. The cDNA-derived amino acid sequence of rat MIP26 showed that it is a highly hydrophobic protein.

Synthesis of large amounts of MIP26 cDNA fragment from the template to study functional analysis was facilitated by using PCR. Unfortunately, ligation of PCR products to the vector is a common problem because of low cloning efficiency. We found that direct ligation of PCR product to pGEM-T is more efficient than blunt-ended ligation of PCR product to pGEM-T in our experiments. The pGEM vector was cleaved with *Eco*RV and 3′-terminal thymidine was added to both ends. These single 3′-thymidine overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product to the vector.

We have developed a novel heterologous expression cell system using *E. coli* as a tool for the production of MIP26 in large quantities to enable study of its biosynthesis and function. Native and mutant cDNA for rat MIP was inserted into the expression vector pPOW containing the $\lambda P_R P_L$ promoters and the *cI857* gene encoding the temperature-sensitive repressor, which provide tight control over protein production. Optimum expression of MIP was found in *E. coli* host strain TOPP 2 in Superbroth (approx. 10 mg of MIP from 1 litre of induced cell culture). No differences were detected in the size or level of synthesis of the native and mutant proteins as assessed by SDS/PAGE.

FLAG peptide fused to the protein was found to be useful for immunological detection and purification of MIP26. The results of Western-immunoblot and immunofluorescence experiments using MIP26 expressed in *E. coli* indicate that the heterologously expressed protein was incorporated into the cytoplasmic membrane using the PelB signal peptide at the N-terminus of the fusion protein. The highly mobile immunopositive component detected in Figure 5(b) might represent some degradation of the FLAG peptide from the FLAG–MIP26 fusion protein. The spheroplast-immunostaining experiments showed that the C-terminus of MIP26 was orientated correctly towards the cytoplasmic side of the membrane in all cases. Immunofluorescence staining shown in Figure 7 indicates that in a minority (about 25%) of the spheroplasts the N-terminus of MIP26 was oriented in the opposite direction; this could have been due to the effect of the signal peptide on the insertion of the protein into the membrane. We have subsequently confirmed the orientation of the conserved NPA-box loop regions using specific antibodies kindly provided by Dr. Ana B. Chepelinsky (National Institutes of Health, Bethesda, MD, U.S.A.) (N. Dilsiz, A. B. Chepelinsky and M. J. C. Crabbe, unpublished work). MIP is closely related to the aquaporins (Chrispeels and Agre, 1994) and its role in the lens may be in regulating water and ion content; changes in protein-bound water may cause crystallin tertiary structural modifications (Goode and Crabbe, 1994a,b), and ultimately lens opacification.

In conclusion, this expression system should prove useful for the isolation and purification of MIP26 and for studying its role in the eye lens, as its role in ion channels can only be meaningfully defined and assayed in a reconstituted membrane system. Our site-directed mutation at Asn^{244} near the C-terminus should enable us to obtain a greater understanding of the age-dependent deamidation of MIP26 in the mammalian lens, and, ultimately, in cataractogenesis. Future experiments will involve examination of calmodulin-binding capacity of native, mutant and glycated (Shaw and Crabbe, 1994a,b) MIP26 in the presence of Ca^{2+}.

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