Single-molecule anatomy by atomic force microscopy and recognition imaging*

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Summary. Atomic force microscopy (AFM) has been a useful technique to visualize cellular and molecular structures at single-molecule resolution. The combination of imaging and force modes has also allowed the characterization of physical properties of biological macromolecules in relation to their structures. Furthermore, recognition imaging, which is obtained under the TRECTM (Topography and RECognition) mode of AFM, can map a specific protein of interest within an AFM image. In this study, we first demonstrated structural properties of purified α Actinin-4 by conventional AFM. Since this molecule is an actin binding protein that cross-bridges actin filaments and anchors it to integrin *via* tailin-

vinculin- α actinin adaptor-interaction, we investigated their structural properties using the recognition mode of AFM. For this purpose, we attached an anti- α Actinin-4 monoclonal antibody to the AFM cantilever and performed recognition imaging against α Actinin-4. We finally succeeded in mapping the epitopic region within the α Actinin-4 molecule. Thus, recognition imaging using an antibody coupled AFM cantilever will be useful for single-molecule anatomy of biological macromolecules and structures.

Introduction

Atomic force microscopy (AFM) can be operated in two different modes; imaging and force modes. The imaging mode has been used to visualize various biological structures and processes (reviewed in Hirano *et al.*, 2008). Nano-scale structures and the dynamics of cells, cytoskeletons (Henderson *et al.*, 1992; Hofmann *et al.*, 1997; Haga *et al.*, 2000; de Jager *et al.*, 2001; Yoshimura *et al.*, 2003; Mucke *et al.*, 2004), DNA-protein interactions (Nettikadan *et al.*, 1996; de Jager *et al.*, 2001; Schnitzler *et al.*, 2001; Yoshimura *et al.*, 2002; Rivetti

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Abbreviations: AFM: atomic force microscope, APTES: 3-aminopropyltriethoxysilane, EDC: 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride, IPTG: isopropyl β -D-1-thiogalactopyranoside, MES: 2-(N-morpholio) ethanesulfonic acid, NHS: N-hydroxysulfosuccinimide, PBS: phosphate buffered saline, PEG: polyethylene glycol, PMSF: phenylmethylsulfonyl fluoride

et al., 2003; Das *et al.*, 2006; Lushnikov *et al.*, 2006; Hizume *et al.*, 2007), and membrane proteins (Saslowky *et al.*, 2002; Czajkowsky *et al.*, 2004; Barrera *et al.*, 2005) have been elucidated. Several technical improvements, such as carbon nanotube probe (Hohmura *et al.*, 2000), and fast-scanning AFM (Viani *et al.*, 2000; Ando *et al.*, 2001; Yokokawa *et al.*, 2006; Crampton *et al.*, 2007) have greatly expanded the application range of AFM in molecular and cellular biology.

The force mode of AFM has contributed to the characterization of physical properties of biological macromolecules such as the elucidation of internal structures of biological macromolecules and intermolecular interaction of protein subunits, and protein-lipid/protein-DNA interactions (Hinterdorfer *et al.*, 1996; Nevo *et al.*, 2003; Afrin *et al.*, 2005). This mode depends on the force measurements between the cantilever and the specimen. Unique ideas for the cantilever modification techniques have been the key for successful force measurement (Ikai *et al.*, 2003; Gamsjaeger *et al.*, 2004; Kamruzzahan *et al.*, 2006; Ebner *et al.*, 2007). Previously, we have developed a novel method for a site-specific attachment of glutathione S-transferase (GST)-fused proteins to the cantilever in a desired direction, and applied to the measurement of interaction between importin β and its interacting proteins (Yoshimura *et al.*, 2006; Otsuka *et al.*, 2008).

Recent development of recognition imaging under the PicoTRECTM mode of AFM (Topography and RECOgnition mode, commercialized by Agilent Technologies, Santa Clara, USA) has further enabled researchers to simultaneously obtain a topographic image together with a recognition signal that is based on the interaction between the specimen and the protein/ligand-coupled cantilever, and thus, to identify a specific molecule within the topographic image. In this imaging mode, an attractive force between a molecule on the cantilever surface and its partner molecules on the specimen can be detected during the imaging (Stroh *et al.*, 2004a). With this technique, histone proteins in reconstituted chromatin and membrane proteins on the cell surface have been successfully recognized (Stroh *et al.*, 2004b; Bash *et al.*, 2006; Chtcheglova *et al.*, 2007).

In this study, we established our own procedure to covalently couple the antibody to the cantilever surface with commercially-available chemical compounds. By using this method, we attached an anti- α Actinin-4 monoclonal antibody to the AFM cantilever and performed recognition imaging against purified α Actinin-4. α Actinin-4 is an actin-binding cytoskeletal protein and contains three functional sub-domains; an N-terminal actin-binding domain composed of two

calponin homology domains, a C-terminal EF hand, and an central rod domain composed of four spectrin repeats (Mimura and Asano, 1987; Davison and Critchley, 1988). Anti-parallel dimerization is necessary for cross-linking of actin filaments (Ylanne *et al.*, 2001). We found that there are several different structures in dimerized α Actinin-4 *in vitro*. In addition, by using antibody-coupled cantilevers, we could successfully map the position of the epitopic region within a single α Actinin-4 molecule.

Materials and Methods

Expression and purification of full-length and coiled- and actin-binding regions of α Actinin-4

DNA constructs for full length α Actinin-4 (corresponding for 1-911 amino acids), actin binding region (1-286 amino acids) and coiled region (287-758 amino acids) were amplified by PCR from a HeLa cDNA pool and cloned into a pGEX-5X-1 vector (GE Healthcare). *Escherichia coli* cells (BL21) harboring the plasmid were cultured in LB medium and the expression of the protein was induced by IPTG (final concentration of 1 mM) at 30°C for 4 h. The cells were harvested and resuspended in a buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM PMSF) and sonicated on ice for a total of 90 sec. After removing the cell debris by centrifugation, the supernatant was mixed with Glutathione-Sepharose 4 B beads (GE Healthcare) in a tube. After washing with the above buffer not containing PMSF several times, the proteins fused to GST were cleaved off from the GST moiety by Factor Xa protease (New England Bio Lab) by following the manufacturer's protocol. To remove free Factor Xa protease in the solution, benzamidine beads (GE Healthcare) were mixed with the solution and precipitated by centrifugation. The supernatant was dialyzed against a buffer (50 mM Hepes-NaOH, pH 7.6, 100 mM NaCl).

Specificity of anti- α Actinin-4 antibody

The amino terminal domain (1-286 amino acids), which contains the actin-binding domain, and the central rod domain (287-758 amino acids), as well as full length α Actinin-4, were expressed as GST-fused proteins in *E. coli* and the cell lysates were subjected to SDS-PAGE followed by Western-blot analyses using the anti- α Actinin-4 monoclonal antibody (Fig. 1). The results showed that the antibody specifically recognized the amino-terminal domain but not the central rod domain.

AFM imaging of α Actinin-4 on the mica surface

Purified α Actinin-4 was diluted to 0.1 ng/ μ l with HMgG buffer (5 mM Hepes-NaOH, pH 7.5, 5 mM MgCl₂, 0.3% glutaraldehyde), incubated at 20°C for 30 min, and dropped onto a freshly cleaved mica surface. After 10 min incubation at room temperature, the mica was washed with Milli Q water and dried by nitrogen gas blowing.

AFM imaging was performed with Multi Mode AFM (Digital Instruments, Santa Barbara, CA, USA), in air using Tapping Mode™. The cantilever (Veeco NanoProbe RTESP7, Tucson AZ, USA) used for the imaging was 125 μ m in length. The scanning rate was set at 2–3 Hz, and images were captured in 512 $\times$ 512 pixel format. To measure the width and length of the structures in AFM images, the “tip effect” was removed by using the apparent size of nucleosomes as a reference (Nettikadan *et al.*, 1996). In this study, DNA, which has the width of 2 nm, was imaged and used as a standard. For the analysis of the volume, the “full width at half-maximum” (Schneider *et al.*, 1998) and height were measured by a procedure published previously (Schneider *et al.*, 1998). Briefly, the specific volume of an assumed globular protein (0.75 cm³/g) and weight of protein Molecular Mass (kDa)/6.02 $\times$ 10²³ g were combined and the volume of the molecule of protein was calculated as Molecular Mass (kDa) $\times$ 1.24 $\times$ 10³ nm³. Based on this formula, molecular mass of the protein in AFM images were estimated.

Preparation of the antibody-coupled cantilever and α Actinin-4 protein

Cantilevers made of silicon nitride (Type VI MAC lever (Agilent Technologies) for recognition imaging) were treated with vaporized 3-aminopropyltriethoxysilane (APTES) (SIGMA) and diisopropyl-ethylamine (SIGMA) for 1 hr inside an argon gas filled chamber to generate amino groups on the cantilever surface, then incubated with heterobifunctional cross linker (5 mg/ml maleimide-dPEG™₁₂-N-hydroxysulfosuccinimide (NHS) ester, (Quanta BioDesign Ltd., Ohio, the USA)) which was dissolved in chloroform containing 0.7% triethylamine for 2 hrs at room temperature. Optionally, the unreacted amino groups were blocked by 5 mg/ml NHS-m-dPEG™ (Quanta BioDesign Ltd) in chloroform containing 0.7% triethylamine. After washing with chloroform, ethanol and ultrapure water, the PEG-modified cantilever was then incubated with another crosslinker carrying thiol group, 1 mg of 5-carboxy-1-pentanethiol, (Dojindo Laboratories, Kumamoto) in PBS (pH 7.2) for 1 h at room temperature. The carboxyl-coupled cantilever was then

Fig. 1. SDS-PAGE and Western blot analysis of α Actinin-4. (His)₆-tagged full-length α Actinin-4 (F), GST-tagged actin-binding domain (A), GST-tagged central rod domain (C) and GST-tagged EF hands domain (E) were run on SDS-PAGE gel (Left). After blotting of the proteins onto PVDF membrane, bands that reacted with anti- α Actinin-4 monoclonal antibody were detected (Right).

mixed with antibody solution (final concentration; 100 μ g/ml) and EDC (final concentration; 500 μ g/ml; Pierce) in an activation buffer (100 mM MES-NaOH, pH 5.0) and kept for 1 h at room temperature. After the reaction, the cantilever was rinsed with PBS, then immediately used for AFM imaging.

Topographic and recognition imaging

For the recognition imaging, purified α Actinin-4 was immobilized on the mica substrate previously mentioned (Lohr *et al.*, 2007). Firstly, freshly cleaved mica was treated with APTES by vapor for 1 h at room temperature inside argon-gas-filled chamber. Then, the mica was treated with glutaraldehyde (final concentration of 1 mM) for 10 min. After washing with ultrapure water, 100 ng/ml of purified α Actinin-4 was immediately dropped on the mica and incubated for 10 min at room temperature. For the blocking of free glutaraldehyde, the mica substrate was treated with 1 mM glycine in PBS for 10 min. After washing with PBS, the mica substrate was immediately used for the recognition imaging. The AFM imaging was performed by PicoPlus AFM equipped with Pico TREC™ module (Agilent Technologies, Tempe, USA) was operated by Pico View software (Agilent Technologies). The image was taken in the magnetic Top MAC mode

Fig. 2. AFM analysis of the purified full-length α Actinin-4. **a:** SDS-PAGE gel of purified full-length α Actinin-4 (F), actin binding domain (A) and central rod domain (C). The SDS-PAGE gel (12%) was stained with Coomassie Brilliant Blue. These proteins were used for AFM imaging. **b:** AFM images of the purified full-length α Actinin-4 imaged by conventional AFM with un-modified cantilever. Observed elliptic structures were indicated by arrowhead, and observed round structures were indicated by arrow. 34% of the all observed structures showed elliptic shape, 62% of them showed round shape without tail, and 10% of them showed round shape with tail. **c:** AFM images of typical elliptic structures (panel **c-i**). Width (**c-ii**), height (**c-iii**), and lengths (**c-iv**) of the elliptic structures were measured in AFM images and tabulated into a frequency distribution. Proposed model of an anti-parallel homodimer of α Actinin-4 is shown in **c-v**. **d:** AFM images of typical lower round structures (**d-i**), taller round structures (**d-ii**), and round structures with tails (**d-iii**). Width (**d-iv**) and height (**d-v**) of the round structures were measured in AFM images and tabulated into a frequency distribution. Proposed model of the possible conformation of α Actinin-4 is shown in **d-vi**; monomer (left), dimer (middle), and dimer with tail (right).

Fig. 3. AFM analysis of actin-binding domain and coiled region of α Actinin-4. **a:** AFM image of actin binding domain. **b:** AFM image of coiled region. **c:** Width (**c-i**) and height (**c-ii**) of the actin binding domain were measured in AFM images and tabulated into a frequency distribution. **d:** Width (**d-i**) and height (**d-ii**) of the coiled region were measured and tabulated into a frequency distribution.

(Han *et al.*, 1996) with Type VI MAC levers (Typical spring constant; 0.1 N/m) (Agilent Technologies) in PBS (pH 7.4). The tune of the cantilever was carried out in PBS. The peak of the amplitude oscillation, about 15-20 kHz, was mainly used for the imaging. Set point and scan speed were set at 2.0–2.5 V and 1 Hz, respectively. The obtained images were analyzed by SPIP™ Ver. 4.6 (Image metrology, Horsholm, Denmark).

Results and Discussion

Structural properties of α Actinin-4 molecule *in vitro*

The overall molecular structure and dimerization properties of purified α Actinin-4 *in vitro* were analyzed by conventional AFM with an un-modified cantilever (Fig. 2 a). As shown in Figure 2 b, full-length α Actinin-4 showed two different structures, elliptic shapes (indicated as arrowheads in Fig. 2b, and typical examples are

shown in Fig. 2 c-i) and round shapes with or without a small “tail” (indicated by arrow in Fig. 2 b, and typical examples with or without tails are shown in Fig. 2 d-i–d-iii). The width and the length of the elliptic structure were 6.08 ± 2.08 nm and 19.2 ± 3.9 nm, respectively. This is well consistent with previous results from X-ray crystallography (Blanchard *et al.*, 1989) that suggested that α Actinin-4 forms an anti-parallel homodimer having a rod-like shape (Fig. 2 c-v). The diameter of the round shapes (Fig. 2 d-i–iii) was 6.70 ± 3.50 nm (Fig. 2 d-iv, d-v). However, the height had two populations; 0.7 nm (Fig. 2 d-i) and 1.4 nm (Fig. 2 d-ii). 14 % of the round structures had tails as shown in Figure 2 d-iii. These results indicate that a certain population of the recombinant full-length α Actinin-4 forms anti-parallel homodimers (Fig. 2 c-i, c-v), but there are a significant amount of miss-folded monomers *in vitro* (Fig. 2 d-i, left of d-vi), dimers (Fig. 2 d-ii, middle of d-vi), and dimers with coiled-coil tails (Fig. 2 d-iii, right of d-vi).

The actin-binding domain (Fig. 3 a) and the coiled

Fig. 4. Recognition imaging of full-length α Actinin-4. **a**: Schematic illustration of antibody-coupling strategy to MAC lever. A MAC lever made of silicon was amino-functionalized by APTES. A hetero-bifunctional PEG linker with NHS at one end and maleimide at the other end was incubated with the amino-functionalized tip. 5-carboxyl-1-pentanethiol was then reacted to produce carboxyl group at the end, then, antibody was coupled to the carboxyl group by EDC. **b-d**: AFM images of full-length α Actinin-4 molecule scanned with unmodified MAC lever. **b**: The α Actinin-4 molecules immobilized on a mica surface were imaged in PBS under the MAC mode by Pico Plus AFM (Bar; 100 nm). **c, d**: Magnified images of **b**. A rod shaped dimer and a round shaped dimer of α Actinin-4 molecules were imaged respectively (Bar; 10 nm). **e-j**: Recognition images of full-length α Actinin-4. The height image (**e**) and the recognition image (**f**) were simultaneously obtained (Bar; 50 nm). In the recognition image, the recognition spots (indicated by white arrows) were detected at the same places in the height image (**e**). In magnified images, two recognition spots were detected (**g-j**). **k, l**: Control imaging of full-length α Actinin-4 with the unmodified cantilever. In the height image (**k**) (Bar; 100 nm), dimerized α Actinin-4 molecules were imaged in the height image (**k**) however, the recognition spots were not detected in the corresponding recognition image (**l**).

region (Fig. 3 b) showed round structures but not elliptic ones. The widths and heights were summarized in Figure 3 c and d. The actin-binding domain had the width of 3.85 ± 1.01 nm and the height of 0.56 ± 0.16 nm (Fig. 3 c-i and c-ii, respectively). The volume was measured to be 31.9 nm³ by using the previously reported method (Schneider *et al.*, 1995, 1998). Since the specific volume of an assumed globular protein is 0.75 cm³/g, 31.9 nm³ corresponds to 25.7 kDa (see Materials and Methods), which is similar to the molecular weight of the actin-binding domain (32 kDa). The coiled region had the width of 7.85 ± 2.15 nm (Fig. 3 d-i) and the height of 0.42 ± 1.7 nm (Fig. 3 d-ii). The volume and molecular weight was calculated to be 49.4 nm³ and 39.8 kDa, respectively, which is far from the size of a dimer (112 kDa) but close to a monomeric size of this region (56 kDa). This indicates that the coiled region does not form a dimer under these conditions.

Monoclonal antibody-coupled cantilever recognizes the actin-binding domain of α Actinin-4.

Attachment of antibodies to the AFM cantilever with low consumption has been achieved by several different methods (Kamruzzahan *et al.*, 2006; Ebner *et al.*, 2007; Wakayama *et al.*, 2008). Some of these methods require a complicated synthesis of chemical compounds. However, using our method, antibodies can be attached easily to the surface of the cantilever using only commercial linkers (See Materials and Methods and Fig. 4 a). The antibody was immobilized on the cantilever by using the procedure described in the Materials and Methods, and the purified full-length α Actinin-4 was covalently cross-linked to the mica surface. Before recognition imaging of α Actinin-4, we confirmed the shape of α Actinin-4 in PBS by AFM imaging on MAC mode (Fig. 4 b–d). To obtain a good resolution image, the imaging was performed with as high gain as possible (Integral and Proportional gain were set at 5 and 8, respectively). In the image, several dimers of α Actinin-4 (indicated by white arrows) were observed within many aggregated α Actinin-4 molecules (Fig 4 b). In the magnified image of those molecules, rod-like (Fig. 4 c, proposed as Fig. 2 c-v) and round shapes (Fig. 4 d, proposed as Fig. 2 d-vi) can be detected.

In the recognition imaging of α Actinin-4 with the antibody-coupled MAC lever, several dimerized α Actinin-4 were observed in the height image (Fig. 4 e). By overlapping the height and recognition images (Fig. 4 f), the dark brown recognition signals can be detected on the head region of α Actinin-4 (Fig. 4 e, f), which is expected to correspond to the interaction spots between the epitope region of α Actinin-4 and the coupled

antibody on the MAC lever. Interestingly, two recognition spots were detected in each dimerized α Actinin-4 in the magnified images (Fig. 4 g–j). As interpreted in Figure 2 a, dimerized α Actinin-4 molecules have two epitope regions (refer to Fig. 2) and the recognition imaging successfully mapped two epitope regions of the dimerized α Actinin-4 molecules. To confirm whether the detected recognition signals were generated from a specific interaction between α Actinin-4 and the antibody, α Actinin-4 dimers were imaged with unmodified AFM cantilever (Fig. 4 k), and specific signals were not detected in the recognition image (Fig. 4 l). These results demonstrated that α Actinin-4 forms a stable dimer with anti-parallel orientation, and the TRECTM mode can be used to identify a specific position of the epitope within a single protein complex.

This study demonstrated that α Actinin-4 forms not only an anti-parallel dimer, but also a parallel dimer *in vitro* (Fig. 3). Among three functional sub-domains of α Actinin-4 (N-terminus actin binding domain, central rod domain and C-terminus EF hand), the central rod domain has been thought to be responsible for the anti-parallel dimerization by hydrophobic binding (Blanchard *et al.*, 1989; Imamura *et al.*, 1988). In the AFM observation of full-length α Actinin-4, the distances between the two head-like structures in the molecule varies among individual dimers, indicating that the actin-binding domains in a dimer can move flexibly around a dimerized coiled region. Since α Actinin-4 is one of the actin-binding and anchoring proteins that link actin molecules to a variety of inter/extracellular structures, the flexibility may give elasticity to the cytoskeletal fibrous structures in cells.

To elucidate behavior of α Actinin-4 and other cytoskeletal proteins inside the cell, we are aiming to map specific proteins within the cellular cytoskeletal structure. When the whole cell was treated with detergent such as Triton X-100, cytoskeletal fibrous network structures appeared (Yoshimura *et al.*, 2003). Several studies have observed these cytoskeletal fibrous architectures by AFM (Hofmann *et al.*, 1997; Yoshimura *et al.*, 2003; Santacrose *et al.*, 2006), however, individual proteins within the fibrous networks have not been identified. For further understanding of the cytoskeletal architecture, identification and mapping of these proteins within the skeletal structure will be important. In this sense, the recognition imaging will be a powerful tool for single-molecule mapping on biological architectures.

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