Fanconi anemia protein, FANCA, associates with BRG1, a component of the human SWI/SNF complex

Tetsuya Otsuki, Yusuke Furukawa1, Keiko Ikeda2, Hitoshi Endo3, Takayuki Yamashita4, Azusa Shinohara5, Akihiko Iwamatsu5, Keiya Ozawa and Johnson M. Liu6,*

Department of Hematology, 1Center for Molecular Medicine, 2Department of Biology and 3Department of Biochemistry, Jichi Medical School, Yakushiji 3311-1, Minamikawachi, kawachi, Tochigi 329-0498, Japan, 4Division of Genetic Diagnosis, Institute of Medical Science, University of Tokyo, Sirokanedai 4-6-1, Minato-ku, Tokyo 108, Japan, 5Section of Protein Chemistry, Central Laboratories for Key Technology, Kirin Brewery Co. Ltd, Fukuura 1-13-5, Kanazawa-ku, Yokohama-shi, Kanagawa 236-0004, Japan and 6Hematology Branch, National Heart, Lung and Blood Institute, Building 10, Room 7C103, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA

Received June 26, 2001; Revised and Accepted September 6, 2001

Fanconi anemia (FA) is a genetic disorder that predisposes to hematopoietic failure, birth defects and cancer. We identified an interaction between the FA protein, FANCA and brm-related gene 1 (BRG1) product. BRG1 is a subunit of the SWI/SNF complex, which remodels chromatin structure through a DNA-dependent ATPase activity. FANCA was demonstrated to associate with the endogenous SWI/SNF complex. We also found a significant increase in the molecular chaperone, glucose-regulated protein 94 (GRP94) among BRG1-associated factors isolated from a FANCA-mutant cell line, which was not seen in either a normal control cell line or the mutant line complemented by wild-type FANCA. Despite this specific difference, FANCA did not appear to be absolutely required for in vitro chromatin remodeling. Finally, we demonstrated co-localization in the nucleus between transfected FANCA and BRG1. The physiological action of FANCA on the SWI/SNF complex remains to be clarified, but our work suggests that FANCA may recruit the SWI/SNF complex to target genes, thereby enabling coupled nuclear functions such as transcription and DNA repair.

INTRODUCTION

Fanconi anemia (FA) is an autosomal recessive genetic syndrome characterized by progressive bone marrow failure, multiple developmental abnormalities and a predisposition to malignancy (1). The diagnostic hallmark of FA cells is a unique hypersensitivity to DNA cross-linking agents such as mitomycin C (MMC) and diepoxybutane (DEB). In FA cells, these agents cause increased cell death, chromosomal breakage and accumulation in the G2 phase of the cell cycle (2–4). Because of associated genomic instability and cancer predisposition, FA has been classified along with Bloom syndrome, ataxia telangiectasia, xeroderma pigmentosa, Cockayne syndrome and trichothiodystrophy as one of the chromosomal instability syndromes (5).

FA is a genetically heterogeneous disorder and complementation analysis using lymphoid cell lines from FA patients initially revealed eight complementation groups (6–8). The reference cell line for complementation group H (EUFA173) was reassigned to group A (9), but the FA-D group was split into FA-D1 and FA-D2. Hence, there are still eight complementation groups: FA-A, FA-B, FA-C, FA-D1, FA-D2, FA-E, FA-F and FA-G. At this time, six FA genes, FANCC, FANCA, FANCG, FANCF, FANCE and FANCD2 have been identified (10–16).

Despite the identification of six FA genes, the function of each protein remains unclear, since each FA gene product has no significant homology to proteins of known function. To identify potential protein–protein interactions, we performed yeast two-hybrid screening using a FANCA fragment as bait. From these experiments, we identified brm-related gene 1 (BRG1) product as a FANCA-binding protein. BRG1 (17), the human homolog of yeast SWI2/SNF2 (18,19) and Drosophila brahma (brm) (20,21), is a key subunit of the SWI/SNF complex and functions through its DNA-dependent ATPase activity (17,22). In Saccharomyces cerevisiae, the products of five genes, switch 1 (SWI1), SWI2/sucrose non-fermentable 2 (SNF2), SWI3, SNF5 and SNF6 were first discovered in connection with the activation of discrete transcriptional responses such as mating and sucrose fermentation (18,19,23). Immunoprecipitation (IP) by antibodies against different SWI–SNF proteins precipitates all five proteins, indicating that they are bona fide subunits of the SWI/SNF complex. Biochemical purification of the complex has shown that it consists of 11 subunits, including TFG III and SNF11 (24–26). In mammals, the SWI/SNF complex purified by immunoprecipitation (IP) with anti-BRG1 antibody contains 9–12 polypeptides, referred to as BRG1-associated factors.

*To whom correspondence should be addressed. Tel: +1 301 496 2452; Fax: +1 301 496 8396; Email: liuj@nhlbi.nih.gov
results

yeast two-hybrid screening using FANCA fragments as bait: interaction between FANCA and BRG1

For yeast two-hybrid screening, four fragments of FANCA were cloned into the pGBT9 vector and used as bait plasmids (Fig. 1A). Initially, all plasmids were tested for intrinsic transcriptional activity (without the library plasmid) as reflected by the X-gal assay. Both full-length FANCA and the N-terminal region of FANCA (FANCA [1]) exhibited positive transcriptional activity. Therefore, we used pGBT9/FANCA (3) as bait in further screening; three independent BRG1 cDNAs were cloned as putative binding partners (clones 3–18, 3–59 and 3–230 in Fig. 1B). A highly conserved region (called domain II) was contained in all three BRG1 clones, suggesting that domain II may be responsible for binding between FANCA and BRG1.

To determine the approximate binding site for BRG1 on FANCA, truncation mutants of FANCA (3) were generated and used for two-hybrid studies with BRG1 (Fig. 1C). Truncation of even the extreme C-terminus (nucleotides 4261–4368 or exon 43) caused loss of interaction between FANCA and BRG1.

binding between FANCA and BRG1 was confirmed by co-IP experiments

Binding between full-length FANCA and full-length BRG1 was confirmed by IP experiments using the lysate of cells co-transfected with plasmids encoding N-terminal Myc-tagged full-length FANCA (FANCA/Myc) and N-terminal HA-tagged full-length BRG1 (BRG1/HA). Cell lysates were immunoprecipitated with anti-Myc antibody (Ab) or anti-HA Ab, and then immunoblotted with anti-HA Ab or anti-Myc Ab, respectively (Fig. 2A). Protein bands of the expected sizes were observed (lanes 1 and 3), confirming that FANCA and BRG1 could specifically interact with each other.

BRG1 is a key molecule in the human SWI/SNF complex. Therefore, interaction between FANCA and BRG1 suggested that FANCA could associate with human SWI/SNF. To test for this, the SWI/SNF complex was immunoprecipitated from lysate of an immortalized lymphoblastoid cell line from a normal individual by using anti-BRG1 Ab and then probed by immunoblotting for FANCA (Fig. 2B). The results showed the presence of a protein band compatible with FANCA among the BRG1-associated factors (BAFs) (lane 1), whereas no FANCA band was observed among those of FANCA-mutant (FA-A) lymphoblastoid cells (HSC72) (lane 2) or of human adenosarcoma SW13 cells, which are null for BRG1 protein expression (lane 4). Interestingly, the FANCA band was observed in HSC72 cells that had been complemented with retrovirus containing wild-type FANCA cDNA (HSC72/FANCA) (lane 3). To confirm that the BRG1 Ab used for the experiments immunoprecipitated the human SWI/SNF complex, the IP samples were analyzed by immunoblotting for BAF170, BAF155 and IN11, showing that these known members of the human SWI/SNF complex were immunoprecipitated together with FANCA by our anti-BRG1 Ab. These results indicate that FANCA interacts with BRG1 at endogenous protein expression levels and suggest that FANCA associates with the human SWI/SNF complex. The immune complex sample prepared with anti-BRG1 Ab was also probed by immunoblotting for FANCC or FANCG: in these studies, FANCC and FANCG could not be detected (data not shown). To further confirm the interaction between FANCA and BRG1, FANCA was immunoprecipitated from each cell lysate and then probed for BRG1 by immunoblotting (Fig. 2C). BRG1 co-immunoprecipitated with FANCA in cells from the normal lymphoblastoid line (lane 1) and HSC72/FANCA (lane 3), but the BRG1 band was not observed in HSC72 cells (lane 2) or SW13 cells (lane 4). The result of this reciprocal blot is completely compatible with that of Figure 2B.

In silver-stained BAFs derived from FANCA-mutant cells, GRP94 was highly expressed

Next, IP with anti-BRG1 Ab was performed from total cell lysates of the normal lymphoblastoid line, HSC72, and HSC72/FANCA, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and silver staining (Fig. 3A). The banding pattern of BAFs in HSC72 cells (lane 2) was markedly different from that seen in normal cells (lane 1), whereas the pattern seen in HSC72/FANCA cells was again similar to that of the normal lymphoblastoid control (lane 3). Taken together with the results from Figure 2B and C, FANCA may affect the assembly of the human SWI/SNF complex, although the content of BRG1, BAF170 and BAF155 is almost the same among these cells. The most dramatic difference in the BAF-banding pattern in HSC72 cells was the presence of an intensely stained protein band of ~100 kDa (indicated by the arrow in lane 2). This band was isolated from large-scale preparations by using SDS–PAGE and Coomassie blue staining, and then analyzed by mass spectrometry: the protein was identified as the molecular chaperone, GRP94. We had previously identified GRP94 as binding to FANCC (44). Differences in the amount of GRP94 present in BAFs of normal, HSC72 and HSC72/FANCA cells were confirmed by immunoblot analysis with specific anti-GRP94 Ab (Fig. 3B). In BAF preparations from HSC72 cells, a large amount of GRP94 was detected, when compared with that found in normal lymphoblastoid and HSC72/FANCA preparations. We
found no difference in GRP94 content in total cell lysates from HSC72, complemented HSC72 or the normal lymphoblastoid lines. GRP94 was not immunoprecipitated from the total cell lysate of HSC72 cells with preimmune rabbit IgG (data not shown). Conversely, IP with anti-GRP94 Ab was performed from each cell lysate, followed by immunoblotting for BRG1 (Fig. 3C). A co-immunoprecipitated band of BRG1 was clearly detected only from the total cell lysate of HSC72 cells (compare lane 2 versus lane 1, normal lymphoblastoid control or lane 3, HSC72/FANCA cells). In HSC72 cells, not only...
Figure 2. Binding between full-length FANCA and full-length BRG1 was confirmed by IP experiments. (A) Myc-tagged full-length FANCA (FANCA/Myc) and HA-tagged full-length BRG1 (BRG1/HA) were co-transfected into 293 cells, and IP was performed with rat anti-HA Ab (lane 1) or mouse anti-Myc Ab (lane 3). The samples with anti-HA Ab (lane 1) and with rat IgG (lane 2) were analyzed by immunoblotting with anti-Myc Ab. The samples with anti-Myc Ab (lane 3) and with mouse IgG (lane 4) were analyzed by immunoblotting with anti-HA Ab. The amount of FANCA/Myc and BRG1/HA in the starting cell lysate is shown under each lane. IP/Hybridization/HA and IP-myc/Hybridization/myc are shown at the bottom. (B) From cell lysates of the normal lymphoblastoid line (lanes 1 and 5), HSC72 (lanes 2 and 6), HSC72/FANCA (lanes 3 and 7), and the BRG1-deficient adenocarcinoma line, SW13 (lanes 4 and 8): the SWI/SNF complex was isolated by IP using rabbit polyclonal anti-BRG1 Ab (lanes 1–4) and the immunoprecipitated samples were analyzed by SDS–PAGE and immunoblotting with anti-FANCA Ab. In lanes 5–8, IP was performed with preimmune rabbit IgG as control. In lane 9, IP from cell lysis buffer only was performed with anti-BRG1 Ab, followed by immunoblot analysis with anti-FANCA Ab. The same membrane was rebotted with anti-BAF170 Ab, anti-BAF155 Ab, anti-INI1 Ab or anti-BRG1 Ab, and the results are shown under each lane. Rabbit IgGs used for the IP are also shown under each lane. (C) From the cell lysates of the normal lymphoblastoid line (lanes 1 and 5), HSC72 (lanes 2 and 6), HSC72/FANCA (lanes 3 and 7) and the BRG1-deficient adenocarcinoma line, SW13 (lanes 4 and 8), IP was performed with anti-FANCA Ab (lanes 1–4) or preimmune rabbit IgG (lanes 5–8), followed by immunoblotting for BRG1. In lane 9, IP from cell lysis buffer only was performed with anti-FANCA Ab, followed by immunoblot analysis with anti-BRG1 Ab. The IP/FANCA–WB/FANCA of the SW13 cell lysate is shown, as well as the BRG1 immunoblot of total cell lysates of positive and negative cell lines.
BRG1 but also BAF170 and BAF155 were co-immunoprecipitated with anti-GRP94 Ab. Thus, although GRP94 may be a minor component of human SWI/SNF in the normal lymphoblastoid line, the amount of SWI/SNF-associated GRP94 was markedly increased with mutation in FANCA. The BAF pattern of FA-C cells (HSC536) and FA-G cells (EUFA316) was also analyzed, but in these two cell lines there were no differences in the banding pattern from that of the normal lymphoblastoid control (data not shown).

Chromatin remodeling assays of the SWI/SNF complexes prepared from FA-A and complemented FA-A cells

To clarify the functional role of FANCA in the SWI/SNF complex, chromatin remodeling assays were performed using SWI/SNF complexes derived from HSC72 and HSC72/FANCA cells. Chromatin was prepared from purified histones and radiolabeled DNA fragments, which were designed to contain an XhoI site that is masked from restriction enzyme digestion when the DNA fragment is compacted by histones. SWI/SNF complexes from both HSC72/FANCA and HSC72 cells exhibited chromatin remodeling activity, implying that FANCA is not absolutely required for SWI/SNF function (data not shown). In addition, DNase I footprinting assays for chromatin remodeling showed nearly equivalent activity between these SWI/SNF complexes (data not shown). These results suggested that FANCA does not directly regulate the activity of SWI/SNF.

Co-localization of FANCA with BRG1 in the nucleus

The intracellular distribution of C-terminal FLAG-tagged FANCA (FANCA/FLAG) and BRG1/HA was investigated by immunohistochemistry and confocal laser microscopy. The results showed that most FANCA and BRG1 co-localized in the nucleus of HeLa cells (Fig. 4C). The possibility of artifactual ‘channel-bleeding’ was ruled out by the control experiments (Fig. 4D–I).

DISCUSSION

Remodeling of chromatin structure has emerged as a key biochemical process in the regulation of transcription, recombination and repair of DNA. One well-established mechanism for chromatin remodeling involves the acetylation or deacetylation of internal lysine residues of core histones...
Figure 4. Co-localization of FANCA with BRG1 in the nucleus of HeLa cells. The intracellular distribution of C-terminal FLAG-tagged full-length FANCA (FANCA/FLAG) and HA-tagged full-length BRG1 (BRG1/HA) was investigated by immunohistochemical staining followed by confocal laser microscopy. (A, D, G and J) FANCA/FLAG (546 nm). (B, E, H and K) BRG1/HA (488 nm). (A–C) Stained with anti-FLAG Ab and anti-HA Ab. (D–F) Stained with anti-FLAG Ab. (G–I) Stained with anti-HA Ab. (A and B), (D and E), (G and H) are merged in (C), (F) and (I), respectively. In (J–L), cells were treated with normal mouse and rat IgG, instead of the first Ab.

Interaction between FANCC and a novel BTB/POZ transcriptional repressor protein, FA zinc finger (FAZF), has been reported (49). FAZF has homology to promyelocytic leukemia zinc finger (PLZF) and is able to bind the same DNA target sequence. PLZF (reviewed in 50) associates in vitro with the Mad corepressor mSin3A and the histone deacetylase HDAC1, and strongly represses transcription initiated from different promoters. Chromatin remodeling is also effected by the SWI/SNF complex, which binds to chromatin and relieves nucleosome-mediated repression of transcription in an ATP-dependent manner, thus providing access to transcriptional activators (31,33–36). Our data show that FANCA associates with the human SWI/SNF complex, probably through interaction with BRG1. These findings suggest that FA proteins, such as FANCA and FANCC, may modulate chromatin remodeling through interaction with BRG1 and FAZF, respectively.

The staining pattern of the SWI/SNF complex derived from HSC72 cells was clearly different from that of normal lymphoblastoid cells, and the pattern was corrected by the retroviral transduction of wild-type FANCA into HSC72 cells. Mass spectrometry data showed that GRP94 was markedly upregulated in the BAF preparation from HSC72 cells. GRP94 (or gp96) is one of the heat shock protein 90 (HSP90) family of chaperones, and has recently attracted considerable attention for its ability to elicit cytotoxic T-cell responses to cancers (51,52). GRP94 was previously identified as a FANCC-binding protein from yeast two-hybrid screening and was reported to regulate the protein level of FANCC (44). Although the intracellular location of GRP94 is usually confined to the endoplasmic reticulum (ER) through interaction between the C-terminal tetrapeptide KDEI and its receptor on the ER (53), GRP94 has been shown to escape KDEL-mediated retention in several cell types (54). Furthermore, GRP94 has been described to localize to the nucleus, particularly following cell stress (55). HSP90, the cytoplasmic homolog of GRP94, has also been reported to directly interact with histones and to induce the condensation of chromatin, suggesting the involvement of HSP90 in chromatin organization (56,57). Although the function of GRP94 in the SWI/SNF complex is still unclear, one possibility is that GRP94 tethers the FA protein complex and the SWI/SNF complex, through binding affinity to FANCC (perhaps across subcellular compartments). We believe that whereas FANCA is a component of the SWI/SNF complex, other FA proteins such as FANCC and FANCN may not be included in the complex, since FANCN and FANCN are not detected in the immune complex sample prepared with anti-BRG1 Ab nor did mutation in FANCC or FANCN influence the pattern of BAF assembly.

Differences in the assembly of the SWI/SNF complex in FA-A cells suggest the possibility of altered chromatin remodeling activity as a result of mutation in FANCA. However, as assessed by in vitro assays, chromatin remodeling by the SWI/SNF complex does occur in HSC72 cells, indicating that FANCA is not indispensable for gross SWI/SNF function. Moreover, we detected no major differences in the activity of the SWI/SNF complex from HSC72 cells when compared to that from complemented HSC72/FANCA cells. Therefore, the functional role of FANCA in the SWI/SNF complex remains unclear. However, one possibility is that FANCA may recruit SWI/SNF to target genes, rather than directly regulate SWI/SNF activity. For example, C/EBPβ, a key transcriptional activator of myeloid-specific genes, induces transcription of the granulocyte-specific mim-1 gene by recruiting the SWI/SNF complex (58). Finally, although in vitro chromatin remodeling assays showed no major differences in SWI/SNF activity between HSC72 and HSC72/FANCA cells, there may still be functional differences in vivo.

Whereas the accumulation of the FA protein complex (at least FANCA, FANCC, FANCE, FANCF and FANCN) appears to be a critical step in the FA pathway, the precise biochemical function of this complex in the nucleus remains unknown (59–63). However, increasing evidence suggests that FA cells are defective in some aspect of DNA repair, and the FA complex may play a direct or indirect role in the repair of DNA damage. The FA protein complex appears to assemble normally in FA-D cells, suggesting that the product of the FANCD gene functions downstream of or independently from the other FA proteins (64). Recently, FANCD2 was cloned.
Human Molecular Genetics, 2001, Vol. 10, No. 23

(16) and its gene product reported to undergo activation from a short (FANCD2-S) to a long (FANCD2-L) form after monoubiquitination, requiring the action of the intact FA protein complex (65). FANCD2-L was found to co-localize with BRCA1 within ionizing radiation-induced nuclear foci (or dots). One group of investigators has reported direct interaction between BRCA1 and BRG1 (66), although the biological significance of this finding is currently under intense investigation.

Recently, interaction between the FA protein complex (FANCA/FANCC/FANCG) and chromatin has been described (67). According to the report, the FA protein complex may be excluded from mitotic (M) chromosomes, although it binds to chromatin in non-mitotic (non-M) phases. This is concordant with our finding that BRG1 is also phosphorylated and excluded from condensed chromosomes during mitosis (68). In non-M phases, chromatin structure may be ‘opened’ by SWI/SNF complexes recruited to specific DNA target sites by chromatin–FA protein complex (FANCA)–BRG1 interactions. This process may allow the repair machinery access to sites of damage, for example (Fig. 5), a hypothesis that is supported by our observation that FANCA co-localizes with BRG1 in the nucleus. Further studies with DNA-damaged templates will be required in order to fully understand the relationship between FANCA, BRG1, BRCA1 and DNA repair.

MATERIALS AND METHODS

Yeast two-hybrid analysis

FANCA cDNA was divided into three overlapping fragments, FANCA (1)–(3) (Fig. 1). Polymerase chain reaction (PCR) fragments encoding FANCA (1)–(3) and full-length FANCA cDNA were inserted into the yeast two-hybrid vector pGBT9 (Clontech) downstream from the GAL4 transcriptional activation domain. Selection was performed on plates lacking tryptophan, leucine and histidine. After cotransfection of the yeast cells, a filter color assay was used to assess the transcriptional activity of lacZ (β-galactosidase), as an indicator of a physical interaction between FANCA and proteins derived from the human B cell cDNA library. The intensity of the color reaction was scored in a semi-quantitative manner by visual inspection.

Transfection, IP and immunoblot analysis of tagged-proteins

Full-length FANCA and BRG1 cDNAs were generated by PCR and cloned into N-terminal Myc-tagged (pMyc) and HA-tagged (pHA) expression vectors (Clontech), respectively. These plasmids were transfected into 293 cells using lipofectamine 2000 (Gibco-BRL) according to the manufacturer’s instructions. After washing with PBS, transfected cells were lysed in lysis buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 50 mM NaF, 100 mg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Protein concentrations of the cell lysate supernatants were determined by using a Bio-Rad protein assay kit. For the IP experiments, preincubation of the supernatant with protein G–Sepharose 4B (Zymed) was performed to exclude non-specific binding. The supernatant was then incubated for several hours with rat anti-HA Ab (Boehringer-Mannheim), followed by precipitation with protein G–Sepharose 4B. After washing in lysis buffer, the sepharose mixture was boiled in 2× Laemmli sample buffer containing reducing agents and the samples were analyzed by SDS–PAGE. Immunoblot analysis was performed with mouse anti-Myc Ab (Clontech) and horseradish peroxidase-conjugated anti-mouse Ab (Amersham), followed by detection with ECL Plus (Amersham).
IP with anti-BRG1 Ab and immunoblotting for FANCA

Total cell lysate was prepared from EBV-immortalized lymphoblastoid cells from a normal individual, EBV-immortalized lymphoblastoid cells from a FA group A patient (HSC72), HSC72 cells retrovirally transduced with normal FANCA cDNA (HSC72/FANCA) and the human adenocarcinoma line SW13, in which BRG1 is defective. The normal lymphoblastoid cell line, HSC72 and HSC72/FANCA were gifts from Dr Christopher E.Walsh, University of North Carolina, Chapel Hill, NC. A total of 200 µg of each cell lysate was incubated with rabbit polyclonal anti-BRG1 Ab (a gift from Dr Tsutomu Ohita, National Cancer Center, Japan), followed by precipitation with protein A–Sepharose (Sigma). After washing in lysis buffer, the precipitation was applied to SDS–PAGE, followed by immunoblotting for FANCA. Anti-FANCA Ab was prepared from a rabbit immunized with a peptide corresponding to the N-terminal region of FANCA, NH2–MSDSWVPNSASGQDPGRRRAC-COOH. The Ab was affinity-purified using specific peptide columns prior to use. Anti-BAF170, anti-BAF155 and anti-IN11 Abs were purchased from Santa Cruz.

IP with anti-BRG1 Ab and silver staining

Immunoprecipitation experiments using anti-BRG1 Ab were performed as described above. The IP samples were analyzed by SDS–PAGE and silver staining with the Silver Stain Plus Kit (Bio-Rad).

Identification of proteins by peptide mass mapping

The samples described above were subjected to electrophoresis on a 7.5% polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore). The immobilized proteins were reduced, S-carboxymethylated and digested in situ with Achromobacter protease I (a Lys-C) (69). Molecular mass analyses of Lys-C fragments were performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using a PerSeptive Biosystem Voyager-DE/RP (70). Identification of proteins was carried out by comparison between the molecular weights determined by MALDI-TOF/MS and theoretical peptide masses from the proteins registered in NCBI (71).

Chromatin remodeling assay

Nucleosomes were reconstituted on a G5E4-5S array fragment by the addition of 100 µl of 10 mM Tris–HCl pH 7.5, 2 mM EDTA, 5 mM DTT, 0.5 mM PMSF, 0.1% nonidet P-40 (NP-40), 20% glycerol and 100 µg/ml BSA. Human SWI/SNF complex was purified from HSC72 and HSC72/FANCA cell lines using rabbit antibody against hBRG1 (36). Briefly, anti-BRG1 antibody was cross-linked to protein A–Sepharose beads equilibrated in Buffer A (BC100) (34) with 0.3% NP-40 and protease inhibitors. Pre-cleared whole cell lysates were mixed with 1/6 volumes of a 50% slurry and rocked overnight at 4°C. Subsequently, the beads were washed twice with 10 volumes of BC700 + 0.1% NP-40, once with BC100 + 0.1% NP-40 and once with 80 mM KCl + 0.1% NP-40. Finally, proteins bound on the beads were eluted with 1.3 volumes of 100 mM glycine pH 2.5, followed by neutralization with 0.14 volumes of 1 M Tris–HCl pH 8.0. Reconstituted chromatin templates or naked array fragments (2500 c.p.m. each) were incubated with a purified SWI/SNF complex in 25 µl of a reaction buffer containing 12 mM HEPES pH 7.9, 60 mM KCl, 6 mM MgCl2, 60 mM EDTA, 2 mM DTT and 13% glycerol in the absence or presence of 4 mM Mg-ATP (34). After incubation at 30°C for 90 min, the samples were treated with DNase I (0.25 U for nucleosome templates, 0.025 U for naked DNA) for 1.5 min at room temperature, followed by the addition of 20 µl of stop solution (20 mM Tris–HCl pH 7.5, 50 mM EDTA, 1% SDS, 0.5 mg/ml tRNA and 0.2 mg/ml protease K) to terminate the reaction. After deproteinization at 50°C for 2 h, template DNAs were precipitated with ethanol and subjected to 2% agarose gel electrophoresis at 75 V for 16 h. Digested fragments were visualized by autoradiography. Chromatin remodeling activity was also determined by the accessibility of XbaI to the G5E4-5S array fragment, which contains an XbaI site within the Gal4-binding domain (74). After the remodeling reaction, the mixture was incubated with 10 U of XbaI for 30 min at 30°C under standard conditions but with 3.5 mM MgCl2 and 60 mM urea. Samples were resolved on 1% agarose gels in Tris–borate–EDTA buffer and analyzed by autoradiography.

Confocal laser microscopy

HeLa cells cultured on a glass-bottom dish were transfected with C-terminal FLAG-tagged FANCA and HA-tagged BRG1 expression plasmids and immunohistochemically stained with mouse anti-FLAG Ab and/or rat anti-HA Ab as the first Ab. After washing with PBS, the cells were stained with anti-rat Ab containing Alexa 488 and/or anti-mouse Ab containing Alexa 546 as the second Ab. Intracellular components accumulating Alexa 488 were observed using confocal laser microscopy (MicroRadiance; Bio-Rad) under a 488 nm excitation light with a 513/30 filter for emission. Samples labeled with Alexa 546 were also observed under a 514 nm excitation light with a 570 long pass filter for emission. The C-terminal FLAG-tagged FANCA plasmid was constructed using pXFLAG-CMV-14 expression vector (Sigma). Anti-FLAG Ab and anti-HA Abs were purchased from Sigma and Boehringer-Mannheim, respectively. All second Abs were purchased from Molecular Probes.

REFERENCES


2660 Human Molecular Genetics, 2001, Vol. 10, No. 23


