Research Article

Interaction between Pheromone and Its Receptor of the Fission Yeast *Schizosaccharomyces pombe* Examined by a Force Spectroscopy Study

Shintaro Sasuga,¹ Ryohei Abe,¹ Osamu Nikaido,¹ Shoichi Kiyosaki,¹ Hiroshi Sekiguchi,² Atsushi Ikai,³ and Toshiya Osada¹

¹ Department of Life Science, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, B-2 4259 Nagatsuta-cho, Midori-ku, Kanagawa, Yokohama 226-8501, Japan

² Department of Advanced Materials Science, Graduate School of Frontier Sciences, The University of Tokyo, #609 Kiban Bldg, 5-1-5 Kashiwanoha, Kashiwa City, Chiba 277-8561, Japan

³ Innovation Laboratory, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Kanagawa, Yokohama 226-8501, Japan

Correspondence should be addressed to Toshiya Osada, tosada@bio.titech.ac.jp

Received 29 August 2011; Revised 22 November 2011; Accepted 26 November 2011

Academic Editor: Peng Xiong

Copyright © 2012 Shintaro Sasuga et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Interaction between P-factor, a peptide pheromone composed of 23 amino acid residues, and its pheromone receptor, Mam2, on the cell surface of the fission yeast *Schizosaccharomyces pombe* was examined by an atomic force microscope (AFM). An AFM tip was modified with P-factor derivatives to perform force curve measurements. The specific interaction force between P-factor and Mam2 was calculated to be around 120 pN at a probe speed of 1.74 $\mu\text{m/s}$. When the AFM tip was modified with truncated P-factor derivative lacking C-terminal Leu, the specific interaction between the tip and the cell surface was not observed. These results were also confirmed with an assay system using a green fluorescent protein (GFP) reporter gene to monitor the activation level of signal transduction following the interaction of Mam2 with P-factor.

1. Introduction

The fission yeast *Schizosaccharomyces pombe* (*S. pombe*) has become a popular tool for analyzing heterologous GPCR due to the tractability of genetic and biochemical manipulation [1–4]. Most yeast systems for the studying of GPCR signaling use reporter constructs to provide readouts for transcription from signal-dependent promoters and are used for clinical and pharmaceutical studies, for example, a drug screening, due to similarities between the yeast mating response pathway and human GPCR signal transduction pathways [5]. The fission yeast has two haploid mating-cell types: P cells and M cells. Under nutrition depletion, the cells cease to divide with the cAMP cascade and conjugate with cells of the opposite mating type to form diploid zygotes. The conjugation between the two mating types is controlled by diffusible peptide pheromones, P-factor and M-factor.

Pheromones are defined as chemical substances that mediate communication between individuals of the same species. The perception of pheromones results in individual changes of physiological conditions and/or behaviors. Mating type-specific pheromones are secreted from cells and are sensed by cells of the opposite mating type [6, 7]. For example, P-factor released from P cells binds to its receptor, Mam2 on M cells, and stimulates M cells for mating. The two types of the fission yeast pheromone receptors belong to G protein-coupled receptors (GPCRs), the largest family of transmembrane receptors. The receptors with seven transmembrane domains are coupled with a heterotrimeric G protein complex and are responsible for transmitting extracellular signals to intracellular responses by stimuli of pheromones. The receptors undergo a conformational change from an inactive form to an active form upon pheromone binding. The active receptors induce $G\alpha$ subunit Gpa1 to facilitate GDP to GTP

exchange and the dissociation of $G\alpha$ from the G protein complex [8]. The $G\alpha$ subunit with GTP then interacts with downstream effectors to engage signaling cascades including the MAP kinase pathway.

Since the invention of the atomic force microscope (AFM) by Binnig et al., it has become a powerful tool to study biological samples not only for imaging at the molecular level but also for measuring their mechanical properties [9–15]. As an AFM tip makes direct contact with the sample, the physical properties as well as the topography of the surface can be examined. For example, interaction force between single molecules can be measured under physiological conditions [16–21]. For these measurements, the AFM tip modified with specific ligands is used to scan over cell surface with its receptors. The tip makes contact with the cell surface allowing binding between ligand and receptor. The tip retraction then induces stretching of the complex molecules followed by forced dissociation of the complex. This technique has already permitted us to quantify unbinding forces of numerous ligand-receptor pairs, either on an artificial surface or on the surface of living cells.

In this study, we investigated the interaction between P-factor and Mam2 by both AFM and the reporter assay. Our study showed that P-factor had specific interaction with Mam2 and was able to induce the signal transduction pathway. The removal of Leu at C terminal of P-factor by carboxypeptidase Sxa2 was reported to result in an inactivation of P-factor function [22]. Our study showed that P-factor lacking C-terminal Leu had no ability to bind Mam2 or induce the signal transduction pathway.

2. Materials and Methods

2.1. Peptides. Peptides used in this study are listed in Table 1. The customized peptides were obtained from Operon Co. Ltd., (Tokyo, Japan). Each peptide was prepared as a stock solution of 1 mM in Milli-Q water and stored at -80°C .

2.2. An AFM Tip Preparation. Coupling of peptides to AFM Si_3N_4 tips (OMCL-TR400PSA, Olympus, Tokyo, Japan; nominal value 0.02 N/m) was done using a heterobifunctional polyethylene glycol (PEG) linker as shown in Figure 1(a) [23, 24]. The AFM tips were cleaned in a UV ozone cleaner (UV/Ozone ProCleaner, Bioforce Nano sciences Inc., IA, USA) under ultraviolet light and exposed for 2 h to APTES (3-aminopropyl triethoxysilane) vapors in a 2-liter desiccator filled with argon and containing 30 μL of APTES and 10 μL of N,N-diisopropylethylamine (Sigma-Aldrich, Tokyo, Japan). The tips were then kept for up to 3 days in an argon-filled atmosphere until use. Amino-group bearing tips were incubated for 60 min with 1 mg/mL of N-hydroxy-succinimide ester-PEG-maleimide (NHS-PEG-MAL, 3400 Da, Nektar Therapeutics, Huntsville, AL) in PBS (phosphate-buffered saline). They were then washed several times with PBS to remove unanchored linker molecules. The final binding step was achieved by a reaction between the linker maleimide end and cysteine residues of peptides. The tips were incubated with each peptide (final concentration of

TABLE 1: List of peptides used in this study.

P-factor	TYADFLRAYQSWNTFVNPDRPNL
C-P-factor	CTYADFLRAYQSWNTFVNPDRPNL
C-P-factor ΔLeu	CTYADFLRAYQSWNTFVNPDRPN

1 μM) in PBS for 30 min and then were abundantly washed with PBS to remove unbound peptides.

2.3. AFM Measurement. Force measurements were carried out at room temperature with an NVB-100 AFM (Olympus, Inc., Tokyo, Japan) which was set on an inverted optical microscope (IX70, Olympus, Inc., Tokyo, Japan) [25–27]. The modified AFM tips were placed on the nitrogen starved cell surface, and force curve measurements were executed on different positions with a scan speed of around 1.74 $\mu\text{m/s}$ and using a relative trigger of 20–40 nm on the cantilever deflection (Figure 1(b)). The force curves from about 1024 positions (32×32) were recorded in each experimental condition to make a histogram of the rupture force in force curves. In the inhibition experiments, the force measurements were performed in an experimental buffer with free P-factor (final concentration of 1 μM). To calibrate the response of the cantilever deflection signal as a function of piezoelectrics, standard force curve measurements were carried out on a bottom of the dish, and the spring constant of the cantilever was calibrated by the thermal vibration method [28].

2.4. Strains and Media. Two *S. pombe* strains used in this study ($\text{h}^- \text{sxa2} > \text{GFP pMAM3G/pAL7}$ and $\text{h}^- \text{mam2::ura4}^+ \text{sxa2} > \text{GFP pMAM3G/pAL7}$) were derived from ARC010 ($\text{h}^- \text{leu1-32 ura4-D18}$). The *S. pombe* cells were grown in YES (0.5% yeast extract, 3% glucose and SP Supplements), YES 10 (0.5% yeast extract, 3% glucose and SP Supplements, 10 $\mu\text{g/mL}$ G418 sulfate), EMM (Edinburgh Minimal Medium, Sunrise Science Product), or YCB (Yeast Carbon Base, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Transformants were plated onto MMA (Minimal Medium Agar, Sunrise Science Product) or MMA supplemented with 1.25% leucine (120 $\mu\text{L/plate}$). To select for the *ura4*[−] cells, transformants were plated onto the YES plates containing 0.1% 5-fluoroorotic acid (YES-FOA). *Escherichia coli* strain DH5 α was used for the subcloning of the plasmid preparation. Oligonucleotide synthesis was performed by Operon Co., Ltd.

2.5. GFP Reporter Strain. The *sxa2* genes were replaced with green fluorescent protein (GFP) by standard homologous recombination method. Briefly, the plasmid was constructed as follows. Three DNA fragments were separately amplified, which were then fused together. The first fragment, the target gene (including upstream, open reading frame, and downstream sequences), was amplified from an *S. pombe* genomic DNA using primers X1, 2 (*sxa2*) introducing NotI restriction site. The *sxa2* fragment was ligated into the multicloning site of pTA2 vector (TOYOBO Co., Ltd., Osaka, Japan).

FIGURE 1: Schematic overview of experiments. (a) AFM tip modification with peptides. Si₃N₄ AFM tips are aminosilanized by exposure to APTES vapors. A heterobifunctional PEG linker is anchored to amino-group bearing tips through its NHS end. Peptide is attached to the PEG linker free end via a maleimide-cysteine bond. (b) Force spectroscopy method. Peptide-modified AFM tips approached the cell surface on a 200 nm z-scan size at a speed of 1.74 $\mu\text{m/s}$ and were retracted at the same speed. (c) The reporter assay. Pheromone binding to its receptors on the cell surface activates the intracellular signaling pathway that leads to the expression of GFP. The released Gpa1 (Galpha) with GTP from a heterotrimeric G protein activates the MAP kinase cascade of Byr2 (MAP3K), Byr1 (MAP2K), and Spk1 (MAPK). Activation of Byr2 also requires Ras1.

Inverse PCR was performed using primers X3, 4 (*sxa2*). The second fragment: GFP-coding sequence was amplified from a Monster Green Fluorescent Protein pHMGFP Vector (Promega, Madison, WI, USA) using primers G1, 2. The third fragment: *ura4* gene and 200 bp downstream of the target gene were amplified from an *S. pombe* genomic DNA using primers U1, 2 and X1, 2 (*sxa2*) introducing XbaI

restriction site. After the fragments were digested by XbaI, they were ligated and performed PCR using primers X5, U2 (*sxa2*). Then three fragments were simultaneously ligated with In-Fusion Advantage PCR Cloning Kit (Clontech, Palo Alto, CA, USA), resulting in replacing GFP vector. The resulting plasmid sequences were confirmed by sequence analysis. The resulting plasmid was treated with NotI, and

TABLE 2: List of primers used in this study.

X1	GCGGCCGC AGCTGTGTTTGTTCGCAATG
X2	GCGGCCGC GGAAGTTAGGCTTGTGTGC
X3	TCAATATCACAAGCT AAGTTTAATATCGGAAAATTTAA
X4	CTTGATCACGCCCAT TGAAGAGAGACAATGA
X5	CGCCAGGCCGGCTAA AAGTTTAATATCGGAAAATTTAA
X6	AAATCTAGA ATTTCTTATTTGGGAACGA
G1	ATGGGCGTGATCAAGCCCGAC
G2	TTAGCCGGCCTGGCGGGG
U1	AAATCTAGA TCTATCTTCTTAATCGCATGGAAG
U2	AGCTTGTGATATTGACGAACTTT
M31	AAAGAATTCTTTTAGAAAGTGTCTATTGTACC
M32	AAAGGTCTCTCATGACGAATTATGGGAAGATCAAG
G3	AAAGGCTCTACATGGGCGTGATCAAGCCC
G4	AAATCTAGATTAGCCGGCCTGGCGGGGTAGT
V1	AAAGAATTTCGAGCTCGGTACCC
V2	AAAGAATTTCGAACTAATGACCCCGTAATTGA
MU1	AAAGGTCTCTGGCCGCATCGGGATTGCATTGAGAGTT
MU2	AAA GGTCTCAAATGTCAGAGGGAGCAAGAACA
MD1	AAA GGTCTCACTTACGCCTGAATGTATCTTTTTTG
MD2	AAAGGTCTCAGGCCGCTCAAAGCCATAACTGTGCATTTATA
UR1	AAACACCTGCTTGTTCATTCTTCTTCTTAATCGCATGGAAG
UR2	AAACACCTGCTTGTTAAGAGCTTGTGATATTGACGAACTTT

subsequently used to transform *S. pombe* strain using a lithium acetate method. Transformed cells were plated onto MMA plates supplemented with leucine. The plates were incubated at 32°C, and, after 2-3 days, positive colonies were selected. To check for correct integration, PCR was performed on a genomic DNA. Then correctly transformed cells were plated onto YES-FOA plates to remove the *ura4* marker. After 2 days, positive colonies were selected and checked by PCR. Thereafter, the resultant transformants were performed on additional gene replacement. Next, we introduced an additional reporter to make the cells more susceptible to P-factor. The second reporter system expressed GFP under the control of *mam3* gene, which was expressed depending on pheromone P-factor. The plasmid was constructed as follows. Three fragments named mam3P, and GFP and pTL2M5-P were amplified by PCR using KOD-plus-Neo. The fragment mam3P including *mam3* upstream region from -1,047 to -1 was amplified from an *S. pombe* genomic DNA using M31 and M32 primers, each introducing EcoRI and BsaI restriction site. The fragment GFP was GFP-coding sequence from pHMGFP Vector using G3 and G4 primers introducing BsaI and XbaI restriction site. The fragment pTL2M5-P containing no hCMV promoter region was amplified from pTL2M5 (Asahi Glass Co., Ltd., Japan) using V1 and V2 primers introducing EcoRI and XbaI restriction site. Then, three fragments were treated with proper restriction enzymes and simultaneously ligated with Ligation-Convenience Kit (Nippon Gene, Japan). The plasmid was named pMAM3G. The sequence of pMAM3G was confirmed by sequence analysis. The pMAM3G was

transformed into *sxa2::GFP* strain with pAL7 vector following the above method. Colonies were selected by YES10 medium containing 10 µg/mL of G418 three times. All DNA fragments used for the plasmid construction were amplified by PCR using the KOD-plus-Neo (TOYOBO) in accordance with the supplier's instructions. All primers used here are listed in Table 2.

2.6. Construction of Mam2Δ Strain. The *mam2* open reading frame was replaced with 1.8 kb *ura4⁺* fragment by standard homologous recombination method. Plasmid was constructed as follows. Three fragments named mam2up, and mam2dw and 1.8 kb *ura4⁺* were amplified by PCR from the *S. pombe* genomic DNA. The fragment mam2up including *mam2* upstream position -1,067 to -1 relative to *mam2* ATG was amplified using MU1 and MU2 primers introducing BsaI restriction site. The fragment mam2dw including *mam2* downstream position +1,048 to +2,055 relative to *mam2* ATG was amplified using MD1 and MD2 primers introducing BsaI restriction site. The fragment 1.8 kb *ura4⁺* position -643 to +1,230 relative to *mam2* ATG was amplified using UR1 and UR2 primers introducing AarI restriction site. Three fragments were digested with BsaI or AarI. Then, we prepared the fragment containing pUC ori. and Km^R gene of pSL6Z digested with NotI. Four fragments were ligated simultaneously with Ligation-Convenience Kit. The sequence of the plasmid was confirmed by sequence analysis. The plasmid was treated with NotI and subsequently used to transform *S. pombe* strain using the lithium acetate method.

FIGURE 2: (a) Typical retraction force curves between P-factor and the cells. Vertical axis indicates cantilever deflections as a function of the cantilever-sample retraction distance. The first curve from the top shows no interaction, and the other curves display specific unbinding events between peptide and the cell. The vertical force jump of events allows access to the specific unbinding force. (b) Force histogram of unbinding events (blue columns) obtained after analysis of 1024 force curves. The mean unbinding force is 120 pN, for a mean loading rate of 1.74 $\mu\text{m/s}$. In the presence of free 1 μM P-factor, the number of unbinding events decreased (red columns).

Transformants were plated onto MMA plates supplemented with leucine. The plates were incubated at 32°C, and, after 3 days, positive colonies were selected. To check for correct integration, PCR was performed on the genomic DNA of transformants.

2.7. GFP Assay for Mam2 Signaling. Reporter strains were grown in YES10 media at 32°C for 24–36 h and were inoculated into 5 mL of the fresh YES10 media. Then the cells were grown at 30°C for 18 h and harvested. After having been washed twice with sterile water, cells were transferred to YCB media at OD₆₀₀ of 1.0 for nitrogen starvation. The cells were incubated at 30°C for 2 h and used for AFM study and Mam2 signaling assay. For the signaling assay (Figure 1(c)), 1 mL aliquots of cells were transferred to 24-well microplate containing 1 μL of peptide solution (final concentration of 1 μM). After incubation at 30°C for 20 h, the cells were washed three times with PBS and resuspended in the same volume of PBS. Fluorescence intensity of GFP was measured by a fluorescence spectrophotometer (Hitachi F-3010, Japan). The cells expressing GFP were excited at 491 nm, and fluorescence emission was detected at 515 nm.

3. Results and Discussion

A force-volume mode of AFM was carried out to examine specific interactions between pheromone and pheromone receptor. Using the AFM tip cross-linked with P-factor derivatives via a heterobifunctional PEG linker, 1024 AFM force curves were then obtained over different spots

on *mam2+* strain cells expressing pheromone receptors. Although most of retraction curves (around 90%) showed no interaction (Figure 2(a), upper curve), some retraction curves presented a downward deflection abruptly ending with a force jump (Figure 2(a), others). Since the PEG linker used to attach the peptide to the AFM tip has a total length of 30 nm, only events occurring after 30 nm extension were considered valid interaction events and were included into further analyses. The distribution of unbinding force is shown in Figure 2(b). To verify the specificity of the unbinding force, force curves were also obtained in the presence of 1 μM -free P-factor where specific interaction was expected to be inhibited. Ranging from 100 to 160 pN, 122 interaction peaks (11.9%) were detected without free P-factor while 33 unbinding events (3.2%) were detected with free P-factor. The number of events clearly decreased and the unbinding probability fell to 3.2 from 11.9%. Next, we carried out force curve measurements to examine interaction force between the AFM tip modified with P-factor and *mam2Δ* strain cells expressing no pheromone receptors, and between the AFM tip modified with P-factor ΔLeu and *mam2+* strain cells. When *mam2Δ* strain cells were used for force curve measurements with P-factor-modified tips, the unbinding probabilities were not affected with or without free P-factor, showing 2.7% without free P-factor and 2.9% with free P-factor (Figure 3(a)). When the AFM tip was modified with truncated P-factor derivative lacking C-terminal Leu, which was reported to have no P-factor function, the specific interaction was not observed, and the unbinding probabilities were almost the same with (3.5%) or without (3.1%) free P-factor (Figure 3(b)). From these three kinds of force curve measurements, the unbinding forces

FIGURE 3: Force histogram of unbinding events with (red columns) or without (blue columns) free 1 μ M P-factor. (a) Unbinding events between AFM tip modified with P-factor and *mam2Δ* strain cells. (b) Unbinding events between AFM tip modified with P-factor Δ Leu and *mam2+* strain cells.

FIGURE 4: GFP production of *mam2+* strain cell (a) and *mam2Δ* strain cell (b). Under nutrition depletion, each cell was treated with Milli-Q water (control), 1 μ M of P-factor, C-P-factor, or P-factor Δ Leu. Values are means with SD of triplicate samples.

observed in the first force curve measurement were supposed to be caused by the specific interaction between P-factor and its receptor.

To examine the interaction of Mam2 with P-factor derivatives with a method other than AFM, we carried out Mam2-signaling assay. Binding of P-factor to Mam2 on the cell surface activates the intracellular signaling pathway that leads to the expression of the genes necessary to bring about cell fusion. Replacing the response genes with a reporter construct, such as GFP, provides a simple readout for signaling. The interaction of Mam2 and P-factor was investigated in an *S. pombe* strain containing *sxa2* > GFP pMAM3G/pAL7 reporter constructs. The expression of GFP in response to P-factor is monitored with a fluorescence spectrophotometer (Figure 4). When 1 μ M P-factor was added to the *mam2+* strain cells, fluorescence intensity of the cells at 515 nm increased, which indicated that GFP was produced in the response of the cells to P-factor (Figure 4(a)). The addition of cysteine at N-terminal of P-factor slightly affect its ability to induce the signal transduction pathway. Since the *mam2+* strain cells showed no response against 1 μ M C-P-factor Δ Leu, this peptide was not able to induce the

signal transduction pathway. The *mam2Δ* strain cells (*sxa2* > GFP pMAM3G/pAL7, Δ mam2) showed no response with the addition of any peptides including P-factor (Figure 4(b)). Without Mam2, P-factor was not able to induce the signal transduction pathway. The reporter assay confirmed the result of AFM study. The specific interaction was only observed by AFM study under the condition in which the reporter assay showed the positive result.

4. Conclusions

We examined here the interaction between P-factor and Mam2 by both AFM and the reporter assay. The unbinding force was calculated to be around 120 pN at probe speed of 1.74 μ m/s. The reporter assay using a fluorescent reporter gene showed results similar to AFM studies. P-factor derivative having no specific interaction to Mam2 by AFM did not activate Mam2-specific signaling pathway. This yeast-based method combined AFM, and the reporter assay will be available for the signaling study of heterologous GPCRs and screening of their ligands.

Acknowledgments

The authors thank Drs Giga-Hama Y and Tohda H for the *S. pombe* strain. This work was supported by a Grant-in-Aid for creative scientific research (no. 19GS0418) from the Japan Society for the Promotion of Science (JSPS) to A. Ikai.

References

- [1] J. Kurjan, "The pheromone response pathway in *Saccharomyces cerevisiae*," *Annual Review of Genetics*, vol. 27, pp. 147–179, 1993.
- [2] J. Davey, "Fusion of a fission yeast," *Yeast*, vol. 14, no. 16, pp. 1529–1566, 1998.
- [3] S. J. Dowell and A. J. Brown, "Yeast assays for G-protein-coupled receptors," *Receptors and Channels*, vol. 552, no. 5-6, pp. 343–352, 2002.
- [4] G. Ladds, A. Goddard, and J. Davey, "Functional analysis of heterologous GPCR signalling pathways in yeast," *Trends in Biotechnology*, vol. 23, no. 7, pp. 367–373, 2005.
- [5] L. Silverman, R. Campbell, and J. R. Broach, "New assay technologies for high-throughput screening," *Current Opinion in Chemical Biology*, vol. 2, no. 3, pp. 397–403, 1998.
- [6] Y. Fukui, Y. Kaziro, and M. Yamamoto, "Mating pheromone-like diffusible factor released by *Schizosaccharomyces pombe*," *EMBO Journal*, vol. 5, no. 8, pp. 1991–1993, 1986.
- [7] U. Leupold, "Sex appeal in fission yeast," *Current Genetics*, vol. 12, no. 7, pp. 543–545, 1987.
- [8] M. Whiteway, L. Houghan, D. Dignard et al., "The STE4 and STE18 genes of yeast encode potential β and γ subunits of the mating factor receptor-coupled G protein," *Cell*, vol. 56, no. 3, pp. 467–477, 1989.
- [9] M. Radmacher, M. Fritz, J. P. Cleveland, D. A. Walters, and P. K. Hansma, "Imaging adhesion forces and elasticity of lysozyme adsorbed on mica with the atomic force microscope," *Langmuir*, vol. 10, no. 10, pp. 3809–3814, 1994.
- [10] K. Mitsui, M. Hara, and A. Ikai, "Mechanical unfolding of α 2-macroglobulin molecules with atomic force microscope," *FEBS Letters*, vol. 385, no. 1-2, pp. 29–33, 1996.
- [11] M. Rief, M. Gautel, F. Oesterhelt, J. M. Fernandez, and H. E. Gaub, "Reversible unfolding of individual titin immunoglobulin domains by AFM," *Science*, vol. 276, no. 5315, pp. 1109–1112, 1997.
- [12] A. Kis, S. Kasas, B. Babić et al., "Nanomechanics of microtubules," *Physical Review Letters*, vol. 89, no. 24, Article ID 248101, 4 pages, 2002.
- [13] R. Afrin, M. T. Alam, and A. Ikai, "Pretransition and progressive softening of bovine carbonic anhydrase II as probed by single molecule atomic force microscopy," *Protein Science*, vol. 14, no. 6, pp. 1447–1457, 2005.
- [14] J. P. Michel, I. L. Ivanovska, M. M. Gibbons et al., "Nanoindentation studies of full and empty viral capsids and the effects of capsid protein mutations on elasticity and strength," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 16, pp. 6184–6189, 2006.
- [15] G. Lee, K. Abdi, Y. Jiang, P. Michaely, V. Bennett, and P. E. Marszalek, "Nanospring behaviour of ankyrin repeats," *Nature*, vol. 440, no. 7081, pp. 246–249, 2006.
- [16] E. L. Florin, V. T. Moy, and H. E. Gaub, "Adhesion forces between individual ligand-receptor pairs," *Science*, vol. 264, no. 5157, pp. 415–417, 1994.
- [17] V. T. Moy, E. L. Florin, and H. E. Gaub, "Intermolecular forces and energies between ligands and receptors," *Science*, vol. 266, no. 5183, pp. 257–259, 1994.
- [18] P. Hinterdorfer, W. Baumgartner, H. J. Gruber, K. Schilcher, and H. Schindler, "Detection and localization of individual antibody-antigen recognition events by atomic force microscopy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 8, pp. 3477–3481, 1996.
- [19] M. Gad, A. Itoh, and A. Ikai, "Mapping cell wall polysaccharides of living microbial cells using atomic force microscopy," *Cell Biology International*, vol. 21, no. 11, pp. 697–706, 1997.
- [20] R. Merkel, P. Nassoy, A. Leung, K. Ritchie, and E. Evans, "Energy landscapes of receptor-ligand bonds explored with dynamic force spectroscopy," *Nature*, vol. 397, no. 6714, pp. 50–53, 1999.
- [21] H. Sekiguchi, H. Arakawa, H. Taguchi, T. Ito, R. Kokawa, and A. Ikai, "Specific interaction between GroEL and denatured protein measured by compression-free force spectroscopy," *Biophysical Journal*, vol. 85, no. 1, pp. 484–490, 2003.
- [22] G. Ladds, E. M. Rasmussen, T. Young, O. Nielsen, and J. Davey, "The *sxa2*-dependent inactivation of the P-factor mating pheromone in the fission yeast *Schizosaccharomyces pombe*," *Molecular Microbiology*, vol. 20, no. 1, pp. 35–42, 1996.
- [23] C. Lesoil, T. Nonaka, H. Sekiguchi et al., "Molecular shape and binding force of Mycoplasma mobile's leg protein Gli349 revealed by an AFM study," *Biochemical and Biophysical Research Communications*, vol. 391, no. 3, pp. 1312–1317, 2010.
- [24] A. Yersin, T. Osada, and A. Ikai, "Exploring transferrin-receptor interactions at the single-molecule level," *Biophysical Journal*, vol. 94, no. 1, pp. 230–240, 2008.
- [25] H. Kim, F. Asgari, M. Kato-Negishi et al., "Distribution of olfactory marker protein on a tissue section of vomeronasal organ measured by AFM," *Colloids and Surfaces B: Biointerfaces*, vol. 61, no. 2, pp. 311–314, 2008.
- [26] H. Kim, H. Uehara, R. Afrin et al., "Application of the atomic force microscopy to the study of expressed molecules in or on a single living cell," in *Applied Scanning Probe Methods IX: Characterization*, B. Bhushan and H. Fuchs, Eds., NanoScience and Technology, pp. 149–175, Springer, Berlin, Germany, 2008.
- [27] H. Kim, H. Arakawa, N. Hatae et al., "Quantification of the number of EP3 receptors on a living CHO cell surface by the AFM," *Ultramicroscopy*, vol. 106, no. 8-9, pp. 652–662, 2006.
- [28] J. L. Hutter and J. Bechhoefer, "Calibration of atomic-force microscope tips," *Review of Scientific Instruments*, vol. 64, no. 7, pp. 1868–1873, 1993.