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Summary

The SWI/SNF enzymes belong to a family of ATP-dependent chromatin remodeling enzymes that have been functionally implicated in gene regulation, development, differentiation and oncogenesis. BRG1, the catalytic core subunit of some of the SWI/SNF enzymes, can interact with known tumor suppressor proteins and can act as a tumor suppressor itself. We report that cells that inducibly express ATPase-deficient versions of BRG1 increase in cell volume, area of attachment and nuclear size upon expression of the mutant BRG1 protein. Examination of focal adhesions reveals qualitative changes in paxillin distribution but no difference in the actin cytoskeletal structure. Increases in cell size and shape correlate with over-expression of two integrins and the urokinase-type plasminogen activator receptor (uPAR), which is also involved in cell adhesion and is often over-expressed in metastatic cancer cells. These findings demonstrate that gene expression pathways affected by chromatin remodeling enzymes can regulate the physical dimensions of mammalian cell morphology.

Key words: Cell size and shape, Nuclear size, SWI/SNF, BRG1, Chromatin remodeling

Introduction

The ATP-dependent SWI/SNF chromatin remodeling enzymes alter histone:DNA contacts on nucleosomes in a manner that promotes interaction of regulatory factors with the chromatin template (Becker and Horz, 2002; Narlikar et al., 2002; Peterson, 2002). The mechanism appears to be an energy-dependent combination of twisting of the DNA helix and displacement of the helix relative to the histone octamer surface (bulging). This is supported by data demonstrating that SWI/SNF can facilitate the binding of a transcription factor in a manner dependent upon the specific orientation of the factor binding site therefore arguing against a simple twisting model (Imbalzano et al., 1994). Regardless of the exact mechanism(s) behind nucleosome remodeling by SWI/SNF enzymes, recent reports indicate that the SWI/SNF enzymes contribute to a diverse collection of cellular gene activation events. In particular, some genes that are turned on in response to developmental or environmental signals seem to show a requirement for SWI/SNF enzymes and their chromatin remodeling activities (Agalioti et al., 2000; Barker et al., 2001; Chi et al., 2002; de la Serna et al., 2000; de la Serna et al., 2001a; DiRenzo et al., 2000; Kowenz-Leutz and Leutz, 1999; Lee et al., 1999; Lemon et al., 2001; Liu et al., 2002; Mudhasani and Fontes, 2002; Pattenden et al., 2002; Pedersen et al., 2001; Soutoglou and Talianidis, 2002; Wang and Hankinson, 2002).

However, the requirement for SWI/SNF enzymes during activation of new gene programs is not absolute; not every gene turned on or up-regulated in response to environmental or differentiation signals requires SWI/SNF enzymes (de la Serna et al., 2000; de la Serna et al., 2001b; Kowenz-Leutz and Leutz, 1999; Roy et al., 2002). In addition, the requirement of genes that are constitutively active during cell culture growth for SWI/SNF enzymes is limited; a microarray analysis of tumor cells lacking or expressing the BRG1 catalytic subunit of some SWI/SNF enzymes identified only 80 genes activated more than three-fold by the presence of BRG1 (Liu et al., 2001). In a limited number of instances, SWI/SNF enzymes have been implicated in gene repression. The microarray analysis identified two genes that were repressed by the presence of BRG1 (Liu et al., 2001). SWI/SNF components have also been implicated in the transcriptional repression of the *c-fos* gene (Murphy et al., 1999) and the *cad* gene (Pal et al., 2003), of neuronal specific gene expression by the REST transcription factor (Battaglioli et al., 2002) and of CD4 silencing during T cell activation (Chi et al., 2002). Repression of cyclin gene expression by BRG1 is essential for Rb-mediated cell cycle arrest (Strobeck et al., 2000; Zhang et al., 2000), and BRG1 also facilitates repression of E2F-mediated transcription (Wang et al., 2002). The mechanism(s) by which SWI/SNF enzymes promote transcriptional repression has not been well defined, though associated histone deacetylase and methyl transferase

activities are required in at least some cases (Battaglioli et al., 2002; Pal et al., 2003; Zhang et al., 2000). How SWI/SNF directly or indirectly represses each of these down-regulated promoters remains to be determined.

A number of cancer cell lines have been shown to be missing one or more SWI/SNF subunits (DeCristofaro et al., 2001; DeCristofaro et al., 1999; Wong et al., 2000), and targeted disruption of at least two SWI/SNF subunits predisposes mice heterozygous for these genes to tumors (Bultman et al., 2000; Guidi et al., 2001; Klochendler-Yeivin et al., 2000; Roberts et al., 2000). The link between mis-regulation of SWI/SNF subunits, changes in cellular gene expression patterns and tumorigenesis is unclear. Changes in physical as well as metabolic properties are hallmarks of cellular transformation. We were therefore intrigued when we noted that cells expressing ATPase-deficient BRG1 increased in size and changed shape.

Early studies manipulating the surface attachment of cultured cells demonstrated that cell shape could regulate proliferation (Folkman and Moscona, 1978) and gene expression (Ben-Ze'ev et al., 1980; Farmer et al., 1978). These effects involve integrin binding to extracellular matrix (ECM) molecules triggering signaling pathways, but integrin occupancy by ECM is not sufficient (Chen et al., 1997; Huang et al., 1998; Huang and Ingber, 1999). Topographies of ECM that force cells into rounded or flattened shapes are also important regulators of cell cycle progression and survival. While cell shape can be experimentally manipulated by specifying ECM geometry on growth surfaces, the normal determinants of shape have been unknown. We show an experimental system in which cell shape can be regulated by manipulations of the chromatin remodeling machinery in proliferating cells. Expression of mutant SWI/SNF chromatin remodeling enzymes causes increases in area of cell attachment and cell volume together with increases in levels of proteins involved in cell signaling, cell adhesion and cancer metastasis.

Materials and Methods

Cell counts and mRNA and protein analyses

Properties of and growth conditions for cell lines expressing mutant BRG1 and the tet-VP16 control cell lines have been described previously (de la Serna et al., 2000). For experiments described here, cells were passaged into media lacking or containing 2 μg/ml tetracycline and assayed as indicated in the figure legends. For cell counting experiments, cells were trypsinized, washed, counted using a Coulter Counter, and plated in the presence or absence of tetracycline at 50,000 cells per 100 mm dish. Whole cell extracts and western blots were performed as described previously (de la Serna et al., 2000) except for the urokinase-type plasminogen activator receptor (uPAR) blot, for which a RIPA buffer was used to generate extracts, and reducing agent was omitted from the extraction buffer and the SDS gel-loading buffer. Antibodies used for westerns included: α5 integrin and αV integrin (Chemicon; Temecula, CA), rabbit antisera against mouse uPAR (Solberg et al., 2001), rabbit antisera against β1 integrin (Heino et al., 1989), N-cadherin and β-catenin (Zymed; South San Francisco, CA), focal adhesion kinase (LabVision/Neomarkers, Inc.; Fremont, CA), paxillin (BD Pharmingen; San Diego, CA), actin (clone C4; Valeant Pharmaceuticals International, Costa Mesa, CA), and PI3K p85 N-SH2 domain (Upstate; Charlottesville, VA).

Northern blotting was done as described previously (Yan et al., 2002) using stringency washes performed with 0.1× SSC, 0.1% SDS at 65°C. The blot was probed at 65°C with random-primed radiolabeled cDNAs corresponding to nucleotides 80-997 of mouse uPAR. Loading efficiencies were checked by reprobing the blot with a random-prime radiolabeled mouse actin cDNA. The following primer sets were used to amplify mouse actin cDNA using RT PCR: 5′ primer – 5′GTGGGCCGCTCTAGGCACCAA and 3′ primer – 5′CTCTTTCATCTCACGCACGATTTC.

Cell labeling

For measurements of cell surface area attachment, cells growing on coverslips were washed with Hanks' balanced salt solution (HBSS), and 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene OR) in warm HBSS was added for 15 minutes at 37°C. Uptake of dye was stopped by washing cells twice with HBSS. Cells were then re-fed and cultured overnight to allow efflux of unbound CSFE but not CSFE covalently bound to intracellular protein. The cells, still attached to the coverslips, were fixed in 4% formaldehyde in CSK buffer (10 mM Pipes, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA) and mounted in ProLong (Molecular Probes). Surface area attachment was determined from three to five fields each containing 15-20 cells; all cells in a given field were measured. For cell volume measurements, cells were labeled as described above, trypsinized, and live cells were imaged. Images of fields were collected with a Zeiss Axioplan 2 fluorescence microscope with a CCD camera and computer workstation running Metamorph version 3.5. Measurements were made in Adobe Photoshop (version 6.0) using the magnetic lasso tool to outline fluorescent cell areas and the histogram function to count pixels in those areas, or using the line tool to measure cell diameters. For statistical analysis of cell attachment area and volume measurements, a non-parametric test (Mann-Whitney) was utilized since the residuals were not normally distributed.

Papanicolau staining was performed as previously described (Fischer et al., 1998b) in duplicate on cells grown to about 75% confluency on glass coverslips, afterwards the cells were rinsed once with PBS and then immediately fixed without air-drying in 95% Flex alcohol (Richard-Allan Scientific, Kalamazoo, MI). Flex is a proprietary mixture of ethanol, methanol and propanol with 5% water widely used in clinical cytopathology laboratories as a fixative. Blinded comparisons of cells with and without tetracycline induction were performed by a board certified cytopathologist to see if differences could be detected, as previously described (Fischer et al., 1998b). To measure nuclear two-dimensional areas, cells were grown to about 75% confluency on glass coverslips, rinsed with PBS, immediately flooded with 95% Flex alcohol, stained for 15 minutes in DAPI dissolved in absolute methanol at 1 μg/ml, rinsed three times with Flex alcohol, and mounted with DAKO Fluorescent Mounting Medium (Carpenteria, CA). No air-drying took place before or after the DAPI staining. After threshholding to remove background fluorescence, Image Tool Version 3.0 (University of Texas Health Science Center, San Antonio, TX) was used to calculate the number of fluorescent pixels per nucleus of the DAPI-stained cells. The effect of dominant-negative BRG1 expression on nuclear areas was evaluated by the Welch-Aspin t-test for unequal variances (Snedecor and Cochran, 1989). The distributional characteristics of nuclear areas were evaluated graphically by inspection of frequency histograms of residuals and by the Kolmogorov-Smirnov goodness of fit test for normality (Siegel, 1956) again on residuals. The residuals were generated by subtracting group means from the observed areas.

Immunofluorescence staining for F-actin and paxillin was performed as described previously (Wagner et al., 2003) on cells grown on coverslips. Coverslips were fixed and permeabilized, then incubated with monoclonal antibody against paxillin (BD Pharmingen, San Diego, CA) at room temperature for 1 hour, followed by incubation for 1 hour at room temperature with rhodamine-conjugated goat anti-mouse IgG (Molecular Probes). For

co-localization of F-actin with paxillin, FITC-phalloidin (Molecular Probes) at 10 μg/ml was added along with the secondary antibody.

Matrigel invasion assay

The invasion assay was performed as recommended by the manufacturer (BD Biosciences, cat. no. 354481, Bedford, MA). In short, B05-1 cells were grown for 3 days with or without tetracycline. After 3 days, cells were resuspended in medium containing or lacking tetracycline and seeded at a density of 1.25×10^5 cells per well containing a Matrigel insert. Invasion chambers were incubated in a humidified tissue culture incubator, at 37°C, 5% CO_2 atmosphere. After 22 hours, chambers were removed; the cells that had not invaded were swabbed with cotton and washed away, and the invading cells were fixed in 4% formaldehyde and stained with 1% Toluidine for 2 minutes. The cells were scored using a Zeiss microscope at 40× magnification. Fields were chosen in the center of the membrane as well as in the periphery of the membrane for true representation of the cell number throughout the membrane. The data on cell invasions is from duplicates of two independent experiments; 502 cells were counted in total. Results were analyzed by a Mann-Whitney non-parametric test.

Results

We previously described NIH3T3-derived cell lines that are tetracycline regulated to inducibly express ATPase deficient, non-functional alleles of BRG1, the ATPase that forms the catalytic core of some SWI/SNF chromatin remodeling enzymes (de la Serna et al., 2000). B22, B24 and B05-1 cells express ATPase-deficient BRG1 upon removal of tetracycline, while tet-VP16 cells inducibly express only the tet-VP16 trans-activator and therefore serve as a control cell line (de la Serna et al., 2000). Expression of ATPase-deficient BRG1 inhibits the ability of these cells to activate the *hsp70* gene in response to some cellular stresses (de la Serna et al., 2000), and prevents prohibitin-mediated repression of E2F-induced transcription (Wang et al., 2002). Expression of ATPase-deficient BRG1 inhibits the ability of cells to activate tissue-specific gene expression and to trans-differentiate into muscle cells upon introduction of MyoD or related muscle regulatory factors (de la Serna et al., 2001a; de la Serna et al., 2001b; Roy et al., 2002). Similarly, activation of adipocyte-specific markers and trans-differentiation into adipocytes upon introduction of PPARγ, C/EBPα or C/EBPβ is blocked by expression of dominant negative BRG1 (Salma et al., 2004). Thus the mutant BRG1 allele functions as a dominant negative with regard to these gene regulation events, however, the expression of some inducible loci as well as several constitutively expressed genes is unaffected by the mutant enzyme (de la Serna et al., 2000; Hill et al., 2004).

We noted that when equivalent numbers of cells were plated in the presence or absence of tetracycline, the cells expressing mutant BRG1 became confluent faster (Fig. 1a). This observation was most obvious for the B05-1 line, which consistently produced the highest level of mutant BRG1 expression among the cell lines isolated. We expected that these cells were dividing at a faster rate after expression of the mutant allele, but by manual counting of the B05-1 cells shown in Fig. 1a we found that there were 609 cells in the sample grown in the presence of tetracycline and 600 cells in the sample grown without tetracycline. Moreover, cell counts for 3 days after removal of tetracycline showed no effect on the

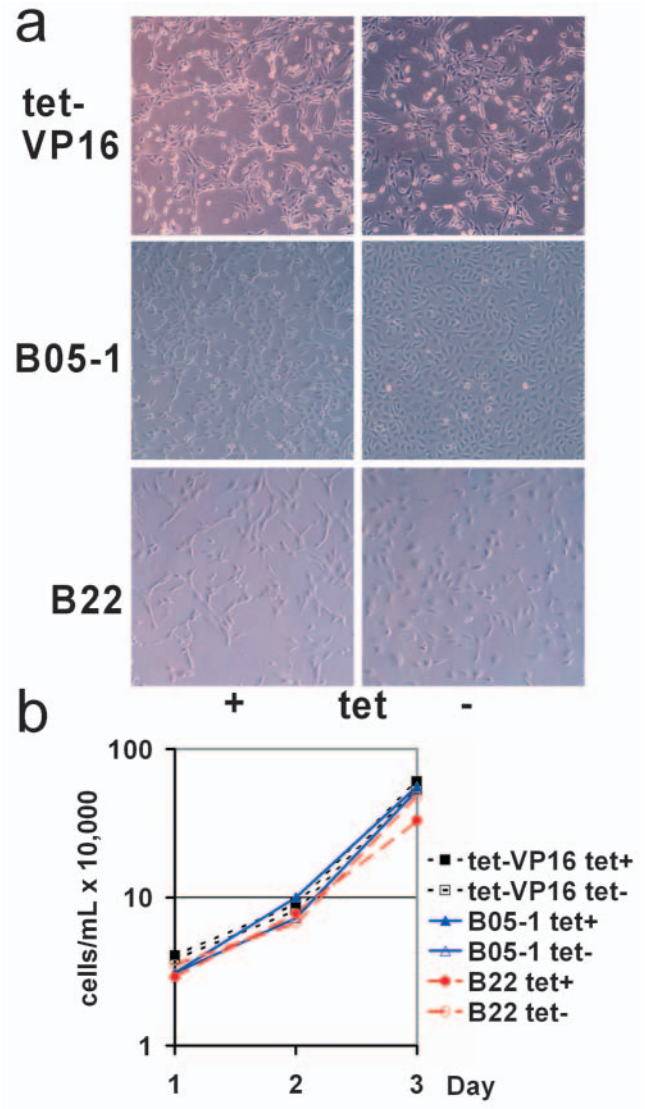

Fig. 1. Changes in cell physical appearance but not in the rate of cell division upon expression of mutant BRG1. (a) Cells expressing mutant BRG1 (–tet) change appearance and reach confluence faster than control cells (+tet). (b) Cell counts for each cell line grown in the presence or absence of tetracycline for 3 days.

rate of cell division (Fig. 1b). FACS analysis confirmed these results (data not shown), however, in some experiments where the cells were lightly trypsinized before fixation, side scatter profiles suggested that cells expressing the mutant BRG1 were larger than those not expressing the mutant allele (data not shown).

To better examine cell size and shape, we grew cells on coverslips in the presence or absence of tetracycline for 3 days. Proteins were fluorescently tagged in living cells with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Fujioka et al., 1994; Lyons and Parish, 1994). Fluorescent images were collected and the area of the attachment surface between the cell and the coverslip was measured using Adobe Photoshop (Fig. 2a; see Materials and Methods). No difference in attachment area was observed for the control tet-VP16 cells in the presence or absence of tetracycline (Table 1). The mean

cell attachment area of B05-1 cells expressing mutant BRG1 (n=61) was 2.96 times larger than that of control B05-1 (Table 1: n=65; $P<10^{-19}$ by Mann-Whitney non-parametric test). The distribution of attachment areas for individual B05-1 cells is presented in Fig. 2b.

Subsequently, we obtained images of similarly stained, trypsinized cells, measured cell diameters and calculated the approximate cell volume using the formula for volume of a sphere. The data indicated a 1.69-fold increase in total cell volume for B05-1 cells expressing mutant BRG1 (n=56) relative to control B05-1 cells (Table 1: n=60; $P<5\times10^{-12}$ by Mann-Whitney non-parametric test) and no difference for the tet-VP16 cells grown in the absence or presence of tetracycline (Table 1). The distribution of cell volumes for individual B05-1 cells is presented in Fig. 2c.

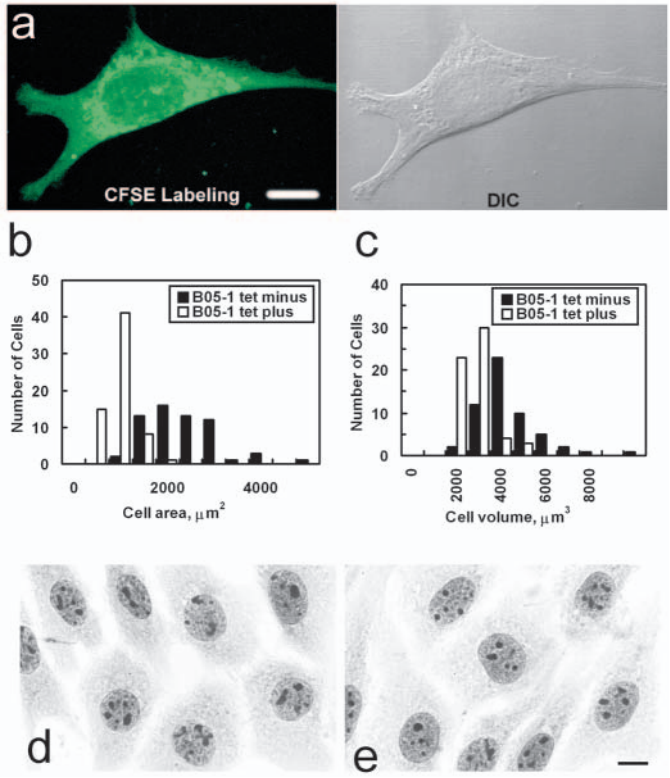

Fig. 2. Mutant BRG1 causes an increase in the size of the cell and the nucleus. (a) Image of a CSFE-labeled cell (left) and the corresponding DIC image (right). Bar, 10 μm. (b) Distribution of cell surface attachment area (in μm^2) for B05-1 cells grown in the presence (n=65) or absence (n=61) of tetracycline for 3 days. Cells were at 20-25% confluence to permit examination of individual cells. (c) Distribution of cell volume in (in μm^3) for B05-1 cells grown to 20-25% confluence in the presence (n=60) or absence (n=56) of tetracycline for 3 days. (d,e) B05-1 cells grown in the presence (d) or (e) absence of tetracycline for 3 days were Papanicolaou stained to examine the appearance and size of the nucleus. Bar, 10 μm.

To examine nuclear size in these cells, we stained cells with DAPI, collected digital images, and measured the nuclear two-dimensional area. Average surface area of the nuclei of cells grown in the presence of tetracycline (n=239) was 198±58 μm^2 and of those grown in the absence of tetracycline (n=158) was 228±51 μm^2. Thus, the nuclear area of cells expressing mutant BRG1 was about 10% larger (Table 1; $P<10^{-7}$ by Welch-Aspin t-test). Detailed examination of heterochromatin staining in Papanicolau-stained cells indicated no differences in large-scale heterochromatin organization due to the expression of mutant BRG1 (Fig. 2d,e) such as has been noted for oncogene-transformed cells (Fischer et al., 1998a; Fischer et al., 1998b). No changes in nucleolar features or in either the basophilia or texture of the cytoplasm were observed. Thus, expression of the mutant BRG1-based SWI/SNF enzymes induced a change in the size of both the cells and the nuclei but did not grossly alter heterochromatin organization or other cytological features.

Given the change in cell size and shape observed in the B05-1 cells, we examined focal adhesions to determine whether they differed in the larger cells. Paxillin is recruited to focal adhesions upon interaction with integrins and is localized at the end of actin stress fibers. Fluorescent images of B05-1 cells doubly stained with phalloidin and an antibody against paxillin revealed that paxillin-containing structures were more numerous, more elongated, and stained more intensely in the larger cells expressing the mutant BRG1 allele (Fig. 3a-b). Nuclear staining was observed with the paxillin antibody; it has been reported that paxillin shuttles between the nucleus and cytoplasm in NIH3T3 cells (Woods et al., 2002). Nuclei staining by both the paxillin antibody and DAPI (Fig. 3g-h) also confirmed that the expression of mutant BRG1 increased nuclear size. The pattern of actin stress fiber staining was largely unchanged (Fig. 3c-d), and western analysis showed that cellular levels of actin and paxillin protein were similar for each cell line irrespective of growth conditions (Fig. 3i). Thus the change in cell attachment area correlates with a change in

Table 1. Effects of mutant BRG1 expression on cell and nuclear size

	Tet-VP16 + tetracycline			Tet-VP16 – tetracycline			
	n	Mean	s.d.	n	Mean	s.d.	P*
Cell area μm^2	43	937	452	31	998	488	NS
Cell vol. μm^3	18	2323	1262	18	2136	1379	NS
Nuc. area μm^2		ND			ND		
	B05-1 + tetracycline			B05-1 – tetracycline			
	n	Mean	s.d.	n	Mean	s.d.	P*
Cell area μm^2	65	703	307	61	2078	800	<10E-19
Cell vol. μm^3	60	2273	227	56	3853	441	<10E-11
Nuc. area μm^2	239	198	58	158	228	51	<10E-7

ND, not determined; NS, not significant.
*See Materials and Methods and text for statistical analysis.

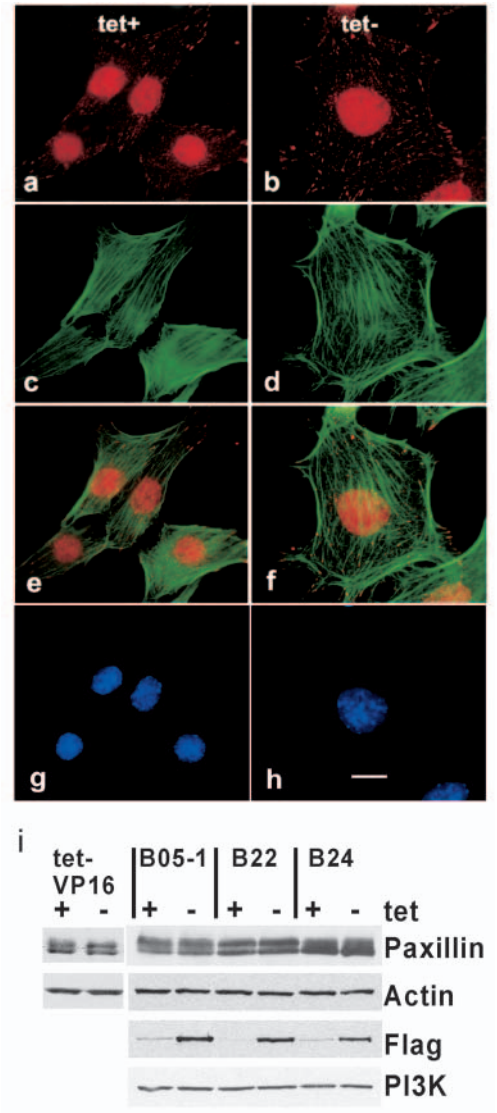

Fig. 3. Expression of mutant BRG1 causes a change in the size and distribution of focal adhesions but not in paxillin or actin protein levels. Immunostaining for (a,b) paxillin, (c,d) phalloidin, (e,f) the merged images of a,c and b,d, respectively, and (g,h) DAPI staining in B05-1 cells grown to approximately 50% confluence in the presence (a,c,e,g) or absence (b,d,f,h) of tetracycline for 3 days. Bar, 10 μm. (i) Western blot demonstrates that total cellular paxillin and actin levels are unchanged by tetracycline or expression of mutant BRG1 in three different cell lines. Flag immunoreactivity demonstrates that the mutant BRG1 was expressed.

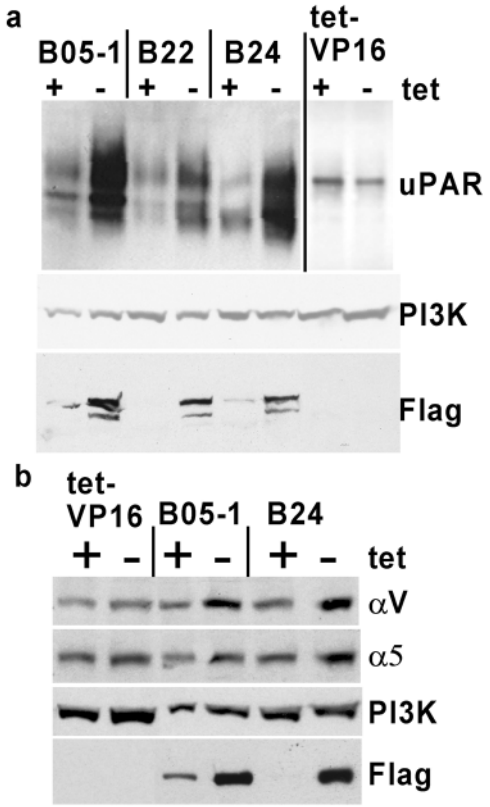

Fig. 4. Cells expressing mutant BRG1 show increased levels of some adhesion proteins. Western blots against urokinase receptor (a) and αV and α5 integrins (b) indicate increased levels in each of the three cell lines expressing mutant BRG1. Phosphatidylinositol 3-kinase (PI3K) levels were monitored as a loading control; Flag levels indicate the level of mutant BRG1 protein expressed in each line. The tet-VP16 samples probed for uPAR levels were run on a separate gel and spliced into the figure.

focal adhesion structure and distribution but not with changes in levels of paxillin.

We next asked whether the larger cells had increased levels of proteins involved in cell adhesion. Surprisingly, we observed that protein levels for the urokinase-type plasminogen activator receptor (uPAR) were greatly increased in the cells expressing mutant BRG1 (Fig. 4a). uPAR binds urokinase, a serine protease that affects multiple components of the extracellular matrix (Wang, 2001). uPAR levels are activated upon drug-induced disruption of the actin or tubulin cytoskeleton (Bayraktutan and Jones, 1995), and uPAR is frequently overexpressed in metastatic tumors. Additionally, uPAR binds to vitronectin and several members of the integrin family, underscoring a role for uPAR in cell adhesion (Wei et al., 1996; Yebra et al., 1996). We therefore examined the levels of several integrins, as well as other attachment proteins. αV and α5 integrins were modestly overexpressed in cell lines after expression of mutant BRG1 (Fig. 4b). No change in expression levels was observed for β_1 integrin, N-cadherin, β-catenin, or focal adhesion kinase (data not shown).

Since uPAR overexpression is associated with metastases, we addressed whether the cells expressing dominant negative BRG1 also might express elevated levels of proteins associated with extracellular matrix invasion. As a functional readout, we utilized a Matrigel invasion assay. B05-1 cells grown in the presence or absence of tetracycline were seeded onto Matrigel-containing chambers and the number of invading cells was determined. Cells expressing dominant negative BRG1 did not show increased invasiveness; in fact, a twofold decrease was observed (Fig. 5). We conclude that the elevated levels of proteins known to be involved in cell adhesion do not correlate with an increase in extracellular matrix invasive properties.

Since the levels of uPAR protein were misregulated in cells expressing dominant negative BRG1, we sought to determine

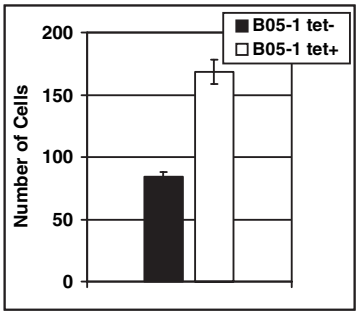

Fig. 5. Expression of ATPase-deficient BRG1 significantly reduces the invasion of Matrigel membrane by B05-1 cells ($P<0.05$, by Mann-Whitney non-parametric test). The average number of invading cells is presented ±s.d.

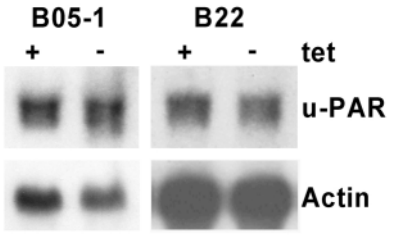

Fig. 6. uPAR mRNA levels are not misregulated by dominant negative BRG1. Northern blot analysis shows levels of uPAR and β-actin mRNAs in two different lines (B05-1 and B22) that express dominant negative BRG1 upon removal of tetracycline.

whether this was a direct consequence of inhibition of uPAR transcription. Northern blot analysis revealed that levels of uPAR mRNA were not significantly affected by the presence of dominant negative BRG1 (Fig. 6). We conclude that the misregulation of uPAR levels in cells expressing dominant BRG1 occurs via an indirect mechanism.

Discussion

Changes in cell size and shape have been correlated with changes in cellular proliferation and gene expression. However, most studies seeking to alter cell size and shape have done so via manipulation of the attachment surface. Here, we present evidence that expression of a mutant chromatin remodeling enzyme, which previously has been documented to act as an inhibitor of several gene activation events, results in an increase in cell size and attachment area that correlates with over-expression of several proteins involved in cell adhesion. To our knowledge, this system for manipulating cell size and shape is novel in that it occurs via manipulation of a specific protein, without the induction of cellular differentiation or transformation or arrest of the cell cycle, and without alteration of the composition or size of the cell attachment surface.

Previous work has demonstrated that re-introduction of BRG1 into the BRG1- and hBRM-deficient, human adrenal adenocarcinoma tumor line, SW13, induces cell cycle arrest and the formation of cells that are larger and flatter than untransfected cells (Asp et al., 2002; Dunaief et al., 1994; Shanahan et al., 1999; Strober et al., 1996). This phenotype was shown to be dependent on the retinoblastoma (RB) tumor suppressor, as BRG1 physically interacted with RB and use of an RB-binding deficient BRG1 allele significantly attenuated the phenotype (Dunaief et al., 1994). Further work demonstrated similar findings for the related hBRM protein, though multiple hBRM mutants, including the ATPase-deficient mutants, caused similar attenuation of the growth-arrested, flat cell phenotype (Strober et al., 1996). Thus, in these experiments, the presence of BRG1, not the absence of Brg1 function, induced cell cycle arrest. The interpretation put forth was that BRG1 and hBRM were demonstrating tumor suppressor activity because of the induced cell cycle arrest. The mechanism resulting in the induction of growth arrest and the flat cell phenotype upon re-introduction of BRG1/hBRM remains undetermined.

However, it has long been appreciated that cell growth continues when cell division is blocked (Johnston et al., 1977). Thus it is possible, if not likely, that transient re-introduction of BRG1 or hBRM under the control of a heterologous promoter resulted in relatively high levels of BRG1 or hBRM that induced cell cycle arrest. The resulting increase in cell size under such conditions is probably a continuation of metabolic pathways controlling cell growth that only cease when cell division fails. Recent studies have shown that cell growth, even in the absence of cell division, can be affected by mammalian TOR protein, a highly conserved member of the phosphatidylinositol kinase family (Fingar et al., 2002). This situation clearly differs from the observations we have made. In our cells, a lack of Brg1 function induces changes in cell size and shape without apparently affecting cell cycle, strongly suggesting that different molecular mechanisms are involved.

Recently, two other groups have reported alterations in cell morphology in response to re-introducing SWI/SNF subunits into tumor cell lines deficient for that specific subunit. Asp et al. succeeded in isolating SW13 clones stably transfected with BRG1 that could continue to divide for a finite number of divisions (Asp et al., 2002). These clones also exhibited flat cell morphology, consistent with the previous studies described above. SW13 clones expressing ATPase-deficient BRG1 could be propagated without inducing cell cycle arrest. These authors noted that the BRG1-expressing SW13 cells formed thick actin filaments that resembled stress fibers in the cell body, whereas SW13 cells and SW13 cells expressing ATPase-deficient BRG1 did not have such structures and showed only thin actin filaments that were abundant at the cell periphery. In contrast, the changes in cell size and shape that we observed were not accompanied by changes in the filamentous actin structure in our cells (Fig. 3i). Again, this suggests that the changes in cell structure observed upon re-introduction of BRG1 in SW13 cells differs from the mechanisms by which BRG1 affects cell size and shape in proliferating fibroblastic cell lines.

In addition, Medjkane and co-workers constructed cell lines that inducibly expressed the hSNF5/INI1 subunit of the SWI/SNF complex in hSNF5/INI1-deficient tumor cells (Medjkane et al., 2004). Upon re-expression of hSNF5/INI1, the cells underwent cell cycle arrest and appeared more rounded and smaller. This observation is consistent with our results: hSNF5/INI1 deficient cells and cells expressing dominant negative BRG1 are larger than their counterparts expressing wild-type hSNF5/INI1 or BRG1. Moreover, these authors observed a disappearance of paxillin-stained focal

adhesions upon expression of hSNF5/INI1. However, as with the results of Asp and co-workers (Asp et al., 2002), these authors observed changes in actin cytoskeleton structure, as restoration of hSNF5/INI1 expression induced a loss of actin stress fibers. The disparate findings regarding differences in actin cytoskeletal staining may reflect cell-type-specific differences.

The absence of a grossly altered large-scale chromatin organization when mutant SWI/SNF enzymes are expressed suggests that SWI/SNF has a more focused role in chromatin remodeling than other factors that cause large-scale changes in heterochromatin organization. The observation that SWI/SNF function is inhibited by the incorporation of linker histone into chromatin templates further supports this idea (Hill and Imbalzano, 2000; Horn et al., 2002). Moreover, recent temporal analyses of inducible gene expression events in mammalian cells indicates that SWI/SNF enzymes function in promoter modification immediately preceding and concurrent with transcription initiation (Agalioti et al., 2000; Salma et al., 2004; Soutoglou and Talianidis, 2002). Collectively, these observations suggest that SWI/SNF enzymes function to alter the structure and accessibility of chromatin at the level of individual genes, not at the level of global chromatin structure.

Throughout the eukaryotic kingdom, SWI/SNF enzymes selectively affect gene expression, both positively and negatively (Holstege et al., 1998; Liu et al., 2001; Sudarsanam and Winston, 2000), though the basis for promoter selectivity is not known. Thus, the fact that some, but clearly not all, adhesion proteins are misregulated upon expression of mutant BRG1-based SWI/SNF enzymes is in agreement with previous results. Over-expression of adhesion proteins upon induction of the mutant SWI/SNF enzymes suggests that the normal function of the enzyme is to negatively regulate levels of these proteins. However, the northern blot presented in Fig. 6 strongly suggests that, at least for the case of uPAR expression, BRG1-containing SWI/SNF enzymes negatively affect gene expression via an indirect mechanism. The pathway(s) through which this is accomplished remain to be determined. Global analysis of changes in gene expression due to the expression of ATPase-deficient BRG1 may help to address this question.

Interestingly, mice heterozygous for BRG1 are susceptible to tumor formation (Bultman et al., 2000), and BRG1 is absent from a number of different tumor cells lines (DeCristofaro et al., 2001; Wong et al., 2000). We hypothesize that the tumor suppressor function of BRG1 may be related to its role in regulating the levels of surface attachment molecules and the interactions they make with their extracellular environment. Misregulation of cell attachment properties may be one step by which BRG1-deficient or mutant cells promote tumorigenesis.

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