Proteomic Analysis of Ischemia-Reperfusion Injury upon Human Liver Transplantation Reveals the Protective Role of IQGAP1

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Ischemia-reperfusion injury (IRI) represents a major determinant of liver transplantation. IRI-induced graft dysfunction is related to biliary damage, partly due to a loss of bile canaliculi (BC) integrity associated with a dramatic remodeling of actin cytoskeleton. However, the molecular mechanisms associated with these events remain poorly characterized. Using liver biopsies collected during the early phases of organ procurement (ischemia) and transplantation (reperfusion), we characterized the global patterns of expression and phosphorylation of cytoskeleton-related proteins during hepatic IRI. This targeted functional proteomic approach, which combined protein expression pattern profiling and phosphoprotein enrichment followed by mass spectrometry analysis, allowed us to identify IQGAP1, a Cdc42/Rac1 effector, as a potential regulator of IRI-induced cytoskeleton remodeling and maintenance of BC integrity. Cell fractionation and immunohistochemistry revealed that IQGAP1 expression and localization were affected upon IRI and related to actin reorganization. Furthermore using an IRI model in human hepatoma cells, we demonstrated that IQGAP1 silencing decreased the basal level of actin polymerization at BC periphery, reflecting a defect in BC structure coincident with reduced cellular resistance to IRI. In summary, this study uncovered new mechanistic insights into the global regulation of IRI-induced cytoskeleton remodeling and led to the identification of IQGAP1 as a regulator of BC structure. IQGAP1 therefore represents a potential target for the design of new organ preservation strategies to improve transplantation outcome. Molecular & Cellular Proteomics 5:1300–1313, 2006.

Orthotopic liver transplantation is the only viable therapeutic option for the treatment of end stage liver diseases. All donor organs experience some degree of preservation damage related to cold ischemia-reperfusion injury (IRI).1 IRI has been linked to graft primary dysfunction (1) and can lead to vascular and biliary complications (2) such as intrahepatic cholestasis, a pathologic state of reduced bile formation or flow, and posthepatic jaundice (3). These conditions involve damage to the biliary tree at the level of either cholangiocyte membrane integrity (4) or the bile canaliculi (BC) (5).

BC are structural tubules that form the most proximal intrahepatic secretory channels and carry bile secreted by the hepatocytes toward the bile duct to the gall bladder. The BC lumen, delimited by the apical membrane of two or more adjacent hepatocytes, is separated from the basolateral membranes of the hepatocytes by the presence of tight junctions supported by adherens junctions (AJ) (6). The cytoplasmic facem of the BC membrane is associated with a dense cytoskeletal network that mostly consists of actin microfilaments and cytokeratin intermediate filaments (7). Treatment of rat liver with compounds that alter actin polymerization, such as cytochalasin B and phalloidin, leads to the alteration of BC structure (canaliculi dilatation and loss of microvilli) as well as contractile properties (8, 9). In addition, a major actin cytoskeleton alteration around the hepatocyte cell membrane has been reported in human liver in response to IRI and has also been related to an impairment of BC contraction, tight junction permeability, and subsequent maintenance of a normal bile flow during the postoperative period (10, 11). An intact actin network is therefore required for BC integrity to ensure proper bile secretion, i.e. both bile polarized transport out of the hepatocytes and maintenance of the blood-biliary barrier. However, although the observed actin cytoskeleton remodeling and alteration of BC structure were reported as primary targets of IRI, critical for graft recovery (5), the underlying molecular mechanisms integrating these events remain largely unknown.

In this work, we combined a targeted functional proteomic analyses of human liver biopsies collected during different phases of the transplantation with functional validation both in

1 The abbreviations used are: IRI, ischemia-reperfusion injury; AJ, adherens junctions; BC, bile canaliculi; F-actin, filamentous actin; shRNA, short hairpin RNA; TRITC, tetramethylrhodamine isothiocyanate; PKA, cAMP-dependent protein kinase; MAPK, mitogen-activated protein kinase.
by chromatography on chelating Sepharose matrices loaded previously as non-significant.

A slope variation between a linear trend line was then calculated for each protein expression per time point summed over all proteins present (in both ischemia used in the calculation considering I0 or R0 as internal standard analyzed by scanning densitometry. Relative quantification values were processed for immunoblot analysis as described previously (15). An-independent protein pools of three liver biopsies. Samples were then processed for immunoblot analysis as described previously (14). Equal protein amounts from three independent ischemia or reperfusion liver biopsies were pooled for each time point to reduce interindividual variability. Immunoblot experiments were carried out in duplicate on three independent protein pools of three liver biopsies. Samples were then processed for immunoblot analysis as described previously (15). Antibodies used in this study are described in Supplemental Table 1.

Protein Extraction and Immunoblot—Proteins were extracted from tissue or cultured cells after solubilization using Triton X-100 as described previously (12–14). This method leads to selective extraction/solubilization of mainly soluble proteins (12). Fractionation experiments were performed as described previously (14). Equal protein amounts from three independent ischemia or reperfusion liver biopsies were pooled for each time point to reduce interindividual variability. Immunoblot experiments were carried out in duplicate on three independent protein pools of three liver biopsies. Samples were then processed for immunoblot analysis as described previously (15). Antibodies used in this study are described in Supplemental Table 1.

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Phosphoprotein Enrichment—Protein extracts were either resolved by chromatography on chelating Sepharose matrices loaded previously with either 0.1 M ZnCl2 or 0.1 M GaCl3 as recommended by the manufacturer (Chelating Sepharose Fast Flow, Amersham Biosciences) and as described previously (16). Following extensive washing (>100 bed volumes), proteins were then eluted using Laemmli sample buffer and resolved by one-dimensional SDS-PAGE.

Mass Spectrometry—Following resolution of the above proteins by one-dimensional SDS-PAGE and Coomassie R-250 staining, each band was excised, transferred to a 96-well tray, dehydrated with acetonitrile, and washed by two cycles of 10 min in 100 mM (NH4)2CO3 before the addition of an equal volume of acetonitrile. The destained gel slices were then treated for 30 min with 10 mM dithiothreitol to reduce cysteiny1 residues and for 20 min with 55 mM iodoacetamide to effect alkylation. After an additional round of (NH4)2CO3 and acetonitrile washes, the slices were extracted with acetonitrile at 37 °C. They were then incubated with trypsin (6 ng/µl in 50 mM (NH4)2CO3) for 5 h at 37 °C, and the peptides were extracted in 1% formic acid, 2% acetonitrile followed by two further extractions with additions of acetonitrile. All treatments were performed robotically using a MassPrep work station (Micromass, Manchester, UK). All extracts from a given gel slice were combined in the corresponding well of another 96-well tray. This tray was transferred to the autosampler of a CapLC system (Waters) for mass spectrometry analysis on a QTOF2 (Micromass) upgraded with Embedded Personal Computer Acquisition System (EPCAS) electronics. A volume of 20 µl of sample was injected at a flow rate of 30 µl/min with pump C to a µ-Precolumn™ (LC Packings, Amsterdam, The Netherlands) filled with C18 PepMap 100 (300-µm inner diameter × 5 mm, 5 µm, 100 Å) held on a 10-port Cheminert® valve (VICI Valco Canada, Brockville, Ontario, Canada). After 5 min of washing, the 10-port valve was actuated so the acetonitrile gradient from pumps A and B eluted the peptides toward the PicoFrit column (New Objective, Woburn, MA) filled with BioBasic® C18 stationary phase (75-µm inner diameter, 10 cm, 5 µm, 300 Å). Gradient solvent was delivered at a flow rate of 1 µl/min and was split to 200 nl/min for the PicoFrit™ column with a splitting tee. Solvent A was water (formic acid, 0.1%), and solvent B was acetonitrile (formic acid, 0.1%). The linear gradient was set from 5% B to 40% in 20 min, from 40% to 70% in 5 min, from 70 to 95% in 5 min, held at 95% for 7 min, and brought back to 5% in 10 min. The PicoFrit column was installed on a nanospray probe so that the spraying tip was near the sampling cone of the mass spectrometer. Voltage on the capillary was adjusted to get a nice plume during elution of peptides. Acquisitions were done in data-directed acquisition mode while a 1-s survey scan was first done from 350 to 1600 m/z. The four most intense doubly and triply charged ions were selected to undergo MS/MS fragmentation in 1-s scans from 50 to 2000 m/z. The collision energies were determined automatically by the instrument based on the m/z values and charged states of the selected peptides. MS/MS fragmentation stopped when the total ion current was lower than three counts or up to a maximum acquisition time of 5 s, whichever came first.

Mass Spectrometry Data Processing and Analyses—The MS/MS data were peak-listed (ProteinLynx, Micromass) and submitted to a local Mascot database search software (Matrix Science) for search analysis against the National Center for Biotechnology Information (NCBI) mammalian non-redundant database with a confidence level of 95% or greater. Specific and shared peptides with an equal or greater score than the identity score were kept and recorded for each band. All queries (MS/MS spectra submitted to Mascot) obtained from zinc and gallium matrices were then parsed, scored, and grouped using the CellMapBase algorithms (17, 18) to reduce redundancy and obtain a minimal list of proteins including all the peptides identified. Only proteins for which at least one unique peptide (i.e. peptide found specifically in their cognate protein) was found in at least two of the three replicates were retained. Nine proteins with known zinc binding properties identified from both data sets (80 and R60) were excluded from the analysis (data not shown). Functional clusters were established using the Gene Ontology annotation (www.geneontology.org).

IQGAP1 and -2 Immunoprecipitation and Pro-Q Staining—IQGAP1 and -2 were respectively immunoprecipitated from 500 µg of biopsy lysate as recommended by the antibody provider (Upstate, Charlottesville, VA). Immunoprecipitates were resolved by SDS-PAGE, and Pro-Q and SYPRO (Invitrogen) staining were performed following the manufacturer’s instructions and as described previously (19).

Plasmid Constructs—pRk5-5myc-IQGAP1 was obtained by amplifying the full-length IQGAP1 cDNA using the following oligonucleotides primers: 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CAT GTC CGC CGC AGAC GAG GGT GAC-G-3′ and 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TTA CCT CCC GTA GAA CTT TTT GGT GAG-G-3′ for Gateway cloning into pDONR201 entry vector (Invitrogen) and subsequent LR recombination in Gateway-converted pRk5myc vector according to the manufacturer’s instructions.

Cell Culture and Transfection—HeP2 cells (HB 8065) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). HuH7 cells were kindly provided by Dr. Lebrun (McGill University, Montreal, Quebec, Canada). HuH7 and HeP2 cells were plated at a density of 2×10⁵ cells/cm² and grown for 16 h in Dulbecco’s modified Eagle’s medium (Wisent, Saint Bruno, Quebec, Canada) containing 10% fetal bovine serum (Invitrogen). Cells were then transiently trans-
stained using Envision munohistochemistry, tissue sections were peroxidase-immunoconjugated phalloidin (Sigma) as described previously (21). For immunofluorescence transplantation, F-actin was labeled in ischemic and reperfusion was then counterstained using hematoxylin (Vector Laboratories, Burlington, Ontario, Canada), then dehydrated, and mounted using Permount (Fisher).

RESULTS

Actin Cytoskeleton Is Subjected to Dramatic Changes during IRI in Hepatocytes—For the purpose of this work, we specifically focused on human liver during the early phases of transplantation. We dissociated ischemia from reperfusion stresses to analyze independently the molecular events occurring during both phases. Biopsies were collected as described under “Experimental Procedures” (Fig. 1A). To characterize cytoskeleton changes occurring in human liver during transplantation, F-actin was labeled in ischemic and reperfused liver tissue sections using phalloloidin-TRITC (Fig. 1B).

Under basal conditions, F-actin most frequently concentrates at hepatocyte junctions surrounding well defined BC (I0, inset, arrow). During the 1st h of the ischemic phase, a progressive polymerization of actin was observed throughout the periphery of hepatocytes (I10 and I60). After longer ischemia, i.e. just before reperfusion (R0), F-actin structures still associate with the plasma membrane, but interestingly, cell-cell junctions appear more disorganized than at I0 with an apparent dilatation of several BC (R0, inset, arrow). The alteration of cell junction integrity was further enhanced during the reperfusion phase, coincident with the apparent disruption of F-actin structures at the cell periphery (R10 and R60). After 1 h of reperfusion, F-actin concentrates only at the hepatocyte junctions, surrounding BC, which present disorganized and dilated structures (R60, inset, arrow). These observations are in agreement with previous morphological studies reporting F-actin remodeling in human transplanted liver during the course of IRI (5, 11). Under these circumstances, the alteration of F-actin structure correlates with the loss of hepatocyte junctions and BC integrity and significantly impacts hepatocyte bile secretion and function (5).

The Expression of Cytoskeletal Regulators Is Altered upon IRI—To characterize the molecular targets involved in the IRI-induced phenotypes described above, the expression pattern of cytoskeleton-related proteins was analyzed during ischemia and reperfusion using an immunoblotting approach. The expression levels of 30 proteins related to this functional machinery were evaluated. The proteins were selected based on the availability of their respective antibodies in the laboratory (see Supplemental Table 1). These proteins were classified into four functional categories: 1) RhoGTPases and small GTPase regulators and 2) trafficking regulators and signal transducers including 3) adaptor molecules and 4) protein kinases, which had been reported to be either directly or indirectly involved in cytoskeleton remodeling. Representative immunoblots of two duplicate experiments on three independent pools of three biopsy protein extracts are shown in Fig. 2A. The blots were quantified by scanning densitometry and analyzed as described under “Experimental Procedures.” Only seven of the 30 proteins tested (p120RasGAP, RhoA, Intersectin, IR51, Src, ERK1, and p38MAPK) did not vary significantly during both ischemia and reperfusion phases. These proteins were therefore excluded from further analysis. The slope of the variation trend line was used to represent the expression patterns for the proteins showing a significant variation upon ischemia (Fig. 2B, left panel, gray bars) or reperfusion (Fig. 2B, right panel, black bars). A positive slope reflects an up-regulation of protein expression, whereas a negative slope reflects a down-regulation of protein expression. The 23 proteins whose expression was significantly modified showed similar expression patterns with a decreased expression during the ischemic phase and an increased expression during the reperfusion phase. These data suggest that the major actin-related phenotypic events occurring during both phases of the transplantation may involve the coordinated expression of numerous cytoskeleton-related proteins. More importantly, these changes occurred in early phases of ischemia or reperfusion, suggesting the rapid activation of major signaling pathways to accommodate both phenotypic changes and expression modulation.

Identification of Total Zinc and Gallium IMAC Enriched Proteins in Ischemic and Reperfused Livers—The expression of a large number of proteins known to be implicated in cytoskeleton remodeling, including several signal transducers (adaptors and kinases), was dramatically affected upon IRI (Fig. 2). A significant proportion of these are protein kinases, which are known mediators of signaling cascades through the modulation of protein phosphorylation. We therefore postulated that the molecular events resulting in the dramatic morphological phenotype observed in Fig. 1B would also include major modifications of liver phosphoproteins at different stages of the transplantation. These phosphoproteins may in turn represent functional links between IRI-induced cytoskeletal reorganization and the related defect in BC structure and...
bile secretion. Intact total phosphoprotein content was enriched on zinc- or gallium-loaded IMAC matrices and processed for mass spectrometry sequencing as described under “Experimental Procedures.” Bands corresponding to the 60-min time points for both ischemia (I60) and reperfusion (R60) were selected because phosphoprotein content was the most abundant at these time points as detected by immunoblot or in vitro phosphorylation (data not shown). We identified 225 proteins at I60 and 365 proteins at R60 that were classified into 15 functional clusters according to Gene Ontology annotation (Fig. 3, A and B, and Supplemental Table 2). A pool of 96 proteins was identified as common to the two conditions (Fig. 3C). The most striking differences between the two data sets concerned proteins involved in blood/immune response and protein biosynthesis with a 3–4-fold increase from ischemia to reperfusion. In contrast, the representation of proteins involved in amino acid and nucleotide metabolism was 50% lower in reperfusion than in ischemia. The representation of the other functional families was not dramatically affected between I60 and R60; however, the nature of the proteins

Fig. 1. Procedure for biopsy collection and F-actin staining of ischemic and reperfused liver. A, description of the protocol for biopsy collection during liver procurement and transplantation. I0, I10, and I60, 0, 10, and 60 min post-cold ischemia; R0, R10, and R60, 0, 10 and 60 min postreperfusion. B, F-actin staining using phalloidin-TRITC on 8-μm liver tissue sections 0, 10, and 60 min post-cold ischemia (I0, I10, and I60) and 0, 10, and 60 min postreperfusion (R0, R10, and R60). Arrows indicate bile canaliculi between adjacent hepatocytes. Scale bar = 30 μm. Insets represent 3× magnification.
identified for both conditions varied as shown by the limited number (only 96) of proteins identified in both data sets (Fig. 3C). This approach, although allowing a significant enrichment of phosphoproteins (16), still provided a low yield in phosphopeptides identified by mass spectrometry (<5% total peptides). This finding was not surprising because phosphopeptides do not generally represent more than 10% of the coverage for full-length proteins (22–24).

Identification of Cytoskeleton-related Zinc and Gallium IMAC Enriched Proteins—Interestingly when all the functional groups related to basic liver metabolic functions (carbohydrate, lipid, amino acid, nucleobase, and bile metabolism) were excluded, proteins related to the cytoskeleton represented one of the remaining major functional groups (8% of identified proteins). This corresponded to 17 proteins identified at the 60-min ischemia time point (Table I) and 28 proteins at the 60-min reperfusion time point (Table II). Only five proteins were common to both conditions (Fig. 3D and bold in Tables I and II), thus suggesting the occurrence of different mechanisms during the two phases of the transplantation. The proteins identified include (i) structural components of the cytoskeleton (actins, tubulins, keratins, and plastin), (ii) proteins involved in filament assembly (microtubule-associated proteins, F-actin capping protein, and members of the Arp2/3 complex), (iii) proteins involved in cytoskeleton anchoring to the plasma membrane (talin, moesin, spectrin, ezrin, and radixin, the latter being directly related to BC functionality (25), and finally (iv) signal transducers of actin cytoskeleton

Fig. 2. Expression profiling of 30 cytoskeleton-related proteins. A, immunoblot analysis of 30 cytoskeleton-related proteins for the three ischemia time points, 0 (I0), 10 (I10), and 60 (I60) min, and the three reperfusion time points, 0 (R0), 10 (R10) and 60 (R60) min. n = 2 on three different pools of three liver biopsy protein extracts. The proteins were classified into four functional families: six GTPases or GTPase regulators, six proteins involved in protein trafficking, five adaptor molecules, and 13 protein kinases. B, histogram representation of the average slope of the variation trend line for the 23 proteins showing a significant variation during the ischemic and reperfusion phases. NSF, N-ethylmaleimide-sensitive factor; FAK, focal adhesion kinase; PI3K, phosphatidylinositol 3-kinase, PAK, p21-activated protein kinase; PLC, phospholipase C; PKC, protein kinase C.
reorganization (PKA, α-actinin, annexins, IQGAPs, α- and β-catenins). Interestingly, some of these proteins are directly involved in the establishment and maintenance of hepatocyte AJ, such as β-catenin (26) and the Cdc42/Rac1 effector IQGAP1 (27, 28). These proteins could therefore represent critical targets linking IRI-induced actin cytoskeleton remodeling

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**Fig. 3. Zinc and gallium IMAC binding proteins.** A and B, pie chart representation of the total number of proteins identified by at least one unique peptide for the 60-min ischemia (A) and the 60-min reperfusion (B). The proteins have been classified into 15 functional families according to their Gene Ontology annotation. C and D, Venn diagrams representing the total number of proteins (C) and number of cytoskeleton-related proteins (D) identified specifically for the 60-min ischemia time point (left circle, I60), for the 60-min reperfusion time point (right circle, R60), or during both phases (overlapping area).
and alteration of the BC structure because BC integrity depends on the maintenance of cell-cell junctions (6).

IQGAP1 and -2 Are Potential Regulators Linking IRI-induced Actin Reorganization and BC Structure—Among the putative targets identified using the proteomic approach, we selected the Cdc42/Rac1 effectors IQGAP1 and -2. IQGAP proteins are very likely to represent critical regulators of hepatocyte intracellular response to IRI. Indeed IQGAP1 is a scaffold protein that modulates cross-talk among diverse pathways including calcium signaling, actin reorganization, and cell-cell junction establishment (29). That (i) each of these signaling pathways has been reported to be affected upon IRI (30–33) and that (ii) the maintenance of adherens junctions is critical for BC integrity in hepatocytes (6) strongly suggest that IQGAP1 could represent a signal integrator linking the IRI-induced actin remodeling and the related alteration of BC structure. Although the exact function of IQGAP2 has been less characterized, this protein shares 62% identity and an overall similarity of 77% with IQGAP1 and contains all of the protein domains identified in IQGAP1 (29), suggesting that IQGAP2 performs a function similar to that of IQGAP1.

Our objective was therefore to validate and characterize the involvement of IQGAP1 and -2 in IRI-induced actin remodeling and the related disruption of BC structure. A peptide specific to IQGAP1 was identified during the ischemic phase (Table I). Peptides specific to IQGAP2 as well as peptides common to IQGAP1 and IQGAP2 were identified after 60 min of reperfusion (Table II). The MS/MS spectrum corresponding to a peptide common to both isoforms is presented in Fig. 4A.

The occurrence of phosphorylated forms of IQGAP1 and IQGAP2 was confirmed at both 60-min ischemia (I60) and 60-min reperfusion (R60) by immunoprecipitation of IQGAP proteins followed by SDS-PAGE resolution and Pro-Q Diamond staining (Fig. 4B, lower panel). When the amount of phospho-IQGAPs was normalized to the total amount of IQGAP proteins as visualized using SYPRO staining, no significant difference in their phosphorylation levels was detected when comparing I60 and R60 (Fig. 4B, upper panel). However, when we evaluated IQGAP1 and -2 expression levels in liver Triton X-100-soluble fractions, we noticed that they both increased during ischemia and decreased during reperfusion (Fig. 4C). The comparison of IQGAP1 and -2 expression with the average value of the expression profiles obtained for the proteins tested in Fig. 2B showed a reverse profile for IQGAP1 and -2 (Fig. 4D). IQGAP1 and -2 atypical expression profiles may therefore reflect a central and active role for these two pro-

### Table I

| Protein name                                                                 | Number of peptides | Unique peptides | Sequence coverage | NCBI accession number | Function                              | Reported phosphorylation sites* |
|-----------------------------------------------------------------------------|--------------------|-----------------|------------------|------------------------|---------------------------------------|---------------------------------|
| 5'-AMP-activated protein kinase, γ-1 subunit                                | 2                  | 2               | 15               | P80385                 | Signal transducer                     |                                 |
| Actin, cytoplasmic 2                                                        | 3                  | 1               | 11               | P63261                 | Structural component                  |                                 |
| Actin-related protein Arp11                                                  | 1                  | 1               | 5                | Q9COK3                 | Structural component                  |                                 |
| α-1 Catenin                                                                  | 1                  | 1               | 3                | P35221                 | Signal transducer (AJ) Ser^641        |                                 |
| α-Actinin 1                                                                  | 6                  | 2               | 10               | Q9Z1P2                 | Signal transducer                     |                                 |
| α-Actinin 4                                                                 | 6                  | 2               | 9                | O43707                 | Signal transducer                      | Ty^266                         |
| Annexin VI isoform 2                                                         | 9                  | 9               | 17               | P08133-2               | Signal transducer                     |                                 |
| F-actin capping protein α-2 subunit                                          | 1                  | 1               | 3                | P47755                 | Filament assembly                     |                                 |
| Guanin nucleotide-binding protein-like 1                                     | 2                  | 2               | 8                | P36915                 | Signal transducer                     |                                 |
| Hypothetical protein Annexin A4                                              | 2                  | 2               | 10               | Q6LES2                 | Signal transducer                     |                                 |
| Plastin-1                                                                   | 1                  | 1               | 3                | Q14651                 | Structural component                  |                                 |
| Microtubule-associated protein-like 4                                       | 1                  | 1               | 1                | Q9HC35                 | Filament assembly                     | Th^899, Ty^226                   |
| Myosin heavy chain                                                          | 3                  | 3               | 2                | Q63731                 | Signal transducer (AJ) Ser^330,1443, Ty^172 |
| Ras GTPase-activating-like protein IQGAP1                                    | 1                  | 1               | 2                | P46940                 | Signal transducer                     |                                 |
| Talin-1                                                                     | 2                  | 1               | 1                | QSNZQ2                 | Plasma membrane anchoring             | Ty^70                          |
| Tubulin α4                                                                  | 4                  | 4               | 15               | P68368                 | Structural component                  |                                 |
| Tubulin β1                                                                  | 7                  | 1               | 22               | QSSU16                 | Structural component                  |                                 |

*Source: Swiss-Prot (ca.expasy.org/) and Human Protein Reference Database (www.hprd.org/).
to the expression level of reported IQGAP-interacting partners identified from the IMAC-purified fractions (Tables I and II), we observed the same atypical pattern for β-catenin (an up-regulation during ischemia and a slight down-regulation during reperfusion) (Supplemental Fig. 1, A and B). As the co-expression of two mRNAs or proteins has been often correlated with their functional association and

| Protein name | Number of peptides | Unique peptides | Sequence coverage | NCBI accession number | Function | Reported phosphorylation sites |
|--------------|--------------------|-----------------|-------------------|-----------------------|----------|-------------------------------|
| Actin, cytoplasmic 1 | 7 | 1 | 50 | P60709 | Structural component | Tyr166,218,294 |
| Actin, cytoplasmic 2 | 15 | 1 | 50 | P63261 | Structural component | |
| Arp2/3 complex, subunit 5-like | 1 | 1 | 8 | Q96PX5 | Filament assembly | |
| Arp2/3 complex, subunit p21 | 1 | 1 | 6 | O15145 | Filament assembly | |
| Hypothetical protein ARPC2 | 1 | 1 | 7 | Q53R19 | Filament assembly | |
| Actin-related protein Arp11 | 1 | 1 | 5 | Q9CO0K3 | Structural component | |
| α -Actinin 4 | 11 | 5 | 6 | Q43707 | Signal transducer | Tyr265 |
| Annexin A2 | 3 | 2 | 12 | Q567R4 | Signal transducer | |
| Annexin VI (isoform 1) | 13 | 10 | 23 | P08133-1 | Signal transducer | |
| β -Catenin (isoform 2) | 1 | 1 | 24 | P35222-2 | Signal transducer (AJ) | Ser23,29,33,37,45, Thr41, Tyr66,654 |
| Cytoskeleton-associated protein 4 | 1 | 1 | 2 | Q07065 | Plasma membrane-anchoring | |
| Ezrin | 4 | 3 | 13 | P15311 | Plasma membrane-anchoring | Tyr145,353 |
| Guanine nucleotide-binding protein-like 1 | 2 | 2 | 9 | P36915 | Signal transducer | |
| Keratin, type I cytoskeletal 18 | 1 | 1 | 3 | P05783 | Structural component | |
| Keratin, type I cytoskeletal 8 | 6 | 2 | 11 | Q6DHW5 | Structural component | |
| Keratin, type I cytoskeletal 9 | 9 | 7 | 19 | P35527 | Structural component | |
| Lamin A | 1 | 1 | 2 | P48679 | Structural component | |
| Moesin | 19 | 13 | 29 | P26038 | Plasma membrane-anchoring | |
| Non-muscle myosin heavy polypeptide 9 | 7 | 6 | 4 | Q6OF82 | Signal transducer | |
| Protein C14orf166 homolog | 1 | 1 | 6 | Q9CQE8 | Filament assembly | |
| Radixin | 7 | 4 | 14 | P35241 | Filament assembly | |
| Ras GTPase-activating-like protein IQGAP2 | 30 | 28 | 24 | Q13576 | Signal transducer (AJ) | |
| Spectrin α chain, brain | 15 | 14 | 7 | Q13813 | Plasma membrane-anchoring | Ser1323 |
| Spectrin β chain, brain | 11 | 8 | 5 | Q01082 | Plasma membrane-anchoring | Ser21,2138,2123,2169,2169 |
| Talin-1 | 3 | 3 | 2 | Q9Y490 | Plasma membrane-anchoring | Tyr260 |
| Tubulin α 6 | 6 | 3 | 11 | Q9BQE3 | Structural component | |
| Tubulin β 5 | 15 | 2 | 38 | Q5TM69 | Structural component | |

* Source: Swiss-Prot (ca.expasy.org/) and Human Protein Reference Database (www.hprd.org/).

teins in IRI-induced cytoskeletal reorganization. Interestingly when we assessed the expression level of reported IQGAP-interacting partners identified from the IMAC-purified fractions (Tables I and II), we observed the same atypical pattern
because IQGAP1 and β-catenin have been reported previously to physically interact, this observation strengthens the potential involvement of a complex including IQGAPs and β-catenin during IRI. In contrast, we did not detect any significant modification in actin level during IRI (Supplemental Fig. 1A). Although actin might be a member of that same complex, our data suggest that the IRI-induced changes involving actin are most likely related to its localization/polymerization (as demonstrated in Fig. 1B) rather than its expression.

**Subcellular Localization of IQGAP1 and -2 in Human Liver upon IRI**—To further characterize IQGAP proteins during IRI, the *in situ* liver distribution/localization of IQGAP proteins was determined upon IRI. Tissue biopsies were homogenized in the absence of detergent with 150 mM KCl. Insoluble (P100) and soluble (S100) fractions were separated by centrifugation. Interestingly, IQGAP1 and -2 show a decreased association with insoluble components during ischemia, whereas this pattern was reversed upon reperfusion (Fig. 5A, upper panel). This observation correlates with the detection of increasing amounts of these two proteins in soluble fractions upon ischemia followed by a decrease upon reperfusion (Fig. 5A, lower panel). These data suggest that IQGAP1 and -2 are subjected to severe localization changes in the liver during ischemia/reperfusion. Immunohistochemical studies on liver sections upon IRI revealed that IQGAP1 followed a redistribution similar to that of F-actin in hepatocytes, concentrating at the cell periphery after 1 h of ischemia (Fig. 5B, I60). This phenomenon was reversed upon reperfusion (Fig. 5B, R60). This apparent discrepancy with the fractionation data suggests that the association of IQGAP1 with hepatocyte plasma membrane structures observed by immunohistochemistry at
IQGAP1 Maintains Bile Canaliculi during Transplantation

IQGAP1 Plays a Central Role in Actin Polymerization at the Apical Pole/Junctions and Confers Resistance to IRI in HepG2 and Huh7 Cells—To determine whether IQGAP1 plays a role in the alteration of BC structure, we used both HepG2 and Huh7 human hepatoma cells. These cell lines have been previously used to study structural and functional properties of BC (35, 36). In these cells, intact BC are characterized by an intense actin ring at the cell-cell contacts. When IQGAP1 expression was silenced by two different shRNA constructs (IQ5 and IQ8) (20), the number of cells displaying an intense actin ring at the apical pole, indicating the formation of BC, was significantly decreased (Fig. 6C) and correlated with the levels of IQGAP1 silencing (Fig. 6A). No significant change was observed when IQGAP1 was overexpressed (Fig. 6, B and D), probably due to its already saturating basal expression level (Supplemental Fig. 2). These results show that IQGAP1 is involved in the formation of actin rings in both HepG2 and Huh7 cells, suggesting that IQGAP1 may participate in an actin-dependent establishment/maintenance of BC structures in these hepatoma cell lines. To further assess the functional consequences of modulation of IQGAP1 expression in the ability of the cells to undergo IRI, HepG2 and Huh7 cells were subjected to IRI as schematically represented in Fig. 6E. Interestingly cell viability under IRI was proportional to IQGAP1 expression and correlated with BC structure integrity (Fig. 6F). Indeed IQGAP1 expression level (and the consequent number of cells displaying intact BC) represented a critical determinant in the ability of both HepG2 and Huh7 cells to resist IRI, thus suggesting that IQGAP1 may play a central role in hepatocyte resistance to IRI via the maintenance of BC integrity.

DISCUSSION

Actin cytoskeleton remodeling and hepatocyte BC integrity were reported as primary targets of IRI and have been shown to have direct functional implications on graft recovery in terms of bile secretion (5, 10, 11). However, the molecular mechanisms underlying these phenotypes remain incompletely characterized. To improve the understanding of these complex mechanisms, we developed an experimental approach that aimed (i) to identify potential molecular targets of IRI using human liver biopsies, (ii) to characterize the identified targets in liver tissue to confirm their involvement in IRI-induced actin remodeling (in situ), and finally (iii) to functionally validate the in situ characterized targets in cell culture models.

Phosphoproteomic Analysis of Liver upon IRI—We demonstrated that IRI induces an important cytoskeleton remodeling (Fig. 1B) involving a common expression pattern for a significant number of signaling components, including adaptors and kinases (Fig. 2). Because signaling events are often linked to protein phosphorylation, we aimed to characterize more extensively the phosphoproteins present during both the ischemic and reperfusion phases. We therefore enriched intact phosphoproteins using zinc- or gallium-loaded IMAC matri-

Fig. 5. IQGAP1 and -2 subcellular localization in human liver upon IRI. A, immunoblot analysis of IQGAP1 and -2 from pellet (P100) and supernatant (S100) fractions obtained after centrifugation of liver biopsies homogenized in the presence of 150 mM KCl and normalized to Ribophorin (Rib) and Intersectin (Int) content respectively for the three ischemia time points, 0 (I0), 10 (I10), and 60 (I60) min, and the three reperfusion time points, 0 (R0), 10 (R10), and 60 (R60) min. n = 2 on three different pools of three liver biopsy protein extracts. B, immunohistochemical detection of IQGAP1 and -2 in 8-μm liver tissue sections at 0 and 60 min post-cold ischemia (I0 and I60) and 60 min postreperfusion (R60). Cells were counterstained with hematoxylin (cell nuclei appear in blue). A representative experiment of the six performed on independent ischemic and reperfused livers is shown. Scale bar = 30 μm. Arrows indicate sinusoidal lining cells.

I60 may be of low affinity as it is disrupted with 150 mM KCl used in our fractionation protocol. In contrast, under basal conditions (I0) and after 1 h of reperfusion (I60), IQGAP1 was most probably associated with a higher affinity to intracellular insoluble structures. Surprisingly although IQGAP2 was initially identified as a liver-specific isoform of IQGAP1 (34), the protein was not detected in hepatocytes under our experimental conditions (Fig. 5B). The localization of IQGAP2 was rather mainly restricted to what appeared to be sinusoidal lining cells in which IQGAP1 was also detected (Fig. 5B, white arrows). In conclusion, although IQGAP isoforms showed similar fractionation profiles upon IRI (Fig. 5A), only IQGAP1 was detected in hepatocytes (Fig. 5B). Because our aim was to identify potential regulators of actin remodeling in hepatocytes in conjunction with disruption of BC, we therefore focused our attention on IQGAP1.
Fig. 6. Involvement of IQGAP1 in actin polymerization at the bile canaliculi and in resistance to IRI. A and B, immunoblot analysis of IQGAP1 in HepG2 and Huh7 cells transfected with either mU6 empty vector (mock) and the two shRNA constructs, mU6-IQ5 and mU6-IQ8 (A), or prK5 empty vector (mock) and prK5-IQGAP1 (B) normalized to IQGAP2 and Intersectin (Int). n = 3. C and D, representative F-actin staining using phalloidin-TRITC in confluent HepG2 and Huh7 cells transfected with mU6 empty vector (mock (mU6)) and the two shRNA constructs, mU6l-Q5 and mU6I-Q5 IQ8 (C) and with prK5 empty vector (mock (prK5)) and prK5-IQGAP1 (D). The arrow indicates bile canaliculi between adjacent cells. Scale bar = 30 µm. E, schematic representation of the ischemia-reperfusion model developed for HepG2 and Huh7 cells. F, histogram representation for the percentage of cells that were adherent and viable (negative trypan blue staining) after IRI simulation for the five conditions described above (total of 2000 initial cells for three independent experiments). *, p < 0.05. I/R, ischemia-reperfusion.
ces followed by mass spectrometry sequencing to identify regulators of the IRI-induced actin remodeling and the related loss of BC integrity. The direct paired comparison by mass spectrometry of two clinical samples corresponding to two distinct physiological conditions (in our case I60 and R60) was limited by the current performances of this technique. However, we still extracted valuable information from our two data sets (Fig. 3). Indeed we first correlated the I60/R60 patterns (Fig. 3, pie charts) with known physiological events. For example, the increased representation of proteins involved in blood/immune response and protein biosynthesis during reperfusion can be related, respectively, to the presence of blood in the R60 biopsies and to the massive restoration of protein synthesis within the 1st h of reperfusion that we have reported previously (13). Similarly the higher representation of proteins involved in amino acid and nucleotide metabolism during ischemia may reflect interconversion of amino acids to glucose and lipid due to nutrient deprivation induced upon blood flow interruption (37). Interestingly we also identified specific cytoskeleton-related targets previously reported to be involved in IRI. For instance, proteins involved in cytoskeleton anchoring to the plasma membrane were mainly identified at R60 and have been previously reported as targets of calpain-mediated degradation postreperfusion in several models of IRI (38, 39). Similarly we identified here the 5′-AMP-activated protein kinase PKA as a signal transducer potentially phosphorylated upon ischemia (Table I). PKA activation has already been demonstrated to be antia apoptotic in liver upon IRI (40). Although our approach, involving direct IMAC purification of protein extracts, less selective for phosphopeptide identification as compared with those involving trypsin digestion prior to IMAC purification, it presents the advantage of allowing the identification of intact phosphoprotein-containing complexes (16). For example, at I60 we identified PKA, α-actinin 1, and IQGAP1, three members of a phosphorylation-dependent complex that has been reported recently to be involved in neurotransmitter-dependent syncapse maturation (41).

Relevant Target Selection Process: Identification and Selection of IQGAP1—IQGAP1 and -2 were first identified by mass spectrometry after IMAC enrichment of phosphorylation-dependent protein complexes in the 60-min ischemia and the 60-min reperfusion liver biopsy lysates, respectively. Phosphorylation mapping of IQGAP1 and -2 could not be determined through our MS/MS analysis probably because of the low representation of IQGAP phosphopeptides. However, we were able to confirm that both IQGAP1 and -2 were phosphorylated at I60 and R60 as they were stained by Pro-Q Diamond. Therefore, IQGAP1 and -2 enrichment on gallium- or zinc-coupled IMAC matrices is most likely due to the fact that these two proteins are phosphorylated rather than being members of phosphoproteins-containing complexes. However, although we demonstrated that, at both I60 and R60, a significant proportion of both IQGAP1 and -2 was phospho-

rylated, we could not determine precisely the nature of the phosphorylation sites.

The relevance of IQGAP1 phosphorylation is further supported by recent evidence revealing that endogenous IQGAP1 is highly phosphorylated and promotes neurite outgrowth in a phosphorylation-dependent manner (16). Incidentally it has been reported that IQGAP1 phosphorylation on Ser^{1443} increases its binding to nucleotide-free Cdc42 leading to the loss of cell-cell contacts (42), an event that could be related to the IRI-induced alteration of BC structure. In addition, our co-expression analysis revealed an atypical expression profile for IQGAP1 and -2 (Fig. 4, C and D), as well as for IQGAP1-interacting partner β-catenin (Supplemental Fig. 1), when compared with the common expression pattern for all other cytoskeleton-related proteins tested. This suggested a peculiar role for these proteins, most likely part of the same complex, in the phenomenon studied. Unfortunately our experimental setting did not allow us to reproducibly confirm the association pattern of IQGAP1 and -2 and their interacting partners during IRI. This might be due to the fact that pools of liver extracts/lysates were frozen, and complexes may have been differentially affected by this conservation procedure. Both validations, in situ in liver biopsies (Fig. 5) and in cell culture models (Fig. 6), allowed us to specifically target potential regulators relevant to our problematic: the IRI-induced actin remodeling and its link to maintenance of BC structure in hepatocytes. Indeed it led us to exclude IQGAP2, whose expression was not detected in hepatocytes. Although HepG2 and Huh7 imperfectly mimic hepatocytes under physiological conditions, they still represent relevant models to rapidly screen the implication of target proteins toward an effective formation/maintenance of BC structure because these mechanisms are well conserved in these cell lines (35, 43). Using this functional validation assay, we were able to demonstrate that IQGAP1 was essential for the integrity of actin structures around BC. Moreover using a chemical simulation of ATP depletion with antimycin A, a classical model of IRI (44) combined with thermal shock, we proved that the integrity of these actin structures was also critical for cell resistance to IRI in our model.

Functional Relevance of IQGAP1—IQGAP1 involvement in maintenance of BC structures can be explained by the demonstrated scaffolding role of IQGAP1 for E-cadherin- and Rac1/Cdc42-mediated signaling pathways (29). Indeed Rac1 and Cdc42 have been recently shown to inhibit the endocytosis of E-cadherin and consequently stabilize AJ through IQGAP1 F-actin cross-linking activity (45, 46). Therefore, we can postulate that, in our in vitro model, IQGAP1 silencing would impact the capacity of HepG2 and Huh7 cells to maintain stable AJ required for the de novo formation of BC structures. This hypothesis may also relate to the association of IQGAP1 with the endocytic machinery (clathrin and AP-2) we
observed in human liver. Indeed this association was only detected before ischemia and 1 h postreperfusion. Interestingly this correlates with our fractionation results (Fig. 5A) where the association of IQGAP1 with endocytic components would result in its presence in the insoluble fraction, whereas its association with plasma membrane during ischemia would be destabilized by a 150 mM KCl treatment. The postreperfusion biliary complications observed in several patients, involving a deterioration of BC structures during the course of reperfusion, could therefore be due to an altered reassociation of IQGAP1 with the endocytic machinery. The maintenance of the hepatocyte bile secretion properties would then depend on their ability to rapidly re-form integral AJ and maintain BC structure upon reperfusion.

In summary, in this work, we were able to identify and functionally validate IQGAP1 as a potential target whose expression and functionality impact on maintenance of BC structure. Our findings confirm the potential of targeted functional proteomic approaches using human samples to identify critical regulators of pathophysiological conditions directly relevant to human health. The elucidation of IQGAP1 involvement in hepatocyte resistance to IRI may eventually allow the design of therapeutic agents that, when present in the preservation solution, should specifically target IQGAP1 and enhance its protective role, thus improving accelerating graft recovery and improving transplantation outcomes.

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