

Phosphorylation and mRNA Splicing of Collapsin Response Mediator Protein-2 Determine Inhibition of Rho-associated Protein Kinase (ROCK) II Function in Carcinoma Cell Migration and Invasion*

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Background: Kinase activity of ROCK II, an important regulator of cell migration, is controlled by its endogenous inhibitor CRMP-2.

Results: GSK3 phosphorylation of CRMP-2 reduces CRMP-2-ROCK II interaction.

Conclusion: GSK3 phosphorylation and mRNA splicing of CRMP-2 regulate ROCK II-dependent carcinoma cell behavior.

Significance: This study leads to an understanding of how ROCK-mediated carcinoma cell migration and invasion are regulated at multiple levels.

The Rho-associated protein kinases (ROCK I and II) are central regulators of important cellular processes such as migration and invasion downstream of the GTP-Rho. Recently, we reported collapsin response mediator protein (CRMP)-2 as an endogenous ROCK II inhibitor. To reveal how the CRMP-2-ROCK II interaction is controlled, we further mapped the ROCK II interaction site of CRMP-2 and examined whether phosphorylation states of CRMP-2 affected the interaction. Here, we show that an N-terminal fragment of the long CRMP-2 splice variant (CRMP-2L) alone binds ROCK II and inhibits colon carcinoma cell migration and invasion. Furthermore, the interaction of CRMP-2 and ROCK II is partially regulated by glycogen synthase kinase (GSK)-3 phosphorylation of CRMP-2, downstream of PI3K. Inhibition of PI3K reduced interaction of CRMP-2 with ROCK II, an effect rescued by simultaneous inhibition of GSK3. Inhibition of PI3K also reduced colocalization of ROCK II and CRMP-2 at the cell periphery in human breast carcinoma cells. Mimicking GSK3 phosphorylation of CRMP-2 significantly reduced CRMP-2 binding of recombinant full-length and catalytic domain of ROCK II. These data implicate GSK3 in the regulation of ROCK II-CRMP-2 interactions. Using phosphorylation-mimetic and -resistant CRMP-2L constructs, it was revealed that phosphorylation of CRMP-2L negatively regulates its inhibitory function in ROCK-dependent haptotactic cell migration, as well as invasion of human colon carcinoma cells. Collectively, the presented data show that CRMP-2-dependent regulation of ROCK II activity is mediated through interaction of the

CRMP-2L N terminus with the ROCK II catalytic domain as well as by GSK3-dependent phosphorylation of CRMP-2.

Protein kinases are important regulators of many cellular processes, for example, cell cycle, motility, metabolism, cell fate and apoptosis (1). Their activities are tightly regulated by various modes, such as phosphorylation, binding of regulatory proteins/molecules, and their subcellular localization. The two homologous 160-kDa Rho-associated protein kinases (ROCK),² ROCK I and ROCK II (2), belong to the AGC protein kinase family of classical serine/threonine kinases (3). These ubiquitously expressed kinases are key regulators of the actin cytoskeleton, downstream of the GTP-Rho, thereby controlling cell shape and behavior. ROCK signaling has been implicated in many widespread human diseases, including cancer, diabetic nephropathy, central nervous diseases, and cardiovascular disorders, and is being considered as a potential target for treatment of these conditions (2, 4–7). Therefore, it is necessary to reveal the activity regulation of these kinases in detail.

The ROCKs are activated through a conformational change. Under quiescent conditions they exist in an autoinhibitory conformation, where the C-terminal Rho binding and pleckstrin homology domains bind and inhibit the N-terminal kinase domain (8). This intramolecular interaction must be disrupted for kinase activity. The main activation mechanism of the ROCKs is binding of GTP-Rho to the Rho binding domain; however, binding of lipids to the pleckstrin homology domain of ROCK II in the absence of Rho-GTP can also lead to increased ROCK II kinase activity (9, 10). The ROCKs regulate the actin-myosin II cytoskeleton through control of myosin

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² The abbreviations used are: ROCK, Rho-associated protein kinase; aa, amino acids; ANOVA, analysis of variance; Cdk5, cyclin-dependent kinase 5; CRMP, collapsin response mediator protein; DMSO, dimethyl sulfoxide; DYRK, dual tyrosine-regulated kinase; GSK3, glycogen synthase kinase; L, long; REF, rat embryo fibroblast; S, short; S2, Schneider 2.

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light chain phosphorylation state and phosphorylation of LIM kinases or ezrin/radixin/moesin, leading to increased actin filament stability (11–15). Despite a range of common substrates, increasing evidence suggests nonredundant functions of the ROCK isoforms. In rat fibroblasts, ROCK I is essential for the formation of stress fibers and focal adhesion, whereas ROCK II is required for phagocytic activity (10). Further evidence for distinct functions of the two ROCKs is the identification of several isoform-specific regulators of ROCK activity (2, 16–21).

Recently, we identified a novel endogenous ROCK II inhibitor, collapsin response mediator protein (CRMP)-2, whose impact on the kinase activity can modulate carcinoma cell adhesion and migration, for example (21). CRMP-2 is a ubiquitously expressed protein belonging to the CRMP family of cytosolic phosphorylated proteins (21–24). CRMP family members were originally identified as mediators of semaphorin signaling, which induce F-actin depolymerization and growth cone collapse (25). However, data have implicated the CRMP family in a range of other cellular functions, such as apoptosis, proliferation, cell migration, and differentiation (21, 23, 26). CRMP-2 exists in two splice variants, named CRMP-2S (short)/CRMP-B and CRMP-2L (long)/CRMP-A, consisting of a common core protein and distinct N-terminal domains (27). Some activities of CRMP-2 are regulated by phosphorylation of multiple serine and threonine phosphorylation sites at the C terminus. In lysophosphatidic acid- or ephrin-, but not semaphorin-3A-, induced growth cone collapse, CRMP-2 is phosphorylated by ROCK at Thr-555 (CRMP-2S), thereby inhibiting CRMP-2 binding to tubulin and Numb (28, 29). The Thr-555 phosphorylation site of CRMP-2 is not conserved in the other CRMPs (30), making it a unique site of regulation for CRMP-2. A major regulator of CRMP-2 function is glycogen synthase kinase 3 (GSK3), which sequentially phosphorylates CRMP-2 at three sites, Ser-518, Thr-514, and Thr-509 (31). Mimicking phosphorylation on Thr-514 correlates with reduced binding of CRMP-2 to tubulin (32). CRMP-2 is primed for GSK3 phosphorylation through phosphorylation by cyclin-dependent kinase 5 (Cdk5) or dual tyrosine-regulated kinase (DYRK) at Ser-22 (31–34). In normal proliferating cells, GSK3 α/β are relatively inactive because of phosphorylation on serine 21/9 by Akt (35).

In this study, we aimed at further understanding of CRMP-2 regulation of ROCK II activity and hypothesized that phosphorylation states of CRMP-2 affect the interaction. Here, we report that GSK3 partly regulates the interaction between CRMP-2 and ROCK II by phosphorylating CRMP-2. This phosphorylation of CRMP-2 is involved in the regulation of human colon carcinoma cell invasion and haptotactic migration. Furthermore, inhibition of ROCK-mediated migration is found to be facilitated by binding of the unique N terminus of CRMP-2L alone to the kinase domain of ROCK II.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies against ROCK II (C-20), ROCK I (K-18), RhoA, and actin (I-19) were purchased from Santa Cruz Biotechnology. Antibody against actin (C4) from Chemicon (Millipore) was used. Monoclonal antibody against CRMP-2 (C4G) was from IBL Co. Ltd. (Gunma, Japan), and anti-V5 monoclonal antibody was from Invitrogen. Monoclonal anti-

TABLE 1
Primers used for cloning of CRMP-2

Primer	Sequence
AYC42	5'-pGTC TGT CTT AGC TGA TGA GGC CGG-3'
AYC44	5'-pCCT GCC AAG CAG CAG GCG CCA CCT GTT-3'
AYC45	5'-pGAC CCG GCC TCA GAT GCT AAG ACA T-3'
AYC46	5'-pGAC CGT CTT GGG ATC CAC AGA CAC CT-3'
AYC47	5'-CGC GAA TTC TAT GGC CGA GAG AAA GCA ATC CGG-3'
AYC48	5'-GCG AAT TCC GCC ACC ATG GCC GAG AGA AAG CAA TCC GGG AAG GC-3'
AYC49	5'-ATA GTC GAC TTA GCC CAG GCT GGT GAT GTT GGC AC-3'
AYC87	5'-GGC TCC CCA CTG GTG GTC ATC AGC CAG GG-3'
AYC88	5'-CCC TGG CTG ATG ACC ACC AGT GGG GAG CC-3'
AYC92	5'-GCA AGC TTA GCC AGG GCC GCC TTG GTT CCT TGG-3'
AYC93	5'-GCA AGC TTG AAA GCC ATG TAC ACG AGG AAG G-3'
AYC94	5'-CGC GAA TTC TAG CGA TCG TCT TCT GAT CAA AGG AGG-3'
AYC95	5'-CGC GAA TTC CCA AGG AAC CAA GGC GGC CCT GGC T-3'
AYC96	5'-ATA GTC GAC TTA GAA AGC CAT GTA CAC GAG GAA GG-3'
AY131	5'-ATA TAA GCT TGC CCA GGC TGG TGA TGT TGG CA-3'
AY197	5'-GCG AAT TCT GGC TCC CCA CTG GTG GTC ATC-3'

GSK3 (4G-1E) antibody was from Millipore. Anti-phospho-GSK3 α/β (Ser-21/9), anti-Akt, anti-pSer473 Akt, and anti-pThr308 Akt antibodies were from Cell Signaling Technology. HRP-labeled secondary antibodies were from Dako (Glostrup, Denmark); and fluorochrome-conjugated secondary antibodies, Alexa Fluor 488 F(ab')₂ fragment of goat anti-rabbit IgG (A11070), Alexa Fluor 488 rabbit anti-goat IgG (A11078), and Alexa Fluor 546 donkey anti-mouse IgG (A10036) were from Invitrogen.

Cell Culture—Rat embryo fibroblasts (REFs) were maintained in MEM α (Cambrex, East Rutherford, NJ) supplemented with 5% heat-inactivated FBS (Hyclone). The human breast tumor cell line, MDA-MB-231, was purchased from ATCC (Manassas, VA) and maintained in DMEM (Invitrogen) supplemented with 10% FBS. The human colon carcinoma cell line SW620 was from ATCC and maintained in DMEM/F12 medium (Invitrogen) supplemented with 10% FBS on collagen I-coated dishes. All mammalian cells were grown at 37 °C in a 5% CO₂ humidified atmosphere. *Drosophila* Schneider 2 (S2) cells were maintained in Schneider's *Drosophila* Medium (Cambrex) with 10% FBS at room temperature.

Immunoprecipitation and Western Blotting—Immunoprecipitation of ROCK II was carried out as described previously (10). Immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blotting with anti-ROCK II and anti-CRMP-2 antibodies. In some experiments, cells were pretreated with PI3K inhibitor wortmannin (500 nM; Calbiochem) or GSK3 inhibitor AR A0114418 (5 μ M, GSK3 inhibitor VIII; Calbiochem) in growth medium for 1 h at 37 °C. Representative Western blots are shown. *Black boxes* and *white lines* indicate that the blots were obtained from different parts of the same membrane. Values are shown as ratio *versus* control.

Plasmids—Construction of cDNAs encoding rat CRMP-2S and human CRMP-2L was described previously (21). Phosphorylation-mimetic mutation at Ser-522 was created by PCR with primer pair AYC44/AYC42 (see Table 1 for primer sequences) and rat wild type CRMP-2 as template. Phosphorylation-mimetic quadruple mutations T509D, T514D, S518D, and S522D

were created using primer pair AYC45/AYC46 and CRMP-2S with S522D mutation as template. The PCR products were digested with DpnI, phosphorylated with T4 polynucleotide kinase, and self-ligated with T4 DNA ligase. CRMP-2 fragment C carrying these mutations was constructed from rat CRMP-2S by PCR using primer pair AY197/AY131 and subcloned into EcoRI and HindIII sites of pET41b(+). Chimeras were constructed using cDNA encoding rat CRMP-2S T509A, T514A, S518A, and S522A (AAAA) and CRMP-2S T509A, T514A, S518A, and S522D (AAAD) in combination with human CRMP-2L WT, using primer pairs AYC87/AYC49, AYC48/AYC88, and AYC48/AYC49. The created cDNAs were subcloned into pIRES2-EGFP vector. cDNA encoding CRMP-2L phosphorylation-mimetic mutations at T619D, T624D, S628D, and S632D (equivalent to CRMP-2S 509, 514, 518, and 522) was created from pIRES2-EGFP CRMP-2L AAAD by whole plasmid PCR using primer pair AYC45/AYC46.

cDNAs encoding human CRMP-2L fragment A1' (aa 1–203), A2' (aa 1–275), A3' (aa 119–275), and A4' (aa 204–275) were subcloned into pET41b(+) or pIRES2-EGFP; creation of cDNA encoding CRMP-2L A' was described previously (21). Primers for pET41b(+) were AYC47/AYC92 (A1'), AYC47/AYC93 (A2'), AYC94/AYC93 (A3'), and AYC95/AYC93 (A4'), and for pIRES2 EGFP they were AYC48/AYC96 (A2'). Construction of bovine ROCK II cDNA and a fragment containing rat ROCK II catalytic domain (aa 1–543) in pMT/V5-His C was described previously (21). All constructs were verified by DNA sequencing.

Plasmid Transfection—Transfections of SW620 cells, with pIRES2-EGFP plasmids encoding CRMP-2L WT, CRMP-2L DDDD, CRMP-2L AAAA, and CRMP-2L A2' were achieved using Lipofectamine2000 according to the manufacturer's protocol (Invitrogen). Stable cell lines of SW620 cells expressing CRMP-2L WT, CRMP-2L DDDD, and CRMP-2L AAAA were obtained by FACS sorting for GFP expression, followed by G418 (1 mg/ml; Sigma) selection. Stable SW620 cells expressing CRMP-2L A2' were obtained by G418 (1 mg/ml; Sigma) selection. Cells were maintained in growth medium supplemented with 1 mg/ml G418 following selection. 24 h prior to assays cells were transiently transfected followed by serum starvation overnight.

Recombinant Protein Expression in Escherichia coli and Binding Assay—Expression of recombinant GST-CRMP-2 proteins and binding assays were described previously (21). Following Western blotting membranes were stained with Coomassie Brilliant Blue. Conventional GTP-RhoA pulldown assays were performed as described previously (10). The amount of bound protein in pulldown from control cells was set at 1.

Immunofluorescence Microscopy—MDA-MB-231 cells were seeded in growth medium on glass coverslips. In some experiments wortmannin (100 nM; Calbiochem) was added in growth medium for 2 h at 37 °C. Cells were fixed with 4% paraformaldehyde for 10 min. To stain cytoplasmic proteins, cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min. Cells were stained with primary antibodies (against ROCK II, CRMP-2 (C4G), pGSK3) at 4 °C overnight, followed by secondary antibodies. Controls for nonspecific cross-reacting of secondary antibodies were included and gave no staining above background. Fluorescence was viewed with a confocal laser

microscope (FV1000D IX81; Olympus, Tokyo, Japan) using an Olympus UPlanSApo 60× NA 1.35 oil objective. Images were processed using Adobe Photoshop (Adobe, San Jose, CA).

Cell Adhesion, Migration, and Proliferation Assays—Cell adhesion and migration assays with transfected SW620 cells were carried out as described previously (21, 36). Cell proliferation assays were performed by seeding transfected SW620 cells in triplicate on collagen I-coated 24-well plates. After 24 h in standard culture conditions, cells were fixed in 2% glutaraldehyde for 20 min, washed with PBS, and stained with 0.1% crystal violet and washed. Bound dye was released using 1% SDS in PBS, and A_{595} was measured. Data are shown as ratio *versus* control.

Cell Invasion—Cell invasion assays with transfected SW620 cells were carried out in 12-well 8.0- μ m pore transparent cell culture Transwell inserts from BD Biosciences. Cell matrix type I-A (Nitta gelatin; Osaka, Japan) was prepared as described previously (37), and inserts were coated by the addition of 200 μ l of matrix solution and removal of 150 μ l, followed by 30-min incubation at 37 °C in cell incubator prior to the assay. Serum-starved transfected SW620 cells were prepared as described previously (21). 5×10^5 cells were seeded on top of the coated filters, and the cells were left to invade the matrix for 24 h toward DMEM:F12 supplemented with 10% serum. The collagen matrix was removed using a cotton swab, and the invading cells were fixed in 4% paraformaldehyde for 20 min. Filters were washed in PBS, and invading cells were stained in 0.1% crystal violet. After washing filters in PBS, invading cells in five random fields for each filter were manually counted using the Olympus IX71 microscope and an Olympus UplanFl 10 × NA 0.30 objective. The number of invasive cells in the five fields counted was summed, and the mean number of invading cells in two filters was calculated for each transfectant. The control was set to 1. The mean number of mock-transfected cells counted per filter was 50.

Statistical Analysis—Western blots were quantified using TotalLAB (Newcastle upon Tyne, UK) and statistical analysis performed using SigmaPlot (Systat Software, Inc., San Jose, CA). Quantification was performed using unsaturated Western blotting.

RESULTS

An N-terminal Fragment of CRMP-2L Alone Is Capable of Inhibiting Haptotactic SW620 Colon Carcinoma Cell Migration and Invasion—The two CRMP-2 splice variants are distinct only in their N-terminal domains. Amino acids 1–13 and 1–118 for CRMP-2S and CRMP-2L, respectively, are different; therefore, ~80% of the sequences of CRMP-2S and -2L are identical (Fig. 1A). It was previously established that both the common C-terminal domain of CRMP-2S/-2L (Fig. 1A, fragment C) and the region containing the unique N-terminal domain of CRMP-2L (Fig. 1A, fragment A') interact with the catalytic domain of ROCK II (21). To further map the interaction site of CRMP-2L for ROCK II in detail, polypeptides derived from CRMP-2L (polypeptides A' (aa 1–380), A1' (aa 1–203), A2' (aa 1–275), A3' (aa 118–275), and A4' (aa 203–275)) were expressed in *E. coli* as GST fusion proteins and immobilized on glutathione-agarose. Using the immobilized proteins, pull-down assays of V5-tagged ROCK II catalytic domain (aa 1–543) expressed in S2 cells were performed (Fig. 1B). CRMP-2L A2'

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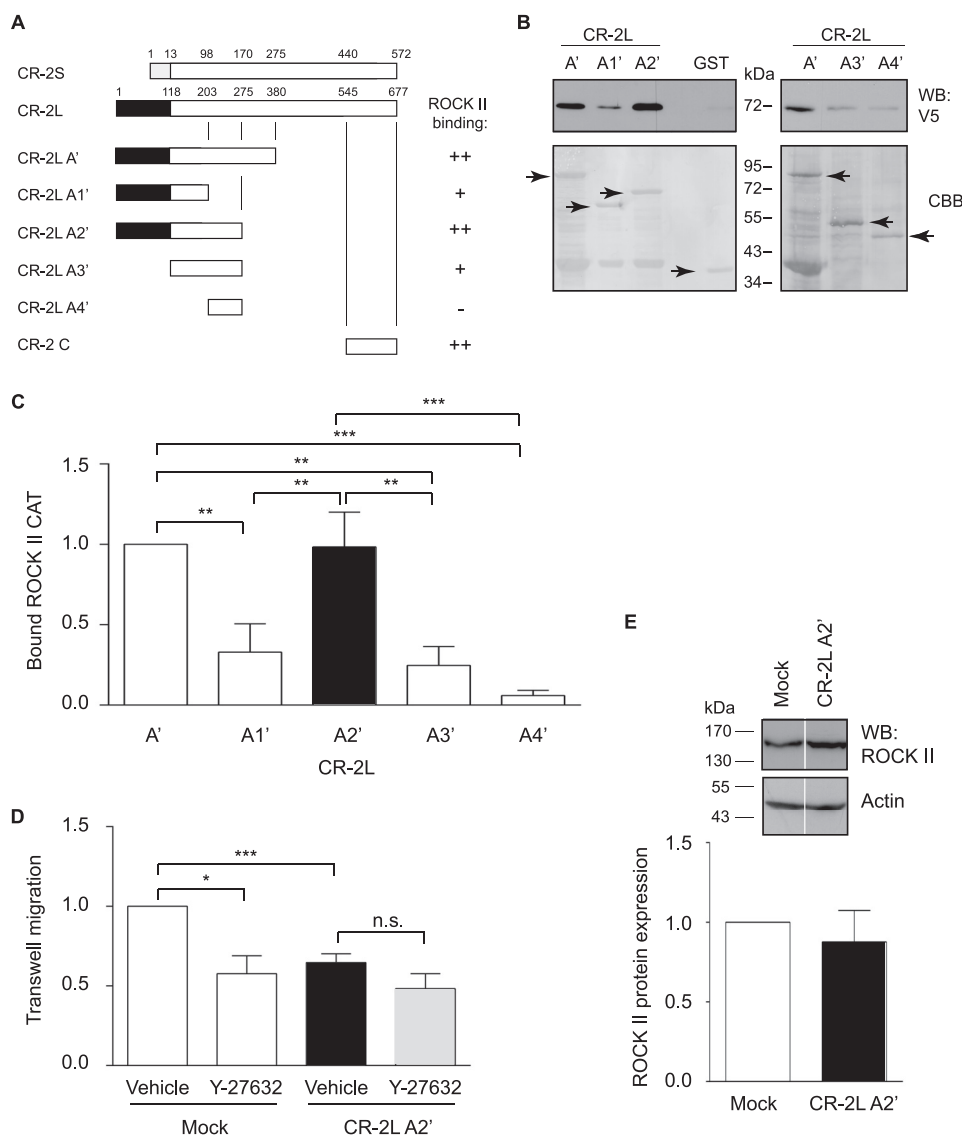


FIGURE 1. An N-terminal fragment of CRMP-2L binds the active form of ROCK II and blocks ROCK-dependent migration as well as invasion. *A*, schematic illustration of CRMP-2 constructs used in pull-down assays. Amino acid numbering from methionine 1 is shown above CRMP-2L and -2S. Shaded or filled boxes show unique regions of CRMP-2S and -2L, whereas white boxes show common core polypeptides. *B*, recombinant V5-tagged ROCK II catalytic domain expressed in S2 cells used for pull-down assays by CRMP-2 GST fusion proteins illustrated in *A*. Representative Western blots are shown. Coomassie Brilliant Blue (CBB)-stained blots show recombinant GST-tagged CRMP-2 proteins used for pull-down. *C*, quantification of Western blots of pull-down assays. Pull-down by CRMP-2L A' was set at 1. Comparison between the different samples was performed by one-way ANOVA with the post hoc Holm-Sidak method. Data are shown as mean \pm S.E. (error bars); $n = 4$. *D*, haptotactic migration of SW620 cell transfectants. Cells were transfected with pIRES2-EGFP CRMP-2L A2' (CRMP-2L A2') or pIRES2-EGFP (mock), and in some experiments treated with 10 μ M ROCK inhibitor Y-27632. Migration by mock transfectants was set at 1. Comparison between mock and mock + Y-27632 was performed by the Mann-Whitney Rank Sum test, whereas others were compared using Student's *t* test. Data are shown as mean \pm S.E.; $n = 4$. *E*, relative expression levels of ROCK II in SW620 cells. Expression was quantified from Western blotting (WB) of total lysates from transfected SW620 cells; $n = 3$. ROCK II expression in mock-transfected cells was set at 1. No statistical difference was observed (Student's *t* test). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., not significant.

pulled down recombinant ROCK II catalytic domain to the same extent as A', whereas A1', A3', and A4' showed significantly less interaction (Fig. 1C). Because neither fragment A' (aa 1–118 of CRMP-2L) nor A (aa 1–275 of CRMP-2S) bound ROCK II (21), data above indicate that both the linker region between the N terminus and the core part of CRMP-2L, and aa 203–275 (CRMP-2L) are important for the unique interaction of N-terminal CRMP-2L with ROCK II.

Previous studies showed that a combination of fragment A' and the C-terminal domain of CRMP-2L significantly reduced migration of a human colon carcinoma cell line SW620, which expresses only the short form of CRMP-2 and exhibits ROCK-dependent haptotactic cell migration (21). We investigated

whether fragment A2' alone is capable of influencing SW620 cell migration. CRMP-2L A2' in pIRES2-EGFP mammalian expression vector was introduced into SW620. Expression of CRMP-2L A2' significantly reduced haptotactic cell migration by ~40% compared with mock-transfected cells (Fig. 1D). The effect of CRMP-2L A2' on migration was similar to that of the ROCK inhibitor, Y-27632, on migration of mock-transfected cells. Moreover, treatment with ROCK inhibitor did not reduce cell migration of CRMP-2L A2' transfectants further (Fig. 1D). These data uphold CRMP-2L as an important regulator of ROCK II-dependent cellular functions. It should be noted that expression of CRMP-2L A2' in SW620 does not affect the relative protein level of ROCK II (Fig. 1E).

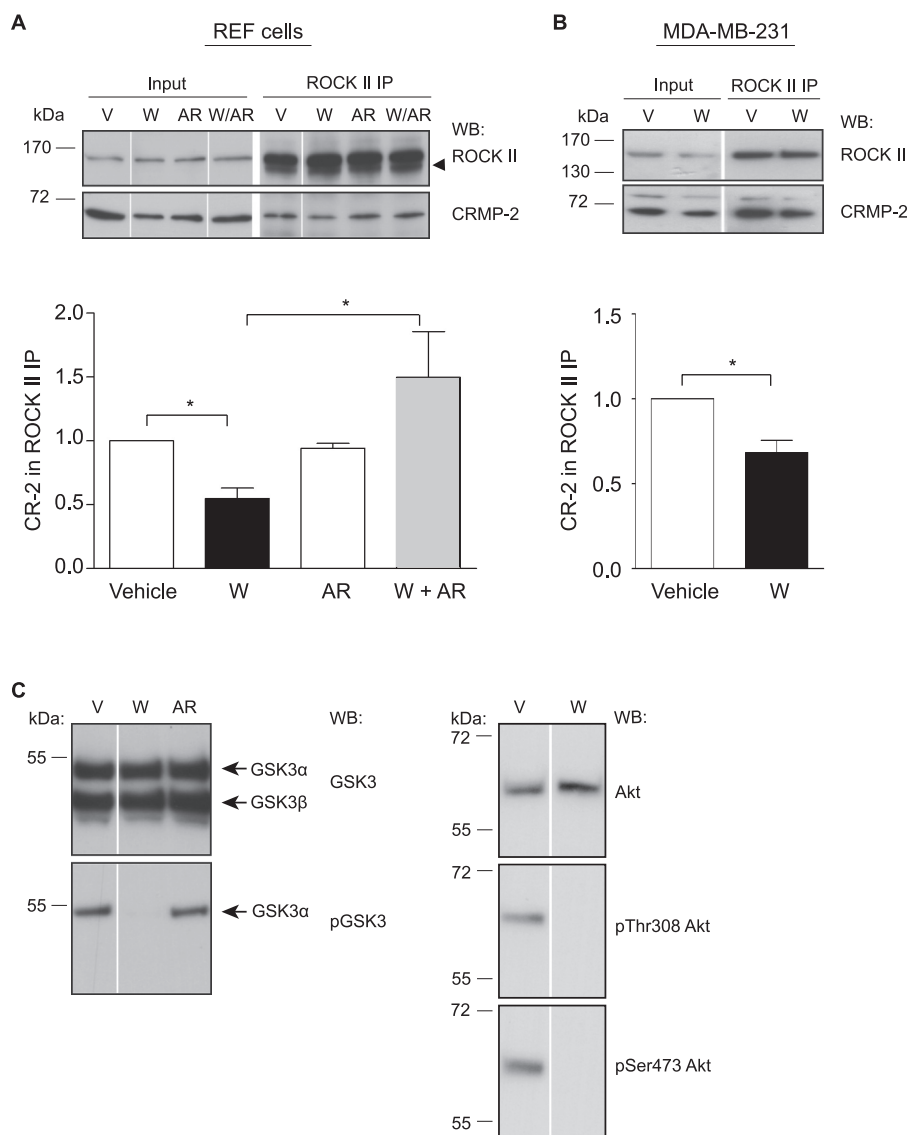


FIGURE 2. PI3K-GSK3 pathway regulates ROCK II-CRMP-2 interaction. *A* and *B*, ROCK II immunoprecipitations (IP) from total cell lysate of REFs (*A*) and MDA-MB-231 (*B*) after treatment with vehicle (V; DMSO), PI3K inhibitor (W; Wortmannin), GSK3 inhibitor (AR) or a combination of wortmannin and GSK3 inhibitor for 1 h at 37 °C. Representative Western blots (WB) are shown. Graphs show relative CRMP-2 amount in ROCK II immunoprecipitates. Immunoprecipitates from DMSO-treated samples were set at 1. *A*, $n = 5$ for vehicle and PI3K inhibitor, $n = 2$ for GSK3 inhibitor and combination. Comparisons were performed using Kruskal-Wallis with post hoc Dunn's test. Arrowhead denotes nonspecific band from antibody. *B*, $n = 3$. Comparison was performed using Student's *t* test. *, $p < 0.05$. *C*, REFs treated with 100 nM wortmannin (W), 40 μ M AR A0114418 (AR), or vehicle (V; DMSO) for 2 h. Total cell lysates were separated by SDS-PAGE, and effects of inhibitor treatments were confirmed by Western blotting analyses of phosphorylation states of GSK3 and Akt.

PI3K-GSK3 Pathway Partially Regulates the ROCK II-CRMP-2 Interaction—Some CRMP-2 activities are regulated through phosphorylation by Cdk5 or DYRK followed by GSK3 phosphorylation (33, 34). A major pathway to regulate GSK3 activity is the PI3K-Akt pathway, which inhibits GSK3 activity through phosphorylation at Ser-9 of GSK3 β (38). Therefore, we examined whether PI3K activity is involved in regulation of the interaction between ROCK II and CRMP-2. REF cells, which express CRMP-2S endogenously (21), were treated with PI3K inhibitor wortmannin, and the amount of CRMP-2 coimmunoprecipitated with ROCK II was analyzed by Western blotting. Cells treated with wortmannin showed significantly decreased (~50%) amounts of CRMP-2 in the ROCK II immunoprecipitates compared with controls (Fig. 2A). The inhibitor also significantly decreased the amount of CRMP-2 coimmunopre-

cipitated with ROCK II (by ~40%) from human breast carcinoma cells (MDA-MB-231 cells), which endogenously express both CRMP-2L and -2S (Fig. 2B). The CRMP-2/ROCK II ratio in total cell lysates of both REF and MDA-MB-231 cells was not altered upon treatment with wortmannin compared with those of vehicle-treated cells (REFs, *t* test, $p = 0.525$; MDA-MB-231, *t* test, $p = 0.196$ for CRMP-2S/ROCK II and $p = 0.622$ for CRMP-2L/ROCK II). In control experiments, decreased phosphorylation levels of Akt and GSK3, downstream of PI3K, by wortmannin treatment were confirmed by Western blotting for phosphorylated Akt at Thr-308 and Ser-473 and phosphorylated GSK at Ser-9 (for β)/Ser-21 (for α) in total cell lysates (Fig. 2C). These results suggested that the interaction between ROCK II and CRMP-2 is not constitutive, but regulated partially by PI3K signaling. To further investigate

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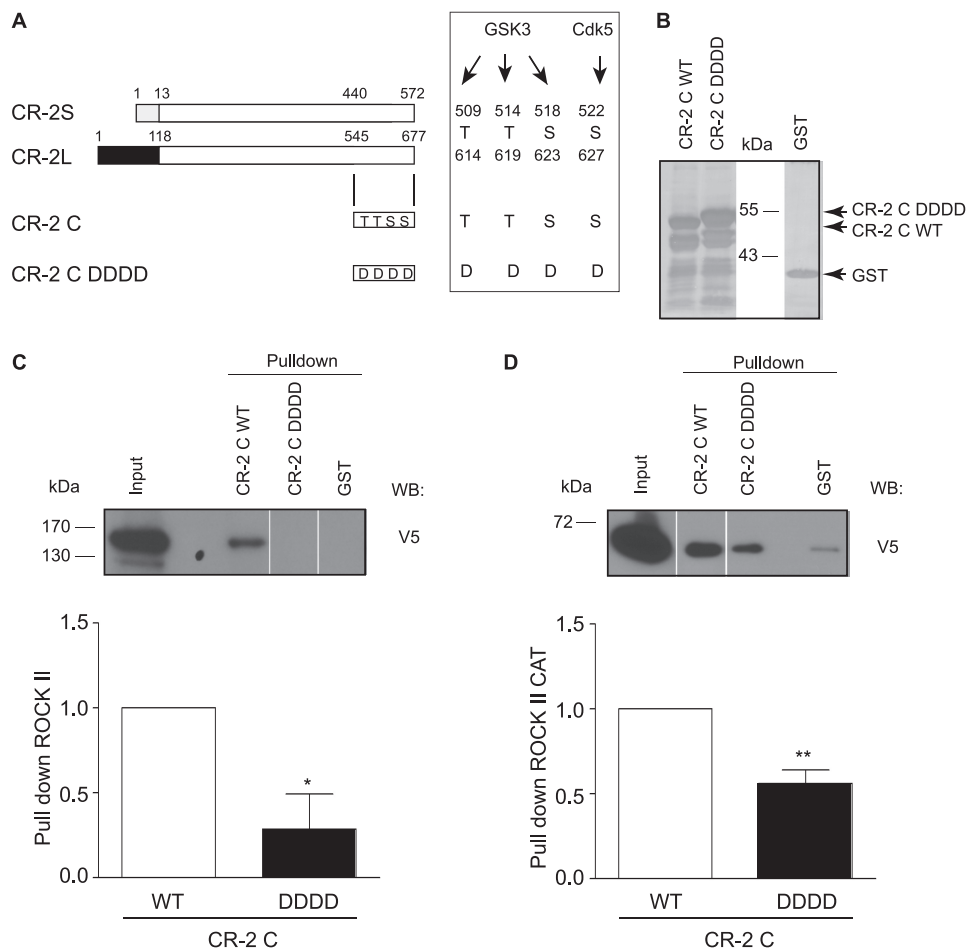


FIGURE 3. GSK3 β phosphorylation of CRMP-2 regulates the ROCK II-CRMP-2 interaction. *A*, schematic illustration shows CRMP-2 fragments C and C DDDD. *B*, recombinant GST fusion proteins eluted from agarose beads used for pull-down assays were stained with Coomassie Brilliant Blue staining. *C* and *D*, S2 cells expressing equivalent amounts of V5-ROCK II full-length (*C*) or ROCK II catalytic domain (CA7; *D*) were lysed, and ROCK proteins were pulled down by CRMP-2 C or CRMP-2 C DDDD. Amount of pulled down ROCK II was quantified from Western blots. Representative Western blots are shown. Graphs show mean \pm S.E. (error bars). Pull-down by CRMP-2 WT was set at 1. *C*, $n = 4$; comparison was performed using Mann-Whitney. *D*, $n = 3$; comparison was performed using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$.

the role of PI3K signaling on ROCK II-CRMP-2 interaction, AR A0114418, an inhibitor of GSK3 (39), was tested in coimmunoprecipitation assays on lysates from REF cells. AR A0114418 itself showed no effect on the interaction of ROCK II with CRMP-2, whereas a mixture of PI3K and GSK3 inhibitors restored the interaction suppressed by PI3K inhibitor (Fig. 2A). This suggested that the effect of PI3K inhibitor was due to the inactivation of Akt followed by the activation of GSK3. Altogether, the results suggest that PI3K-Akt-GSK3 pathway is involved in regulating the ROCK II-CRMP-2 interaction.

Because CRMP-2 is a GSK3 substrate in neuronal cells (31), we further investigated whether the ROCK II-CRMP-2 interaction is affected by GSK3-dependent phosphorylation of CRMP-2. Phosphorylation-mimetic mutations (substitution of Thr or Ser with Asp) in CRMP-2 at aa 509, 514, and 518 and the Ser-522 priming site in the C fragment of CRMP-2 (numbering according to CRMP-2S, Fig. 3A) were introduced, and recombinant GST fusion proteins were expressed in *E. coli*. Recombinant V5-tagged ROCK II (full-length or its catalytic domain) expressed in S2 cells was subjected to pull-down assay using CRMP-2 wild type (WT) and mutant C-terminal domain immobilized on glutathione beads (Fig. 3B). The interactions of both full-length and

catalytic domain of ROCK II with mutant CRMP-2 C fragment were significantly reduced, by ~ 70 and $\sim 45\%$, respectively, compared with the interaction with CRMP-2 C WT (Fig. 3, C and D). These data suggest that CRMP-2 phosphorylation by GSK3 regulates the ROCK II-CRMP-2 interaction.

pGSK3, ROCK II, and CRMP-2 Are Enriched at the Cell Periphery—In human breast carcinoma MDA-MB-231 and human colon carcinoma SW480 cells, ROCK II and CRMP-2 colocalized at the cell periphery (21). In addition, the phosphorylated, and thereby inactive, GSK3 has been detected at the leading edge of MDA-MB-231 cells (40). As ROCK II membrane localization could be regulated by PI3K (10), the role of PI3K in ROCK II and CRMP-2 colocalization was investigated. Upon treatment with wortmannin, pGSK3 could no longer be detected at the cell periphery of MDA-MB-231 cells, showing specificity of pGSK3 periphery staining in control cells (Fig. 4A). Colocalization of ROCK II and CRMP-2 at the cell periphery in vehicle-treated MDA-MB-231 cells was confirmed by confocal microscopy (Fig. 4, B and C), whereas treatment with wortmannin led to a significant reduction in colocalized ROCK II and CRMP-2 at the cell periphery (Fig. 4B). The observed

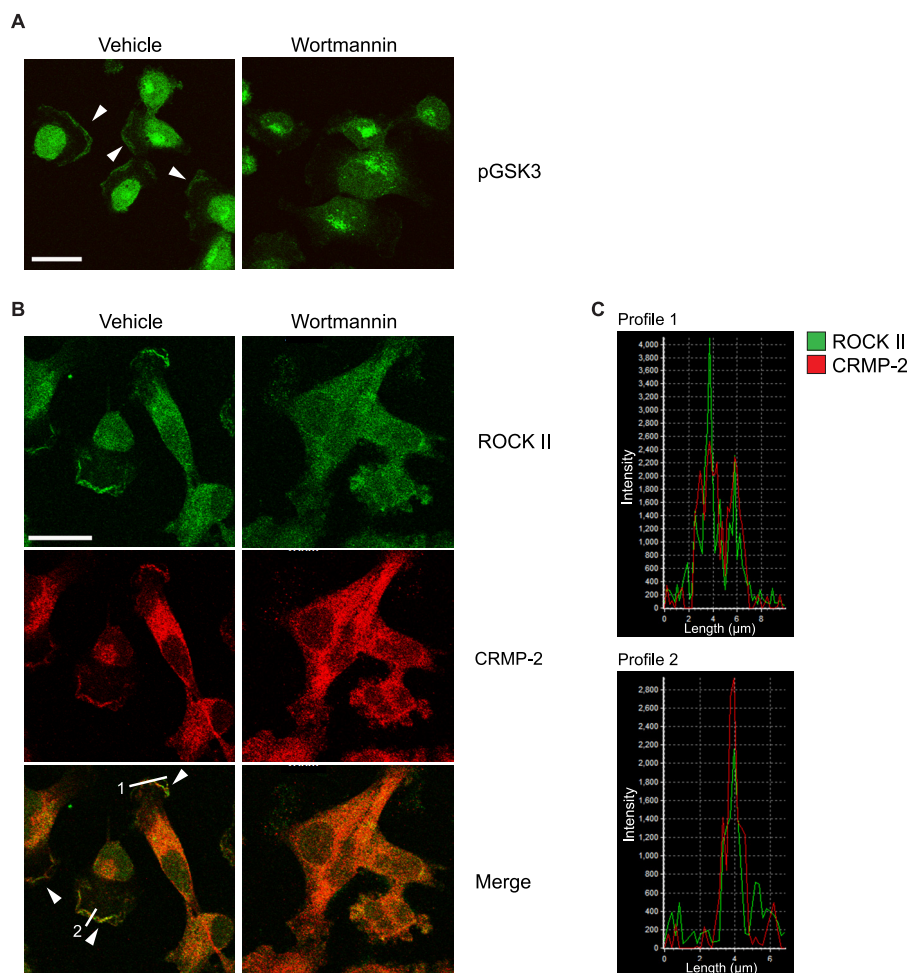


FIGURE 4. Treatment with PI3K inhibitor attenuates pGSK3 localization and ROCK II and CRMP-2 colocalization at the cell periphery of human breast carcinoma cells. MDA-MB-231 cells were treated with vehicle (DMSO) or wortmannin (100 nM) as described under "Experimental Procedures." *A*, fixed cells were stained for phospho-GSK3. *Arrowheads* show localization of pGSK3 at ruffling membrane. *B*, fixed MDA-MB-231 cells were stained for ROCK II (green) and CRMP-2 (red). *Arrowheads* show colocalization of ROCK II and CRMP-2. *Lines* indicate colocalization profiles seen in *C*. All images were acquired by confocal laser scanning microscope. *Scale bars*, 20 μm. Images are representative of two independent experiments.

reduction in colocalization is in good agreement with the immunoprecipitation analysis (Fig. 2).

CRMP-2L Phosphorylation State Regulates Cell Migration of Human Carcinoma Cells—ROCK II is a key regulator of several important cellular functions, including adhesion and migration. We previously showed that CRMP-2 influences ROCK II-dependent cellular functions, including carcinoma cell migration (21). The current data suggest that phosphorylation of CRMP-2 by GSK3 sensitively regulates the CRMP-2 and ROCK II interaction and thereby influences ROCK II-dependent cellular functions. Therefore, we investigated the impact of GSK3 phosphorylation of CRMP-2 on carcinoma cell adhesion and migration. Phosphorylation-mimetic or phosphorylation-resistant mutations were introduced in CRMP-2L at Thr-614, Thr-619, Ser-623, and Ser-627. The created cDNAs were expressed in SW620 cells. Expression of CRMP-2L WT, CRMP-2L DDDD (phosphorylation-mimetic), or CRMP-2L AAAA (phosphorylation-resistant) had no impact on the ability of cells to adhere to collagen I compared with mock-transfected cells (Fig. 5A).

However, and similar to previous findings, expression of CRMP-2L WT in SW620 cells significantly reduced haptotactic cell migration (by ~40%, Fig. 5B and Ref. 21). A reduction, of

~25%, in migration was also observed with SW620 cells expressing CRMP-2L AAAA (Fig. 5B). Expression of CRMP-2L DDDD, however, had no impact on migration compared with mock-transfected cells (Fig. 5B). The reductions in migration were not a result of altered Rho activity because expression of the CRMP-2L constructs did not affect GTP-RhoA levels (Fig. 5C). No significant differences in CRMP-2 or ROCK II expression levels were observed either (Fig. 5, D and E). The expression levels of CRMP-2L in SW620 cells show a high degree of variance. However, CRMP-2L WT, with the lowest expression level, significantly reduced migration of SW620 cells, whereas CRMP-2L DDDD had no effect. Collectively, this suggests that phosphorylation of CRMP-2L by GSK3 affects ROCK-dependent colon carcinoma cell migration.

CRMP-2L Is Involved in the Regulation of Matrix Invasion by Human Colon Carcinoma Cells—CRMP family proteins have been linked to the progression of cancer by regulating the invasive potential of cancer cells (41, 42). Because both the CRMP-2L N-terminal domain and phosphorylation of the C-terminal domain are involved in regulation of migration, their potential role in invasion of collagen gels was investigated. Here, expression of CRMP-2L WT and phosphorylation-resistant CRMP-2L AAAA

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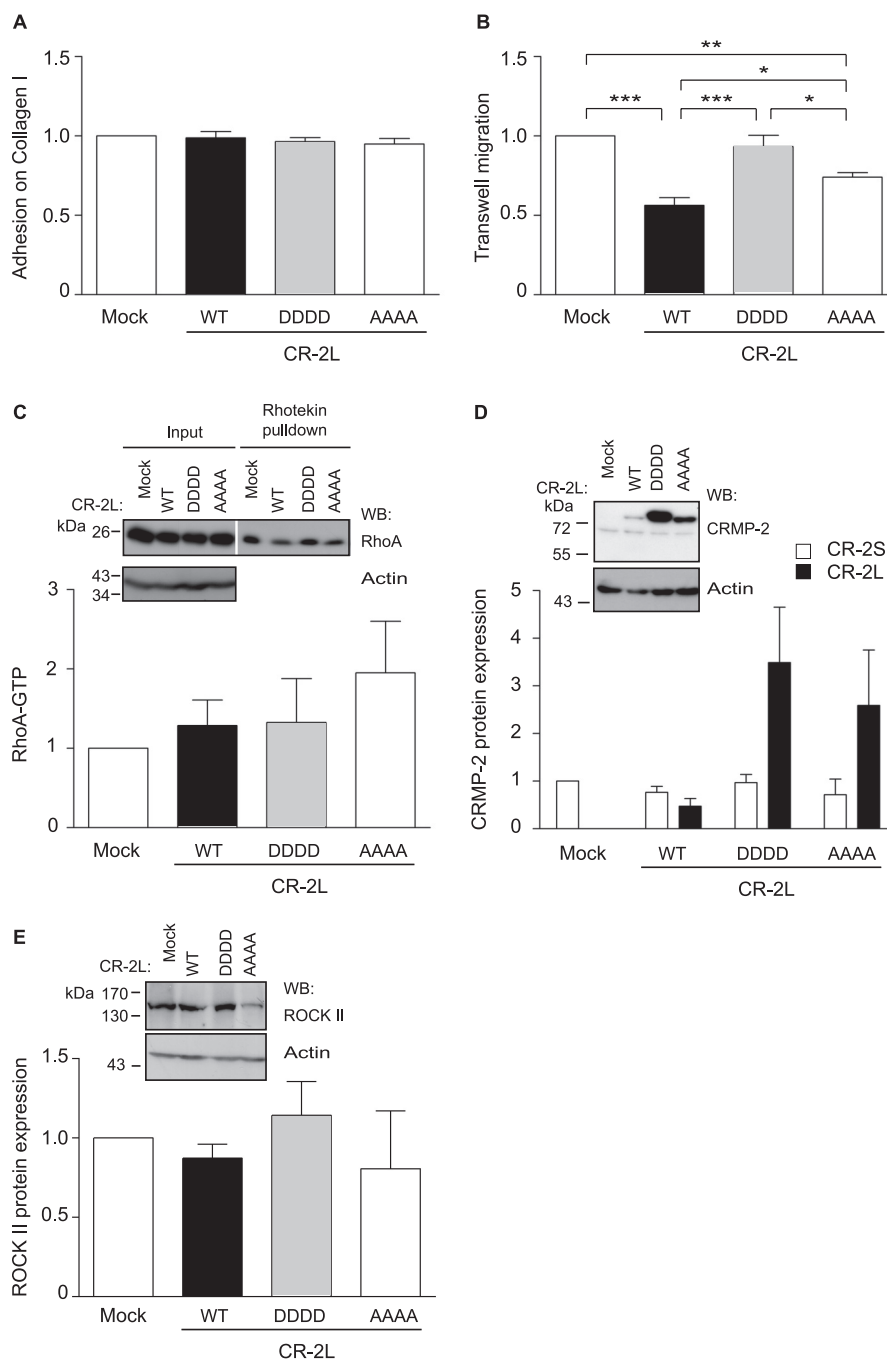


FIGURE 5. CRMP-2L phosphorylation state affects migration of SW620 colon carcinoma cells toward collagen I. SW620 colon carcinoma cells were either mock-transfected or transfected with plasmids encoding CRMP-2L WT, CRMP-2L DDDD, or CRMP-2L AAAA. *A*, relative adhesion of transfected SW620 cells to collagen I. Adhesion by mock transfectants was set at 1; $n = 3$. No significant difference was observed (one-way ANOVA). *B*, relative migration of transfected SW620 cells in Transwells coated with collagen I. $n = 5$ for mock, CRMP-2L WT, and CRMP-2L DDDD. $n = 3$ for CRMP-2L AAAA. Migration by mock transfectants was set at 1. Comparison between the different treatments was performed by one-way ANOVA with a post hoc Holm-Sidak test. *C*, relative GTP-RhoA levels in SW620 transfectants. GTP-Rho level in mock transfectants was set at 1. $n = 3$. No significant difference was observed (one-way ANOVA). *D*, relative expression levels of CRMP-2S and -2L in SW620 cells. Expression was quantified from Western blots of total lysates from transfected SW620 cells. $n = 3$. CRMP-2S expression in mock-transfected cells was set at 1. No statistical difference was observed (one-way ANOVA with post hoc Holm-Sidak test). *E*, relative expression levels of ROCK II in SW620 cells. Expression was quantified from Western blot of total lysates from transfected SW620 cells. $n = 3$. ROCK II expression in mock-transfected cells was set at 1. No statistical difference was observed (one-way ANOVA). Data in graphs show means \pm S.E. (error bars). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

significantly reduced SW620 colon carcinoma cell invasion, whereas expression of phosphorylation-mimetic CRMP-2L DDDD had no effect (Fig. 6A). Furthermore, expression of CRMP-2L A2' reduced colon carcinoma cell invasion to the same level as CRMP-2L WT (Fig. 6C). To ensure that the effect seen on invasion was not due to differences in prolifer-

ation, the relative proliferation of SW620 cells expressing CRMP-2L WT, CRMP-2L A2', or CRMP phosphorylation mutants was determined. No significant difference in proliferation compared with mock-transfected cells was observed (Fig. 6, B and D). Combined, the results suggest a role for CRMP-2L in not only ROCK-dependent migration, but also in invasion.

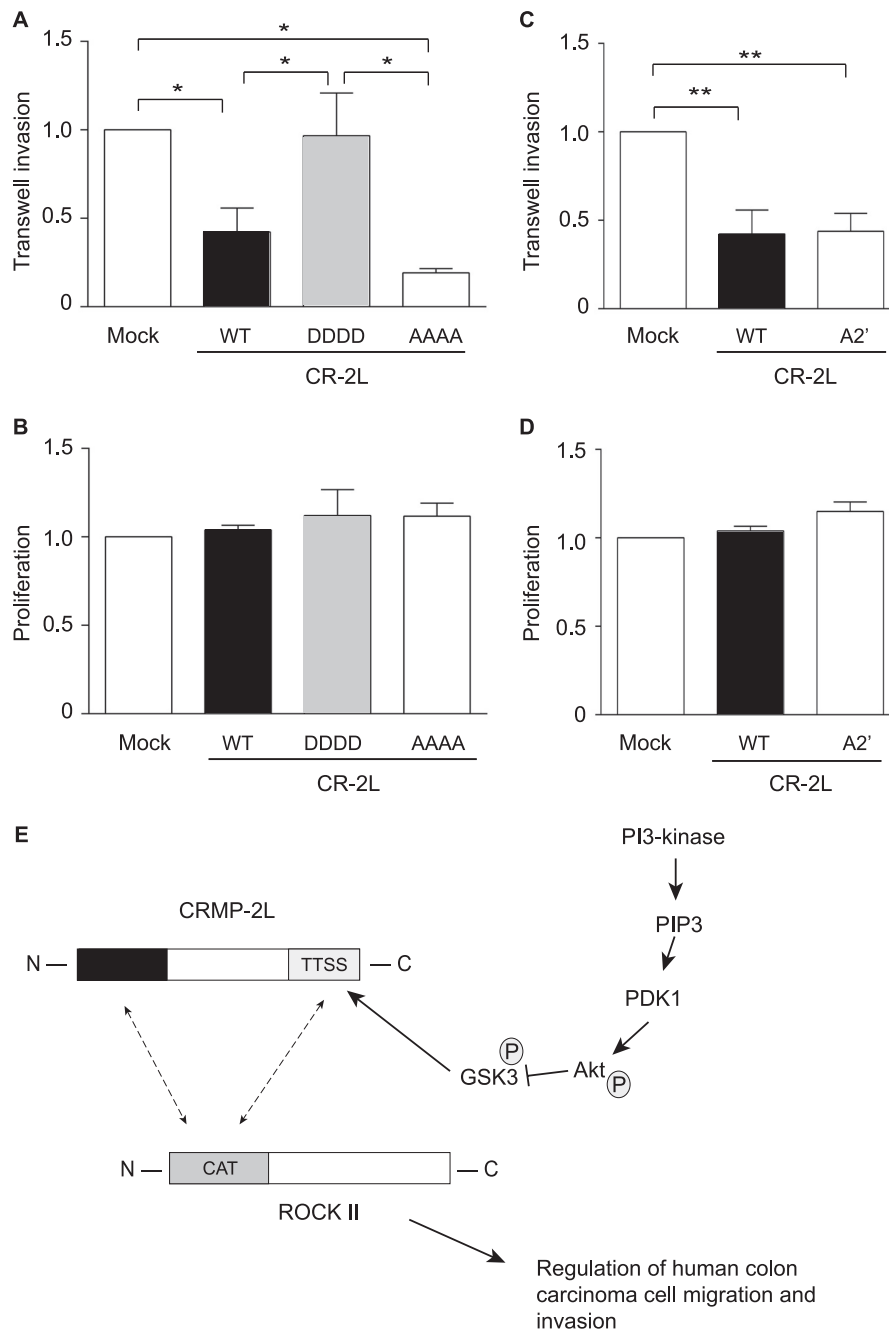


FIGURE 6. CRMP-2L is involved in the regulation of cell invasion of human carcinoma cells. *A*, relative invasion of transfected SW620 cells in Transwells coated with Cell matrix type I-A. $n = 4$. Invasion by mock transfectants was set at 1. For mock-transfected cells a mean of 50 invading cells was counted per filter. Comparison between the different treatments was analyzed by Kruskal-Wallis with a post hoc Student-Newman-Keuls method. *B*, relative proliferation of transfected SW620 cells. Proliferation by mock transfectants set at 1. $n = 3$. No statistical difference was observed (one-way ANOVA). *C*, relative invasion of transfected SW620 cells in Transwells coated with Cell matrix type I-A. $n = 4$. Invasion by mock transfectants was set at 1. Comparison between the different treatments was analyzed by one-way ANOVA with a post hoc Holm-Sidak test. *D*, relative proliferation of transfected SW620 cells. Proliferation by mock transfectants was set at 1. $n = 3$. No statistical difference was observed (one-way ANOVA and post hoc Holm-Sidak test). Data in graphs show means \pm S.E. (error bars). *, $p < 0.05$; **, $p < 0.01$. *E*, schematic illustration of the main findings. CRMP-2L interacts with ROCK II catalytic domain (CAT) through two distinct sites, one in the N terminus and one in the C terminus of CRMP-2 (shown as broken lines). The N-terminal region of CRMP-2L alone regulates ROCK II-dependent migration, as well as colon carcinoma cell invasion. The interaction between ROCK II and CRMP-2 is partly regulated through phosphorylation of the C terminus of CRMP-2. These sites are known to be phosphorylated by GSK3 downstream of PI3K.

DISCUSSION

Previously, we established CRMP-2 as an endogenous inhibitor of ROCK II kinase activity and therefore as a regulator of ROCK II-dependent cellular functions (21). ROCK II is a major regulator of myosin II activity, but additionally has pleiotrophic roles in actin cytoskeleton dynamics (2). Therefore, its activity is

under tight spatio-temporal control. Our current data demonstrate that GSK3 phosphorylation of CRMP-2 is important for regulation of the interaction between ROCK II and CRMP-2.

PI3K, a key signaling molecule in cell survival, proliferation, and growth, is activated following stimulation of multiple membrane-bound receptors such as the integrins, G protein-

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coupled receptors, and receptor tyrosine kinases (43, 44). Downstream of PI3K, active Akt phosphorylates and inactivates GSK3 (45), a serine/threonine kinase known to influence CRMP-2 activities (31). Here we show that inhibition of PI3K significantly reduces the interaction between ROCK II and CRMP-2, whereas inhibition of GSK3 had no effect on the interaction (Fig. 2). However, a combination of PI3K and GSK3 inhibitors rescued the effect of PI3K inhibitor alone, suggesting that the association of ROCK II and CRMP-2 is partly regulated by GSK3 downstream of PI3K. In addition to Akt, other kinases can inactivate GSK3, by phosphorylation of GSK3 α Ser-21 or GSK3 β Ser-9, including protein kinase C and ribosomal S6 kinase (46–48). This could explain the partial inhibition of the ROCK II-CRMP-2 interaction upon inhibition of PI3K, as PI3K-Akt only is responsible for a portion of GSK3 inactivation.

GSK3 sequentially phosphorylates CRMP-2 at Ser-518, Thr-514, and Thr-509 (31), following Cdk5 or DYRK phosphorylation of CRMP-2 Ser-522. Introduction of phosphorylation-mimetic mutations to these four sites in the C-terminal domain of CRMP-2L significantly reduced the ability of CRMP-2 to interact with the catalytic domain of ROCK II, supporting the importance of GSK3 and Cdk5/DYRK phosphorylation of CRMP-2 in the regulation of the ROCK II-CRMP-2 interaction. In this connection, the multiple phosphorylation sites in the C-terminal domain of CRMP-2 are involved in the regulation of colon carcinoma migration and invasion (Figs. 4B and 6A).

It was shown previously that ROCK II and CRMP-2 colocalize to the cell periphery of human MDA-MB-231 breast carcinoma cells and that their subcellular localization is independent of each other (21). Here we show that the ROCK II/CRMP-2 colocalization at the cell periphery of human breast carcinoma cells is dependent on PI3K activity. This is consistent with previous findings showing that ROCK II localization at the cell periphery is partly dependent on PI3K activity (10, 49). Inhibition of PI3K also reduced the amount of inactive GSK3 associated with the periphery (Fig. 4). GSK-mediated phosphorylation of CRMP-2 inhibits CRMP-2 localization to primary cilia (50), implicating GSK3 in the regulation of CRMP-2 localization by an unknown mechanism.

The interaction between ROCK II kinase domain and CRMP-2 is facilitated through two separate domains of CRMP-2, one in the common CRMP-2S/-2L core part and one within amino acids 1–275 of CRMP-2L (CRMP-2L A2'), covering its splice variant-specific N-terminal domain (Figs. 1 and 3). Expression of CRMP-2L A2' alone significantly reduced the haptotactic colon carcinoma cell migration, as well as colon carcinoma cell invasion (Figs. 1D and 6C). Furthermore, the pan-ROCK inhibitor Y-27632 had no additional effect on migration of CRMP-2L A2' transfectants. This indicates that expression of CRMP-2L A2' can trigger a complete inhibition of ROCK-dependent cell migration, via inhibition of ROCK II (21). This is consistent with previous findings that CRMP-2L, but not CRMP-2S, has an inhibitory effect on ROCK II, even though both splice variants can participate in ROCK II-interacting complexes (Fig. 2) (21). Collectively, the data indicate that although the N terminus of CRMP-2L is required for ROCK II kinase inhibition, the interaction of endogenous proteins can be regulated via the C-terminal domain of CRMP-2 (Fig. 6E). Phosphorylation of

the CRMP-2 C-terminal domain by Cdk5/DYRK and GSK3 might alter the conformation of CRMP-2L, making the protein less potent for binding to the catalytic domain of ROCK II (Fig. 6E). However, as only truncated CRMP-2S (aa 13–490) has been crystallized, little is known about the differences in overall conformation or post-translational modifications of the two splice variants (51, 52).

Increased GSK3 β expression and kinase activity and reduced levels of inactivating phosphorylation have been observed in colon and pancreatic cancer cell lines as well as in colorectal cancer patients compared with normal counterparts (53, 54). Very recently, phosphorylated CRMP-2 (Thr-509/Ser-518/Ser-522) was described to correlate with breast cancer progression (41). Following from the observations presented here, increased cancer-associated GSK3 activity can lead to increased CRMP-2 phosphorylation, decreased CRMP-2-ROCK interaction and colocalization, and consequently increased ROCK II-dependent migration and invasion. This may be relevant to understanding disease progression and metastasis.

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