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Selective glucocorticoid control of Rho kinase isoforms regulate cell-cell interactions

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Abstract

The two Rho kinase isoforms ROCK1 and ROCK2 are downstream effectors of the small GTPase RhoA, although relatively little is known about potential isoform specific functions or the selective control of their cellular activities. Using Con8 rat mammary epithelial cells, we show that the synthetic glucocorticoid dexamethasone strongly stimulates the level of ROCK2 protein, which accounts for the increase in total cellular ROCK2 activity, whereas, steroid treatment down-regulated ROCK1 specific kinase activity without altering ROCK1 protein levels. In Con8 cells, the glucocorticoid induced formation of tight junctions requires the steroid-mediated down-regulation RhoA and function of the RhoA antagonist Rnd3. Treatment with the ROCK inhibitor Y-27632 ablated both the glucocorticoid-induced and Rnd3-mediated stimulation in tight junction sealing. Taken together, our results demonstrate that the expression and activity of ROCK1 and ROCK2 can be uncoupled in a signal-dependent manner, and further implicate a new function for ROCK2 in the steroid control of tight junction dynamics.

Keywords

Rho kinase isoforms; ROCK1; ROCK2; glucocorticoids; differential regulation; tight junction dynamics

Introduction

Adherens junctions and the tight junctions are located at the points of contact between cells and are indispensable to epithelial cell polarity and normal tissue architecture [1–4]. These junctions control intercellular adhesion and permeability properties and have a highly dynamic structure [1,5]. Using rodent Con8 mammary epithelial tumor cells, we have uncovered a glucocorticoid hormone mediated signaling cascade that stimulates adherens junction organization and induces tight junction sealing as well as polarization of cell monolayers [6–9]. We further established that the glucocorticoid down-regulation of the small GTPase RhoA is required for formation of organized intercellular junctions [10]. Our recent evidence shows that the functional ratio of RhoA to Rnd3/RhoE, a recently discovered natural RhoA antagonist, controls both adherens junction formation and tight junction sealing [11], suggesting that key downstream effectors of RhoA and Rnd3/RhoE signaling may be regulated in a steroid-dependent manner.

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Two downstream effectors of RhoA signaling that have been implicated for a role in the dynamics of epithelial cell morphology are the Rho kinase isoforms ROCK1 and ROCK2; ROCK1 is also referred to as ROCK-I, ROK-beta or p160 ROCK [12,13], whereas ROCK2 is also named ROCK II, ROK-alpha, or p150 ROCK [14]. ROCK1 and ROCK2 are serine/threonine protein kinases that are 92% homologous in their kinase domains, contain pleckstrin homology domains for protein interactions, and have carboxy-terminal domains with an auto-inhibitory function that can reduce their kinase activity [15]. The Rho kinases were the first effectors of Rho family of small GTPases (RhoA, RhoB, RhoC) to be identified, and were found to mediate stress fiber formation and focal adhesion by phosphorylation of the Myosin Light Chain [16]. Rho kinase signaling has also been implicated in actin stress fiber assembly, cytoskeleton remodeling, myosin incorporation into polymerized actin bundles, and centrosome positioning [17] as well as in maintaining tight junction integrity in endothelial cells [18], and in human intestinal epithelial cells [19].

Even though ROCK1 and ROCK2 are not highly homologous outside of their kinase domains [13], studies up to now have not distinguished the potential functions of the Rho kinase isoforms. In addition, nothing is known about the signal-dependent control of the individual Rho kinase isoforms. Using rodent mammary epithelial tumor cells, we show in this study that glucocorticoid hormones stimulate total cellular activity of ROCK2 and simultaneously decrease ROCK1 specific activity and that this differential regulation of Rho kinase isoform function plays a role in the steroid induction of tight junction sealing and cell-cell interactions.

MATERIALS AND METHODS

Reagents

Permeable filter inserts were supplied by Nunc. Puromycin, and Dexamethasone were purchased from Sigma Chemical Comp. Rabbit polyclonal anti-ROCK1, anti-ROCK2, and anti-phospho-MYPT1 and anti-MYPT antibodies, as well as recombinant MYPT protein, were purchased from Upstate Signaling Technologies. Mouse monoclonal anti-ROCK1, goat polyclonal anti-ROCK 1 and anti-ROCK2, rabbit polyclonal anti-RhoA, goat polyclonal anti-actin, monoclonal anti- α -tubulin, and monoclonal anti c-Myc (clone 9E10) antibodies, as well as the Rho kinase inhibitor (Y-27632) were supplied by Santa Cruz Biotechnology.

Cell Culture and Transepithelial Electrical Resistance Measurement

Con8 cells are rat mammary epithelial tumor cells that have been previously described [9,10, 20,21]. Cells were cultured at 37°C in DMEM/F-12 medium with 10% calf serum in the presence of penicillin/streptomycin, and the transepithelial electrical resistance (TER) measurements were recorded using an EVOM Epithelial Voltohmmeter (World Precision Instruments) as described previously (6–9). Raw TER values were normalized by subtracting the background resistance values from an empty filter and multiplying the difference by the area of the monolayer of cells.

Immunoblot Protocol and Analysis

Cells were grown to confluence in 10 cm plates, serum-starved for 24 hours, and treated with DMEM/F-12 medium containing penicillin and streptomycin with or without 1 μ M Dex daily for the remainder of the experiment. Cells were then rinsed with PBS at 4°C, and extracted using 1% NP-40 lysis buffer (50 mM Tris, pH=7.5, 150 mM NaCl, 20 mM Magnesium Chloride, 1% Nonidet P-40), and protease inhibitors. Samples were normalized for protein content with the Bradford assay (BioRad). Cell lysates were fractionated on SDS-polyacrylamide gels. Proteins were electrophoretically transferred to a nitrocellulose membrane (Micron Separations Inc., Westboro, MA). Blots were blocked in 5% non-fat dry milk in washing buffer (0.1 M Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for at least

30 min at room temperature, and then incubated with primary antibodies overnight at 4°C. After three washes for 10 min each in 1% non-fat dry milk in washing buffer, the blots were incubated with horseradish-peroxidase-conjugated secondary antibodies (BioRad, Hercules, CA) for 1 hr. The blots were then washed twice with 1% non-fat dry milk in washing buffer and once with washing buffer. The blots were developed by NEN Life Science Products chemiluminescence reagent kit (Arlington Heights, IL). Anti-ROCK1, anti-ROCK2, and anti-phospho MYPT1 were used at 1:500 dilution, anti-actin and anti-MYPT1 at a 1:1,000 dilution.

Rho Kinase Assay

Con8 cells were grown to confluence on six 10 cm plastic dishes each and then treated with or without Dexamethasone for 5 days, in serum-free medium. To assay Rho kinase activity, cells were lysed in immunoprecipitation (IP) buffer (10 mM Tris-HCl at pH 7.5, 1% Triton X-100, 0.5% NP-40, 150mM NaCl, 2mM CaCl₂, 0.1 mM sodium orthovanadate, 10 µg/ml aprotinin/leupeptin, and 1 mM PMSF, all from Sigma), then centrifuged at 14,000 rpm for 4 min. Protein was normalized using the Bradford Assay (BioRad). Equal aliquots of lysates were incubated with 5 µL of either goat anti-ROCK1 antibody, goat anti-ROCK2 antibody, goat IgG, or no antibody as a control for 30 min. on a nutator at 4°C. To all lysate samples except those not containing antibodies or IgG, 100 µL of washed protein G sepharose beads (Amersham) diluted in IP buffer (1:1 slurry) was added, and samples were nutated at 4°C for 60 min. Beads were gently spun down at 2000 rpm for 2 min, and washed four times with IP buffer, then resuspended in kinase assay buffer (50mM HEPES [pH 7.4], 150 mM NaCl, 1mM MgCl₂, 1 mM MnCl₂, 10mM NaF, 1mM sodium orthovanadate, 5% glycerol, 1% NP-40, 1 mM dithiothreitol, and 1 mM PMSF, all from Sigma). ROCK kinase assay was performed using 10 µM ATP (Sigma), and recombinant MYPT1 substrate (Upstate), incubated with the bead-kinase assay buffer slurry in a reaction volume of 50 µL at 37°C. To each of the duplicated antibody-containing samples 10 µM of the Rho kinase inhibitor Y-27632 (Santa Cruz) was added as a control prior to incubation at 37°C. The reaction was stopped by the addition of SDS-PAGE buffer, followed by boiling for 10 min at 100°C. Kinase assay activity was detected by SDS gels, followed by Western blotting using anti-phospho-MYPT1 and anti-MYPT antibodies (Upstate Cell Signaling).

Results and Discussion

Glucocorticoids selectively stimulate the level of ROCK2 protein without affecting ROCK1 protein levels

To test the potential effects of glucocorticoids on the two Rho Kinase isoforms (ROCK1 and ROCK2), which are downstream effectors of RhoA and Rnd3/RhoE signaling [14,15,22], Con8 mammary epithelial cells were grown to confluence, serum-starved, treated for 5 days with or without 1 µM dexamethasone, and then total cell lysates were analyzed by western blots using monoclonal anti-ROCK1 or anti-ROCK2 antibodies. This time point was chosen because approximately 3-4 days of steroid treatment is needed for complete apical junction organization and formation of highly sealed tight junctions [6–8,20,23]. As shown in Figure 1, dexamethasone strongly induced the levels of ROCK2 protein, but had no effect on ROCK1 protein levels. Actin protein levels were used as a control for protein loading. No changes were observed in the level of ROCK2 transcripts suggesting that glucocorticoid signaling targets this Rho kinase protein (data not shown). The levels of ROCK1 and ROCK2 protein detected in three independent experiments were quantified by densitometry and the results shown in the bar graph in Figure 1. This result demonstrates for the first time that ROCK1 and ROCK2 can be differentially regulated in a signal-dependent manner.

Glucocorticoids differentially regulate ROCK1 and ROCK2 Kinase Activity

To assess if ROCK1 and ROCK2 kinase activity is regulated by glucocorticoids, *in vitro* Rho kinase activity assays were performed for both ROCK1 and ROCK2. Con8 cells were grown to confluence, serum-starved, treated with or without dexamethasone for 5 days, and then total cell lysates were immunoprecipitated with either ROCK1-specific, ROCK2-specific, or IgG control antibodies. *In vitro* activity of both kinases was determined using myosin phosphatase targeting subunit (MYPT1) as a substrate, which is phosphorylated on Thr-696 [24]. The *in vitro* kinase assay mixtures included the immunoprecipitated material, MYPT1, and ATP, and were incubated in the presence or absence of 10 μ M of the Rho kinase inhibitor Y-27632 as a control for Rho kinase specificity in the assay. Phosphorylation of MYPT1 was detected using anti-phospho-MYPT1 antibodies in western blots of the kinase reactions, and a parallel set of western blots were probed with anti-MYPT1 antibodies. As shown in Figure 2, ROCK1 protein immunoprecipitated from dexamethasone treated cells displayed significantly less *in vitro* kinase activity compared to ROCK1 in cells not treated with steroids (IP: ROCK1 -Dex vs. ROCK1 +Dex). Similar to the western blots shown in Fig. 1, both immunoprecipitated samples contained the same level of total ROCK1 protein (data not shown). The level of active ROCK1 detected by this assay was slightly higher than the background signal in the assay (Fig. 2, IP with IgG), indicating that ROCK1 activity is not completely inhibited in glucocorticoid-treated cells. The *in vitro* production of phospho-MYPT1 was abolished when the Rho kinase inhibitor Y-27632 was added to the kinase reaction mixtures (Fig. 2, +Y-27632), demonstrating the specificity of the assay.

In contrast to ROCK1 kinase activity, the overall ROCK2 kinase activity was up-regulated by dexamethasone treatment (Fig. 2, IP with ROCK2 -Dex vs. ROCK2 +Dex). The observed ROCK2 kinase activity is specific for the ROCK2 antibodies (Fig. 2, IP with ROCK2 vs. IgG) and is ablated in the presence of the Y-27632 Rho kinase inhibitor (Fig. 2 +Y-27632). The kinase assays from three independent experiments were quantified (Fig. 2 bar graphs) and compared to the protein studies described in Figure 1. Our studies show that glucocorticoids down-regulate total ROCK1 activity due to the inhibition of ROCK1 specific activity in that the total level of ROCK1 protein is not altered by steroid treatment. In contrast, glucocorticoids strongly stimulate total detectable ROCK2 cellular kinase activity that can be accounted for by the steroid induction of ROCK2 protein levels. These results demonstrate that the predominant functioning Rho kinase in glucocorticoid-treated cells is ROCK2, thus for the first time uncoupling the potential actions of the two known Rho kinase isoforms.

Inhibition of Rho kinase activity ablates both the glucocorticoid-induced and Rnd3/RhoE-mediated tight junction sealing

We have previously shown that tight junctions sealing of monolayers of Con8 mammary epithelial tumor cells can be stimulated by either treatment with glucocorticoids [6–10,20,23] or by expression of the Rnd3/RhoE antagonist of RhoA [11]. To determine whether Rho kinase activity plays a role in the stimulation of cell-cell interactions by either glucocorticoids or Rnd3/RhoE, the effects Y-27632, a relatively specific pharmacological inhibitor of Rho kinase activity [25], was analyzed on tight junction sealing. The transepithelial electrical resistance (TER) provides a direct measure of tight junction sealing in cultured mammary epithelial cells [20,23]. Con8 cells or its transfected cell derivative C8-Rnd3 cells, which was designed to constitutively express high levels of Rnd3/RhoE, were plated onto filter inserts and the TER was monitored in cells treated in the presence or absence of the indicated combinations of 1 μ M dexamethasone and 10 μ M Y-27632. The cell monolayers were treated with dexamethasone until a high TER had formed (4 days), and then Y-27632 was added to the cell cultures for 24 hours. Under the steroid treated conditions, the primary functioning Rho kinase is ROCK2.

As shown shown in the upper panel of Figure 3, in Con8 cells the steroid-increased TER was reversed to background levels within 24 hours of inhibitor treatment (Fig. 3, +Dex/+Y-27632) and became indistinguishable from cells not treated with steroid (Fig. 3, -Dex, -/+Y-27632). Treatment of cells with the Rho kinase inhibitor in the absence of dexamethasone had no further effect on the basal TER. When the kinase inhibitor was removed but dexamethasone treatment continued, Con8 cells recovered the high TER, demonstrating that the effects on Rho kinase are reversible and that treatment with Y-27632 is not toxic to the cells (data not shown). Similarly, in C8-Rnd3 cells, which express exogenous Rnd3, the Y-27632 Rho kinase inhibitor ablated the high TER observed in Rnd3-expressing cells in the absence or presence of dexamethasone (Fig. 3, lower panel). Immunofluorescence analysis of the ZO-1 tight junction protein revealed that the highly organized apical junctions observed in dexamethasone treated or untreated C8-Rnd3 cells were disrupted by an inhibition Rho Kinase activity (data not shown).

Due to their high homology in the kinase domain (92%) [13] and many common downstream effectors, ROCK1 and ROCK2 are often studied as a pair. Our study provides the first evidence that the cellular activities of these two Rho kinase isoforms can be uncoupled in that glucocorticoids induce an increase in ROCK2 protein levels while down-regulating ROCK1 specific kinase activity. Regardless of the precise cellular process that under control of glucocorticoid hormones that enhances ROCK2 protein levels and reduces ROCK1 specific enzymatic activity, our results implicate distinct functions for each Rho kinase isoform. In glucocorticoid-treated cells, the predominant active Rho Kinase isoform is ROCK2, and we propose that ROCK2 signaling plays a role in the steroid-control of cell-cell interactions in mammary epithelial cells. Consistent with this concept, treatment with a relatively selective Rho kinase inhibitor blocks the glucocorticoid-induced formation of tight junction sealing. We further propose that ROCK1 plays either no role or perhaps an inhibitory role in this process, explaining its down-regulation by dexamethasone.

We previously showed that ectopic expression of the RhoA antagonist Rnd3/RhoE induces apical junction organization and tight junction sealing of mammary epithelial tumor cells in the absence of glucocorticoids [11]. Treatment with Rho kinase inhibitor Y-27632 disrupted the Rnd3/RhoE induced cell-cell interactions suggesting that the Rho kinases are down-stream effectors of Rnd3/RhoE. Because increasing the Rnd3/RhoE to RhoA ratio mimics the effects of glucocorticoids on apical junction formation and tight junction sealing [11], and ROCK2 is the predominant active Rho kinase in glucocorticoid-treated cells, we further propose that ROCK2 is likely to be the Rho kinase isoform that is downstream of Rnd3/RhoE. Consistent with our overall observations that Rho kinase activity is important for the glucocorticoid-regulated cell-cell interactions, in endothelial cells and in intestinal epithelial cells the pharmacological inhibition of Rho kinase activity lead to loss of tight junction integrity and increased intercellular leakiness [18,19]. It is tempting to consider that key downstream targets of Rho kinase, in particular ROCK2, ultimately target structural components of the adherens junction and tight junctions, either directly or through other cellular processes such as the cytoskeleton [26]. Thus, an important issue will be to eventually delineate the precise interactions and signaling pathways that selectively mediate the steroid induced ROCK2 function in mammary epithelial tumor cells.

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Fig. 1.

Dexamethasone-regulated expression of ROCK1 and ROCK2 protein in Con8 mammary tumor cells. Top Panel: Con8 cells were grown to 100% confluence, and treated with or without dexamethasone for 5 days. Western blots of electrophoretically fractionated cell extracts were probed with antibodies against ROCK1 and ROCK2, respectively, and actin as a loading control. Bar Graphs: The relative production of ROCK1 and ROCK2 protein was quantified as the detected levels of ROCK1 and ROCK2 proteins in comparison to the detected actin protein at a significantly shorter film exposure. The band intensities were quantified using densitometry, and the bars graphs represent the average of three independent experiments.

Rho Kinase enzymatic activities

ROCK1

ROCK2

Fig. 2.

Dexamethasone regulation of ROCK1 and ROCK2 kinase activity. Top Panel: Con8 cells were treated with or without dexamethasone for 3 days in the presence or absence of the Rho kinase inhibitor Y-27632. Cell lysates were incubated with ROCK1 polyclonal antibodies or IgG control antibodies, bound to Protein G sepharose beads. A kinase assay using the Rho kinase substrate MYPT1 was performed with the immunoprecipitated samples as described in Materials and Methods. The kinase assay mixture was electrophoretically separated on an SDS gel and subjected to Western blot analysis for both phospho-MYPT1 or total MYPT1. Middle panel: The experiment was performed as described for the top panel except that ROCK2 polyclonal antibodies were employed for the kinase-specific immunoprecipitations. Lower

Panel: The relative ROCK1 and ROCK2 specific enzymatic activities were quantified as the level of detected phospho-MYPT1 in immunoprecipitates using the anti-ROCK antibodies and then subtracting the basal signal using the IgG control antibodies. The band intensities were quantified using densitometry, and the bar graphs represent the average of three independent experiments.

Fig. 3.

Inhibition of Rho kinase activity disrupts the glucocorticoid-induced and Rnd3/RhoE mediated tight junction sealing. Con8 mammary tumor cells or a Con8-derived cell line that stably expresses Rnd3/RhoE (C8-Rnd3 cells) were cultured in the presence or absence of 1 μ M dexamethasone (Dex) for 4 days and then each cell culture treated with or without 10 μ M Y-27632, a Rho kinase inhibitor. The monolayer transepithelial electrical resistance (TER) was monitored to examine tight junction sealing. The results are an average of three independent experiments.