RACK1, a New ADAM12 Interacting Protein
CONTRIBUTION TO LIVER FIBROGENESIS*

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ADAM12 belongs to a disintegrin-like and metalloproteinase-containing protein family that possesses multidomain structures composed of a pro-domain, a metalloprotease domain, disintegrin-like, cysteine-rich, epidermal growth factor-like, and transmembrane domains, and a cytoplasmic tail. Overexpression of several ADAMs has been reported in human cancer, and we recently described the involvement of ADAM12 in liver injury (Le Pabic, H., Bonnier, D., Wewer, U. M., Coutand, A., Musso, O., Baffet, G., Clement, B., and Théret, N. (2003) Hepatology 37, 1056-1066). In this study, we used a yeast two-hybrid screening of a cDNA library from human hepatocellular carcinoma to analyze binding partners of ADAM12. We identify RACK1, a receptor for activated protein kinase C (PKC), as a new ADAM12 interacting protein. RACK1 is up-regulated in patients with hepatocellular carcinoma and is highly expressed by activated hepatic stellate cells. We demonstrate the involvement of RACK1 in mediating the PKC-dependent translocation of ADAM12 to membranes of activated hepatic stellate cells. In particular, treatment of cells with phorbol esters enhances ADAM12 immunostaining in the membrane fractions and the co-immunoprecipitation of ternary complexes containing RACK1, ADAM12, and PKC. By using RNA interference, we demonstrate that inhibition of RACK1 expression diminishes the phorbol 12-myristate 13-acetate-dependent translocation of ADAM12 to membranes of hepaticstellate cells. Finally, hepatic stellate cells cultured on coated type 1 collagen induces relocalization of ADAM12 in the membrane, suggesting that this major matrix component in liver cancer and fibrogenesis might stimulate ADAM12 translocation to the cell membrane where its shedding activity takes place.

ADAMs (a disintegrin and metalloproteinase domain) are a family of cell surface multidomain proteins involved in ectodomain shedding, cell adhesion, and cell signaling (2–5). Common domains to ADAM proteins include propeptide, metalloprotease, disintegrin, cysteine-rich, EGF-like, transmembrane and cytoplasmic domains. More than 30 members have been identified in the ADAM family with broad tissue distribution and have been implicated in highly diverse biological processes, including spermatogenesis/fertilization, neurogenesis, and inflammatory response. In recent years, increased expression of several ADAMs has been reported in human cancers (6–11) and biological properties of ADAMs suggest an important role in cancer processes including cell adhesion, migration, survival, and proliferation (12, 13). Thus, we have recently described the up-regulation of ADAM12 mRNA levels in patients with hepatocellular carcinoma and the association of ADAM12 expression with liver tumor aggressiveness (1).

ADAM12 is expressed as two spliced forms, the secreted form (ADAM12S) has been described as an active metalloprotease that cleaves IGFBP-3 and -5 (14–16). In addition, by shedding the membrane-bound HB-EGF, ADAM12 was shown to promote cardiac hypertrophy (17) and by cleaving gelatin, fibronecin, and gelatinase IV, ADAM12 was suggested to facilitate tumor invasion in breast cancer (8). The role of ADAM12 in cell-cell and cell-matrix interactions has been supported by the interaction with β1-integrin (18, 19) and syndecans (20). The membrane-anchored long form of ADAM12 has a cytoplasmic tail that interacts with several SH3 domain containing proteins including the Src-kinase SRC and YES1 (21), the adapter proteins Grb2 (21) and Fish (22), the regulatory subunit of phosphatidylinositol 3-kinase, p85α (17), and PACSIN3, a cytoplasmic phosphoprotein that plays a role in vesicle trafficking (23). In addition, the ADAM12 tail interacts with eve-1, a EEN binding protein that acts as an adaptor module to inhibit the Ras stimulating activity of Sos2 (24) and α-actinin-2, an actin cross-linking protein highly expressed in skeletal and cardiac muscle (25). More recently, the role of ADAM12 in modulating the transforming growth factor-β signaling pathway has been suggested by the interaction of ADAM12 with both FRLG (follistatin-related gene), binding proteins to transforming growth factor type II receptor (TβRII) leading to an increase in transforming growth factor-β-dependent transcriptional activity (27).

In the present study, we screened a human hepatocellular carcinoma cDNA two-hybrid library using ADAM12 as bait, and identified RACK1 (Receptor of activated protein kinase C) as a new partner of ADAM12 in liver cancer. RACK1 is an homologue of the β-subunit of heterotrimeric G proteins (28), involved in membrane anchorage of multiple proteins including protein kinase C (29, 30). Our data indicate that, similarly to
ADAM12, the mRNA steady state level of RACK1 was increased in hepatocellular carcinomas and the underlying fibrotic livers. In addition we showed that interaction between endogenous ADAM12 and RACK1 occurred in hepatic stellate cells. We further demonstrated that RACK1 is required for the PKCe dependent translocation of ADAM12 to the hepatic stellate cell membrane. Evidence for the role of type I collagen as a potential physiological inducer of ADAM12 translocation strongly suggested that the extracellular matrix environment modulates ADAM12 activity during liver injury.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screening**—Full-length cDNA of ADAM12 was prepared as bait by PCR amplification and cloned into the pGBK7 vector, which contains the DNA-binding domain of Gal4. For screening, a cDNA library from a pool of 10 human hepatocellular carcinomas was constructed in the EcoRI site of the pGADT7 vector, which includes the Gal4 activation domain. A total of 4 × 10^6 independent cDNA clones with an average insert size of ~1.45 kb pairs (range of 0.5–4.0 kb pairs) were obtained. An aliquot of the resulting plasmid cDNA library was then transformed into AH109 yeast cells that had been transformed with pGBK7 containing a full-length ADAM12 bait fused to the 1–147 amino acids of the Gal4 protein and screened on selection plates lacking leucine, tryptophan, histidine, and containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). After 3–5 days of incubation, positive clones were picked, and plasmid DNA was retransformed into Escherichia coli strain DH10B for further cloning and sequencing.

**GST Fusion Protein Binding Assay**—Cultures of E. coli BL21 containing pGEX RACK1 plasmids were incubated with 0.1 mM isopropyl β-D-thiogalactopyranoside overnight at 25 °C and GST fusion proteins were purified as previously described (31). Purified GST-RACK1 was incubated with in vitro translated ADAM12 products for 3 h at 4 °C and protein complexes were collected with addition of glutathione beads, washed three times in wash buffer containing 20 mM Tris, pH 8.0, 100 mM sodium chloride (NaCl), and 0.02% Tween, and boiled in SDS sample buffer. Proteins were resolved by SDS-PAGE and subjected to immunoblot analysis.

**In Vitro Translation of Proteins**—cDNA encoding full-length human ADAM12S and ADAM12L, cDNA encoding the prodomain, the metalloprotease domain, and the disintegrin domain (A12-PMD) (amino acids 1–512), cDNA encoding the prodomain and the metalloprotease domain (A12-PM) (amino acids 1–512), and cDNA encoding the disintegrin, the cysteine-rich domain, and the EGF-like domain (A12-DC) (amino acids 417–707) (32) were transcribed and translated in vitro using the TNT coupled rabbit reticulocyte lysate system (Promega) according to the procedure recommended by the manufacturer. In vitro translated products labeled or not with EasyTag™ L-[35S]methionine (PerkinElmer Life Sciences) were incubated with 1 μg of purified GST-RACK1 or GST as described above.

**Cell Culture and Transfection**—Human hepatocytes and hepatic stellate cells were isolated from histologically normal specimens of partial hepatectomy from patients undergoing hepatic resection for liver metastases. Hepatocytes, activated hepatic stellate cells (HSC), and enriched fractions in liver macrophages were isolated as previously described (1). The human hepatoma cell lines (HepG2 and Hep3B) were maintained in Williams’ E medium containing 0.1% albumin, 1 mM l-glutamine, 5 μg/ml insulin, 5 units/ml penicillin-streptomycin, hydrocortisone, and 10% fetal bovine serum. The human hepatic stellate cells and COS7 cells were maintained in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum and 10 units/ml penicillin-streptomycin. Human hepatic stellate cells were transfected using AMAXA technologies (Amansa Biosystems, Cologne, Germany) according to the manufacturer’s instructions. Pmax GFP-green vector encoding GFP (Amaxa) was used to monitor transfection efficiency. When specified, cells were plated on collagen type I dishes or on laminin, fibronectin, and collagen type IV-coated multwell plates (BD Biosciences). All expression constructs for wild-type ADAM12 and chimeras of ADAM12 and enhanced green fluorescence protein (pEFP-GFP-N1, Clontech) were prepared as previously described (33). The expression vector for HA-RACK1 was a gift from Dr. Cartwright (Stanford University). To knock down RACK1 expression, a pool of 3 chemically synthesized double-stranded RACK1 siRNA were used, these 3 target-specific siRNAs and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Tissue Samples**—Lever tissue samples were obtained from 27 patients with hepatocellular carcinoma undergoing surgical resection of the tumor as previously described (34). The intensity of necroinflammatory lesions were graded according to the METAVIR score: AO, no activity; A1, mild; A2, moderate; A3, severe. All tissue sections were routinely analyzed after staining with hematoxylin-eosin-saffran and Sirius red. Access to this material was in agreement with French laws and satisfied the requirements of the local Ethics Committee.

**Antibodies**—For RACK1, a rabbit polyclonal antibody H-187 (Santa Cruz Biotechnology) was used for immunoprecipitation and a mouse monoclonal antibody from BD Transduction Laboratories (Lexington, KY) was used for immunoblot analyses. Mouse monoclonal antibodies recognizing the ADAM12 disintegrin domain (6E6, 8F8, 6C10) or polyclonal antibodies against the prodomain (rb132), the metallocprotease domain (rb128), or the cysteine-rich domain (rb122) were a gift from Prof. U. Wewer (Department of Biomedicine and BRIC, University of Copenhagen, Denmark). Rabbit antibodies against PKCε, HA peptides, and mouse monoclonal antibodies to Hsc70, actin, and β1-integrin-blocking antibody (PSD2) were from Santa Cruz Biotechnology. Rabbit antibodies against C termini of Delta-like 1 (DLL1) were a generous gift from Prof. Logeat (35).

**Immunoprecipitation and Western Blotting**—Hepatic stellate cells were transfected with the indicated expression vectors or siRNAs by the AMAXA method and cultured for 36 h. After one night in serum-starved medium, cells were treated for 15 min with 0.1 μM PMA and then lysed at 4 °C in RIPA buffer containing 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 25 mM HEPES, 150 mM NaCl, 0.2% deoxycholate, 5 mM MgCl2, 1 mM Na3VO4, 1 mM sodium fluoride, and a protease inhibitor mixture (Roche Applied Science). Membrane fraction enrichment was performed as previously described (36). Briefly, cells were washed with phosphate-buffered saline, scraped into buffer...
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(16.8 mM HEPES, pH 8.0, 2.0 mM MgCl2, 0.88 mM EDTA, 1 mM Na2VO4, 1 mM NaF, and protease inhibitors), homogenized in a Dounce homogenizer on ice, and centrifuged at 200 × g for 10 min. Supernatants were further centrifuged at 200,000 × g for 30 min. The resulting membrane-enriched pellets were dissolved in RIPA buffer supplemented with protease inhibitor mixtures (Roche Applied Science). For immunoprecipitation, cell extracts were preincubated for 1 h with Sepharose-coupled protein-G beads (Amersham Biosciences) alone to reduce non-specific protein binding and followed by adsorption overnight to Sepharose-coupled protein G prebound with 2 μg of specific or control rabbit IgG. The beads were washed five times in buffer and samples were analyzed by SDS-PAGE and immunoblotting. Proteins were electrophoretically transferred to nitrocellulose membrane (Amersham Biosciences) and probed with the appropriate antibodies. The bound antibodies were visualized with horseradish peroxidase-conjugated anti-rabbit (Pierce) or anti-mouse (Bio-Rad) IgG antibodies using an enhanced chemiluminescence system.

Cell Surface Biotinylation—Hepatic stellate cells, treated or not with PMA for 15 min at 4 °C were further incubated with 1 mg/ml N-hydroxysulfosuccinimidobiotin (sulfo-NHS-biotin; Sigma), and then washed with phosphate-buffered saline. Cellular proteins scraped into RIPA buffer (1 ml to a 100-mm Petri dish) were incubated for 1 h at 4 °C with streptavidin beads (Mag Select SA, Sigma). The magnetic beads were washed five times with extraction buffer, eluted with SDS sample buffer, and samples analyzed by SDS-PAGE.

Immunostaining and Imaging—To detect ADAM12, hepatic stellate cells were plated on Permanox chamber slides supporting cell attachment (NUNC, Rochester, NY) for 24 h. Cells were treated with PMA for the indicated time, fixed with 4% paraformaldehyde. When indicated, cells were permeabilized with 0.1% Triton X-100 and further incubated with ADAM12 polyclonal antisera (rb122). The bound antibodies were visualized with horseradish peroxidase-conjugated anti-rabbit antibodies (Pierce). For immunofluorescence studies, 4 h post-transfection, COS7 cells or subconfluent hepatic stellate cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 and further incubated with ADAM12 polyclonal antisera (rb122). The bound antibodies were visualized with horseradish peroxidase-conjugated anti-rabbit antibodies (Pierce). For immunofluorescence studies, 48 h post-transfection, COS7 cells or subconfluent hepatic stellate cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were incubated for 1 h at room temperature with rabbit anti-HA antibodies and then for 90 min with TRITC-conjugated anti-rabbit IgG antibodies. The cover-slips were washed, mounted in phosphate-buffered saline containing 50% glycerol and 1 mg/ml diazabicyclo[2.2.2]octane, and viewed on a Leica laser scanning confocal microscope or the Automated microscope Leica DMRXA2 equipped with Photometrics CoolSnapES N&B camera driven by MetaMorph software (Universal Imaging Corp.). Immunohistochemistry in liver tissues was performed on frozen sections with either ADAM12 antibodies common to the long and short variants as described (7), or RACK1 monoclonal antibodies (BD Transduction Laboratories) as described (37).

Flow Cytometry—Cells were detached with trypsin/EDTA, incubated for 5 min in medium supplemented with 10% fetal bovine serum, and then transferred to 4 °C. After two washes with ice-cold phosphate-buffered saline, 1% bovine serum albumin, cells were incubated with ADAM12 antibodies (rb122) or isotype controls for 30 min. Cells were washed and incubated with phycoerythrin conjugates for 20 min and finally rinsed, resuspended in phosphate-buffered saline and used for flow cytometry analyses by using FACS ARIA (BD Biosciences).

Real Time PCR—Total RNA were extracted by the guanidinium thiocyanate/cesium chloride method and first strand cDNA synthesis was achieved with SuperScript reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed by the fluorescent dye SYBR Green methodology using the qPCR™ Core Kit for SYBR™ Green I from Eurogentec and the ABI Prism 7700 (PerkinElmer Life Sciences). Primer pairs for target genes were RACK1, sense 5′-TGGGATGGAA-CCTGCG-3′, antisense 5′-TGATGCCCCACCATTCCGC-3′; ADAM12, sense 5′-GGTTTGCTTTTGGAGAAGCAG-3′ and antisense 5′-TGAACCAGCAAGGCTTCTGAG-3′; 18S, sense 5′-CGCCGCTAGAGGTGAAATT-3′; antisense 5′-TTGGCAATCGTCTCGTC-3′. Using the comparative Ct method, the amount of target sequence in unknown samples normalized to the 18S reference, was expressed relative to a calibrator (mean of control in our assay).

Results

Identification of RACK1 as a Binding Partner of ADAM12—Using the full-length of human ADAM12 as bait, we performed a yeast two-hybrid screen of a human hepatocellular carcinoma cDNA library. An homology search in GenBank™ using the blast program showed that the cDNA fragments from several positive clones encode polypeptides containing 244 amino acid residues spanning five of the seven WD domains of GNB2L1, guanine nucleotide-binding protein subunit β2-like 1 (GenBank accession number BC032006, 100% identity) (Fig. 1A). GNB2L1, also named RACK1 has been involved in intracellular signaling by recruiting and anchoring many proteins in specific subcellular compartments facilitating protein interaction in signaling complexes.

To confirm the results obtained with the yeast two-hybrid assay, we investigated the ADAM12-RACK1 interaction with in vitro binding experiments. RACK1 was expressed as a bacterial fusion protein with GST, purified on glutathione-agarose beads, and incubated with the in vitro translated transmembrane form, ADAM12L (110 and 90 kDa for the pro- and pro- processed forms, respectively) and the spliced secreted form, ADAM12S (90 and 68 kDa for the pro- and processed forms, respectively). We observed that GST-RACK1 bound in vitro translated ADAM12S and ADAM12L, whereas the GST control did not (Fig. 1B, upper panel). These in vitro binding studies demonstrated that RACK1 interacts with ADAM12 and that the interaction did not involve the cytoplasmic tail.

To further define the ADAM12 domains involved in RACK1 interaction, we generated different peptides including the prodomain, metalloprotease domain, and disintegrin domain (A12-PMD) (amino acids 1–512), the prodomain and the metalloprotease domain (A12-PM) (amino acids 1–419), or the disintegrin, the cysteine-rich domain, and the EGF-like domain (A12-DC) (amino acids 417–707) as previously described (32). The A12-PMD, A12-PM, and A12-DC peptides did not show significant interaction with GST-RACK1 suggesting that specific structural conformation of the protein combining at
least metalloprotease, disintegrin, and cysteine domains is required (Fig. 1B, bottom panel).

In accordance with these observations, a fragment of ADAM12, which includes the metalloproteinase, disintegrin, and cysteine-rich domains (amino-acids 142–739), was tagged with FLAG and cotransfected into COS7 cells alone or in combination with HA-RACK1. Immunoprecipitation with anti-HA followed by immunoblotting with anti-FLAG revealed that RACK1 can interact with ADAM12 (Fig. 1C). To provide further evidence that ADAM12 interacts with RACK1, we examined their colocalization by immunofluorescence in cells coexpressing GFP-ADAM12 and HA-RACK1. As previously described (27), ADAM12 is localized predominantly in patched areas in the cell. Interestingly, we found that RACK1 extensively colocalized with ADAM12 (Fig. 1D).

To show whether the interaction between ADAM12 and RACK1 occurs under physiological conditions, we used activated HSC previously described to express detectable endogenous ADAM12 (1). In immunoprecipitates prepared with preimmune antisera, no ADAM12 was coprecipitated. However, in the anti-RACK1 immunoprecipitates, we could clearly detect ADAM12 coprecipitating with RACK1, indicating that interaction can occur with physiological levels of these proteins (Fig. 1E). Accordingly, RACK1 and ADAM12 exhibited regions of colocalization within the cytoplasm as demonstrated by immunofluorescence analyses (Fig. 1F).

Up-regulation of RACK1 and ADAM12 in Patients with Hepatocellular Carcinomas and Association with Fibrogenesis—The finding of interaction between ADAM12 and RACK1 in human hepatic stellate cells and the previous observation of association of ADAM12 expression with fibrogenesis and liver cancer raised the question of whether the expression of RACK1 is induced in liver cancers because activated hepatic stellate cells play a key role in host-stroma response and contribute to matrix remodeling, facilitating tumor invasion (34). We therefore investigated the steady-state mRNA levels of RACK1 in 27 patients with hepatocellular carcinoma (T) and their adjacent non-tumor livers (NT) and 10 normal liver controls. Patients were 24 men and 3 women with a median age of 59 ± 9, 4 being positive for RACK1 Interacts with ADAM12.
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Figure 2. RACK1 expression is up-regulated in patients with hepatocellular carcinomas (n = 27). A, RACK1 and ADAM12 mRNA were determined by real-time PCR in tumor (T), adjacent non-tumor areas (NT), and normal liver controls (n = 10). Data are shown as mean ± S.D. from a representative experiment. Results are presented as -fold increase of control. B, RACK1 and ADAM12 mRNA expression in NT livers was increased in cirrhosis (F4) compared with fibrosis (F1–F3). C, ADAM12 and RACK1 mRNA expression was analyzed in isolated HSC, Kupffer cell (KC)-enriched fraction, hepatoma cell lines (HC), fresh (0 h) and cultured hepatocytes (HH) (24 h) and compared with expression in control livers. D, localization of RACK1, ADAM12, and α-smooth muscle actin in fibrotic liver tissues from patients with hepatocellular carcinomas. a and d, RACK1; b and e, ADAM12; c, and f, α-smooth muscle actin. Specific antibodies were omitted in negative control serial sections (g) and immunoperoxidase was counterstained with hematoxylin. Original magnification: a–c, ×25; d–g, ×100.

By using immunohistochemistry analysis (Fig. 3, B–E), we showed that ADAM12 immunostaining was very low at the cell surface in subconfluent cultures. When cells were permeabilized with Triton X-100 before immunostaining with ADAM12 antibodies, the intensity of labeling was increased suggesting a high intracellular pool of ADAM12. To examine the role of PKC activation on ADAM12 distribution, cells were treated with PMA for 30 min before immunostaining. In these conditions, we observed an increase in labeling intensity at the cell surface, more specifically confined to cellular processes of non-permeabilized cells showing protein at the external cell membrane and a large redistribution of ADAM12 staining in permeabilized cells. We further tested whether the increase in ADAM12 cell surface expression could be confirmed by FACS analysis and we examined the cell surface expression of ADAM12 stained with phycoerythrin antibodies. PMA treatment induced a notable increase in ADAM12 expression in fibrotic liver might originate from HSC (Fig. 2C). As we have previously described, ADAM12 expression was mainly associated with hepatic stellate cells (1). In addition, RACK1 expression was increased in the hepatocellular cell line HepG2 and Hep3B as compared with human hepatocytes suggesting contribution of the tumor cell to RACK1 up-regulation in liver cancer. To further investigate the cellular localization of RACK1 and its interaction with ADAM12, we performed immunohistochemistry analyses in liver tissues from patients with hepatocellular carcinoma. RACK1 and ADAM12 staining was localized in the region enriched in α-smooth muscle actin positive cells, suggesting ADAM12 and RACK1 are coexpressed by hepatic stellate cells in vivo (Fig. 2D).

PMA Treatment Increases ADAM12 Cell Surface Expression through PKCe Activation in Hepatic Stellate Cells—To explore the functional significance of the interaction between RACK1 and ADAM12, we investigated the role of PKCe, a major RACK1-binding partner (39, 40) that was also previously reported as an ADAM12 interacting protein (17). To test whether ADAM12, which is highly expressed in activated hepatic stellate cells, interacts with PKCe, immunoprecipitation of endogenous PKCe was performed from crude hepatic cell extracts and anti-ADAM12 monoclonal antibodies were used for detection. Endogenous ADAM12 binds PKCe in hepatic stellate cells (Fig. 3A). Because PKCe was recently shown to mediate ADAM12 membrane translocation (36) upon PMA stimulation, a general activator of PKC, we next examined the distribution of ADAM12 in hepatic stellate cells in the presence or absence of PMA.

Heptatitis virus A, 6 for hepatitis virus B. 20 patients were diagnosed with cirrhosis (F4) and 7 with fibrosis (F1–F3) in the underlying liver with a necroinflammatory activity ranging from A1 to A3 according to the METAVIR score. Similarly to ADAM12, RACK1 expression was increased both in hepatocellular carcinoma (T, p < 0.001) and the adjacent non-tumor liver (NT, p < 0.001) compared with control liver (Fig. 2A). The enhanced mRNA expression for ADAM12 and RACK1 in the non-tumor liver (NT) was associated with the score of fibrosis because both genes were up-regulated in cirrhosis (F4) compared with the early step of fibrogenesis (F1–F3) (Fig. 2B). By using isolated liver cells, we showed that activated hepatic stellate cells, the key cellular element involved in the development of hepatic fibrosis, highly expressed RACK1 compared with human hepatocytes and Kupffer cells suggesting that the increase in RACK1 expression in fibrotic liver might originate from HSC (Fig. 2C). As we have previously described, ADAM12 expression was mainly associated with hepatic stellate cells (1). In addition, RACK1 expression was increased in the hepatocellular cell line HepG2 and Hep3B as compared with human hepatocytes suggesting contribution of the tumor cell to RACK1 up-regulation in liver cancer. To further investigate the cellular localization of RACK1 and its interaction with ADAM12, we performed immunohistochemistry analyses in liver tissues from patients with hepatocellular carcinoma. RACK1 and ADAM12 staining was localized in the region enriched in α-smooth muscle actin positive cells, suggesting ADAM12 and RACK1 are coexpressed by hepatic stellate cells in vivo (Fig. 2D).
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Cell Surface Localization of ADAM12 Is Induced by Interaction of Hepatic Stellate Cells with Type I Collagen—By linking integrin to PKCe, RACK1 has been suggested to play a role in integrin-dependent signaling pathways involved in cell adhesion, spreading, and motility (43). Thus RACK1, β1-integrin and PKCe form a complex interactive network in which ADAM12 takes place by interacting with β1-integrin (44), PKCe (45), and RACK1 (this paper) (Table 1). Because β1-integrin mediates the interaction of HSC with type I collagen, the major matrix component in injured liver (46), we reasoned that activation of β1-integrin by type I collagen might be involved in ADAM12 translocation to the membrane fraction through second messenger release and activation of PKCe. To determine whether type I collagen used as substratum affected ADAM12 localization in hepatic stellate cells or not, we performed immunostaining of hepatic stellate cells cultured for 24 h on the type I collagen-coated surface. Interestingly, an increase in ADAM12 staining was observed at focal adhesion-like structures in HSC, cultured on type I collagen (47) (Fig. 5A). Further

were detectable in membrane fractions of hepatic stellate cells at the basal state (Fig. 3G). Upon PMA treatment, a marked increase in PKCe and ADAM12 was observed in membrane fractions. Pre-treatment of cells with the PKC inhibitor calphostin C inhibited the PMA-dependent PKCe activation in human hepatic stellate cells. Cultured on type I collagen (47) (Fig. 5A). Treatment of PMA-for immunoprecipitation, a marked increase in RACK1 interaction pathways. When anti-RACK1 antibodies were used for immunoprecipitation, a marked increase in RACK1-ADAM12 complexes was found upon PMA treatment (Fig. 4B), which was partially abolished by calphostin C. The evidence for ternary complexes associating ADAM12 both to PKCe and RACK1 was further shown by PKCe staining in RACK1-ADAM12 immunoprecipitates.

The role of RACK1 in ADAM12 translocation was further evidenced by using an RNA interference approach. RACK1 was targeted in hepatic stellate cells by using a pool of 3 chemically synthesized double-stranded RACK1 siRNA. In these conditions, the steady state level of the RACK1 protein was significantly reduced in hepatic stellate cells showing RNA interference efficiency (Fig. 4C). Thus, the PMA-dependent accumulation of ADAM12 in the membrane fraction from cells treated with RACK1 siRNA was decreased, suggesting a RACK1 requirement for ADAM12 translocation to the membrane fraction (Fig. 4D). Accordingly, by using immunoprecipitation of PKCe in membrane cell fractions, we showed that the amount of ADAM12 was reduced in RACK1 siRNA-treated cells (Fig. 4E). Furthermore, cell surface expression of ADAM12 was evaluated by FACS analysis of siRNA-transfected cells treated or not by PMA. Increase in ADAM12 cell surface expression observed upon PMA treatment was significantly reduced by transfection of RACK1 siRNA (Fig. 4F). Taken together these data demonstrated that RACK1 is involved in the PMA-dependent translocation of ADAM12 to cell membrane fractions, thereby affecting ADAM12 activity at the cell surface. In support of this hypothesis, we investigated the proteolytic processing of Delta-like 1 that was recently demonstrated to be a substrate for ADAM12 (42). Lysates from hepatic stellate cells treated or not by PMA were analyzed by Western blotting using antibodies specific for the C termini of Dll1. PMA treatment induced the cleavage of Delta-like 1 protein suggesting that ADAM12 might contribute to the processing of Delta-like 1 at the cell surface of activated hepatic stellate cells (Fig. 4G).
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evidence for increased ADAM12 cell surface expression upon type I collagen stimulation was observed using FACS and cell membrane fraction analyses. FACS analysis demonstrated that hepatic stellate cells cultured on type I collagen exhibited higher amounts of ADAM12 protein at the cell surface (Fig. 5B). In addition this effect was blocked by using siRACK1 suggesting that type I collagen-dependent translocation of ADAM12 require RACK1 expression. In accordance with these observations, membrane fractions from cells cultured on type I collagen showed a higher amount of ADAM12 proteins compared with cells cultured on plastic (Fig. 5C). The total amount of ADAM12 proteins in crude cell extracts was not significantly modified suggesting that type I collagen coating did not induce ADAM12 expression but affect only ADAM12 translocation. We next explored the specificity of type I collagen in inducing ADAM12 translocation to the cell surface by analyzing the effect of other matrix components including fibronectin, laminin, and type IV collagen. A marked increase in ADAM12 was observed in the membrane fraction of cells cultured on type I collagen and not on type IV collagen, fibronectin, and laminin (Fig. 5D).

Finally, the mechanism underlying type I collagen-dependent translocation of ADAM12 was investigated by analyzing the involvement of β1-integrin because it has been previously described as the receptor for type I collagen in hepatic stellate cells (46). Pretreatment of HSC with β1-integrin blocking antibodies reduced significantly the amount of ADAM12 in membrane fractions suggesting that β1-integrin mediated the effect of type I collagen in ADAM12 translocation (Fig. 5E). Taken together these data strongly support the hypothesis that interaction of hepatic stellate cells with type I collagen through β1-integrin recruitment induced relocalization of ADAM12 in membrane fraction.

**DISCUSSION**

The current study investigated new partners of ADAM12, a disintegrin and metalloprotease, which has been recently involved in tumor progression. Yeast two-hybrid screening using the full-length ADAM12 as bait resulted in the isolation of the protein RACK1, first identified as an intracellular receptor of protein kinase C (28). RACK1 belongs to the family of proteins that contain WD repeats, a domain involved in protein-protein interactions. The evolutionary conserva-

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**FIGURE 4.** RACK1 mediates the PKCe-dependent ADAM12 translocation in hepatic stellate cell membranes. Immunoprecipitation (IP) and Western blot analysis were performed to detect complexes between ADAM12, RACK1, and PKCe in membrane fractions from cells pre-treated with PMA and/or calphostin C. After immunoprecipitation of endogenous PKCe (A) or RACK1 (B), Western blotting was performed using ADAM12 and RACK1 antibodies (A) or ADAM12 and PKCe antibodies (B). ADAM12-PKCe and ADAM12-RACK1 complexes were both immunoprecipitated in cells stimulated by PMA, C–F. Human hepatic stellate cells were transfected with 2 nm non-targeted siRNA (Control siRNA) or a pool of RACK1 siRNA (RACK1 siRNA), as described under "Experimental Procedures." After 24 h, Western blotting was used to confirm the efficiency of RNA interference in cell extracts (C). Both Western blotting analysis of ADAM12 and RACK1 expression are shown (D). ADAM12-PKCe and RACK1-PKCe complexes in membrane fractions (E) and FACS analysis (F) showed that PMA-dependent ADAM12 translocation to the membrane was decreased in cells transfected with RACK1 siRNA. G, PMA treatment of hepatic stellate cells increases the cleavage of full-length Delta-like 1 (FL Dll1) as shown by the release of the C-terminal fragment (CTF Dll1). IB, immunoblot.

**TABLE 1**

Complex interactions between ADAM12, ITGB1, PRKCE, and RACK1 (adapted from Human Protein Reference Database)

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<th>Name of interactors</th>
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<th>Interaction type</th>
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<td>Lillienal and Chang (44)</td>
<td>9,442,085</td>
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<td>ITGB1-ADAM12</td>
<td>In vitro</td>
<td>Direct</td>
<td>Kawaguchi et al. (19)</td>
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<td>ADAM12-RACK1</td>
<td>In vivo; in vitro; yeast two-hybrid</td>
<td>Direct</td>
<td>Kawaguchi et al. (19)</td>
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Membrane targeting, trafficking, and signaling including receptors, integrin, PKCs, phospholipase C, GTPase, and cytoskeleton-associated proteins (49, 50). It is likely that ADAM12, a membrane-anchored protein that was previously reported to interact with signaling proteins, adhesion molecules, and actin network, might interact with RACK1 in these pathways. The interaction between ADAM12 and RACK1 was confirmed by an in vitro binding assay using the purified RACK1 fused to glutathione S-transferase along with in vitro translated ADAM12. This interaction did not involve the cytoplasmic domain but required the metalloprotease, disintegrin, and cysteine domains.

By using an overexpression system, we further provided evidence of a direct interaction between GFP-tagged ADAM12 and HA-tagged RACK1 in cells. Microscopy showed that both proteins mainly colocalized in intracellular vesicle-like structures with partial distribution in the plasma membrane. These data are in accordance with the previous report showing an association of exogenous ADAM12 with endoplasmic reticulum, trans-Golgi networks, and the plasma membrane (33, 51). To demonstrate that interaction between ADAM12 and RACK1 occurs under physiological conditions we first analyzed the expression of both proteins in liver tissues and determined the cell source of RACK1. As we have previously described for ADAM12 (1), we showed that the RACK1 mRNA level was increased in patients with hepatocellular carcinomas, both in tumor and underlying fibrotic livers. According to this observation, we showed that activated hepatic stellate cells, which plays a critical role in fibrogenesis and tumor progression (34, 52), highly express RACK1. In addition, we could clearly detect an interaction between endogenous ADAM12 and endogenous RACK1 in hepatic stellate cells, suggesting a functional relationship in vivo. In support of this, ADAM12 and RACK1 were colocalized in isolated hepatic stellate cell and human tissues. Alteration of RACK1 expression has been previously reported in human cancer and an increase in mRNA levels was observed in lung, colon, breast carcinoma (53), and ovarian cancer cells (54). Similarly, we showed that hepatocellular carcinoma cell lines expressed RACK1 suggesting that hepatic cancer cells contribute to RACK1 expression in patients with hepatocellular carcinoma.

Interestingly, RACK1 expression has been reported to be stimulated by growth promoting extracellular stimuli (55) suggesting involvement of RACK1 in the cell adaptive response to the microenvironment. Thus, RACKs are the main adaptors of PKC signaling pathways that play a critical role in transduction of signals for cell proliferation, differentiation, and apoptosis (56). In addition, the requirement for PKC activation by extracellular signals including phorbol esters, has been demonstrated for RACK1-PKC interaction and translocation of PKC to the membrane fraction (57). Consistently, we demonstrated that ADAM12 is constitutively expressed and localized inside stellate cells and that PMA stimulation of HSC induced translocation to the membrane fraction of protein complexes containing RACK1, PKC, and ADAM12, suggesting that RACK1 acts as an adaptor protein bridging PKC to ADAM12. In accordance with these data, ADAM12 was previously shown to interact with the PKCe (17) thereby facilitating translocation of

**Figure 5.** The matrix component, type I collagen, induces ADAM12 redistribution through β1-integrin stimulation. Hepatic stellate cells were seeded on uncoated (control) or coated dishes. After 24 h, ADAM12 redistribution was analyzed by using immunohistochemistry (A), FACS (B), and Western blotting analyses (C–E). A, cells cultured on type I collagen showed higher ADAM12 labeling in the focal adhesion structure. The panels represent two representative fields. B, FACS analysis using ADAM12 antibodies, rb122, or control isotype IgG shows that type I collagen coating induces ADAM12 cell surface expression (black line, uncoated; and red line, collagen coated). C and D, cells were seeded on an increased amount of collagen I (C) or other matrix component including collagen IV, laminin, or fibronectin (D). Western blotting showed increased amounts of ADAM12 in membrane fractions from cells cultured on type I collagen. E, cells were preincubated without or with anti–β1-integrin blocking antibody or control mouse IgG1 before seeding on type I collagen. Blocking antibodies diminished ADAM12 translocation to the membranes. Ab, antibody; IB, immunoblot.
RACK1 Interacts with ADAM12

ADAM12 to the cell surface (36). Taken together these data suggest that activity of ADAM12 at the cell surface is tightly regulated by extracellular stimuli-dependent translocation. In addition, both the rapid translocation of ADAM12 in response to PMA stimulation and localization of ADAM12 in vesicular-like structures near the cell membrane suggest that ADAM12 is stored in secretory vesicles unless specific extracellular signaling induces redistribution of ADAM12 toward the membrane structure where its activity takes place. In accordance with this hypothesis, we showed that PMA treatment induced the cleavage of Delta-like 1 that was shown to be a new substrate for ADAM12 (42). Similarly, breast cancer-associated mutations in ADAM12 were recently demonstrated to interfere with translocation of the protein to the cell surface resulting in loss of Delta-like 1 proteolysis (58). Interestingly, the human Delta-like 1 homologue has been previously reported in activated hepatic stellate cells and implicated in the progression of liver fibrosis (59). Taken together these data suggest that ADAM12 activity at the cell surface might contribute in Notch signaling in hepatic stellate cells.

The physiological relevance of this phenomenon was demonstrated by analyzing the role of the microenvironment on ADAM12 translocation in hepatic stellate cells. Indeed, during chronic liver disease, hepatic stellate cells undergo “activation” that consists in transition from quiescent vitamin A-rich cells into proliferative, fibrogenic, and contractile myofibroblasts (60). The normal basement membrane-like matrix present within the space of Disse converts to a matrix rich in type I collagen and modification of the microenvironment affects in return HSC activation mainly supported by engagement of integrins. Our data showed that type I collagen coating mimics the phorbol ester effects by promoting ADAM12 translocation to the membrane where ectodomain shedding activity and/or interaction with adhesion molecules take places. We proposed a model in which type I collagen binds its receptor, β1-integrin leading to PKC activation, recruitment of RACK1, and further ADAM12 translocation to the HSC membrane fraction. Consistent with this hypothesis, it was previously shown that integrin stimulation by ligands (61) activates phospholipase C leading to inositol 1,4,5-trisphosphate-dependent Ca\(^{2+}\) mobilization and diacylglycerol release, which further stimulates protein kinase C activation (62). Once activated, PKCε interacts with RACK1, which mediate the association of PKCε with β1-integrin (43). Interestingly, Kawaguchi et al. (19) have previously reported the interaction between ADAM12 and β1-integrin that could result from RACK1-dependent translocation of ADAM12 to the membrane fraction.

In summary, we have identified the scaffolding protein RACK1 as a new ADAM12 interacting protein, thereby modulating the PKC-dependent translocation of ADAM12 to the membrane. In addition we showed that type I collagen, the main matrix component in chronic liver disease, is a physiological inducer of ADAM12 translocation through binding to integrin receptors and activation of a PKC/RACK1-dependent pathway. Once, at the cell surface of hepatic stellate cells, ADAM12 may modulate cell adhesion and spreading through interaction with β1-integrin (19), affect transforming growth factor-β signaling through association with TGFβRII (27) or modulate Notch signaling through Dll1 processing (42).

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RACK1, a New ADAM12 Interacting Protein: CONTRIBUTION TO LIVER FIBROGENESIS
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