Transformation mediated by RhoA requires activity of ROCK kinases

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Background: The Ras-related GTPase RhoA controls signalling processes required for cytoskeletal reorganisation, transcriptional regulation, and transformation. The ability of RhoA mutants to transform cells correlates not with transcription but with their ability to bind ROCK-I, an effector kinase involved in cytoskeletal reorganisation. We used a recently developed specific ROCK inhibitor, Y-27632, and ROCK truncation mutants to investigate the role of ROCK kinases in transcriptional activation and transformation.

Results: In NIH3T3 cells, Y-27632 did not prevent the activation of serum response factor, transcription of c-*fos* or cell cycle re-entry following serum stimulation. Repeated treatment of NIH3T3 cells with Y-27632, however, substantially disrupted their actin fibre network but did not affect their growth rate. Y-27632 blocked focus formation by RhoA and its guanine-nucleotide exchange factors Dbl and mNET1. It did not affect the growth rate of cells transformed by Dbl and mNET1, but restored normal growth control at confluence and prevented their growth in soft agar. Y-27632 also significantly inhibited focus formation by Ras, but had no effect on the establishment or maintenance of transformation by Src. Furthermore, it significantly inhibited anchorage-independent growth of two out of four colorectal tumour cell lines. Consistent with these data, a truncated ROCK derivative exhibited weak ability to cooperate with activated Raf in focus formation assays.

Conclusions: ROCK signalling is required for both the establishment and maintenance of transformation by constitutive activation of RhoA, and contributes to the Ras-transformed phenotype. These observations provide a potential explanation for the requirement for Rho in Ras-mediated transformation. Moreover, the inhibition of ROCK kinases may be of therapeutic use.

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Received: 16 October 1998
Revised: 22 December 1998
Accepted: 5 January 1999

Published: 28 January 1999

Current Biology 1999, 9:136–145
http://biomednet.com/elecref/0960982200900136

 ISSN 0960-9822

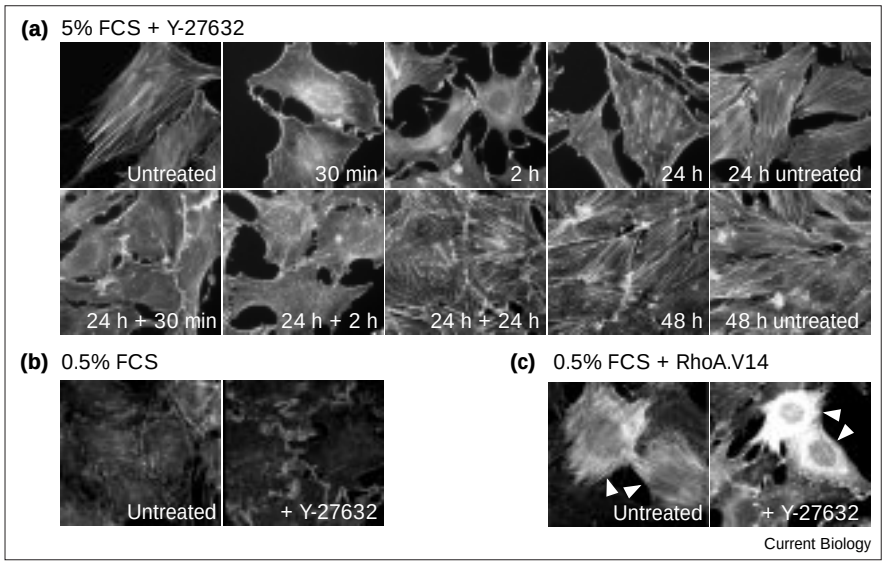
Background

The RhoA GTPase controls diverse cellular processes ranging from cytoskeletal reorganisation to transcription. Many studies have demonstrated the involvement of RhoA signalling in cell cycle progression and transformation. Activated forms of the Rho family GTPases RhoA and Rac synergise strongly with Raf in transformation assays, and interfering forms of these GTPases inhibit Ras transformation; in addition, Rho protein guanine-nucleotide exchange factors (GEFs) exhibit potent transforming activity *in vitro* (see [1] for review). Activated Rho can induce DNA synthesis in Swiss 3T3 cells, and Rho signalling has also been implicated in the degradation of the cyclin-dependent kinase (CDK) inhibitors p21 and p27 during cell cycle re-entry and progression in response to activated Ras [2–5]. The relationship between these observations and transformation, however, remains unclear.

As is the case for other small GTPases, GTP-bound RhoA acts by regulating the activity of effector proteins. Numerous RhoA effectors have been identified, including at least three families of protein kinases, the ROCKs, PRKs and Citron kinase [1,6]. We previously used RhoA effector-loop mutants, which are selectively defective in their interaction with different effectors, to investigate the relationship between these effectors and RhoA-controlled processes. We found that transformation by activated RhoA mutants correlated with their ability to bind ROCK kinases rather than to potentiate transcriptional activation by serum response factor (SRF) [7]. However, ROCKΔ3, an 'activated' ROCK derivative, whose expression is sufficient to induce aberrant rearrangement of the actin cytoskeleton, remained inactive in the focus formation assay [7].

A pyridine-derived smooth muscle relaxant, Y-27632, which can be specifically photo-crosslinked to ROCKs in cell extracts, has recently been identified [8]. This compound exhibits selectivity for ROCK *in vitro* and does not inhibit other kinases tested, including both Rho effectors, such as PRK1/PKN and Citron kinase, and others including protein kinase C and protein kinase A [6,8] (T.I. and S.N., unpublished observations). Y-27632 inhibits processes

Figure 1



The effect of Y-27632 on the actin cytoskeleton. (a) NIH3T3 cells maintained in 5% serum (FCS) were treated with 10 μM Y-27632 for the indicated times; where samples are labelled with two time points, this indicates the effect of treatment for 24 h followed by the subsequent readdition of inhibitor for the time indicated. (b) NIH3T3 cells maintained in 0.5% serum were treated with 10 μM Y-27632 for 2 h or left untreated. (c) NIH3T3 cells maintained in 0.5% serum for 24 h were microinjected with a RhoA.V14 expression plasmid (injected cells are indicated with arrowheads) and incubated for 3 h, after which time they were either left untreated or treated with Y-27632 for a further 2 h, as indicated. The cells were fixed and filamentous actin (F-actin) visualised using TRITC–phalloidin.

previously shown to be ROCK-dependent *in vivo*, such as stress fibre bundling and focal adhesion assembly [8–12], Na^+/H^+ exchange [13,14] and invasion of MM1 tumour cells [15]. Here, we have used Y-27632 and additional ROCK truncation mutants to investigate the role of ROCK in the establishment and maintenance of transformation. Our results show that in NIH3T3 cells ROCK activity is selectively required for both establishment and maintenance of transformation by RhoA and its GEFs Dbl and mNET1, but not for transformation by Src, or for normal growth control in nontransformed NIH3T3 cells.

Results

Chronic treatment with Y-27632 disrupts the cytoskeleton

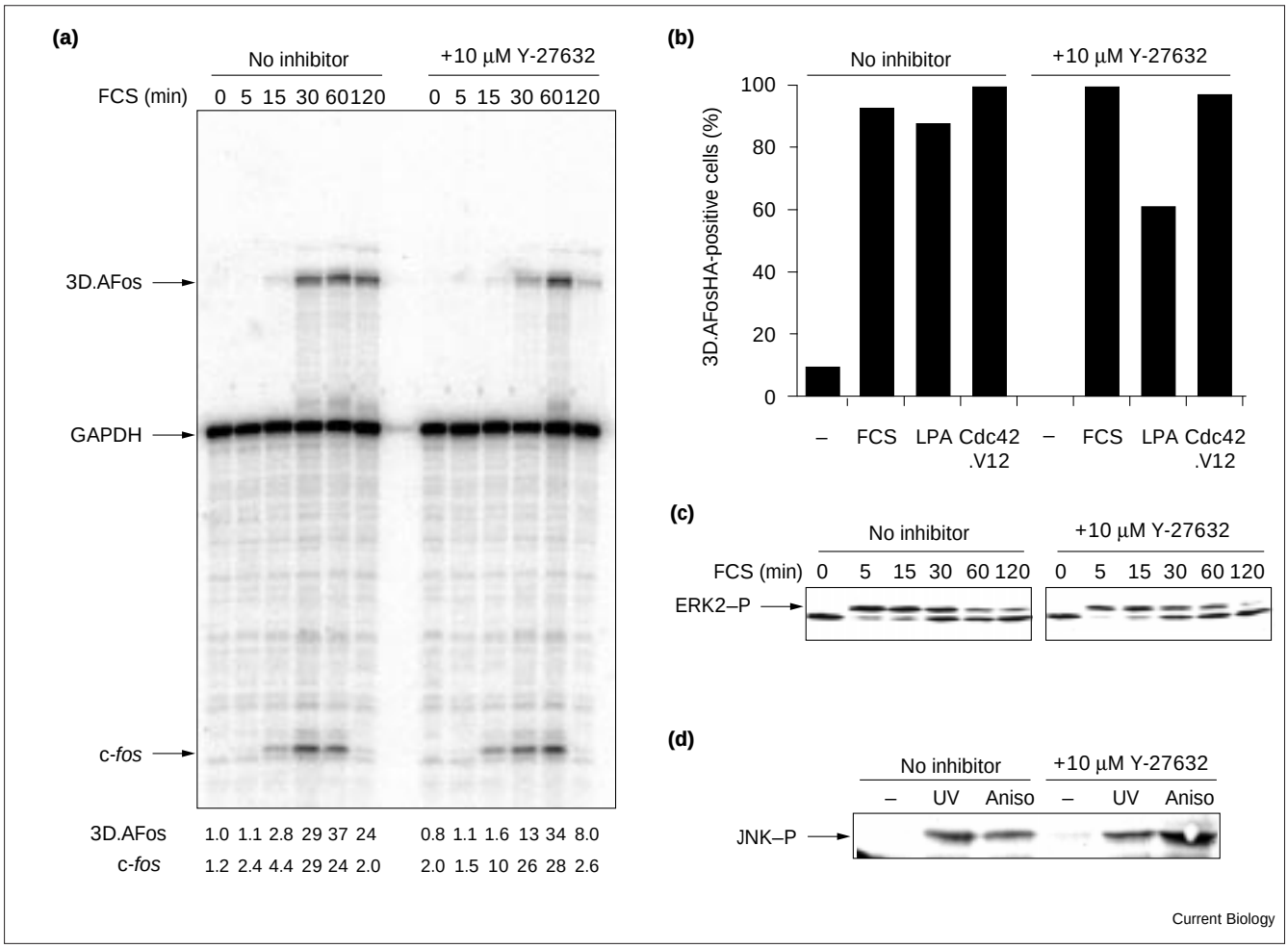
We first used staining with TRITC–phalloidin to assess the effect of repeated treatment with Y-27632 on the actin cytoskeleton of NIH3T3 cells (Figure 1a). In agreement with a previous study in HeLa cells [8], we found that treatment of cells growing in 5% serum with 10 μM Y-27632 was sufficient to substantially disrupt the actin fibre network within 30 minutes. This effect persisted for several hours, although substantial recovery of the cytoskeleton was apparent after 24 hours (Figure 1a). Although re-addition of Y-27632 at this time induced similar changes to those observed following the initial treatment (Figure 1a), repeated treatment with Y-27632 did not affect the cells' growth properties, as will be described below. Cells maintained in 0.5% serum exhibited similar cytoskeletal changes upon Y-27632 administration (Figure 1b) and, as in HeLa cells, this treatment prevented the induction of stress fibres by the activated Rho mutant, RhoA.V14 (Figure 1c). Given that 2 μM Y-27632 had only limited effects whereas 25 μM Y-27632 slightly impaired cell growth (data not shown), we used daily administration of 10 μM Y-27632 in the experiments presented below.

Y-27632 does not inhibit signalling to SRF or to mitogen-activated protein kinases

Studies using RhoA effector-loop mutants and ROCK overexpression suggested that, although deregulated ROCK is able to activate SRF weakly, it is not required for serum-induced signalling to SRF [7]. We used RNase protection assays to test whether Y-27632 treatment impaired serum induction of either a transfected SRF-controlled reporter plasmid, 3D.AFosHA, or the endogenous c-*fos* gene. Activation of neither gene by serum was prevented, although activation of the SRF reporter gene was somewhat reduced (Figure 2a). We also tested the effect of Y-27632 treatment on the induction of SRF activity using a microinjection assay. Y-27632 had no effect on the activation of SRF induced by serum or the activated Cdc42 mutant Cdc42.V12, but slightly reduced SRF activity induced by lysophosphatidic acid (LPA) in this assay (Figure 2b). We did not evaluate the effect of Y-27632 on RhoA.V14-induced SRF activity in these assays because Y-27632 interfered with the synthesis or accumulation of RhoA.V14 expressed from microinjected plasmids although immunoblotting experiments showed that inhibitor treatment did not affect endogenous RhoA levels (data not shown).

We used immunoblotting experiments to monitor the effects of Y-27632 on the activation of cellular mitogen-activated protein (MAP) kinase pathways. Neither the magnitude nor the kinetics of serum-induced activation of the MAP kinase ERK2 were affected by 10 μM Y-27632

Figure 2



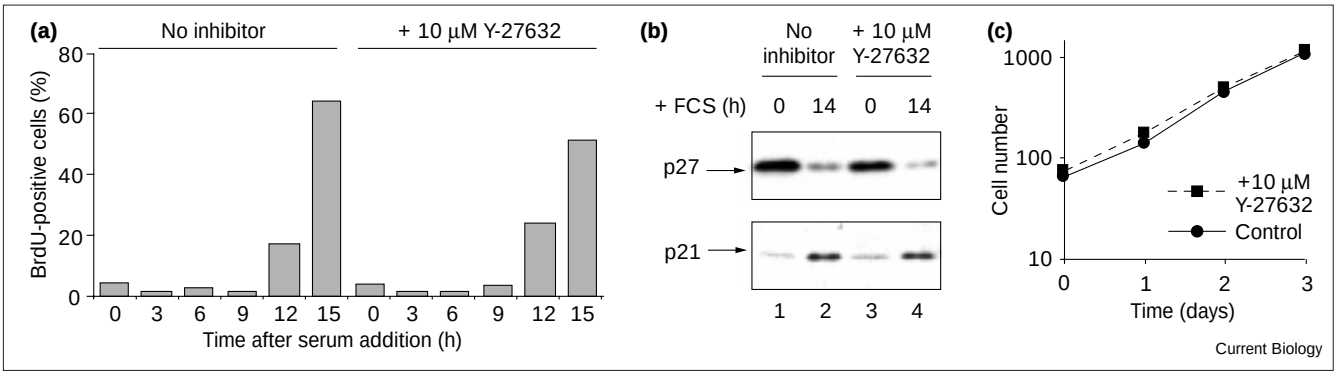
The effect of Y-27632 on nuclear signalling. (a) NIH3T3 cells were transfected with the SRF reporter plasmid 3D.AFosHA, maintained in 0.5% serum for 40 h and stimulated with 15% serum (FCS) for the times indicated. Where indicated, 10 μM Y-27632 was added 30 min before stimulation. RNase protection assays were used to analyse FosHA, mouse c-*fos* and GAPDH mRNA levels; c-*fos* and FosHA mRNA levels normalised to those of GAPDH are shown numerically at the bottom. After 30 min, the serum-induced FosHA mRNA level was 75% of that measured in untreated cells (standard deviation, 24%; $n = 5$). (b) NIH3T3 cells maintained in 0.5% serum were microinjected with the 3D.AFosHA reporter plasmid and stimulated with either 15% serum or 10 μM LPA or by co-injection of the Cdc42.V12 expression plasmid. (c) Cells were stimulated as in (a) and cell lysates were analysed by SDS–PAGE and immunoblotting for ERK2; phosphorylated ERK2 (ERK2–P) is indicated. (d) NIH3T3 cells were maintained in 0.5% serum for 24 h prior to stimulation with 50 ng/ml anisomycin (Aniso) or ultraviolet radiation (UV); 10 μM Y-27632 was added to samples 30 min before stimulation and total cell lysates were analysed by immunoblotting for phosphorylated JNK (JNK–P).

(Figure 2c). Similarly, the activation of the MAP kinase JNK by stress stimuli was unaffected by 10 μM Y-27632 (Figure 2d). Taken together, these data show that ROCK activity is not required for serum-induced signalling to MAP kinase pathways, SRF or c-*fos*.

Y-27632 does not inhibit cell cycle re-entry or progression

We next examined the effects of Y-27632 on cell cycle re-entry and progression. Y-27632 treatment did not prevent re-entry of quiescent NIH3T3 cells into the cell cycle, as assessed by measuring the incorporation of bromodeoxyuridine (BrdU) 20 hours after serum stimulation (data not shown). Pulse-labelling experiments showed that Y-27632 treatment did not significantly affect the kinetics of entry into S phase, which commenced some 12 hours after serum stimulation (Figure 3a), nor did it affect serum-induced changes in p27 and p21 levels (Figure 3b). Although the Rho effector Citron kinase is involved in cytokinesis, 10 μM Y-27632 did not affect the proliferation rate of subconfluent NIH3T3 cells maintained in 5% serum, indicating that Citron kinase must be unaffected (Figure 3c). These data establish that repeated Y-27632 treatments do not affect cell cycle re-entry and progression by nontransformed NIH3T3 cells.

Figure 3



The effect of Y-27632 on cell cycle progression and subconfluent growth. (a) NIH3T3 cells maintained in 0.5% serum for 48 h were stimulated with 15% serum. Where indicated, 10 μM Y-27632 was added 30 min before serum addition. Cells in S phase were detected at various times after stimulation by measuring BrdU incorporation following a 2 h BrdU pulse. (b) Immunoblot analysis of p21 and p27 levels 14 h after cells were serum-stimulated, as described in (a). (c) Subconfluent NIH3T3 cells, maintained in 5% serum with or without daily addition of 10 μM Y-27632, were counted daily.

The involvement of ROCK in focus formation

We next used focus formation assays to assess the effect of Y-27632 on transformation by different oncogenes. RhoA.V14 is weakly active in this assay, but cooperates with the activated Raf protein ΔNRaf; in both cases transformation was strongly inhibited by Y-27632 (Figure 4a; Table 1). The inhibitor also completely prevented focus formation induced by oncogenic forms of two RhoGEFs, Dbl and mNet1, and substantially inhibited focus formation induced by RasR12, an activated Ras protein. In contrast, Y-27632 had no effect on focus formation induced by v-Src (Figure 4a; Table 1). These data indicate that Y-27632 does not non-specifically inhibit focus formation *per se* but selectively inhibits transformation by RhoA and its GEFs.

Ras effector-loop mutants able to activate either only Raf-1, Ral guanine-nucleotide dissociation stimulator (RalGDS) or only phosphatidylinositol 3-kinase (PI 3-kinase) are not transforming when tested alone, but cooperate to cause transformation [16]. The low numbers of foci produced in our cells by these mutant Ras proteins alone or in combination, however, precluded their use in assessing the potential role of ROCK in focus formation (data not shown). We therefore examined the effects of Y-27632 on transformation by combinations of signalling molecules known to mediate Ras signalling. Transformation by ΔNRaf and an activated form of the catalytic subunit of PI 3-kinase, p110K→E, was substantially inhibited by Y-27632 (Table 1). Although Rac1 is a PI 3-kinase target, Y-27632 had no significant effect on cooperation between activated Rac, Rac1.V12, and ΔNRaf in focus formation assays, suggesting that PI 3-kinase but not Rac1 acts upstream of ROCK in transformation by Ras (Table 1; also see Discussion).

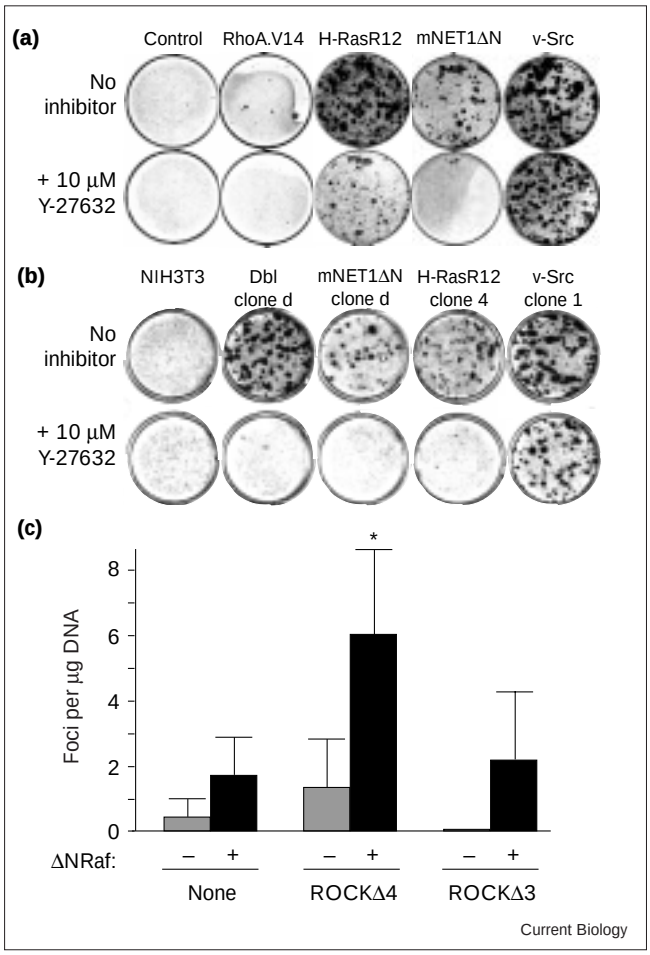
Together with our previous effector-loop mutant data [7], these results strongly implicate ROCK in transformation. We previously found that ROCKΔ3, a ROCK deletion derivative that induces gross disturbances in the actin fibre network, was inactive in focus formation [7]. Recently, however, the ROCK mutant ROCKΔ4, which is less active in cytoskeletal rearrangement, was found to substitute for active RhoA in invasion by MM1 tumour cells [15]. We therefore tested whether ROCKΔ4 could cooperate with activated Raf in transformation. In contrast to ROCKΔ3, this ROCK truncation mutant exhibited weak but significant focus formation activity in NIH3T3 cells, providing direct evidence that deregulated ROCK activity can promote transformation (Figure 4c).

Y-27632 treatment restores normal growth control to cells transformed with RhoAGEFs or H-Ras

The data presented above demonstrate that Y-27632 inhibits the establishment of transformation by activated forms of RhoA, RhoAGEFs and H-Ras, but do not address the potential relevance of ROCK signalling for the maintenance of the transformed state. To investigate the latter, we derived cell lines from foci transformed by RhoA, Dbl, mNET1ΔN, H-RasR12 and v-Src and examined the effects of Y-27632 on their growth properties. Each of eight lines examined had doubling times up to 20% shorter than the parental NIH3T3 cells, but their growth rates were unaffected by Y-27632 treatment (data not shown).

We tested whether Y-27632 would prevent the formation of secondary foci upon reseeding of transformed cells among nontransformed NIH3T3 cells. One thousand transformed cells were plated with 30,000 parental nontransformed

Figure 4



The involvement of ROCK in transformation. (a) NIH3T3 cells were transfected with the indicated expression plasmids and maintained in 5% serum for 14 days with or without daily addition of 10 μM Y-27632. (b) Cells from the indicated cell lines were seeded among nontransformed NIH3T3 cells and maintained in 5% serum for 9 days with or without daily addition of 10 μM Y-27632. (c) NIH3T3 cells were transfected with the indicated ROCK expression plasmids with and without a plasmid encoding activated Raf (ΔNRaf), as indicated, and maintained in 5% serum for 14 days. The asterisk indicates significance compared to mock transfection at $p = 0.007$, significance compared to the ΔNRaf at $p = 0.02$ (Student *t* test).

NIH3T3 cells in 35 mm dishes, and maintained for nine days in 5% serum with or without 10 μM Y-27632. Cell lines transformed by Dbl, mNET1ΔN, or H-RasR12 formed between 10 and 72 foci per thousand cells, whereas two Src-transformed cell lines formed more than 100 foci per thousand cells. In contrast, RhoA.V14-transformed cells formed secondary foci only very inefficiently. Thus, Y-27632 treatment blocked the ability of cells transformed by Dbl, mNET1ΔN, H-RasR12 or RhoA.V14 to form secondary foci, but had no effect on Src-transformed cells (Figure 4b; Table 2). The RhoA-transformed cell lines were not studied further.

Table 1

Focus formation assays.

Plasmid	Number of foci per 0.5 μg DNA			
	No inhibitor		+ 10 μM Y-27632	
Control (4)	0.4	(0.5)	0	
ΔNRaf (2)	1	(1.4)	0	
RhoA.V14 (3)	6	(2)	0.3*	(0.6)
RhoA.V14 + ΔNRaf (3)	18	(8.1)	2.6	(2.8)
Dbl (3)	5.7	(0.5)	0*	
mNET1ΔN (4)	30	(12.8)	0*	
H-RasR12 (3)	208	(39)	60*	(18)
v-Src (3)	465	(96)	465	(120)
Rac1.V12 (6)	1.4	(1.5)	0	
Rac1.V12 + ΔNRaf (6)	27.5	(7.9)	21	(12.7)
p110K→E (1)	2		1	
p110K→E + ΔNRaf (2)	14	(2.8)	3	(1.4)

NIH3T3 cells were transiently transfected with the indicated expression plasmids and maintained in 5% serum with or without daily addition of 10 μM Y-27632. The medium was changed every two days. After 14 days, cells were stained with crystal violet and foci larger than 1.5 mm in diameter were counted. The number of independent experiments and standard deviations are indicated in parentheses next to plasmid names and average number of foci, respectively. An asterisk (*) indicates significance at 1% level (Student *t* test).

Although the inhibition of secondary focus formation by Y-27632 is consistent with the restoration of cell cycle control by contact inhibition, it might also arise as a consequence of increased apoptosis at high cell density. We therefore tested directly whether Y-27632 treatment restores cell cycle exit at confluence. Transformed cells were seeded in 35 mm dishes and maintained in 5% serum for eight days either in the absence or presence of 10 μM Y-27632. The cell densities reached by each cell line were then compared (Figure 5a), and the proportion in cycle estimated by pulse-labelling with BrdU for the 24 hours prior to harvest (Figure 5b). Parental NIH3T3 cells grew to about 650 cells per mm^2, with approximately 10% labelling with BrdU and, as expected, Y-27632 treatment had no effect on either value (Figure 5). All the transformed cell lines grew to significantly higher densities than the nontransformed parental cells (Figure 5a), and, at these densities, retained a greater proportion of BrdU-positive cells (Figure 5b). In the presence of Y-27632, both Dbl and mNET1ΔN cell lines grew to a density similar to that of the parental NIH3T3 cells, and the proportion of cells in cycle was reduced in parallel (Figure 5b). In striking contrast, Y-27632 had no effect on the saturation density or cycling of the two Src cell lines (Figure 5a,b). In the case of the Ras-transformed cells, however, Y-27632 treatment caused a substantial reduction in saturation density, but the number of cells in cycle at this density remained substantially greater than for the parental cells (Figure 5a,b). Taken together, these results show that Y-27632 restores growth control to cells transformed by RhoAGEFs but not to cells transformed by Src.

Table 2

Secondary focus formation assays.

Cell line	Number of foci per 1,000 cells			
	No inhibitor		+ 10 μM Y-27632	
NIH3T3 (3)	0		0	
RhoA-2 (3)	7	(6.5)	0	
RhoA-4 (3)	1	(1)	0	
Dbl-d (3)	72	(15)	3.6	(4.7)
Dbl-e (3)	10	(1.5)	0	
mNET1-d (3)	32	(22)	0	
mNET1-e (3)	23	(12)	0.7	(1.2)
Ras-2 (3)	36	(22)	1.3	(1.5)
Ras-4 (3)	28	(19)	2	(3.5)
Src-1 (2)	121	(44)	126	(44)
Src-4 (2)	316	(63)	322	(105)

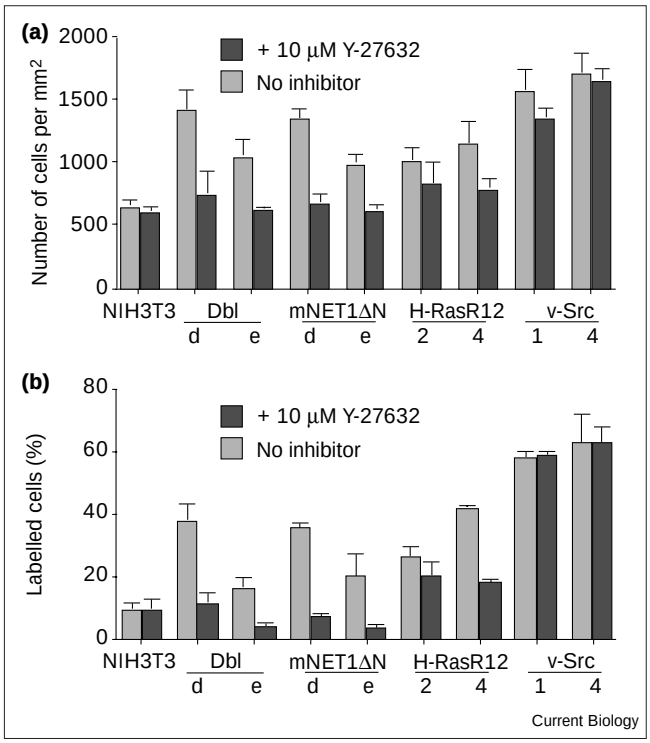
One thousand cells from each of the indicated cells lines were plated among 30,000 nontransformed parental NIH3T3 cells and maintained in 5% serum with or without daily addition of 10 μM Y-27632. The medium was changed every two days. After 9 days, cells were stained with crystal violet and foci larger then 1 mm in diameter were counted. The number of independent experiments and standard deviations (or half range) are indicated in parentheses next to plasmid names and average number of foci, respectively.

The effect of Y-27632 on transformed cell morphology

RhoA has been implicated in the formation of stress fibres, focal adhesions, and cadherin-mediated cell–cell adhesion. We therefore examined the effect of Y-27632 on the cytoskeleton of both parental and transformed NIH3T3 cells to determine whether its effects on growth control correlated with its effect on any aspects of cell morphology. NIH3T3 cells grown in 5% serum with 10 μM Y-27632 exhibited reduced size and abundance of focal adhesions, as judged by immunostaining for paxillin and integrin β1; their predominantly peripheral location reflected the disorganisation of actin fibres (Figure 6a). Similar effects were observed in the transformed cell lines (data not shown). Immunoblotting with anti-phosphotyrosine antibodies showed that Y-27632 treatment affected neither the basal level of tyrosine phosphorylation nor that induced by serum-stimulation of starved cells. Moreover, Y-27632 did not affect the spectrum of tyrosine-phosphorylated proteins detectable at confluence in the various transformed cell lines (data not shown). The cadherin-associated protein β-catenin is localised to sites of cell–cell contact, which are distinct from focal adhesions (Figure 6a). Treatment with 10 μM Y-27632 reduced the level of β-catenin staining at these sites, but this occurred both in the parental NIH3T3 cells and the transformed cell lines with the exception of those transformed by Src, in which β-catenin did not localise to sites of cell contact (Figure 6a and data not shown).

We also examined the effect of Y-27632 on filamentous actin (F-actin) structures in normal and transformed NIH3T3 cells. The morphology of the transformed cell lines was markedly different from that of the parental NIH3T3 cells: Dbl- and mNET1ΔN-transformed cells had large lamellipodia-like structures with relatively few actin fibres, which were not sensitive to Y-27632 (Figure 6b; compare with Figure 1a). In contrast, both the H-RasR12- and Src-transformed cells had fewer actin fibres than the parental cells, and the Src-transformed cells also possessed F-actin patches; however, neither structure was sensitive to Y-27632. Thus, the effects of Y-27632 on cell cycle control cannot be correlated with gross effects on F-actin structures or focal adhesions.

Figure 5

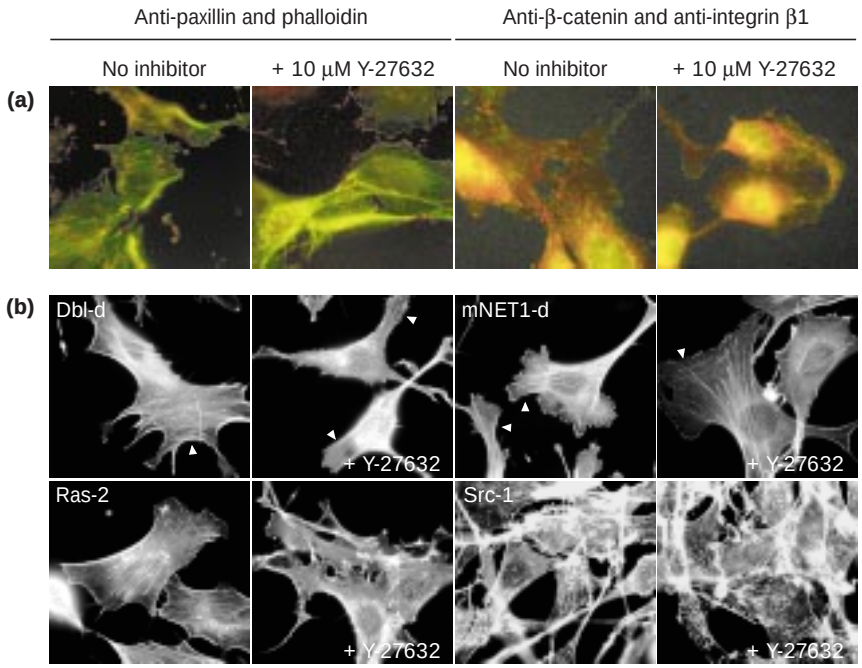


Y-27632 restores growth control to transformed cell lines. 30,000 cells of the indicated cell line were seeded in 35 mm wells and maintained in 5% serum for eight days with or without 10 μM Y-27632; fresh Y-27632 was added daily and the medium was changed every two days. 24 h prior to harvesting BrdU was added to the medium and upon harvesting both the total number of cells per mm^2 and the percentage of BrdU-positive cells were calculated. Black and grey bars represent cells treated with and without 10 μM Y-27632, respectively. The data shown are the average of two or three independent experiments ± half range or standard error. (a) Effect of Y-27632 treatment on total cell number per mm^2. (b) Effect of Y-27632 treatment on the percentage of BrdU-labelled cells at confluence.

Inhibition of anchorage-independent growth by Y-27632

Finally, we tested whether Y-27632 treatment could inhibit anchorage-independent growth. Colony formation in soft agar by parental NIH3T3 cells was very inefficient and was insensitive to Y-27632. In contrast, the cells transformed by Dbl, mNET1ΔN, or H-RasR12 grew in soft

Figure 6



The effect of Y-27632 on the cytoskeleton. **(a)** NIH3T3 cells maintained in 5% serum were fixed and stained for paxillin (red) and F-actin (coumarin–phalloidin; green; left panels) or integrin β1 (green) and β-catenin (red; right panels). Where indicated, cells were treated with Y-27632 30 min before fixation. **(b)** Transformed NIH3T3 cell lines maintained in 5% serum were stained with TRITC–phalloidin to visualise F-actin. Where indicated, cells were treated with Y-27632 30 min before fixation.

agar. Colony formation by the Dbl- and mNET1ΔN-transformed cell lines was substantially inhibited by Y-27632, however. Both Ras-transformed cell lines were also sensitive to Y-27632, although Ras-2 cells were only partially affected. Colony formation by three Src-transformed cell lines tested was both efficient and not significantly inhibited by Y-27632 (Table 3).

Taken together, our data suggest that deregulation of ROCK activity mediates both loss of contact inhibition and anchorage-independent growth in NIH3T3 cells transformed *in vitro* by RhoAGEFs or oncogenic H-Ras. To test whether cell lines selected for altered growth properties *in vivo* also exhibited dependence on ROCK activity, we tested whether Y-27632 would inhibit anchorage-independent growth of four human epithelial cell lines derived from colorectal tumours, each of which contains an activating Ras mutation. Of the cell lines tested, HCT15, HCT116 and LS174T cells formed colonies within 17 days, while SW620 cells did not. Treatment with 10 μM Y-27632 dramatically reduced the ability of HCT116 and LS174T cells to grow in soft agar but had little effect on the growth of HCT15 cells (Table 3). Y-27632 did not affect the growth rate of any of these cell lines. Moreover, as with the NIH3T3-derived cell lines, although Y-27632 treatment brought about changes in the distribution of cadherin, β-catenin and paxillin, these changes occurred regardless of whether cell growth was affected by the compound (data not shown). These data suggest that ROCK activity can also be important for the transformed phenotype of cells selected during tumorigenesis *in vivo*.

Discussion

In this work, we investigated the role of ROCK kinases in transformation by RhoA and its regulators, using a specific ROCK inhibitor, Y-27632, and truncated derivatives of ROCK. We found that Y-27632 had no effect on the growth of nontransformed cells but selectively blocked transformation by RhoA, its GEFs and Ras. Contact inhibition in RhoGEF-transformed cells was restored by treatment with Y-27632, which also inhibited the anchorage-independent growth of RhoGEF-transformed cells and two epithelial tumour cell lines. Moreover, a truncated ROCK derivative, ROCKΔ4, exhibited weak activity in focus-formation assays with activated Raf. These results strongly suggest that ROCK activity is important for the establishment and maintenance of transformation, and are consistent with our previous demonstration that transformation by activated RhoA effector-loop mutants correlates with their ability to bind ROCK [7]. Although we cannot exclude the possibility that Y-27632 acts by inhibiting targets other than ROCK, several lines of evidence argue that this is not the case. Previous studies have demonstrated the selective inhibition of ROCK-dependent processes by Y-27632, including the formation of focal adhesions and stress fibres, Na^+/H^+ exchange, and invasion by MM1 tumour cells [8,13–15]. Biochemical studies have shown that, at the concentration

Table 3

Y-27632 inhibits anchorage-independent growth.

Cell line	Colony formation			
	No inhibitor		+ Y-27632	
	Expt 1	Expt 2	Expt 1	Expt 2
NIH3T3	5	0	0	5
Dbl-d	63	65	0	5
Dbl-e	33	4	2	0
mNET1-d	15	71	2	10
mNET1-e	20	18	5	5
Ras-2	49	46	20	19
Ras-4	24	17	4	6
Src-1	420	143	421	80
Src-4	409	487	452	240
NIH3T3	0	0	0	1
Src-1	308	312	323	319
Src-2	155	140	140	72
SW620	0	0	0	0
HCT15	316	135	207	142
HCT116	39	57	0	0
LS174T	296	85	56	11

Five thousand parental NIH3T3 cells or transformed NIH3T3 cell lines were maintained in suspension in soft agar in DME/10% serum with or without 18 μM Y-27632; medium and inhibitor were changed three times per week. Human colorectal tumour cells (1000 cells per cell line) were maintained in suspension in soft agar in DME/10% serum with or without daily administration of Y-27632 to 10 μM. Colonies visible to the naked eye (~100 cells) after neutral-red staining were counted after 24 days (NIH3T3 derivatives) or 18 days (tumour cell lines). The results of two independent experiments for each group of cell lines are shown.

used in our experiments, Y-27632 does not inhibit other RhoA effector kinases such as Citron kinase and PKN [6,8] (T.I. and S.N., unpublished observations), and that Y-27632 can be specifically photo-crosslinked to ROCK in cell extracts [8]. Finally, we found that Y-27632 treatment did not impair activation of the ERK and JNK MAP kinase pathways.

Our studies with RhoA effector-loop mutants suggested that ROCK binding is not required for SRF activation [7]. Although overexpression of truncated ROCK proteins does potentiate SRF activity [7,17], this is RhoA-dependent, and we therefore think that it arises either as a result of aberrant cytoskeletal rearrangements or through cooperation with other Rho effectors [7]. The failure of Y-27632 to significantly impair SRF activation at concentrations sufficient to disrupt the cytoskeleton suggests that ROCK is not an obligatory intermediate in signalling to SRF, but may affect the efficiency of signal transmission. Activity of the c-*fos* promoter is dependent on both SRF and additional signal-regulated transcription factors including the ternary complex factors, which are regulated by MAP kinases. Y-27632 treatment did not inhibit either ERK activation or c-*fos* induction following serum stimulation.

Cell cycle progression is dependent on adhesion to matrix and is inhibited by cell–cell contact (for recent reviews, see [18,19]). Our data suggest that constitutive RhoA activation either mimics signals generated by cell anchorage or inhibits those occurring at confluence. Although the nature of such signals remains unclear, ROCK is intimately involved in these processes. ROCK is required for signal-induced assembly of focal adhesion complexes in several cell types [9–12,20], and has also been implicated in Rho-dependent focal adhesion assembly following integrin–fibronectin interaction, possibly through its effects on the NHE1 Na^+/H^+ exchanger [13,14,21]. In addition, RhoA also controls cadherin-mediated cell–cell adhesion [22–25] and insoluble matrix assembly [26,27]. Given that Src-family kinases are associated with focal adhesions, the lack of sensitivity of Src transformation to Y-27632 is consistent with their acting downstream of ROCK in transformation. We were unable to identify Y-27632-induced alterations in tyrosine phosphorylation, F-actin structures or β-catenin staining specific to transformed cells sensitive to the compound, although this may merely reflect the low resolution of our methods. It will be interesting to examine whether transformation alters the phosphorylation state of ROCK substrates such as myosin light chain (MLC) [11], the MLC phosphatase [28], the Na^+/H^+ exchanger NHE1 [14], and ezrin–radixin–moesin (ERM) proteins [29].

Studies of the role of Rho signalling in cell cycle transit have revealed links between Rho-mediated signalling and accumulation of the CDK inhibitors p21 and p27 [2–5]. In Swiss 3T3 cells expressing activated Ras, RhoA signalling inhibits the accumulation of p21 [3]. In contrast, in our Y-27632-sensitive transformed cells, whether transformed by RhoGEFs or Ras, p21 levels were either unchanged or actually decreased following the inhibition of ROCK (E.S., unpublished observations). We also found that the inhibition of ROCK by Y-27632 did not affect changes in p21 and p27 levels occurring during cell cycle re-entry of serum-stimulated NIH3T3 cells. Further work is necessary to clarify the connection between RhoA signalling, CDK inhibitor accumulation and transformation.

Both RhoA and Rac1 are required for transformation by Ras (reviewed in [1]), but although Y-27632 significantly inhibited focus formation by activated Ras, we were unable to determine whether a specific Ras effector pathway is involved. Focus formation assays with activated forms of PI 3-kinase and Rac1 suggested that ROCK activity is specifically required for transformation by PI 3-kinase but not by Rac1; PI 3-kinase may thus control Rho activity independently of Rac1, as suggested by others [30,31]. Curiously, cells transformed by Ras, but not those transformed by NET1 or Dbl, failed to efficiently exit the cell cycle at confluence in the presence of Y-27632; preliminary experiments, in which the TUNEL technique was used to monitor DNA fragmentation,

indicate that these cells exhibit increased apoptosis (E.S., unpublished observations).

It is intriguing that Y-27632 also significantly inhibited the anchorage-independent growth of two out of the four epithelial tumour cell lines examined. The altered growth properties of these cells are at least partly the result of mutational changes selected *in vivo*, so our results demonstrate that the involvement of RhoA-controlled signalling in transformation is not limited only to *in vitro* models using cultured cells. Moreover, a recent study indicated that ROCK may also play a role in Rho-dependent tumour cell invasion [15]. It is therefore likely that at least some tumour cells may exhibit sensitivity to inhibition of ROCK kinases *in vivo*, and drugs that target the ROCK kinases might be useful as anti-tumour agents.

Conclusions

The ROCK family of RhoA effector kinases is required for the establishment and maintenance of transformation, both in RhoA-mediated transformation of NIH3T3 fibroblasts and in a subset of cell lines derived from colorectal tumours. The failure of Y-27632 to inhibit the growth of normal nontransformed cells, coupled with its ability to restore growth control at confluence, suggests that constitutive Rho activation may contribute to transformation either by mimicking growth promoting signals generated by cell anchorage or by blocking growth-inhibitory signals induced by confluence. Use of inhibitors of ROCK activity may thus provide novel therapeutic strategies.

Materials and methods

Cell culture, extract preparation and analyses

NIH3T3 and human colorectal tumour cell lines were gifts from Chris Marshall. Cells were routinely maintained in DMEM with donor calf serum (DCS; Gibco). Y-27632 was from Yoshitomi Pharmaceutical Industries Ltd. Cells were stimulated with 10 μM lysophosphatidic acid (Sigma), 15% DCS, 100 ng/ml anisomycin or UV irradiation, as described [32]. Immunoblotting was by standard methods. Antibodies were as follows: pan-ERK, Transduction Laboratories; phospho-JNK, Santa Cruz; p21, Santa Cruz; p27, Santa Cruz; β-catenin (VB2) and integrin β1 (9EG7), gifts from Fiona Watt, ICRF; paxillin, Transduction Laboratories. TRITC–phalloidin was from Sigma. NIH3T3 cells were transfected using DEAE dextran or lipofectamine; RNase protection assays were performed as described [32]. Microinjection assays were performed as described [7]; Y-27632 was added immediately after injection.

Immunofluorescence

Cells were fixed with fresh 4% formaldehyde in PBS for 15 min followed by extraction in 0.3% Triton X-100, except for focal adhesion staining where the cells were simultaneously fixed and permeablised using 4% formaldehyde and 0.2% Triton X-100. Antibodies were diluted 1:100 in PBS + 8% calf serum and the cells were washed four times in PBS after antibody incubations and prior to mounting. Images were obtained using a Zeiss Axiovert microscope and Smart Capture system (Vysis) and processed as PICT files using Adobe Photoshop 3.0.

Focus formation assays

Focus formation assays were performed as described previously [7], except that following transfection cells were split into two 6 cm dishes, one of which was treated with 10 μM Y-27632 daily. For secondary focus formation assays, 1000 cells were plated into 30,000 parental NIH3T3 cells in a 35 mm well. The cultures were maintained for 10 days in DMEM + 5% DCS, which was changed three times a week; 10 μM Y-27632 was added daily. Cells were harvested by washing once with PBSA, followed by staining with 0.1% crystal violet (Sigma) in 10% methanol and PBSA. Foci larger than 1 mm in diameter were counted.

Cell cycle assays

Following maintenance in 0.5% serum for 48–60 h, NIH3T3 cells were stimulated with 15% DCS; p21 and p27 levels were analysed by immunoblotting and cells in S phase were detected by 2 h pulse-labelling with BrdU (Boehringer Mannheim). The growth rate of subconfluent cells maintained in 5% serum was measured by counting the number of cells daily over a 4 day period. To evaluate cell cycle exit at confluence approximately 30,000 cells were seeded in a 35 mm well, maintained in 5% serum and 10 μM Y-27632 for 8 days, and pulse-labelled with BrdU for 24 h prior to staining for BrdU and DNA (using Hoechst 33258).

Soft agar assays

Bottom agar (2ml; 0.9% ultra-pure LMP agarose (BRL life technology), 1 × DMEM, 10% DCS) was poured into a 35 mm well and allowed to set. Either 1000 or 5000 cells were mixed with top agar (2 ml; 0.5% LMP agarose, 1 × DMEM, 10% DCS) at 40°C and the mixture poured onto the bottom agar. Cells were maintained for 21 days, with 200 μl of DMEM + 10%DCS added three times per week. For visualisation, colonies were incubated with 1 ml 0.01% neutral red (Sigma) in PBSA for 1 h. Colonies visible to the naked eye (~100 cells) were counted.

Acknowledgements

We thank Yoshitomi Pharmaceuticals Ltd (Saitama, Japan) for supplying Y-27632. We thank Art Alberts, Chris Marshall and members of the Watt and Downward laboratories for cells, reagents and experimental protocols, and Denise Sheer for use of her microscope. We thank Art Alberts, Chris Marshall, Fiona Watt and members of the Transcription Laboratory for helpful discussions and comments on the manuscript.

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