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Unfolding individual Als5p adhesion proteins on live cells

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Abstract

Elucidating the molecular mechanisms behind the strength and mechanics of cell adhesion proteins is of central importance in cell biology, and offers exciting avenues for the identification of potential drug targets. Here we use single-molecule force spectroscopy to investigate the adhesive and mechanical properties of the widely expressed Als5p cell adhesion protein from the opportunistic pathogen *Candida albicans*. We show that the forces required to unfold individual tandem repeats of the protein are in the 150–250 pN range, both on isolated molecules and on live cells. We also find that the unfolding probability increases with the number of tandem repeats and correlates with the level of cell adherence. We suggest that the modular and flexible nature of Als5p conveys both strength and toughness to the protein, making it ideally-suited for cell adhesion. The single molecule measurements presented here open new avenues for understanding the mechanical properties of adhesion molecules from mammalian and microbial cells, and may help us to elucidate their potential implications in diseases like inflammation, cancer and infection.

Keywords

AFM; *Candida albicans*; cell adhesion proteins; pathogens; protein unfolding; single-molecule force spectroscopy

Cell adhesion mediated by specific cell surface molecules plays a pivotal role in life sciences.[1–7] Neuronal interactions,[1] cellular communication,[2] tissue development,[3] inflammation,[4] cancer[5] and microbial infection[6,7] are just a few examples of cellular processes regulated by cell adhesion molecules. Microbial infection is generally initiated by the specific adhesion of the pathogens to host tissues.[6,7] A prominent example is found in *Candida albicans*, the most common agent causing opportunistic infections in immunocompromised patients. Adhesion of *C. albicans* yeast cells to host tissues is a key factor in the maintenance of commensal and pathogenic states, which is mediated by a family of cell surface proteins known as the agglutinin-like sequence (Als) proteins.[6,8–11] Of the eight different Als proteins, Als5p is one of the most extensively characterized.8 Als proteins possess four functional regions (Fig. 1a), i.e. an N-terminal immunoglobulin (Ig)-like region, which initiates cell adhesion, followed by a threonine-rich region (T), a tandem repeat (TR) region that participates in cell-cell aggregation, and a stalk region projecting the

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molecule away from the cell surface. Microscopic assays using *Saccharomyces cerevisiae* surface display models have revealed that Als-mediated adherence involves two steps, i.e. initial adhesion via the specific binding of the Ig region to peptide ligands, followed by cell-cell aggregation mediated by the TR regions.[10,12–14] Despite the vast amount of data available on the molecular biology of Als proteins, the molecular details underlying their strength and mechanics have not been elucidated yet.

With its ability to observe and manipulate single molecules at work, atomic force microscopy (AFM)15–18 has provided a wealth of novel opportunities in life sciences. Notably, progress in single-molecule force spectroscopy (SMFS) has allowed researchers to measure the specific binding strength of adhesion molecules,19,20 and to elucidate the folding/unfolding mechanisms of water-soluble or membrane proteins.21–24 However, studying the molecular elasticity of proteins on live cells, i.e. in their fully functional state, remains a challenging task. Here, we demonstrate the ability of SMFS to study the adhesive and mechanical properties of Als5p proteins, both on isolated molecules and on cell surfaces. Single Als5p proteins are localized on the surface of living yeast cells and their individual TR domains unraveled. The unfolding probability is found to correlate with cell adherence, suggesting that protein mechanics may play an important role in fungal adhesion.

RESULTS AND DISCUSSION

We first investigated the mechanical unfolding of isolated Als5p proteins. To this end, soluble Ig-T-TR_6 fragments terminated with a His-tag were attached at low density on gold surfaces modified with nitrilotriacetate groups (Fig. 1b). Ig-T-TR_6 proteins were picked up and stretched by their terminal Ig domain using AFM tips modified with Ig-T fragments (Fig. 1b). Force extension curves for Ig-T-TR_6 proteins showed a sawtooth pattern with well-defined force peaks (Fig. 1c). These periodic features represent the characteristic fingerprint of modular proteins,21,22 in which each peak corresponds to the force-induced unfolding of an individual domain. In unfolding experiments, proteins are generally picked up anywhere along their length using bare tips, meaning the number of unfolding peaks per curve varies. Here, the use of an Als5p Ig-T-tip allowed us to reproducibly observe sawtooth patterns with seven well-defined force peaks. It is interesting to note that force peaks were never (or hardly) seen using a bare tip, indicating that the specific Ig-Ig interaction is required in order to unfold the protein domains.

The first six peaks were equally spaced and showed ascending forces (Fig. 1c), ranging from 160 ± 10 pN to 240 ± 11 pN (mean ± s.e.m.; n = 100 curves), that are consistent with the sequential unfolding of the six TR repeats of Als5p. The seventh peak showed a much higher force, 350 ± 25 pN, and larger spacing. Although the origin of this last peak is not fully understood, it may be attributed to the stretching of Ig domains, leading eventually to the rupture of the Ig-Ig complex. We note that most sawtooth patterns were preceded by a few poorly defined peaks, varying widely in magnitude and spacing. We suggest these events reflect the unfolding of the T regions for two reasons. First, molecular modeling has revealed these regions may fold into an ensemble of structures of different sizes. Second, T regions are known to partly unfold under native (unstressed) conditions to allow amyloid formation.[25] In agreement with molecular modeling and CD studies revealing that Ig, T and TR regions are antiparallel β-sheet-rich structures, our measured forces are in the range of unfolding forces reported for β-folds domains, such as Ig and fibronectin domains in titin21 and tenascin,22 and larger than the forces needed to unfold α-helical domains.26 In the muscle protein titin, it is well-known that the mechanical properties of the Ig domains are essential for the biological function of the protein. Similarly, the mechanical response of Als5p may be function-related. We suggest that the modular and flexible nature of Als5p conveys both strength and toughness to this cell-adhesion molecule.[27]

The force-extension curves obtained by stretching Ig-T-TR_6 proteins were well-described by the worm-like-chain model (Fig. 1c, inset), supporting further our interpretation of the sawtooth pattern. For peaks 1 to 6, the change in contour length between consecutive peaks was constant, ΔL_c = 8.4 ± 1.6 nm (n=100). Assuming that each amino acid contributes 0.36 nm to the contour length of a fully-extended polypeptide chain and that the folded length of a TR repeat is 4.3 nm, we found that the measured 8.4 nm increment corresponds to the expected 36 amino acids of a single TR repeat. We also observed that urea strongly alters the shape of the unfolding peaks (Fig. 1c), confirming that disruption of the protein hydrogen bonds leads to a loss of mechanical stability. This observation correlates with cellular behavior since Als5p-mediated adhesion is known to be reversibly inhibited by urea and formamide.[12]

We then explored the dependence of the unfolding forces on the loading rate, i.e. the rate at which the load is applied to the molecules.[21,22,28] We found that the average unfolding forces of each TR domains increases linearly with the logarithm of the loading rate (Fig. 2a). From these dynamic force spectroscopy data, the width of the unfolding potential (x_u) and the unfolding rate constant (k_u) were estimated, and found to be $x_u \approx 0.2$ nm and $k_u \approx 6 \times 10^{-2}$ s^{-1}. The width of the unfolding potential obtained here compares well with that reported for other proteins, usually in the range of 0.17–0.25 nm. Interestingly, we also found that the unfolding probability (number of curves showing sawtooth patterns) increases exponentially with interaction time to reach a plateau after 4 s (Fig. 2b). This behavior is reminiscent of that of cadherins, i.e. transmembrane glycoproteins known to play an important role in cell-to-cell adhesion, except that bond formation is much faster for cadherin.[29] Our results are consistent with the notion that Als5p-mediated adhesion is a time-dependent process.[14] They are also in line with the slow kinetics of bonding of the Als homolog alpha-agglutinin.[30]

Next, we could localize and stretch single Als5p proteins directly on live cells. Using an Ig-T-tip, force-extension curves were recorded across the surface of yeast cells expressing Als5p with different numbers of TR repeats (Fig. 3a). The curves obtained on cells expressing six repeats (TR_6) displayed sawtooth patterns similar to those found on isolated Ig-T-TR_6, i.e. they generally showed six force peaks varying from 150 to 250 pN and spaced by 9.1 ± 1.2 nm (n=25), followed by a last peak of 350–400 pN magnitude (Fig. 3b). Cells expressing a smaller number of repeats showed patterns with fewer force peaks (Fig. 3a) and lower unfolding probability (Fig. 3c), the unfolding frequency decreasing in the order TR_6>TR_4>TR_2>TR_0. Further work is needed to understand the molecular origin of this behavior. One may argue that a lower unfolding frequency may simply reflect lower expression of the proteins. However, this interpretation is unlikely since the intensity of cell surface immunofluorescence with anti-Als antibody was shown to be similar for each of these proteins, thus implying similar cell surface concentrations.[12] Alternatively, lower unfolding frequencies may indicate that the shorter proteins are less accessible for surface interaction.

To demonstrate that these nanoscale measurements correlate with microscale cellular behaviour, yeast adherence was measured using bead adherence assays (Fig. 3c).[12] Fibronectin-coated beads and yeast cells expressing different numbers of TR repeats were agitated in buffer resulting in adhesion of single yeast cells to the beads, followed by cell-to-cell aggregation, eventually forming small microcolonies of aggregated cells. Fig. 3c shows that the level of yeast adherence (number of cells per bead) was correlated with the unfolding probability, suggesting that the mechanical properties of the TR region are important for yeast adherence.

We also explored the distribution of unfolding forces over the cell surface (Fig. 4a). For cells expressing Als5p with six TR repeats, unfolding patterns were detected in about 25% of the locations (bright pixels), revealing that the proteins were exposed at the surface. Although the distribution of unfolding events was generally homogeneous, in some localized regions they seemed to be organized into nanoscale domains, possibly reflecting clustered Als molecules. Whether these clusters are indeed due to multiple proteins or to one protein interacting with the tip multiple times remains to be clarified. As shown before, the probability of unfolding decreased when decreasing the number of repeats (Fig. 4a). In addition, we found that the unfolding probability increases with interaction time (Fig. 4b), consistent with the notion that Als5p-mediated adhesion is a time-dependent process. Thus, we could localize single Als5p proteins on the surface of yeast cells, showing they are surface exposed, and we were able to unfold their individual TR domains, revealing that their mechanical properties may be essential for yeast adherence.

CONCLUSIONS

Our experiments demonstrate that SMFS provides new insights into the mechanics of cell adhesion proteins, both on isolated molecules and on cell surfaces. Stretching of single Als5p proteins leads to the unfolding of their individual TR domains. Owing to its modular and flexible nature, Als5p is able to resist high mechanical force. We believe that this modular elongation mechanism conveys both strength and toughness to the protein, making it ideally-suited to function as an adhesion molecule. The unfolding probability increases with the number of TR repeats and is correlated with yeast adherence, suggesting that this process is relying on the mechanical properties of Als5p proteins. This finding may have broad implications since modular TR structures are found in cell-adhesion molecules from other pathogens.[6,8]

METHODS

Expression and purification of Als5p

Ig-T and Ig-T-TR_6 fragments were expressed and purified as described elsewhere.[12]

Microorganisms, cultures and aggregation assays

S. cerevisiae YPH499 harboring different plasmids (pGK114 (6TR), pDG100 (2TR), pDG101 (0TR), pDG102 (4TR)) were grown as previously described.[17] Briefly, two or three colonies from the SC-trp plate used as inoculum were transferred into YPRG medium (10 g/L yeast extract, 20 g/L peptone, 10 g/L raffinose, and 10 g/L galactose). Cells were agitated at 30°C, grown up to the late logarithmic phase, and harvested by centrifugation. They were washed three times with sodium acetate buffer and resuspended in 10 mL buffer to a concentration of ~10^6 cells per mL. The protocol that we used for bead adherence assays is similar to that described elsewhere.10,[12] Briefly, Als5p-expressing cells were mixed with fibronectin-coated magnetic beads at a cell-to-bead ratio of 100:1 in Tris-EDTA buffer, vortexed, and incubated at room temperature with gentle shaking for 30 to 45 min. Each tube was vortexed briefly and immediately placed into a magnetic separator (Dynal). Adherent and aggregated cells were gently washed three times with buffer while the tube remained within the magnet. The cells were resuspended in buffer, and a sample was placed onto a microscope slide for examination.

Atomic force microscopy

AFM measurements were performed at room temperature (20°C) in buffered solutions (sodium acetate; pH 4.75), using a Nanoscope IV Multimode AFM (Veeco Metrology Group, Santa Barbara, CA) and oxide sharpened microfabricated Si_3N_4 cantilevers

(Microlevers, Veeco Metrology Group). Cells were immobilized by mechanical trapping into porous polycarbonate membranes (Millipore), with a pore size similar to the cell size. After filtering a concentrated cell suspension, the filter was gently rinsed with buffer, carefully cut (1 cm × 1 cm), attached to a steel sample puck (Veeco Metrology Group) using a small piece of double face adhesive tape, and the mounted sample was transferred into the AFM liquid cell while avoiding dewetting. The spring constants of the cantilevers were measured using the thermal noise method (Picoforce, Veeco Metrology Group), yielding values ranging from 0.01 to 0.025 N/m. Unless otherwise specified, all force measurements were recorded with a maximum applied force of 250 pN and a loading rate of 10,000 pN/s, calculated by multiplying the tip pulling velocity (nm/s) by the slope of the unfolding peaks (pN/nm). For interaction time experiments, delays of 500 ms, 1 s, 2 s, 4 s or 6 s were applied between tip approach and tip retraction, while keeping constant the maximum applied force.

To attach purified Ig-T and Ig-T-TR_6 fragments onto AFM tips and supports, AFM cantilevers and silicon wafers (Siltronix) were coated using electron beam thermal evaporation with a 5-nm thick chromium layer followed by a 30-nm thick gold layer. The gold-coated surfaces were cleaned for 15 min by UV and ozone treatment, rinsed with ethanol, dried with a gentle nitrogen flow and immersed overnight in ethanol containing 0.05 mM of nitrilotriacetate-terminated (5%) and triethylene glycol-terminated (95%) alkanethiols. After rinsing with ethanol, the tips and supports were immersed in a 40 mM aqueous solution of $NiSO_4$ (pH 7.2) for 1h, rinsed with water, incubated in acetate buffer containing 10 μg/mL Ig-T or Ig-T-TR_6 for 2h, and finally rinsed with buffer.

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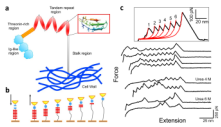

Figure 1.

Unfolding isolated Als5p proteins. (a) Representation of an Als molecule projecting outward from the *C. albicans* cell wall by means of the stalk region. The tandem repeat (TR) region is comprised of multiple glycosylated 36-amino acid repeats that are arranged in anti-parallel β-sheets. As shown in the inset, modelling with ROSETTA and LINUS consistently predicted independently folded three-stranded anti-parallel β−sheet domains for each repeat. Thus, the TR region consists of 6 such repeats, which would unfold independently. The amyloidogenic Threonine-rich region (T) is the most conserved sequence in Als proteins and connects to the ligand-binding Ig-like region which possesses three equal-sized β-sheet rich domains. (b) Principle of the SMFS experiments. Ig-T-TR_6 fragments were attached on a gold surface and stretched via their Ig domains using an Ig-T-tip. (c) Force-extension curves obtained by stretching single Ig-T-TR_6 showed periodic features reflecting the sequential unfolding of the TR domains (upper traces). Force peaks were well-described by the worm-like-chain model (inset; red line), using a persistence length of 0.4 nm: $F(x) = k_bT/l_p$ [0.25(1-$x/L_c)^{-2} + x/L_c - 0.25$], where L_c and l_p are the contour length and persistence length of the molecule, k_b is the Boltzmann constant and T the absolute temperature. Addition of urea dramatically altered the unfolding peaks due to hydrogen bond disruption (lower traces). All curves were recorded using a loading rate of 10,000 pN/s and an interaction time of 500 ms. Similar data were obtained using more than ten different tips and four independent samples.

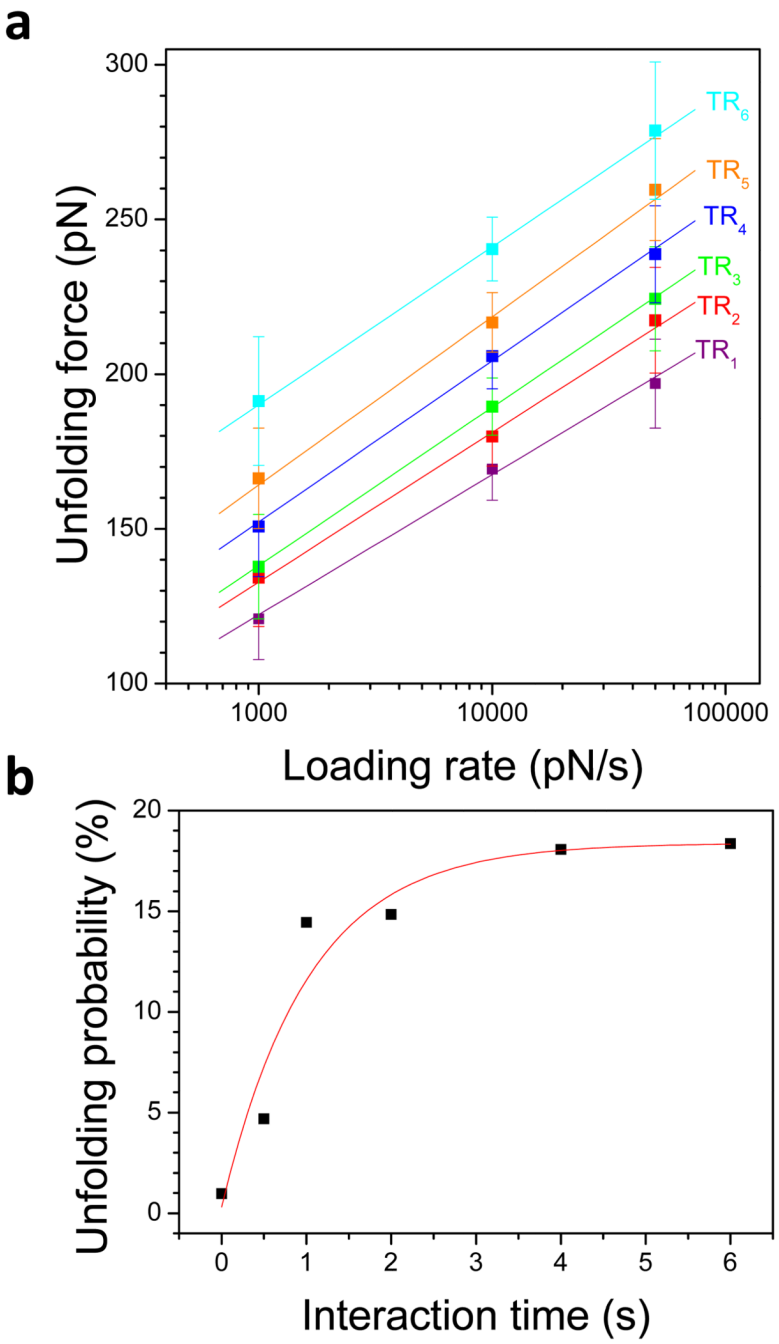

Figure 2.

Unfolding forces depend on pulling speed and interaction time. (a) Unfolding forces of individual TR domains as a function of the loading rate (constant interaction time of 500 ms). The unfolding force F is related to the loading rate ($k_c v$) by: $F = \frac{k_b T}{x_u} \ln\left(\frac{k_c v x_u}{k_b T K_u^0}\right)$ where k_c is the spring constant, v is the pulling speed, x_u is the width of the potential barrier, and k_u^0 is the mechanical unfolding rate constant under zero force. Thus, x_u can be determined from the slope of this plot and k_u^0 from the intercept. Data represent the mean ± s.e.m. (n = 50). (b)

Dependence of the unfolding probability on the interaction time (constant loading rate of 10,000 pN/s). The data can be fitted with an exponential function (red line).

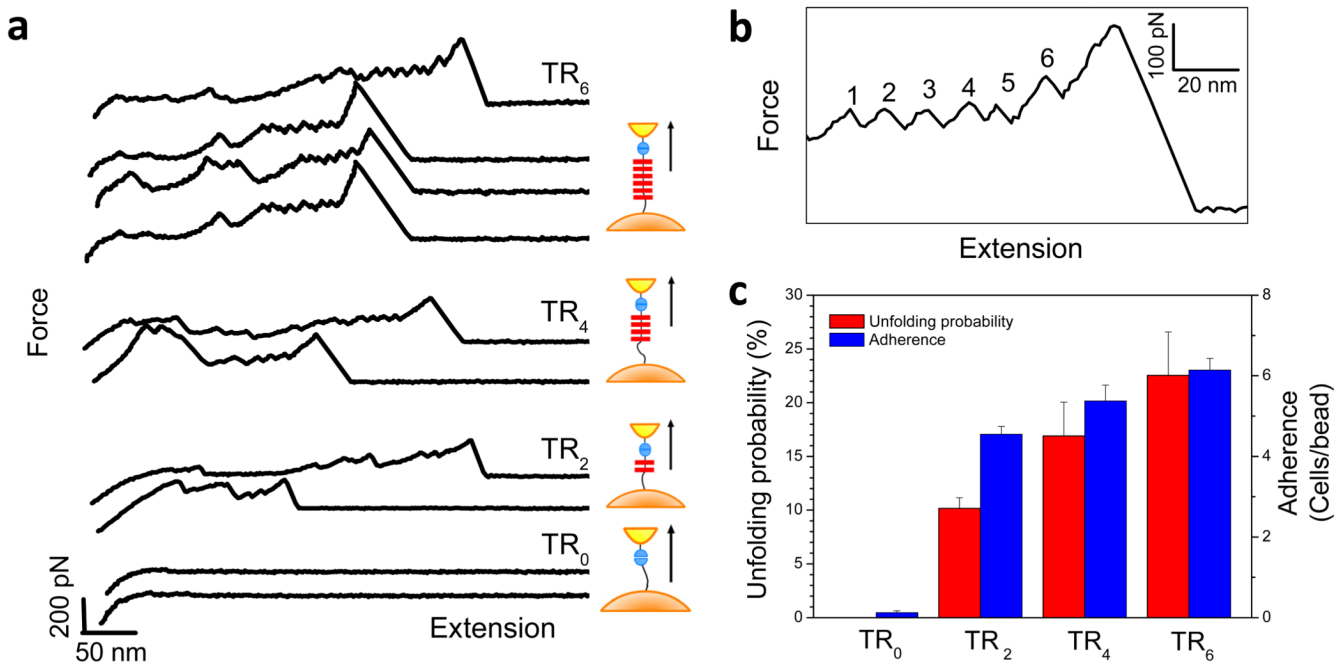

Figure 3.

Unfolding Als5p proteins on live cells. (a) Force-extension curves recorded between an Ig-T-tip and the surface of *S. cerevisiae* cells expressing Als5p with six, four, two and no TR repeats. All curves were recorded using a loading rate of 10,000 pN/s and an interaction time of 500 ms. Similar data were obtained using more than ten different tips and ten cells from independent cultures. (b) Higher magnification curve documenting the sequential unfolding of six TR domains. (c) Both the unfolding probability and the level of yeast adherence increased with the number of TR repeats, indicating there was a good agreement between single-molecule and microscale assays.

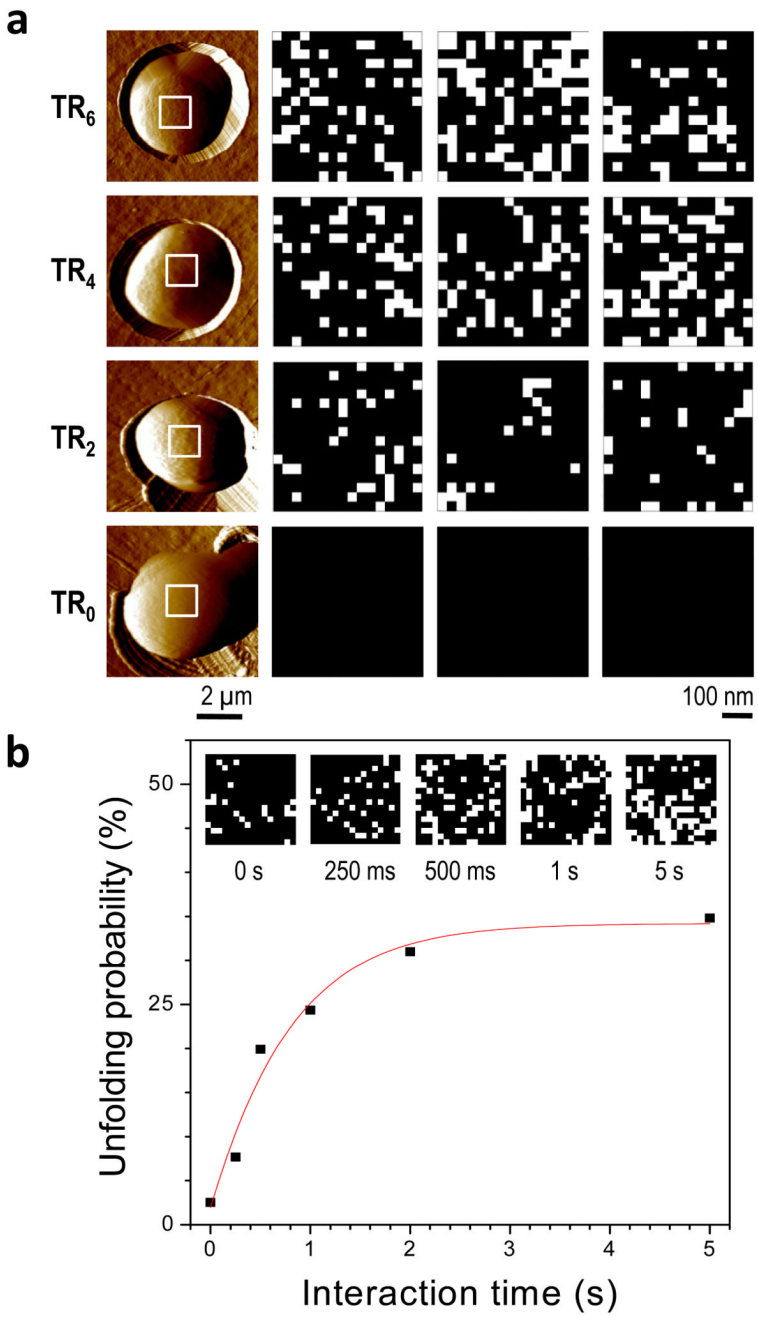

Figure 4.

Mapping unfolding forces on cell surfaces. (a) Left column: AFM topographic images recorded in buffer with an Ig-T-tip showing single *S. cerevisiae* cells expressing different numbers of TR repeats. Cells were trapped into the pores of a porous polymer membrane for in vivo imaging. Columns 2–4: representative unfolding maps (256 force curves; $n = 256$) recorded on 500 nm × 500 nm areas on top of the cells (loading rate of 10,000 pN/s, interaction time of 500 ms). Each bright pixel reflects the detection and unfolding of single Als5p proteins. The unfolding probability is clearly proportional to the number of TR repeats. (b) The probability of unfolding increased with interaction time (inset: unfolding maps obtained at increasing times).