

Isolation of an scFv targeting BRG1 using phage display with characterization by AFM

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Abstract

Remodeling of chromatin is a vitally important event in processes such as transcription and replication. Brahma-related gene 1 (BRG1) protein is the major ATPase subunit in the human Swi/Snf complex (hSwi/Snf), an important example of the family of enzymes that carry out such remodeling events. We have used a recently developed technique, recognition imaging, to better understand the role of BRG1 in remodeling chromatin. In such experiments, a specific antibody against BRG1 is needed. However, we have found that the commercially available polyclonal (CAP) antibodies interact non-specifically with nucleosomes, making it impossible to identify hSwi/Snf (BRG1) in their presence. Here antibody phage display technology is employed for development of an antibody specifically targeting BRG1. The Tomlinson I and J single chain variable fragment (scFv) libraries were used for successful isolation of an anti-BRG1 scFv. We demonstrate that the scFv binds more strongly and with less nonspecific interactions than the CAP antibody. This work lays the groundwork for future studies involving chromatin remodeling.

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The activities of the members of the large family of ATP-dependent nucleosome remodeling complexes provide a major *in vivo* mechanism to relieve the repressive effects of nucleosomes on transcription, replication, repair, and recombination [1–11], and also are factors in cancer and other disease states [12,13]. Their global role in genome processes makes understanding how they work a high priority.

The Brahma-related gene 1 (BRG1) protein-containing human Swi/Snf complex (hSwi/Snf) has major biological importance. These complexes are potent tumor suppressors; *brg1* mutations are strongly associated with cancer, being found in primary tumors and in tumor-derived cell lines [13]. BRG1 is essential for mouse embryonic development [14]. This reflects a specific developmental role

because mutant *brg1* cell lines are viable and in fact grow faster than wild type. BRG1 complexes also function in cell-cycle control and are required for differentiation of specific cell types [14,15]. Thus, BRG1-containing hSwi/Snf complexes function globally and are of great importance.

A recent study from this laboratory looked at the interactions of the hSwi/Snf complex with mouse mammary tumor virus (MMTV) DNA and chromatin [16]. The goal of that study was to follow the movements of hSwi/Snf in its interaction with chromatin, in order to understand the precise mechanisms of how chromatin-remodeling enzymes work in general. Recognition imaging technology was employed to track the movements of the hSwi/Snf complex. This technology, developed by Stroth et al. [17], allows for the recognition of a specific type of single molecule within a complex sample using the atomic force microscope (AFM) with an antibody against the target antigen coupled to the AFM tip. While the typical topographical

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data are collected, recognition data are simultaneously collected via detection of minute changes in the absolute (dc) level of the cantilever-deflection signal caused by the binding of the antibody to its antigen. The elegance of this technique is that it allows for the examination of a molecule before and after activation of the hSwi/Snf complex (by addition of ATP) so that changes can be tracked on individual molecules ultimately leading to insights concerning the mechanisms that this protein utilizes to perform its remodeling functions.

In recognition imaging, the antibody is tethered to the atomic force microscope probe by a short (6 nm) oligoethylene glycol tether, so the antibody is confined to a small volume (ca. 10^{-22} L) near the sample surface. In consequence, its effective concentration is a significant fraction of a mole [18]; much more than would be used in a blotting experiment. Therefore, crossreactions become much more evident. In recognition imaging, the commercially available polyclonal (CAP) antibody that targets BRG1 also binds to nucleosomes. Thus, we could use it to characterize hSwi/Snf complexes alone but could not use it in the desired application, to recognize hSwi/Snf when bound to and acting on chromatin. Here, we combine antibody phage display and atomic force microscopy to isolate and characterize a single chain variable fragment (scFv) antibody that can specifically recognize BRG1 within the chromatin-remodeling complex hSwi/Snf.

The advent of phage display technology has allowed researchers to quickly isolate antibodies against a range of different target antigens for unique purposes. These antibodies can be used as diagnostics [19–22] or potential therapeutics [23–26]. Developed in the early 1990s [27,28], antibody phage display technology allows for rapid isolation of monoclonal antibodies through a process called biopanning. The target antigen for our study is a peptide that represents a portion of the BRG1 protein. We demonstrate that the monoclonal antibody that was isolated through phage display biopanning performs better than the CAP antibody. The merger of these two powerful techniques generates a new approach for following molecular composition on the nanometer scale.

Materials and methods

Libraries, bacteria, and helper phage. The human single-fold scFv libraries I + J (Tomlinson I + J), *Escherichia coli* TG1 and HB2151, and KM13 helper phage were all kindly provided by the Medical Research Council (MRC).

Biopanning. Antibody-coated phage produced using the Tomlinson I + J libraries were used to pan against a peptide (KRDS DAGSSTPT; synthesized at Protein Analysis and Synthesis Laboratory, ASU) corresponding to residues 1414–1425 of BRG1. The panning protocol used was essentially as described by the MRC protocol (<http://www.geneservice.co.uk/products/proteomic/datasheets/tomlinsonIJ.pdf>). Briefly, immunotubes (Nalge Nunc International, USA) were coated with 10–100 μ g/ml of the peptide in PBS. After blocking and washing, tubes were loaded with 10^{12} phages. After extensive washing, bound phages were eluted with 100 mM triethylamine, neutralized, treated with trypsin (final concentra-

tion of 1 mg/ml) for 30 min, and further propagated and amplified in *E. coli* TG1.

Monoclonal phage ELISA. Phage ELISAs were also performed according to the producer's protocol (<http://www.geneservice.co.uk/products/proteomic/datasheets/tomlinsonIJ.pdf>). Briefly, after three rounds of panning, individual clones were grown in microtiter wells and phages were produced by addition of KM13 helper phages. Phage was then added to corresponding wells of a BRG1 peptide-coated (10 μ g) plate and detected by an anti-M13 antibody.

Phagemid DNA sequencing. Isolation of the pIT2 phagemid vector was performed using the Qiagen Plasmid Isolation Kit (Qiagen, Valencia, CA). The phagemids were sequenced by the DNA Laboratory at Arizona State University using the pHEN seq (5'-CTA TGC GGC CCC ATT CA-3') or LMB3 (5'-CAG GAA ACA GCT ATG AC-3') sequence primers.

Site-directed mutagenesis. Primers were designed to change the premature amber codon (TAG) to CAG (glutamine) by using the 21 nucleotides flanking the amber codon in each chain: Heavy chain forward (5'-AGT ACG CGG GGT CGG ACG ACA CAG TAC GCA GAC TCC GTG AAG GGC-3') and reverse (5'-GCC CTT CAC GGA GTC TGC GTA CTG TGT CGT CCG ACC CCG CGT ACT-3'); Light chain forward (5'-CTC CTG ATC TAT AAT GCA TCC CAG TTG CAA AGT GGG GTC CCA TCA-3') and reverse (5'-TGA TGG GAC CCC ACT TTG CAA CTG GGA TGC ATT ATA GAT CAG GAG-3'). The following mix was created in a PCR tube on ice: 33 μ l of water, 2 μ l of 50 mM MgSO₄, 5 μ l of 10 \times Pfx amplification buffer, 4 μ l dNTPs, 1 μ l of each mutagenesis primer, 5 μ l of isolated plasmid, and 2 μ l Platinum Pfx polymerase. The following thermocycler conditions were used: initial cycle for 60 s and 94 $^{\circ}$ C, then 12 cycles of 30 s at 94 $^{\circ}$ C, 30 s at 55 $^{\circ}$ C, and an extension time of 10 min at 68 $^{\circ}$ C (extension time is based on 2 min/kbp of plasmid). A 1 μ l aliquot of DpnI and 5.7 μ l of NEBuffer 4 were added to the PCR mixture and incubated at 37 $^{\circ}$ C for 1 h. A 200 μ l aliquot of chemically competent HB2151 cells was added to the mixture. The mixture was placed on ice for 30 min, heat shocked for 3 min at 42 $^{\circ}$ C, and then iced for 3 min. A 100 μ l aliquot of the mixture was plated onto LB agar plates (supplemented with 100 μ g/ml of ampicillin) and grown overnight at 37 $^{\circ}$ C.

Dot blot. A 2 μ l aliquot of sample was placed on a nitrocellulose membrane. The membrane was blocked with 2% milk in PBS, pH 7.4, for one hour at room temperature. The membrane was washed once with PBS and then stained with anti-myc-tag mouse antibody (Santa Cruz Biotechnology, USA) at 1:500 dilution in PBS for one hour at room temperature. The membrane was washed 3 times with PBS and then stained with a goat anti-mouse IgG HRP conjugate antibody at 1:1000 dilution in 2% MPBS for one hour at room temperature. The membrane was then washed 3 times with PBS and developed using DAB substrate (Sigma, USA).

Purification of anti-BRG1 scFv. A 1 L culture of the HB2151 containing the anti-BRG1 scFv phagemid was grown (shaking at 250 rpm at 37 $^{\circ}$ C) in 2 \times TY and 100 μ g/ml ampicillin to OD₆₀₀ = 0.5. One molar IPTG was added to the culture to induce scFv expression. The culture was grown overnight (shaking at 250 rpm) at 30 $^{\circ}$ C. The following day, the culture was spun down at 10,000g for 10 min. The pellet was resuspended in 50 ml of the osmotic shock buffer TSE (50 mM Tris, 20% sucrose, and 1 mM EDTA, pH 7.5) for 30 min at 4 $^{\circ}$ C. The buffer was spun down at 10,000g for 30 min. The supernatant was added to the sample loop of the ÄKTA FPLC system (Amersham Pharmacia Biotech, Piscataway, NJ) and the scFv was purified using the HiTrap Protein A (5 ml) column. Glycine (0.2 M, pH 3.0) was used to elute the scFv from the column. After collection of eluted peaks, the pH was immediately neutralized by addition of NaOH. The purified sample was then dialyzed overnight in PBS and purity was checked by SDS gel electrophoresis. The scFv concentration was determined by the BCA protein assay kit (Pierce, Rockford, IL).

Sample preparation. Nucleosomal arrays containing the mouse mammary tumor virus (MMTV) promoter region were salt-reconstituted as described [29]. Human Swi/Snf was prepared as described [30]. The nucleosomal arrays or hSwi/Snf were deposited on glutaraldehyde aminopropyltriethoxysilane (GD-AP TES)-treated mica, derivatized at 1 μ M levels with GD [31], and allowed to adsorb for 40 min.

Atomic force microscopy recognition imaging. Commercially, available polyclonal (CAP) anti-BRG1 antibodies (Abcam, Cambridge, MA) or monoclonal scFvs were tethered to silicon nitride cantilevers and used to generate recognition images just as described [17]. The recognition signal was obtained by PicoTREC (Molecular Imaging, Tempe, AZ). Magnetized cantilevers are driven by a MacMode dynamic-force microscope (Molecular Imaging, Tempe, AZ). Images were taken in 10 mM NaCl/5 mM phosphate buffer, pH 7.5, with 3-nm peak-to-peak amplitude oscillation at 8 kHz, imaging at 70% set point, and scanning at 1 Hz. The recognition and topographical image were obtained simultaneously.

Results

The key component for efficient recognition of BRG1 within hSwi/Snf is a targeting molecule that binds with a high efficiency. To that end, phage display techniques were employed to develop a specific antibody. The antibody was then attached to an AFM tip and used for recognition imaging of hSwi/Snf, MMTV chromatin, or a mixed sample of both.

Isolation of scFv against BRG1

Biopanning against the BRG1 peptide was performed using the Tomlinson I and J libraries. After three rounds of panning, eluted phage was used to infect TG1 *E. coli* bacteria and 32 individual clones were picked from a plate and screened by monoclonal phage ELISAs. Ten clones were selected as strong binders since the binding values detected in the wells coated with BRG1 peptide were at least twice that of wells with no antigen (data not shown). The phagemids of these 10 clones were isolated and the nucleotide sequences of the scFvs were obtained. All 10 of the sequences were identical, and they all contained premature amber stop codons (TAG) in both the heavy and light chains of the scFv.

Mutagenesis, soluble expression, and purification of scFv

Mutagenesis of the premature amber stop codons in the anti-BRG1 scFv phagemid from TAG to CAG was performed since TG1 was a suppressor E strain of *E. coli* bacteria that recognized the amber codon as glutamine, and phage ELISAs detected binding of the scFv/phage chimera. Forward and reverse primers for the amber codon in the heavy/light chains were created by using the 21 nucleotides before the amber codon, CAG instead of TAG, and then the 21 nucleotides after the amber codon. The protocol given for the mutagenesis of the amber codon created entire new phagemids with the amber codon changed to glutamines. *DpnI* was used to cleave dam methylated GATC sites of DNA after PCR was performed. Bacteria frequently methylates its DNA and cleavage of these GATC sites ensures that the only phagemid DNA left is that created as a PCR product.

The newly synthesized phagemids were transformed into HB2151 bacteria and grown on LB agar plates, supplemented with 100 µg/ml of ampicillin. Individual colonies

were picked, grown to log phase ($OD_{600} = 0.5$), and induced with 1 mM (final concentration) of IPTG. Dot blots were performed on supernatants and periplasmic fractions of several clones to check for expression. A double mutagenesis of both the heavy and light chains was initially performed. However, none of the 15 clones that were picked showed expression of the scFv. Mutagenesis was then performed first on the light chain. Ten clones were picked to verify that the amber codon was changed to glutamine. Then, the single mutant was used to perform the mutation of the heavy chain. Dot blot analysis of the double mutants was then performed to determine which clones had scFv expression. A one-liter culture of the double mutant was grown and used for expression and purification of 300 µg of anti-BRG1 scFv.

Recognition imaging of BRG1 within hSwi/Snf

Atomic force microscopy recognition imaging was used to identify BRG1 in the hSwi/Snf complex. Fig. 1 shows representative topographic (A and D) and recognition (B, C, E, and F) images of the hSwi/Snf complex, deposited on glutaraldehyde aminopropyltriethoxysilane (GD-APTES)-treated mica. The scans were done with AFM tips containing either the CAP antibody (A–C) or the customized scFv (D–F). Both tip-bound antibodies recognize the hSwi/Snf complex. In order to determine the recognition efficiency of each antibody, it is necessary to compare the number of recognition events with the total number of hSwi/Snf complexes present in the same field. The hSwi/Snf sample also contains bovine serum albumin (BSA), so we measured heights in order to identify bona fide hSwi/Snf complexes in the topographic image. Based on a previous analysis [16], particles greater than 2 nm in height were considered to be hSwi/Snf. A customized program, Recognition Image Analysis, was used to determine the recognition, as evidenced by the green dots overlaying the topographical images. In a representative scan shown here (Fig. 1A), 11 hSwi/Snf molecules are present in the topographic image. Four of these were recognized by the CAP antibody (Figs. 1B and C). In the field shown in Fig. 1D, 3 hSwi/Snf complexes are present in the topographic image and the customized scFv antibody recognized all 3 complexes (Figs. 1E and F). In Table 1, data from a larger set of images are presented (a total of 39 hSwi/Snf molecules imaged topographically with the CAP antibody; and a total of 24 hSwi/Snf molecules with the customized scFv). BRG1 is recognized almost twice as efficiently with the customized scFv antibody (83.3%) as with the CAP antibody (43.6%).

Non-specific recognition of MMTV chromatin

The major drawback of the CAP antibody is its non-specific recognition of MMTV chromatin. Fig. 2 shows an example of recognition imaging of MMTV nucleosomal arrays using tips containing the CAP antibody (A–C) or

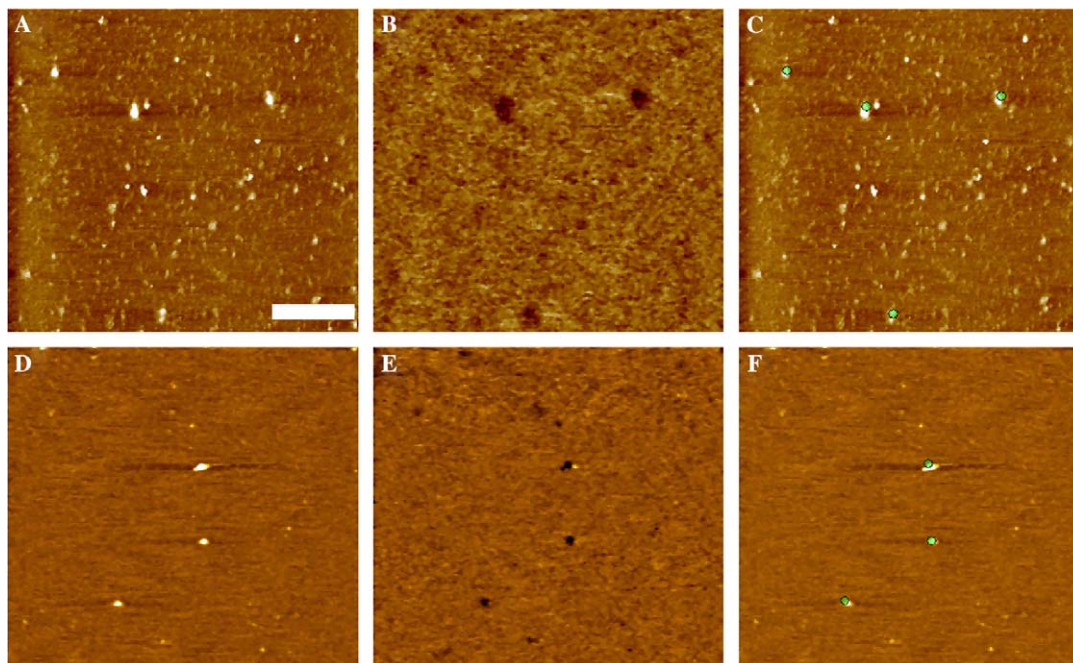


Fig. 1. Specific recognition imaging of Swi/Snf. Top panels show a representative scan of 11 Swi/Snf molecules placed on mica and imaged (A) topographically in white with (B) antibody-antigen recognition by the CAP antibody as a black recognition event or (C) as green dots showing recognition overlaying the topographical image. The bottom panels show a representative scan of 3 Swi/Snf molecules placed on mica and imaged (D) topographically in white with (E) antibody-antigen recognition by the scFv as a black recognition event or (F) as green dots showing recognition overlaying the topographical image. The scale bar is 500 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

Table 1
Comparison of characterizations of CAP antibody and customized scFv

	CAP antibody properties	Customized scFv properties
Recognition frequency (%)	43.6	83.3
Non-specific recognition frequency (%)	15.6	<3.1
Specific-binding force (pN)	67.4 ± 35.4	88.1 ± 38.6
Non-specific-binding force (pN)	73.4 ± 18.8	23.5 ± 7.7

the customized scFv (D–F). The Recognition Imaging Analysis software program was again used to determine recognition efficiency for this nonspecific target. Fig. 2A has ~32 chromatin areas topographically imaged, with 5 of those areas recognized when using the CAP antibody (Figs. 2B and C). This is a nonspecific recognition frequency of 15.6%. Fig. 2D shows >32 chromatin areas topographically imaged and only 1 of those areas is recognized by the scFv antibody (Figs. 2E and F). This is a nonspecific recognition frequency of <3.1%. The data are summarized in Table 1.

Specific and nonspecific force curve data

To understand why recognition imaging using the customized scFv was better than using the CAP antibody, force curves using both antibodies and both molecules (hSwi/Snf and MMTV chromatin) were examined (Fig. 3). These data were obtained at a pulling rate of

30 nN/s. The bar graph shows that an average force of 67.4 ± 35.4 pN ($n = 137$) is required to break the bond between the CAP antibody and the BRG1 subunit within the hSwi/Snf complex. An average force of 73.4 ± 18.8 pN ($n = 112$) is required to break the bond between the CAP antibody and MMTV chromatin. There is no significant difference between these values. On the other hand, an average force of 88.1 ± 38.6 pN ($n = 158$) is required to break the bond between the customized scFv and the BRG1 subunit within the hSwi/Snf complex but an average force of only 23.5 ± 7.7 pN ($n = 35$) is required to break the bond between the scFv and MMTV chromatin. Thus, there is a significant force difference between the specific and nonspecific targets when the scFv antibody is used. Moreover, the relatively low force value for the scFv antibody-MMTV chromatin case suggests there is little interaction between these proteins. The differences between the force curves of the two antibodies (CAP vs. scFv) targeting MMTV are significant ($p < 0.0001$). The data are summarized in Table 1.

Recognition imaging of BRG1 within a mixture of hSwi/Snf and MMTV chromatin

The CAP antibody could be used to characterize hSwi/Snf complexes alone but could not be used for its desired application, to recognize hSwi/Snf when bound to and acting on chromatin [16]. In Figs. 4A and D, mixed samples of hSwi/Snf and MMTV chromatin are shown

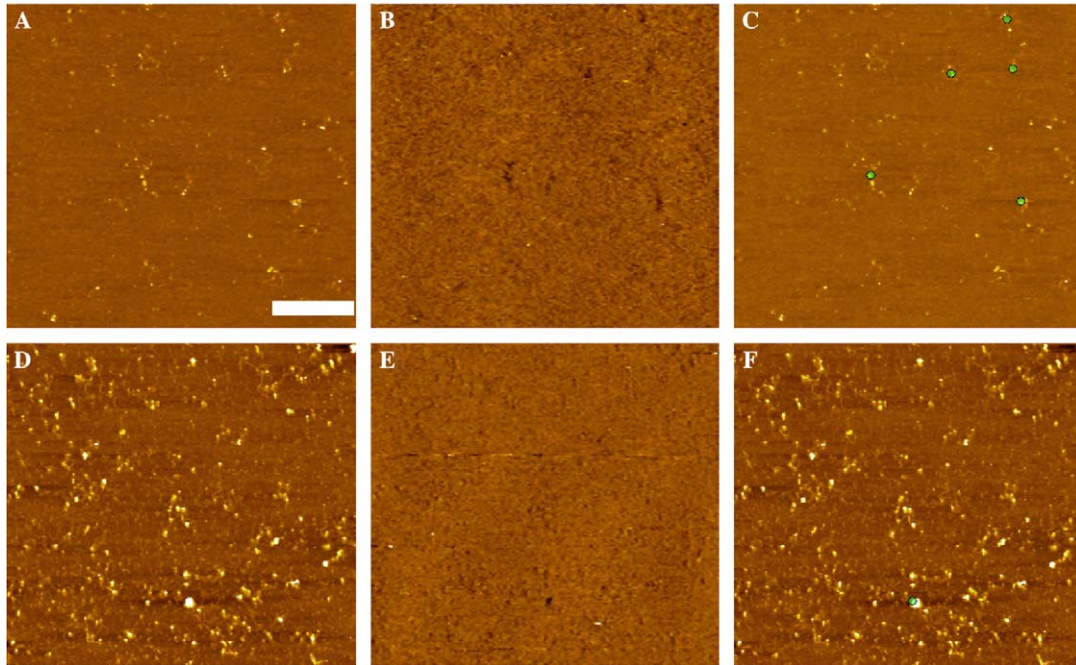


Fig. 2. Non-specific recognition imaging of MMTV chromatin. Top panels show a representative scan of MMTV chromatin placed on mica and imaged (A) topographically in white with (B) antibody-antigen recognition by the CAP antibody as a black recognition event or (C) as green dots showing recognition overlaying the topographical image. The bottom panels show a scan of MMTV chromatin placed on mica and imaged (D) topographically in white with (E) antibody-antigen recognition by the scFv as a black recognition event or (F) as green dots showing recognition overlaying the topographical image. The scale bar is 500 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

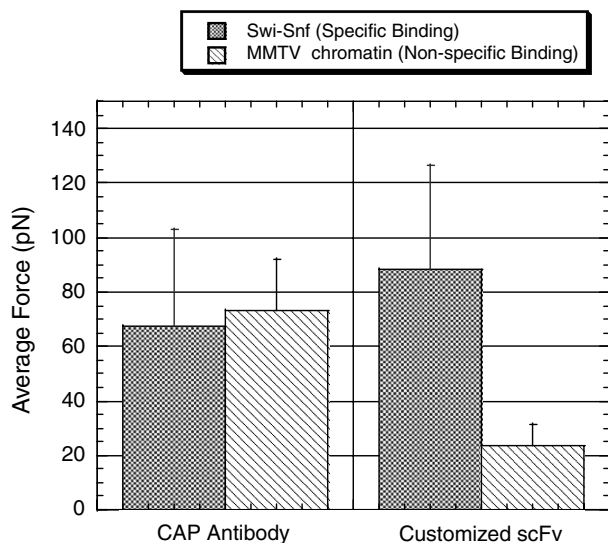


Fig. 3. Average force curve data for specific and non-specific interactions using the CAP antibody or customized scFv. Error bars are standard deviations.

topographically. The mixed sample shows more overall recognition by the CAP antibody (40 events; Figs. 4B and C) than the mixed sample recognized by the scFv (5 events; Figs. 4E and F). Although there is more hSwi/Snf in Fig. 4A than D, it is evident that the additional recognition is attributed to the nonspecific interaction of the CAP antibody with the MMTV chromatin.

Discussion

Precisely how the hSwi/Snf complex works to remodel chromatin is not completely understood. Atomic force microscopy recognition imaging is a powerful tool that can allow us to understand the functional mechanisms of this important complex. However, to apply this approach, one needs an antibody that can efficiently and specifically recognize its target, in this case the BRG1 subunit of the hSwi/Snf complex. BRG1 is the major ATPase subunit and is crucial for the function of hSwi/Snf. The CAP antibody was previously used to image BRG1 [16]. However, this antibody interacts non-specifically with chromatin, making it unsuitable for imaging hSwi/Snf in the presence of chromatin. Therefore, antibody phage display methods were utilized to develop a monoclonal scFv against BRG1.

Two important facets of antibody phage display are addressed by this research. First, by using a peptide that represents a unique region of BRG1 as an antigen, we were able to develop this monoclonal scFv. As more antibody targets for chromatin remodeling are discovered, it may not be possible to always isolate and purify these antigens. Also, even if isolation and purification were possible, the full protein may share significant sequence homology with other proteins, leading to nonspecific interactions. The use of peptides representing a specific and exposed region of the antigen will become an increasingly useful strategy to utilize when performing biopanning. Second, the incorporation of randomly generated amber stop codons (TAG)

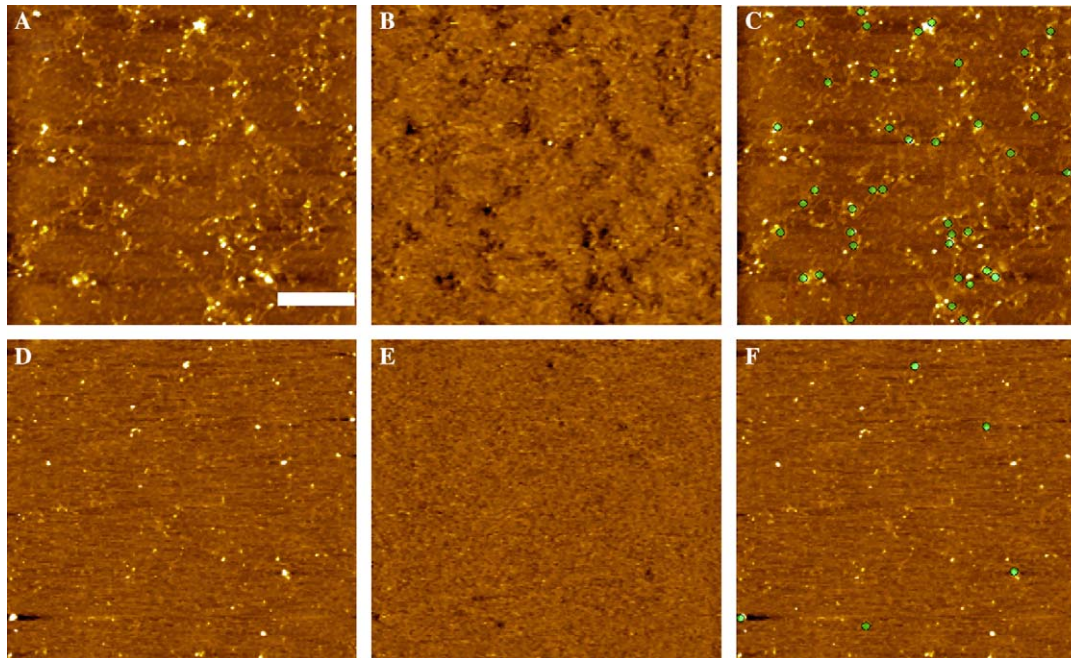


Fig. 4. Mixed Swi/Snf and MMTV chromatin sample images. Top panels show a scan of Swi/Snf and MMTV chromatin placed on mica and imaged (A) topographically in white with (B) antibody-antigen recognition by the CAP antibody as a black recognition event or (C) as green dots showing recognition overlaying the topographical image. The bottom panels show a representative scan of Swi/Snf and MMTV chromatin placed on mica and imaged (D) topographically in white with (E) antibody-antigen recognition by the scFv as a black recognition event or (F) as green dots showing recognition overlaying the topographical image. The scale bar is 500 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

into the phagemid containing the scFv is an important issue that has not been addressed previously. We have found that the amber codon confers a growth advantage to the *E. coli* suppressor strain TG1 used during the bio-panning process. Therefore, after the several rounds of panning, clones that bind to the target antigen that contains the amber codon will be preferentially selected. Once the phagemid of these clones are put into non-suppressor cells for soluble antibody secretion, the randomly generated amber codon will prematurely terminate translation of the antibody. The monoclonal scFv isolated against BRG1 had two of these randomly generated amber codons. This research outlines a method for isolating positive binding clones, regardless of the presence of randomly generated amber codons.

This work also highlights how antibody phage display may be used to develop antibodies that can work better than commercially available antibodies. We were interested in finding an antibody that would bind specifically to BRG1. The CAP antibody, although recognizing the BRG1 antigen (Figs. 1B and C and Table 1), clearly recognizes an epitope within chromatin, as evidenced by recognition imaging of MMTV chromatin (Figs. 2B and C and 4B and C) and by force curve data (Fig. 3). These nonspecific interactions are likely due to the fact that the CAP antibody is polyclonal and may recognize different amino acid segments of the BRG1 antigen, including a segment that may share sequence homology and be readily accessible in MMTV chromatin. In contrast, the monoclonal scFv

not only strongly recognized BRG1 (Figs. 1E and F), but it had very little nonspecific binding to chromatin (Figs. 2E and F, 3, and 4E and F). Although 32 molecules were used for analyzing nonspecific interactions of the scFv to chromatin, many more were on the topographical scan (Fig. 2D) that were not recognized. Therefore, the 3.1% nonspecific interaction is a gross overestimate.

Therefore, the combination of strong specific binding and little nonspecific interactions makes the anti-BRG1 scFv a suitable tool for use in further recognition imaging to elucidate the mechanisms of hSwi/Snf and BRG1 action on chromatin. This work clearly demonstrates how phage display and atomic force microscopy recognition imaging can be used together as a tool for studying other factors involved in chromatin remodeling.

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