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p190RhoGAP negatively regulates Rho activity at the cleavage furrow of mitotic cells

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Abstract

Previous studies demonstrated that p190RhoGAP (p190) negatively affects cytokinesis in a Rho-GAP-dependent manner, suggesting that regulation of Rho may be a critical mechanism of p190 action during cytokinesis. P190 localizes to the cleavage furrow (CF) of dividing cells, and its levels decrease during late mitosis by an ubiquitin-mediated mechanism, consistent with the hypothesis that high RhoGTP levels are required for completion of cytokinesis. To determine whether RhoGTP levels in the CF are affected by p190 and to define the phase(s) of cytokinesis in which p190 is involved, we used FRET analysis alone or in combination with time lapse microscopy. In normal cell division activated Rho accumulated at the cell equator in early anaphase and in the contractile ring, where it co-localized with p190. Real-time movies revealed that cells expressing elevated levels of p190 exhibited multiple cycles of abnormal CF site selection and ingression/regression, which resulted in failed or prolonged cytokinesis. This was accompanied by mislocalization of active Rho at the aberrant CF sites. Quantified data revealed that in contrast to ECT2 and dominate negative p190 (Y1283Ap190), which resulted in hyper-activated Rho, Rho activity in the CF was reduced by wild type p190 in a dose-dependent manner. These results suggest that p190 regulates cytokinesis through modulation of Rho-GTP levels, thereby affecting CF specification site selection and subsequent ring contraction.

Keywords

p190RhoGAP; Rho; mitosis; cytokinesis; cleavage furrow; ECT2; FRET

Introduction

Cytokinesis, the final step of cell division, is initiated at the metaphase to anaphase transition by specification of the cleavage plane, followed by furrow assembly and ingression, midbody formation, and cell separation. This process results in the production of two daughter cells, each with an equal complement of chromosomes and membrane [1,2]. The molecular requirements for these events appear to be conserved in animal cells, since a core set of proteins have been found to be important for cytokinesis [3,4]. Among them are the two major

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cytoskeleton components, microtubules and actin, as well as their motor proteins, kinesins and myosins. Early studies demonstrated by physical manipulation of the central spindle apparatus that selection of the cleavage plane initiation site and contractile ring assembly are mediated by mitotic spindle microtubules [5,6]. While multiple reports substantiate these findings, evidence suggests that astral microtubules may also play an important role in these events [7–12]. Cleavage furrow (CF) assembly and contraction involve actin filaments, myosin II, septins, and actin-interacting proteins, such as profilin and cofilin [3].

Accumulating evidence suggests that RhoGTPases and their regulated local activities are also critical for successful completion of mitosis [13,14]. Cells depleted of Rho protein by siRNA or of Rho activity by treatment with C3 transferase, show cytokinesis defects [15–27]. Rho is important not only for CF specification through microtubules but also for furrow formation and contraction through actinmyosin via its downstream targets, including ROCK, citron kinase, LIM kinase, and formin-homology proteins [3]. The molecular mechanisms by which Rho is localized and activated to mediate CF specification, ring assembly and contraction are still poorly understood. While multiple models have been proposed to delineate these events [10,13,28], the current consensus is that initial positioning of the furrow is dependent on local enrichment and activation of Rho [13,14,22]. This process can be regulated by the centralspindlin complex, composed of a Rho family GTPase activating protein (GAP), CYK4/MgcRacGAP, and a kinesin protein, MKLP1. ECT2, a guanine nucleotide exchange factor (GEF) for Rho, is also recruited by centralspindlin to this region where it can activate Rho [26,27,29,30]. Until recently, it has been a challenge to visualize the active pool of Rho at the CF. Now, a number of different probes have permitted detection of the active population, but the results are conflicting and differ by method and cell type used [22,26,31]. The study reported here utilizes a single chain FRET biosensor that detects conformational changes in Rho that occur upon GDP-GTP exchange, yet still maintains Rho's sensitivity to its regulators [32,33]

At any given point in time and space, the activity of Rho represents a balance between its GTP- and GDP-bound states, which is mediated by the combined action of guanine nucleotide dissociation inhibitors (GDIs), GEFs, and GAPs. ECT2 and CYK-4/mgcRacGAP are candidates for performing the Rho GEF and GAP functions, as both localize to the cytokinetic apparatus and are critical for cytokinesis [26,29,34–38]. MgcRacGAP is the human homolog of CYK-4 and required for cytokinesis. Since its primary target is Rac in vitro, whether MgcRacGAP has Rho GAP activity in vivo during cytokinesis is unclear [39,40].

However, previous studies have revealed another RhoGAP, p190RhoGAP (p190), that exhibits specific GAP activity for Rho in vivo and localizes to the CF [41,42]. P190 is a multidomain and multifunctional protein that plays a crucial role in regulating actin stress fiber dynamics by negatively regulating Rho and promoting actin stress fiber disassembly [42–48]. Endogenous p190 is degraded in late mitosis by a ubiquitin-proteasome-dependent pathway. Gene silencing strategies to further define the role of p190 in cytokinesis have been uninformative, because p190-silenced cells become highly adherent and flattened and fail to even enter mitosis. In contrast, studies involving overexpression of p190 have shown that p190 induces multinucleation and mis-localization of the cleavage plane specification site, suggesting that it functions in the early stages of cytokinesis. These effects of p190 are observed in multiple mammalian cell lines and are dependent on its RhoGAP domain. Together, these characteristics of p190 suggest that its effects on cytokinesis may be mediated in part through Rho.

To test this hypothesis, we used time lapse microscopy and fluorescence resonance energy transfer (FRET) technology to analyze the effect of p190 on living cells and on Rho activity during cytokinesis. In normally dividing cells, FRET analysis demonstrated that activated Rho

localized to the cell equator at the furrow specification plane in early anaphase and to the CF during cytoknesis, where it co-localized with p190. Real-time microscopy showed that overexpression of p190 resulted in defective and delayed mitosis through abnormal and/or failed specification of the CF plane and cycles of ingression/regression in both HeLa and MDA-MB 468 breast cancer cells. These abnormalities were accompanied by aberrant localization and reduced levels of active Rho in the CF. P190 overexpression reduced, while ECT2 and dominant negative p190 (Y1283A p190) increased Rho activity in the CF in a dose-dependent manner. Together, these data support the conclusion that p190 affects selection of the cleavage plane and ingression of the contractile ring through regulation of Rho activity in the CF during cytokinesis

Materials and methods

Tissue culture and transfection

Tet-on HA-tagged p190RhoGAP MDA-MB-468 human breast cancer cells [41] were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum and antibiotics and induced to overexpress p190 by addition of 1 ug/ml doxcycline (DOX) to the culture medium for the indicated time. HeLa cells, cultured in DMEM with 10% serum, were transfected with Rho biosensor variants and p190 variants or ECT2 using Polyfect (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. Rho biosensors were introduced into 468 cells by the same method and simultaneously treated with DOX to induce expression of p190. Cells were fixed and stained 24 hrs post transfection and visualized on a fluorescent microscope (Orthoplan; E. Leitz, Inc., Rockleigh, NJ).

Plasmids

The pkH3190 plasmid, which encodes an N-terminal triple HA-tagged full-length, wild type (wt) p190, and GFP-p190 were gifts from Ian Macara [47]. Mutant P190Y1283A, previously shown to be deficient for RhoGAP activity [48], was cloned into the pKH3 vector at the BamH1 and EcoR1 sites. RFPp190 was cloned into the pTriEXmRFP vector at the BamH1 and EcoR1 sites. The various Rho biosensors (wtRho, T19NRho and Q63LRho) were cloned into the pTriEx vector as described [32]. Each biosensor consists of a CFP-RBD (Rho binding domain from rhotekin) and YFP-Rho (wtRho, RhoQ63L or RhoT19N) connected by a linker. Constituitively active ECT2 (ECT2C {residues 414–883} hereafter referred to as ECT2) was cloned into the pcDNA3Flag vector at the EcoR1 and BamH1 sites. Expression of HA-tagged p190 and Flag-tagged ECT2 was verified by Western immunoblotting and immunofluorescence.

Generation of HeLa cells stably expressing the Rho biosensor

HeLa cells were co-transfected with the Rho biosensor and pBabe (puromycin) plasmids at a molar ratio of 10:1, respectively, and selected with 400ng/ml puromycin in tissue culture medium for 6 days. YFP positive cells were sorted by Becton Dickinson FACSVantage SE Turbo Sorter at 488nm. Stable transfectants were cultured in DMEM medium supplemented with 400ng/ml puromycin and 10% FBS and utilized for live FRET imaging.

Immunofluorescence

Cells were prepared for immunofluorescence as described in [44]. Two ug/ml anti-HA monoclonal antibody (mAb), (HA11 {Covance, Berkeley, CA}) and 2 ug/ml anti-Flag M2 mAb (Sigma, St. Louis, MO) were used as primary antibodies to stain p190 and ECT2, respectively. Two ug/ml Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, Molecular Probes, Inc., Eugene, OR) served as the secondary antibody. Coverslips were mounted on microscope slides, and cells were viewed on a Nikon TE200 inverted microscope with a 60× 1.3 NA lens

for FRET images or on a Zeiss LSM 510 UV confocal microscope equipped with a Plan Apochromat 100×/1.4 NA oil immersion lens for confocal images.

Movies and images

Hela or MDA-MB-468 cells were seeded in 0.17 mm Delta T dishes (Fisher Scientific, Pittsburgh, PA) and cultured in Leibovitz's L-15 Medium (Gibco/Invitrogen) supplemented with 10% fetal bovine serum. P190 expression was induced by addition of 1ug/ml Dox to MDA-MB-468 tet-on cells or by transient transfection of pKH3p190, GFP p190, or RFPp190 into Hela cells. Both 468 and HeLa cells were transfected with the indicated biosensor as described above. Movies were taken 24 hrs after exogenous p190 induction or transfection. Ten ug/ml OxyFluor (Oxyrase, Inc, Mansfield, OH) was added to the medium before taking movies to facilitate the capture of fluorescent images. Images were taken with a Nikon TE200 inverted microscope, equipped with a 60× 1.3 NA lens and a Hamamatsu camera controller, and analyzed with OpenLab software. Temperature in the dish was controlled by a Bioptechs Delta T4 Culture Dish Controller. For FRET movies of living cells, RFPp190- and/or biosensor-positive mitotic cells were selected by FACS sorting, and CFP/YFP, CFP/CFP and DIC images were taken every min.

FRET analysis

HeLa cells were synchronized (see below) and transfected with the various Rho biosensors or cotransfected with either the pKH3p190 or ECT2 constructs as described above. Cells were fixed and stained for either p190 or ECT2 and visualized by fluorescence microscopy. For FRET analysis, the following wave length filters were used: CFP excitation - 436/10nm; CFP emission - 470/30nm; YFP excitation - 493/17nm; YFP emission - 535/30nm; Alexa Fluor 594 excitation - 575/25 and emission - 630/60. Three images were captured for each field: CFP excitation and CFP emission (CC), CFP excitation and YFP emission (CY) and YFP excitation and YFP emission (YY). Fluorescence ratio images are presented as CY/CC, showing the relative Rho specific activity in cells. The ratio range is depicted by the colored bar, with red showing the highest and blue the lowest ratio. Digital densitometric measurements of fluorescence intensity (CFP, YFP, or Alexa Fluor 594) in each cell (or designated part of each cell) include background subtraction. OpenLab software was used for the image analysis.

Cell cycle synchronization

Cells were synchronized in mitosis by incubation in 2 mM thymidine (Sigma) in tissue culture medium for 12–16 hrs, washed and released from the block by incubation in normal medium for 8–10 hrs. During release, cells were transfected with different Rho biosensors or cotransfected with pKH3-p190 or Flag-ECT2 constructs. Cells were then fixed, stained, and visualized by fluorescence microscopy.

Results

Ectopic expression of p190RhoGAP induces a mitotic defect in living cells

Our previous studies showed that overexpression of p190 induced multinucleation in both MDA-MB-468 breast cancer cells and HeLa cells, suggesting a role for p190 in cytokinesis. Employing conventional immunofluorescence of fixed cells, we obtained initial evidence that p190 can affect cleavage plane specification. To visualize the effect of p190 overexpression in real time and to gain additional insights into its mechanism of action, we produced movies of 468 cells that inducibly overexpress p190 in response to tetracycline (doxycycline) or of HeLa cells transiently transfected to express GFP-tagged p190. In movies S1 and S2, recordings were started 24 hrs after exogenous p190 was induced by 1ug/ml doxcycline (DOX) in 468 cells. Figure 1a depicts selected images in sequence over a 12 hr period from movie S1.

In these asynchronous cultures, the arrows identify four cells attempting to undergo mitosis. These cells either took an abnormally long time to transit mitosis (at least 12 hours, see cells 2, 3, and 4), as compared to control cells (about 3 hrs) in Figure 1c, or appeared to undergo apoptosis (cells 1 and 3). All suffered mitotic failure. When cells entered mitosis, it appeared that the division planes could be positioned at anaphase (sometimes abnormally – see cells 3 and 4) and ring contraction was initiated. But the rings retracted, and several cells tried to reposition the furrow, in some instances repeatedly (cells 2, 3, and 4). Movie S2 and Figure 1b show at higher magnification one cell that attempted multiple times to define the cleavage plane and divide. Quantitation from a 20 hrs movie (total of 30 cells) revealed that about 30% of the cells failed to complete mitosis, while 16% eventually divided into two daughter cells after a prolonged time. All of the mitotic cells made multiple attempts to define the cleavage plane and underwent partial ingression. About 17% became apoptotic, as determined by morphological changes. This abnormal phenomena is consistent with our previous observations in fixed cells, in which large, multinucleated and multi-lobed cells were seen in p190 overexpressors [41]. Figure 1c and movie S3 depict cultures of tet-on 468 cells undergoing normal division in the absence of Dox (see cells 1, 2, and 3). Together, these data suggest that p190 overexpression can result in multiple mitotic phenotypes, including abnormal division plane selection, restricted CF contraction followed by regression, and cycles of faulty site selection and unsuccessful division, all resulting in delayed or unproductive mitosis. Membrane blebbing, cell rounding, and condensed chromatin were also observed, consistent with the cells undergoing apoptosis.

To determine whether this phenomena was cell type-specific, we transiently transfected GFPp190 into Hela cells and started recording 24 hrs after transfection. Figure 1d and movie S4 feature four GFPp190 expressing cells (noted by arrows and numbers). None of these four cells initiated CF contraction or cell division within the 40 hr time-frame of the movie, although all four exhibited the rounded morphology of mitotic cells. Two of them (cells 3 and 4) detached from the dish at the end of movie, and two others (cells 1 and 2) failed to divide. However, the majority of non-GFPp190 expressing cells near the GFPp190 positive cells underwent cell division. These data further support our hypothesis that overexpression of p190 disrupts the initiation of the cleavage plane and demonstrate that this phenomenon is not cell type-specific.

p190RhoGAP and Rho colocalize at the CF during cytokinesis

Our previous data showed that endogenous p190 co-localized with actin to the CF specification site in early anaphase and to the CF during ring contraction [41]. To determine whether p190 and Rho co-localized at these sites, we examined by confocal microscopy their subcellular localization in HeLa cells throughout mitosis. Figure 2 shows that HA-p190 (red) colocalized with wtRho (green) at the CF throughout the various stages of cytokinesis, further supporting the idea that p190 regulates Rho in cytokinesis.

Localization of active Rho to the equatorial zone and the CF during cytokinesis

Rho GTPase, the major in vivo target of p190RhoGAP [42], is required for CF formation, ingression and stabilization, but its spatio-temporal regulation during cytokinesis is unclear. To further study the function of p190 in cytokinesis and its effect on Rho activity, we employed a recently developed Rho biosensor which detects RhoA conformational changes [32]. The biosensor is a single, genetically encoded chain consisting of RBD (Rho binding domain from rhotekin) linked to RhoA (either wtRho, constitutively active [ca] RhoQ63L or dominant negative [dn] RhoT19N). In the linker between these molecules are two mutants of GFP that undergo FRET (CFP and YFP). When the Rho is in an active, GTP-bound form, it binds the RBD, causing the orientation to change and the distance between the GFP mutants to diminish, thereby inducing FRET. The ratio of FRET/CFP provides a measure of localized Rho

activation. The GFP mutants are placed in the middle of the chain to leave the C terminus free for normal regulation by GDI [32].

Using the Rho biosensor, we examined the subcellular localization of active Rho at different stages of mitosis. The various Rho biosensors (wtRho, RhoQ63L, and RhoT19N) were transfected separately into HeLa cells. Initially, a dose-dependency study was conducted to determine the lowest amount of transfected plasmid that would generate a FRET signal while avoiding potential artifacts of high overexpression. This amount was used throughout the study. Following transfection, cells were synchronized in different stages of mitosis by release from a thymidine block for varying lengths of time (8–11 hrs), fixed and imaged. The fluorescence ratio and controls for protein distribution and bleaching were carried out as previously described [32]. Figure 3 shows that active Rho was distributed throughout the cytoplasm in metaphase cells but concentrated at the initial points of furrow formation (furrow specification site) in early anaphase and at the CF throughout ring contraction. At the end of cytokinesis, when the two daughter cells were separated, RhoGTP re-localized to the cytoplasm. The fluorescence ratio and levels of caQ63LRho and dnT19NRho are shown for comparison. A similar localization of the active Rho biosensors was also observed in living cells (see Figure 5), suggesting that the localization was not affected by the fixation process. This pattern of Rho activation is consistent with recent publications, in which a single fluorescence-conjugated RBD was used as a probe to follow the spatiotemporal regulation of Rho [22,26]. These results further support the involvement of Rho in CF site specification and contraction.

P190RhoGAP and ECT2 co-regulate Rho activity in the CF during cytokinesis

Our previous studies showed that the GAP domain of overexpressed p190 was required for induction of multinucleation, suggesting that p190 might regulate Rho activity during cytokinesis. To more rigorously test this hypothesis, we co-transfected HeLa cells with HA-tagged p190 and each of the various Rho bio-sensors. Cells were synchronized in mitosis, fixed, stained with anti-HA mAb, and visualized by immunofluorescence microscopy for levels of p190. Although individual cells expressed varying levels of HA-p190 as visualized by immunofluorescence, Western blotting of extracts prepared from populations of cells revealed no greater than 2–3 fold overexpression relative to endogenous p190 (Fig. 4b and data not shown). Rho biosensor expression was detected by YFP excitation (493nm) and emission (535nm) (YY). Rho activity was assessed by ratio imaging of the CF (area delineated by the oval) during cytokinesis (i.e., FRET signal normalized to the amount of Rho biosensor per cell {CY/CC}). Figure 4a, upper panels, shows two cells undergoing cytokinesis, both of which co-express HA-p190 and wtRho biosensor. The cell expressing the higher level of p190 can be seen to generate the lower Rho activity at the CF, suggesting a dose-dependency of p190 action. The middle and lower series of panels show that neither caRho nor dnRho activity was affected by p190 co-expression.

Quantitative analysis of biosensor signals from several independent experiments (a minimum of 10 cells per treatment group) revealed that p190 significantly reduced Rho activity at the CF as compared to cells expressing the biosensor alone (Fig. 4b, upper panel). In contrast, overexpression of ECT2, an established activator of Rho in cytokinesis, significantly increased levels of Rho activity in the CF. Similar to ECT2, a high-level (>5 fold wt p190) of a GAP inactive mutant of p190 (Y1283A) significantly increased Rho activity at the CF, while expression of the mutant at the same level as transfected wt p190 had no effect. Moreover, the subcellular location of Y1283Ap190 in different stages of mitosis mimicked that of wt-p190 (supplemental figure S9) and co-localized with Rho throughout mitosis. These results suggest that the GAP deficient mutant functioned as a dominant negative, interfering with the activity of endogenous p190 (Fig. 4b, upper panel). The lower panel of Fig. 4b shows the expression levels of wt and mutant p190 used in the experiments. caRho and dnRho biosensors generated

the highest and lowest activation levels, respectively, among all the treatment groups, but these signals were not altered by either p190 or ECT2. In comparison, Rho activity in interphase cells was slightly reduced by p190 but not significantly changed by ECT2 (data not show). Figure 4c demonstrates that the extent to which p190 downmodulated and ECT2 increased the activity of Rho was indeed dependent on the amount of p190 or ECT2 expressed. Together, these data suggest that p190 functions upstream of Rho to downregulate Rho signaling during cytokinesis in a dose-dependent manner.

FRET analysis of RhoGTP in living cells expressing ectopic p190: Reduced and mislocalized RhoGTP is associated with mitotic defects

To study the effect of p190 on the dynamic activity of Rho during cytokinesis in living cells, the Rho biosensor and p190 were co-expressed in 468 and HeLa cells, and the distribution of Rho activity was followed by FRET analysis. Both cell lines exhibited a defect in CF site selection, as evidenced by the oscillations of the Rho activity zone and furrow ingression/regression. The upper panels of Figure 5a and movie S5 show a 468 cell induced to overexpress p190 and undergoing cytokinesis, juxtaposed to cells in interphase. The phenotype of the cell was similar to that seen in Figure 1 and movies S1 and S2, in which cells attempted multiple times to specify a CF. At the beginning of the movie, the CF was sited near the equator, where high Rho activity could be seen (arrows, frame 1). A new CF region was then defined by a new Rho activity zone, and the original Rho activity zone disappeared (arrows, frame 2). Finally, the CF returned to the original site (frames 3–5). Cell separation was not observed during the 41 mins of the movie, and Rho activity in the CFs appeared to be suppressed. In contrast, cells lacking exogenous p190 displayed normal progression through cytokinesis (Figure 5a, lower panels and movie S6). Rho activity was concentrated at the equatorial region and remained until the two cells separated. The process took less than 10 min.

Figure 5b (upper and lower panels), movies S7 and S8, show Rho activity changes during cytokinesis in HeLa cells with or without RFPp190 expression. Without exogenous p190 (lower panel and S8), Rho activity was cortically distributed in metaphse then concentrated to the CF area and both poles during anaphase and telophase until the two cells separated. The process was completed in ~10 min. However, in RFPp190 expressing cells (upper panel and S7), Rho activity failed to concentrate in the equatorial CF area, CF sites migrated back and forth between the two daughter cells, and eventually the two daughter cells rejoined, but failed to divide. These data strongly support the hypothesis that p190 affects CF specification in early anaphase by reducing Rho activity at the CF and inhibiting cytokinesis. As such, p190 functions as an important negative regulator of mitotic progression.

Discussion

One of the mysteries of cytokinesis is the molecular mechanism by which the cleavage plane is established. Much evidence suggests that the cleavage plane is specified by the anaphase mitotic spindle, perhaps through regulation of Rho GTPase. In support of this model are the findings that active RhoA becomes concentrated at the cleavage plane specification site before ring contraction and that this accumulation is independent of actin and myosin II, but dependent on ECT2 and the centraspindlin complex [22,26,27,30].

Data in this paper together with our previous observations indicate that p190 may also play a role in CF site selection. Specifically, using immunofluorescence of fixed cells, p190 overexpression was shown to induce abnormal positioning of CF specification sites and formation of large, multi-lobed cells [41]. In the current study we used time lapse microscopy of living cells alone or in combination with FRET analysis and observed that p190 overexpression resulted in mitotic defects that arose through inappropriate selection of the cleavage plane, failure to concentrate and retain high RhoGTP levels at the selected sites, and

an inability to complete ring contraction. These defects resulted in abnormal cell shapes, including membrane blebbing, a mark of apoptosis. All of these data suggest that without the appropriate amount of p190, especially in the early stages of cytokinesis, the process can go awry. This conclusion is consistent with our previous findings that p190 is degraded in late mitosis, perhaps after it has fulfilled its function in early and mid-cytokinesis.

An alternative approach utilized by our laboratory to investigate the role of p190 in cytokinesis was gene silencing. Unexpectedly, knock-down of p190 resulted in failure of cells to enter mitosis. Cells with reduced p190 exhibited a highly flattened morphology and were unable to release from the substratum and round up, thereby preventing initiation of mitosis. While uninformative with regard to cytokinesis, these results suggested that p190 plays multiple roles in the cell cycle, one at the G2/M border (early prophase, as revealed by gene silencing) and another in cytokinesis (as revealed by overexpression). In fact, Burridge and coworkers have suggested a role for p190 in early prophase that involves contraction of the actinomyosin network at the cell cortex, a process involved in cell rounding [49].

While the GAP domain of p190 is critical to its function in cytokinesis, other domains within the molecule may also contribute to the process. P190 is a large protein that contains multiple protein-protein interaction motifs, including an N-terminal GTP binding domain [50], FF domains that mediate interactions with RNA binding proteins [51], polyproline regions that bind SH3 domains, and phosphotyrosine, phosphoserine, and phosphothreonine that can also mediate binding with proteins containing SH2 or PTB (phosphotyrosine binding) domains [52]. Such interactions could serve as scaffolding functions in assembly of the actinomyosin complexes that form the contractile ring or in associations with the mitotic spindle that determine the cleavage plane.

Although several Rho regulators and effectors are involved in cytokinesis [3,13,15], the signaling pathway(s) related to this process are not completely defined. An emerging model suggests that localization and activation of RhoA during cytokinesis is regulated by the ECT2-centraspindlin complex [26,27,30]. Both positive and negative regulation of Rho are necessary, since CF assembly and ring contraction are dynamic processes [53]. Several models have suggested that cytokinesis is initiated by activation of Rho at the site of CF assembly and terminated by inactivation of Rho, presupposing that Rho activation and inactivation are temporally uncoupled. However, recent studies, including the findings in this paper, support the concept that Rho's GTPase cycle undergoes rapid and repetitive turnover during the entire process of cytokinesis and that this cycling is necessary for normal cytokinesis [28,54]. This model implies that both RhoGEFs and RhoGAPs are highly activated and remain associated with the Rho activation zone at the CF. It is well established that ECT2 localizes to the CF and is a positive regulator of Rho in cytokinesis. The following evidence suggests that p190RhoGAP is a strong candidate for fulfilling the negative regulatory role. First, the GAP activity of p190 is specific for Rho *in vivo*, as compared to Rac and Cdc42 [42]. Second, p190's involvement in cytokinesis is GAP domain-dependent [41], and a dominant negative GAP-domain mutant enhances Rho activity at the CF above that of control, suggesting that measured negative regulation of Rho activity is mediated during cytokinesis by p190 (Fig. 4). Third, p190 localizes to the CF and co-localizes with Rho throughout the entire process of cytokinesis (Fig. 2). Fourth, FRET analysis demonstrated that p190 reduces Rho activity at the CF, that this reduction is dependent on the dose of p190, and that reduced Rho activity correlates with aberrant localization of the cleavage plane (Figs. 3–5). Lastly, p190 and ECT2 are physically associated during mitosis [55]. Together, these data strongly support p190 as an upstream negative regulator of Rho activity during cytokinesis.

Our findings regarding the spatio-temporal regulation of Rho activity during cytokinesis are consistent with other studies in which alternative probes and cell types were used to follow

activated Rho. Bement et al. (2005) utilized an eGFP-conjugated Rhotekin Binding Domain (eGFP-RBD) construct to explore the active pool of Rho GTPases in sea urchin and frog embryos [22]. They found that the eGFP-RBD concentrated at the equator of blastomeres in narrow zones that coincided with the sites of furrow formation and that these zones contracted in synchrony with ingressing CFs. Yuce et al. (2005) transfected YFP-tagged, full-length C. elegans RhoA (Ce-Rho) into HeLa cells, and like eGFP-RBD in sea urchin and frog embryos, found that CeRhoA accumulated at sites of furrow initiation early in anaphase, a concentration that was sustained in the CF throughout ring contraction [26]. A similar localization of endogenous RhoA was found in trichloroacetic acid-fixed HeLa cells stained with specific RhoA antibodies [26]. However, using a single chain Rho FRET probe, designed differently than the one used in the current study, Yoshizaki et al (2003) found that RhoGTP levels increased only at the end of cytokinesis [31], suggesting that activated Rho does not play a significant role in cleavage plane specification or contractile ring assembly and contraction. In contrast to this study, we show that Rho activity increases at the equatorial region before and during CF contraction. The discrepancies between these studies might be due to the different biosensor constructs or the sensitivity of the detection systems. Also, unlike the biosensor used in Yoshizaki et al (2003), the biosensor used here is subject to normal regulation by GDI and has been used to study Rho activity in situ in both interphase cells and cells undergoing cytokinesis [32,33].

Our data clearly suggest that overexpression of p190 affects specification of the CF through inhibition of Rho activity at the potential CF. It is generally agreed that anaphase spindle microtubules define the furrow position. In addition to regulating actin dynamics, Rho has been shown to regulate microtubules through its substrates, formin-homology proteins [56,57]. Live cell FRET imaging (Fig. 5) showed a concentration of active Rho at potential cleavage sites, but it is not clear whether this effect is mediated through p190 directly or indirectly through Rho, or whether the effect is on microtubules or actin. Such questions warrant further investigation.

In summary, the results in this paper suggest that active Rho accumulates at the cell equator in early anaphase before ring contraction and remains in the activated state throughout cytokinesis. Overexpression of p190 results in dose-dependent downregulation of Rho activity at the CF, thereby altering the dynamics of cleavage plane specification and furrow contraction. Endogenous p190 co-localizes with Rho throughout cytokinesis. From these results, we postulate that p190 acts as a negative regulator of Rho during cytokinesis in juxtaposition to RhoGEFs to ensure normal progression through cytokinesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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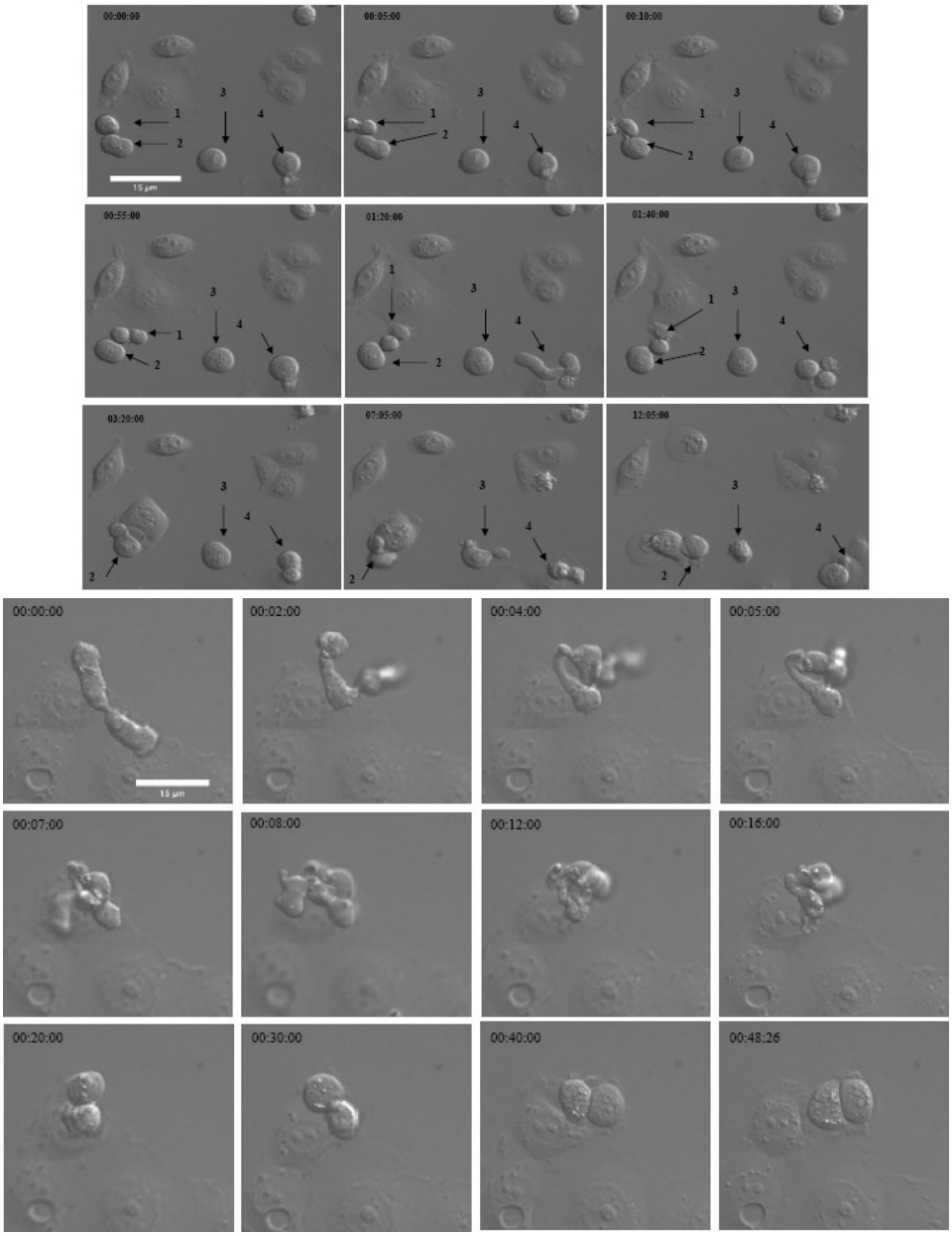


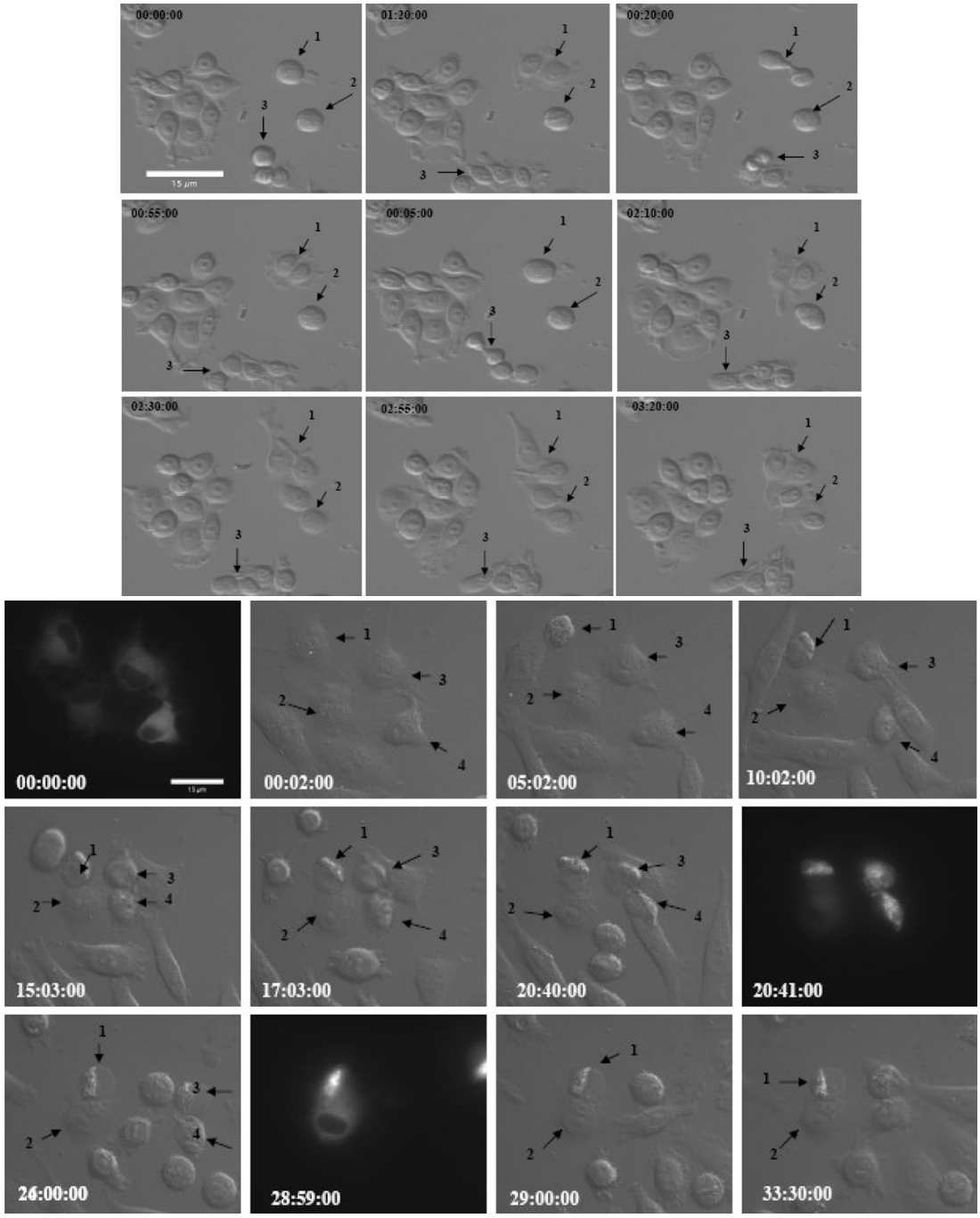


Figure 1.

p190RhoGAP overexpression results in a mitotic defect in tet-on MDA-MB-468 breast cancer and HeLa cells. (a) Selected images of 12 hr time-lapse recordings of multiple mitotic 468 cells inducibly overexpressing p190 in the presence of 1ug/ml Dox (movie S1). Cells exhibit a defect in cytokinesis. (b) p190 overexpression disturbs furrow specification in anaphase. Enlarged, selected images of one hour time-lapse recordings of a p190-overexpressing 468 cell in the presence of 1ug/ml Dox (movie S2) (anaphase to telophase). (c) Selected images of 3 hr time-lapse recordings of tet-on 468 cells during mitosis in absence of Dox (movie S3). All movies started recording 24 hrs after Dox treatment. Upper right corners show the time frame of the

image. Arrows and numbers beside the arrows indicate the mitotic cells. (d) Selected images of 40 hr time-lapse recordings of HeLa cells transiently overexpressing GFP190 (movie S4). Lower left corner shows the time. Arrows and numbers beside the arrows in DIC images and black background images show GFP190 positive cells. The movie started recording 24 hrs after transfection. Scale bars, 15μm.

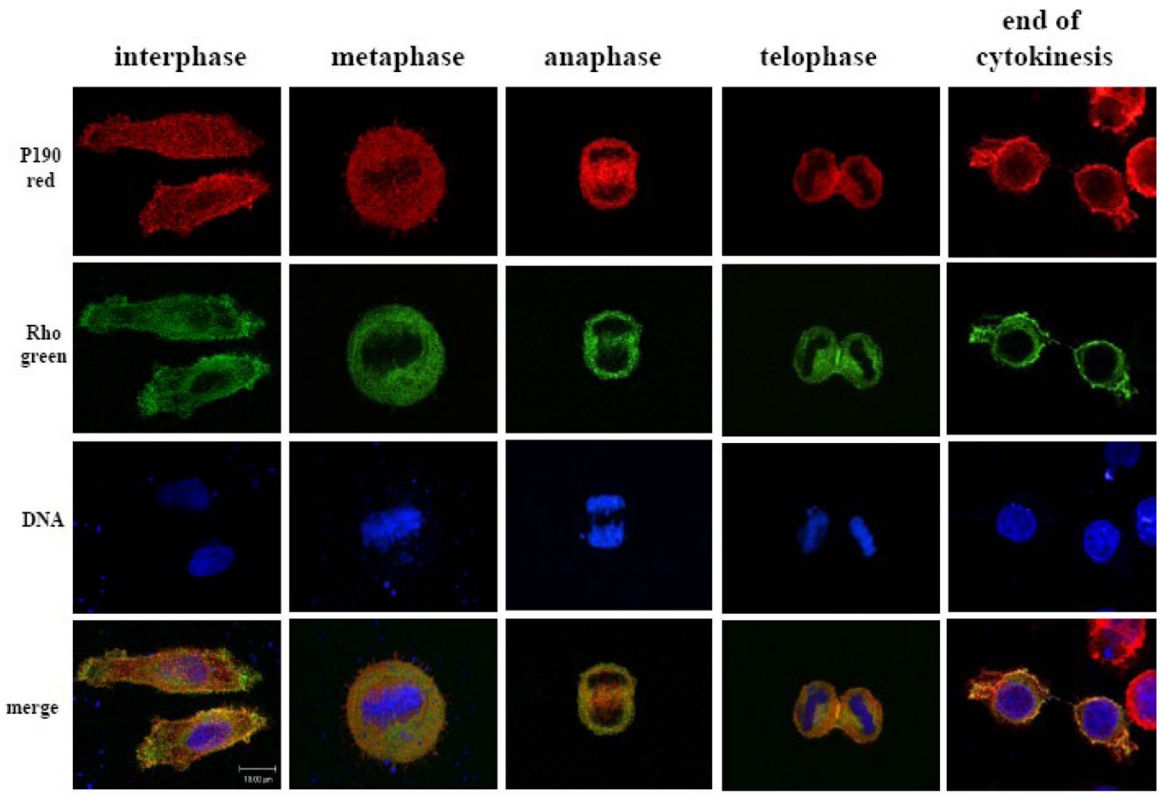


Figure 2.

p190RhoGAP and Rho co-localize in mitosis. HeLa cells were synchronized by thymidine block, transfected with pKH3p190 and wtRho biosensor, and released for 8 to 11 hrs to enter mitosis. In various stages of mitosis, cells were fixed and immunostained for p190 with anti-HA mAb/Alexa red 594 goat anti-mouse IgG and visualized by confocal fluorescence microscopy. Total Rho biosensor was visualized in the same cells by YFP excitation/emission. Scale bars, 10μm.

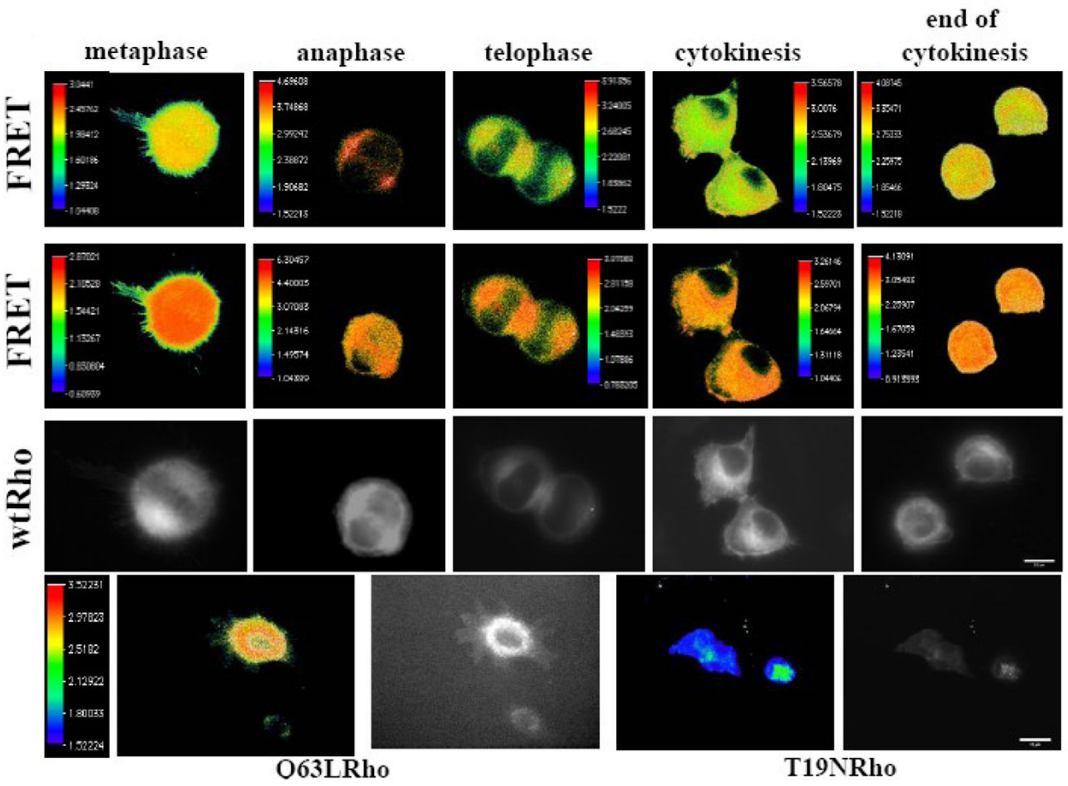
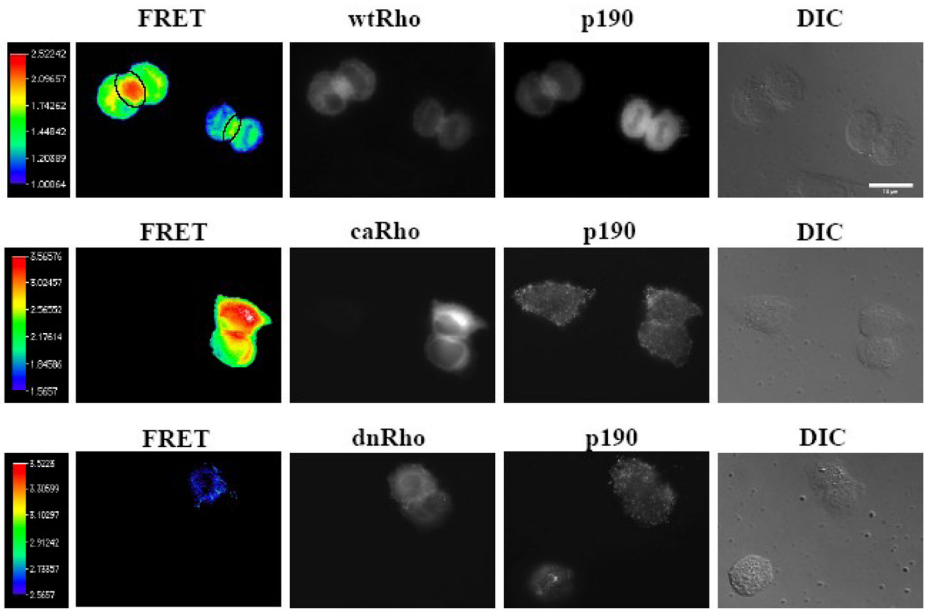
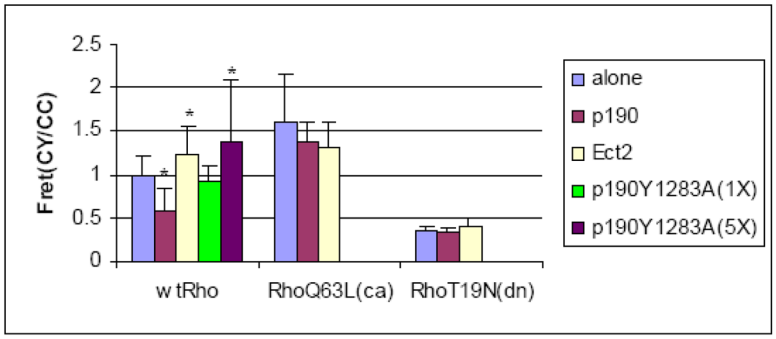
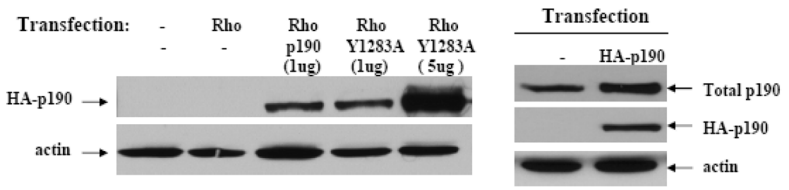


Figure 3.

Localization of RhoGTP in mitotic cells by FRET analysis. HeLa cells were transfected with wtRho or mutant Rho biosensors and 24 hrs later were visualized by fluorescence microscopy. Color images showing the subcellular localization of Rho activation are represented by the ratio of CY/CC (CFP excitation and YFP emission/CFP excitation and CFP emission) calculated with OpenLab software. The upper panel presents Rho activity in the various cells, when images were adjusted to similar ratio ranges. The second panel shows the highest Rho activation levels in each cell by modifying the ratio range respective to the upper panel. The third panel shows the Rho expression levels in cells depicted in the upper panels, visualized by YFP excitation and YFP emission. Lower panels show fluorescence ratio generated by the positive and negative Rho biosensor controls. Scale bars, 10μm.







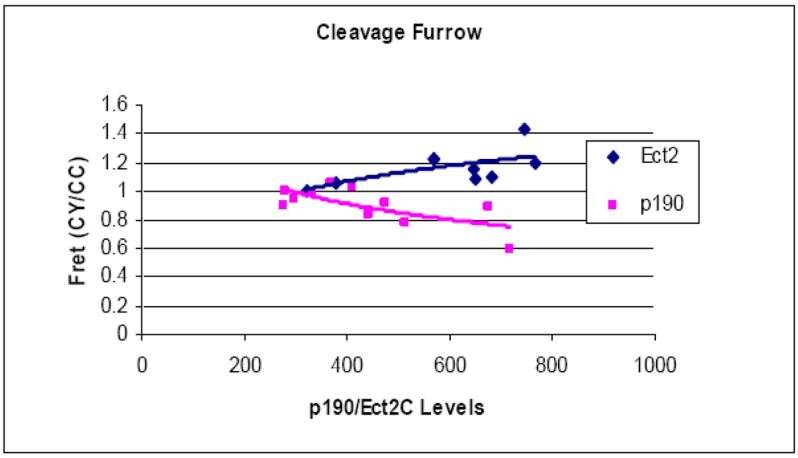


Figure 4.

p190RhoGAP and ECT2 co-regulate Rho activity in the CF during cytokinesis. (a) Effect of p190RhoGAP on Rho activity during cytokinesis. HeLa cells were synchronized into mitosis by a thymidine block, co-transfected with pkH3p190 and one of the Rho biosensor mutants (wtRho, Q63LRho or T19NRho), and released from the block for 8 to 10 hrs. At various times after release, Rho expression levels were visualized by YFP excitation/emission, while p190 levels were determined by Alexa red 594 excitation/emission, as described in Methods. Rho activity was quantified by CY/CC ratioing, using OpenLab software. Circles around the CF show the areas used for quantitation in (b). Scale bar, 10 μm. (b) Upper panels, quantitation of Rho activation. The Y axis represents the densitometry units of each interested area in a CY image divided by the densitometry units of the same area of the CC image. More than 10 cells were analyzed per individual group. Error bars represent the standard error of the mean. *$p<0.05$ compared to the wtRho biosensor only. Only the CF area was quantified for cells undergoing cytokinesis. Lower panels, relative protein expression levels of wt or mutant p190 used in (a) and (b), as analyzed by Western blot. Data from (c) show the mean effect of different levels of p190 or ECT2 on Rho activation. The X axis represents arbitrary densitometry units of p190 or ECT2 in individual cells, while the Y axis denotes the CY/CC ratio from the same cells. Rho activity from the lowest p190 or ECT2-expressing cell was set at 1, to which all other values were normalized.

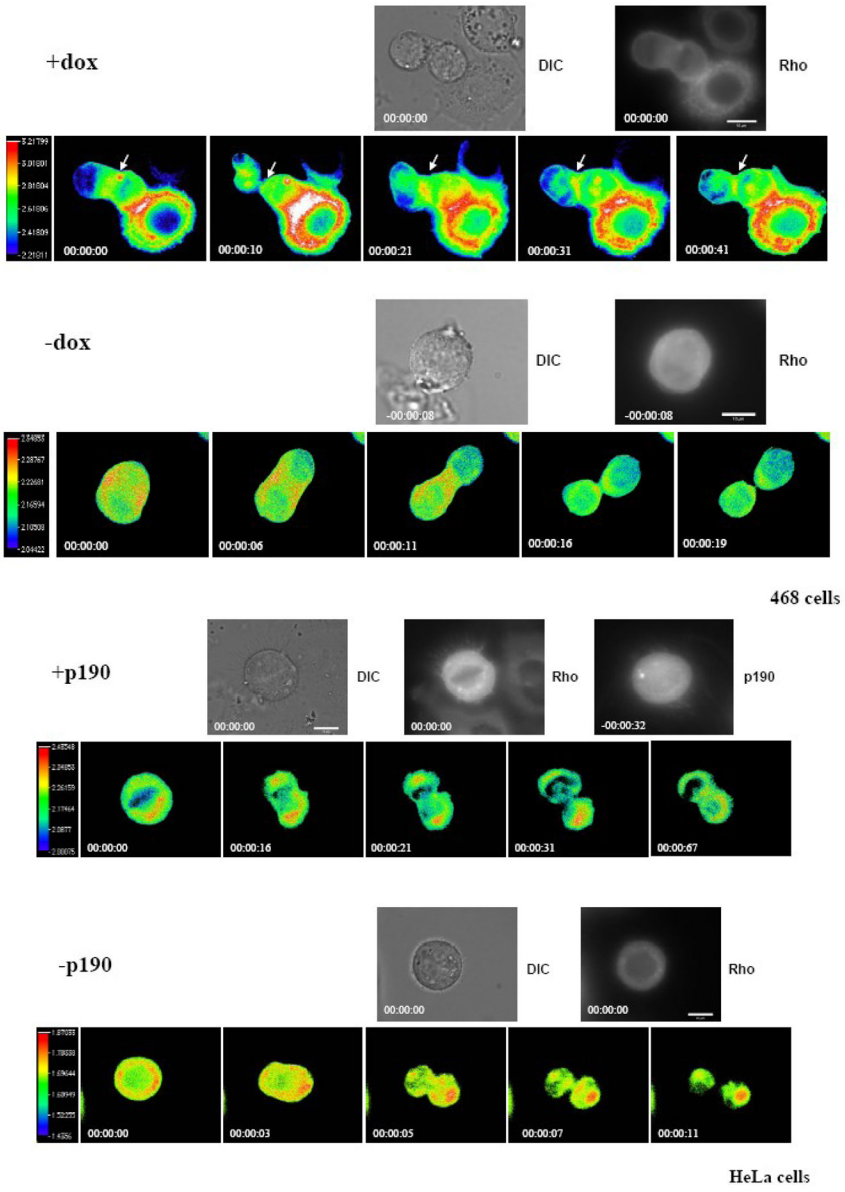


Figure 5.

Effects of p190RhoGAP on Rho activity in live mitotic cells. (a) Upper panels, selected FRET ratio images from a 40 min time-lapse recording of mitotic 468 tet-on p190 expressing cells transfected with wtRho biosensor and induced to overexpress p190 (movie S5). The movie was recorded 24 hrs after transfection and addition of 1ug/ml Dox. Lower panels, selected FRET ratio images from a 20 min time-lapse recording of a mitotic 468 cell transfected with wtRho biosensor and not treated with Dox (movie S6). The movie was recorded 24 hrs after transfection. (b) Upper panels, selected FRET ratio images from a 100 min time-lapse recording of a mitotic HeLa cell stably expressing wtRho biosensor and transfected with RFPp190 (movie S7). The movie was recorded 24 hrs after transfection. Lower panels, selected FRET ratio images from an 11 min time-lapse recording of a mitotic HeLa cell stably expressing wtRho biosensor only (movie S8). The DIC image selected from each movie represents frame 1. Black background images are YY showing the Rho biosensor expression levels. Lower left corner shows the time. Scale bars, 10μm.