

December 2004

Translocation of Hepatocellular Mrp2 to the Canalicular Membrane Via Activation of PKC- delta by cAMP

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Project Number: MQP-BC-DSA-3917
MQP-BIO-DSA-9804

**TRANSLOCATION OF HEPATOCELLULAR Mrp2 TO THE CANALICULAR
MEMBRANE VIA ACTIVATION OF PKC δ BY cAMP**

A Major Qualifying Project Report

submitted to the Faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degrees of Bachelor of Science in

Biochemistry

and

Biology and Biotechnology

by

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January 12, 2005

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ABSTRACT

Mrp2 is an organic anion and bile salt export pump that must be translocated from the cytoplasm to the hepatocyte tubule canalicular membrane to transport its substrate. Although cAMP has been shown to induce both Mrp2 translocation and PKC δ activation, the interdependency of those two events has not been established. Hepatocytes were treated with a cAMP analogue and/or bistratene A (a known activator of PKC δ) at varying concentrations, followed by biotinylation of cell surface protein, preparation of cell lysates, immunoprecipitation by streptavidin, and western analysis of Mrp2 levels. Like cAMP, bistratene A also induced Mrp2 translocation, and the combined effects were not additive, indicating that cAMP translocates Mrp2 by activating PKC δ .

TABLE OF CONTENTS

Abstract	2
List of Tables and Figures	4
Acknowledgements	5
Background Information	6
Bile Salts	6
<i>Bile Salt Physiology</i>	6
<i>Bile Salt Uptake into Hepatocytes</i>	7
<i>Bile Salt Secretion from Hepatocytes via the Canalicular Membrane</i>	9
Cholestasis	11
Mrp2	13
<i>Characterization of Structure</i>	14
<i>Role of Mrp2 in Bile Acid Transport</i>	17
<i>Mrp2 in Cholestasis</i>	18
<i>Role of cAMP and PKCδ in Translocation of Mrp2</i>	19
Purpose of the MQP	22
Materials and Methods	23
Hepatocyte Harvesting.....	23
<i>Cell Acquisition</i>	23
<i>Cell Treatment</i>	23
Cell Biotinylation and Lysis	24
<i>Cell Biotinylation</i>	24
<i>Preparation of Cell Lysate</i>	24
<i>Protein Concentration Determination by Lowry Assay</i>	24
Purification of Biotinylated Proteins	25
Immunoblot (Western) Analysis.....	25
<i>SDS/PAGE</i>	25
<i>Detection of Proteins by Antibodies</i>	26
<i>Detection of Proteins via ECL</i>	27
Results	28
Discussion	34
Bibliography	41

LIST OF TABLES AND FIGURES

TABLE 1: SUMMARY OF BILE SALT TRANSPORTERS.....	8
FIGURE 1: RAT BILE SALT TRANSPORTERS	9
FIGURE 2: HUMAN BILE ACID TRANSPORTERS	11
TABLE 2: SUMMARY OF TYPES OF CHOLESTASIS	13
FIGURE 3: Mrp2 AMINO ACID SEQUENCE.....	14
TABLE 3: TRANSMEMBRANE DOMAINS OF Mrp2.....	15
FIGURE 4: Mrp2 GENE STRUCTURE.....	16
FIGURE 5: Mrp2 GENE PRODUCTS	16
FIGURE 6: cAMP REVERSES TLC-INDUCED INTERNALIZATION OF Mrp2.....	20
FIGURE 7: cAMP ACTIVATES PKC δ	20
FIGURE 8: AN INHIBITOR OF PKC δ INHIBITS cAMP-INDUCED TRANSLOCATION OF Mrp2 AND PKC δ	21
FIGURE 9: SIGNALING PATHWAY OF Mrp2 TRANSLOCATION.....	21
FIGURE 10: PRECIPITATION OF BIOTINYLATED Mrp2 FROM THE HEPATOCYTE CELL SURFACE.....	28
TABLE 4: RELATIVE INTENSITIES OF PRECIPITATED Mrp2 BANDS	29
FIGURE 11: HISTOPLLOT OF THE Mrp2 SIGNALS QUANTITATED IN TABLE 4	29
FIGURE 12: DIRECT QUANTITATION OF PROTEIN Mrp2 LEVELS IN THE HEPATOCYTE LYSATES	30
TABLE 5: QUANTITATION OF Mrp2 PROTEIN LEVELS FROM FIGURE 13.....	31
FIGURE 13: HISTOPLLOT OF THE DATA FROM TABLE 5	31
FIGURE 14: BIS-A TRANSLOCATES Mrp2 TO THE HEPATOCYTE MEMBRANE	32
FIGURE 15: THE TRANSLOCATION OF Mrp2 BY cAMP AND BIS-A IS NOT ADDITIVE	33
FIGURE 16: SUPPORTIVE AND ALTERNATIVE HYPOTHESES FOR THE EFFECTS OF cAMP AND BIS-A ON Mrp2 TRANSLOCATION.....	35
FIGURE 17: cAMP TRANSLOCATES Mrp2 VIA PKC δ ACTIVATION	37

ACKNOWLEDGEMENTS

We would like to thank Dr. Sawkat Anwer, our Major Advisor, for allowing us to make use of his personal laboratory (Tufts University School of Veterinary Medicine; North Grafton, MA), and for all his guidance and assistance throughout the entire course of our project. Additionally, we would like to thank Cory Gillin, Dr. Marie McConkey, Holly Jameson, and Dr. Cynthia Webster (all from Tufts) for their technical assistance in the lab, and for helping us obtain background information relevant to the project. Their day-to-day assistance helped us improve our lab skills as the project progressed. We would also like to thank Dr. Keppler for donating the primary antibody used to detect Mrp2 on the Western blots, without which we would not be able to study this protein. Finally, we thank Dr. David Adams, our project advisor from WPI, for assisting in the choosing of a project, guidance of the project along the way, and the editing of the project report.

BACKGROUND INFORMATION

Bile Salts

Bile Salt Physiology

The liver has many important functions, one of which is to form bile and recycle bile acids (Ganong, 1977). Bile plays an important physiological role in the digestion of fats. Stored in the gallbladder between meals, bile is released into the small intestine when CCK, an enzyme activated by the presence of food in the duodenum, stimulates the gallbladder to contract (Cunningham, 1992). Bile acids are a major constituent of bile, and in conjunction with pancreatic lipase and other solutes in the bile, they help to emulsify fat by forming micelles, thereby helping lipid digestion and absorption (Cunningham, 1992). Bile is also a major route by which cholesterol, bilirubin, hormones, some drugs, and some toxins are eliminated from the body (Nathanson and Boyer, 1991; Boyer and Nathanson, 1999).

These bile salts are recycled through the intestine, liver, and gallbladder, and this is referred to as enterohepatic circulation; as much as 95% of bile salts in the intestine are reabsorbed by the liver (Ettinger, 1983). Bile salts are absorbed back into the hepatocytes from portal blood via transport proteins in the basolateral membrane of hepatocytes (Anwer, 1993). The absorbed bile salts are then secreted into the canaliculi by another transporter.

Hepatocytes secrete early bile into a network of tubules between hepatocytes called canaliculi, and this early bile is modified as it makes its way into the bile ducts and the gallbladder (Trauner and Boyer, 2003). This secretion is driven by the active

transport of organic solutes (bile salts, phospholipids, cholesterol) into the canaliculi, and the other components of bile (water, electrolytes, non-electrolytes) soon follow (Sperber, 1959). The secretion of bile salts into the canaliculi is one of the strongest choleric agents, providing an osmotic driving force to produce more bile (Swenson, 1977; Trauner et al, 1999).

Bile Salt Uptake into Hepatocytes

In order to maintain the flow of enterohepatic circulation, bile salts must be taken up into the hepatocytes, lowering their concentration in enterohepatic blood. This movement occurs across the basolateral (or sinusoidal) membrane of the hepatocytes. Because these bile salts are moving against a concentration gradient, this transport must be coupled with energetically favorable transport in order to provide the energy necessary to achieve the transport of the bile salts; this energy can be provided by coupling the transport to the sodium gradient achieved by the sodium-potassium ATPase pump, or by coupling the transport to anion exchange (Kullak-Ublick et al, 2000A).

Table 1 below shows a list of the main transporter proteins involved in the enterohepatic circulation of bile.

TABLE 1: SUMMARY OF BILE SALT TRANSPORTERS. A summary of all of the basolateral and canalicular protein transporters involved in enterohepatic circulation of bile (Trauner and Boyer, 2003).

Name	Abbreviation (Gene Nomenclature)	Function
Basolateral membrane of hepatocyte		
Sodium-taurocholate cotransporting polypeptide	NTCP (<i>SLC10A1</i>)	Primary carrier for sodium-dependent conjugated bile salt uptake from portal blood
Organic anion transporting proteins	OATPs (<i>SLC21A</i>)	Multispecific organic anion transporters for sodium-independent uptake of bile salts and a broad range of other organic anions and cations
Multidrug resistance-associated protein 3*	MRP3 (<i>ABCC3</i>)	Multispecific organic solute transporter, weakly expressed in normal liver but highly upregulated on basolateral membrane of hepatocytes in cholestasis; capable of extruding bile salt conjugates from liver
Canalicular membrane of hepatocyte		
Multidrug resistance-1 P-glycoprotein*	MDR1 (<i>ABCB1</i>)	ATP-dependent excretion of various organic cations, xenobiotics, and cytotoxins into bile
Multidrug resistance-3 P-glycoprotein (phospholipid transporter)*	MDR3 (<i>ABCB4</i>)	ATP-dependent translocation of phosphatidylcholine from inner to outer leaflet of membrane bilayer; the phospholipid export pump
Multidrug resistance-associated protein-2 (Canalicular conjugate export pump)*	MRP2 (<i>ABCC2</i>)	Mediates ATP-dependent multispecific organic-anion transport (e.g., bilirubin diglucuronide, sulfates, glutathione conjugates) into bile; major determinant of bile salt-independent bile flow by GSH transport
Canalicular bile salt-export pump*/(sister of P-glycoprotein)	BSEP (SPGP) (<i>ABCB11</i>)	ATP-dependent transport of monovalent bile salts into bile; stimulates bile salt dependent bile flow
Familial intrahepatic cholestasis-1	FIC1 (<i>ATP8B1</i>)	Potential aminophospholipid translocating ATPase (function not known); gene defect in Byler's disease
Chloride-bicarbonate anion exchanger isoform-2	AE2 (<i>SLC4A2</i>)	Acid loader. Excretes bicarbonate into bile and stimulates bile salt-independent bile flow

Sodium taurocholate cotransport peptide (Ntcp) is the main protein responsible for uptake of conjugated bile salts (Hagenbuch and Meier, 1944). The transporter brings in two sodium ions for every molecule of bile acid, using the sodium gradient established by the sodium/potassium ATP pump (Takikawa, 2002).

A family of polypeptide transporters, named the Organic Anion Transporting Polypeptides (OATP), is responsible for the rest of basolateral uptake of bile salts and organic solutes involved in the production of bile (Jacquemin et al, 1994). In the rat liver, the OATP family is made up of Oatp1 and Oatp2 (Kayko et al, 1999A), of which the human orthologs are OATP2 (Konig et al, 2000) and LST-1 (Abe et al, 1999). Oatp1 helps to transport bile salts, conjugated steroids, and some types of drugs (Kullak-Ublick et al, 1994; Eckhardt et al, 1999). The driving force for this transport is utilizing the energy of transporting glutathione in the opposite direction (Li et al, 1998). Oatp2, in the same family as Oatp1, helps to transport bile salts, cardiac sugars, and cyclic peptides,

(Noe et al, 1997; Reichel et al, 1999) whereas LST-1 helps in the uptake of taurocholate (Kayko et al, 1999B). Oatp4, another member of the family, is a full length version of LST-1, and it is involved in transport of other organic solutes in addition to taurocholate (Cattori et al, 2000). Figure 1 shows a diagram of the function of proteins Ntcp, Oatp-1, and Oatp-2. Human correspondents exist to all of the rat transporters, but since this project focuses on Mrp2, the human correspondents of the other proteins are not discussed in this paper.

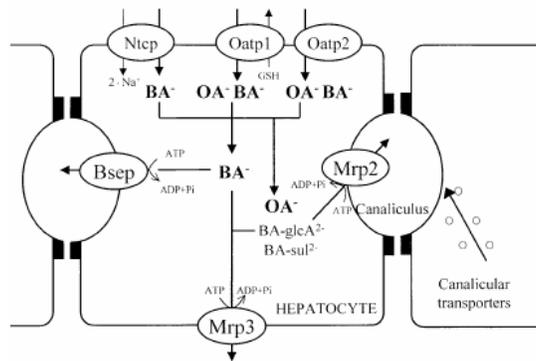


FIGURE 1. RAT BILE SALT TRANSPORTERS. Illustrates the role of each transporter in the enterohepatic circulation of bile, as well as demonstrating the interactions between the transporters. OA = organic anion, BA = bile acid, BA-glcA²⁻ = bile acid glucuronide, GSH = reduced glutathione, BA-sul²⁻ = bile acid sulfate (Takikawa, 2002).

Bile Salt Secretion from Hepatocytes via the Canalicular Membrane

As stated earlier, the hepatocyte secretes bile salts and organic solutes into the canalculus, a tubular network that drains into the bile duct. This transport occurs at the canalicular membrane. Because the concentration of bile salts in the bile itself is very high, these bile salts are being actively transported against a steep concentration gradient, and this transport is the rate limiting step in the transhepatic transport of bile salts

(Trauner and Boyer, 2003). The murine transporters that accomplish this are the bile salt export pump (Bsep), as well as Mdr1, Mdr2, and Mrp2 (Trauner and Boyer, 2003).

The transporters on the canalicular membrane are members of the ATP binding cassette family (ABC transporters), which hydrolyze ATP to ADP and P_i to provide energy to transport solutes against a concentration gradient (Takikawa, 2002). Bile salt export pump (Bsep) is the primary transporter of amidated bile acids (Gerloff et al, 1998), and in line with the protein being an ABC, the energy provided for this transport comes from the hydrolysis of ATP (Suchy et al, 1997; Stieger and Meier, 1998). Bile acids transported by this protein include taurocholate, glycocholate, taurochenodeoxycholate, and tauroursodeoxycholate (Kullak-Ublick et al, 2000B; Meier and Stieger, 2002). Mutations that prevent this protein from functioning normally result in progressive familial intrahepatic cholestasis type 2 (PFIC2) (Jansen et al, 1999).

As mentioned earlier, the bile in the canaliculus is an early form of bile that is modified in order to become the bile that is used in the intestines. Other solutes are added to this initial bile, one of which is the lipid phosphatidylcholine, and this lipid is excreted from the rodent canalicular membrane via Mdr2 (Oude Elferink et al, 1997), an ATP dependent flippase (Smit et al, 1993; Smith et al, 1994). Another solute hepatocytes secrete via the canalicular membrane is bicarbonate, and bicarbonate is secreted via the Cl^-/HCO_3^- anion exchanger two (AE2) (Medina, 1997).

The final canalicular transporter is multidrug resistance protein 2 (Mrp2), the protein of interest to this MQP report. Thus, specifics of this protein will be discussed later in the background of the paper. In short, Mrp2, an ABC transporter (Keppler and Konig, 1997; Stieger and Meier, 1998), is expressed in the liver and localized to the

canalicular membrane. It is responsible for the secretion of non-bile salt organic anions (Paulusma and Oude Elferink, 1997), as well as glutathione conjugates, glucuronides, leukotriene C₄ and divalent (but not monovalent) bile salts (Madon et al, 1997; Ito et al, 1998). Further details of Mrp2, including its sequence, structure, physiological role, changes under cholestatic conditions, and activation will be discussed later in the paper. The human proteins involved in bile acid transport are shown below in figure 2.

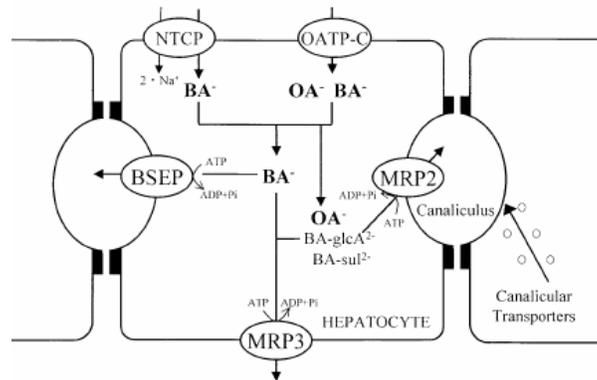


FIGURE 2: HUMAN BILE ACID TRANSPORTERS. Illustrates the human orthologs of the murine bile acid transporters illustrated in figure 1 (Takikawa, 2002).

Cholestasis

Cholestasis, in its most general terms, is a disease state characterized by decreased bile formation. It can have many physiological effects, such as the accumulation of hepatocellular bile acids and cholesterol, which can have toxic effects (Jansen, 2000). At high concentrations, bile acids can be cytotoxic, damage mitochondria, stimulate the formation of hepatic stellate cells, which produce collagen, induce apoptosis (programmed cell death), and have a direct effect on transcriptional regulation of enzymes and transporters (Jansen, 2000).

Cholestasis is usually associated with decreased transhepatic transport of bile acids at the basolateral or canalicular membrane by specific transporters present there (Meier and Steiger, 2000). These transporters include Ntcp, Bsep, Mrp3, and Mrp2. Cholestasis is associated with the upregulation of Mrp3 (Soroka et al, 2001; Donner and Keppler, 2001), the down-regulation of Ntcp and Mrp2 (Trauner et al, 1997; Lee et al, 2000), and relatively normal expression of Bsep (Lee et al, 2000). Changes to transporter activity can be caused by two different events, one event being mutation in the gene encoding the transporter, and the other event being a change in the translocation of the transporters responsible for the movement of bile acids through the hepatocyte.

A mutation in the gene encoding Bsep results in type 2 progressive familial intrahepatic cholestasis (PFIC2) (Strautnieks et al, 1998), while mutations in Mdr3 and Mrp2 result in type 3 progressive familial intrahepatic cholestasis and Dubin-Johnson syndrome, respectively (Paulusma et al, 1997; De Vree et al, 1998). Cholestasis can also be induced using different chemicals, which include tauroolithcholate (TLC) and estradiol-17 β -D-glucuronide, which aid in the removal of Bsep and Mrp2 from the canalicular membrane (Mottino et al, 2002; Crocenzi et al, 2003). Thus, when hepatocytes are treated with these chemicals, the proteins are unable to transport bile salts, and cholestasis occurs.

In addition to decreased movement of bile acids due to a change in translocation of transporters to the basolateral and canalicular membranes, cholestasis can also be caused by disturbing the flow of bile through damaged intrahepatic bile ductules (Jansen, 2000). This is the case in primary biliary cirrhosis (PBC), as the bile ducts are damaged in this disease. Jaundice is a symptom of PBC, and is a result of cholestasis and the

inability of the hepatocytes to secrete bilirubin (Jansen, 2000). This type of cholestasis is experimentally induced via bile duct ligation.

TABLE 2: SUMMARY OF TYPES OF CHOLESTASIS. A summary of the known types of cholestasis, along with the cause of each type (Trauner et al., 1999).

Disease	Molecular Change
<i>Acquired</i>	
Primary Biliary Cirrhosis	AE2 mRNA and protein reduced; hepatocytes and cholangiocytes are affected;MDR3 mRNA levels unchanged
Extrahepatic Biliary Atresia	NTCP mRNA reduced. Inverse correlation with serum bilirubin levels increase after successful Kasai procedure
Primary Sclerosing Cholangitis	OATP mRNA increased
Biliary Obstruction	MDR1 and MDR3 mRNA increased. Direct correlation with serum 104 bilirubin levels
<i>Hereditary</i>	
Progressive Familial Intrahepatic Cholestasis (PFIC)	
PFIC-1	Mutation of FIC1 gene (chromosome 18q21--22): low y-CT
PFIC-2	Mutation of BSEP gene (chromosome 2q24); low y-CT; canalicular BSEP protein absent
PFIC-3	Mutation of A4DR3 gene (chromosome 7q21); high y-CT; canalicular MDR3 protein absent
Benign Recurrent Intrahepatic Cholestasis (BRIC)	Mutation of FIC1 gene (chromosome 18q21122)
Dubin-Johnson Syndrome	Mutation of MRP2 gene (10q23-24) Canalicular MRP2 protein absent

Mrp2

Mrp2 stands for multidrug resistance associated transporter 2. It is also known as cMOAT, or canalicular membrane organic anion transporter.

Characterization of Structure

This protein is a part of a superfamily of transporter proteins known as the ATP-binding cassette proteins (ABC). These proteins hydrolyze ATP to ADP and P_i to provide energy to transport solutes against a concentration gradient (Takikawa, 2002). Within this large family of proteins, there are seven subfamilies, which include ABC1, MDR/TAP, MRP, ALD, OABP, GCN20, and White groups (Borst et al, 2000). Mrp2 is a part of the multidrug resistance protein (MRP) subfamily. Proteins in this family are responsible for carrying a wide range of drugs out of cells, making them suspects in many unexplained drug-resistance cases, though no clear connection has been identified (Borst et al, 2000). Mrp2 is 1541 amino acids long and contains 6 different regions (Genbank Protein ID: NP 036965). The amino acid sequence of Mrp2 is shown in figure 3. It also contains 16 transmembrane domains (SOSUI), shown in table 3.

```
1   mdkfcnstfw dlsllespea dlplcfteqv lwwiplgflw llapwqlysv yrsrtkrssi
61  tkfylakqvf vvfllilaa dslslatedt gqatvppvry tnpilylctw llvlavqhsr
121 qwcvrknsfw lsflwilsvl cgvfqfqtli rallkdsksn maysylffvs ygfqivllil
181 tafsgpsdst qtpsvtasfl ssitfswydr tvlkgykhpl tledvwdide gfktrsvtsk
241 feaamtkdld karqafqrrl qksqrkpeat lhglnkksq sqdvlvlea kkksekttkd
301 ypkswliksl fktfhvilk sfilklihd1 lvflnpqllk lligfvkssn syvwfgyica
361 ilmfavtliq sfclqsyfgh cfvlgmcvrt tvmssiykka ltlsnlarkq ytigetvnlm
421 svdsqklmda tnymqlwss vigitlsiff lwrelgpsil agvgvmvlli pvngvlatki
481 rniqvqnmkn kdkrkrkimne ilsgikilky fawepsfgeq vqgirrkkelk nllrfgqlqs
541 llifilqitp ilvsvvtfsv yvlvdsanvl naekaftsit lfnilrfpls mlpmvtssil
601 qasvsvdrle rylggddldt sairrvsnfd kavkfseasf twdpdleati qdvnldikpg
661 qlvavvgtvq sgksslvsam lgemenvhgh itiqgstayv pqqswiqngt ikdnilfgse
721 ynekkyqqvl kacallpdle ilpggdmaei gekginlsgg qkqrsvlara ayqdadiyil
781 ddplsavdah vgkhifnkvv gpngllagkt rifvthgihf lpqvdeivvl gkgtilekgs
841 yrdlldkkgv farnwktfmk hsgpegeatv nndseaeddd dgliptmeei pedaaalamr
901 renslrtrls rssrsssrrg kslknslkik nvnvlkekek evegqklikk efvctgkvkf
961 siylkylqav gwsilfiil fyglnnvafi gsnlwlawsawt sdsdnlngtn nssshrdmri
1021 gvfgalglqg gicllistlw siyacrnask alhgqlltni lrapmrffdt tptgrivnrf
1081 sgdistvddl lpqtlrswmm cffgiagtlv micmatpvfa iiiiplsily isvqvfyvat
1141 srqlrrldsv tkspiyshfs etvtglpiir afehqqrfla wnekqiding kvfswitsn
1201 rwlairlelv gnlvfvfcsal llviyrktlt gdvvgfvlsn alnitqtlnw lvrmtseae
1261 nivaverise yinveneapw vtdkrppadw prhgeiqfnn yqvryrpeld lvlkgitcni
1321 ksgekvvgv rtagksslt nclfrilesa ggqiiidgid vasiglhdlr erltiipqdp
1381 ilfsgslrmn ldpfnkysde evwralelah lrsfvsglql gllsevtegg dnlsigqrql
1441 lclgravlkr skilvldeat aavdletdsl iqttrkefs qctvitiahr lhtimdsdki
1501 mvlndngkive ygspeellsn rgsfylvmake agienvnhte 1
```

FIGURE 3: Mrp2 AMINO ACID SEQUENCE. (Genbank Protein ID: NP 036965)

TABLE 3: TRANSMEMBRANE DOMAINS OF Mrp2. The amino acid sequences of the Mrp2 transmembrane segments, and their locations within the overall protein (SOSUI).

No.	N terminal	Transmembrane region	C terminal	Type	Length
1	27	EQTVLVWVIPLGFLWLLAPWQLYS	49	Primary	23
2	63	FYLAQVFFVFLILAAIDLSLA	86	Primary	23
3	99	RYTNPILYLCTWLLVLAVQHSRQ	121	Primary	23
4	131	LSLFWILSVLCGVFQFQTLIRAL	153	Primary	23
5	163	YSYLFFVSYGFQIVLLILTAFS	184	Primary	22
6	319	LKSFILKLIHDLVFLNPQLLKL	341	Primary	23
7	352	YVWFGYICAILMFAVTLIQSFCL	374	Primary	23
8	431	TNYMQLVWSSVIQITLSIFFLWR	453	Secondary	23
9	458	SILAGVGVMLLIPVNGVLATK	479	Primary	22
10	542	LIFILQITPILVSVVTFVSVYVLV	564	Primary	23
11	580	TLFNILRFPLSMLPMVTSSILQA	602	Secondary	23
12	975	ILFIILFYGLNNVAFIGSNLWLS	997	Primary	23
13	1020	IGVFGALGLAQGICLLISTLWSI	1042	Primary	23
14	1095	LRSWMMCFFGIAGTLVMICMATP	1117	Primary	23
15	1121	IIIIPLSILYISVQVFYVATSRQ	1143	Primary	23
16	1213	LVVFCSALLLVIIYRKTLTGDEVVG	1235	Primary	23

The Mrp2 protein is 174.19 kD in size (Gillin et al, 2004). The ABC transporter transmembrane region is found from amino acids 318-589. The ABC-type multidrug transport system, ATPase and permease components are found from amino

acids 306-857, and the ABC transporter nucleotide-binding domain is found from 633-841. Another set of these three components completes the six regions; these are found from amino acids 975-1252, 1020-1534, and 1296-1514 respectively. The ABC transporter region is made up of six transmembrane helices, and Mrp2 has two ABC transporter regions used to bind ATP to provide energy for solute transport, much like other transporters in the ABC family (Genbank Protein ID: NP 036965).

The ABC-type multidrug transport system, ATPase, and permease components are made up of many transmembrane domains, and may take part in a defense system. The ABC transporter nucleotide-binding domain is made up of a “signature motif, Q-loop, and H-loop/switch region in addition to the Walker A motif/P-loop and Walker B motif commonly found in a number of ATP- and GTP-binding and hydrolyzing proteins” (Genbank Protein ID: NP 036965).

The gene for Mrp2 (Gene ID: 25303) is located on chromosome one, and it is called Abcc2. The following figures show both the gene and its transcripts and products; the gene is located at the 1q51-q55 location of the chromosome (Genbank Gene ID: NP 25303).



FIGURE 4: Mrp2 GENE STRUCTURE. Illustrates the Abcc2 gene encoding Mrp2 (Genbank, GeneID: 25303).

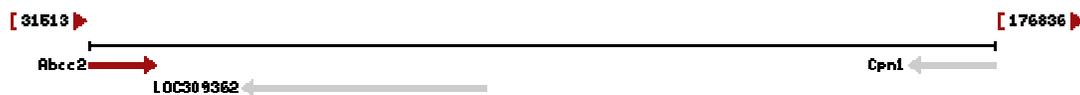


FIGURE 5: Mrp2 GENE PRODUCTS. Shows the products of the Abcc2 gene (Genbank).

Role of Mrp2 in Bile Transport

Mrp2 is localized to the canalicular membrane of the hepatocyte, and its substrates include dinitrophenyl-S-glutathione, sulfotaurolithocholate, bilirubinmonoglucuronide and diglucuronide, 17 β -estradiol glucuronide, glutathione conjugates, leukotriene C₄, and glutathione disulfide (GSSG) (Meier and Steiger, 2000). It is responsible for transporting glutathione (GSH) and other glutathione, glucuronide, and sulfate conjugates into bile (Mottino et al, 2002). In addition, although Mrp2 is not responsible for transporting taurocholic acid, a substitution of leucine from arginine at either position 586 or 1096 can make the protein able to transport it (Ito et al, 1998). This could indicate that all of the bile acid transporters are similar in structure.

In order for Mrp2 to secrete any of these substances into bile, it must first be translocated to the canalicular membrane, since it is inactive when present in the cytosol. This translocation is mediated by cyclic AMP (cAMP), shown in 1998 when treatment of hepatocytes with DBcAMP caused Mrp2 to move to the apical membrane (Roelofsen et al, 1998). A mechanism for this movement was proposed. At one hour after treatment, Mrp2 is present at points in the plasma membrane or is located in vesicles just underneath it. These vesicles are believed to contain Mrp2. Mrp2 is then spread throughout the plasma membrane when vesicles migrate to the membrane and fuse with it. At three hours after treatment, Mrp2 is spread throughout the apical membrane (Roelofsen et al, 1998).

Mrp2 in Cholestasis

A mutation in Mrp2 is indicated in Dubin-Johnson syndrome, a type of cholestasis. This has been shown in a study involving Wistar rats which are transport deficient (TR-). These rats show many similar symptoms to Dubin-Johnson patients (Zimniak, 1993). The first of these symptoms is chronic conjugated hyperbilirubinemia, along with the inability to clear injected bromosulphothalein from the liver, and regurgitation of glutathione conjugate into the cytosol (Mandema, 1960). There is an increased urinary secretion of coproporphyrin I, a byproduct of heme synthesis (Jansen, 1985). Finally, liver biopsies of Dubin-Johnson patients show lysosomal accumulation of black pigment, which is also seen in rats whose diet is supplemented with aromatic amino acids (Zimniak, 1993).

In addition to mutations in the DNA encoding the protein, cholestasis can also be induced by a variety of agents and methods. Bile duct ligation, endotoxin, TLC, and estradiol-17 β -D-glucuronide (E217G) cause the internalization of Mrp2 in the cell (Trauner et al, 1997), which in turn causes cholestasis.

E217G has been shown to cause removal of Mrp2 from the plasma membrane, resulting in an accumulation of Mrp2 in the cytosol (Mottino et al, 2002). However, when the cells are pretreated with cAMP, the effects of E217G are reversed within three hours of the treatment. This suggests that more Mrp2 is present in the plasma membrane as a result of the treatment with cAMP, so there is more to be removed when treated with E217G (Mottino et al, 2002).

Role of cAMP and PKC δ in Translocation of Mrp2: Anwer Lab Experiments

Protein kinase C (PKC) is a family of at least 12 isozymes (Newton, 2003). Within this family, there are different subgroups, such as the conventional PKCs (PKC α , β , β I, β II, and γ), novel PKCs (PKC δ , ϵ , η and θ), atypical PKCs (PKC ζ and λ), and PKC μ . These different families are designated cPKC, nPKC, and aPKC respectively. The cPKCs are dependent on Ca²⁺ and diacylglycerol, while nPKCs are Ca²⁺ independent, and aPKCs are independent of both calcium and phospholipids. The PKC of importance in this study was nPKC δ , which is present in rat hepatocytes (Beuers et al, 1999). It is thought that PKC δ is activated by a PI3K signaling pathway, although this has not been proven.

It has already been shown that cAMP is involved in the translocation of Mrp2 to the plasma membrane; however, the biochemical pathway by which it does so is unknown. The aim of a study completed in the Anwer lab was to determine whether cAMP reversed the effects of TLC-induced internalization of Mrp2, whether cAMP activates PKC δ , and whether PKC δ mediates the translocation of Mrp2 with cAMP (Gillin et al, 2004).

In the first part of this Anwer lab study, rat hepatocytes were isolated and treated first with cAMP and then TLC. The results showed that cAMP reversed TLC-mediated internalization of Mrp2, which is shown in figure 6.

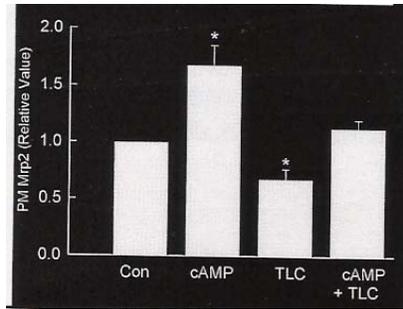


FIGURE 6: cAMP REVERSES TLC-INDUCED INTERNALIZATION OF Mrp2. Shows the amount of Mrp2 present in the plasma membrane increases as a result of treatment with cAMP or cAMP + TLC relative to TLC alone (Gillin et al, 2004).

Additionally, hepatocytes were treated with increasing amounts of cAMP, and then the amount of PKC present in the plasma membrane was analyzed. The results showed that cAMP does indeed activate PKC, summarized in figure 7.

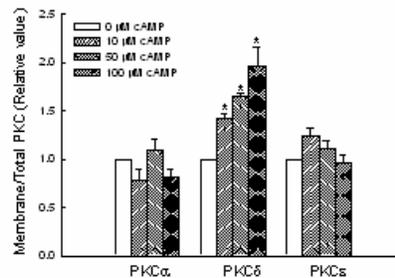


FIGURE 7: cAMP ACTIVATES PKCδ. Shows the relative values of membrane and total PKC as a result of treatment with increasing concentrations of cAMP (Anwer, 2001).

Finally, hepatocytes were treated with Rottlerin, a known inhibitor of PKCδ. Both the amount of membrane/total PKCδ and the amount of Mrp2 present in the plasma membrane were analyzed. The results showed that Rottlerin inhibited both translocation of PKCδ and translocation of Mrp2, suggesting that PKCδ plays a role in the translocation of Mrp2 to the plasma membrane. A summary of these results is shown in figure eight.

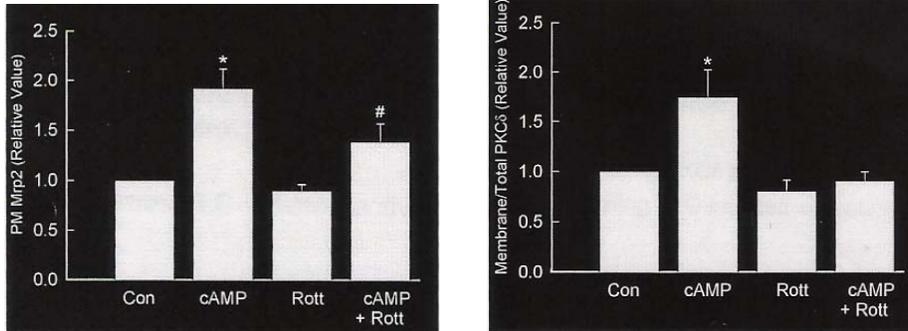


FIGURE 8: AN INHIBITOR OF PKC δ INHIBITS cAMP-INDUCED TRANSLOCATION OF MRP2 AND PKC δ . Shows the effect of cAMP +/- Rottlerin on plasma membrane and the relative membrane/total PKC δ (Gillin et al, 2004).

Based on these results, the authors hypothesized cAMP-induced MRP2 translocation may be mediated via activation of PKC δ . Although some of the pathways are not yet known, the hypothesis is summarized in figure 9.

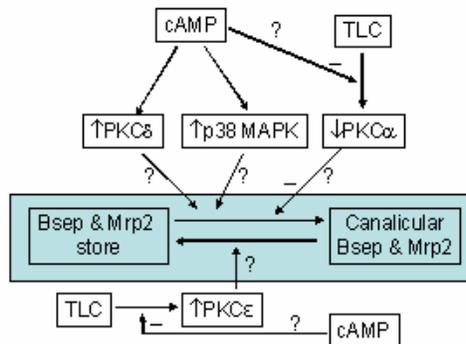


FIGURE 9: SIGNALING PATHWAY OF MRP2 TRANSLOCATION. Shows the proposed signaling pathway for the translocation of MRP2 to the canalicular membrane (Anwer, 2001).

PURPOSE OF THE MQP

The study that we performed was directly related to this proposed mechanism pathway of Anwer, 2001, as we attempted to determine whether cAMP induced translocation of Mrp2 to the plasma membrane through the activation of PKC δ . Bistratene A (BisA), isolated from the marine sponge *Lissoclinium bistratum*, is a known selective activator of PKC δ (Frey et al, 2001) and was used to treat primary hepatocytes in culture; then, the cells were biotinylated to label surface proteins. If cAMP induces translocation of Mrp2 by activating PKC δ , then an activator of PKC δ should also independently induce Mrp2 translocation. This can be detected by precipitating the biotinylated membrane proteins by using streptavidin beads, which show an affinity for biotin (Manufacturer's Information: purchased from Novagen). These proteins can be separated via SDS/PAGE and transferred to a nitrocellulose membrane. Then, the levels of Mrp2 can be quantified by Western blotting specifically for Mrp2. In addition, Mrp2 will be probed for in the whole cell lysate (non-precipitated with streptavidin beads) to make sure there is approximately equal amounts of Mrp2 protein in each cell sample.

If BisA is shown to translocate Mrp2, then the hypothesis that PKC δ translocates Mrp2 would be supported. If the translocation effect of BisA and cAMP are not summative, then it is likely that they act via the same pathway, and since BisA is known to be an activator of PKC δ , it can be extrapolated that cAMP translocates Mrp2 via PKC δ .

MATERIALS AND METHODS

Hepatocyte Harvesting

Cell Acquisition

Hepatocytes were harvested from the livers of Male Wistar rats (200-300 g) obtained from Charles River Laboratories. Hepatocytes were isolated from the rat livers through collagenase perfusion (Anwer et al, 1976). The collagenase was purchased from Sigma.

Cell Treatment

Prior to cell treatment, cells were suspended in a HEPES assay buffer at pH 7.4 for thirty minutes at 37°C. The buffer contained each of the following in mM: 20 HEPES, 140 NaCl, 5 KCl, 1 MgSO₄, 1.0 CaCl₂, 0.8 KH₂PO₄, and 5 glucose.

There were eight different treatment groups for the cells. The first group was treated with DMSO, the second with DMSO and 100 µM cAMP (purchased from Sigma), the third with 50 µM Bistratene A (BisA) (purchased from Sigma) and DMSO, the fourth with 50 µM BisA and 100 µM cAMP, the fifth with 100 µM BisA and DMSO, the sixth with 100 µM BisA and 100 µM cAMP, the seventh with 200 µM BisA and DMSO, and the eighth with 200 µM BisA and 100 µM cAMP. Cells were treated for 30 minutes with 1 mL of treatment solution, at which point the treatments were stopped by washing the cells in ice cold PBS.

Cell Biotinylation and Lysis

Cell Biotinylation

0.5 mg/mL biotin was made by dissolving biotin in PBS pH 8.0. This biotin was ice cold. Cells were biotinylated by adding 1.5 mL of this solution to the cells and rocking the cells at 4°C for forty-five minutes. Afterwards, the cells were washed three times with ice cold PBS to remove the excess biotin.

Preparation of Cell Lysates

After biotinylation, the cells were lysed with one mL of cell lysis buffer (CLB+) per sample. The cell lysis buffer contained each of the following (all constituents of CLB+ purchased from Sigma): 20 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, 500 nM okadaic acid, and 1 mM orthovanadate. After the lysis buffer was added to the cells, the cells were vortexed every ten to fifteen minutes for forty-five minutes, sitting on ice in between vortexing. Afterwards, these tubes were centrifuged at 14,000 rpm for 10 minutes, and the supernatants were removed into new tubes. These supernatants were the lysates used for the experiment.

Protein Concentration Determination by Lowry Assay

The concentration of total protein for each cell treatment sample was determined by performing a Lowry Assay (Lowry et al, 1951). In this assay, a standard curve for protein of known concentration (protein standard 60 g/L) was constructed, and the

unknown concentrations of sample proteins were determined by plotting them against the standard curve.

Purification of Biotinylated Proteins

Streptavidin beads (purchased from Novagen) were pre-cleared by washing them four times in an excess of PBS pH 8.0, centrifuging between each wash. After the washes, the beads were transferred to a new tube and adjusted to a 50:50 slurry of streptavidin beads: PBS pH 8.0.

300 μg of lysate protein for each sample was added to a different tube, and 50 μL of pre-cleared streptavidin bead slurry was added to each sample. Then, 100 μL of PBS pH 8.0 was added to each sample. Samples were rotated at room temperature for thirty minutes and then centrifuged at 14,000 rpm for one minute at room temperature. Samples were then washed three times with one mL of PBS pH 8.0, after which all PBS was removed, leaving only the bead-protein conjugate behind.

Immunoblot (Western) Analysis

SDS/PAGE

10 μL of β -mercaptoethanol (purchased from BIORAD) was added to 500 μL of SDS gel loading buffer, and 30 μL of this solution was added to each precipitated protein sample. Samples were allowed to incubate for five minutes so that the proteins would separate from the streptavidin beads.

Whole cell lysates, whose biotinylated proteins had not been precipitated with streptavidin beads, were also prepared for gel loading. 100 μg of total protein was loaded

for each of these samples, and β -mercaptoethanol with 3X SDS was added to the protein in such a way that it was one third of the total protein volume.

Two ten lane, six percent SDS polyacrylamide were made. Precipitated proteins were loaded to one gel, keeping the beads left behind in the sample tube. Non-precipitated lysates were loaded to another gel. After loading, 10 more μ L of β -mercaptoethanol with 3X SDS was added to each lane in the gel. High molecular weight and kaledoscope protein standards were used for size markers (both purchased from BIORAD). Then, the proteins were subjected to SDS/PAGE at 180 Volts for 55 minutes.

After SDS/PAGE was complete, gels were placed into a transfer apparatus. The proteins were transferred to a nitrocellulose membrane (purchased from BIORAD) at 100 Volts for 75 minutes. After the transfer, the gels were placed in a Coomassie Blue stain, and the immunoblots were labeled and subjected to antibody treatments.

Detection of Proteins by Antibodies

The nitrocellulose membranes were blocked for one hour at room temperature with bovine serum albumin (BSA) 5% (purchased from SIGMA) and TBST (distilled water, 10% TBS 10X, and 0.1% Tween (purchased from ACROS)). TBS consists of 200 mM Tris base and 1370 mM NaCl. Afterwards the blots were washed three times with TBST.

The blots were incubated with rabbit anti-Mrp2 (donated by Dr. D. Keppler at the German Cancer Research Center in Heidelberg, Germany) diluted at 1:3000 with 5% BSA/TBST overnight at 4°C. Afterwards the blots were washed three times with TBST.

The blots were incubated with goat-anti rabbit HRP (purchased from BIORAD) diluted at 1:1000 with 5% non-fat milk (purchased from BIORAD) / TBS for two hours at room temperature. Afterwards, the blots were washed three times with TBSR.

Detection of Proteins via ECL

10 mL of ECL A was combined with 10 mL of ECL B (fluids purchased from Santa Cruz), and the blots were bathed in this solution for one minute. Then, the blots were placed into a transparent plastic folder, and the air bubbles were pressed out as the folder was sealed. This folder was taped into an X-Ray cassette.

X-ray films were exposed to the blots for varying amounts of time (1 second to 2 minutes) and subsequently developed. Ideal exposures were marked and scanned into Adobe Acrobat (Adobe System Inc, San Jose, CA). Protein signal intensities were quantified by the program SigmaGel (Jandel Scientific Software, San Rafael, CA).

RESULTS

Rat primary hepatocyte cultures were stimulated with various treatments, then the cell surface proteins were biotinylated and purified by streptavidin precipitation. When subject to SDS/PAGE and Western blotting (primary blot rabbit anti-Mrp2, secondary goat-anti-rabbit HRP, detect via ECL), protein MRP2 shows up strongly at 174.19 kD (Figure 10, arrow on right side).

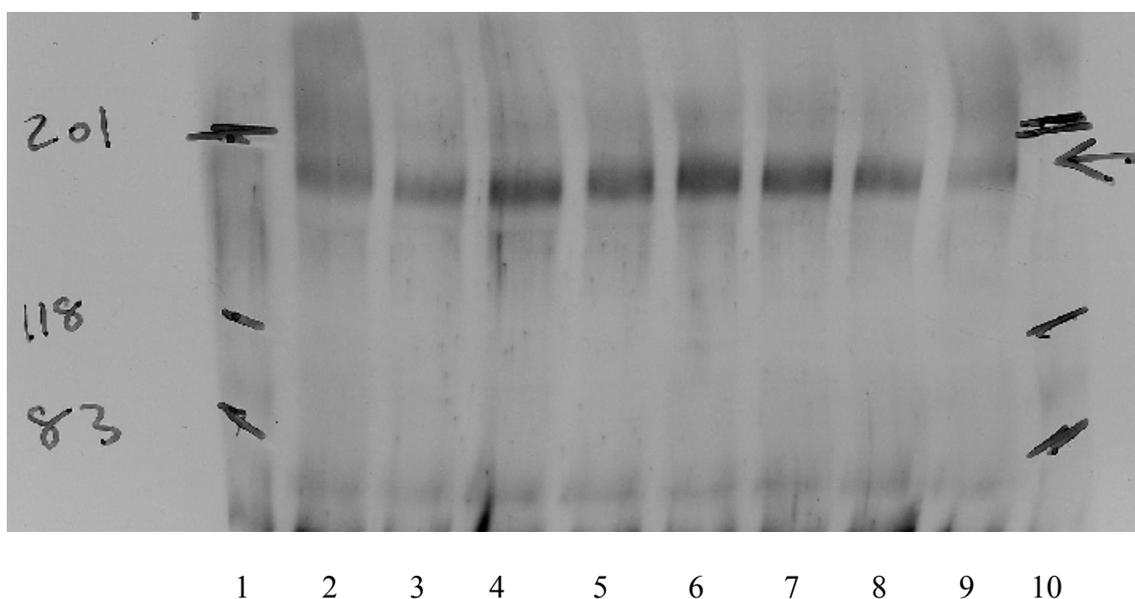


FIGURE 10: PRECIPITATION OF BIOTINYLATED Mrp2 FROM THE HEPATOCYTE CELL SURFACE (Example western 1 of 3). Radiograph developed after exposing a precipitated Mrp2 Western blot to ECL and exposing the blot to film for five seconds. Primary rat hepatocyte cells were biotinylated, and the proteins were precipitated by streptavidin beads. The precipitated proteins were subjected to SDS/PAGE on a 6% gel. The arrow on the right denotes the MRP2 band at 174.19 kD. Molecular sizes are indicated in kD at the left. Lanes 1 and 10: High Molecular Weight Marker, Lane 2: DMSO/DMSO, Lane 3: DMSO/100 uM cAMP, Lane 4: 50 uM BisA/DMSO, Lane 5: 50 uM BisA/100 uM cAMP, Lane 6: 100 uM BisA/DMSO, Lane 7: 100 uM BisA/100 uM cAMP, Lane 8: 200 uM BisA/DMSO, Lane 9: 200 uM BisA/100 uM cAMP.

Program SigmaGel was used to quantitate the Mrp2 signals from 3 independent immunoblots (table 4 and figure 11).

TABLE 4: RELATIVE INTENSITIES OF PRECIPITATED Mrp2 BANDS. Quantitative description of the effect of BisA and cAMP on translocation of Mrp2 to the canalicular membrane by listing the relative intensities of the bands from the blots of precipitated Mrp2. The data from each experiment, the dates of each experiment, and the average values are all shown.

Precipitated Samples	11/9/2004	11/10/2004	11/16/2004	Average
Sample				
DMSO/DMSO	1.00	1.00	1.00	1.00
DMSO/100 μ M cAMP	0.70	1.39	6.65	2.92
50 μ M Bis A/DMSO	1.20	1.64	4.24	2.36
50 μ M Bis A/100 μ M cAMP	0.98	1.29	4.28	2.18
100 μ M Bis A/DMSO	1.09	1.37	6.37	2.94
100 μ M Bis A/100 μ M cAMP	1.04	1.29	5.34	2.56
200 μ M Bis A/DMSO	0.75	0.95	4.02	1.91
200 μ M Bis A/100 μ M cAMP	0.42	0.81	1.27	0.83

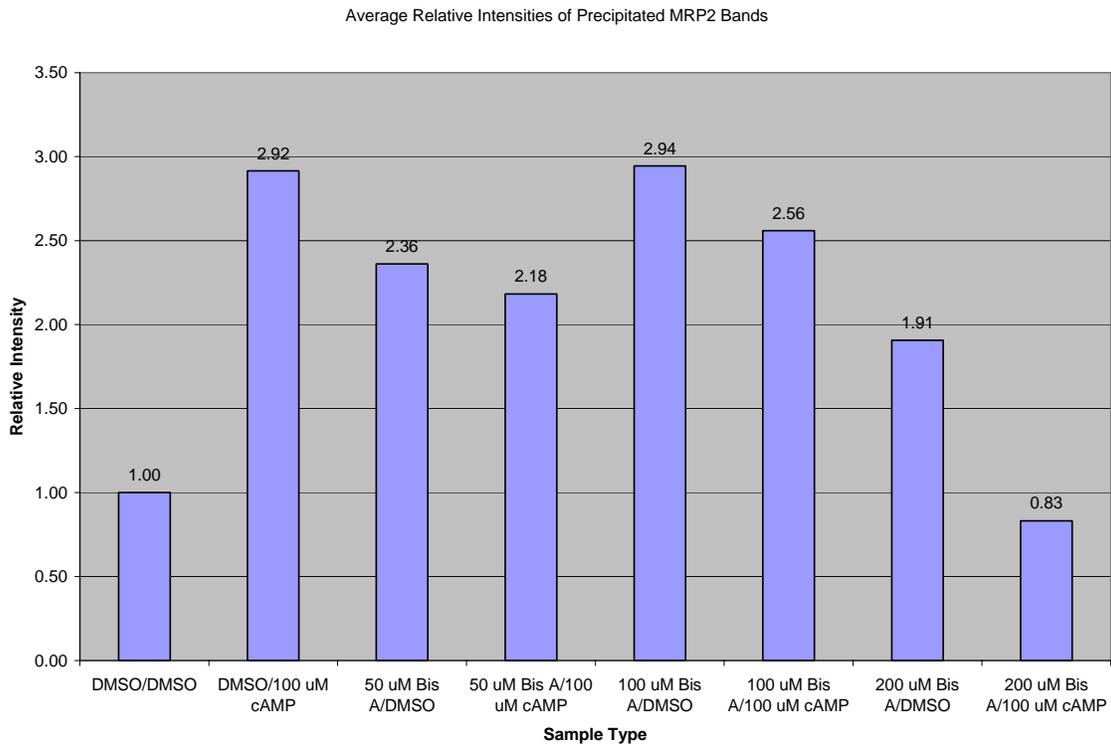


FIGURE 11: HISTOPLLOT OF THE Mrp2 SIGNALS QUANTITATED IN TABLE 4.

The quantitated data show that cAMP stimulates translocation of Mrp2, that BisA stimulates translocation of Mrp2 in a dose dependent fashion, and that the effects of BisA and cAMP are not summative, suggesting they act via the same pathway. Additionally, at around 200 μ M of BisA, BisA becomes less successful at translocating protein. This could be due to interference with cAMP, or perhaps BisA is toxic when in high concentrations. This will be elaborated upon in the discussion.

An important part of the experimental procedure was to establish that the total amount of Mrp2 in each hepatocyte lysate sample was approximately the same. If this was not the case, then it would be possible that an increase in Mrp2 intensity in the membrane was not due to increased translocation. Figure 12 is a close-up of a Western blot of Mrp2 from the whole cell lysate (non-streptavidin-precipitated protein). Visually, it appears that the Mrp2 intensities are similar. Table five and figure 13 support this idea, showing that there is only a 13% variation of total Mrp2 among all of the samples. There was no statistical difference between the intensities of the protein (T-test, $p = 0.98$).

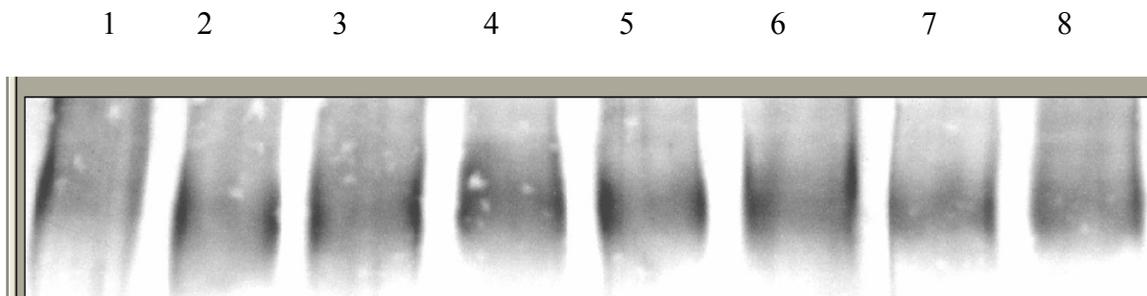


FIGURE 12: DIRECT QUANTITATION OF PROTEIN Mrp2 LEVELS IN THE HEPATOCYTE LYSATES. Image of a radiograph developed after exposing an Mrp2 whole cell lysate Western blot to ECL and exposing the blot to film for ten seconds. The whole cell lysates were subject to SDS/PAGE on a 6% gel for 55 minutes at 180 V. See the method section for more detail regarding the experimental techniques. Lane 1: DMSO, Lane 2: DMSO/100 μ M cAMP, Lane 3: 50 μ M BisA/DMSO, Lane 4: 50 μ M BisA/100 μ M cAMP, Lane 5: 100 μ M BisA/DMSO, Lane 6: 100 μ M BisA/100 μ M cAMP, Lane 7: 200 μ M BisA/DMSO, Lane 8: 200 μ M BisA/100 μ M

cAMP. Bands appear to have similar intensities of darkness, suggesting the Mrp2 protein was loaded in similar amounts.

TABLE 5: QUANTITATION OF Mrp2 PROTEIN LEVELS FROM FIGURE 13. Table five shows a quantitative description of the intensities of MRP2 bands from whole cell lysate samples. The dates of each experiment are shown. Although somewhat variable, the standard deviation of the values is only 0.14 (the average is 0.77). Statistically, there is no difference (t test, p = 0.98)

Whole Cell Lysates Sample	11/9/2004	11/10/2004	11/16/2004	Average
DMSO/DMSO	1.00	1.00	1.00	1.00
DMSO/100 μ M cAMP	0.65	1.25	0.66	0.86
50 μ M Bis A/DMSO	0.94	1.24	0.50	0.90
50 μ M Bis A/100 μ M cAMP	0.80	1.01	0.39	0.73
100 μ M Bis A/DMSO	0.68	1.05	0.35	0.69
100 μ M Bis A/100 μ M cAMP	0.89	0.88	0.30	0.69
200 μ M Bis A/DMSO	0.50	1.05	0.25	0.60
200 μ M Bis A/100 μ M cAMP	0.70	0.96	0.38	0.68

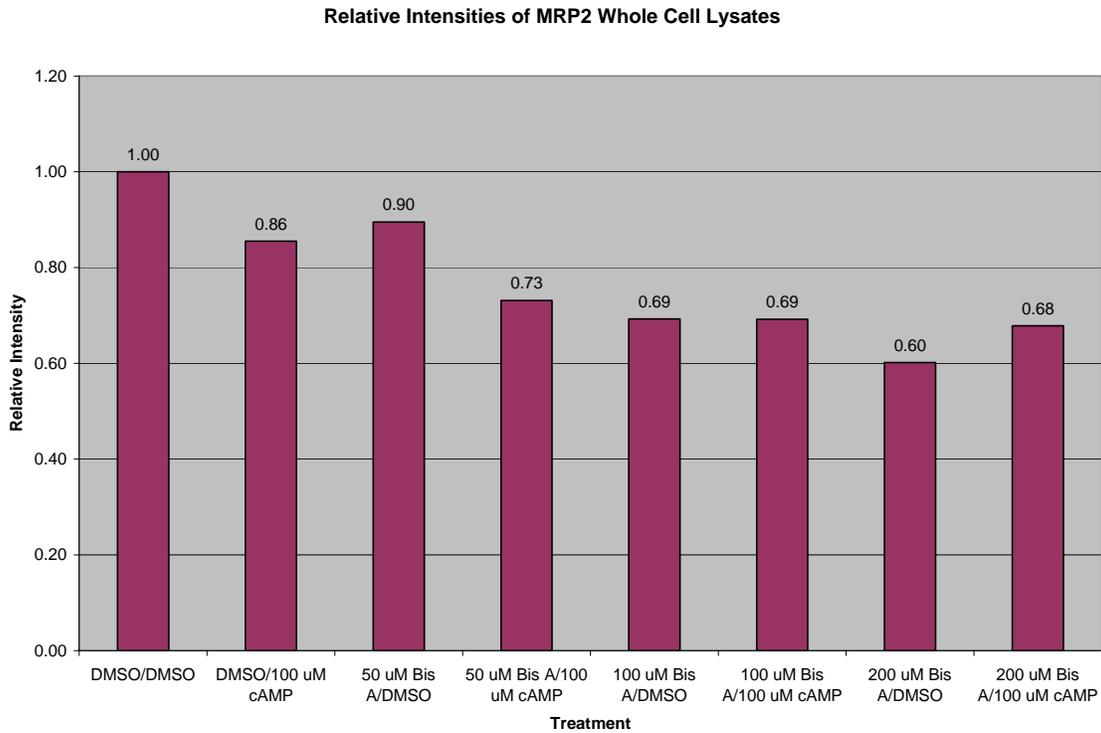


FIGURE 13: HISTOPLLOT OF THE DATA FROM TABLE 5. Represents the data from table 5 graphically showing approximately equal loading of protein Mrp2 input for streptavidin precipitation.

As stated in the project purpose, a fundamental part of this project was to show that BisA stimulates the translocation of Mrp2 to the hepatocyte cell surface. Figure 14 represents a plot of the average Mrp2 protein levels versus BisA concentration taken from the previous data. The data very clearly illustrates that BisA can indeed stimulate translocation of Mrp2 from the cytosol to the membrane in a dose dependent fashion. For some reason, between 100 uM and 200 uM of BisA, BisA starts losing its ability to translocate Mrp2. The reasons for this are unknown, but it is possible that at such high concentrations, BisA can be toxic or losing its specificity.

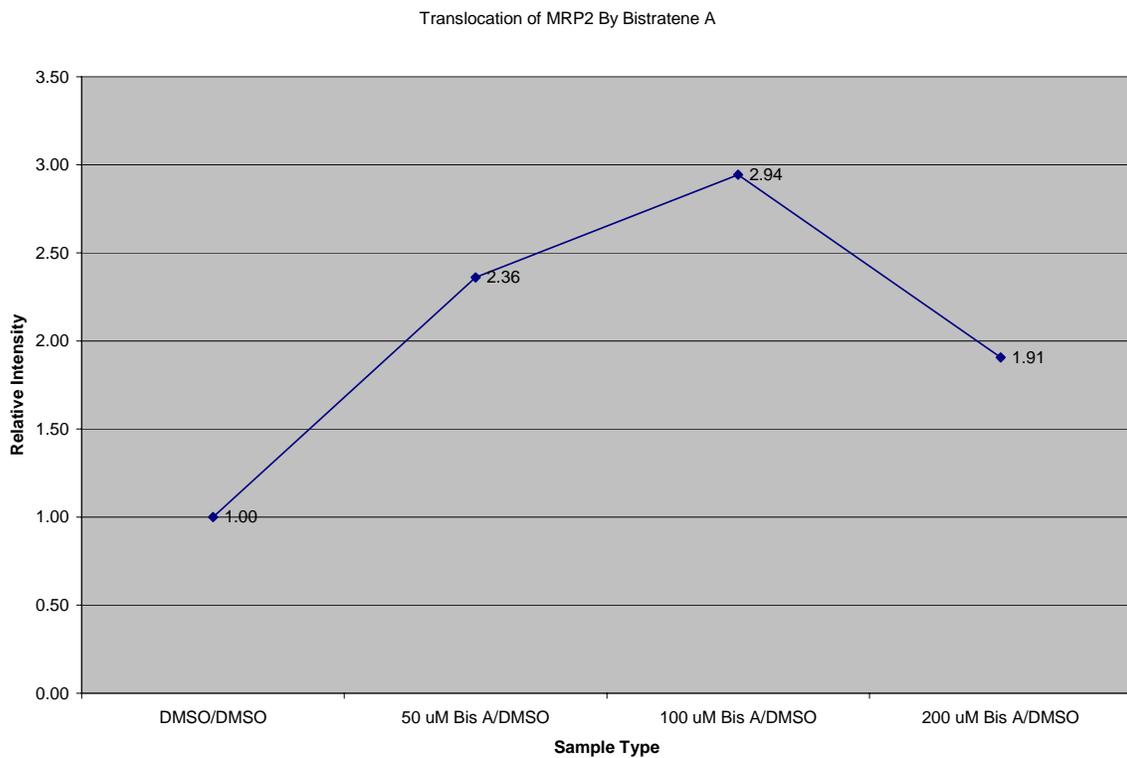


FIGURE 14: BIS-A TRANSLOCATES Mrp2 TO THE HEPATOCYTE MEMBRANE. Plot of the average of 3 independent experiments discussed previously. BisA activates translocation up through 100 μ M, but at 200 μ M, it begins to lose this ability.

If BisA and cAMP act by separate pathways, one would expect treating a cell with both chemicals to double the translocation of Mrp2. However, if they acted via the same pathway, the translocation when treating the cells with both chemicals would be the same as if treating with one of the chemicals. The data (figure 15, replotted from figure 13) supports the latter, as there is no statistical difference between the translocation from BisA alone or BisA with cAMP (2 sample T test, $p = 0.42$). Thus, the data support that these two activators translocate Mrp2 by the same pathway. Since it is already known that BisA activates PKC δ , and it has been shown that cAMP translocates PKC δ (Gillin et al, 2004), it can be deduced that cAMP translocates Mrp2 by activating PKC δ .

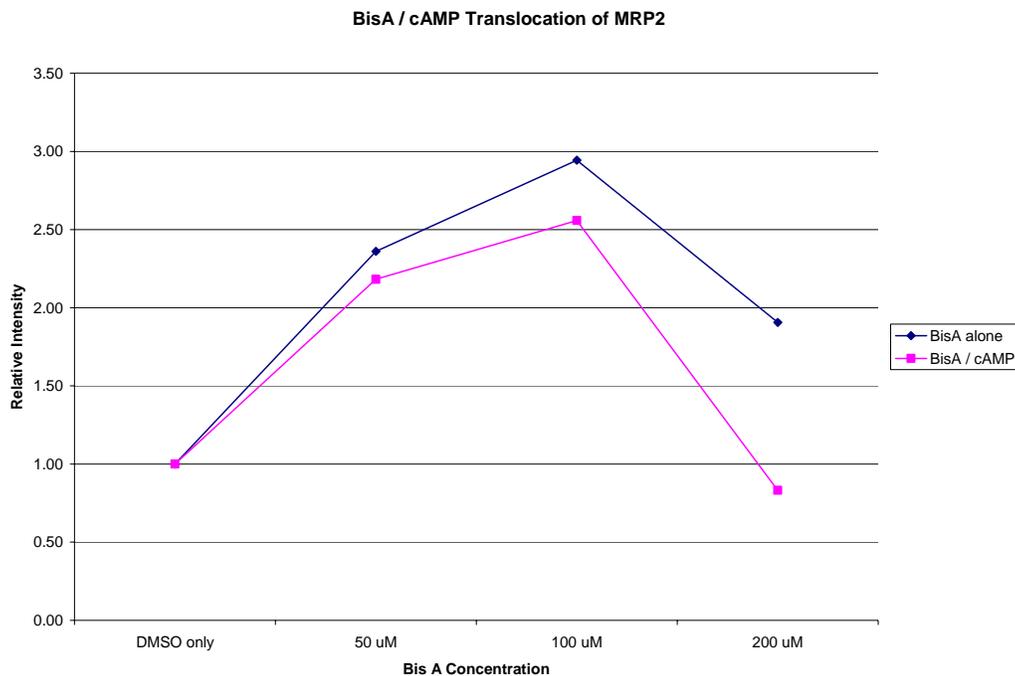


FIGURE 15: THE TRANSLOCATION OF Mrp2 BY cAMP AND BIS-A IS NOT ADDITIVE. This figure compares the translocation of Mrp2 to the hepatocyte membrane by BisA alone and BisA plus cAMP. As already shown, BisA activates translocation of Mrp2 to the membrane up until 200 uM BisA. BisA, when combined with 100 uM cAMP, also stimulates translocation of Mrp2 to the membrane up until 200 uM BisA. It is important to note that the effects of BisA and cAMP are not summative; they are the same as BisA alone (T test, $p =$

0.42), suggesting that BisA and cAMP are acting through the same pathway.

DISCUSSION

The purpose of this report was to investigate whether cAMP stimulates the translocation of Mrp2 by activating PKC δ . The Anwer lab's model of Mrp2 translocation from the hepatocyte cytosol to the canalicular membrane suggested that this is the case. It had already been shown that Mrp2 is downregulated in cholestasis (Trauner et al, 1997; Beuers et al, 2003). It had also been shown that cAMP can not only stimulate translocation of Mrp2 to the canalicular membrane under normal conditions (Roelofsen et al, 1998), but also that cAMP can also restore Mrp2 translocation under estradiol-induced cholestasis (Mottino et al, 2002).

Anwer's lab at Tufts University School of Veterinary Medicine was able to build upon this information already presented by associating the cAMP-induced translocation of Mrp2 with the translocation of a protein kinase named PKC δ . Their experiment showed that cAMP stimulated translocation of PKC δ to the canalicular membrane in a dose dependent fashion (Gillin et al, 2004). Thus, they hypothesized that cAMP may translocate Mrp2 to the canalicular membrane by activating PKC δ . Further experiments performed by the Anwer lab supported this idea, because Rottlerin, an inhibitor of PKC δ , prevented cAMP from translocating Mrp2 (Gillin et al, 2004). In effect, they had provided evidence cAMP acts via PKC δ , because when the inhibitor of PKC δ was used to treat the cells, cAMP's action was hindered.

This Anwer lab experiment provided reason to test the hypothesis that cAMP translocates Mrp2 via activation of PKC δ by treating the cells with a known activator of PKC δ , Bistratene A. This was the goal of the MQP. Rat hepatocyte cell membrane

proteins were detected by biotinylating the cells (biotin binds only to surface proteins under the experimental conditions used). Mrp2 was detected from that solution via specific antibodies through Western blotting.

Figure 16 represents the working hypothesis (and the alternate hypothesis, which would be that PKC δ and cAMP act via separate pathways to translocate Mrp2). If PKC δ and cAMP acted via separate pathways, then if both were used to treat the cells, they would have summative effects on the translocation of Mrp2. This is represented by the pink line in the figure, which is a curve with the expected relative intensity (sum of cAMP and respective BisA translocations). If PKC δ and cAMP acted via the same pathway, the translocation should not differ if only BisA were used to treat the cells or if BisA and cAMP were used together to treat the cells.

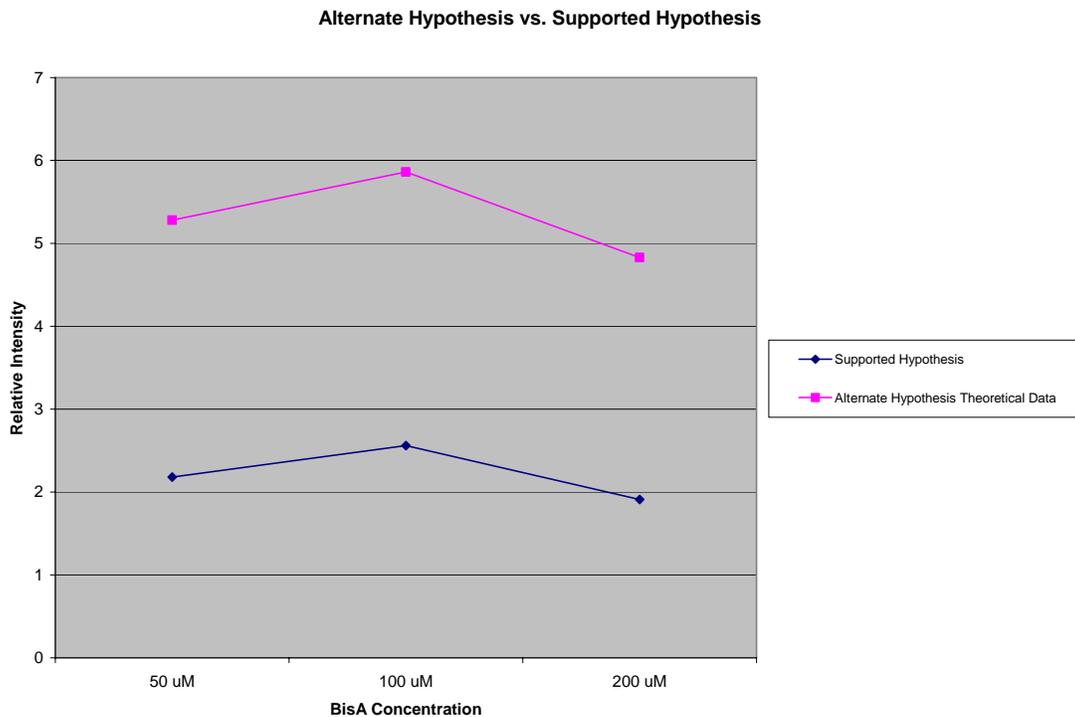


FIGURE 16: SUPPORTIVE AND ALTERNATIVE HYPOTHESES FOR THE EFFECTS OF cAMP AND BIS-A ON Mrp2 TRANSLOCATION. The main hypothesis of this MQP is that cAMP induces the translocation of Mrp2 to the hepatocyte membrane using PKC-delta, thus the effects of cAMP (which translocates Mrp2) plus BisA (a known PKC-delta activator) should not be additive (blue line). The alternative hypothesis (pink line) would be that BisA and cAMP activate translocation of Mrp2 to the membrane via different pathways, so if both BisA and cAMP were used to treat the cells, then the effects would be summative, and there would be twice as much translocation as if BisA were used alone. Our data support the former hypothesis.

Table four, figure 11, and figure 14 were successful in showing that BisA does stimulate translocation of Mrp2 into the hepatocellular membrane until a concentration of 200 μ M. Table five and figure 13 show that the amounts of whole cell lysate did not vary. This indicates that the amounts of Mrp2 loaded onto the gel are equal. Furthermore, the amounts of total Mrp2 in the protein are equal. Therefore, the differences in the amount of Mrp2 present in the membrane are indeed due to translocation.

The reason why BisA starts to lose its effect is unclear; there is the possibility that at a higher concentration, BisA can become toxic to the cell or lose its specificity and start interacting with other processes in the cell. It is likely that there is some kind of interaction between BisA and cAMP at high concentrations of BisA, because at 200 μ M of BisA only, the membrane presence of Mrp2 is 1.91, but at 200 μ M of BisA and 100 μ M of cAMP, the membrane presence is only 0.83.

Nevertheless, results obtained with 50 and 100 μ M BisA are consistent with the idea that PKC δ alone is responsible for the translocation of Mrp2. More importantly, in figure 15, there is no significant difference between the translocation of Mrp2 by BisA alone or by BisA and cAMP combined, suggesting that BisA and cAMP are translocating

Mrp2 via the same pathway. Since BisA is the known activator of PKC δ , and PKC δ translocates Mrp2, it can be assumed that cAMP translocates Mrp2 via PKC δ , especially since it has already been shown that cAMP translocates PKC δ to the membrane (Gillin et al, 2004). The specifics of the interaction between the translocation of PKC δ and the translocation of Mrp2 have yet to be determined, and this would be interesting to examine in a future experiment. Figure 17 provides a schematic of the pathway proposed by this paper.

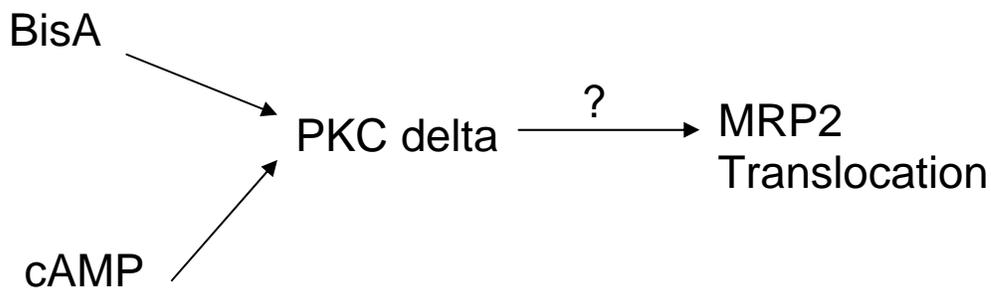


FIGURE 17: cAMP TRANSLOCATES Mrp2 VIA PKC δ ACTIVATION. Schematic of the pathway that the results of this MQP support. The data support the idea that BisA and cAMP activate Mrp2 translocation via the same pathway. Since BisA is an activator of PKC δ , and since previous studies have shown that cAMP translocates PKC δ to the membrane, this same pathway can be assumed to be PKC δ . The interaction between PCK δ and Mrp2 translocation is still unknown.

There is one weakness of this paper. The data for this is limited, as only three experiments were performed on one cell lysate. However, even though there are only three experiments performed, the data was replicated three times, providing strength to the conclusions. The reason for this low amount of experiments is because the MQP team originally was working on a different experiment that aimed to determine the dephosphorylation site of Ntcp. After a month of training and learning experimental techniques, the team spent roughly two months on the Ntcp project. However, the team

was unable to obtain protein concentrations high enough to produce valid results, because the protein signals on the Western blots were too weak. The lab thought the reason for this was poor transfection success of the protein. Since Mrp2 was obtained straight from rat liver and not via transfection, protein levels were much higher, and the group was able to obtain data from the Mrp2 experiments. The team realizes that the data is limited, but because of the lack of success in obtaining Ntcp data, an experiment had to be quickly performed before the graduation deadline. However, even though the earlier Ntcp experiments were not successful, the team did learn invaluable lab techniques.

This experiment should be continued in order to provide more data to support the hypothesis with more strength. Currently, the Anwer lab is studying PKC δ 's association with Mrp2 during the translocation, and results of these experiments are still pending. Since PKC δ is a protein kinase, it is likely that it phosphorylates a protein that continues downstream to translocate Mrp2, or it phosphorylates Mrp2 itself. This mechanism by which PKC δ causes translocation of Mrp2 should be the focus of future studies. Also, a cell viability study should be performed in order to validate the idea that BisA is toxic at high concentrations, as well as studies showing that the activation of PKC δ still occurs with BisA concentrations of 200 μ M, and whether 200 μ M BisA and 100 μ M cAMP together may actually inhibit PKC δ .

In conclusion, this MQP has provided preliminary data that Mrp2 is translocated from the hepatocellular cytosol to the canalicular membrane when cAMP activates PKC δ . These data provide a foundation for the support of this hypothesis, helping to provide more knowledge about the disease state of cholestasis. Hopefully, with these

data, the molecular pathophysiology of cholestasis will be better characterized, and more successful treatments for the disease will be developed.

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