Functional expression of Multidrug Resistance Protein 4 MRP4/ABCC4

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Abstract

To study the function and structure of membrane proteins high quantities of pure and stable protein are needed. One of the first hurdles in accomplishing this is expression of the membrane protein at high levels and in a functional state. Membrane proteins are naturally expressed at low levels so finding a suitable host for over expression is imperative. Multidrug resistance protein 4 (MRP4) or ATP-binding cassette subfamily C member 4 (ABCC4) is a multi-transmembrane protein which is able to transport a range of organic anionic compounds (both endogenous and xenobiotic) out of the cell. This versatile transporter has been linked with extracellular signalling pathways and cellular protection, along with conferring drug resistance in cancers. Here we report the use of MRP4 as a case study to be expressed in three different expression systems: mammalian, insect and yeast cells to gain the highest yield possible. Interestingly, using the baculovirus expression system with *Sf*9 insect cells produced the highest protein yields. Vesicular transport assays were used to confirm MRP4 expressed in *Sf*9 was functional using a fluorescent cAMP analogue (fluo-cAMP) instead of the traditional radiolabelled substrates. MRP4 transported fluo-cAMP in an ATP dependent manner. Specificity of functional expression of MRP4 was validated by the use of non-hydrolysable ATP analogues and MRP4 inhibitor MK571. Functionally expressed MRP4 in *Sf*9 cells can now be used in downstream processes such as solubilisation and purification in order to better understand its function and structure.

Introduction

One of the limitations of membrane protein structural biology is expressing the membrane protein of interest. The challenge lies not only in expressing the protein of interest but also expressing it to a high level in its native conformation(s). Most membrane proteins are naturally expressed in low levels, and so obtaining sufficient amounts of the native membrane proteins to conduct functional and structural studies requires large amounts of resources and is really only realistic for proteins which are naturally abundant in certain cell types such as rhodopsin in the retina[1].

To overcome the problem of low natural expression, recombinant overexpression can be performed, increasing the yield per cell[2]. Another advantage of recombinant expression is the ability to easily add tags to enable efficient separation of the target protein from the other membrane proteins. Common purification tags include histidine, strep and flag tags which can increase the purity and yield though affinity purification[3]. However, it is important these tags do not interfere with the function of the protein. Recombinant membrane protein expression is also a means of producing more stable membrane proteins through the use of mutagenesis and protein engineering but the native conformation will be altered and therefore the correct function and structure will not be discovered[4].

Effective recombinant membrane protein expression requires finding a suitable host. If the membrane protein is a prokaryotic protein then *E. coli* could potentially be used. The advantages of using *E. coli* for recombinant overexpression of membrane proteins is that it can be carried out quickly as they have a high growth rate, high quantities of cells are easily achieved and are cost effective[5]. If the target protein is eukaryotic, such as human membrane proteins, a eukaryotic host such as yeast, insect or mammalian cells can be used.

Insect cell expression is a commonly used expression system for recombinant mammalian membrane proteins. It requires the production of a recombinant baculovirus carrying the gene of interest, and infection of insect cells, such as *Spodoptera frugiperda* (*Sf*9), with this virus leads to protein expression[6]. Inclusion bodies are rarely formed with the baculovirus expression system in insect cells unlike in *E.coli*[7]. This system has also been beneficial in the production of multiprotein subunit complexes[8-10].

Two main strains of yeast have been used for membrane protein expression, *Pichia pastoris* and *Sacchromyces cerevisiae*. *Pichia pastoris* requires the integration of the recombinant gene of interest into the yeast genome allowing a stable strain to be produced but it is not possible to control the number of copies or location of the recombinant gene. Whereas *Sacchromyces cerevisiae* expression tends to use plasmids containing the gene of interest, similarly to *E. coli*. However the advantages of using *Pichia pastoris* are the high cells densities it can grow to with exceptionally high yields of correctly folded protein meaning large amount of recombinant protein can be produced[11], which is why *Pichia pastoris* was chosen for this study.

Mammalian cell expression offers potentially the most relevant cellular environment for human membrane proteins. Two of the most common mammalian cell lines used are human embryonic kidney (HEK) and Chinese hamster ovary (CHO) cells[6, 12]. The HEK cell line was chosen for this study as it has increasingly been used for membrane protein expression[13]. Proteins expressed in HEK cells are usually fully glycosylated compared to *Sf*9 cells[8]. HEK cells can be made to overexpress recombinant membrane proteins by either producing a transient or stable cell lines[14]. Whilst transient expression can give considerable batch-to-batch variability, creating

stable cells often reduces the expression yield. Thus transient transfections were utilised in this study.

ATP Binding Cassette (ABC) transporters are integral membrane proteins that are found in all types of organisms from prokaryotes to humans. They utilise energy from ATP binding and hydrolysis to transport a variety of substrates across the biological lipid bilayer[15]. In humans, the 48 different ABC transporters can be separated into seven different subfamilies ABCA- ABCG of which Multidrug Resistance Protein 4 (MRP4/ABCC4) is part of the C subfamily[16].

MRP4 can be found in a wide range of cells all over the human body including blood cells, neurons, testis, ovaries, adrenal glands, prostate tubuloacinar cells and renal proximal tubule cells[17]. Endogenously MRP4 is able to transport substrates which are involved in inflammation, such as prostaglandins and leukotrienes[18] and cell signalling, including cyclic nucleotides such as cyclic AMP (cAMP) and cyclic GMP (cGMP)[19]. It has also been shown to transport a wide range of drugs and their metabolites including anticancer, antiviral and antibiotic molecules[20].

How MRP4 is able to transport such a wide variety of substrates is not well known. In particular how it can recognise, bind and transport both relatively hydrophilic molecules like cAMP and hydrophobic molecules such as bile salts or drugs like methotrexate is unclear. This could be due to the lack of structural knowledge about the transmembrane domains (TMD) of MRP4, which are responsible for transporting substrates. Therefore functional and structural studies will help reveal the intricacies of this membrane protein.

In this study we investigated the functional overexpression of MRP4 by examining which approach gave the best expression yield and then characterized the function with a fluorescent vesicular transport assay.

Materials and Methods

Sf9 expression

Expression of the recombinant human MRP4-his$_6$ within *Sf*9 cells was conducted using a baculovirus encoding for recombinant MRP4 generated from a pFastBac-MRP4-his$_6$ construct as described previously[21]. Cells were grown in shaker cultures using Insect Xpress media (Lonza). To find the optimal expression conditions cells at a density of either 1 or 2 million per ml were infected with baculovirus using a multiplicity of infection (MOI) of either 2 or 4, and cells harvested after 24, 48 or 72 hours.

Pichia Pastoris expression

Growth media: BMGY (Buffered Glycerol-complex Medium) and BMMY (Buffered Methanol-complex Medium) was made using 10 g of yeast extract and 20 g peptone was dissolved in 700 mL water and autoclaved. To which: 100 mL 1 M potassium phosphate buffer, pH 6.0, (13.2 mL of 1M K_2HPO_4 and 86.8 mL of 1M K_2HPO_4), 100 mL 10X YNB (13.4% Yeast Nitrogen Base with Ammonium Sulphate without amino acids), 2 mL 0.02% Biotin, 100 mL 10% Glycerol for BMGY or 100 mL 5% Methanol for BMMY was added after filter sterilisation.

The recombinant pPICZαC-MRP4-his$_6$ construct was created using a double digest of the pFastBac MRP4-his$_6$ plasmid and pPICZαC with EcoRI, followed by ligation of MRP4-his$_6$ into the pPICZαC plasmid, at a plasmid to insert molar ratio of 1:3, overnight at 16^{o}C. pPICZαC MRP4-his$_6$ was linearized using PmeI and transformed into *P. pastoris* X33 using electroporation. Colonies containing integrated MRP4 were grown essentially as described previously for *Pichia* expression of a membrane protein[22]. Briefly, colonies were grown in 25 mL BMGY in sterile 250 mL flasks at

30°C in a shaking incubator (250-300 rpm) until the culture reached an OD_{600} of 2-6. Cells were harvested by centrifugation at 3000 g for 5 minutes, all BMGY removed, then washed with BMMY and resuspended in BMMY at an OD_{600} of 1.0 before being returned to the shaking incubator at 22 or 30°C. Sterilized pure methanol was added every 24 hours to a final concentration of 0.5% (v/v) methanol. Samples were taken every 24 hours over a 72h period.

HEK293T expression

pcDNA3.1-MRP4-his_6 plasmid was constructed by restriction digestion of MRP4-his_6 out of the pFastBac plasmid and ligation into a pcDNA 3.1 Zeo + plasmid. pOPINE-MRP4–3C-flag-his_8 was made by the OPPF (Oxford Protein Production Facility). pcDNA3.1-MRP4 without a his-tag was a kind gift from Professor Susan Cole (Queens' University, Kingston). HEK293T cells were seeded in a 6 well plate with 300,000 cells/well in DMEM containing 10% FBS and 1% penicillin/streptomycin 24 hours prior to transfection. 3 hours prior to transfection the media was replaced with low serum DMEM containing 2.5% FBS and 1% penicillin/streptomycin. For transfection, 4µg of plasmid DNA was combined with 18µL of 10mM linear polyethylenimine (PEI, Polysciences) and 100µL reduced serum media (OPTIMEM) and added to each well. 24 hours after transfection the media was replaced with DMEM containing 10% FBS 1% penicillin/streptomycin. Samples were taken every 24 hours over a 72 hour period.

Cell lysis and membrane preparation

For both *Sf*9 and HEK293T, cells were harvested by centrifugation (5000g for 10 minutes) and cell pellets were resuspended in buffer 1 (50mM Tris-HCl pH 7.4, 250mM Sucrose, 0.25mM $CaCl_2$) containing protease inhibitors (1.3µM benzamidine,

1.8µM leupeptin and 1µM pepstatin). Cells were disrupted through nitrogen cavitation at 500 psi for 15 minutes, 4°C. The cell lysate was centrifuged at 750g for 10 minutes to remove cell debris; the supernatant was then ultracentrifuged at 100,000 g for 20 minutes, 4°C. The membrane pellet was resuspended in buffer 2 (50mM Tris-HCl pH 7.4, 250mM Sucrose) and stored at -80^{o}C.

P. pastoris cells were pelleted via centrifugation at 2500g for 30 minutes and then resuspened in buffer 3 (5.5 % w/v glycerol, 2 mm EDTA, 100 mM NaCl, 50 mM NaH_2PO_4, 50 mM Na_2HPO_4) containing EDTA free protease inhibitor tablets (Roche). Cells were resuspened at a buffer (mL) to cell pellet weight (g) ratio of 3:1.Resuspended cell pellets were homogenised by passing them through the Emulsi Flex C3 machine (Avestin) three times. The homogenized cells were centrifuged at 5000g for 5 minutes, the supernatant was then centrifuged at 13,000g for 15 minutes and finally the supernatant was centrifuged for 1 hour at 100,000g. Membrane pellets were resuspended in buffer 4 (20 mM HEPES pH 8, 50 mM NaCl, 10 % w/v glycerol) containing protease inhibitor (Roche) and stored at -80^{o}C.

Analysis of expression

Expression of MRP4 was monitored by Western blot. Total protein concentration of membranes was measured using a BCA assay kit (Pierce). Specified amounts (µg) of total protein were loaded on 8% SDS-PAGE, transferred to PVDF membrane and blocked with 5% (w/v) BSA in TBS-T (20mM Tris pH 7.5, 150mM NaCl, 0.01% (v/v) Tween-20). Blots were probed either with a mouse anti-his antibody (R & D Systems) at a dilution of 1:500 or a rat anti-MRP4 antibody (M4I-10, Enzo) at 1:100, followed by anti-mouse HRP (Cell Signalling, 1:3000) or anti-rat HRP (sigma,

1:3000). All were visualised using chemiluminescence (Pierce) and a C-Digit Western Blot scanner (Licor).

Vesicular Transport Assays (VTAs)

VTAs were based upon the study by Reichel *et al* [23], and performed using *Sf*9 control and *Sf*9 MRP4 expressing cell membrane vesicles from the optimised expression conditions ($1x10^6$ cells/ml, MOI of 2, 48 hour incubation). 10 – 100 µg of total protein membrane protein were incubated with 10mM ATP (plus an ATP regenerating system: 100µg/mL creatine kinase and 10mM creatine phosphate) or AMP and 10mM $MgCl_2$ and 1 -100µM 8- (2-[Fluoresceinyl]aminoethylthio) adenosine- 3', 5'- cyclic monophosphate (fluo-cAMP) (Biolog). VTA were conducted in buffer 2 in a 50µL volume and incubated at room temperature for 10 minutes. This time period was chosen since previous kinetic studies showed it to be within the linear range[23]. For vanadate inhibition, 500µM sodium orthovanadate was added along with ATP. AMP-PNP inhibition was conducted by replacing the ATP with 10mM AMP-PNP. 0.01 – 10uM MK571 was added along with ATP to measure MK571 inhibition.

After incubation transport was stopped by the addition of 950µL of ice cold buffer 2. Samples were either filtered using a PVDF filter (Millipore 0.45µM) or centrifuged at 14,000 g for 5 minutes. The filter was washed with 5mL of ice cold buffer 2 or the pelleted vesicles washed with 1 mL ice cold buffer 2. The filter or pellet was solubilised with 1mL of SDS/HEPES buffer (1% (w/v) SDS and 7.5mM HEPES) for 15 minutes. The amount of fluo-cAMP transported was measured by the fluorescence signal (RFU) of the solubilised sample measured on a Perkin Elmer LS55 Fluorescence Spectrometer (excitation 480 ± 5nm, emissions 500-600

±20nm). Samples were run in triplicate and an average of 5 scans was taken for each sample.

Data fitting for concentration curves in the VTA was performed by fitting a Michaelis-Menten and for MK571 inhibition used a dose-response curve. Statistical analysis was performed using an un-paired two-tailed t-test or one-way ANOVA. Data fitting and statistical analysis was carried out using GraphPad prism.

Results

MRP4 Expression

The first step of the investigation was to determine the optimal conditions for MRP4 expression in each of the three expression systems, *Sf*9 insect cells, *P. Pastoris* yeast cells and HEK293T mammalian cells. For *Sf*9 insect cell expression the cell density, multiplicity of infection and infection period were altered. Western blots in Figure 1A show the expression of MRP4 within *Sf*9 cells was successful. As reported previously MRP4 expressed in *Sf*9 cells migrated at approximately 150kDa[24]. After 48 hours an increased expression was seen compared to 24 hours (Supplementary Figure 1A), however after 72 hours the expression level decreased again, possibly due to viral lysis of the cells. Increasing the cell density from $1x10^6$/ml to $2x10^6$/ml did not significantly improve the expression yield, and changing the MOI had little effect. It should be noted that a lower molecular weight band is also visible in several lanes, particularly those showing higher levels of expression, however this band is not specific to these highly expressing conditions and is visible in all samples if the exposure time is increased, and has been observed previously when MRP4 was expressed in *Sf*9 cells[24]. Therefore the optimal expression conditions in *Sf*9 cells were 48 hours with 1-2 x 10^6 insect cells/mL at either an MOI of 2 or 4.

After successful integration of MRP4 into *P. pastoris* the temperature and time were altered to gain the highest yield possible in shaker flasks. Figure 1B shows the expression of MRP4 within *P. pastoris.* Notably the MRP4 from *P. pastoris* runs at a higher molecular weight than the *Sf*9 expressed MRP4. At a lower temperature (22^{o}C) the highest expression level was achieved after 24 hours and then decreased over the 72 hour period. At higher temperature (30^{o}C) the expression level increased

over time reaching the highest expression level after 72 hours. The use of a 2L bioreactor for *P. pastoris* expression was also investigated (Supplementary Figure 1B), however this gave a lower yield of MRP4 expression than the shaker flasks. The optimal conditions for *P. pastoris* expression were therefore obtained using shaker flasks at 30°C for 24 hours. However it should be noted that this still gave a lower level of expression than the *Sf*9 cells.

Transient transfections were performed in HEK293T cells using PEI as a transfection reagent. As shown in Figure 1C, in contrast to *Sf*9 and *P. pastoris,* HEK293T cells express MRP4 endogenously. Transfection of the HEK293T cells with pcDNA3.1-MRP4$_{his}$ gave only marginally increased levels of MRP4 expression. Similarly transfection with pOPINE-MRP4-3C-flag-his8 led to very little overexpression of MRP4 (Supplementary Figure 1C). In contrast transfection with pcDNA-MRP4 without a his-tag gave a substantial time-dependent overexpression of MRP4 (Supplementary Figure 1C).

MRP4 was successfully overexpressed in all three expression systems. However in HEK293T cells it was only achieved in the absence of a his-tag, which would make downstream purification challenging. The yield obtained with *Sf*9 cells was higher than that achieved with *P. pastoris*. In addition the MRP4 from *Sf*9 cells migrated at a lower molecular weight than in the other two expression systems, possibly related to the degree of glycosylation. Extensive glycosylation can be problematic for downstream structural biology, thus this was perceived as another benefit of the *Sf*9 cell system. Therefore the *Sf*9 expression system was taken forward to assess if the MRP4 expressed was functional.

Vesicular Transport

Finding the balance between overexpression and quality needs to be obtained. Therefore it is vital to ascertain that the protein is functional following overexpression. To facilitate this a fluorescent vesicular transport assay (VTA) was used. cAMP is a known substrate for MRP4[25] and this assay utilises a fluorescent analogue of cAMP: 8- (2- [Fluoresceinyl]aminoethylthio)adenosine- 3', 5'- cyclic monophosphate (fluo-cAMP). This substrate had previously been reported to be transported by MRP4 within renal proximal tubules and by MRP4 overexpressed in *Sf*9 membrane vesicles[23]. By measuring the amount of substrate transported into the vesicle when ATP is present compared to AMP the specific transport activity can be determined (Figure 2).

Figure 3A shows a significant increase in the transport of fluo-cAMP in the presence of ATP compared to AMP in *Sf*9 MRP4 vesicles, showing ATP dependent transport of fluo-cAMP. MRP4 was shown to be responsible for the transport of fluo-cAMP as there is an increase in ATP dependent specific activity of *Sf*9 MRP4 vesicles compared to *Sf*9 control vesicles (Figure 3B). There was a positive correlation of ATP dependent specific activity in *Sf*9 MRP4 vesicles with increased total membrane protein content, again indicating that MRP4 was responsible for the transport of fluo-cAMP. Whereas the *Sf*9 control vesicles had a steady background fluorescence with increasing total membrane protein content. Figure 3C demonstrates a concentration dependent transport of fluo-cAMP with a K_m of 5.8μM, which is comparable to previously reported values[23].

ATP hydrolysis is needed for the transport of substrates with MRP4 and inhibiting ATP hydrolysis should therefore inhibit transport. As shown in Figure 4A in the

presence of vanadate or the non-hydrolysable ATP analogue AMP-PNP, the uptake is reduced to the same level as with AMP indicating that ATP is the driving force behind the transport of fluo-cAMP. MK571, a known inhibitor of MRP4, was also used to demonstrate the functionality of MRP4. MK571 inhibits transport of substrates by binding within the transmembrane domains rather than the nucleotide binding sites like vanadate and AMP-PNP[26]. MK571 inhibits the transport of fluo-cAMP in a concentration dependent manner with an IC_{50} of 0.39μM.

These results verify that MRP4 expressed in *Sf*9 cells is functional as it is responsible for the transport of fluo-cAMP in a concentration and ATP dependent manner and was prevented by inhibiting either ATP hydrolysis or substrate binding.

Discussion

The need for good starting material is paramount in elucidating the function and structure of membrane proteins. To address this we investigated MRP4 expression in three different systems, *Sf*9 insect cells, *P. pastoris* yeast and HEK293T mammalian cells. All three of these systems have been successfully utilised in the past for overexpression of mammalian ABC transporters for functional and structural studies.

P. pastoris has been successfully used for the overexpression of mouse MRP1/ABCC1[27, 28], mouse P-glycoprotein/ABCB1[29] and human TAP1/2[30]. In this study we found that human MRP4 could also be successfully overexpressed using *P. pastoris*. Surprisingly, the level of expression achieved was lower when using a bioreactor rather than shaker flask cultures (Supplementary Figure 1B). With a bioreactor it is possible to continuously monitor and respond to the conditions within the culture such as oxygenation and pH, thus it might be considered to be more optimal for cell growth. Although we were able to grow the yeast to very high cell densities within the bioreactor, this didn't translate into high expression levels of MRP4. Following optimisation of the shaker flask conditions the level of MRP4 expression achieved was still lower than that obtained when using *Sf*9 insect cells (Figure 1B). It might be that codon optimisation of the construct could help improve this further in the future[31].

The expression of MRP4 within *Sf*9 cells has been reported previously[21, 23, 24, 32, 33], however this has predominantly been utilised for functional assays to date, rather than with the aim to develop an expression system for future purification. Here we showed that MRP4 with a his-affinity tag could be successfully overexpressed in *Sf*9 cells, and the expression level could be optimised by changing the time of infection

(Figure 1A). Insect cells have previously been utilised for the expression, purification and structural study of human P-glycoprotein/ABCB1[34], although this used High Five (*Trichoplusia ni*) cells rather than *Sf*9 cells. Insect cells have also been proven to be especially useful for structural studies on GPCRs (G-protein coupled receptors), which have shown a preference for Sf9 cells[35].

The overexpression of MRP4 in HEK cells has also been reported many times previously[32, 36-38], but again to date this has mainly been for the purposes of functional studies. Transient transfection of HEK cells has been carried out using the transfection reagent Lipofectamine[36]. In this study we have successfully shown overexpression of MRP4 in HEK cells using the much cheaper reagent PEI (Supplementary Figure 1C). PEI has also been successfully utilised for the transfection of HEK cells with the related protein ABCG2[39]. However interestingly this only worked successfully for the un-tagged MRP4 construct (Supplementary Figure 1C). For two different constructs containing MRP4 with a C-terminal his-tag, only minor, if any, overexpression was achieved (Figure 1C and Supplementary Figure 1C). It is unclear at this point if this could be improved with the use of an alternative transfection reagent. It is known that MRP4 contains a PDZ motif at its C-terminal which is important for interaction with other proteins and localization within mammalian cells[40] and perhaps the his-tag interferes in some way. An alternative approach to transfection, that has been successfully utilised for HEK expression of other ABC transporters for structural studies, is transduction of HEK cells with a recombinant baculovirus containing a mammalian promoter[41, 42].

Notably both the *P. pastoris* and HEK expressed MRP4 migrated at higher molecular weights than the *Sf*9 MRP4 (Figure 1B and 1C). It is known that MRP4 is glycosylated[36] and *Sf*9 cells are only able to carry out simple mannose

glycosylation[6], so this difference is likely to be due to differential glycosylation in the three systems. Glycosylation can be problematic for structural studies since it adds heterogeneity.

Taken together the higher yield of MRP4, potential lower levels of glycosylation and ease of scale up led us to choose *Sf*9 cell expression to proceed with.

The next step was to check that the *Sf*9 overexpressed MRP4 was functional. Typically function is assessed by vesicular transport assays (VTAs) using radiolabelled substrates, however a fluorescent based assay can be both cheaper and easier. It was previously shown that MRP4 can transport the fluorescent analogue of cAMP, fluo-cAMP[23]. We found that crude membranes of *Sf*9 cells expressing MRP4 were able to transport fluorescent cAMP in an ATP dependent manner (Figure 3). The K_m of fluo-cAMP was found to be very similar to that found in the previous study[23] showing that this method is a robust way of determining the functionality of MRP4 using fluorescent analogues. Transport was also inhibited by MK571 and ATP analogues confirming its functionality (Figure 4).

During this study, both a rapid filtration and centrifugation technique were tested for separating free fluo-cAMP from the vesicles (Figure 2). Rapid filtration is typically used with radiolabelled substrates, however with the fluorescent assay particles from the filters caused an increase in light scattering which decreased the signal to noise ratio. PVDF filters were better than glass fibre filters, however the centrifugation method improved this even further, as well as increasing the efficiency of the transport assay.

In conclusion we have successfully demonstrated functional overexpression of MRP4 in *Sf*9 cells that can now be taken forward for solubilisation and purification to enable mechanistic and structural studies.

Figure Legends

Figure 1: Overexpression trials for MRP4 in *Sf*9 insect cells, *P. pastoris* yeast cells and HEK293T mammalian cells. A; Western blot of MRP4 in *Sf*9 insect cell membranes after 48 and 72 hours using an MOI of 2 or 4 with 1 or 2 x 10^6 *Sf*9 cells/mL. 5µg of total protein was loaded for each condition. +ve represents a control sample of MRP4 expressed in *Sf*9 cells which was quantified, aliquoted and frozen to be used as a control/standard on all Western blots to allow reliable comparison across different experiments. B; membrane expression levels in *P. pastoris* yeast cells after 24, 48 and 72 hours at 22^{o}C and 30^{o}C. 5 or 20µg of total protein was loaded, and compared to the *Sf*9 control expression levels (*Sf*9, 5µg total protein). C; Expression of MRP4 in HEK293T cell after 24, 48 and 72 hours. Controls include untreated HEK293T cells (-ve), treatment with PEI only or with an empty pcDNA3.1 vector (VC), and the *Sf*9 control expression sample (*Sf*9). Each HEK sample contained 20µg total protein, whereas the *Sf*9 control contained 10µg. A & B were probed with an anti-his primary antibody and an anti-mouse HRP secondary antibody. C was probed with an anti-MRP4 primary antibody and an anti-rat HRP secondary antibody.

Figure 2: Schematic of the steps in the vesicular transport assay. The first step is the incubation of the fluorescent cAMP substrate (green stars) with the membrane vesicles (orange circles) in the presence of AMP or ATP (along with an ATP regenerating system). The fluorescent cAMP is transported into the membrane vesicles via ATP hydrolysis. The vesicles are then either filtered or centrifuged to remove all excess fluo-cAMP. The vesicles are then solubilised and the amount of fluorescent cAMP transported into the vesicles is measure on a fluorescent

spectrometer. The difference between ATP and AMP is calculated giving the specific transport activity.

Figure 3: Vesicular uptake of fluo-cAMP is both ATP and MRP4 dependent. A; The relative fluorescence of membrane vesicles containing MRP4 when incubated with fluo-cAMP in the presence of ATP or AMP. 20µg of total membrane protein, with a 10 minute incubation period and 10µM fluo-cAMP. Data are mean ± SEM (n=3) Un-paired two-tailed t-test **** P<0.001. B; Specific transport activity of *Sf*9 control vesicles (*Sf*9) and *Sf*9 vesicles overexpressing MRP4 (*Sf*9 MRP4) using 10 -100µg total membrane proteins and 10µM fluo-cAMP, with a 10min incubation time. Data are mean ± SEM (n=3) Two-way ANOVA** P=0.01 ****P<0.001. C; Specific transport activity of *Sf*9 MRP4 membrane vesicles (50µg protein) using 0 – 50 µM fluo-cAMP showing a concentration dependent increase. Data are mean ± SEM, n≥2, V_{max} 64 RFU, K_m 5.8 µM, Michaelis-Menten curve fitted.

Figure 4: Vesicular uptake of fluo-cAMP is inhibited by non-hydrolysable ATP analogues and MK571. A; fluo-cAMP transported (RFU) in the presence of ATP (10mM), AMP (10mM), ATP (10mM) plus vanadate (500 µM) (ATP + V) or the non-hydrolysable ATP analogue AMP-PNP (10 mM). 30µg total membrane protein (*Sf*9 MRP4) with an incubation period of 10 minutes and 10µM fluo-cAMP. Data are mean ± SEM (n=3), 1 way ANOVA, multiple comparisons**** P<0.001. B; Dose dependent inhibition of fluo-cAMP (10µM) transport by MRP4 *Sf*9 membrane vesicles (50µg protein) in the presence of 0.01 - 5µM MK571, with a 10 minute incubation time. The percent of ATP specific transport was measured using ATP as 100%. Data are mean ± SEM, n=3, IC_{50}=0.39µM MK571, [inhibitor] vs normalised response curve fitted.

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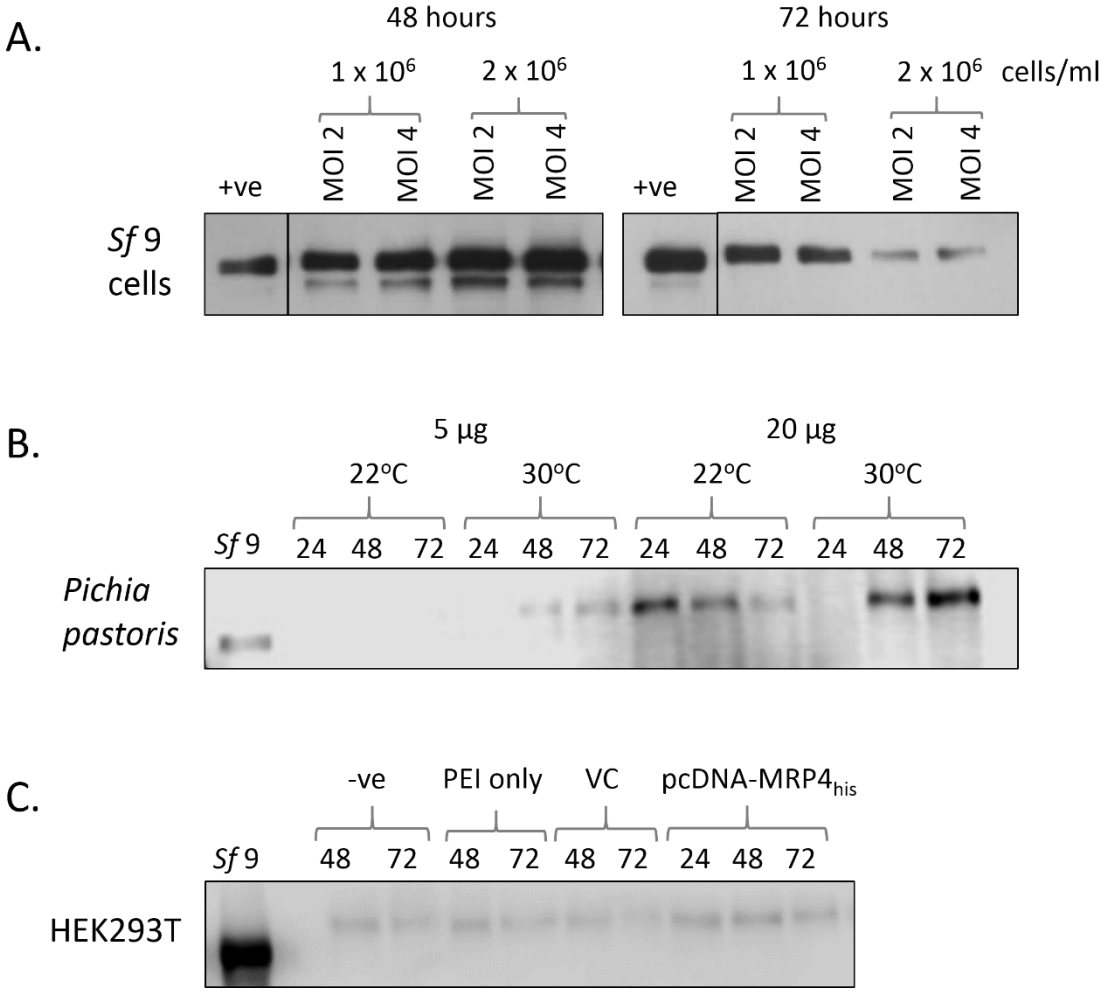
Figure 1


A.
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72 hours
1 x 10^6
2 x 10^6
1 x 10^6
2 x 10^6
cells/ml
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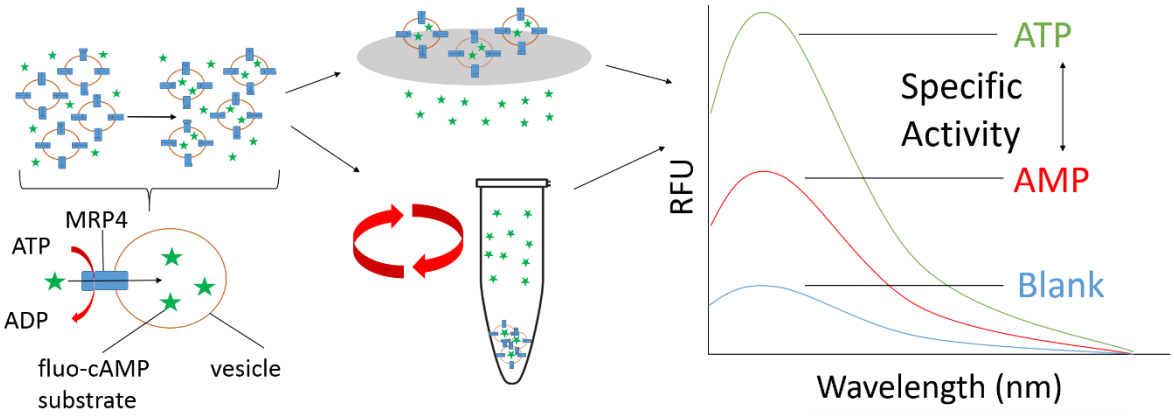
MOI 4
+ve
+ve
MOI 2
MOI 4
MOI 2
MOI 4
Sf 9 cells
B.
5 µg
20 µg
22°C
30°C
22°C
30°C
Sf 9
24 48 72 24 48 72 24 48 72 24 48 72
Pichia pastoris
C.
-ve
PEI only
VC
pcDNA-MRP4his
Sf 9
48 72 48 72 48 72 24 48 72
HEK293T

Figure 2


MRP4
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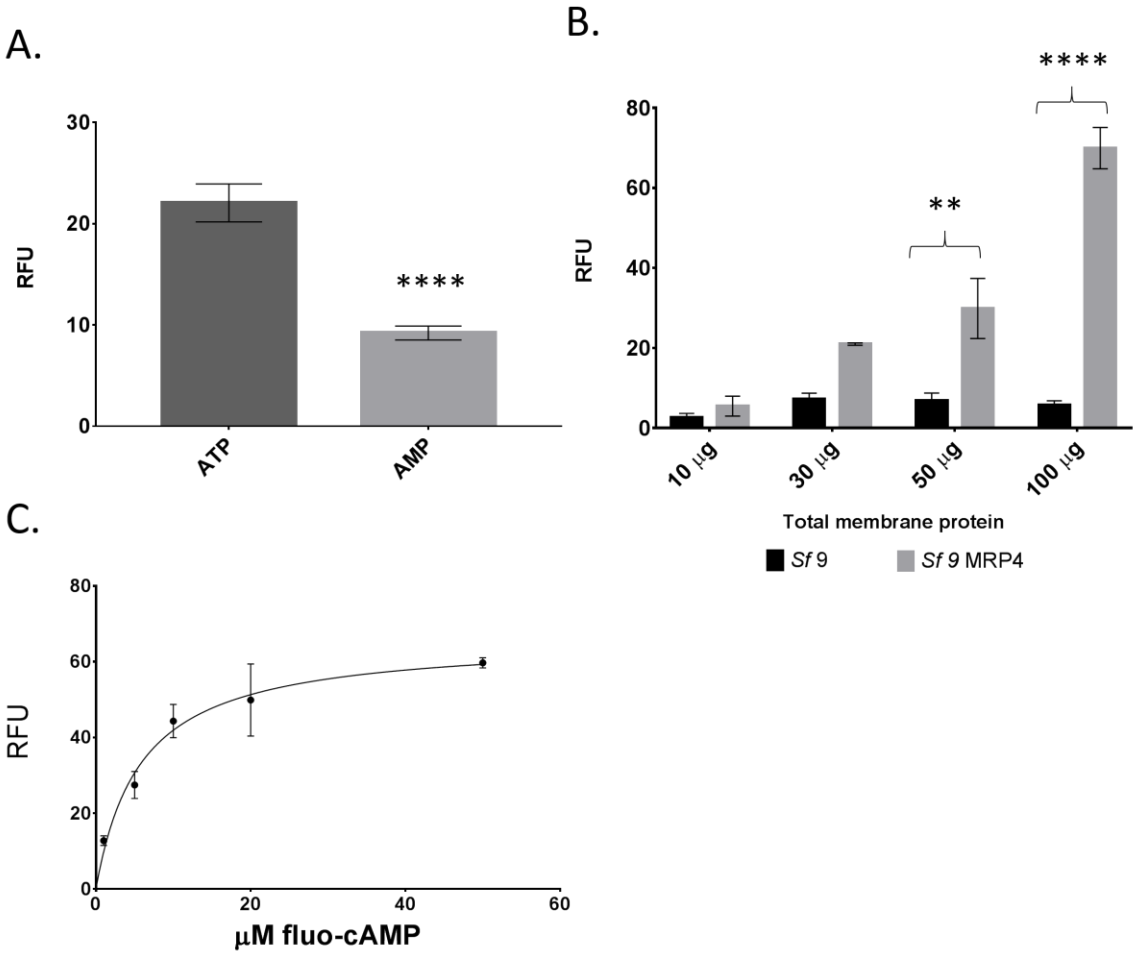
ADP
fluo-cAMP
substrate
vesicle
RFU
ATP
Specific
Activity
AMP
Blank
Wavelength (nm)

Figure 3


A.
RFU
30
20
10
0
****
ATP
AMP
B.
RFU
80
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40
20
0
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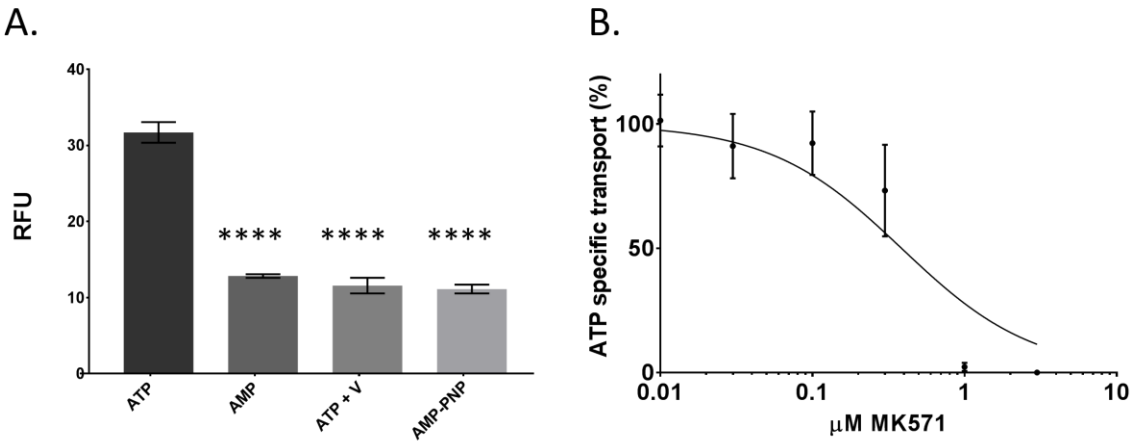
**
10 µg
30 µg
50 µg
100 µg
Total membrane protein
Sf 9
Sf 9 MRP4
C.
RFU
80
60
40
20
0
0
20
40
60
µM fluo-cAMP

Figure 4


A.
RFU
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30
20
10
0
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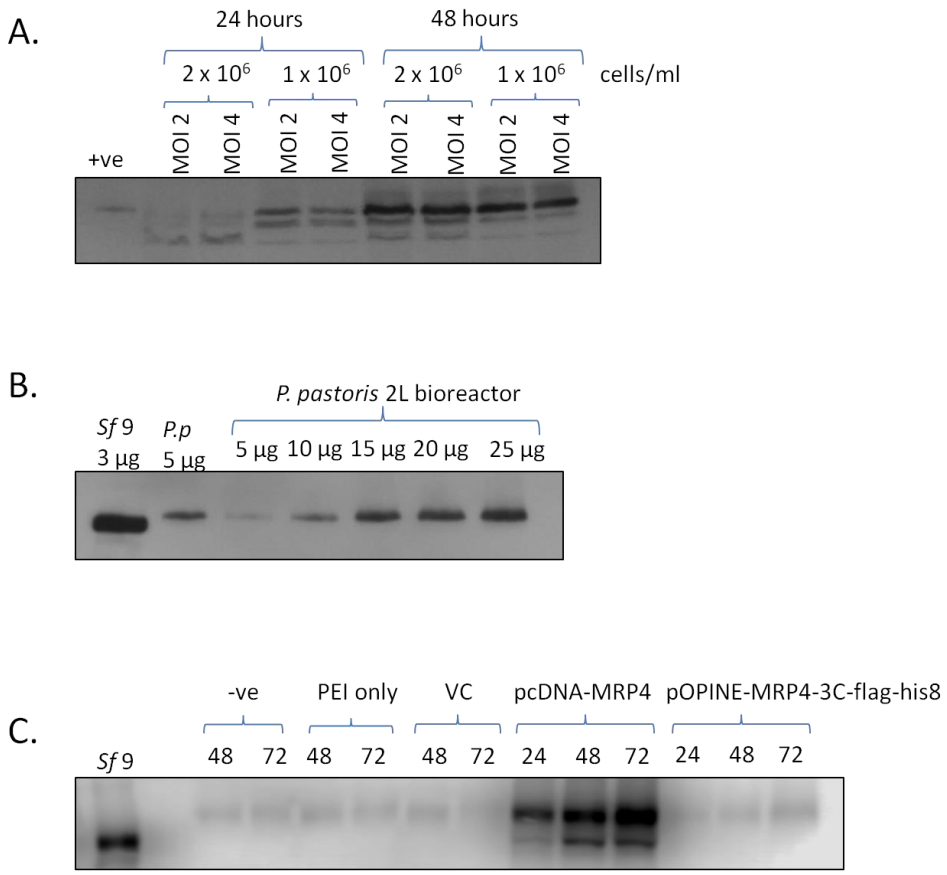
****
****
ATP
AMP
ATP + V
AMP-PNP
B.
ATP specific transport (%)
100
50
0
0.01
0.1
1
10
µM MK571

Functional expression of MRP4/ABCC4

David Hardy[1,2], Roslyn Bill[1], Anass Jawhari[2] & Alice Rothnie[1*]*

Supplementary Information



Supplementary Figure 1: Overexpression trials for MRP4 in *Sf*9 insect cells, *P. pastoris* yeast cells and HEK293T mammalian cells. A; Western blot of MRP4 in *Sf*9 insect cells (whole cell lysates) after 24 and 48 hours using an MOI of 2 or 4 with 1 or 2 x 10^6 Sf 9 cells/mL. +ve represents a control/standard sample of MRP4. B; membrane expression levels in *P. pastoris* yeast cells grown in a 2L bioreactor in comparison to *P. Pastoris* grown in shaker flasks. Specified amounts (µg) of total protein were loaded, and compared to control *Sf*9 expression levels (*Sf*9, 3µg total protein). C; Expression of MRP4 in HEK293T cells after 24, 48 and 72 hours using the pcDNA3.1-MRP4 un-tagged construct or the pOPINE-MRP4-3C-flag-his_8 construct. Controls include untreated HEK293T cells (-ve), treatment with PEI only or with an empty pcDNA3.1 vector (VC) and an *Sf*9 expressed MRP4 positive control (10µg). All HEK samples contain 20µg total protein. A & B were probed with an anti-his primary antibody and an anti-mouse HRP secondary antibody. C was probed with an anti-MRP4 primary antibody and an anti-rat HRP secondary antibody.