Two related ARID family proteins are alternative subunits of human SWI/SNF complexes

Xiaomei WANG*, Norman G. NAGL, JR*, Deborah WILSKER*, Michael VAN SCOY[1], Stephen PACCHIONE*, Peter YACIUK†, Peter B. DALLAS*[1] and Elizabeth MORAN*[2]

*Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, PA 19140, U.S.A., and †St. Louis University School of Medicine, St. Louis, MO 63104, U.S.A.

p270 (ARID1A) is a member of the ARID family of DNA-binding proteins and a subunit of human SWI/SNF-related complexes, which use the energy generated by an integral ATPase subunit to remodel chromatin. ARID1B is an independent gene product with an open reading frame that is more than 60 % identical with p270. We have generated monoclonal antibodies specific for either p270 or ARID1B to facilitate the investigation of ARID1B and its potential interaction with human SWI/SNF complexes *in vivo*. Immunocomplex analysis provides direct evidence that endogenous ARID1B is associated with SWI/SNF-related complexes and indicates that p270 and ARID1B, similar to the ATPase subunits BRG1 and hBRM, are alternative, mutually exclusive subunits of the complexes. The ARID-containing subunits are not specific to the ATPases. Each associates with both BRG1 and hBRM, thus increasing the number of distinct subunit combinations known to be present in cells. Analysis of the panels of cell lines indicates that ARID1B, similar to p270, has a broad tissue distribution. The ratio of p270/ARID1B in typical cells is approx. 3.5:1, and BRG1 is distributed proportionally between the two ARID subunits. Analysis of DNA-binding behaviour indicates that ARID1B binds DNA in a non-sequence-specific manner similar to p270.

Key words: ARID family, ARID1B, BRG1, p270, SWI/SNF.

INTRODUCTION

SWI/SNF-related complexes are chromatin-remodelling complexes that play fundamental roles in the regulation of gene expression during cell growth and development in all eukaryotes. Individual human SWI/SNF complexes contain at least eight identified proteins, but these complexes are not completely defined and they can exist in multiple forms (see [1–4] for recent reviews). However, an essential component of the complexes is an ATPase of the SWI2/SNF2 family. Human complexes can contain either of the two related but distinct ATPases, BRG1 and hBRM [5,6].

Human SWI/SNF complexes also include a large subunit that contains a DNA-binding domain of the ARID family. This 270 kDa protein, p270, shares antigenic specificity with the chromatin-modifying histone acetyltransferases p300 and CBP [7,8], although p270-containing complexes do not show histone acetyltransferase activity [8]. Analysis of p270-associated proteins revealed that p270 is a component of human SWI/SNF complexes, and cloning of the p270 cDNA suggested that p270 is an orthologue of yeast SWI1 [8,9]. Cloning of a BRG1-associated factor, designated BAF250, with a cDNA sequence co-linear with p270 independently confirmed the presence of p270 in the complexes [10].

p270 is expressed in all the human tissues examined [9,10]. The most prominent feature seen in the p270 open reading frame is the presence of an ARID DNA-binding domain. This is a recently defined helix–turn–helix-based domain, typical of a family that includes at least 15 distinct human proteins suggested to play a role in the regulation of development, tissue-specific gene expression and/or cell proliferation (reviewed in [11–13]). p270 binds linear duplex DNA depending on the integrity of the ARID consensus sequence [9]. Some ARID family members bind selectively to AT-rich sequences, a behaviour that prompted the acronym ARID (AT-rich interactive domain); in contrast, p270 and its closest *Drosophila* counterpart, Osa, do not select oligonucleotides of any preferred sequence from a random pool [9,13,14]. Other recognizable features in p270 include glutamine-rich regions and multiple LXXLL motifs (where L stands for leucine and X for any amino acid), which are generally indicative of a potential for association with liganded nuclear hormone receptors [15,16]. The p270 (synonym: BAF250) subunit is one of many components of SWI/SNF complexes that appear to interact directly with the glucocorticoid receptor [10,17], and the exogenously introduced expression of p270 can stimulate expression from a glucocorticoid receptor-dependent reporter construct [10,18].

A specific search for large open reading frames expressed in the human brain revealed a partial cDNA, designated KIAA1235, which is closely related to p270 [19]. This cDNA has been isolated and its characterization has begun in several laboratories under various names (p250R, hELD/OSA1, hOSA2 or BAF250B; [18,20–22]). The Human Genome Organization Gene Nomenclature Committee and the Mouse Genomic Nomenclature Committee have recently recommended that ARID family members carry gene designations that reflect their relationship. According to this scheme, the p270 and KIAA1235 genes, which map to the chromosomal loci 1p35.3 and 6q25.1 respectively, are designated *ARID1A* and *ARID1B* respectively. Since the *ARID1B* gene product does not have a widely accepted common name, we adopt the latter designation in the present study for both the gene and the protein.

Abbreviations used: Dri, *Drosophila* deadringer; CMV, cytomegalovirus; DTT, dithiothreitol; GST, glutathione S-transferase; mAb, monoclonal antibody; NP40, Nonidet P40; poly(A)$^+$, polyadenylated.

[1] Present address: TVW Telethon Institute for Child Health Research and the Center for Child Health Research, University of Western Australia, Subiaco, Western Australia 6008, Australia.

[2] To whom correspondence should be addressed (email betty@temple.edu).

The apparent full-length sequence of ARID1B is described in Nie et al. [22]. ARID1B and p270 are more than 60 % identical across their entire lengths. The ARID consensus is intact in ARID1B, but the exons encoding the glutamine-rich regions are shorter, essentially eliminating the pattern of glutamine enrichment. The pattern of LXXLL motifs is also somewhat different. Regardless of these differences, ARID1B can activate the expression from androgen, oestrogen and glucocorticoid receptor-dependent reporter constructs in co-transfection assays [18].

Although p270 and ARID1B behave similarly in transient transfection assays, their patterns of expression differ during early primate development, suggesting that the proteins have different functions *in vivo* [23]. A central question in the composition of the complexes is whether p270 and ARID1B are present together, similar to the closely related proteins BAF170 and BAF155, or whether they are mutually exclusive, similar to the alternative ATPases, BRG1 and hBRM. This issue has been examined, but not resolved. One report [18] concluded from *in vitro* association assays and immunocomplex analysis that both ATPases associate with ARID1B (synonym: hOSA2). However, this study did not establish whether p270 and ARID1B are in separate complexes. Another study, using immunocomplex analysis, concluded that p270 and ARID1B (synonym: BAF250b) are in separate complexes, but MS analysis of the associated proteins led these investigators to conclude that ARID1B associates only with BRG1 and not with hBRM [22].

We have generated mAbs (monoclonal antibodies) specific for either p270 or ARID1B to facilitate comparative analysis of these proteins *in vivo*. The immunocomplexes demonstrate directly that endogenous ARID1B is associated with SWI/SNF complexes and indicate that p270 and ARID1B, similar to BRG1 and hBRM, are alternative, mutually exclusive subunits of the complexes. The ARID-containing subunits are not specific to the ATPases. Each associates with both BRG1 and hBRM, thus increasing the number of distinct subunit combinations known to be present in cells. Analysis of panels of cell lines indicates that ARID1B, similar to p270, has a broad tissue distribution. The ratio of p270 to ARID1B in typical cells is approx. 3.5:1, and BRG1 is distributed proportionally between the two ARID subunits. Analysis of DNA-binding behaviour indicates that ARID1B binds DNA in a non-sequence-specific manner similar to p270.

MATERIALS AND METHODS

Immunoprecipitation

Cells were harvested in PBS, pelleted and lysed in Tris lysis buffer [250 mM NaCl, 0.1 % NP40 (Nonidet P40), 40 mM Tris (pH 7.4) and 1 mM DTT (dithiothreitol), supplemented with the following protease inhibitors at a final concentration as indicated: aprotinin (1.0 μg/ml), leupeptin (1.0 μg/ml) and pepstatin (1.0 μg/ml)]. Immunoprecipitation was performed as described previously [8].

Immunoblotting

Preconfluent cells were harvested in PBS. Cells were lysed, and proteins were separated on 8 % gels, transferred on to an Immobilon-P membrane (Millipore) and visualized as described previously [8] or by using a chemiluminescence system (NEL602; NEN).

Northern blots

Poly(A)$^+$ (polyadenylated)-selected RNA was prepared from appropriate cell cultures using TRI Reagent (Sigma), and PolyATract mRNA Isolation System (Promega) according to the supplier's instructions. Then, 18 μg of RNA was loaded per lane and fractionated by electrophoresis on a 0.7 % formaldehyde–agarose gel. The RNA was transferred on to a Hybond-N nylon membrane (Amersham Biosciences, Arlington Heights, IL, U.S.A.) and cross-linked by UV irradiation and baking at 80 °C. ^{32}P-labelled probes were prepared using a random-primed labelling kit (Boehringer Mannheim). Between successive probes, blots were stripped by boiling in 0.1 % SDS. The specificity of the probes was verified by hybridization with plasmid DNA under the same conditions.

Probes

A p270 probe hybridizing to the middle region of the 270 kDa protein was generated by PCR from the plasmid pNHXSS98, which contains the p270 cDNA sequence reported in [9], using the primer sequences TACCAGCAGAACTCCATGGGGAGCTAT and TTTCTTGGGTTTTCCGGTTCATGC. An ARID1B probe hybridizing to the corresponding region of ARID1B was generated by PCR from the plasmid pfh08704 (a gift from Dr T. Nagase, Kazusa DNA Research Institute, Kisarazu, Chiba, Japan), which contains the ARID1B cDNA sequence from residues 1 to 5834 (according to accession number AB033061) in a pBluescript II vector, using the primer sequences TTCAGCAGAGTAACTCAAGTGGGAC and TTACGGTTCACAGTTGGCATT. The β-actin probe was described previously [24].

Cell lines

HeLa, C33A, SW13, MCF7 and MDA-MB-435s cells were obtained from the A.T.C.C. PC-3, DU-145 and TSU-Pr1 cells were gifts from B. Lokeshwar (University of Miami School of Medicine, Miami, FL, U.S.A.). Saos-2, U2-OS, MG 63, and OHS 50 cells were gifts from M. F. Hansen (Center for Molecular Medicine, University of Connecticut Health Center Graduate School, Farmington, CT, U.S.A.). All cell lines were cultured according to A.T.C.C. recommendations.

Antibodies

The p270 mAb, PSG3, was raised against the pNDX2 GST (glutathione S-transferase)-fusion protein described previously [9]. The fusion protein contains 419 residues from the mid-portion of p270 and also contains the ARID. The BRG1-specific mAb, mAb 320.7, was raised against an N-terminal peptide sequence, STPDPPLGGTPRPG(T), corresponding to residues 2–15 of human BRG1. The BAF155-specific mAb, DXD7, was raised against a peptide sequence, EKPVDLQNFGLRTDIYSK(C), corresponding to residues 591–608 in the BAF155 sequence. The ARID1B mAb, KMN1, was raised against a GST-fusion protein product containing a portion of ARID1B roughly analogous to the p270 antigen. The fusion protein includes an optional additional ARID1B exon sequence noted in some cDNA versions; the amino acid sequence corresponds to residues 1–422 of accession number BAA86549, which is a middle region of ARID1B containing the ARID consensus. All hybridoma isolation work was performed at the St. Louis University Hybridoma Facility. A BRM-derived antibody was purchased from Transduction Laboratories (B36320).

Expression plasmids

The *in vitro* translated constructs used in the antibody tests are pNHXSS98 (which expresses p270 residues 471–2285 according to accession number NM_006015) and pKM5 (which expresses the C-terminal portion of ARID1B corresponding to residues 1–1485 in the partial-sequence accession number BAA86549

described above). The p270 *in vitro* translation plasmid NE9-B2 used for the DNA-binding assay has been described previously [13]; it expresses amino acid residues 901–1187, including the ARID consensus, which extends from residue 1013 to 1107. The ARID1B *in vitro* translation plasmid KM20 used for the DNA-binding assay contains an insert derived by PCR from the KM15 plasmid described previously [13] in the pCR2.1-TOPO vector (Invitrogen). The KM20 insert extends from 2003 to 2864 bp and expresses amino acids 658–944, including the ARID consensus, which extends from residue 768 to 864 (accession number AF253515). The GST-fusion protein constructs are p410 [*Drosophila* deadringer (Dri)] [25], pNDX (p270) [9] and pKM19. Plasmid KM19 contains an ARID1B insert in a pGEX-4T-1 vector (Amersham Biosciences); the insert extends from 2174 to 3292 bp and expresses residues 715–1087, according to the same accession numbers cited above. The CMV (cytomegalovirus)-promoted mammalian expression constructs used were pNXCMV, which expresses p270 residues 471–2285 (according to accession number NM_006015), and pKM12. The pKM12 plasmid was generated by ligating a 3530-bp *Sal*I–*Not*I restriction fragment of plasmid KIAA1235 (accession number AB033061; a gift from Dr T. Nagase) into the backbone of *Sal*I–*Not*I-digested plasmid HRC04412 (accession number AK025945, a gift from Dr S. Sugano, Institute of Medical Science, The University of Tokyo). The resulting pKM12 plasmid expresses a 1706-residue C-terminal portion of ARID1B, and includes the optional additional 52-residue sequence described above.

In vitro expression

p270 and ARID1B cDNA fragments in appropriate plasmid vectors were used to generate [^{35}S]methionine-labelled polypeptides using the TNT-coupled reticulocyte system (Promega).

Sequence-specific selection of DNA

GST-fusion protein pull-down assays were performed as described previously [13,14]. Restriction fragments were filled in with [α-^{32}P]dATP. Labelled DNA (0.8 μg) was incubated with 100 ng of GST-fusion protein bound to glutathione–agarose beads for 1 h at 4 °C in Lambda DNA-binding buffer [20 mM Hepes, pH 7.6, 1 mM EDTA, pH 8, 10 mM $(NH_4)_2SO_4$, 0.2 % Tween 20, 1 mM DTT, 25 μg/ml BSA and 25 μg/ml poly(dI-dC)·(dI-dC)] containing varying amounts of KCl, as indicated in the text. The beads were washed three times with Lambda DNA-binding buffer without DTT, BSA and poly(dI-dC)·(dI-dC). Bound DNA was eluted by boiling in formamide loading buffer (90 % formamide, 1 × TBE, 0.04 % Bromophenol Blue and 0.04 % xylene cyanol), separated on a 6 % sequencing gel and visualized by autoradiography.

DNA–cellulose chromatography

In vitro translated proteins were diluted in 1 bed volume (0.5 ml) of column loading buffer [10 mM potassium phosphate, pH 6.2, 0.5 % NP40, 10 % glycerol, 1 mM DTT, 1 mg/ml aprotinin, 1 mg/ml pepstatin and 1 mg/ml leupeptin], and applied to native DNA–cellulose columns (Amersham Biosciences). The protein sample was passed through the column four times. Unbound material is designated as flow-through. The columns were then washed several times with 1.0 bed volume of column loading buffer containing 50 mM NaCl (these are the 50 mM wash fractions) and then eluted stepwise with column loading buffer adjusted to contain increasing concentrations of NaCl from 100 to 800 mM, as indicated in the text. Fractions were analysed by SDS/PAGE. The signal on the dried gel was quantified using a PhosphoImager (Fuji) and associated software. The signal in each fraction was plotted as a percentage of the total recovered.

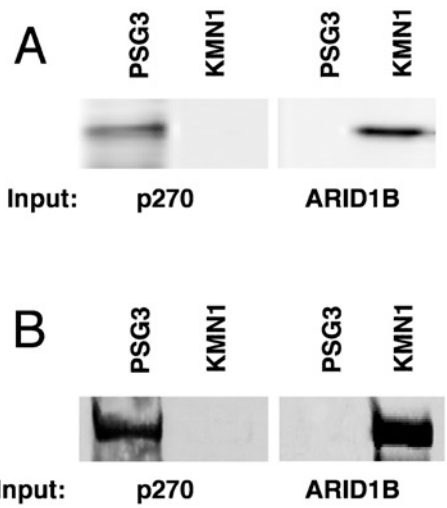

Figure 1 Specificity of mAb raised against p270 and the ARID1B protein

(**A**) *In vitro* translated peptides containing the antigenic regions used to generate each of the mAbs were immunoprecipitated with either mAb PSG3 or mAb KMN1 as indicated. The origin of the *in vitro* translated product, either p270 or ARID1B, is shown as 'input' below each lane. (**B**) CMV-promoted expression vectors for portions of p270 and ARID1B, containing the antigenic regions used to generate each of the mAbs, were transiently expressed in HEK-293 cells (human embronic kidney 293 cells). Cell lysates were collected and immunoprecipitated with mAb PSG3 or KMN1, as indicated above each lane. The immunocomplexes were separated by SDS/PAGE and transferred for Western blotting. The specificity of the mAb used in the Western blot matches the origin of the mammalian expression product, shown as 'input' below each lane.

RESULTS

Generation of mAbs that distinguish the p270 and ARID1B proteins

The high degree of identity (over 60 % at the amino acid level) between p270 and ARID1B means that polyclonal antibodies may not distinguish these products clearly *in vivo*. Therefore we raised mAbs to each using GST-fusion proteins as antigens. After preliminary characterization of the resulting hybridoma cell lines, a p270-specific antibody-secreting line designated PSG3 and an ARID1B-specific antibody-secreting line designated KMN1 were selected. Immunoprecipitation and Western blotting of the respective products expressed *in vivo* and *in vitro* verified that the antibodies do not cross-react (Figure 1).

Expressions of p270 and ARID1B

Antibodies were used to screen the expression of p270 and ARID1B in a panel of common laboratory cell lines (Figure 2). These included HeLa (cervical carcinoma), SW13 (adenocarcinoma), PC-3 and DU145 (prostate cancer), Saos-2, MG 63, U2OS and OHS 50 (osteosarcomas), MCF-7 and MDA-MB-435s (breast carcinomas) and TSU-Pr1 (formerly considered to be a prostate carcinoma cell line; recently identified by Van Bokhoven et al. [26] as a derivative of the T24 bladder carcinoma cell line). ARID1B is detectable in each of these cell lines, but the signal is weaker than the p270 signal. Reaction of each of the antibodies against the respective purified GST-fusion proteins (not shown) indicates that the antibody signals are proportional to the respective amounts of proteins. The Western-blot signals, therefore, indicate that expression levels of ARID1B are generally

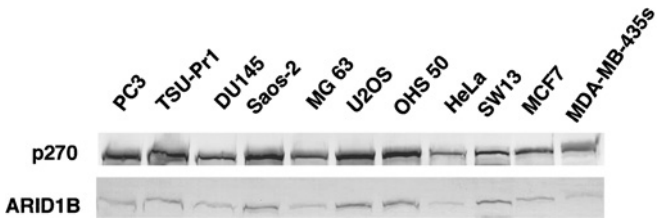

Figure 2 Western-blot analysis of total cell lysates of selected tumour cell lines: distribution of ARID1B expression

Aliquots (150 μg) of each of the various cell lysates were separated by SDS/PAGE, transferred on to a PVDF membrane and probed with antibodies specific to either p270 or to the ARID1B protein, as indicated in the Figure.

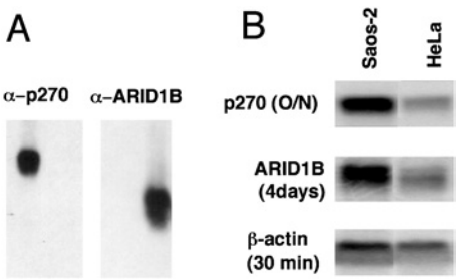

Figure 3 Northern-blot analysis of ARID1B expression

(**A**) p270 plasmid NHXSS98 (100 ng; lane 1) and ARID1B plasmid pKM10_v1 (lane 2) were separated in duplicate by agarose-gel electrophoresis, transferred on to a Hybond-N nylon membrane and hybridized separately with either the p270- or the ARID1B-specific probes, as indicated, to verify the specificity of the probes. (**B**). Aliquots of poly(A)$^+$-selected RNA were separated by electrophoresis, transferred on to membranes and hybridized with the p270- or ARID1B-specific probes, as indicated in the Figure.

lower than p270, although tissue distribution is widespread. Expression of ARID1B is generally strongest in the osteosarcoma cell lines, but there is variability.

To obtain a more quantitative comparison, expression levels in representative cell lines were also examined by Northern blotting (Figure 3). Probes of comparable size from directly comparable regions of each cDNA were used to maximize the utility of the comparison. The specificity of the Northern-blot probes was verified against isolated cDNA under the same conditions as the Northern blot (Figure 3A). RNA was prepared by poly(A)$^+$ selection to optimize the clarity of the signal. The same blot was probed successively for p270, ARID1B and β-actin. To obtain a comparable signal, the ARID1B probe had to be exposed approx. four times longer than the p270 probe (Figure 3B). The RNA signals were roughly proportional to the protein signals, with expression consistently higher in Saos-2 cells when compared with, for example, HeLa cells. From quantification of these and other cell lines (not shown), we estimate the message level of p270 to be 3–4-fold higher than that of ARID1B. The Northern blots indicate a message size for ARID1B approx. 1 kb shorter than that of p270.

ARID1B and p270 are mutually exclusive components of human SWI/SNF-related complexes

Saos-2 cells were used to probe directly whether the endogenous ARID1B protein is associated with human SWI/SNF complexes. An antibody to BAF155, a ubiquitous component of the SWI/SNF family of complexes (composition reviewed in [4]), was used to pull down the complexes from Saos-2 cells where ARID1B is well

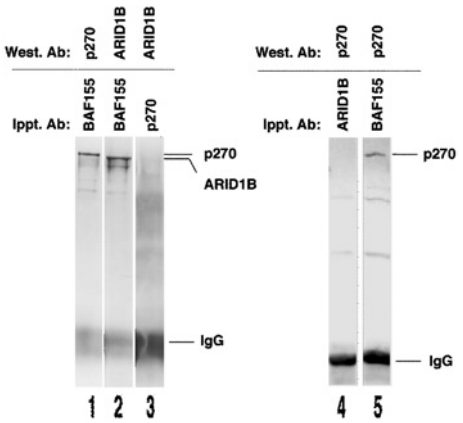

Figure 4 ARID1B and p270 are mutually exclusive subunits of human SWI/SNF complexes

Saos-2 cell lysate (2 mg/lane) was precipitated with antibodies of specificity indicated in the 'Ippt. Ab' lanes. Immunocomplexes were separated by SDS/PAGE and transferred on to a PVDF membrane. The membrane was cut to separate the individual lanes, which were then probed in Western blots with antibodies of specificity indicated as 'West. Ab'. Since the samples were immunoprecipitated before Western blotting, a prominent signal corresponding to the IgG heavy chain is apparent in each lane.

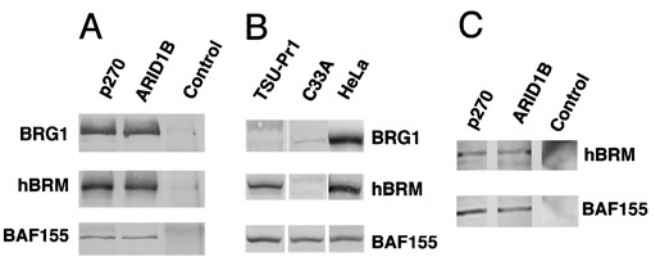

Figure 5 The ARID subunits are not specific to the ATPase subunits

p270- or ARID1B-specific immunocomplexes were isolated from Saos-2 cells (**A**) or TSU-Pr1 cells (**C**), separated by SDS/PAGE, transferred on to membranes and analysed by Western blotting for the presence of BRG1, hBRM or BAF155, as indicated. Control immune reactions were performed with a monoclonal antibody specific to an irrelevant viral protein. Direct Western blots shown in (**B**) confirm that TSU-Pr1 cells lack BRG1 expression, but express relatively normal levels of hBRM. Western-blot results are also shown in this panel for C33A cells (which express low levels of BRG1 and undetectable hBRM), as well as HeLa cells, which express both proteins.

expressed. The isolated immunocomplexes were reactive with both the p270-specific and ARID1B-specific antibodies (Figure 4, lanes 1 and 2). In contrast, when the complexes were isolated using the p270-specific antibodies, ARID1B was not detected (lane 3). Similarly, when the complex was immunoprecipitated with ARID1B-specific antibodies, no p270 could be detected (lane 4), although a p270 signal is present in complexes isolated in parallel with the BAF155-specific antibody (lane 5). Additional controls confirm the presence of other major complex components in the ARID1B and p270-specific lanes (see e.g. Figure 5). The immunocomplex analysis demonstrates, first, that endogenous ARID1B associates with human SWI/SNF complexes and, secondly, ARID1B is a mutually exclusive alternative to p270 in the complexes. The assay shown in Figure 4 also permits a direct comparison of the relative migration rates of p270 and ARID1B (lanes 1 and 2). ARID1B migrates ahead of p270, consistent with their deduced amino acid lengths (accession numbers AF253515 for ARID1B and NM_006015 for p270). We estimate the relative molecular mass of ARID1B to be approx. 240 kDa.

p270 and ARID1B can partner with either ATPase

Human SWI/SNF complexes can contain either of two closely related ATPases, BRG1 and hBRM. Despite their close structural relationship, mouse genetics indicates that BRG1 and mammalian BRM are functionally distinct. *Brm*-null mice are viable and fertile, and heterozygotes are not prone to tumorigenesis [27]. In contrast, *Brg1*-null mice die at a pre- or peri-implantation stage, and the corresponding heterozygotes have heightened tumour susceptibility [28]. Expression of *BRG1* and *hBRM* is activated at different times during early primate embryogenesis [23], consistent with the evidence that they play different roles during development. Recent evidence indicates that BRG1 and hBRM associate with different promoters during cellular proliferation and differentiation, and interact preferentially with distinct classes of transcription factors [29]. The mutual exclusion of p270 and ARID1B raises the possibility that each ATPase has a specific associated ARID family subunit. To explore this question, endogenous p270-specific or ARID1B-specific complexes were immunoprecipitated from Saos-2 cell lysates and probed with antibodies targeted to either BRG1 or hBRM. The results show that each of the ARID-containing subunits partner with both ATPases *in vivo* (Figure 5A). The BRG1-specific antibody used in the present study is an mAb raised against an N-terminal peptide sequence that is not present in hBRM and is not cross-reactive. The hBRM-targeted antibody is a commercially prepared mAb, which may be cross-reactive *in vivo* [30]. To ensure that the antibody is detecting authentic hBRM, immunocomplexes were isolated from the TSU-Pr1 cell line. This line lacks BRG1 expression as a result of bi-allelic mutation [31], but has relatively normal levels of hBRM (Figure 5B) as well as p270 and ARID1B (as shown in Figure 2). The antibody signal in TSU-Pr1 cells (Figure 5C) confirms that hBRM is associated *in vivo* with both the ARID proteins. The ability of each ARID protein to partner with both classes of ATPase-containing complexes expands the diversity of subunit combinations known to be present in cells.

Ratio of BRG1 in p270 versus ARID1B complexes

As discussed above, BRG1 is essential for tumour suppression and embryonic viability, whereas BRM is not [27,28]. Therefore it is of particular interest to know how BRG1 complexes are specifically apportioned with respect to the p270 and ARID1B subunits. To address this question, saturating levels of antibody were used to immunoprecipitate either ARID1B complexes or p270 complexes from aliquots of Saos-2 cell lysate. The complexes were assayed by Western blotting using a chemiluminescence method for the presence of BRG1. A range of lysate amounts and a range of exposure times were included to ensure that signal would be obtained in a linear range (Figure 6). Quantification of the signal obtained in the 1 mg of lysate series at 15, 30 and 60 s exposure yielded ratios of BRG1 in p270 versus ARID1B complexes of 3.3, 3.7 and 2.8 respectively. By 3 min exposure, the signal from this series was no longer within the linear range. From this titration, we estimate that approx. 3.3-fold more BRG1 is associated with p270 compared with ARID1B in these cells. This is consistent with the relative amounts of p270 and ARID1B present in the cells, implying that the ARID subunits compete equally well for the complexes.

DNA-binding activity of ARID1B

The role of the ARID DNA-binding region in p270 and ARID1B is not clearly established. p270 binds DNA in a non-sequence-specific manner [9,13]. Deletion of the ARID region of p270 moderately reduces its ability to enhance glucocorticoid receptor-mediated transcription in a co-transfection reporter assay [10]. Deletion of the ARID-containing region from ARID1B abrogates its activity in a similar assay [18]. It is not clear whether differences in the severity of the effect result merely from assay conditions as opposed to true physiological differences in the role of the domain in the context of protein function. ARID1B binds DNA with an affinity comparable with that of p270 or the prototypical sequence-specific ARID family member Dri [13], but experiments addressing the potential for sequence-specific binding by ARID1B have not been reported. Within the 94-residue ARID consensus, p270 and ARID1B differ at 17 amino acid positions; several of these are non-conservative changes. The potential of ARID1B for sequence-specific DNA-binding behaviour was evaluated in the present study in a DNA pull-down assay. Lambda phage DNA was cut with restriction enzymes to produce a large pool of DNA fragments of different sizes and a wide range of sequences. GST-fusion proteins were used to probe for preferential binding within the lambda DNA restriction fragment pool. The control ARID family protein Dri shows selectivity in this assay, as in other approaches [13,25]. Increasing the stringency of the interaction by adjusting the salt concentration results in increasingly more specific preference for selected fragments (Figure 7A, lanes 2–4). In contrast, a p270 fusion protein binds the fragments with no obvious selectivity (lanes 5–7). Increasing stringency does not reveal a preference for specific fragments, except for the eventual selection of longer fragments over shorter ones, probably because there are more binding surfaces on longer pieces of DNA. An ARID1B fusion protein, identical in length with the p270 fusion protein, behaves like p270 in this assay, showing no selectivity for specific fragments except for increasing the selection of longer DNA pieces (Figure 7B, lanes 2–4).

In the salt titration shown in Figure 7, relatively little DNA remains bound to the ARID1B protein after the 200 mM salt wash, raising the possibility that ARID1B has a weaker DNA-binding affinity when compared with p270. However, a previous analysis on DNA–cellulose affinity columns showed indistinguishable elution profiles for p270 and ARID1B [13]. The *in vitro* translated ARID1B peptide used in that assay was longer than the p270 protein with which it was compared and also longer than the respective GST-fusion protein segment analysed here. To control the possibility that sequences outside the ARID region contribute to DNA-binding affinity, a smaller expression vector, designed to be identical in length with p270, was constructed. The ^{35}S-labelled *in vitro* translated peptides were applied to native DNA–cellulose

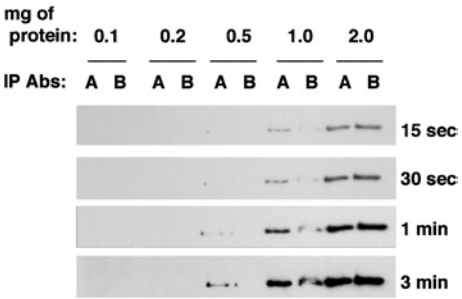

Figure 6 Ratio of BRG1 in p270 versus ARID1B complexes

The indicated amounts of Saos-2 cell lysates were immunoprecipitated with saturating amounts of p270-specific mAb PSG3 (lanes A) and ARID1B-specific mAb KMN-1 (lanes B). Immunocomplexes were separated by SDS/PAGE (8 % gel), transferred on to a PVDF membrane, Western-blotted with anti-BRG1 mAb and visualized by chemiluminescence assay. Different exposures were obtained at the indicated time points, and the ratio of the amount of BRG1 in the p270 complex to that in the ARID1B complex was quantified by densitometry.

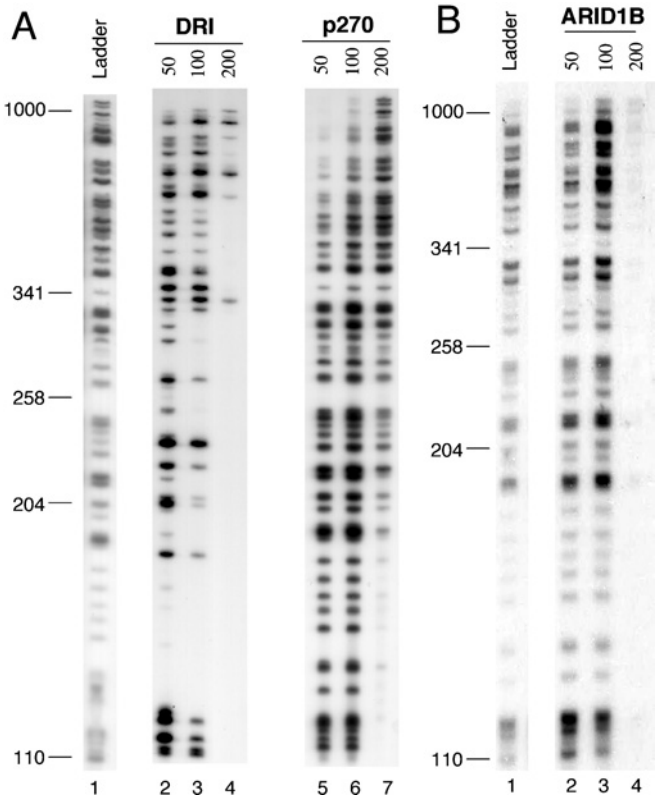

Figure 7 ARID1B binds DNA non-sequence-specifically

Lambda phage DNA was digested with *Eco*RI, *Hind*III and Sau3A1 to generate a large DNA oligonucleotide pool predicted to contain 128 fragments ranging in size from 12 to 2225 bp. The fragments were filled in with [^{32}P]dATP, incubated with GST-fusion proteins containing the ARID regions of p270, Dri or ARID1B as indicated, pulled-down with glutathione beads and analysed by PAGE. Lane 1 in each panel shows the unselected pool of DNA fragments. The remaining lanes show the fragments selected in Lambda DNA-binding buffer with increasing KCl concentrations as indicated.

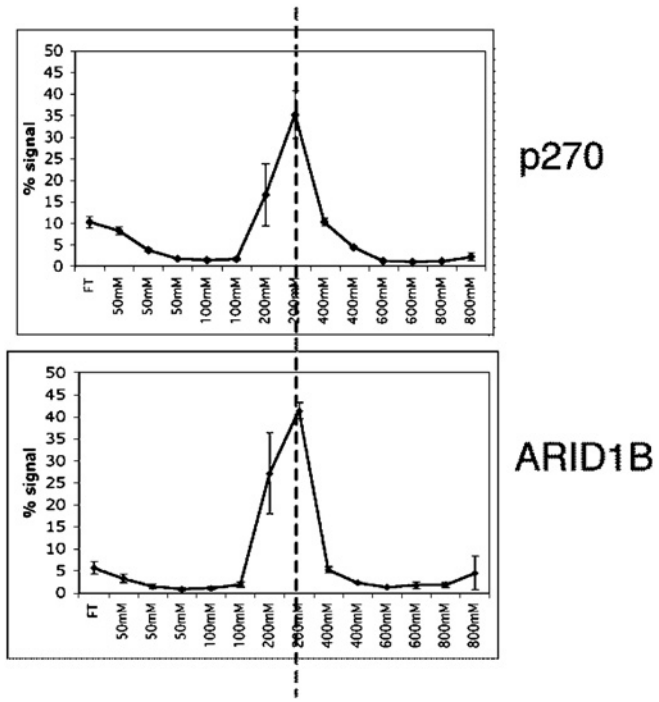

Figure 8 DNA-binding affinity of p270 and ARID1B

In vitro translated [^{35}S]methionine-labelled peptides were applied to native DNA–cellulose columns as described in the Materials and methods section. Bound protein was eluted stepwise with loading buffer adjusted to contain increasing concentrations of NaCl from 100 to 800 mM, as indicated in the Figure. Fractions were separated by SDS/PAGE and the p270 signal in each fraction was quantified by phosphoimaging. The results are plotted as the percentage of signal in each fraction relative to the entire signal recovered. Error bars represent S.D. Graphs are aligned for ease of comparison.

columns and washed with increasing salt concentrations. The p270 and ARID1B proteins again show similar elution profiles (Figure 8), reaching a peak at the same fraction determined previously. Both the proteins begin to be eluted at the 200 mM salt concentration; therefore, the difference seen in the pull-down assay might reflect slightly different sensitivities over this range.

DISCUSSION

Generation of mAbs that react selectively with either p270 or ARID1B has allowed us to probe the expression of ARID1B and its potential interaction with human SWI/SNF complexes *in vivo*. These screens indicate that ARID1B levels are normally low relative to p270. A survey of tumour cell lines of various tissue origins, including cervix, adrenal cortex, breast, prostate, bladder and bone, indicates that ARID1B is widely expressed and that its expression level relative to p270 is fairly constant. Analysis with mAbs whose signal equivalence was checked by reaction against purified p270 and ARID1B GST-fusion proteins, combined with Northern-blot results using directly comparable probes, suggest that the ratio of p270 to ARID1B in most cells is approx. 3.5:1. The immunocomplexes confirm that endogenous ARID1B is associated with SWI/SNF complexes and show that p270 and ARID1B, similar to BRG1 and hBRM, are alternative, mutually exclusive subunits of the complexes. The ARID-containing subunits are not specific to the ATPases. Each associates with both BRG1- and hBRM-containing complexes, thus increasing the number of distinct subunit combinations known to be present in cells. Titration of the ARID subunit-associated BRG1 signal in Saos-2 cells indicates that BRG1 is distributed proportionally between the two ARID subunits.

The mAbs described here are unique reagents. Previous reports were unable to clarify whether p270 and ARID1B are present in distinct complexes [18,20,21] or conflicted in their conclusions about the associations between the two ARID proteins and the two ATPases [18,22]. All of the interactions discussed in the present study were probed with endogenous proteins under normal physiological conditions, under conditions permitting a relatively quantitative analysis not possible previously. The *in vivo* analysis does not address the question whether the association of the ARID proteins and ATPases is direct, but *in vitro* association data indicate that the interaction is direct and requires the C-terminal portion of the ARID proteins [18].

The functional distinction between p270 and ARID1B-containing complexes is not yet known. The expression profiles of p270 and ARID1B are distinguishable during early primate development, similar to BRG1 and hBRM [23], implying that the ARID-containing subunits also have distinct functions. Expression patterns of p270 (synonym: Osa1) and ARID1B (synonym: BAF250b) have been examined during mouse development, but have not been compared directly, although differences

do appear to exist [22,32]. Transient transfection assays reveal similar abilities to enhance expression from steroid hormone-responsive promoters; however, it is clear that more physiologically relevant assays are needed to distinguish the roles of each protein. Knockdown studies are in progress in our laboratory and they should offer an indication of the respective biological roles of p270 and ARID1B in differentiating cells.

We thank W. Long (Department of Microbiology, Temple University) for help with hybridoma development and culture, T. Nagase and S. Sugano for gifts of plasmids and B. Lokeshwar and M. F. Hansen for gifts of cell lines. D. Haines, X. Graña-Amat, D. Dhanasekaran, B. Hoffman, C. Sapienza and S. Shore offered advice and critical comments. Other members of our laboratory, in particular A. Patsialou and D. Zweitzig, offered additional insight, advice and support. This work was supported by Public Health Service grant no. RO1CA53592 (E. M.) and by a Shared Resources grant to the Fels Institute (1R24CA88261). X. W., N. G. N. Jr and D. W. are recipients of Department of Defense BCRP (Breast Cancer Research Program) fellowships, namely DAMD-17-00-1-0453 (X. W.), DAMD-17-02-1-0577 (N. G. N. Jr) and DAMD-17-01-1-0407 (D. W.). X. W. and D. W. were also supported by Daniel Swern Fellowships from Temple University.

REFERENCES

1 Vignali, M., Hassan, A. H., Neely, K. E. and Workman, J. L. (2000) ATP-dependent chromatin-remodeling complexes. Mol. Cell. Biol. **20**, 1899–1910
2 Muchardt, C. and Yaniv, M. (2001) When the SWI/SNF complex remodels . . . the cell cycle. Oncogene **20**, 3067–3075
3 Tsukiyama, T. (2002) The *in vivo* functions of ATP-dependent chromatin-remodelling factors. Nat. Rev. Mol. Cell. Biol. **3**, 422–429
4 Martens, J. A. and Winston, F. (2003) Recent advances in understanding chromatin remodeling by Swi/Snf complexes. Curr. Opin. Genet. Dev. **13**, 136–142
5 Wang, W., Cote, J., Xue, Y., Zhou, S., Khavari, P. A., Biggar, S. R., Kalpana, G. V., Goff, S. P., Yaniv, M., Workman, J. L. et al. (1996) Purification and biochemical heterogeneity of the mammalian SW-SNF complex. EMBO J. **15**, 5370–5382
6 Sif, S., Saurin, A. J., Imbalzano, A. N. and Kingston, R. E. (2001) Purification and characterization of mSin3A-containing Brg1 and hBrm chromatin remodeling complexes. Genes Dev. **15**, 603–618
7 Dallas, P. B., Yaciuk, P. and Moran, E. (1997) Characterization of monoclonal antibodies raised against p300: both p300 and CBP are present in intracellular TBP complexes. J. Virol. **71**, 1726–1731
8 Dallas, P. B., Cheney, I. W., Liao, D. W., Bowrin, V., Byam, W., Pacchione, S., Kobayashi, R., Yaciuk, P. and Moran, E. (1998) p300/CREB binding protein-related protein p270 is a component of mammalian SWI/SNF complexes. Mol. Cell. Biol. **18**, 3596–3603
9 Dallas, P. B., Pacchione, S., Wilsker, D., Bowrin, V., Kobayashi, R. and Moran, E. (2000) The human SWI-SNF complex protein p270 is an ARID family member with non-sequence-specific DNA binding activity. Mol. Cell. Biol. **20**, 3137–3146
10 Nie, Z., Xue, Y., Yang, D., Zhou, S., Deroo, B. J., Archer, T. K. and Wang, W. (2000) A specificity and targeting subunit of a human SWI/SNF family-related chromatin-remodeling complex. Mol. Cell. Biol. **20**, 8879–8888
11 Kortschak, R. D., Tucker, P. W. and Saint, R. (2000) ARID proteins come in from the desert. Trends Biochem. Sci. **25**, 294–299
12 Wilsker, D., Patsialou, A., Dallas, P. B. and Moran, E. (2002) ARID proteins: a diverse family of DNA binding proteins implicated in the control of cell growth, differentiation, and development. Cell Growth Differ. **13**, 95–106
13 Wilsker, D., Patsialou, A., Zumbrun, S. D., Kim, S., Chen, Y., Dallas, P. B. and Moran, E. (2004) The DNA-binding properties of the ARID-containing subunits of yeast and mammalian SWI/SNF complexes. Nucleic Acids Res. **32**, 1345–1353
14 Collins, R. T., Furukawa, T., Tanese, N. and Treisman, J. E. (1999) Osa associates with the Brahma chromatin remodeling complex and promotes the activation of some target genes. EMBO J. **18**, 7029–7040
15 Le Douarin, B., Nielsen, A. L., Garnier, J. M., Ichinose, H., Jeanmougin, F., Losson, R. and Chambon, P. (1996) A possible involvement of TIF1 alpha and TIF1 beta in the epigenetic control of transcription by nuclear receptors. EMBO J. **15**, 6701–6715
16 Heery, D. M., Kalkhoven, E., Hoare, S. and Parker, M. G. (1997) A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature (London) **387**, 733–736
17 Hsiao, P. W., Fryer, C. J., Trotter, K. W., Wang, W. and Archer, T. K. (2003) BAF60a mediates critical interactions between nuclear receptors and the BRG1 chromatin-remodeling complex for transactivation. Mol. Cell. Biol. **23**, 6210–6220
18 Inoue, H., Furukawa, T., Giannakopoulos, S., Zhou, S., King, D. S. and Tanese, N. (2002) Largest subunits of the human SWI/SNF chromatin-remodeling complex promote transcriptional activation by steroid hormone receptors. J. Biol. Chem. **277**, 41674–41685
19 Nagase, T., Ishikawa, K., Kikuno, R., Hirosawa, M., Nomura, N. and Ohara, O. (1999) Prediction of the coding sequences of unidentified human genes. XV. The complete sequences of 100 new cDNA clones from brain which code for large proteins *in vitro*. DNA Res. **6**, 337–345
20 Kato, H., Tjernberg, A., Zhang, W., Krutchinsky, A. N., An, W., Takeuchi, T., Ohtsuki, Y., Sugano, S., De Bruijn, D. R., Chait, B. T. et al. (2002) SYT associates with human SNF/SWI complexes and the C-terminal region of its fusion partner SSX1 targets histones. J. Biol. Chem. **277**, 5498–5505
21 Hurlstone, A. F., Olave, I. A., Barker, N., Van Noort, M. and Clevers, H. (2002) Cloning and characterization of hELD/OSA1, a novel BRG1 interacting protein. Biochem. J. **364**, 255–264
22 Nie, Z., Yan, Z., Chen, E. H., Sechi, S., Ling, C., Zhou, S., Xue, Y., Yang, D., Murray, D., Kanakubo, E. et al. (2003) Novel SWI/SNF chromatin-remodeling complexes contain a mixed-lineage leukemia chromosomal translocation partner. Mol. Cell. Biol. **23**, 2942–2952
23 Zheng, P., Patel, B., McMenamin, M., Paprocki, A. M., Schramm, R. D., Nagl, Jr, N. G., Wilsker, D., Wang, X., Moran, E. and Latham, K. E. (2004) Expression of genes encoding chromatin regulatory factors in developing rhesus monkey oocytes and preimplantation stage embryos: possible role in genome activation. Biol. Reprod. **70**, 1419–1427
24 Beck, Jr, G. R., Sullivan, E. C., Moran, E. and Zerler, B. (1998) Relationship between alkaline phosphatase levels, osteopontin expression, and mineralization in differentiating MC3T3-E1 osteoblasts. J. Cell. Biochem. **68**, 269–280
25 Gregory, S. L., Kortschak, R. D., Kalionis, B. and Saint, R. (1996) Characterization of the dead ringer gene identifies a novel, highly conserved family of sequence-specific DNA binding proteins. Mol. Cell. Biol. **16**, 792–799
26 Van Bokhoven, A., Varella-Garcia, M., Korch, C. and Miller, G. J. (2001) TSU-Pr1 and JCA-1 cells are derivatives of T24 bladder carcinoma cells and are not of prostatic origin. Cancer Res. **61**, 6340–6344
27 Reyes, J. C., Barra, J., Muchardt, C., Camus, A., Babinet, C. and Yaniv, M. (1998) Altered control of cellular proliferation in the absence of mammalian brahma (SNF2alpha). EMBO J. **17**, 6979–6991
28 Bultman, S., Gebuhr, T., Yee, D., La Mantia, C., Nicholson, J., Gilliam, A., Randazzo, F., Metzger, D., Chambon, P., Crabtree, G. et al. (2000) A Brg1 null mutation in the mouse reveals functional differences among mammalian SWI/SNF complexes. Mol. Cell **6**, 1287–1295
29 Kadam, S. and Emerson, B. M. (2003) Transcriptional specificity of human SWI/SNF BRG1 and BRM chromatin remodeling complexes. Mol. Cell **11**, 377–389
30 Reisman, D. N., Strobeck, M. W., Betz, B. L., Sciariotta, J., Funkhouser, Jr, W., Murchardt, C., Yaniv, M., Sherman, L. S., Knudsen, E. S. and Weissman, B. E. (2002) Concomitant down-regulation of BRM and BRG1 in human tumor cell lines: differential effects on RB-mediated growth arrest vs CD44 expression. Oncogene **21**, 1196–1207
31 Wong, A. K., Shanahan, F., Chen, Y., Lian, L., Ha, P., Hendricks, K., Ghaffari, S., Iliev, D., Penn, B., Woodland, A. M. et al. (2000) BRG1, a component of the SWI-SNF complex, is mutated in multiple human tumor cell lines. Cancer Res. **60**, 6171–6177
32 Kozmik, Z., Machon, O., Kralova, J., Kreslova, J., Paces, J. and Vlcek, C. (2001) Characterization of mammalian orthologues of the Drosophila osa gene: cDNA cloning, expression, chromosomal localization, and direct physical interaction with Brahma chromatin-remodeling complex. Genomics **73**, 140–148

Received 30 March 2004/18 May 2004; accepted 1 June 2004
Published as BJ Immediate Publication 1 June 2004, DOI 10.1042/BJ20040524