Expression of BRG1, a human SWI/SNF component, affects the organisation of actin filaments through the RhoA signalling pathway

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Summary

The human BRG1 (brahma-related gene 1) protein is a component of the SWI/SNF family of the ATP-dependent chromatin remodelling complexes. We show here that expression of the BRG1 protein, but not of an ATPase-deficient BRG1 protein, in BRG1-deficient SW13 cells alters the organisation of actin filaments. BRG1 expression induces the formation of thick actin filament bundles resembling stress-fibres, structures that are rarely seen in native SW13 cells. BRG1 expression does not influence the activity state of the RhoA-GTPase, which is involved in stress-fibre formation. We find that RhoA is equally activated by stimuli, such as serum, in BRG1-expressing cells, ATPase-deficient BRG1-expressing cells and native SW13 cells. However, the activation of RhoA by lysophosphatidic acid and serum does not trigger the formation of stress-fibre-like structures in SW13 cells. Activation of the RhoA-GTPase in BRG1-expressing cells induces stress-fibre-like structures, indicating that the BRG1 can couple RhoA activation to stress-fibre formation. At least two downstream effectors are involved in stress-fibre formation, Rho-kinase/ROCK and Dia. BRG1 expression, but not the expression of the ATP-deficient BRG1, increases the protein level of ROCK1, one form of the Rho-kinase/ROCK. That this is of importance is supported by the findings that an increased Rho-kinase/ROCK activity in SW13 cells, obtained by overexpressing wild-type ROCK1 and ROCK2, induces stress-fibre formation. No specificity between the two Rho-kinase/ROCK forms exists. Our results suggest that the BRG1 protein affects the RhoA pathway by increasing the protein level of ROCK1, which allows stress-fibre-like structures to form.

Key words: BRG1, SWI/SNF, Actin filament organisation, RhoA, Rho kinases

Introduction

The BRG1 protein (brahma related gene 1 or SNF2β) (Khavari et al., 1993; Randazzo et al., 1994; Chiba et al., 1994) is the functional ATPase subunit of the mammalian SWI/SNF family of ATP-dependent chromatin remodelling complexes (Kwon et al., 1994; Wang et al., 1996a; Zhao et al., 1998; Xue et al., 2000; Sif et al., 2001). It is closely related to the mammalian brahma protein (BRM or SNF2α) (Muchardt and Yaniv, 1993; Chiba et al., 1994), which also functions as an ATPase in separate SWI/SNF complexes (Wang et al., 1996a; Wang et al., 1996b; Sif et al., 2001). The BRG1 and BRM proteins are subunits of separate complexes that share most subunits. Further purification and characterisation have revealed three different types of human SWI/SNF complex (Xue et al., 2000; Sif et al., 2001). The BRG1 protein is present in the BAF (SWI/SNF complex A) and PBAF (SWI/SNF complex B) complexes, and the BRM is present in one SWI/SNF complex. Each of these complexes has some unique subunits and differs in chromatin remodelling activity of mononucleosomes and nucleosomal arrays (Xue et al., 2000; Sif et al., 2001). The different cellular functions of these distinct complexes are still unknown. Evidence is now emerging that the mammalian SWI/SNF complexes are involved in transcriptional regulation. Direct involvement of BRG1-containing complexes has been shown for the switch to the adult form of the β-globin gene (Armstrong et al., 1998; Lee et al., 1999). Furthermore, a wide gene screen to find genes that are dependent on the mammalian BRG1-containing complexes has identified approximately 80 genes that are activated or repressed (Liu et al., 2001). Both of the BRG1 and the BRM proteins are close orthologues of the yeast SWI2/SNF2, which is the central ATPase in the yeast SWI/SNF complex (Cairns et al., 1994; Peterson et al., 1994), and to STH1 of the RSC complex (Cairns et al., 1996). The yeast SWI/SNF complex is clearly involved in transcription by remodelling the nucleosomal structure (reviewed by Workman and Kingston, 1998; Wolffe and Hayes, 1999), and it is required for the activation and repression of many genes (Holstege et al., 1998; Sudarsanam et al., 2000). The function of the yeast RSC is less clear, but it is essential for viability (Cairns et al., 1996). Nevertheless, genomic screens suggest a role in transcriptional regulation (Angus-Hill et al., 2001). RSC localises to the centromere of mitotic chromosomes suggesting that it is involved in mitosis (Tsuchiaya et al., 1998). The human PBAF contains a unique subunit, BAF180 (which is

homologous to RSC subunits), and localises to mitotic chromosomes (Xue et al., 2000).

Several studies in BRG1- and BRM-deficient cell lines, such as the human adrenal adenocarcinoma cell line SW13 and a human cervix carcinoma cell line C33A (Muchardt and Yaniv, 1993; Wang et al., 1996b; Wong et al., 2000), have shown that the BRG1 and the BRM proteins are involved in the regulation of proliferation, differentiation and cell growth. Expression of the BRG1 or the BRM protein in SW13 cells induces growth arrest and a change in morphology to flat cells (Dunaief et al., 1994; Strober et al., 1996; Shanahan et al., 1999). These effects have been linked to an interaction with the retinoblastoma protein (Rb) (Dunaief et al., 1994; Singh et al., 1995; Strober et al., 1996), an important cell cycle control protein and tumour suppressor (for reviews, see Knudsen et al., 1998; Dyson, 1998). It has been suggested that the association of Rb with the BRG1 or BRM protein is needed for full repression of the transcription of genes under the control of the E2F transcription factor (Trouche et al., 1997). BRG1 expression in SW13 cells reduces the level of proteins such as cdc2, cyclin A and cdk2, which are responsible for cell cycle progression (Zhang et al., 2000). These cell cycle proteins are also reduced in C33A cells if BRG1 is expressed together with the Rb-protein (Strobeck et al., 2000; Zhang et al., 2000). Furthermore, the BRG1 protein introduced into SW13 cells interacts with the G1/S-phase-specific cyclin E, which inactivates the BRG1 protein by phosphorylation (Shanahan et al., 1999). The phosphorylation of BRG1 excludes it from chromatin in late G2/M-phase and inactivates the SWI/SNF complex (Muchardt et al., 1996; Sif et al., 1998). The BRM protein is also phosphorylated, which causes it to be not only excluded from chromatin but also degraded (Muchardt et al., 1996; Muchardt et al., 1998). Further evidence for a role of SWI/SNF complexes in the control of proliferation is the finding that BRM knockout mice are larger than normal (Reyes et al., 1998). The BRG1 protein is essential for early development and *Brg1* null homozygots die before the preimplantation stage (Bultman et al., 2000).

Many cellular processes are involved in cell cycle progression. The morphology of cells changes during the cell cycle, a process that involves rearrangement of the actin filaments (reviewed by Van Aelst and D'Souza-Schorey, 1997). Actin filaments are responsible for the shape of differentiated cells, spreading, anchorage, cell movement, and cytokinesis (reviewed by Van Aelst and D'Souza-Schorey, 1997; Small et al., 1999). Actin filaments are highly dynamic structures, and rearrangements are essential for these cellular processes. Therefore, regulation of the organisation of actin filaments is highly controlled and several signal transduction pathways affect the actin filament organisation. The family of small Rho GTPases, which includes RhoA, Rac and cdc42, mediates many of the external signals and the effects of the substratum by switching to the active GTP-bound state upon stimulation (reviewed by Hall, 1998; Small et al., 1999; Bishop and Hall, 2000). Most studies have been conducted on fibroblasts, in which the activation of individual Rho family members results in different actin filament organisation; RhoA induces stress-fibre formation and the assembly of focal contacts, Rac induces lamellipodia, and cdc42 induces filopodia (Small et al., 1999; Hall, 1998). The activated Rho-GTPases bind to and activate in turn downstream effectors. The formation of stress fibres in fibroblasts requires at least two classes of the RhoA effectors, Dia and Rho associated kinase (Rho-kinase/ROCK) (reviewed by Bishop and Hall, 2000).

In this study we investigate the role of the BRG1 protein in the organisation of actin filaments in SW13. BRG1 expression induces morphological changes in SW13 cells and we present evidence that BRG1 expression induces the formation of thick actin filament bundles in the cell bodies (stress-fibre-like structures) by affecting the RhoA signalling pathway. We present evidence that the RhoA GTPase is not directly affected, but BRG1-expression results in an elevated protein level of ROCK1, one of the isoforms of Rho-kinase/ROCK. The balance between Rho-kinases/ROCK and Dia proteins is important for proper stress-fibre formation in fibroblasts (Watanabe et al., 1999), and we suggest that the elevated ROCK1 protein level affects this balance, coupling RhoA activation to stress-fibre formation.

Materials and Methods

Chemicals

Y-27632 was a gift from the Welfide Corporation (formerly Yoshitomi Pharmaceutical Industries). Lysophosphatidic acid (LPA) and Trichostatin A (TSA) were purchased from Sigma.

Vector constructs and DNA cloning

Standard molecular biology cloning techniques were used for all cloning steps. All vector constructs were verified by extensive sequencing.

The pORSVI-BRG1 expression vector was constructed by ligating a *Sal*I/*Spe*I fragment from pSVhSNF2β (Chiba et al., 1994) into the *Spe*I/*Xho*I sites of pORSVI. pORSVI-BRG1-K798R was constructed by exchanging a *Xho*I/*Bgl*II fragment with the same fragment taken from the expression vector pBJ5-BRG1-K798R (Khavari et al., 1993).

A specific BRG1 antisense vector was constructed by ligating a 1625 base-pair, blunt-ended, N-terminal PCR fragment from the brg1 cDNA, in the antisense direction into the *Eco*RV site of the pORSVI expression vector of the LacSwitch system (Stratagene).

PCR primer 1: ATG CAT GCG GGA TCC CAG ACC CAC CCC TGG GCG GAA CTC C.

PCR primer 2: CGT ACG TAG GGG ATC CCC CTT CTG GTC GAT GAG CTT GCG GTA CCC C.

GST-Rhotekin-RBD (rho-binding-domain) was constructed by ligating a PCR-generated fragment of the human rhotekin cDNA, corresponding to amino acids 24-106, into the *Eco*RI/*Xho*I sites of the pGEX-4T-1 vector (Amersham/Pharmacia), generating an in-frame fusion protein. The human rhotekin fragment corresponds to amino acids 8-89 of the mouse rhotekin. cDNA was generated from total RNA isolated from untreated SW13 cells using MMLV reverse transcriptase and random primers (Life Technologies) according to the manufacturer's instructions.

PCR primer 1: ATGC ATGC GAA TTC GCA CAC CCC TGC CTT CCT CTC.

PCR primer 2: ATGC ATGC CTC GAG GCT TGT CTT CCC CAG CAC CTG.

Cell cultures

SW13 cells were cultivated in 5% fetal calf serum in 50% Dulbecco's modified Eagle's medium (DMEM)/ 50% Ham's F12 medium supplemented with 50 μg/ml each of streptomycin and penicillin.

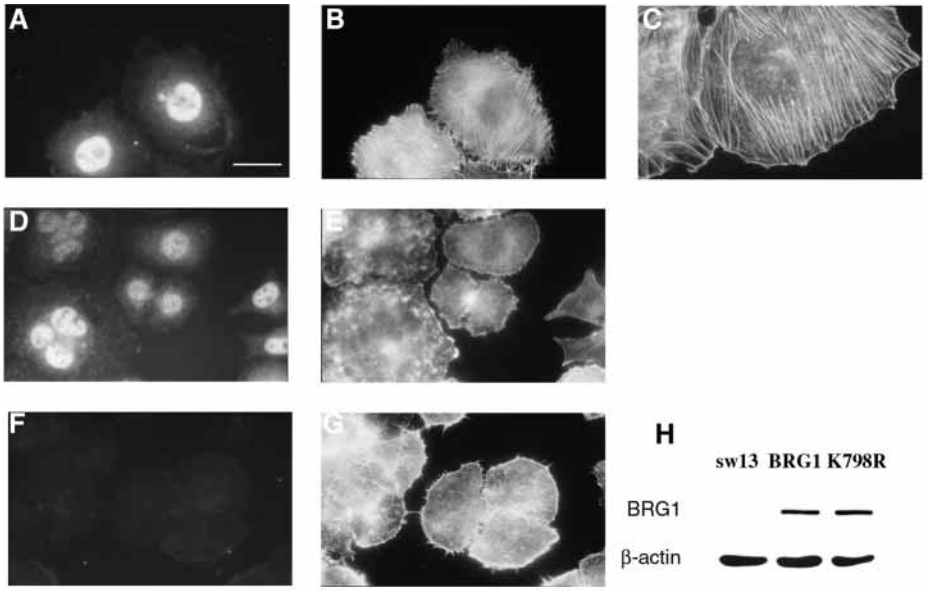

Fig. 1. The formation of stress-fibre-like structures in BRG1-expressing clones. Immunofluorescence images show a BRG1-expressing clone of SW13 cells seeded for 2 days before being fixed and stained with affinity-purified anti-BRG1 antibodies against the N-terminal part of the protein visualised by TRITC-conjugated secondary anti-rabbit antibodies (A) and FITC-conjugated phalloidin (B) to visualise actin filament structures from the same cells. In (C), an immunofluorescence image of a second BRG1-expressing clone grown for 5 days before fixation and staining with phalloidin is shown. Immunofluorescence images of a BRG1-K798R-expressing clone stained with affinity-purified anti-BRG1 antibodies as in A (D), and the same cells stained with phalloidin (E). Immunofluorescence images of SW13 cells fixed and stained with affinity-purified anti-BRG1 antibodies as in A (F), and the same cells stained with phalloidin (G). Bar, 20 μm; the magnification is the same in A-G. (H) Cell lysates were made from untransfected SW13 cells and from clones expressing the BRG1 protein or the BRG1-K798R protein. The proteins (20 μg/sample) were separated on a 7% SDS-PAGE for detection of BRG1 and a 15% SDS-PAGE for the detection of actin, and transferred to a membrane that was subsequently probed with anti-BRG1 antiserum or monoclonal β-actin antibodies. The lane marked 'sw13' is lysate from untransfected cells, 'BRG1' is from the BRG1-expressing clone and 'K798R' is from the BRG1-K798R-expressing clone.

Transfection of cells

SW13 cells were transfected with the pBJ5 vector containing the cDNA for *BRG1* or a form of BRG1 with a non-functional ATPase (*BRG1-K798R*, with a lysine to arginine mutation at position 783 in the human *BRG1* cDNA (Khavari et al., 1993) corresponding to position 798 in the *SWI2/SNF2* cDNA in the ATPase motif), together with a vector containing the gene for *neomycin/G418* resistance. The transfection was performed using Lipofectamin plus (Life Technologies). SW13 cells were also transfected with the *BRG1* or the *BRG1-K798R* cDNA in the pORSVI expression vector. Selection with 0.3 mg/ml of G418 was started 2 days after transfection and colonies were collected 2-3 weeks after selection had been started.

Transient transfections were performed using Lipofectamin plus and cells were lysed for immunoblots, or fixed for immunofluorescence from 6 to 72 hours after transfection, as described below.

Immunofluorescence

Cells were fixed with 2% formaldehyde (Sigma) for 20 minutes at 37°C and lysed in 0.2% Triton X-100 for 20 minutes at 37°C. Actin filaments were visualised using 0.6 μM TRITC- or FITC- conjugated phalloidin (Sigma) in phosphate-buffered saline (PBS). The BRG1 protein was visualised using affinity-purified rabbit antibodies against an N-terminal fragment of the human BRG1 protein (a kind gift from Emma Lees, DNAX Research Institute, Uppsala, Sweden) or against a C-terminal fragments of the rat BRG1 protein (Östlund Farrants et al., 1997). The myc-tagged RhoA proteins were detected using anti-myc 9E (Santa Cruz) and the Rho protein was detected using monoclonal anti-RhoA (Santa Cruz). TRITC- and FITC-conjugated secondary antibodies used for visualisation were obtained from Jackson Immunochemical. The specimens were mounted in Vectashield (Vector Laboratories). Immunofluorescence images were recorded using a Leitz Aristoplan microscope and confocal images were recorded using a Leica confocal laser scanning microscope, with an objective lens of of magnification 63× and a numerical aperture of 1.4.

Immunoblots

The cells were lysed in 20 mM Tris-Cl at pH 8.0, 0.7 M NaCl, 0.5% Nonident 40, frozen and thawed, and then centrifuged at 11,700 ***g*** for 20 minutes to remove DNA and cell debris. The proteins of cell lysates (20 μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to an Immobilone membrane (Millipore). The membrane was probed with anti-BRG1 serum, monoclonal anti-β-actin (Sigma), monoclonal anti-RhoA (Cytoskeleton), anti-ROCK1 antibodies (Santa Cruz), anti-ROCK2 antibodies (Santa Cruz), anti-Dia1 antiserum (Immunoglobe), anti-histone deacetylase 2 antibodies (Abcam), anti p65 of NFκB antibodies (Abcam) and monoclonal anti-α-tubulin (Amersham-Pharmacia).

RNA analysis

Total RNA was prepared using Trizol (Life Technologies) and 2 μg of total RNA was converted to cDNA using 200 U MMLV reverse transcriptase and 100 ng random primer (Life Technologies). One tenth of the reaction volume was used for target-specific PCR reactions to detect the presence of BRG1, GAPDH, ROCK1, ROCK2 and Dia1 mRNAs.

RhoA activation assay

The GST pull-down assay was performed essentially as described (Ren and Schwartz, 2000), except that the RhoA-binding-domain from human rhotekin was used instead of that from the mouse. They differ by seven amino acids in the N-terminal portion of the protein. In order to load equal amounts of total protein for each pull-down and input control, the cells were grown in single dishes at equal densities (~60% confluence) and then the protein concentrations of the crude lysates were measured.

Protein analysis

The protein concentration was determined using Bradford reagent (Bio-Rad).

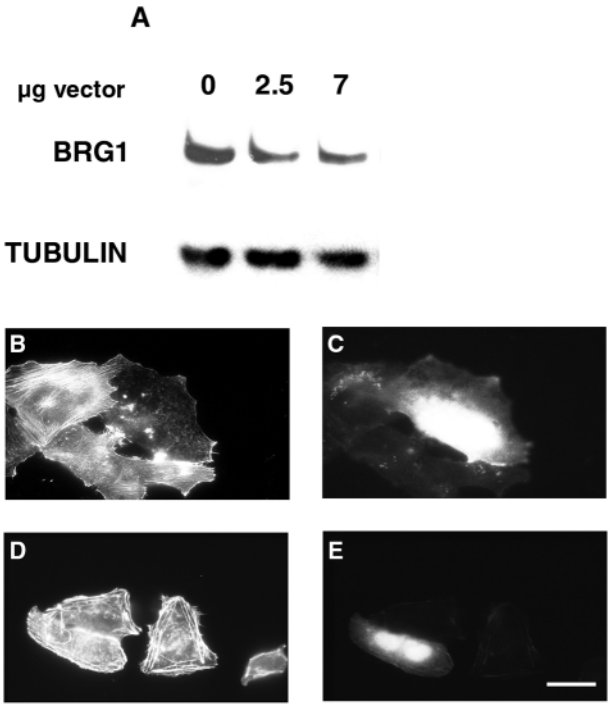

Fig. 2. The effect of a specific antisense BRG1-RNA on the formation of stress-fibre-like actin filament structures. Cells were transfected with a vector expressing a specific BRG1 antisense RNA and the green fluorescent protein (GFP) as a marker. (A) Cell lysates (20 μg/sample) from BRG1-expressing cells grown for 72 hours after transfection and from untransfected BRG1-expressing cells were separated using 7% SDS-PAGE and probed with anti-BRG1 serum (top) and anti-α-tubulin (bottom). The amounts of antisense vector (μg) used for transfection of 0.6×10^6 BRG1-expressing cells are indicated above the lanes. (B) An immunofluorescence image shows BRG1-expressing cells transfected with the antisense BRG1-vector stained with TRITC-conjugated phalloidin. (C) The same cells visualised by GFP-fluorescence as a marker for transfected cells. BRG1-expressing cells were also transfected with the GFP-vector without the antisense insert and stained with TRITC-conjugated phalloidin (D). The same cells were visualised by GFP-fluorescence as a marker (E). Bar, 20 μm; the magnification is the same in B-E.

Results

BRG1-expressing SW13 cells change morphology and form actin filament bundles in their cell bodies

To investigate the effect of the BRG1 protein on the organisation of actin filaments, SW13 cells were transfected with BRG1 expressed from either of the two different expression vectors, pBJ5 (driven by the SRα promoter) (Khavari et al., 1993) and pORSVI (driven by the RSV-promoter). Several clones with a flat appearance were obtained after three weeks of selection with both vectors, but only a few of these survived the cloning step. The clones that had been isolated went into cell cycle arrest after some time, and could be passaged only a limited number of times. The cells expressing the BRG1 protein from the pORSVI vector were more stable and could be passaged more times than those expressing the BRG1 protein from the pBJ5 vector. We also made clones that expressed a BRG1 protein mutated in the ATPase motif (BRG1-K798R) (Khavari et al., 1993) from the RVS promoter, and these did not go into cell cycle arrest. No clones that expressed the BRG1-K798 protein were obtained when using the pBJ5 vector, which might be caused by higher expression from the SRα promoter in SW13 cells. High levels of a BRG1 mutated in the ATPase motif might be toxic to the cells on long-term exposure (Bultman et al., 2000).

In the BRG1 and the BRG1-K798R-expressing clones, but not in native SW13 cells, a clear BRG1 protein signal was observed, which was located in the nucleus (compare Fig. 1A and D with 1F). The BRG1-positive clones that were isolated formed thick actin filament bundles stretching mostly from the plasma membrane into the cell body (Fig. 1B,C), but could also appear in short stretches within the cell body. These stress-fibre-like actin structures were absent both in clones that expressed the BRG1-K798R protein (Fig. 1E) and in native SW13 cells (Fig. 1G). Instead, a thin actin filament network was seen in the cell bodies of these cells, and actin filaments were abundant at the edges (Fig. 1E,G). The BRG1-expressing cells developed flat cell morphology and grew larger than native SW13 cells and cells expressing the BRG1-K798R protein after 4-5 days of growth, although some of the cells in the BRG1-K798R clone were slightly larger than native SW13 cells (Fig. 1E). Furthermore, SW13 cells that expressed the BRG1 protein were thinner (approximately 5 μm) than native SW13 cells (approximately 20 μm), measured by viewing the depth of focus in the confocal microscope. The expression of the BRG1 protein in the clones was confirmed by immunoblots (Fig. 1H), while no expression of the BRG1 protein was detected in SW13 (Fig. 1H). The change in actin organisation observed in the BRG1-expressing clones was not caused by differences in β-actin levels, since native SW13 cells and the BRG1-expressing clones contained approximately the same levels of β-actin (Fig. 1H).

Many of the large BRG1-expressing cells had multiple nuclei (not shown), indicating a disturbance in mitosis and cytokinesis. However, multinuclear cells were observed not only in cells that expressed the BRG1 protein, but also in cells expressing the mutated BRG1-K798R protein (Fig. 1D). These cells were also larger than mononuclear cells, but they were without any thick actin filament bundles in the cell body. This suggests that some of the effects caused by the BRG1 protein are achieved without the ATPase activity.

The formation of thick actin filaments in the cell body in BRG1-expressing cells depends on the BRG1 protein

In order to confirm that the formation of actin filament bundles in the cell body in BRG1-expressing clones depends upon the expression of the BRG1 protein, we used a vector co-expressing a specific antisense BRG1-RNA fragment and the GFP (Green Fluorescent Protein). The expression of the antisense BRG1 reduced the protein level in BRG1-expressing cells by approximately 50% 72 hours after transfection, but did not affect the protein level of α-tubulin (Fig. 2A). The BRG1-antisense vector at amounts higher than 5 μg was toxic and resulted in cell death, and no further reduction could be detected in cells transfected with the vector above these amount. The expression of the antisense BRG1-RNA fragment in BRG1-expressing cells abolished the thick actin bundles in the cell body (Fig. 2B). The expression of a GFP protein expressed from the same vector was used as a marker for cells

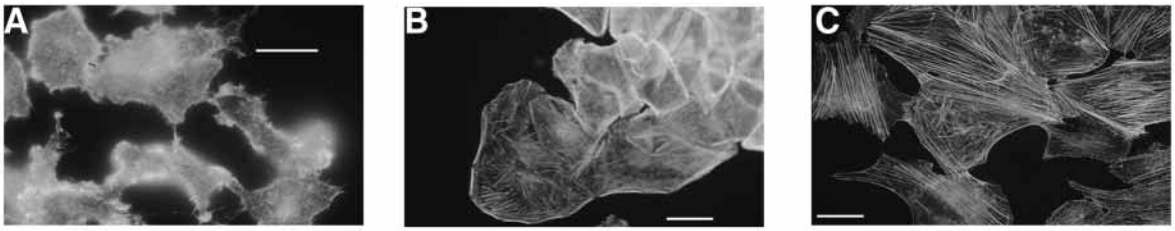

Fig. 3. The effect of TSA-treatment on the actin filament organisation. Immunofluorescence images show SW13 cells treated for 24 hours with 125 ng/ml of TSA, fixed and stained with FITC-phalloidin (A), BRG1-expressing cells selected for 10 days and stained with FITC-phalloidin (B), and BRG1-expressing cells selected for 10 days, treated with 125 ng of TSA/ml for 24 hours and stained with FITC-phalloidin (C). Bars, 20 μm (A); 100 μm (B,C).

expressing the antisense BRG1-RNA fragment (Fig. 2C). The same vector expressing only the GFP protein was used as a control, and these cells still had stress-fibre-like actin filaments (Fig. 2D,E). The disappearance of thick actin filament bundles in BRG1-expressing cells in response to a reduced level of BRG1 protein strongly suggests that this protein is responsible for the induction of thick actin bundles in the cell body.

Several protein complexes in addition to the BRG1-containing SWI/SNF complexes can remodel chromatin and may be involved in transcriptional regulation. Modification of the histone tails by acetylation alters the structure of chromatin, and is required for many cellular processes (Kingston and Narlikar, 1999). Therefore, we next examined whether chromatin remodelling by acetylation in the absence of the BRG1 protein resulted in a similar actin bundle formation as that found in BRG1-expressing cells. SW13 cells were treated with the histone deacetylase inhibitor trichostatin A (TSA) at concentrations of 50 ng/ml, 150 ng/ml and 450 ng/ml for 24 hours, which leads to hyperacetylation of the histones. None of these concentrations induced stress-fibre-like actin filament bundles in SW13 cells, but the actin filaments at the edges were affected at the 150 ng/ml (Fig. 3A). Several protrusions resembling retraction fibres were observed. TSA at high concentrations is toxic and, as expected, the highest concentration caused cell death. BRG1-expressing cells treated with TSA at the same concentrations for 24 hours had a larger

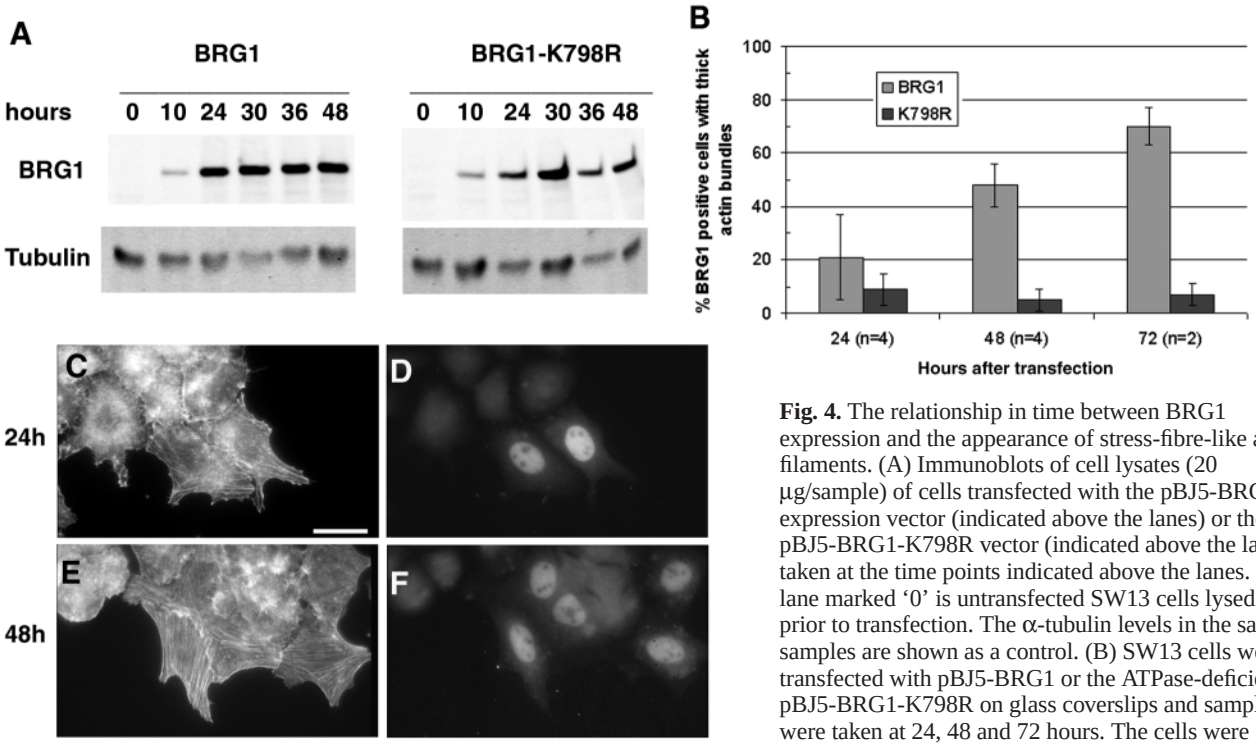

Fig. 4. The relationship in time between BRG1 expression and the appearance of stress-fibre-like actin filaments. (A) Immunoblots of cell lysates (20 μg/sample) of cells transfected with the pBJ5-BRG1 expression vector (indicated above the lanes) or the pBJ5-BRG1-K798R vector (indicated above the lanes) taken at the time points indicated above the lanes. The lane marked '0' is untransfected SW13 cells lysed just prior to transfection. The α-tubulin levels in the same samples are shown as a control. (B) SW13 cells were transfected with pBJ5-BRG1 or the ATPase-deficient pBJ5-BRG1-K798R on glass coverslips and samples were taken at 24, 48 and 72 hours. The cells were double-stained with FITC-phalloidin and anti-BRG1 antiserum visualised by TRITC-secondary antibodies after fixation. Between 30 and 50 cells expressing the BRG1 protein or the mutated BRG1-K798R protein in the nucleus were counted blindly in each separate experiment for each group (four separate experiments for 24 hours and 48 hours). The percentage of BRG1/mutated BRG1-K798R-expressing cells that had formed thick actin bundles in the cell body was determined for each sample. The standard deviation is given for each group; *n* is the number of separate experiments in the group. The two values for the 72 hour point were 65 and 72% for BRG1-expressing cells, and 3 and 13% for BRG1-K798R-expressing cells. Immunofluorescence images of cells transfected with the BRG1-vector stained with phalloidin (C) and with the BRG-antiserum against the N-terminal (D) 24 hours after transfection and stained with phalloidin (E) and the anti-BRG1 antiserum as in D (F), 48 hours after transfection are shown. Bar, 20 μm.

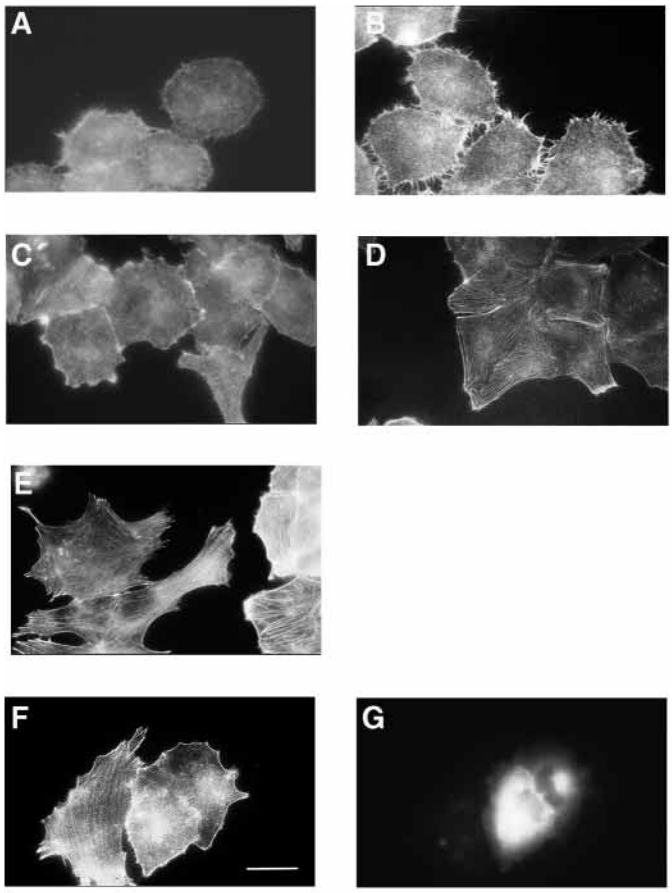

Fig. 5. Activation of the RhoA pathway by LPA and serum stimulation. Immunofluorescence images show SW13 cells starved (0.25% fetal calf serum) for 6 hours (A) and exposed to 10 μM LPA after 6 hours of starvation (B) before being fixed and stained with FITC-phalloidin. Immunofluorescence images show BRG1-expressing cells starved (0.25% fetal calf serum) for 18 hours (C), exposed to 5 μM LPA for 20 minutes after starvation (D) and given 5% serum for 20 minutes after starvation (E). Immunofluorescence images show BRG1-expressing cells expressing a myc-tagged RhoA(N19) protein stained with FITC-phalloidin (F) and the anti-9E-myc antibody (G) for detection of cells expressing the RhoA(N19) protein. All images are at the same magnification; bar, 20 μm.

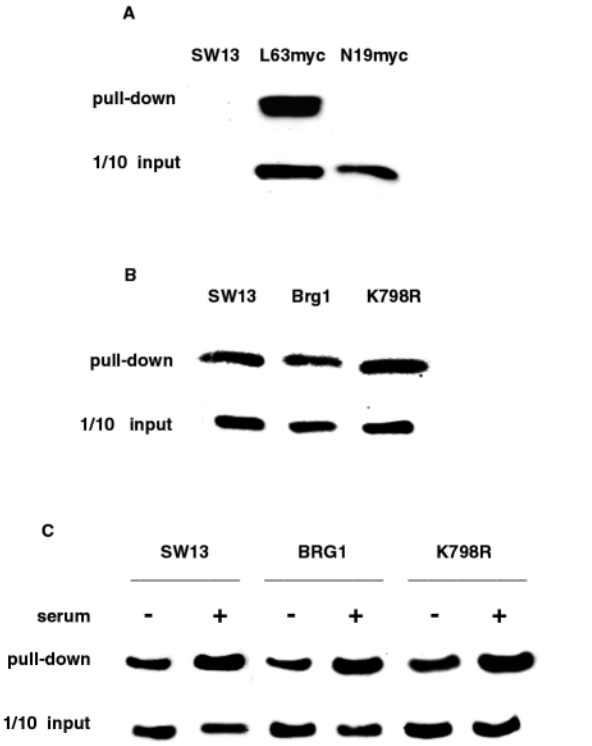

Fig. 6. The activation state of the RhoA-GTPase. (A) Immunoblots of control pull-downs using the human form of rhotekin-RBD showing that it is specific for the activated form of RhoA. Approximately 10^4 SW13 cells were seeded in 5 cm dishes and left untreated or transfected with the vector as indicated above the lanes for 24 hours prior to the pull-down. Myc-tagged mutant forms of RhoA were detected using the anti-myc-9E antibody. (B) The activation states of endogenous RhoA in SW13 cells, a BRG1-expressing clone and a BRG1-K798R-expressing clone were measured 24 hours after seeding at ~60% confluence. The protein concentration of each sample was measured in the crude lysates and the protein input adjusted accordingly. The RhoA protein was detected using anti-RhoA antibodies. (C) The activation state of RhoA in SW13 cells, BRG1 and BRG1-K798R-expressing clones seeded at ~60% confluence, starved for 18 hours in serum-free medium and stimulated with 5% serum for 20 minutes as indicated above the lanes is shown. (The experiment presented is representative of three experiments.)

number of thick actin bundles in their cell bodies than BRG1-expressing cells, indicating that hyperacetylation of histones increases the effect of the BRG1 protein on the actin filament organisation in the cell body (compare Fig. 3C with 3B).

Thick actin filament bundles appear between 24 hours and 48 hours after BRG1 transfection of SW13 cells

We next examined the relationship in time between BRG1 expression and the appearance of thick actin bundles in the cell body in SW13 cells by transfecting SW13 cells with either the pBJ5-BRG1 or the pBJ5-BRG1-K798R expression vector. Samples were taken at 10, 24, 30, 36 and 48 hours after transfection. The BRG1 and the ATP-deficient BRG1-K798R proteins were first detected in immunoblots 10 hours after transfection, with a peak at 30 hours (Fig. 4A). The protein levels expressed from both vectors were in the range of the levels found in HeLa cells and NIH 3T3 cells (not shown). Actin filament bundles in the cell bodies could be clearly detected by immunofluorescence in many of the BRG1-expressing cells at 24 hours, and at 48 hours after transfection 48% of the BRG1-expressing cells had thick actin bundles in the cytoplasm (Fig. 4B). Few cells with actin bundles in the cytoplasm were detected in cells expressing the BRG1-K798R protein, and the level did not increase with time (Fig. 4B). There was no clear difference in the morphology of the filaments after 24 hours and 48 hours, but the number of filaments was lower at 24 hours (compare Fig. 4C and D with 4E and F).

Expression of BRG1 affects the RhoA pathway

To gain further insight into the effect of the BRG1 protein on the formation of actin filament bundles, we investigated

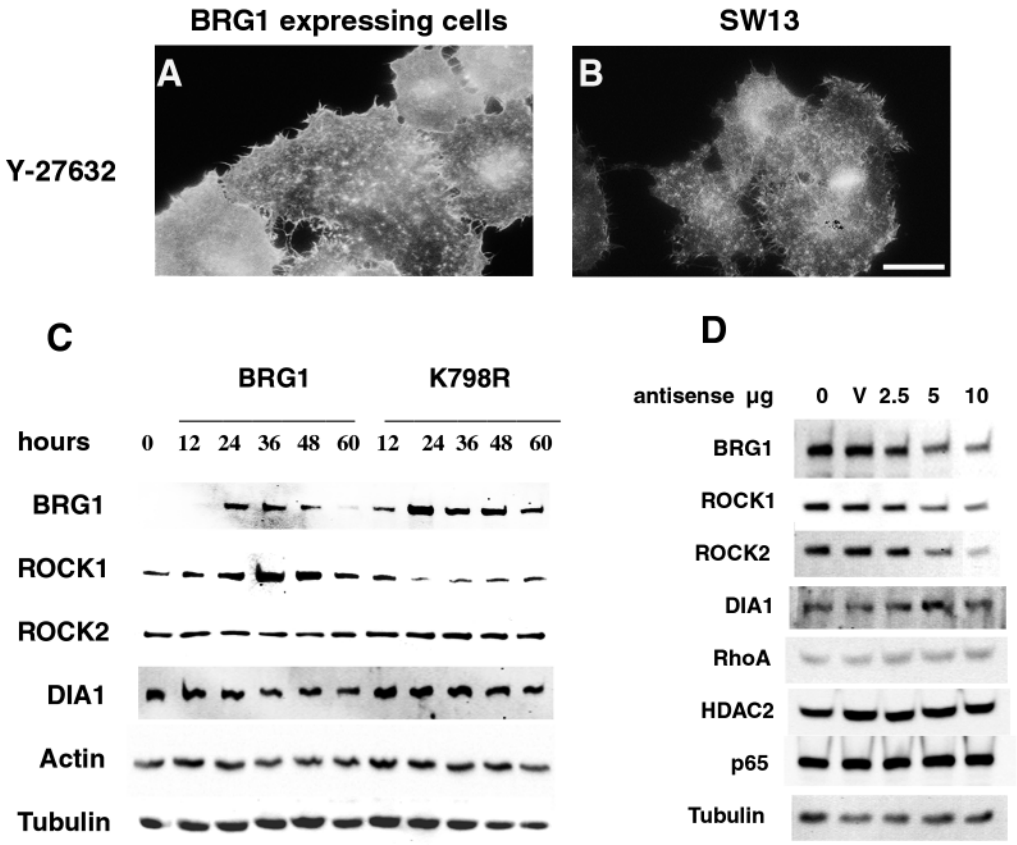

Fig. 7. The effect of BRG1 expression on the protein levels of Rho-kinase/ROCK proteins. Immunfluorescence images of BRG1-expressing cells exposed to the Rho-kinase/ROCK inhibitor Y-27632 for 1 hour, fixed and stained with FITC-phalloidin (A) and native SW13 cells exposed to the same treatment as BRG1-expressing cells (B). Bar, 20 μm. (C) Immunoblots of cell lysate (20 μg/sample) of SW13 cells transfected with the pBJ5-BRG1 or the pBJ5-BRG1-K798R expression vector. Samples were taken at the time points indicated above the lanes. The membranes were probed with a C-terminal antiserum against BRG1, ROCK1 antibodies, ROCK2 antibodies, a Dia1 antiserum, β-actin antibodies and α-tubulin antibodies as indicated on the left. The BRG1-transfection is shown on the left and the BRG1-K798R on the right as indicated (representative of one experiment of five). (D) Immunoblots of the level of ROCK1, ROCK2, Dia1, RhoA, HDAC2, P65 of NFκB and α-tubulin in cell lysate (20 μg/lane) of BRG1-expressing cells (labelled '0'), cells that were transfected with a 5 μg GFP-expressing vector (labelled 'V') or a GFP-expressing vector co-expressing a specific antisense BRG1-fragment. The amount of GFP-BRG1-antisense vector used is given above the lanes.

whether the BRG1 protein affected the RhoA pathway, the major signalling pathway leading to stress-fibre formation. We exposed SW13-cells and BRG1-expressing cells to LPA (lysophosphatidic acid) and serum, two stimuli that are known to activate the RhoA pathway and induce stress-fibre formation in fibroblasts (Ridley and Hall, 1992). SW13 cells were unable to respond to LPA and serum treatment by forming stress-fibre-like actin filament bundles. Because the SW13 cells were sensitive to starvation, we starved these cells only for 6 hours (Fig. 5A). No thick actin filaments were induced by LPA concentrations of 0.1, 10 or 90 μM for 5 minutes, 20 minutes, 6 hours or 18 hours. Fig. 5B shows cells exposed to 10 μM LPA for 20 minutes. Serum (5%) did not have an effect on the actin filament organisation in native SW13 cells (Fig. 1G), nor could other stimuli that induce stress-fibres in fibroblasts, such as different substrata, induce the formation of stress-fibre-like actin filament bundles (not shown). BRG1-expressing cells that were starved for 18 hours had lost most of their thick actin filament bundles in the cell body (Fig. 5C), but in contrast to native SW13 cells, clear filament bundles in the cell body reformed when the cells were exposed to 5 μM LPA for 20 minutes (Fig. 5D). Exposure to 5% serum for 20 minutes resulted in a stronger effect, and the thick actin filament bundles in the cell body were fully restored (Fig. 5E). These results indicate that BRG1 expression in SW13 establishes an active RhoA pathway that can respond to stimuli and thereby induce the formation of stress-fibre-like structures.

We next transfected BRG1-expressing cells with a vector containing a myc-tagged, dominant negative *RhoA* cDNA, RhoA(N19), which causes loss of stress-fibres in fibroblasts when expressed. Expression of RhoA(N19) in BRG1-expressing cells caused disassembly of the thick actin bundles in the cell body 18 hours after transfection (Fig. 5F), leaving behind diffusely stained cells. The cells that expressed the RhoA(N19) protein were detected with the myc-9E antibody (Fig. 5G).

The RhoA GTPase is activated in both SW13 cells and BRG1-expressing cells

The different response between native SW13 cells and BRG1-expressing cells to stimuli that activate the RhoA pathway

prompted us to analyse directly the activity state of the RhoA-protein in these cells. The human rhotekin-GST fusion, which differs from the commonly used mouse rhotekin by 15 amino acids in the N-terminal, was used, and Fig. 6A shows that it was specific for the RhoA-GTP form in a pull-down assay. The rhotekin protein fragment bound the constitutively active RhoA(L63) protein from cells lysate, but not the dominant negative RhoA(N19) protein (Fig. 6A). When the RhoA-GTP level was compared in SW13 cells and clones expressing the BRG1 protein or the BRG1-K798R protein continuously grown in the presence of 5% serum, no difference was detected between the cell populations (Fig. 6B). Similarly, the RhoA protein was activated to the same extent in native SW13 cells, BRG1-expressing cells and cells expressing the K798R-BRG1 when exposed to 5% serum for 20 minutes after 18 hours of starvation (Fig. 6C). Thus, the RhoA GTPase was equally activated by serum in all three cell types, but serum induced only thick actin filaments in the cell body in BRG1-expressing cells (compare Fig. 5C and D with 5A and Fig. 1G). Therefore, the formation of thick actin filament structures in BRG1-expressing cells cannot be caused by an upregulation of the amount of RhoA-GTP, but must be regulated by effectors downstream of the RhoA-GTPase.

The BRG1 protein increases the expression of the ROCK1 protein

The RhoA small GTPase has several downstream effector targets, at least two of which are involved in the induction of stress-fibres in fibroblasts, the Rho-kinase/ROCK (Leung et al., 1996; Amano et al., 1997; Sahai et al., 1998) and the profilin-binding protein Dia (Watanabe et al., 1997; Watanabe et al., 1999). To determine whether downstream effectors were involved in formation of thick actin filament bundles in BRG1-expressing cells, we treated BRG1-expressing cells with Y-27632, a specific inhibitor of the Rho-kinase/ROCK. Inhibiting the Rho-kinase/ROCK by adding 10 μM of Y-27632 to the culture medium for 1 hour led to the disruption of the thick actin bundle formation in the BRG1-expressing cells (compare Fig. 7A with Fig. 1B and C). These cells displayed a thin network of actin filaments in the cell body similar to that seen in untreated SW13 cells (compare Fig. 7A with Fig. 1G). The actin filament structure in SW13 cells exposed to 10 μM of Y-27632 remained unaltered (Fig. 7B). This indicated a role for the Rho-kinase/ROCK in the formation of thick actin bundles in BRG1-expressing cells, and we next determined the protein levels of RhoA effectors involved in stress-fibre formation. The balance between the two RhoA effectors, Rho-kinase/ROCK and Dia, is important for the formation and appearance of the actin filament bundles (Watanabe et al., 1999). The protein levels of the two Rho-kinase/ROCK isozymes, ROCK1 and ROCK2 (ROKα), and Dia1 were determined in cells transiently transfected with either the *BRG1* vector or the ATPase-deficient *BRG1-K798R* vector, and samples were taken at 12, 24, 36, 48 and 60 hours after transfection. The protein level of ROCK1 was slightly higher in BRG1-expressing cells after 24 hours, whereas the BRG1-K798R-expressing cells showed no increase (Fig. 7C). The protein levels of ROCK2 and Dia1 remained essentially unaltered in all three cell types (Fig. 7C), as were those of actin and α-tubulin (Fig. 7C). We also transfected the BRG1-expressing clone with the vector from which the specific antisense BRG1-RNA fragment was expressed in order to interfere with the BRG1 expression. The 0.6×10^6 cells were transfected with increasing amounts of vector, 2.5, 5 and 10 μg, for 72 hours. The BRG1expression decreased following the increase in antisense BRG1 vector (Fig. 7D), and the same pattern was displayed by ROCK1 and also by ROCK2. The protein levels of these proteins were not affected by the vector without the antisense BRG1 fragment (Fig. 7D). The protein level of Dia1 remained unaltered, as did the protein levels of α-tubulin and actin (Fig. 7D). Two proteins unrelated to actin filament organisation, histone deactylase (HDAC) and p65 in NFκB, remained unaltered by the BRG1-antisense expression (Fig. 7D). We conclude that the effect of BRG1-expression in SW13 cells affects the protein level of Rho-kinase/ROCK, but seems to have a stronger effect on that of ROCK1.

We also determined the mRNA levels of ROCK1, ROCK2 and Dia1 in transient transfected SW13 cells (not shown), but there were no significant changes in mRNA levels between native SW13 cells, BRG1-expressing cells and BRG1-K798R-expressing cells, suggesting that the increase in ROCK1 protein is not caused by direct transcriptional regulation by the BRG1 action on the ROCK1 gene.

Transiently expressed ROCK1 and ROCK2 induce stress-fibre like structures in SW13 cells

The elevated protein level of ROCK1 protein in BRG1-expressing cells would result in an alteration in the balance between the Rho-kinase/ROCK and Dia, which in turn leads to changes in actin filament organisation. To examine whether an elevated Rho-kinase/ROCK activity induced stress-fibre-like structures in SW13 cells, we expressed a myc-tagged fragment of the catalytical kinase domain of ROCK2. SW13 cells that expressed the catalytical fragment clearly formed thick actin filaments in the cell body (Fig. 8A,B), indicating that an increased Rho-kinase/ROCK activity was sufficient to form stress-fibre like structures in these cells. The myc-tagged, wild-type protein of both isoforms of Rho-kinase/ROCK, ROCK1 and ROCK2, were also expressed in SW13 cells, and both proteins induced thick actin filaments (Fig. 8C,E, respectively). Cells expressing the proteins were detected with anti-9E-myc antibodies (Fig. 8D,F). Furthermore, BRG1-expressing cells were transfected with myc-tagged, dominant-negative forms of ROCK1 or ROCK2. The ROCK1 construct (KD-IA) carried mutations in both the RhoA binding site and the kinase catalytical site (Ishizaki et al., 1997), and the cells expressing this protein lost their actin stress-fibres (Fig. 8G,H). Cells expressing ROCK2 mutated in the RhoA binding site and with a deleted kinase domain (pEF-BOS-myc-RB/PH(TT) (Amano et al., 1997) also abolished stress-fibre-like structures when expressed (not shown). These results suggest that the Rho-kinase/ROCK level in SW13 is involved in regulating the formation of stress-fibre-like actin structures.

Discussion

The expression of the BRG1 protein affects the actin filament organisation in the cytoplasm, and is involved in the formation of thick stress-fibre-like actin filament bundles in the cell body.

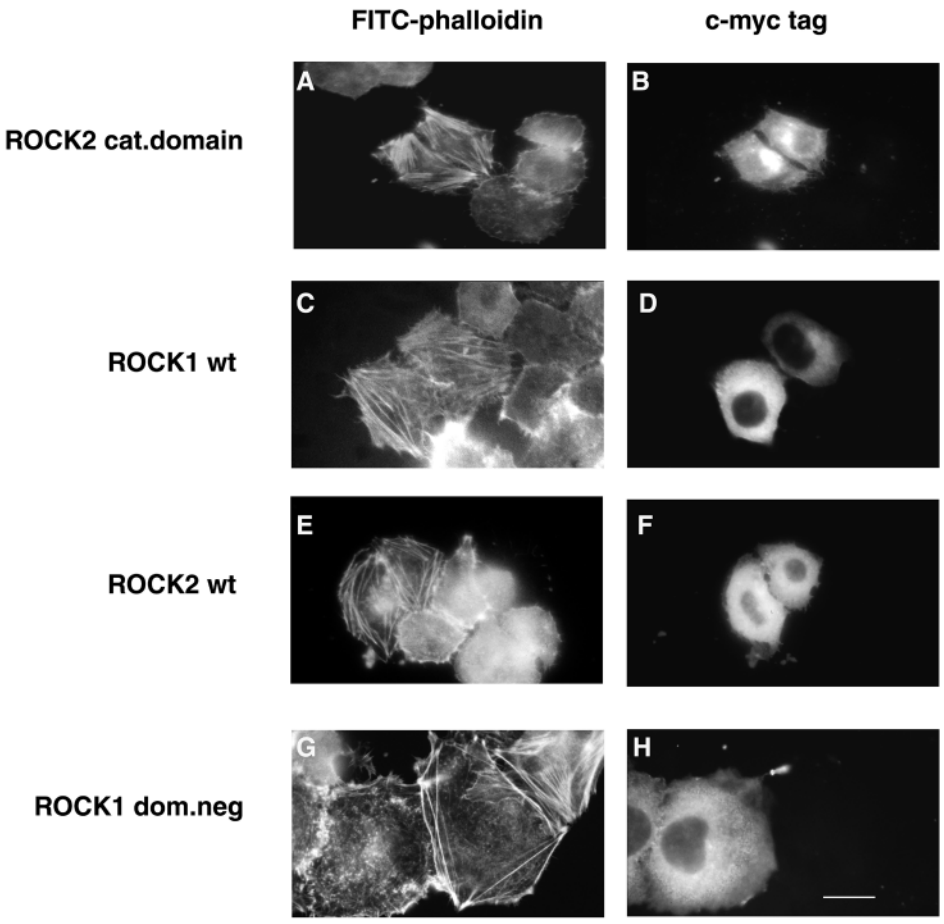

Fig. 8. The effect of Rho-kinase/ROCK expression in SW13 cells. (A,B) Immunofluoroscence images show SW13 cells expressing a myc-tagged, constitutively active Rho-kinase/ROCK fragment habouring the kinase domain, which were fixed and stained with FITC-phalloidin (A) and the anti-9E-myc antibody (B) for detection of cells expressing the Rho-kinase/ROCK fragment. Immunofluorescence images of cells expressing a wild-type, myc-tagged ROCK1 protein stained with FITC-phalloidin (C) and anti-9E-myc antibodies (D), and SW13 cells expressing a wild-type, myc-tagged ROCK2 protein stained with FITC-phalloidin (E) and anti-myc-9E-antibodies (F). Immuofluorescence images of BRG1-expressing cells transfected with a dominant negative, myc-tagged ROCK1 (KD-IA), stained with FITC-phalloidin (G) and anti-9E-myc antibodies (H). Bar, 20 μm; the same magnification was used in A-H.

The BRG1 protein is a strictly nuclear protein (Khavari et al., 1993; Muchardt et al., 1996; Reyes et al., 1997; Reyes et al., 1998; Zhao et al., 1998; Shanahan et al., 1999; Machida et al., 2001) according to several indirect immuflourescence studies using different fixation techniques. It is one of two possible central ATPases in the SWI/SNF complexes, the other being the homologous BRM protein. The ATPase activity of the BRG1 protein is absolutely required for the chromatin remodelling activity of the BRG1-containing SWI/SNF complexes in vitro (Kwon et al., 1994; Östlund Farrants et al., 1997; Phelan et al., 1999), and for the transcriptional regulation that these complexes are responsible for in vivo (Kwon et al., 1994; Murphy et al., 1999). We demonstrate that a functional ATPase activity of BRG1 is also required for the formation of stress-fibre-like structures, which indicates that BRG1 affects the actin filament structures on the transcriptional level. This study was performed in SW13 cells, which are deficient in the BRG1 and the BRM proteins, but other SWI/SNF core components, such as BAF 170, BAF 155 and SNF5, are expressed (Wang et al., 1996a; Zhao et al., 1998; DeCristofaro et al., 2001). Since the ATPase subunit is lacking, SW13 cells have no functional SWI/SNF complexes, but several reports indicate that exogenously expressed BRG1 proteins are incorporated into functional SWI/SNF complexes in SW13 cells (Zhao et al., 1998; Shanahan et al., 1999; Murphy et al., 1999). In addition, we found that both the BRG1 protein and the ATP-deficient BRG1protein in stable clones are parts of complexes with a molecular mass of 2 MDa, together with the other SWI/SNF core component BAF 155 (P.A., E. Cavallán and A.-K.Ö.F., unpublished).

Control of the actin filament organisation is complex and several factors and signal transduction pathways are involved. These include actin regulatory proteins, cell adhesion signal pathways and signal transduction pathways that are activated in response to growth factors. In particular, signal pathways involving small GTPases of the Rho family play a major role in the reorganisation of actin, and studies in fibroblasts have implicated RhoA in stress-fibre formation. Our results suggest that BRG1 protein affects the RhoA signalling pathway, but not by regulating the activity state of the RhoA GTPase. The RhoA GTPase in native SW13 cells and in cells expressing the ATP-deficient BRG1-K798R protein is equally activated when exposed to stimulatory factors, such as serum, as the RhoA GTPase in BRG1-expressing cells after starvation, but the activation of RhoA in native SW13 cells and in those expressing the BRG1-K798R protein does not trigger formation of stress-fibre-like structures. Uncoupling of activated RhoA from stress-fibre formation has been observed in ras-transformed fibroblasts with a sustained activated MAPK pathway, which have lost their stress-fibres (Sahai et al., 2001; Pawlak and Helfman, 2002). These cells have a downregulated protein level of the RhoA downstream effector Rho-kinase/ROCK; both a downregulation of one of the isoforms, ROCK1, accompanied by an altered subcellular distribution (Sahai et al., 2001), and a downregulation of both ROCK1 and ROCK2 (Pawlak and Helfman, 2002) have been

reported. We observed an upregulation of the ROCK1 protein in BRG1-expressing cells, which gained the ability to form stress-fibres. We could not find any difference in subcellular distribution of these proteins between BRG1-expressing cells and SW13 cells, with RhoA, ROCK1, ROCK2 and Dia1, another RhoA effector involved in stress-fibre formation, found in the soluble Triton X-100 fraction (P.A. and A.-K.Ö. F., unpublished).

Both Rho-kinase/ROCK and Dia contribute to the formation of and to the maintenance of the stress-fibres (Leung et al., 1996; Amano et al., 1996; Watanabe et al., 1997; Sahai et al., 1998; Watanabe et al., 1999), and studies have shown that they co-operate in the formation of RhoA-activated stress-fibres (Watanabe et al., 1999; Nakano et al., 1999). The mechanisms of Rho-kinase/ROCK and Dia function are not fully understood. The Rho-associated kinase/ROCK induce stress-fibres by activating myosin (Leung et al., 1996; Amano et al., 1997; Chihara et al., 1997; Ishizaki et al., 1997), most probably both by a direct activating phosphorylation of the myosin light chain (Amano et al., 1996; Kureishi et al., 1997) and by an inactivating phosphorylation of the myosin light chain phosphatase (Kimura et al., 1996; Kawano et al., 1999). In addition, cofilin, with an actin-depolymerising activity, is inactivated by phosphorylation by the Rho-kinase/ROCK downstream target LIM kinase (Maekawa et al., 1999; Yang et al., 1998). The Dia protein is an FH protein that binds profilin and these proteins may work in cooperation to induce stress-fibres upon activation (Watanabe et al., 1997). It has been shown that different ratios of Rho-kinase/ROCK to Dia contribute to different types of actin structures; overexpression of an active Dia1 gives thin disorganised filaments, similar to those seen in untreated SW13 cells, whereas overexpression of an active Rho-kinase/ROCK gives very thick condensed fibres (Watanabe et al., 1999).

We propose that the elevated ROCK1 level in BRG1-expressing cells couples the activation of RhoA to stress-fibre formation by altering the balance between the Rho-kinase/ROCK activity and the Dia activity. Although we could detect only an increased ROCK1 protein level in BRG1-expressing cells, both forms were downregulated when interfering with BRG1-expression, which indicates that both are affected. However, the ROCK1 is affected more strongly. The fact that increasing the Rho-kinase activity in SW13 cells by transient expression of the kinase domain induced thick actin filamnent formation further supports this. No specificity between the two isoforms of Rho-kinase/ROCK could be seen when transiently expressing wild-type ROCK1 or ROCK2, since both induced thick actin filaments that resemble those in BRG1-expressing cells. In addition, both nonfunctional, dominant-negative forms of ROCK1 and ROCK2 were able to abolish the thick actin filament formation in BRG1-expressing clones. The lack of specificity could be explained by the similarity between these isoforms. The kinase domains are 92% identical on the amino acid level (Nakagawa et al., 1996), and they have the same targets. The elevated ROCK1 protein level observed in BRG1-expressing cells would result in an increase in overall phosphorylation of myosin and cofilin, which subsequently would lead to the formation of stress-fibre-like actin filaments.

We could not find evidence for a transcriptional regulation of the *ROCK1* gene directly, since the mRNA levels in BRG1-expressing cells were not significantly different from those in native SW13 cells. Instead, the effect of BRG1 on the ROCK1 level may be at the post-transcriptional or the post-translational level. Transcriptional downregulation has been reported for ROCK2 in ras-transformed cells (Zuber et al., 2000), but it has been suggested that post-transcriptional mechanisms exist for ROCK1 and ROCK2 regulation. The ROCK1 protein that is obtained when expressed exogenously in ras-transformed cells is significantly lower than that found in the corresponding native cells, although the mRNA-level increases (Sahai et al., 2001). In a separate study of ras-transformed fibroblasts, both the ROCK1 and ROCK2 protein level were lower than in native cells, but no decrease in the mRNA-levels could be observed (Pawlak and Helfman, 2002). Our results suggest that the BRG1 protein affects the stability of the ROCK1 protein and, to a lesser extent, ROCK2. Since the ATPase domain of the BRG1 protein is required for the increase of ROCK1 protein level, it is tempting to speculate that the BRG1 is involved in transcriptional regulation of components involved in regulating protein stability.

TSA treatment, which leads to hyperacetylation of the histone tails by inhibiting histone deacetylases, did not induce the thick actin bundles in the cell body in SW13 cells. This means that the formation of thick actin filament bundles in the cell body does not depend on histone acetyltransferases. Nevertheless, TSA clearly potentiated the thick actin bundle formation in the cell bodies of BRG1-expressing cells, indicating a co-operation between the BRG1 protein and histone acetylation. Co-operation between histone acetylation and ATP-dependent chromatin remodelling has been observed in gene activation of several genes in yeast and *Xenopus* (Sudarsanam et al., 1999; Cosma et al., 1999; Li et al., 1999; Krebs et al., 1999). Interestingly, TSA treatment of HeLa cells, which express the BRG1 and BRM proteins, increases the number of stress-fibres (Hoshikawa et al., 1994), which could reflect a co-operation between the BRG1 protein as a component of the SWI/SNF complex and histone acetyl transferases, similar to the effect displayed in BRG1-expressing SW13 cells. Recently, it was shown that the BRG1 protein is a component of two nuclear complexes with slightly different subunit compositions and chromatin remodelling activities, the BAF complex (SWI/SNF A) and PBAF (SWI/SNF B) (Xue et al., 2000; Sif et al., 2001). Therefore, the effects of the BRG1 protein expressed in SW13 cells may arise from its role in different nuclear complexes with different functions. This may also explain the multiple effects of the BRG1 protein when expressed in SW13 cells, including the induction of multinuclear cells (Zhang et al., 2000). However, we also found cells that expressed the ATP-deficient BRG1-K798R protein that were multinuclear, indicating that these cells arise independently of a functional ATPase activity. It remains to be clarified which of the BRG1-containing SWI/SNF complexes is responsible for inducing the change in actin filament organisation. The post-transcriptional mechanism involved in regulating the ROCK1 protein level also needs to be characterised in order to determine at which step or steps in the regulation of the actin filament structure the BRG1 protein is directly involved.

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expresson vectors for RhoA were a kind gift from Pontus Aspenström (The Ludwig Institute, Uppsala, Sweden) and Alan Hall (University College London, UK), the ROCK1 vectors (pCAG-myc-p160^{ROCK}(wt) and pCAG-myc-p160^{ROCK}(KD-IA)) from S. Narumiya (Kyoto University, Faculty of Medicine, Kyoto, Japan) and the ROCKII vectors (pE-BOS-myc-Rho-kinase(wt) and pE-BOS-myc-RB/PH(TT)) from K. Kaibuchi (Nagoya University, Nagoya, Japan). We are also grateful to George Farrants for helpful discussions and for editing the manuscript. This work was supported by grants from the Natural Scientific Research Council in Sweden, the Magnus Bergvall's Foundation, the Åke Wiberg Foundation, the Nilsson-Ehle Foundation, the Trygger Foundation and the Lars Hierta Foundation.

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