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Citron Kinase Is a Cell Cycle-dependent, Nuclear Protein Required for G_2/M Transition of Hepatocytes*

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Citron Kinase (Citron-K) is a cell cycle-dependent protein regulating the G_2/M transition in hepatocytes. Synchronization studies demonstrated that expression of the Citron-K protein starts at the late S and/or the early G_2 phase after that of cyclin B1. Expression of Citron-K is developmentally regulated. Levels of Citron-K mRNA and protein are highest in embryonic liver and gradually decrease after birth. Citron-K exists in interphase nuclei and begins to disperse into the cytoplasm at prophase. It concentrates at the cleavage furrow and midbody during anaphase, telophase, and cytokinesis, implicating a role in the control of cytokinesis. However, studies with knockouts show that Citron-K is not essential for cytokinesis in hepatocytes. Instead, loss of Citron-K causes a significant increase of G_2 tetraploid nuclei in one-week-old rat and mouse liver. In addition, Citron-K deficiency triggers apoptosis in a small subset of embryonic liver cells. In summary, our data demonstrate that Citron-K has a distinct cell cycle-dependent expression pattern and cellular localization as a downstream target of Rho-GTPase and functions in the control of G_2/M transition in the hepatocyte cell cycle.

The small GTPase Rho is required for cytokinesis as a regulator of the actomyosin contractile ring. Immunofluorescence showed that RhoA accumulates at the cleavage furrow during cytokinesis in Swiss 3T3 cells and HeLa cells (1, 2). Inactivation of Rho blocked cytokinesis as demonstrated in several experimental systems including *Drosophila* embryos, *Xenopus* embryos, mammalian cells, etc. (3–9). RhoA is also critical for cytokinesis in hepatocytes. Overexpression of the dominant negative mouse Ect2, a guanine nucleotide exchange factor (GEF) that activates RhoA, caused cytokinesis failure and the formation of binucleated cells in cultured mouse hepatocytes (10).

Rho regulates cytokinesis through its downstream targets. The Rho-associated kinases ROCK[1] I and II and Citron kinase (Citron-K) bind to Rho-GTP and have been shown to be involved in the regulation of cytokinesis (2, 11–15). ROCKs and Citron-K are serine/threonine kinases, and they probably regulate cytokinesis by phosphorylating downstream targets. ROCKs activate the regulatory myosin light chain (RMLC) directly by phosphorylating its Ser-19 and indirectly by inhibiting myosin phosphatases (12). Citron-K also phosphorylates RMLC at Ser-19 and Thr-18, but it does not phosphorylate myosin phosphatases (16).

The gene encoding Citron kinase has two major transcripts, the full-length Citron-K and a form of Citron that does not contain the kinase domain (2, 11). Citron is highly expressed in differentiated neuronal cells (17, 18). In contrast, the expression of Citron-K mRNA is limited to the proliferating neuroblasts, as revealed by *in situ* hybridization (15). Citron-K mRNA is detected by Northern blots in adult murine tissues including brain, kidney, spleen, thymus, skin, lung, and at very high levels in testis, but it is not detected in liver, heart, and skeletal muscle (11). Considering its strong expression in highly proliferative tissues such as testis or embryonic neuronal cells, it is possible that the expression of Citron-K is cell cycle-dependent rather than tissue-specific. Therefore, the absence of Citron-K in adult mouse liver could be a consequence of the quiescent state of the tissue.

The major function of Citron-K is the control of cytokinesis. Citron-K localizes to the cleavage furrow and midbody in anaphase and telophase in HeLa cells (2, 9). Overexpression of truncated or kinase-dead constructs of Citron-K induces formation of binucleated HeLa cells (2). The requirement of Citron-K for cell division is cell type-specific as revealed by examining the knockout animals. Loss of Citron-K causes failure of cytokinesis and therefore triggers apoptosis in the male germ cells and a specific population of neuroblasts (15, 19, 20). Whether Citron-K is involved in the control of hepatocyte cell cycle and cytokinesis is not clear.

MATERIALS AND METHODS

Reagents—All chemicals were purchased from Sigma except where otherwise indicated.

Animals—Male adult Fisher 344 rats (2 months old) and pregnant Fisher 344 rats were purchased from the Charles River Breeding Laboratories (Raleigh, NC). One pair of heterozygous Wistar Kyoto rats with a mutation at the Citron-K gene was a gift from Dr. Lo Turco, University of Connecticut, Storrs, CT. They were bred to generate the Flathead (FH) rats, the heterozygous rats, and the homozygous wild type (WT) rats. The autosomal recessive mutant FH rat is a spontaneous Citron-K knockout. It was named because of an easily recognized phenotype, a flattened skull of the neonate (21). The mutation was confirmed to be a single base deletion in the exon 1 of the *Citron* gene, resulting in an early stop codon, and no Citron-K protein is synthesized (20, 21). Genotypes of the littermates were determined by PCR with genomic DNA followed by restriction enzyme cutting. A fragment of exon 1 containing the mutation site was amplified by PCR followed by *Ban*II (Promega, Madison, WI) cutting of the PCR products. The wild

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[1] The abbreviations used are: ROCK, Rho-associated kinase; Citron-K, Citron kinase; RMLC, regulatory myosin light chain; FH flathead; WT, wild type; EGF, epidermal growth factor; PBS, phosphate-buffered saline; RT, reverse transcription; PCNA, proliferating cell nuclear antigen; PH, partial hepatectomy; E*n*, embryonic day (*e.g.* E15, embryonic day 15).

type gene has a *Ban*II cutting site lost in the mutant. The primers to exon 1 of the Citron-K gene in rat were based on the sequence of mouse exon 1 and were 5′GAGATGTTGAAGTTCAAGTA-3′ and 5′CCTGGAAGAAGAGATTTAGC-3′ (20). Phenotypes of FH rats are very similar to those observed in the Citron-K −/− mice with minor differences. Failure of cytokinesis causes the formation of binucleated neuronal cells. Massive apoptosis is detected in the developing central nervous system, which leads to the small brain size at birth (40% of normal) (22). FH rats begin to have seizures around postnatal day 7 and die in the first month (23). All animal studies were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Experiments have been conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Liver Perfusion—Single cell suspensions of hepatocytes were prepared with a modified two-step collagenase perfusion method (24). Cell viability was >90% as measured by trypan blue exclusion.

Primary Cultures of Rat Hepatocytes—Freshly isolated hepatocytes were seeded onto type I collagen (5–10 $\mu g/cm^2$)-coated tissue culture plates or coverslips at a density of 15,000 cells/cm^2 to 20,000 cells/cm^2 for maximal cell proliferation (25). Cells were seeded in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 5 μg/ml insulin with 100 μg/ml streptomycin and 100 units/ml penicillin for 4 h. The culture was then changed to a serum-free medium; RPMI 1640 was supplemented with 10 μg/ml iron-saturated transferrin, 100 nM dexamethasone, 5 μg/ml insulin, 10 ng/ml epidermal growth factor (EGF), 1×10^{-7} M copper ($CuSO_4$), 5×10^{-11} M zinc ($ZnSO_4 \cdot 7H_2O$), 3×10^{-10} M selenium ($NaSeO_3$), 100 μg/ml streptomycin, and 100 unit/ml penicillin. Both epidermal growth factor and insulin are hepatic mitogens and provide an additive stimulus for cell proliferation in culture.

Binucleation Ratio of Hepatocytes—Single cell suspensions of rat hepatocytes were fixed with 3.7% formaldehyde in PBS for 10 min and then stained with Hoechst 33342 (5 μg/ml in PBS and 0.1% Triton X-100, Molecular Probes, Eugene, OR). Cells were then checked under a fluorescence microscope. At least 2000 cells were counted for each sample, and the binucleation ratio of hepatocytes was calculated as the percentage of binucleated cells per total number of hepatocytes.

Nuclei Isolation and Flow Cytometric Analysis—Nuclei of rat hepatocytes were prepared by a modified trypsin-detergent method (26). A total of 20,000 events was collected for each sample with a FACScan (BD Biosciences). Quantitative analysis of cell cycle parameters was performed using the WinMDI program.

RT-PCR—A human fetal cDNA panel was purchased from Clontech and used as the template for PCR. Total RNA from liver samples were isolated using the RNeasy Mini kit (Qiagen, Valencia, CA). The cDNA was synthesized from 5 μg of total RNA using the SuperScript™ first strand synthesis system for RT-PCR (Invitrogen). To detect the specific signal for Citron-K, the forward primer was designed to locate within the kinase domain, and the reverse primer would be in the non-kinase domain of the molecule. The forward primer was designed according to the sequence of the rat *Citron* kinase domain nucleotides 883–906, *i.e.* 5′-TGGACTGTGACTGGTGGTCTGTCG-3′, whereas the reverse primer corresponds to rat *Citron* 1154–1177, *i.e.* 5′-TGGCCTCTGTGCTGGCTTTTACAG-3′. The primers amplify a 1.1-kb PCR fragment according to the sequence information of mouse *Citron-K*. These two primers are separated by at least one intron. Thus, a positive PCR signal with the right length should reflect the existence of the Citron-K mRNA. The PCR product was purified and sequenced to confirm that it is an amplified fragment of rat Citron-K cDNA. Semi-quantitation of PCR products is performed according to the method of Relative RT-PCR (Ambion, Austin, TX) by using β-actin as an internal standard with Ambion's QuantumRNA™ β-actin internal standards kit.

In Situ Hybridization—*In situ* hybridization was performed as described (15), with antisense RNA probes transcribed from plasmids containing fragments of *Citron-K* (nucleotides 911–2056) (11).

Hep3B Cell Culture and Transfection—Hep3B cells were cultured on 22-mm^2 coverslips in RPMI 1640 supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. Transfection was performed using FuGENE 6 transfection reagent (Roche). The cDNA of mouse *Citron-K* was cloned in-frame with an N-terminal FLAG tag in the pcDNA3 expression vector (Invitrogen) (11). Cells were cultured for 24–48 h after transfection to allow gene expression.

Immunofluorescence—Rat hepatocytes and Hep3B cells cultured on coverslips were fixed in methanol/acetone (1:1) for 5 min, and incubated with blocking buffer (phosphate-buffered saline, 2% goat serum, 0.1% Triton X-100) for 10 min. Primary antibodies were diluted with blocking buffer and used as follows: mAb mouse anti-α-tubulin 1:100 (Oncogene, Cambridge, MA); mouse anti-FLAG 1:200 (M2, Sigma); mouse anti-phospho-histone H3 (Ser-10) 1:100 (Cell Signaling, Beverly, MA); and a polyclonal rabbit anti-Citron antibody at 1:4000 (prepared by immunization of rabbits with a purified fragment of mouse Citron-K amino acids 454–637) (15). The secondary antibodies Alexa 488 conjugated goat anti-mouse IgG and Alexa 594 conjugated goat-anti-rabbit IgG (Molecular Probes) were diluted 1:500 with blocking buffer and incubated with the fixed cells for 1 h. Cells were then stained with Hoechst 33342 (Molecular Probes) to visualize the DNA. The stained cells were examined with an Olympus IX70 microscope (Olympus, Melville, NY) or a Leica confocal microscope.

Immunohistochemistry—Embryos were fixed by immersion in 4% paraformaldehyde in PBS for over 12 h, embedded in paraffin, and sectioned at 5 μm. Sections containing liver were stained with hematoxilin-eosin. Activated Caspase-3 was detected with an affinity-purified rabbit polyclonal antiserum (dilution 1:1000), which recognizes the p17 subunit of cleaved caspase-3 (kindly provided by Dr. A. Nelsbach, New England Biolabs, Beverly, MA).

Immunoprecipitation and/or Western Blotting—Immunoprecipitation and Western blotting were performed as described previously (11). Liver tissue samples were homogenized in a high stringency buffer (120 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40) with supplemented protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 45 μg/ml aprotinin, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 1 mM EDTA, 2 μg/ml antipain, and 1 μg/ml pepstatin A). Immunoprecipitation was performed with the rabbit polyclonal anti-Citron antibody (15) at a dilution of 1:2000. Cultured Hep3B cells and primary cultures of hepatocytes were harvested in radioimmune precipitation (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl, pH8.0, with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with the same set of protease inhibitors. Protein lysates or precipitates from immunoprecipitation were run on SDS-PAGE gels and transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA). Citron and Citron-K were detected with the same anti-Citron antibody at 1:4000. Other antibodies used were monoclonal antibody against cyclin B1, 1:200 (NeoMarker, Fremont, CA), and proliferating cell nuclear antigen (PCNA), 1:10,000 (Sigma). The secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse (Amersham Biosciences) and goat anti-rabbit (Jackson Laboratories, West Grove, PA) used at a dilution of 1:10,000 and 1:40,000, respectively. The signal was detected with an ECL plus kit (Amersham Biosciences) according to the manufacturer's instructions. The results shown are representative of at least three independent experiments. Densitometric analysis of Citron-K protein levels during development was quantified with the software Scion Image (Scion Corporation, Frederick, MD). Relative expression levels were determined by dividing the mean densitometric value of each stage by that found in the livers of first postnatal week animals.

Partial Hepatectomy—Two-thirds partial hepatectomy (PH) was performed on 2-month-old male F344 rats as described previously (27). Liver samples removed during surgery were saved as time 0 quiescence controls. Animals were euthanized by CO_2 asphyxiation at the following hourly intervals after PH: 20, 22, 24, 26, 28, and 30 h. Portions of livers were snap frozen in liquid nitrogen and stored at −80 °C for protein extraction. A sham operation, including laparotomy and mobilization of liver without tissue removal, was performed as a control.

Statistical Analysis—Significance of differences between FH rats and WT control was assessed by the Student's t test. $p < 0.05$ means significant difference.

RESULTS

Expression of Citron Kinase Is Not Tissue-specific but Cell Cycle-dependent—Expression of Citron-K has been considered tissue-specific, because Citron-K mRNA is not detected in adult mouse liver, heart, and skeletal muscle by Northern blots (11). *In situ* hybridization studies have shown that Citron-K mRNA was detected only in proliferating but not postmitotic neuronal cells (15), a phenomenon that could occur in other tissues. If so, the negative signal in adult mouse liver, heart, and skeletal muscles could be a result of their quiescent states. A positive signal of Citron-K should be detected in these tissues if undergoing proliferation. To address this question, Citron-K expression in proliferating cells was evaluated. RT-PCR detected Citron-K mRNA in human fetal heart, liver, spleen, kidney, thymus, skeletal muscle, and lung (Fig. 1*A*). *In situ* hybridization detected a positive signal of Citron-K mRNA in mouse fetal

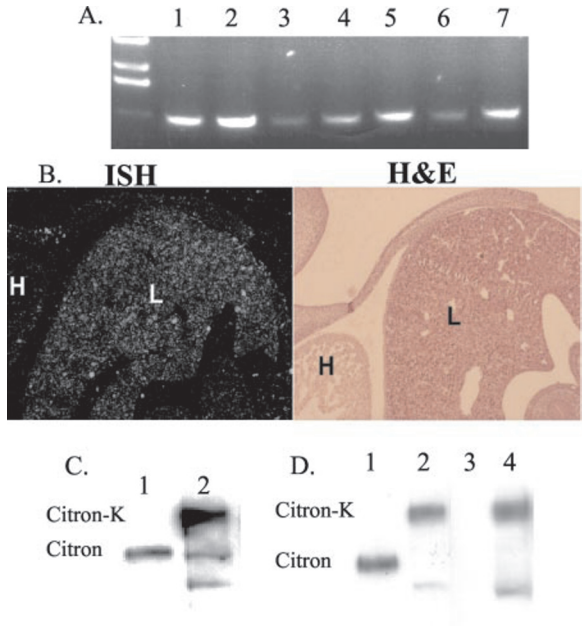

FIG. 1. **Expression of Citron kinase is not tissue-specific but cell cycle-dependent.** *A*, RT-PCR showed expression of Citron-K mRNA in human fetal tissues. *Lane 1*, thymus; *lane 2*, kidney; *lane 3*, skeletal muscle; *lane 4*, lung; *lane 5*, spleen; *lane 6*, heart; and *lane 7*, liver. *B*, *in situ* hybridization (*ISH*) detected a positive signal of Citron-K mRNA in embryonic mouse liver. *H*, heart; *L*, liver; *H&E*, hematoxilin-eosin. *C*, Western blots detected Citron-K protein expression in embryonic day 15 rat liver. *Lane 1*, adult rat cerebellum; *lane 2*, embryonic day 15 rat liver. *D*, Western blots detected Citron-K protein expression in the cultured Hep3B cell line and in cultured proliferating rat hepatocytes. *Lane 1*, adult rat cerebellum; *lane 2*, cultured Hep3B cell line; *lane 3*, freshly isolated hepatocytes from 2-month-old rat; *lane 4*, hepatocytes in culture for 72 h under the stimulation of insulin and epidermal growth factor.

liver (Fig. 1*B*). Citron-K is detected by Western blots in cultured Hep3B cells, embryonic day 15 (E15) rat liver, and cultured proliferating rat hepatocytes but not in quiescent adult rat hepatocytes (Fig. 1, *C* and *D*). These results strongly argue that Citron-K expression is cell cycle-dependent rather than tissue-specific.

The expression pattern of Citron-K was further characterized in regenerating liver after two-thirds PH because the first round of DNA synthesis by hepatocytes is naturally synchronized, providing a convenient model for studying the cell cycle expression pattern of proteins. Rat hepatocytes reach first peak of DNA synthesis at 24 h after PH as revealed by BrdUrd labeling and PCNA expression (27–30). Thus, we followed the protein expression ~24 h after surgery. Previous studies have demonstrated that cell proliferation induced by PH is predominantly non-binucleating (31, 32). Thus, the expression of Citron-K in the regenerative liver after PH will provide important information as to the relationship between Citron-K and liver cell cytokinesis.

The expression of two well studied cell cycle markers was examined to define stages of the cell cycle. PCNA starts to appear at the end of G_1, peaks in S, and declines at the G_2/M phases (33, 34). It has been shown that the protein level of PCNA parallels DNA synthesis as revealed by BrdUrd or [^{3}H]thymidine incorporation in the regenerating liver after PH (27, 30). Cyclin B1 is a critical regulator of mitosis. Expression of cyclin B1 starts at late S phase and accumulates until metaphase (35). After that, it is degraded quickly by a ubiquitin-dependent mechanism.

The protein level of Citron-K was detected using immunoprecipitation followed by Western blotting. The protein level of PCNA and cyclin B1 were analyzed using Western blots di-

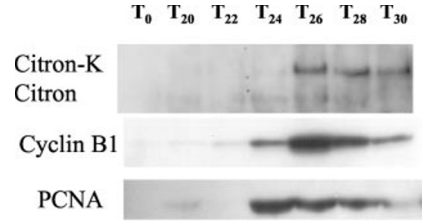

FIG. 2. **Expression of PCNA, cyclin B1, and Citron-K proteins in regenerating liver after two-thirds partial hepatectomy.** Expression of cyclin B1 and PCNA protein was analyzed by Western blotting. Total protein (50 μg) was loaded from each samples. Immunoprecipitation followed by Western blots was used to detect Citron-K. Total protein (3 mg) of each sample was used to pull down Citron-K. Liver tissue removed during the surgery was saved as the time zero (T_o) control. *T20–30* represented 20–30 h after PH. Shown is a representative result of three independent experiments.

rectly. PCNA protein level reached its first peak at 24 h after PH, a finding that is consistent with the literature. Citron-K was detected as early as 26 h after PH when cyclin B1 reached its first peak (Fig. 2*B*). These protein expression changes were not observed in the sham-control animals (data not shown).

The data presented here demonstrate that expression of Citron-K is cell cycle-dependent, not tissue specific. Its expression starts after that of cyclin B1 in the mitotic cell cycle, probably at late S and/or early G_2 phase.

Cellular Localization of Citron Kinase—Comparing the immunofluorescence signals from WT and FH hepatocytes permitted the identification of the specific signal derived from Citron-K. An evenly distributed positive nuclear signal was detected in a small subset of interphase nuclei in WT hepatocytes but not FH cells (Fig. 3*A*). A positive nuclear signal was also detected in cultured Hep3B cells, and this was further confirmed by XZ scanning with a confocal microscope (Fig. 4*A*). An examination of transfected FLAG-tagged Citron-K in Hep3B cells showed that overexpressed exogeneous Citron-K presented itself as puncta in interphase cells, and some puncta were localized in the nuclei as confirmed by confocal microscopy (Fig. 4*B*). Double staining with an antibody against the phosphorylated histone H3 at serine 10 allowed the recognition of mitotic cells. Citron-K began to disperse into cytoplasm at prophase (Fig. 5, *A* and *B*) and was distributed to the whole cytosol during prometaphase (Fig. 5*C*), metaphase (Fig. 3*B*), and early anaphase (Fig. 3*A*). It moved to the cleavage furrow during anaphase (Figs. 3*B* and 6*A*) and concentrated at midbody during telophase and cytokinesis (Fig. 6*B*), which is consistent with previous reports on HeLa cells (2, 9). A punctate signal was detected in some interphase and mitotic cells (Figs. 3*B* and 6, *C* and *D*). Double staining with anti-α-tubulin antibody showed that the intracellular puncta resembled the signal from the midbody residue after cytokinesis (Fig. 6, *C* and *D*). The intracellular puncta could be detected in any part of the cytosol, including cleavage furrow. In most cases, only 1–3 puncta existed in a single hepatocyte.

Expression of Citron Kinase Declines Gradually during Development—The mRNA level of Citron-K was detected with RT-PCR and further quantified with β-actin serving as an internal standard. The mRNA level of Citron-K was detected in embryonic day 14 (E14) rat livers and increased to its highest level in E15 and E16 rat livers. Its expression gradually declined after birth and was beneath the detection limit of RT-PCR after the third postnatal week (Fig. 7*A*).

Protein expression of Citron-K showed a similar pattern to that of its mRNA. The level of Citron-K protein was highest in E15 rat liver; it decreased after birth and stayed stable for the first week, then it decreased again and stayed at that level until weaning, when a third decrease happened. After that,

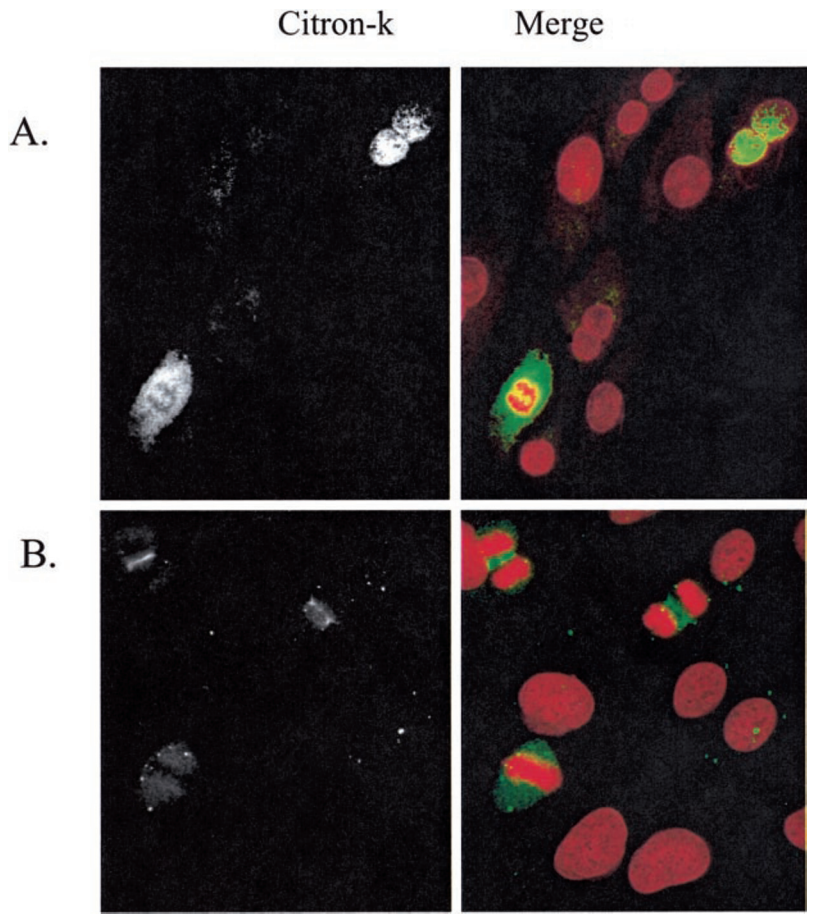

FIG. 3. **Cellular localization of Citron-K.** The merged image is a double staining of Citron-K (*green*) and DNA (*red*). *A*, cultured rat hepatocytes. *B*, cultured Hep3B cells.

Citron-K was no longer detected by Western blots (Fig. 7*B*). In addition, expression of Citron was also detected in rat liver and mirrored that of Citron-K with a gradually decreasing pattern during the first 4 weeks of postnatal life. A very low and constant level of both Citron and Citron-K protein was detected in rat livers at weeks 4 and 5 with immunoprecipitation followed by Western blots (Fig. 7*C*). Densitometric analyses of the Western blots indicated that Citron-K protein levels in E15 rat livers were as much as 8.25-fold higher than those observed in the livers in the first postnatal week (Fig. 7*D*). In addition, by the time of weaning Citron-K had declined to a level that was only 5% of that in the first postnatal week. Finally, the ratio of Citron-K *versus* Citron decreased from four at E15 to one after weaning. In summary, both mRNA and protein expression of Citron-K decrease gradually after birth, reaching the lowest level after weaning.

Citron-K Knockout Mice and Rats (Flathead Rats)—The relevance of Citron-K to the cell cycle and cytokinesis control in hepatocytes was examined by comparing the nuclear pattern and ploidy level of hepatocytes in FH rats with that in WT littermates. Based on the developmental expression pattern of Citron-K, neonates were examined in their first postnatal week when Citron-K is still expressed at a relatively high level. This stage also provides a better match of the body weight of the animals because FH rats grow slower than their littermates, and the differences in body weight and body size become more apparent after 1 week. Finally, interference from the natural binucleating process of hepatocytes is reduced to a minimal level at this age of the animals. Western blotting confirmed that no Citron-K was detected in either the freshly isolated or the cultured proliferating hepatocytes from FH rats (Fig. 8*A*). Isolated hepatocytes were cultured and checked under phase contrast microscope after 24 h.

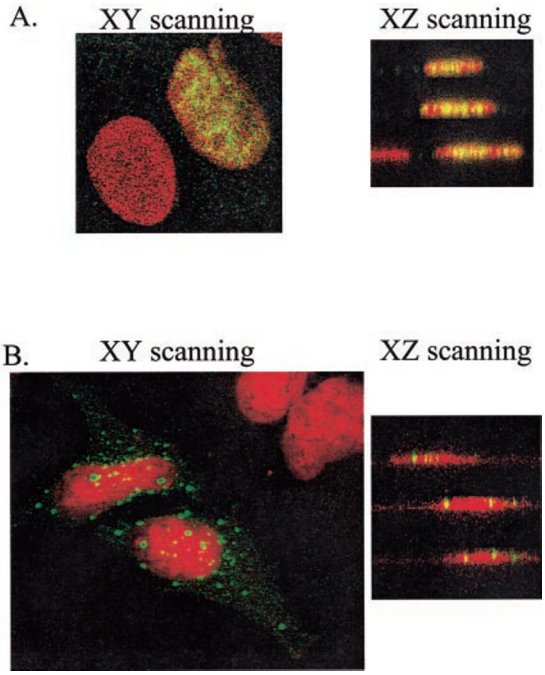

FIG. 4. **Confocal microscopy to confirm the nuclear localization of Citron-K.** *A*, endogenous Citron-K localizes in a small subset of interphase Hep3B cells. *Green*, Citron-K; *red*, DNA. *B*, exogenous Citron-K exists in the nuclei of Hep3B cells transfected with FLAG-Citron-K plasmid. *Green*, FLAG-Citron-K; *red*, DNA.

Cells from WT and FH rats presented a similar morphology in culture (data not shown). A low percentage of binucleated hepatocytes do exist in both WT and FH animals but with no

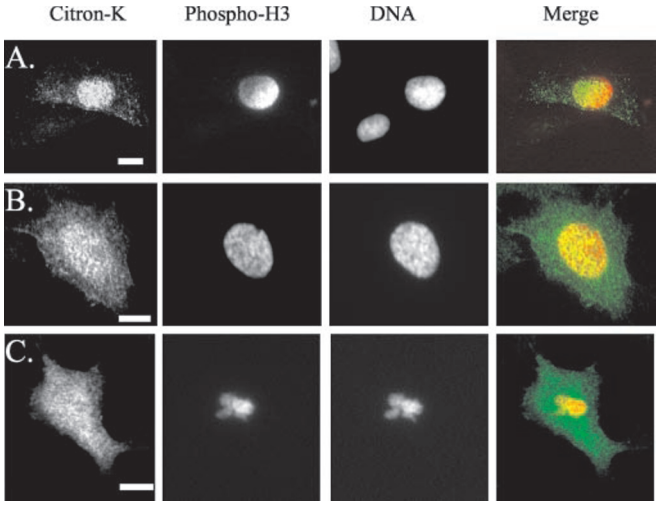

FIG. 5. **Cellular localization of Citron-K in cultured rat hepatocytes during prophase and prometaphase.** *A*, Citron-K began to move to cytoplasm at early prophase. *B*, Citron-K localized to both nucleus and cytosol during prophase, *C*, Citron-K dispersed to the whole cytosol during prometaphase. The merged image is a double staining of Citron-K (*green*) and phospho-histone H3 (*Phospho-H3*) at Ser-10 (*red*). *Bars*, 5 μm.

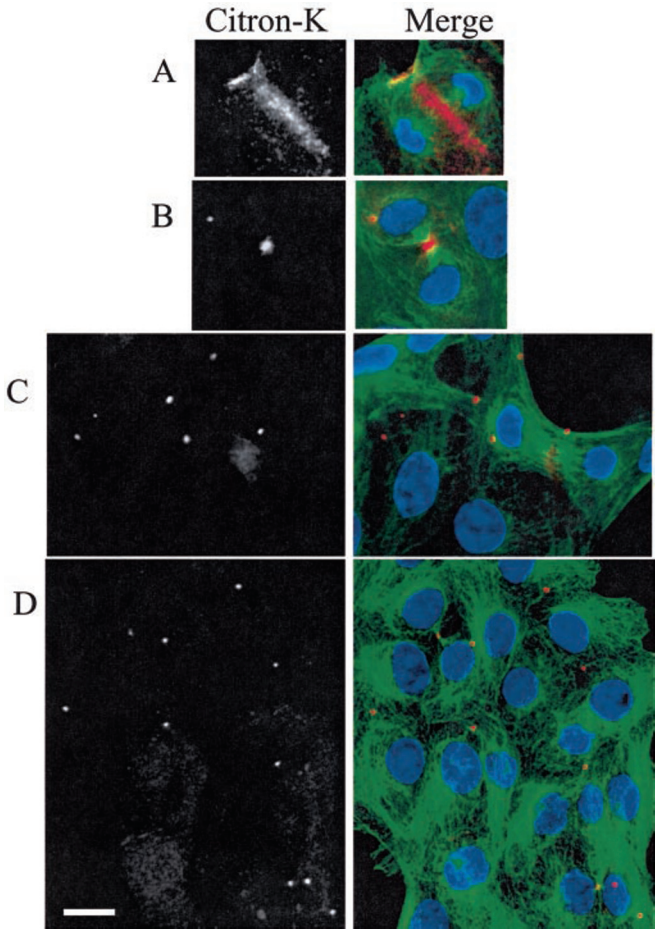

FIG. 6. **Cellular localization of Citron-K in cultured rat hepatocytes.** The merged image is a triple staining of α-tubulin (*green*), Citron-K (*red*), and DNA (*blue*). *A*, Citron-K localizes to cleavage furrow during anaphase. *B*, Citron-K localizes to midbody during telophase and cytokinesis. *C*, Citron-K presents itself as puncta in some interphase and mitotic cells. *D*, Citron-K puncta exist at midbody at the end of cytokinesis and in some interphase cells.

significant difference (2.8 ± 0.35% for FH and 2.6 ± 0.53% for WT, $n = 4$, $p > 0.05$) (Fig. 8*B*). These results suggest that Citron-K is not essential to the cytokinesis of rat hepatocytes and that some functionally redundant molecules exist in these cells. Flow cytometric analysis of hepatocyte nuclei from one-week-old rats revealed a significant 50% increase of the G_2 tetraploid nuclei in FH rat livers over controls (5.0 ± 0.22% for FH *versus* 3.3 ± 0.28% for WT, $n = 5$, $p < 0.05$) (Fig. 8*C*). There were no significant differences among the diploid G_0/G_1 nuclei percentages between FH and WT animals (WT 93.3 ± 0.44% *versus* FH 91.6 ± 0.53%, $n = 5$, $p > 0.05$) and those in S phase (WT 3.4 ± 0.20% *versus* FH 3.4 ± 0.34%, $n = 5$, $p > 0.05$). Identical results have been obtained with Citron-K −/− mice (data not shown). These data indicate that Citron-K influences the G_2/M phase transition of the cell cycle in hepatocytes. Moreover, increased apoptosis was detected in embryonic livers of the Citron-K −/− mice. Activated caspase 3 immunostaining, a marker for apoptotic cells, was obvious in the liver of E14.5 Citron-K −/− mice but not WT animals (Fig. 8*D*), suggesting that Citron-K is essential to some embryonic liver cells.

The structural and functional similarity between ROCKs and Citron-K make ROCKs the possible candidates to compensate the lost function of Citron-K. Protein levels of ROCKs in FH rats and the inhibition of ROCK function in Citron-K knockout cells might reveal whether the dispensability of Citron-K is due to the up-regulation and/or existence of ROCKs. Western blotting detected similar protein levels of ROCK I and ROCK II in both freshly isolated and cultured hepatocytes from WT and FH rats (Fig. 8*E*). Thus, the expression of ROCKs was not up-regulated in the Citron-K deficient situation. The requirement of Citron-K and ROCKs for hepatocyte cytokinesis was further investigated with the application of the specific inhibitor of ROCKs, Y-27632, to the primary culture of isolated hepatocytes from both WT and FH rats. The efficacy of Y-27632 was confirmed by blocking the formation of stress fibers in NIH 3T3 cells at 10 μM after 1 h and after 24 h of treatment (data not shown). Adding Y-27632 at four different concentrations (10, 25, 50 and 100 μM) to the cultured cells did not show easily detectable changes in cytokinesis in both WT and FH cells (data not shown).

DISCUSSION

This study demonstrates that Citron-K is the first known Rho-GTP downstream target with a cell cycle-dependent expression pattern and has a nuclear localization at interphase. In addition to a possible involvement in cytokinesis control, it is required for the G_2/M transition of hepatocytes.

Citron-K Has a Distinct Cell Cycle-dependent Expression Pattern and Cellular Localization—The expression of Citron-K is not tissue-specific but cell cycle-dependent. This result extends the previous observations on the central nervous system, *i.e.* that mRNA of Citron-K is detected only in proliferating but not postmitotic neuronal cells (15). It also explains that the absence of Citron-K mRNA in adult liver, and presumably also skeletal muscle and heart, is due to the quiescent state in these organs. The developmental expression data further confirms the cell cycle-dependent expression pattern of Citron-K, as it was shown previously that the percentage of cycling cells is quickly reduced in rat liver after birth (36). On the other hand, the decline of both Citron-K mRNA and protein after weaning is inversely correlated with the postweaning binucleation of hepatocytes. Considering the function of Citron-K in the control of cytokinesis, this observation might provide some indirect evidence to support the long believed hypothesis that acytokinesis is the basis of hepatocyte binucleation.

The small GTPases of the Rho family members are critical regulators in many important cellular functions. At present,

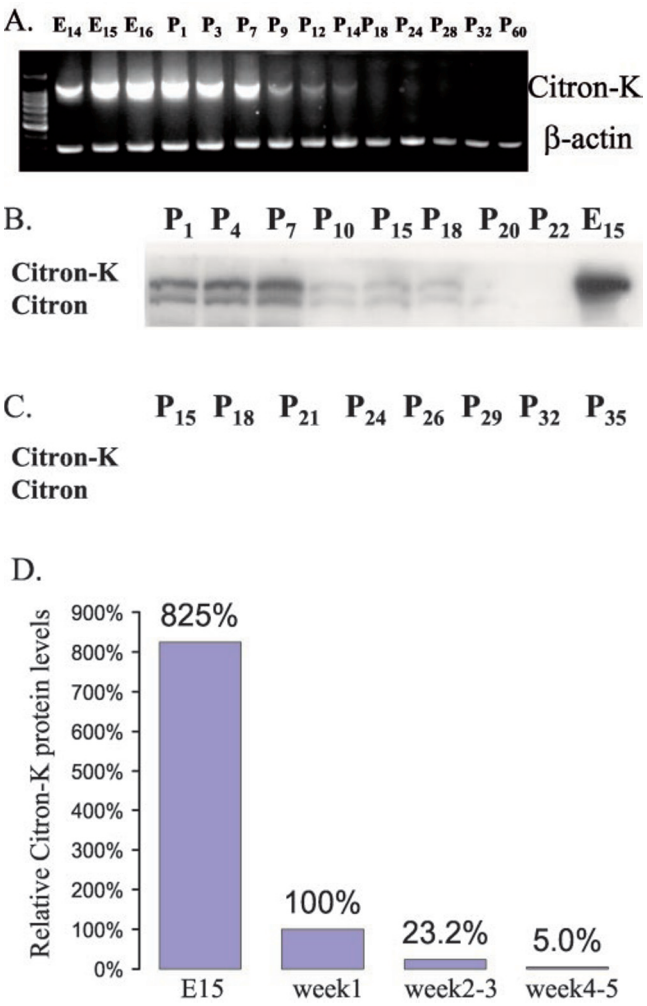

FIG. 7. **Citron kinase expression decreases gradually during development in rat liver.** *A*, relative RT-PCR confirms the quantitative changes of Citron-K mRNA during rat liver development. Semiquantitative analysis of the RT-PCR products was achieved by using β-actin as an internal standard. *E14-16*, embryonic days 14–16. *P1–60*, postnatal days 1–60. *B*, Citron-K protein level gradually decreased during development as revealed by Western blots. *E15*, embryonic day 15; *P1–22*, postnatal days 1–22. Total protein loaded in E15 samples (50 μg) was only half of that after birth (100 μg). *C*, immunoprecipitation followed by Western blotting to show the low levels of both Citron-K and Citron-N in the liver from P15-P35 day-old rats. Total protein used for immunoprecipitation was 6 mg. *P15–35*, postnatal days 1–35. Shown is a representative result of three independent experiments. *D*, densitometric analysis to show the relative level of Citron-K protein expression during development in rat liver. Each stage consisted of 4–10 samples.

Citron-K is the only known downstream target of Rho that presents a cell cycle-dependent expression pattern, which makes it extremely possible that Citron-K might act to switch Rho from its other cellular functions to its role in cytokinesis. In support of this idea, a reduced RhoA signal at the cleavage furrow was detected in mitotic neuronal cells isolated from Flathead rats, suggesting that localization of RhoA to the cleavage furrow is partly Citron-K-dependent (20).

The finding of increased G_2 nuclei in the liver of the Citron-K knockouts implies that Citron-K functions in the G_2/M transition, which is consistent with observations on the central nervous system in Citron-K knockout mice (15). The small difference in the numbers of G_2 nuclei would be more meaningful considering that there is only a small percentage of cycling cells, whereas the majority of the diploid nuclei are actually in the quiescent G_0 state.

The immunofluorescence antibody staining shows that Cit-

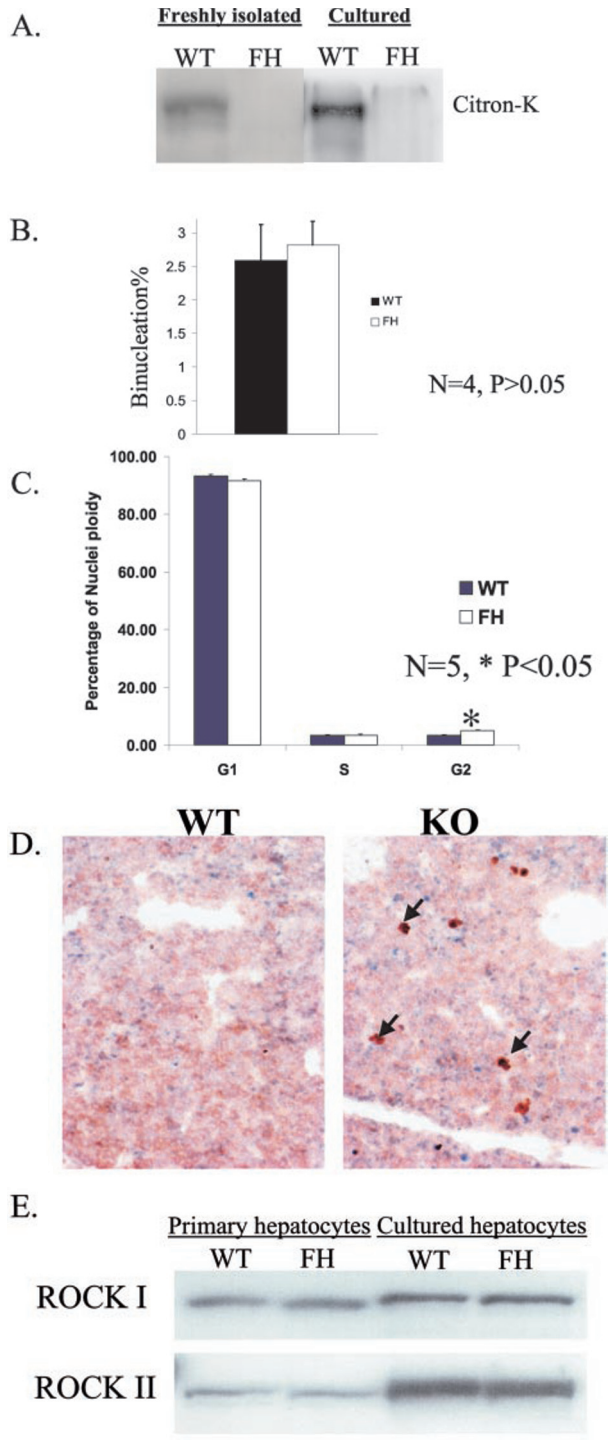

FIG. 8. **Analysis of the liver from Citron-K knockouts.** *A*, Citron-K protein is not detected in both freshly isolated and cultured hepatocytes of FH rats by Western blotting. Total protein loaded for each sample was 50 μg. *B*, binucleation ratio of hepatocytes isolated from Citron-K −/− and +/+ rats. Binucleated cells were counted, and binucleation percentage was calculated as 2.8 ± 0.35% for FH and 2.6 ± 0.53% for WT, $n = 4$, $p > 0.05$. *C*, flow cytometrical analysis of hepatocyte nuclear ploidy from WT and FH rats during the first week after birth. There was no significant difference between the WT and FH group of diploid G_0/G_1 (WT 93.3 ± 0.44% *versus* FH 91.6 ± 0.53%) and S phase (WT 3.4 ± 0.20% *versus* FH 3.4 ± 0.34%) nuclei, but there is a 50% increase of G_2 population (5.0 ± 0.22% for FH *versus* 3.3 ± 0.28% for WT) ($n = 5$, $^*p < 0.05$ for G_2). *D*, increased apoptosis in embryonic liver of Citron-K −/− mice. Sections of the E14.5 liver of Citron-K +/+ and −/− mice were analyzed by anti-activated caspase-3 immunohistochemistry to detect apoptotic cells. *KO*, knockout. *Bar*, 200 μm. *Arrow* points to activated caspase-3 staining. *E*, Protein levels of ROCK I and II in both freshly isolated and cultured hepatocytes from 1-week old WT and FH rats examined by Western blots. An equal amount of 50 μg of total protein was loaded for each sample.

ron-K is a nuclear protein in a small percentage of interphase cells. Confocal microscopy confirms that both endogenous Citron-K and some exogenous FLAG-tagged Citron-K proteins localize in the nuclei. The fact that overexpressed FLAG-tagged Citron-K exists in the cytosol and the nucleus suggested that the nuclear import of Citron-K requires a specific condition. By searching the Prosite data base, a potential bipartite nuclear targeting sequence has been found in the Citron-K protein (39). Double staining with the anti-phospho-histone H3 Ser-10 antibody shows that cells at later G_2 and prophase have a nuclear positive signal for Citron-K, which is consistent with our Western blot data on the expression pattern of Citron-K. The nuclear localization of Citron-K may provide useful information for understanding the mechanism of Citron-K in the control of the G_2/M transition. Thus, Citron-K is a downstream target of Rho with a nuclear localization. Because Rho is a cytoplasmic protein, the function of Citron-K in the nucleus may not be Rho-dependent. An interesting note to mention is that mouse Ect2/Drosophila pebble, an upstream stimulator of Rho, also has a cell cycle-dependent expression pattern, a nuclear localization at interphase, and a function in cytokinesis regulation (37).

An additional observation in our study is that Citron-K still diffuses to the whole cytosol at the beginning of anaphase, right after the sister chromatids dissociate from each other. Citron-K then quickly moves to the cortex of the cleavage furrow, a time point we speculate to be the initiation of cytokinesis. Whether this transition is a precondition or a consequence of the formation of the cleavage furrow, it suggests that Citron-K is probably involved in the earliest step of cytokinesis, even though a truncated Citron-K construct only interfered with the final stages of cytokinesis in transfected HeLa cells (2).

In a previous paper it was argued that Citron-K existed as puncta in interphase cells (9). These punctas dispersed into the whole cytosol during prometaphase in order to conduct their function during cytokinesis. We question this interpretation for several reasons. These punctate signals resemble the midbody residue after cytokinesis. In most situations, only 1–3 punctas were observed in a single hepatocyte, whereas a similar number of puncta were observed in both interphase and mitotic cells. According to our results, Citron-K is a cell cycle-dependent protein expressed after cyclin B1. However, no obvious pattern was found between the number and size of the puncta with the cell cycle progress. In addition, it is hard to believe that the Citron-K protein that exists in this state could be functionally active in controlling the G_2/M transition. As reported previously, abscission occurred at the intercellular bridge on one side of the midbody, and the midbody residue was then left in the other side's daughter cell after cytokinesis (38). We would propose that some if not all punctas are more likely the residue of the midbody left in one daughter cell.

Citron-K Regulates Cytokinesis in Hepatocytes—The importance of Citron-K in the control of cytokinesis in hepatocytes is implicated by several observations. Citron-K localizes to the cleavage furrow and midbody during cytokinesis. The expression of Citron-K is highly up-regulated in proliferating dividing hepatocytes both *in vivo* and *in vitro*. In contrast, its expression is down-regulated during the postweaning binucleation of hepatocytes, a process believed to be caused by acytokinesis. The knockout studies indicate that Citron-K is not essential for cytokinesis in postnatal hepatocytes. Several possibilities should be considered. There might be unidentified functionally redundant molecules in these cells, considering the importance of cytokinesis. The control mechanism of cytokinesis could be different in varied cell types, and Citron-K might regulate a dispensable step of cytokinesis in hepatocytes. There could be distinct isoforms of Citron-K that are functional in embryonic *versus* adult developmental stages. Thus, the susceptibility to apoptotic death could be different in embryonic *versus* postnatal cells as observed in the central nervous system and in the testis (15, 19, 22). The knockout shows only a low penetrance cytokinesis phenotype due to the elimination of the binucleated hepatocytes by apoptosis during embryonic stages as suggested by increased activated caspase-3 staining in the fetal liver of Citron-K −/− mice.

ROCKs, the homologues of Citron-K and also the downstream targets of Rho-GTP involved in the regulation of cytokinesis, were the most promising functionally redundant alternatives. Both Citron-K and ROCKs phosphorylate the RMLC during cytokinesis (12, 16). However, the absence of Citron-K did not result in up-regulation of ROCKs in the neural cells (15) or the liver cells. In the Citron-K-sensitive neuronal cells, ROCKs do not cover the lost function caused by deficiency of Citron-K (15). Furthermore, our results show that inhibition of the kinase activities of ROCKs by its specific inhibitor Y-27632 does not block cytokinesis in cultured Citron-K −/− hepatocytes. This confirms that the lost function of Citron-K is not compensated by the kinase activity of ROCKs. In addition, even if RMLC is a substrate of both Citron-K and ROCKs during cytokinesis, it must be activated by at least one other kinase such as the myosin light chain kinase in these cells.

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