# Heterologous expression and topography of the main intrinsic protein (MIP) from rat lens

K. Dawn Drake[a], Diana Schuette[a], Ana B. Chepelinsky[b], M. James C. Crabbe[a,*]

[a] *Division of Cell and Molecular Biology, School of Animal and Microbial Sciences, The University of Reading, P.O. Box 228, Whiteknights, Reading, Berkshire RG6 6AJ, UK*
[b] *National Eye Institute, National Institutes of Health, Bethesda, MD, USA*

Received 11 December 2001; revised 27 December 2001; accepted 27 December 2001

First published online 23 January 2002

Edited by Gunnar von Heijne

**Abstract Wild type rat lens main intrinsic protein (MIP) and MIP mutated (F73I, F75L) to resemble the glycerol facilitator of *Escherichia coli* in the region of the NPA1 box were used to investigate the topology of MIP in the membrane of *Spodoptera frugiperda* (Sf21) cells using the baculovirus expression system and expression in mouse erythroid leukaemia cells (MEL C88). Differential fixation for staining was used, with paraformaldehyde for externally exposed antigenic sites, and acetone for both externally and internally exposed protein antigenic sites. Immunofluorescence using antibodies to synthetic MIP peptides showed that wild type MIP had a six transmembrane topography. The N- and C-termini were intracellular in both expression systems, and both NPA boxes were found to be extracellular. These results show that residues around the NPA1 box can influence the folding of the MIP in the membrane, and provide structural evidence for the poor water transport properties of MIP, as the NPA boxes lie outside the plane of the membrane.** 

*Key words:* Confocal microscopy; Cataract; Lens; Antibody

## 1. Introduction

Main intrinsic protein (MIP) is a 26 kDa protein found exclusively in the membranes of lens fibre cells. It was first isolated as the most abundant protein of the fibre cell membrane [1], and since then the cDNA has been cloned and sequenced [2]. Screening of cDNA libraries revealed a wide variety of organisms that possessed homologous membrane protein showing 30–60% sequence similarity with MIP, including the aquaporins (AQP1) and glycerol facilitators. Aquaporins can be further divided into the true aquaporins, which only transport water, and the aquaglyceroporins, which transport water and glycerol. The model by Gorin and colleagues proposed that the protein crossed the membrane six times with both the N- and C-termini being cytoplasmic. It was also proposed that the highly conserved NPA (Asn-Pro-Ala) regions were at either side of the membrane. In nearly all cases MIP consists of a two-fold repeat [3] which is thought to have arisen as a result of gene duplication. Each of the repeats contains a NPA box repeat (NPA1 or NPA2), which has been highly conserved throughout evolution, from prokaryotes to vertebrates [3–5]. The first repeat corresponds to the first exon of the *mip* gene whereas the second repeat corresponds to exons 2–4 providing further evidence that the *mip* gene has resulted from gene duplication. Regulation of *mip* expression is thought to involve the transcription factor Sp3 [6]. MIP has been found to undergo selective proteolysis during cataractogenesis and ageing. The product of proteolysis is a 22 kDa protein (MIP22) that has been cleaved at the C-terminus; its loss appears to affect the regulation of the protein [7,8]. The highest levels of MIP22 are found in the nucleus of the lens, which contains the oldest fibre cells [9]. There is also evidence that the N-terminus undergoes proteolytic cleavage at residues 2–8, 29, 35 and 37 and that truncation of the N- and C-termini was seen in human lenses as young as 7 years [10].

Expression of MIP in oocytes has been shown to increase the osmotic water permeability of the cells by approximately two-fold [11–13]. However, this is significantly lower than the 42-fold increase achieved by introduction of AQP1 in the 28 kDa homologous water channel of erythrocytes [14]. Comparison of single channel water permeabilities of AQP1 and MIP showed that MIP was again significantly lower than that of AQP1 [14,15], showing that MIP was a poor transporter of water and there may be some other transport function allocated to this protein. It has been suggested that MIP may be involved in the metabolism of glycerol acting as an activator of glycerol kinase [16] by allosteric interaction with the enzyme in a similar fashion to GlpF. Other members of the aquaporin family are also known to be involved in the metabolism of glycerol – GlpF [17] and FPS1, a glycerol facilitator of yeast [18]. The evidence of a current found in planar lipid bilayers incorporating MIP has been found to be substantially greater than that measured in the normal lens [19,20] and although the results in the planar bilayers have been reproducible the relevance to normal lens function still remains uncertain.

The work presented here elucidates the topography of MIP using heterologous expression and immunofluorescence mapping, with differential fixation for staining (paraformaldehyde for externally exposed antigenic sites, and acetone for both externally and internally exposed protein antigenic sites). Our work shows that small sequence changes can result in large protein folding changes, and provides evidence underlying the poor water transport properties of the protein.

*Corresponding author. Fax: (44)-189-318894.
*E-mail address:* m.j.c.crabbe@rdg.ac.uk (M.J.C. Crabbe).

0014-5793/02/$22.00 
PII: S0014-5793(02)02256-1

## 2. Materials and methods

### 2.1. Sub-cloning of wild type MIP into pNV

Wild type *mip* had previously been sub-cloned into the prokaryotic expression vector pET28a [21]. This construct was used as a template for the amplification of *mip* by the polymerase chain reaction (PCR) for sub-cloning into the unique *Eco*RI site in the mammalian expression vector pNV [22].

Oligonucleotide primers containing the endonuclease site *Eco*RI were obtained from Genosys (UK). Purification of the PCR product was carried out using a Promega Wizard PCR DNA purification kit in accordance with the manufacturer's instructions.

### 2.2. Ligation of wild type mip into pNV

Wild type *mip* was ligated into the expression vector pNV, which had previously been cut with the restriction enzyme *Eco*RI using the following conditions: 80 ng pNV, 320 ng wild type *mip* insert, 3 U Promega T4 ligase, 2 μl of 10× Promega T4 ligase buffer. The reaction was carried out in a final volume of 20 μl at room temperature for 4 h.

### 2.3. Preparation of DNA for transfection of mouse erythroleukaemia (MEL C88) cells

Prior to transfection, recombinant plasmid containing wild type *mip* was digested using *Pvu*I, cutting within the $amp^r$ gene; this produced an enhanced uptake of foreign DNA by MEL C88 cells.

Mixtures contained: 50 μg recombinant DNA, 50 U *Pvu*I (Gibco BRL), 30 μl of 2× proprietary buffer, made up to a final volume of 150 μl with nano pure water. The reaction mixture was incubated at 37°C for 2 h.

### 2.4. Mammalian tissue culture procedures

MEL C88 cells were cultured in growth medium containing Dulbecco's modified Eagle's medium (DMEM), 10% foetal calf serum (FCS), L-glutamine (200 mM) and antibiotics penicillin (2%) and streptomycin (2%). Cells were seeded at $1\times10^4$ in 5 ml of growth medium and incubated in vented tissue culture flasks (Iwaki) at 37°C with 5% $CO_2$. Cells were passaged every 4 days.

### 2.5. Transfection of MEL cells

Both cells and DNA were gently mixed and transferred to an electroshock cuvette (Bio-Rad gene pulser cuvette 0.4 cm electrode), and shocked at 250 V, 960 μF using a Bio-Rad gene pulser. Cells were left to recover at room temperature for 10 min and then diluted to a concentration of $10^4$ and $10^5$ cells/ml with growth medium. Cells (1 ml) were placed into each well of a 12 well plate and incubated at 37°C for 24 h, after which 1 ml of selective medium (growth medium+geneticin 1.6 mg/ml, giving a final concentration in each well of 0.8 mg/ml geneticin) was added to each well. Cells were incubated for a further 10 days under the same conditions, after which clones were harvested. Each clone was placed in 2 ml of growth medium in a 25 ml tissue culture flask and incubated in an upright position. After 24 h 3 ml of growth medium was added and the flask incubated horizontally. Recombinant cells were passage after 4 days with the addition of 0.4 mg/ml geneticin G418 for plasmid maintenance. For induction, cells were resuspended in induction medium (growth medium+2% dimethyl sulphoxide (DMSO)) and incubated at 37°C, 5% $CO_2$ for 5 days. Induction was monitored by the production of human β-globin (red).

### 2.6. Insect tissue culture procedures: routine passage and storage conditions

The cell line used was derived from *Spodoptera frugiperda* (Sf21) ovarian tissue (fall army worm). Cells were seeded at a density of $1\times10^6$ in 15 ml of growth medium (TC100 insect tissue culture medium (Gibco BRL)+10% FCS, 2% penicillin/streptomycin) in 75 $cm^2$ plastic tissue culture flasks. Flasks were incubated at 25°C until cells formed a confluent monolayer.

### 2.7. Co-transfection using lipofectin

All co-transfections were performed in duplicate. Cells were seeded at a density of $1\times10^6$ in 35 mm plastic petri dishes and allowed to grow overnight at 25°C. Triple cut linearised viral DNA (150 ng) (Bac Vector 2000, Novagen) and transfer vector (750 ng) were mixed with an equal volume of diluted lipofectin (2:1) and left to incubate at room temperature for 15 min until a liposome–DNA complex had formed. Cells were washed twice with serum-free medium and 1 ml of serum-free medium and DNA liposome complex added. Cells were incubated overnight at 28°C, after which a further 1 ml of growth medium containing 10% FCS was added and the cells incubated for a further 48 h. Recombinant virus was harvested.

### 2.8. Plaque purification of recombinant viruses

All assays were performed in duplicate. Cells were seeded at a density of $1\times10^6$ in 35 mm petri dishes and grown overnight at 25°C. Recombinant virus was diluted in growth medium (serum-free) at the following concentrations: undiluted, $10^{-1}$, $10^{-2}$, $10^{-3}$. Medium was removed from the cells and 100 μl of the appropriate dilution added, unused virus was stored at 4°C. Infected cells were incubated for 1 h at room temperature (rocking to ensure virus covered all cells). Following incubation the viral inoculum was removed and the cells were covered with 2 ml low gelling temperature agarose (Sigma type VII)/TC100 growth medium (1 ml of each). A further 1 ml of liquid overlay (growth medium) was added to the dishes and cells incubated for 4 days at 28°C, after which the cells were stained for the presence of plaques.

### 2.9. Amplification of wild type (AcMIPN) and mutant (AcMIPVK3) virus

A spinner flask (Techne, Cambridge, UK) was seeded with $1\times10^5$ cells/ml in 50 ml of growth medium and incubated (stirring 70 rpm) until the cell density had reached $5\times10^5$ cells/ml. Cells were infected with 5 ml of the seed virus stock and incubated at 25°C for 5 days. Virus was harvested by centrifugation of the cell suspension and the decanted supernatant aliquoted into 10 ml samples and stored at 4°C or −70°C for long-term storage. The final working stock titre of AcMIPVK3 was $1\times10^8$ pfu (plaque forming units) and AcMIPN $1.8\times10^7$ pfu.

### 2.10. Expression of mutant and wild type MIP in Sf21 cells

Cells were seeded at a density of $5\times10^5$ cells/ml in a 25 $cm^2$ tissue

Table 1
Sequence of synthetic peptides to which primary antibodies were raised

| MIP peptide sequence | Amino acid position | Antibody name |
|---|---|---|
| ELRSASFWRA | 3–12 | SAS |
| SLRWAPGPLHVLQ | 31–43 | RWA |
| QTVGHISGAH | 57–66 | QTV |
| SGAHVNPAVT | 63–72 | HNPA1 |
| LVGSQMSLLR | 76–85 | LVG |
| RAFCYIAAQL | 85–94 | RAF |
| AVRGNLALNT | 111–120 | AVR |
| HAGVSVGQATTVE | 122–134 | TTV |
| EIFLTLQFVLCIFATYDERRN | 134–154 | EIF |
| DERRNGRMGSVAL | 150–162 | DER |
| SLTLGHLFGMY | 167–177 | GM |
| TGAGMNPARS | 179–188 | NPAR2 |
| GSLLYDFLLFPRLKS | 215–229 | LYD |
| SNGQPEGTGEPVEL | 245–258 | VEL |

culture flask and incubated overnight at 25°C. Cells were infected at a multiplicity of infection (m.o.i.) of 10 with either AcMIPN or AcMIPVK3 and incubated for 1 h at 25°C (rocking). Virus was removed and 5 ml of growth medium+5% FCS added, cells were incubated for 48 h at 28°C. Cells were harvested, pelleted by centrifugation and washed in twice in phosphate buffered saline (PBS) to remove residual medium and finally resuspended in 100 μl of PBS. 10 μl of cell suspension was lysed with 10 μl of loading buffer prior to SDS–PAGE and Western blot analysis.

*2.11. MIP peptide antibodies*

Peptides corresponding to MIP amino acid sequences (indicated in Table 1) were synthesised and injected into rabbits to obtain polyclonal antibodies. Antiserum was purified by affinity chromatography using the same peptide the antibody was raised against.

*2.12. SDS–PAGE and Western blot analysis*

These, together with other standard molecular biology procedures, were performed as described previously [21,23–26].

*2.13. Immunofluorescence (MEL cells)*

Immunofluorescence was used to determine the topology of MIP in the membrane of MEL cells transfected with pRATMIP or pRATMIPVK3. Cells transfected with either pRATMIP, pRATMIPVK3 or pNV (control cells) were induced and incubated for 5 days at 37°C with 5% $CO_2$. Harvested cells were centrifuged (20 500×*g*/5 min) and washed in PBS×2, prior to fixing in 3% paraformaldehyde for 20 min at room temperature. Fixed cells were washed 3×5 min in PBS and incubated with one of the primary antibodies (1:100 dilution) in PBS+1% FCS for 1 h at 25°C (by rocking). Primary antibodies were raised in rabbits to synthetic peptides of specific regions of MIP (Tables 1 and 2). Following incubation, cells were washed in PBS (3×5 min) before incubating with secondary antibody, goat anti-rabbit FITC conjugated IgG (obtained from Sigma) (1:500 dilution) in PBS buffer with 1% FCS for 1 h in the dark at 25°C. Before mounting cells were again washed in PBS and resuspended in 20 μl PBS. Resuspended cells (10 μl) were smeared onto slides and allowed to air dry in the dark. Citifluor (Agar) was added to mounted cells to preserve fluorescence. Coverslips were placed over the cells and sealed with nail varnish. Slides of MELratmip cells were examined by fluorescence microscopy (×100 lens). MELratmipvk3 cells were examined using confocal microscopy, using a Leica TCSNT microscope and Leica TCSNT software.

*2.14. Immunofluorescence (Sf21 cells)*

Immunofluorescence was used to determine the topology of wild type and mutant MIP in the membrane of insect cells (Sf21) infected with AcMIPN or AcMIPVK3. Sf21 cells were seeded at a density of $2\times10^6$ cells/ml onto sterile glass coverslips in 35 mm petri dishes and incubated at 25°C for 1 h to ensure cells had adhered to coverslips. Cells were infected at a m.o.i. of 5 with either AcMIPN, AcMIPVK3 or wild type virus (AcMNPV) and then incubated at 25°C for 1 h (rocking). Virus was then removed and replaced by 2 ml of medium (TC100+5% FCS) after which dishes were incubated for 24 h at 28°C.

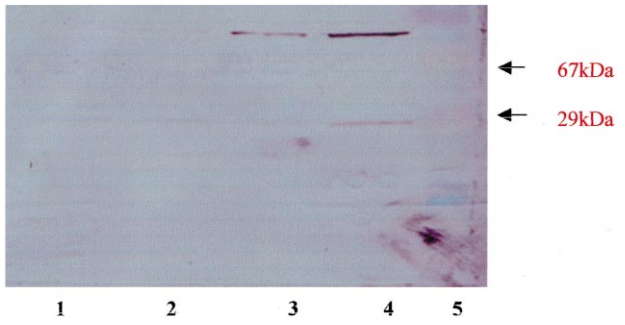



Fig. 1. Western blot of expression of wild MIP in MEL cells transfected with rat MIP. Lane 1 was loaded with lysed MEL cells, lanes 2–4 were loaded with lysed MELratmip cells expressing MIP. Lane 5 contains molecular weight markers.

Following incubation, medium was removed and the infected cells washed with PBS (3×5 min). Cells were fixed with 3% paraformaldehyde for 10 min (to stain externally exposed protein antigenic sites) or ice-cold acetone for 3 min (to stain both externally and internally exposed protein antigenic sites), in order to distinguish between external and internal localisation. Fixed cells were washed with PBS (3×5 min) prior to incubation with one of the primary antibodies listed in Table 1. The primary antibodies were diluted in PBS+2% FCS (1:100) and incubated with the infected cells for 1 h. Cells were again washed in PBS (3×5 min) before incubating with secondary antibody, goat anti-rabbit Texas red conjugated IgG (1:1000) in PBS+2% FCS for 1 h in the dark. Texas red was the fluorochrome of choice due to Sf21 cells auto fluorescing at the wavelength of light used to excite FITC (518 nm). Before mounting cells were washed in PBS (3×5 min), coverslips removed from the petri dishes and one drop of Vectashield added to preserve fluorescence. A microscope slide was then placed over the coverslip and sealed with nail varnish. Slides were examined by confocal microscopy, using a Leica TCS NT microscope and Leica TCSNT software.

## 3. Results

Production of recombinant protein in the LCR/MEL expression system was achieved by the addition of 2% DMSO to the growth medium. Expression from the β-globin promoter resulted in production of the recombinant protein and human β-globin. Production of human β-globin allowed induction to be monitored by the change in cell colour to red. Induced cells were grown for 5 days and then harvested for analysis by Western blotting. Fig. 1 shows a Western blot of the recombinant clone containing the plasmid pRATMIP and

Table 2
MIP expressing MEL cells fluorescence with MIP antibodies

| Antibody name | Antibody to MIP amino acids | Fluorescence | | Predicted orientation |
|---|---|---|---|---|
| | | Formalin fixed | Acetone permeabilised | |
| SAS | 3–12 | − | + | internal |
| RWA | 31–43 | − | + | internal |
| QTV | 57–66 | + | + | external |
| HNPA1 | 63–72 | + | + | external |
| LVG | 76–85 | + | + | external |
| RAF | 85–94 | − | − | membrane |
| AVR | 111–120 | + | + | external |
| TTV | 122–134 | + | + | external |
| EIF | 134–154 | + | + | external |
| DER | 150–162 | − | + | internal |
| GM | 167–177 | − | − | membrane |
| NPAR2 | 179–188 | + | + | external |
| LYD | 215–229 | − | + | internal |
| VEL | 245–258 | − | + | internal |

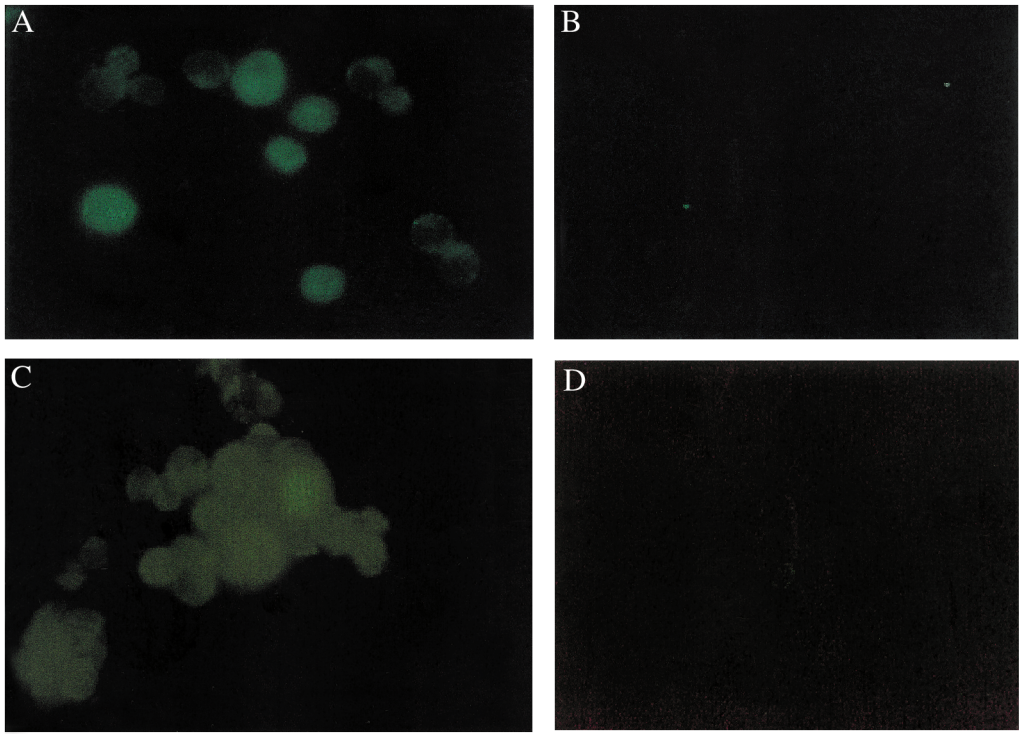



Fig. 2. Typical immunofluorescence photographs. A,B: External view of MEL cells incubated with antibody LVG. A shows MELratmip cells fluorescing, suggesting that this region is extracellular. B shows MELnv control cells. C,D: Internal view of MEL cell lines incubated with antibody LYD. C shows MELratmip cells fluorescing, suggesting that this region of MIP is situated on the cytoplasmic side of the cell membrane. D shows MELnv control cells.

showed that MIP was being expressed. Of the two bands corresponding to MIP, the lower band at 28 kDa is the correct size for monomeric MIP; the higher band may be an undissociated dimer or a monomer that has undergone post-translational modification. Expression of the mutant protein gave similar results. Immunofluorescence was used to establish the incorporation and topology of both wild type and mutant MIP in the membrane of MEL cells. Both control and test cells were labelled with one of the antibodies listed in Table 1.

Initially immunofluorescence microscopy was used to visualise the antibody labelled MELratmip and MELnv cells. Fig. 2 shows a typical immunofluorescence photomicrograph. The increased intensity of fluorescence in MIP expressing cells compared with that of control cells suggested that the NPA1 box was accessible externally. A topographical map is shown in Fig. 3A, while Table 2 presents the results for the individual antibodies.

Mutating the two residues (F73I, F75L) around the NPA1 box resulted in changes to the topology of the protein in the membrane (Fig. 3B). These changes were not just restricted to the area around the NPA1 box but also affected the folding of the protein downstream. As with the wild type protein the mutant appears to have six transmembrane regions and the N-terminus was found to be intracellular. The C-terminus, i.e. the region corresponding to VEL, no longer reacted with the antibody. This could be a result of the cleavage noted on Western blotting or the protein has refolded in such a way as to render the binding site inaccessible. However, the region of residue 214–228 to which the antibody LYD was raised did react intracellularly suggesting that the C-terminus was still intracellular as in the wild type protein. The NPA2 box also appears to be extracellular as in the wild type protein. Another major difference between wild type and mutant protein is the extracellular location of the region 149–161 (DER) which was only found intracellularly in the wild type protein.

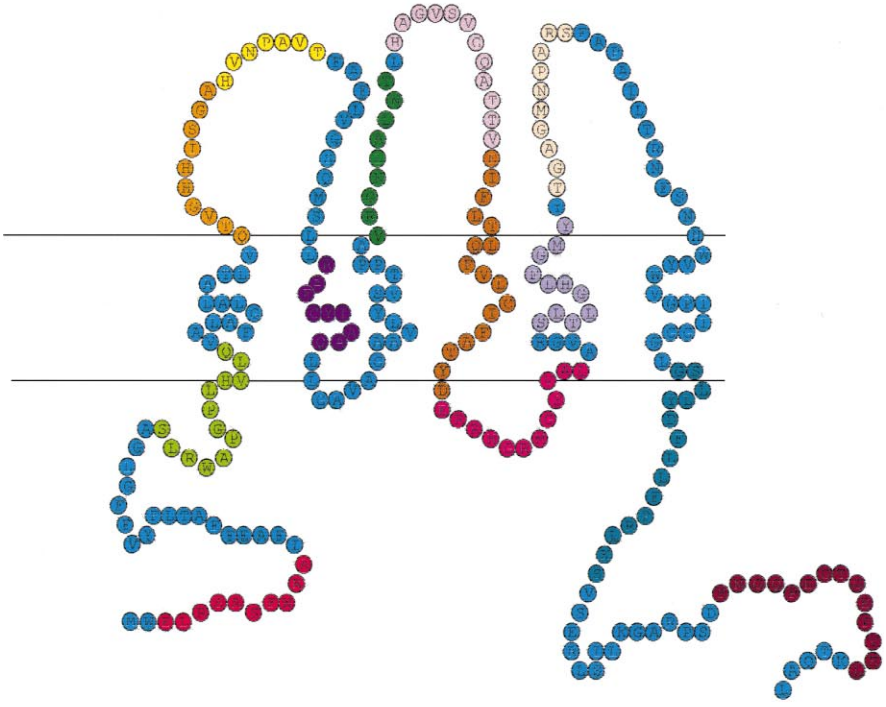
Sf21 cells were seeded at a density of $5\times10^5$ cells/ml in 25 $cm^2$ flasks prior to inoculation with either AcMIPN, AcMIPNK3 or AcMNPV virus at a m.o.i. of 10. Harvested cells were lysed and analysed by SDS–PAGE and Western blotting, the result of which can be seen in Fig. 4. There appears to be no difference in levels of expression between the mutant and wild type protein and no cross-reaction between antibody and the wild type virus (control). The molecular weight of the both proteins appears to be higher than would be expected (28 kDa). This was either a result of incomplete denaturation on SDS–PAGE analysis and the quaternary structure of the protein was not disrupted or there was a high level of post-translational modification. Swamy-Mruthinti and Schey [27] noted an increase in molecular weight of MIP when expressed

→

Fig. 3. A: Diagram to show the relative positions of the immunoreactive regions of wild type rat MIP in MEL cell membranes. The blue coloured circles are regions of the protein to which no antibody has been raised. Other regions can be identified by the colour of the circles: red = SAS, green = RWA, orange = QTV, yellow = HNPA1, pale blue = LVG, purple = RAF, dark green = AVR, pink = TTV, coffee = EIF, dark pink = DER, lilac = GM, peach = NPAR2, turquoise = LYD, dark brown = VEL. B: Diagram to show the relative positions of the immunoreactive regions of mutant rat MIP in MEL cell membranes. Colour coding as in A.

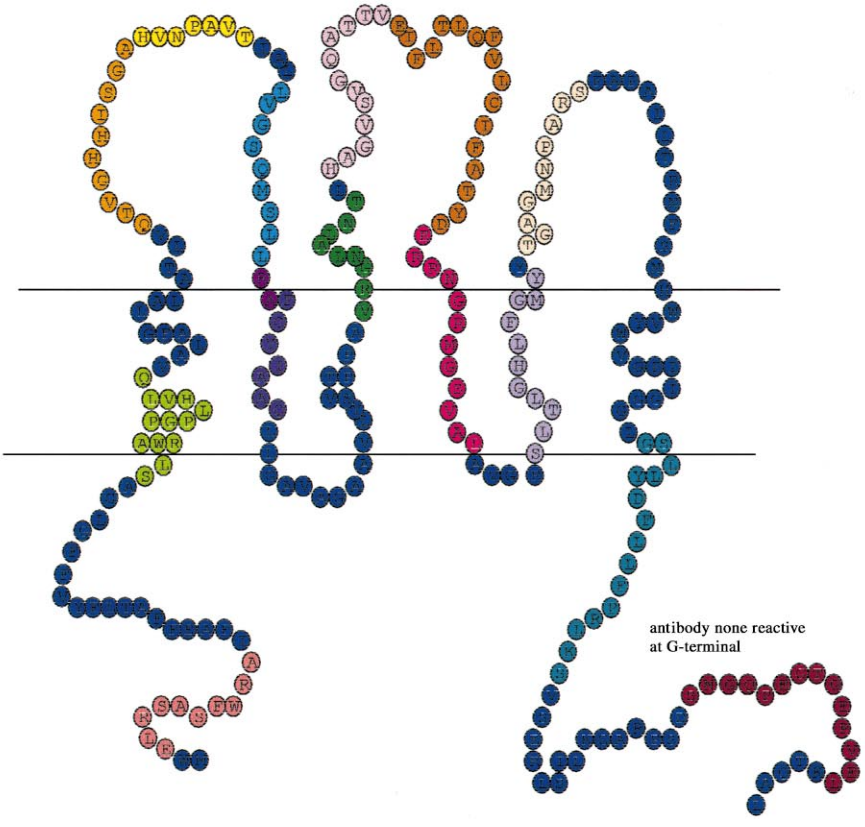
A

Topology of Native MIP in the membrane of Mouse Erythroid Leukaemia Cells



B

Topology of Mutant (F72I,F74L) MIP in the membrane of Mouse Erythroid Leukaemia Cells



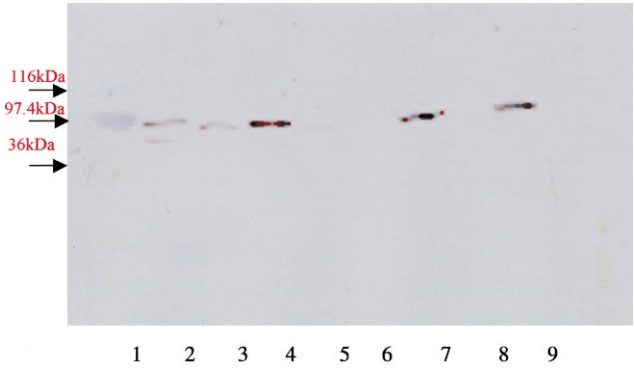



Fig. 4. Western blot of wild type and mutant MIP using the viral expression vectors AcMIPN and AcMIPVK3. Lane 1: wide range coloured molecular weight markers. Lanes 2–5, 7: mutant MIP. Lane 9: wild type MIP. Lane 8: cells infected with wild type virus. Lane 6 is empty.

in Sf9 cells using the baculovirus system; however, the increase in molecular weight was only 4 kDa not the much higher molecular weight seen here, which probably represents retention of the tetrameric structure of the protein. A less intense band of lower molecular weight can be seen in all lanes containing cells infected with recombinant virus. This is likely to be a product of proteolytic cleavage. The most likely candidate for this would be the C-terminus, which is cleaved in lenses during maturation.

Cells were incubated for 24 h rather than 48 due to increased friability of the cells. The longer period of infection led to an increase in cell lysis and therefore erroneous results. Following incubation both control and test cells were labelled with one of the antibodies listed in Table 1.

Immunofluorescence confirmed that MIP had been incorporated into the membrane of Sf21 cells and that there was a high degree of similarity with the predicted model of the structure of MIP [2]. The results suggest a six transmembrane spanning structure with the N- and C-termini being intracellular: this was consistent with the predicted model and the 2D crystal structure [28]. The effect of mutating the two aromatic residues to aliphatic ones (F73I, F75L) appears to have had no significant effect on the topology of MIP expressed in insect cells as typified in the confocal images in Fig. 5. Topological results for both wild type and mutant proteins are shown in Fig. 6A,B.

## 4. Discussion

Froger and colleagues [29] compared the sequences of members of the MIP family and showed that in glycerol facilitators the second extracellular loop is larger than the second extracellular loop of the aquaporins. From the results obtained here it would appear that the mutation of the two residues (F73 and F75) to those found in a glycerol facilitator have changed the structure of the protein to resemble that of a glycerol facilitator in the region of the second extracellular loop [30].

The hourglass model proposed by Agre and found in the crystal structure of AQP1 [31–34] proposes that the NPA boxes are on opposite sides of the cell membrane and that these two regions form a water channel by protruding into the membrane. Our results, using differential fixation for staining, show that as the NPA boxes are extracellular, they cannot form a water channel; this can explain the poor water transport properties of rat lens MIP. The C-terminus domain (from the second extracellular loop onwards) of our model is consistent with work published by Schey and colleagues for MIP [10] although there are some differences in the N-terminal region; these differences may be due to the heterologous expression system used and the possible differences in the lipid composition of the membranes.

*Acknowledgements:* We thank the British Diabetic Association, the University of Reading Research and Endowment Trust Fund and the Institute of Food Research for funds, and Dr. O. de Peyer for helpful conversations.

## References

[1] Broekhuyse, R.M., Kuhlmann, E.D. and Stols, A.L. (1976) Exp. Eye Res. 23, 365–371.
[2] Gorin, M.B., Yancey, S.B., Cline, J., Revel, J.P. and Horwitz, J. (1984) Cell 39, 49–54.
[3] Chepelinsky, A.B. (1994) in: Handbook of Membrane Channels (Peracchia, C., Ed.), Academic Press, New York.
[4] Pao, G.M., Wu, L.F., Johnson, K.D., Hofte, H., Chrispeels, M.J., Sweet, G., Sandal, N.N. and Saier, M.H. (1991) Mol. Microbiol. 153, 171–180.
[5] Harding, J.J. (1991) Cataract: Biochemistry, Epidemiology and Pharmacology, Chapman and Hall, London.
[6] Kim, S., Ge, H., Ohtaka-Maruyama, C. and Chepelinsky, A.B. (1999) Mol. Vis. 5, 12–17.
[7] Peracchia, C., Girsch, S.J., Bernardini, G. and Peracchia, L.L. (1985) Curr. Eye Res. 4, 1155–1169.

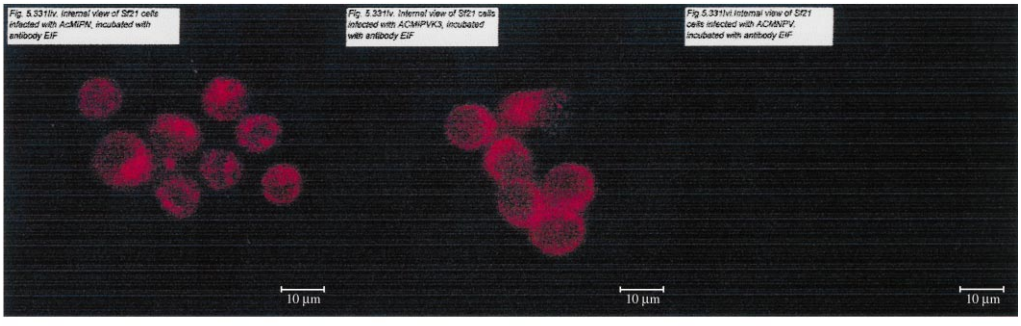



Fig. 5. Typical confocal micrographs of mip is Sf21 cells. A: Internal view of Sf21 cells infected with AcMIPN, incubated with antibody EIF. B: Internal view of Sf21 cells infected with AcMIPVK3, incubated with antibody EIF. C: Internal view of Sf21 cells infected with AcMNPV, incubated with antibody EIF.

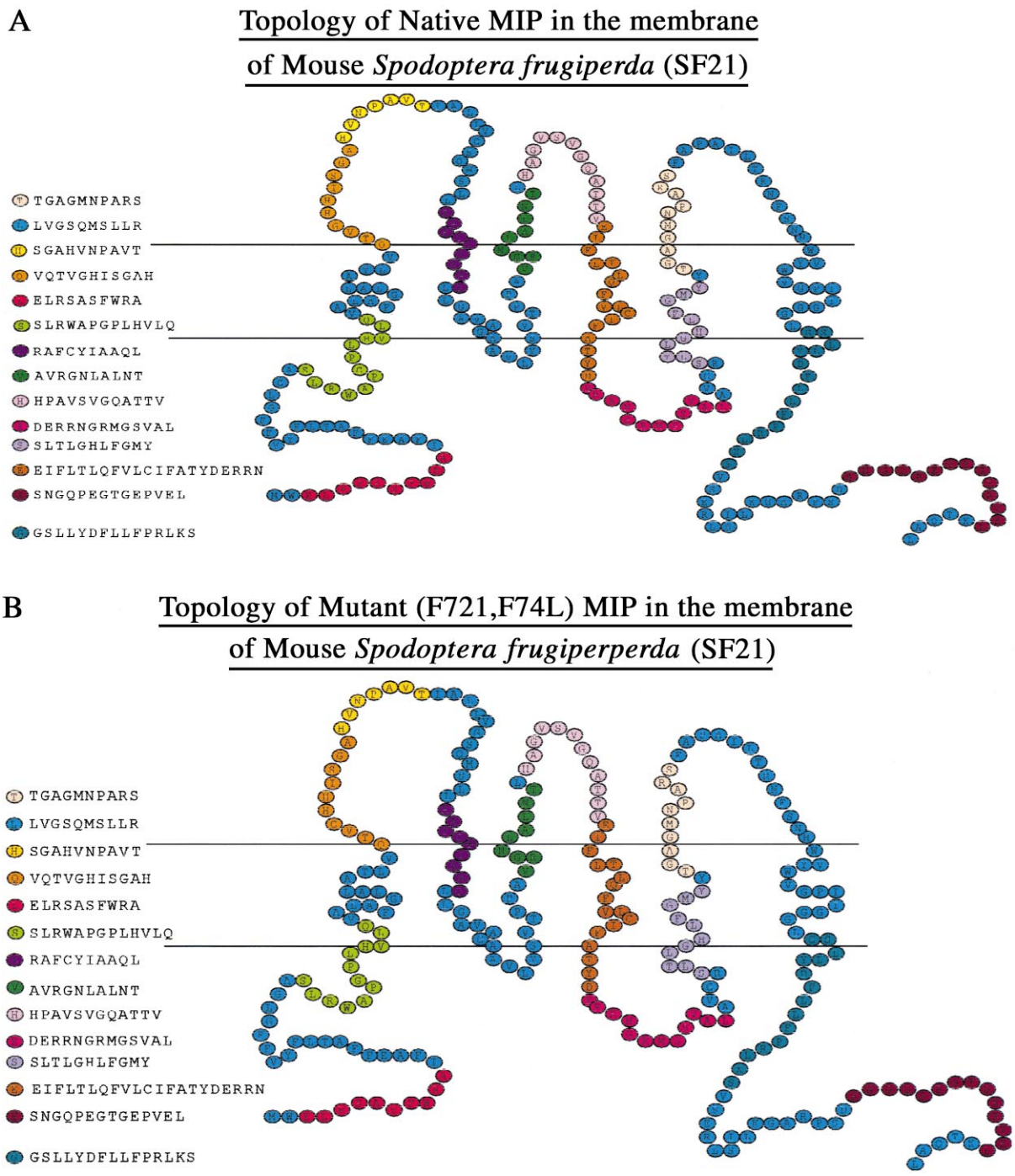



Fig. 6. A: Topological diagram of the insertion of wild type MIP in the membrane of Sf21 cells. Blue circles are regions to which no antibodies have been raised. B: Topological diagram of the insertion of mutant MIP into the membrane of sf21 cells. Blue circles are regions to which no antibodies have been raised.

[8] Girsch, S.J. and Peracchia, C. (1991) Curr. Eye Res. 10, 839–849.
[9] Takemoto, L.J., Hansen, J.S. and Horwitz, J. (1985) Exp. Eye Res. 41, 415–422.
[10] Schey, K.L., Little, M., Fowler, J.G. and Crouch, R.K. (2000) Invest. Ophthal. Vis. Sci. 41, 175–182.
[11] Chandy, G., Kreman, M., Laidlow, D.C., Zampighi, G.A. and Hall, J.E. (1995) Biophys. J. 68, A35.
[12] Mulders, S.M., Preston, G.M., Deen, P.M.T., Guggino, W.B., van Os, C.H. and Agre, P. (1995) J. Biol. Chem. 270, 9010–9016.
[13] Kushmerik, C., Rice, S.J., Baldo, G.J., Haspel, H.C. and Mathias, R.T. (1995) Exp. Eye Res. 61, 362–531.
[14] Chandy, G., Zampighi, G.A., Kreman, M. and Hall, J.E. (1997) J. Membr. Biol. 159, 29–39.
[15] Yang, B. and Verkman, A.S. (1997) J. Biol. Chem. 272, 16140–16146.
[16] Kushmerik, C., Varadaraj, K. and Mathias, R.T. (1998) J. Membr. Biol. 161, 9–19.
[17] Weissenborn, D.L., Wittekindt, N. and Larson, T.J. (1992) J. Biol. Chem. 267, 6122–6131.
[18] Luyten, K., Albertyn, J., Skibbe, W.F., Prior, B.A., Ramos, J., Thevelein, J.M. and Hohman, S. (1995) EMBO J. 14, 1360–1371.
[19] Mathias, R.T., Riquelme, G. and Rae, J.L. (1991) J. Gen. Physiol. 98, 1085–1103.

[20] Mathias, R.T., Rae, J.L. and Eisenberg, R.S. (1979) Biophys. J. 25, 181–201.
[21] Dilsiz, N. and Crabbe, M.J.C. (1995) Biochem. J. 305, 753–759.
[22] Needham, M., Gooding, C., Hudson, K., Grosveld, F. and Hollis, M. (1992) Nucleic Acids Res. 20, 997–1003.
[23] Derham, B.K., van Boekel, M.A.M., Muchowski, P.J., Clark, J.I., Horwitz, J., Hepburne-Scott, H.W., Crabbe, M.J.C. and Harding, J.J. (2001) Eur. J. Biochem. 268, 713–721.
[24] King, L.A. and Posse, R.D. (1992) The Baculovirus Expression System; A Laboratory Guide, 1st edn., Chapman and Hall, London.
[25] Wiesmann, K.E.H., Coop, A., Goode, D., Hepburne-Scott, H. and Crabbe, M.J.C. (1998) FEBS Lett. 438, 25–31.
[26] Plater, M.L., Goode, D. and Crabbe, M.J.C. (1996) J. Biol. Chem. 271, 28558–28566.
[27] Swamy-Mruthinti, S. and Schey, K.L. (1997) Curr. Eye Res. 16, 936–941.
[28] Hasler, L., Walz, T., Tittmann, P., Gross, H., Kistler, J. and Engel, A. (1998) J. Mol. Biol. 279, 855–864.
[29] Froger, A., Tallur, B., Thomas, D. and Delamarche, C. (1998) Protein Sci. 7, 1458–1468.
[30] Nollert, P., Harries, W.E.C., Fu, D., Miercke, L.J.W. and Stroudet, R.M. (2001) FEBS Lett. 504, 112–117.
[31] Agre, P., Lee, M.D., Devidas, S., Guggino, W.B., Sasaki, S., Uchida, S., Kuwahara, M., Fushimi, K., Marumo, F., Verkan, A.S., Yang, B., Deen, P.M.T., Mulders, S.M., Kansen, S.M., van Os, C.H., Fischbarg, J., Kuang, K., Li, J., Iserovich, P., Wen, Q., Patil, R.V., Han, Z. and Wax, M.B. (1997) Science 275, 1490–1492.
[32] Mathia, J.C. and Agre, P. (1999) Biochemistry 38, 923–928.
[33] Agre, P., Mathai, J.C., Smith, B.L. and Preston, G.M. (1999) Methods Enzymol. 294, 550–572.
[34] Walz, T., Hiral, T., Murata, K., Heymann, J.B., Mitsuoka, K., Fujiyoshi, Y., Smith, B.L., Agre, P. and Engel, A. (1997) Nature 387, 624–626.