Bile Formation: Do Not Ignore the Role of Plasma Membrane–Cytoskeleton Linking Proteins

Kikuchi S, Hata M, Fukumoto K, Yamane Y, Matsui T, Tamura A, Yonemura S, Yamagishi H, Keppler D, Tsukita S, Tsukita S. Radixin deficiency causes conjugated hyperbilirubinemia with loss of Mrp2 from bile canalicular membranes. Nat Genet 2002;31:320-325. (Reprinted with permission.)

Abstract

The ezrin-radixin-moesin (ERM) family of proteins crosslink actin filaments and integral membrane proteins. Radixin (encoded by Rdx) is the dominant ERM protein in the liver of wildtype mice and is concentrated at bile canalicular membranes (BCMs). Here we show that Rdx(−/−) mice are normal at birth, but their serum concentrations of conjugated bilirubin begin to increase gradually around 4 weeks, and they show mild liver injury after 8 weeks. This phenotype is similar to human conjugated hyperbilirubinemia in Dubin-Johnson syndrome, which is caused by mutations in the multidrug resistance protein 2 (MRP2, gene symbol ABCC2), although this syndrome is not associated with overt liver injury. In wildtype mice, Mrp2 concentrates at BCMs to secrete conjugated bilirubin into bile. In the BCMs of Rdx−/− mice, Mrp2 is decreased compared with other BCM proteins such as dipeptidyl peptidase IV (CD26) and P-glycoproteins. In vitro binding studies show that radixin associates directly with the carboxy-terminal cytoplasmic domain of human MRP2. These findings indicate that radixin is required for secretion of conjugated bilirubin through its support of Mrp2 localization at BCMs.

Comments

In mammals, bile exerts a range of vital physiologic functions from solubilization and digestion of dietary lipids and lipid-soluble nutrients to elimination of lipid-soluble substances, drugs, and toxic compounds.[1] To this end, bile formation has unceasingly kindled the interest of both the clinical and the experimental hepatologist. Bile is a primarily aqueous but complex solution, comprised of electrolytes, organic anions (*i.e.*, bile acids, bilirubin), lipids (*i.e.*, cholesterol), proteins, amino acids and peptides, nucleotides, vitamins, heavy metals, and drugs. Bile secretion represents an intricate process reflecting the structural and functional interplay of the 2 epithelial cells of the liver: the hepatocytes and the cholangiocytes (*i.e.*, the cells that line the intrahepatic bile ducts). Formation of bile is initiated at the canalicular (*i.e.*, apical) domain of the hepatocyte because this polar epithelial cell expedites the excretion into bile ducts of osmotically active substances such as bile acids and other organic anions via specific plasma membrane transport proteins against their concentration gradient.[1] The primary bile is then modified as it percolates through the bile ducts via a combination of secretory and absorptive processes performed by cholangiocytes that contribute ~40% of total bile daily production in humans.[2]

One of the cloned transporters of the canalicular membrane of hepatocytes is the canalicular multispecific organic anion transporter (cMOAT) or multidrug resistance protein 2 (MRP2 in human, Mrp2 in animals; gene symbol ABCC2).[3] Mrp2 is a member of the ABC family of transporters.[3] Studies have reported a microtubule-dependent, cyclic adenosine monophosphate–stimulated sorting of Mrp2 intracellular vesicles to the apical domain in hepatocyte couplets.[4] Mrp2 facilitates the efflux of different amphiphilic molecules from hepatocytes into bile. Such substances include bilirubin diglucuronides, glutathione conjugates, glutathione disulfide, sulfated and glucuronidated products, including bile salts, drugs, and exogenous compounds. Thus, Mrp2 is an important hepatocyte plasma membrane carrier that contributes to bile formation and affects bile composition. The interesting observation that a naturally occurring mutant Wistar rat (*i.e.*, the TR-rat) has a functionally defective Mrp2 gene associated with conjugated hyperbilirubinemia[5] led to the discovery that mutations of MRP2 cause Dubin-Johnson syndrome in humans.[6]

The findings of Kikuchi et al.,[7] recently reported in *Nature Genetics*, indicate that radixin (Rdx), a plasma membrane–cytoskeleton linking protein, is critical for secretion of conjugated bilirubin in bile by influencing the cellular localization of Mrp2. Radixin is highly homologous to 2 other plasma membrane-cytoskeleton linking proteins, namely, ezrin and moesin. In fact, this family of proteins known as ezrin-radixin-moesin (ERM) functions as a cross-linker between actin filaments and integral plasma membrane proteins, such as cell adhesion molecules and transporters.[8] In rodent liver, radixin represents the predominant protein among the ERM family and is primarily localized at the canalicular domain of hepatocytes.[9] To better understand the role of radixin, Kikuchi et al. used homologous recombination in embryonic stem cells to generate 2 phenotypically identical lines of mice, which were homozygous for a targeted disruption of the

Rdx locus. The radixin deficient (Rdx−/−) mice grew without evidence of weight, size, or reproductive abnormalities up to 13 months in comparison with the wild-type animal. Of interest, however, in the Rdx−/− mice the serum concentrations of conjugated bilirubin started to increase progressively at about 4 weeks of age and reached approximately a 15-fold elevation at 16 weeks compared with the wild-type animal. At 16 weeks, the mean serum concentration of alkaline phosphatase and aspartate transaminase in the Rdx−/− rodent were almost 4-fold higher than the wild-type animal. During the same time period, liver histology of the Rdx−/− mice revealed moderate degenerative changes of hepatic morphology.

In humans, a comparable phenotypic equivalent of the Rdx−/− mice is the Dubin-Johnson syndrome, an autosomal-recessive disorder characterized by chronic, conjugated hyperbilirubinemia. However, compared with the Rdx−/− mice, these patients usually have normal liver enzymes and lack notable hepatocyte damage on liver biopsy apart from a characteristic, coarse-granular, brown pigment deposition of hepatocytes. Granted that mutations of the MRP2 gene cause Dubin-Johnson syndrome,[6] Kikuchi et al. postulated that the Rdx−/− mice develop conjugated hyperbilirubinemia via loss of Mrp2 from the hepatic canalicular membranes. To test their hypothesis, the investigators executed a number of experiments. First, they showed almost undetectable Mrp2 immunofluorescence on the bile canaliculi of the Rdx−/− rodent compared with the wild-type animal at 4 weeks. This observation was noted in the absence of structural damage of the hepatocyte canalicular membrane as indicated by normal immunofluorescence microscopy of markers of canalicular integrity. Moreover, the concentration of P-glycoproteins—a family of bile canaliculi transporters—and CD26—an apical marker of hepatocytes—were not declined significantly in the bile canaliculi of the Rdx−/− mice at 4 weeks. Second, to address whether radixin deficiency decreased the total amount of Mrp2 in hepatocytes or solely affected its apical distribution the investigators performed Northern and Western blotting experiments. In brief, they showed that in young Rdx−/− mice (up to 4 weeks) Mrp2 messenger RNA and its protein expression level were not affected; however, radixin deficiency caused mistargeting of Mrp2 to canalicular hepatocyte membranes, in a relatively selective fashion as compared with P-glycoproteins and CD26. After 4 weeks, though, progressive hepatocyte canaliculi destruction affected the localization of marker molecules (*i.e.*, P-glycoproteins and CD26) thus, preventing any further experimentation. Third, ultra-thin–section electron and scanning electron micrographs of the Rdx−/− mice liver revealed lack of canaliculi microvilli, resulting in flattened apical surfaces as compared with the wild-type rodent. This finding was conforming with the evidence that radixin is the predominant of the ERM proteins at the canalicular microvilli and that the latter are significant for the creation of apical microvilli.[8]

Furthering these novel observations, Kikuchi et al. then pursued *in vitro* experiments to unravel the mechanism by which the loss of radixin specifically alters the expression of Mrp2 on the bile canaliculi in young Rdx−/− mice. First, using immunofluorescence microscopy they showed that Mrp2 and radixin are colocalized at the hepatocyte canalicular membranes of wild-type rodents. In addition, isolated bile canaliculi derived from wild-type mice were immunoprecipitated with a polyclonal Mrp2 antibody and radixin was detected on the Mrp2, but not on the CD26 (control)-derived, immunoprecipitates. Second, by using MDCK cells, the investigators showed that the C-terminal cytoplasmic domain of Mrp2 is required for the apical targeting of Mrp2 in hepatocytes. Third, binding studies revealed that the C-terminal cytoplasmic domain of Mrp2 couples to the N-terminal half, but not the C-terminal half, of radixin. The findings suggest that radixin may bind either directly to the C-terminal tail of Mrp2 at the bile canaliculi or indirectly via the PDZ domain-containing proteins such as the ERM-binding phosphoprotein 50 (EBP50), NH3 kinase A regulatory protein (E3KARP), or both. Because the specific localization of Mrp2 at the hepatocyte canalicular membranes was influenced in a relatively selective fashion in the liver of the Rdx−/− mice, it appears that either a direct or indirect interaction of radixin with C-Mrp2 is necessary for the Mrp2 protein to target to its accurate localization at the apical domain of hepatocytes.

All together, these observations suggest that radixin is important in Mrp2 sorting to the canalicular hepatocyte domain. Apical loss of Mrp2 in the Rdx−/− mice causes apparent conjugated hyperbilirubinemia despite normal Mrp2 protein structure and expression. Kikuchi et al.'s study generates additional questions. Is radixin affecting the function of other hepatic or cholangiocyte plasma membrane transporters apart from Mrp2? Moreover, what is the importance of ERM family in bile secretion? Of interest, interruption of the EBP50 signaling complex by overexpession of PDZ1 inhibits cyclic adenosine monophosphate–dependent Cl^- secretion in a cholangiocarcinoma cell line.[9] Beuers et al.[10] has reported that tauroursodeoxycholic acid facilitates insertion of Mrp2 into canalicular hepatocyte membranes and stimulates organic anion secretion in a protein kinase C–dependent fashion. It now will be interesting to study whether this effect of tauroursodeoxycholic acid on Mrp2 is radixin

dependent. Finally, are phenotypes of human conjugated hyperbilirubinemia (*i.e.*, a variant of Dubin-Johnson syndrome or other comparable entities) caused by mutations of radixin? Relevant to this possibility is the recent work by Groman et al.[11] in which variant cystic fibrosis phenotypes were reported in the absence of CFTR mutations.

With the introduction of the concept of hepatic epithelial-cell polarity, the advent of cellular and molecular techniques, and development of novel experimental models, we have witnessed an unparalleled progress in unraveling the mechanisms of bile formation. We now appreciate that molecules, such as the plasma membrane transporters/exchangers/channels, a number of cytoplasmic proteins and vesicles, as well as the cytoskeleton of hepatic epithelia, all interact in a harmonized fashion to form bile. Disarray of even one component of this complex machinery can cause cholestasis and liver damage. Over the past decade, efforts at discovering and cataloguing the structure and topography of bile formation-related molecules in hepatic epithelia have been fruitful. As we move ahead, however, many questions remain. How do the molecules involved in bile formation interact with each other in hepatic epithelia? Are there any pivotal players among them? What is the best way to estimate their contribution in bile formation? And last but not least, with the concepts and experimental tools of genetic variation (*i.e.*, single nucleotide polymorphisms) available to us, are specific variants of these molecules or of their regulatory domains significant in affecting bile production in humans in either health or disease? Until we can address these queries, however, and certainly afterward, elegant animal model studies such as the one by Kikuchi et al. will be critical.

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