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Force profiles of protein pulling with or without cytoskeletal links studied by AFM

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Abstract

To test the capability of the atomic force microscope for distinguishing membrane proteins with/without cytoskeletal associations, we studied the pull-out mechanics of lipid tethers from the red blood cell (RBC). When wheat germ agglutinin, a glycophorin A (GLA) specific lectin, was used to pull out tethers from RBC, characteristic force curves for tether elongation having a long plateau force were observed but without force peaks which are usually attributed to the forced unbinding of membrane components from the cytoskeleton. The result was in agreement with the reports that GLA is substantially free of cytoskeletal interactions. On the contrary, when the Band 3 specific lectin, concanavalin A, was used, the force peaks were indeed observed together with a plateau supporting its reported cytoskeletal association. Based on these observations, we postulate that the state of cytoskeletal association of particular membrane proteins can be identified from the force profiles of their pull-out mechanics.

Keywords: Atomic force microscopy (AFM); Red blood cell (RBC); Glycophorin A (GLA); Wheat germ agglutinin (WGA); *Psathyrella velutina* lectin (PVL); Band 3 protein; Cytoskeleton; Interaction force; Lipid tether; Concanavalin A (Con A)

Interaction between glycophorin A (GLA) on the red blood cell (RBC) and lectins, in particular, wheat germ agglutinin (WGA), has been intensively studied and the specificity of the interaction has been well documented [1–3], and so has been for Band 3 protein–concanavalin A (ConA) interactions [4,5]. Although a small sialoglycoprotein with 131 amino acid residues, GLA, containing 60% carbohydrate on the weight basis, is known as a prominent contributor to the glycocalyx layer and its negative charges on the RBC surface. It has a single stretch of α-helical membrane spanning domain flanked by the N-terminal, carbohydrate bearing extracellular domain and the C-terminal cytoplasmic domain which is substantially free from the interaction with cytoskeletal components. It has been postulated as a specific receptor for Sendai virus [6], for malaria parasites (*Plasmodium falciparum*) [7], and for *Escherichia coli* hemolysin [8] as three representative pathogenic agents. Band 3 protein has been characterized as an anion exchange channel and has small extracellular and relatively large cytoplasmic domains on the two sides of multiple membrane spanning domains. The cytoplasmic domain of Band 3 is non-covalently associated with the cytoskeletal structure through ankyrin proteins [9,10]. Approximately 1.2 and 1 million copies of Band 3 and GLA, respectively, have been found per human RBC [11,12]. Stomatin, tentatively assigned as a regulator of glucose transporter, is probably the third major membrane protein on RBC with approximately a half a million copies per RBC [13].

Whether a particular membrane protein is in association with the cytoskeletal structure or not is biochemically an important issue because the signal transduction from the extracellular to intracellular compartments is often mediated by the linkage between ligand bearing membrane proteins to the cytoskeletal protein components. Interaction

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of membrane proteins with cytoskeletal proteins has been studied by, e.g., detergent extraction of unassociated proteins together with membrane lipids leaving those with association with cytoskeleton on the delipidated remains of the cell [14], or from the measurement of the rotational diffusion coefficient of labeled proteins [15]. In this paper, we postulated that, in the case of GLA and Band 3 on the RBC membrane, the pull-out force curves determined by AFM can distinguish the presence or absence of their cytoskeletal associations. And with further studies this proposition may be extended to probe the cytoskeletal association of other membrane proteins in the future.

Materials and methods

Proteins. WGA and Con A were purchased from Sigma Chemicals (St. Louis, MO) and PVL was supplied by Wako Chemicals (Tokyo, Japan). FITC (fluorescein isothiocyanate) conjugate of WGA was from Dojin (Tokyo, Japan). Other chemicals were purchased from Sigma or Wako. All the lectins and chemicals were used without further purification.

RBC. Freshly supplied human RBCs were used throughout this study after five times washing by centrifugation in phosphate-buffered saline (PBS)(−) but supplemented with 2 mM ATP. An aliquot of diluted cell suspension in PBS(−) was left on a clean and untreated or poly-L-lysine-coated slide glass for 30 min and then gently rinsed with the same buffer.

Chemicals. 3-Aminopropyl triethoxylsilane (APTES), disuccinimidylsuberate (DSS), and *N*-succinimidyl 3-[2-pyridyldithio]-propionate (SPDP) were purchased from Pierce Chemical (Rockford, IL). Deglycosylation enzyme kit, Glyko, containing *N*-glycanase, *O*-glycanase, and sialidase A was purchased from Prozyme (San Leandro, CA).

AFM experiments. A BioScope AFM (Veeco Instruments, Santa Barbara CA) was used throughout this work enabling us to monitor the location of the AFM cantilever over the cell surface through an optical microscope equipped with a CCD camera system. OMCL TR400BP-1 (both side gold coated with a nominal force constant k of 0.02 nN/nm) and NP (Si_3N_4 with $k = 0.06$ nN/nm) cantilevers were purchased from Olympus (Tokyo, Japan) and Veeco Japan (Tokyo Japan), respectively. Modification of NP cantilevers with APTES and DSS was performed according to the method described previously [16], whereas OMCL cantilevers were directly modified with SPDP after cleaning with chloroform, ethanol, and MilliQ water. The cantilever force constant was determined as described in ref. [17]. All experiments were performed at room temperature (25 ± 1 °C) using a liquid cell for BioScope. The scan rate was changed between 0.1 and 1 Hz but all the data presented in this paper were obtained at 0.3 Hz (~1 μm/s in terms of pulling speed) as a compromise of reliability and speed of data collection.

Deglycosylation of RBC. For the removal of carbohydrates from RBC, specified amounts of each of *N*-glycanase, *O*-glycanase, and sialidase A by the supplier of the Glyko kit were added to 1 mL RBC suspension containing 100 U/ml penicillin and 0.1 mg/ml streptomycin, and the suspension was left at room temperature for one to two days with stirring at regular intervals. The extent of deglycosylation was monitored from the fluorescence intensity of treated and untreated RBC after labeling them with FITC conjugate of WGA.

Results

Classification of force curves obtained on RBC

In Fig. 1, an AFM image of RBC under physiological conditions as adsorbed on poly-L-lysine coated glass is given. Cells were almost confluently adsorbed to the glass surface ensuring a high probability of setting the AFM tip on

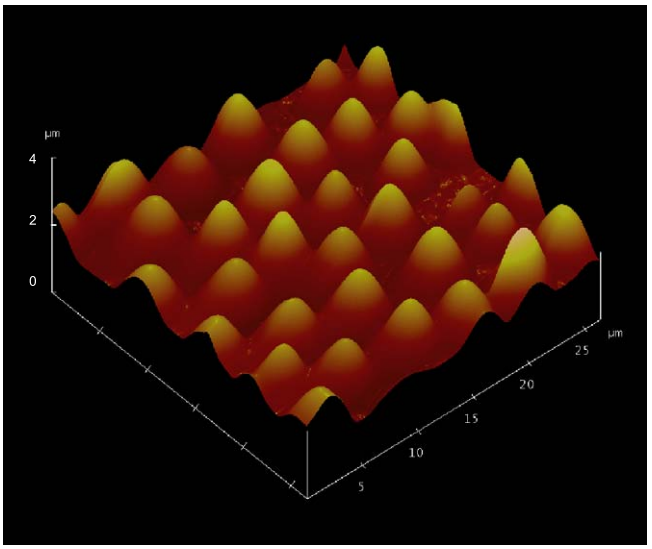

Fig. 1. An AFM image of RBC in contact mode (height image) under physiological conditions as adsorbed on a poly-L-lysine-coated glass surface. Due to strong interaction with the substrate, the shape of RBC was changed from the original biconcave shape. Scales are in micrometers.

RBC surface. On an unmodified glass surface, cells were less firmly adsorbed but still workable under the AFM.

Fig. 2a explains our schematic view of pulling process of a protein from the cell membrane followed by the formation of a lipid tether. Such a process is known to give a force curve with a plateau [18]. In Fig. 2b, the membrane protein is initially anchored to a part of the cytoskeletal structure with a rubber like elasticity, and, when it is pulled up, a nonlinear increment of the force is expected (inset curves).

Force spectroscopy with WGA and PVL

By using OMCL or NP tips, modified first with SPDP or DSS, respectively, and then with WGA, force curves were obtained after keeping a modified tip in contact with RBC for 0.5–2 s. About 70% of all the force curves showed positive indication of tether forming interaction between the probe and the cell surface.

A collection of force-extension curves which were converted from the force curves are given in Fig. 3a. All the curves showed force plateaus with varying lengths of extension suggesting a process explained in Fig. 2a. Plateaus were abruptly terminated with a jump of cantilever either to a next plateau level or to the free level. By multiplying the cantilever deflections in such vertical jumps, the rupture force, F_R, of the interacting pairs was calculated.

A histogram for F_R given in Fig. 3b has a major peak with mean ± SD values of 75 ± 27 pN. Almost all the F_R values under the major peak were obtained as termination force after long tether elongation. A minor peak with a mean of 35 pN was also observed for force curves with short or almost no extension of tether formation. We considered them as representing premature break of tether or extraction of glycolipids, e.g., G_{M1} having a specificity to WGA.

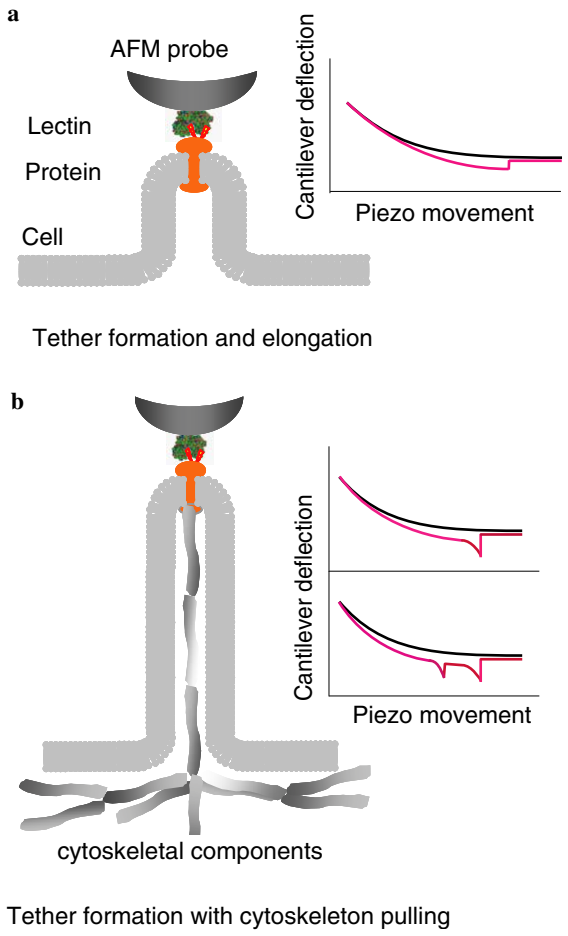

Fig. 2. Conceptual presentation of the pulling process of a membrane protein from the lipid bilayer. (a) As the probe pulls up the protein, a part of lipid bilayer trails behind the protein as a tether. The inset force curve on the right (black for approach and purple for retraction) has a long plateau of the cantilever deflection representing tether elongation followed by a snap back to the free position. (b) When the membrane protein is associated with a part of the intracellular components i.e., cytoskeleton, the tether elongation is overlapped with a nonlinear extension of rubber like material giving inset composite curves on the right.

Similar force curves were obtained when modified probes with PVL were used giving a distribution of F_R as given in Fig. 3c with a mean of 68 ± 30 pN for the major peak. Since PVL has a 40× higher affinity to GLA than WGA, we hypothesized that GLA was transferred from RBC to the AFM tip at the end of tether elongation. A similar value ~50–60 pN has been reported for tether elongation from RBC [19]. Assignment of the other peak in Fig. 3c with a mean of 125 pN is left for future studies.

The above hypothesis was supported by the following experiment using the covalent crosslinker, SPDP, on deglycosylated RBC.

Force curves obtained with Con A modified probes

Force curve acquisition using AFM probes modified with Con A was much less efficient compared with similar

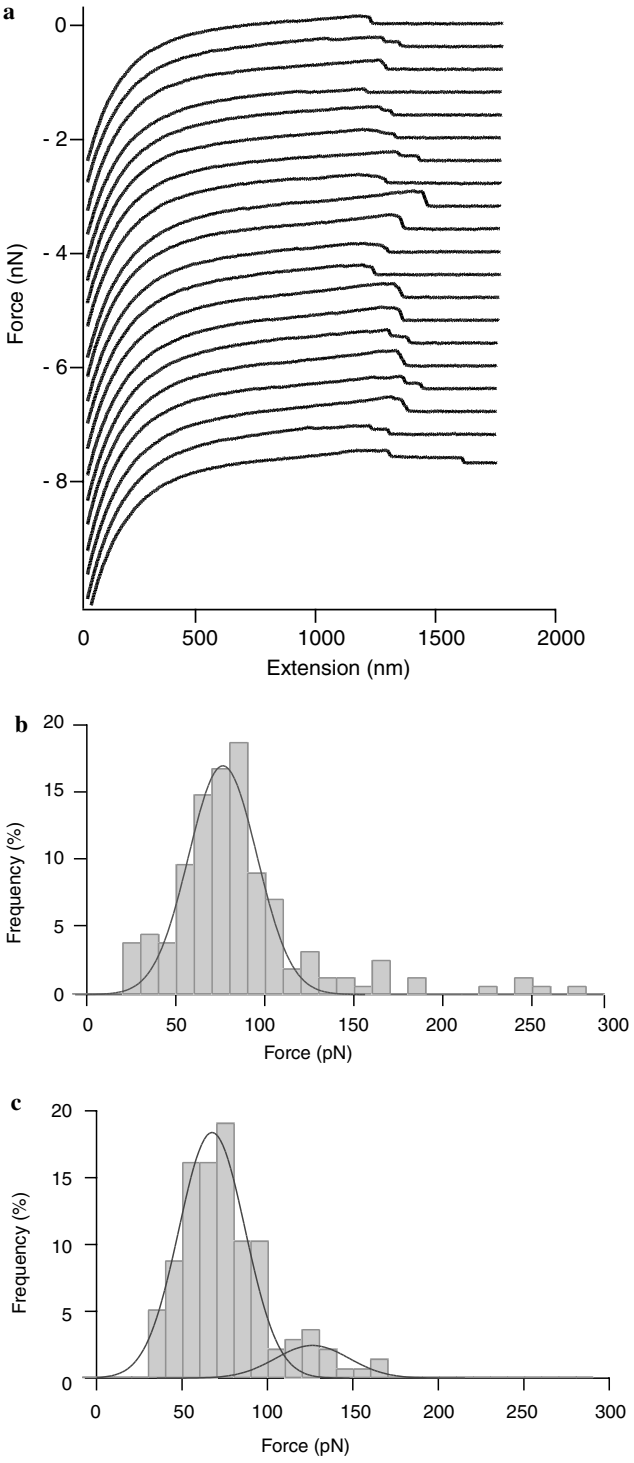

Fig. 3. (a) A collection of force-extension curves obtained on RBC using modified probes with WGA. (b) Histogram of F_R collected from such force curves, and (c) histogram of F_R obtained by using probes modified with PVL.

attempts with WGA or PVL presumably because of the presence of thick glycocalyx layer due mainly to GLA. It has been documented that Con A binding is negatively interfered by the presence of GLA sugar moiety, especially by the presence of sialic acid [20]. Nevertheless, we

collected force curves with similar as well as different features from the ones given in Fig. 3a as presented in Fig. 4a and b. The top five curves had similar whereas the bottom 5 curves had different features from those in Fig. 3a. The similarity and difference were determined for the shape of the force curves in the beginning and toward the end of the plateau. In the top five, plateaus are terminated without change of the height, whereas the bottom five curves involved nonlinearly increasing force peaks in addition to the plateau. We call the former curves as Type I and the latter one as Type II. Considering the reported fact that ~70% of GLA are not associated with [21,22], whereas ~50% of Band 3 are in association with the cytoskeletal structure [23,24], it is tempting to assign the difference in the two types of force curves to the presence and absence of interaction with cytoskeletal structures. To test the specificity of the force curves presented in Figs. 3a and 4a to the lectins used for probe modification, inhibition experiments using free sugars, *N*-acetylglucosamine for WGA and α-methyl mannose for Con A, were performed resulting in a substantial decrease of the efficiency of obtaining force curves with positive indications of probe-RBC interaction. The result was much more clear when deglycosylated RBCs were used as the sample, where almost total disappearance of force curves having positive interaction between the probe and the sample was observed.

We then performed the following experiment where AFM probes modified with amino group reactive bifunctional covalent crosslinkers so that all possible force curves involving membrane protein extraction would be obtained.

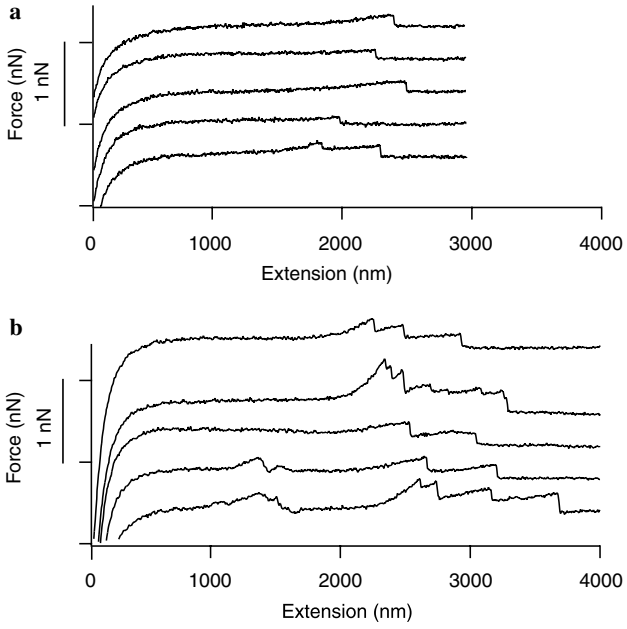

Fig. 4. Typical force curves obtained with AFM probes modified with Con A. (a) Force-extension curves similar to those in Fig. 3a (Type I) and (b) those with non-linear force peaks (Type II).

Force spectroscopy with covalent crosslinkers

We performed pulling experiments using AFM probes modified with the amino reactive covalent crosslinkers such as SPDP, after deglycosylation of RBC. Before deglycosylation, the efficiency of obtaining force curves with positive indication of probe-RBC interaction was low, again presumably due to the coverage of amino groups of membrane proteins by glycocalyx. After removal of glycocalyx layer from the RBC by deglycosylation, the probability of bond formation between covalent crosslinkers and membrane proteins was substantially increased. In this case, covalent bond formation with SPDP was open to all the amino group bearing proteins and lipids on the cell surface with the three most abundant proteins on RBC, i.e., Band 3, GLA, and stomatin as major candidates. We obtained many composite force curves with characteristics of Type II together with a smaller number of Type I curves as given in Fig. 5a and b but not other types than I and II.

We first collected F_R from tether-ending curves of Type I and summarized the result as a histogram and obtained the mean force as $\sim 70 \pm 18$ pN in agreement with those in Fig. 3b and c. We concluded that these curves represented pulling events of proteins not in association with the cytoskeletal structure, i.e., mainly GLA. Since the covalent crosslinker was used in this case, it was certain that GLA molecules were transferred from the cell surface to the AFM tip after tether elongation and breakage including a case of complete denuding of lipids from the protein. Thus, in all three cases of using WGA, PVL, and covalent crosslinkers with widely different bond strengths to GLA, we obtained ~70 pN as the average value of F_R. In our separate experiment, we confirmed that unbinding force of WGA from immobilized GLA on a solid surface was close to ~200 pN under similar experimental conditions to the present ones (data not shown).

A majority of the force curves obtained on deglycosylated RBC were of the Type II feature and had $F_R = 151 \pm 38$ pN (The histogram in Fig. 5c shows the results for both Type I and Type II curves). Proteins responsible for these Type II curves must be Band 3, stomatin, and other membrane proteins with direct or indirect association with the cytoskeletal structure. Although this hypothesis must be pursued further with more careful treatment of RBC, after a short heat treatment of deglycosylated RBC at 50 °C for 10 min, only Type I force curves were observed (data not given). Since such a mild heat treatment has been known to selectively denature spectrin [25], involvement of cytoskeletal structure was strongly supported. We propose the following as the possible mechanisms for the origin of Type I and Type II force curves.

Type I curves are similar to commonly reported tether forming force curves for having a long force plateau [26–28]. Our Type I curves were, however, completely devoid of usually observed force peaks attributed to the forced unbinding of membrane components from the cytoskeletal structure presumably because WGA pulled out

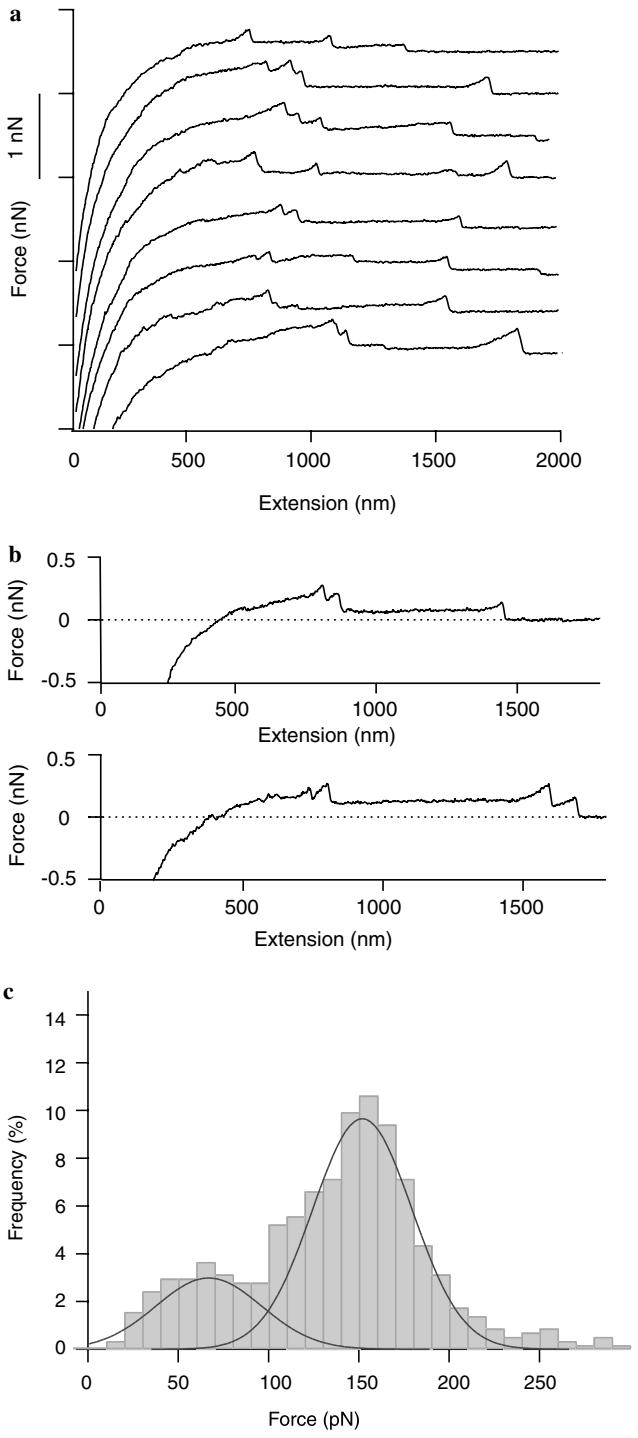

Fig. 5. (a) Typical force-extension curves obtained on deglycosylated RBCs using AFM probes modified with covalent crosslinkers themselves. (b) Zooming in of two representative curves of (a). (c) Histogram of F_R collected from the force-extension curves.

tethers behind GLA molecules which were not associated with cytoskeleton.

Evans et al. [29] recently obtained tether forming force curves similar to those in Fig. 3a without having conspicuous force peaks when P-selectin was used to pull out tethers from leukocytes using a biomembrane force probe. They analyzed the force curves in terms of the initial linear increase of the force followed by a force plateau. The estimated crossing point of the two curves of different regimes was considered to represent unbinding of the membrane components from the cytoskeletal structure. If we accept their interpretation, Type I curves represent not only tether elongation without unbinding from cytoskeleton but also involve cases where the unbinding from cytoskeleton required less force than that required for tether elongation.

Tether formation itself has been studied by both AFM and micropipette force devices and an extensive theoretical analysis has also been developed. One very interesting theoretical result is the reciprocal relationship between the equilibrium force of tether maintenance (F_t) [28] and the radius of the tether (R_t) as below where B is the membrane bending modulus which is about $2–3 \times 10^{-19}$J [30].

$$R_t = \frac{2\pi B}{F_t}$$

From the plateau force of 70 pN, we obtain about 22 nm for R_t which is not an unreasonable value for a single walled lipid tether. Whether the tether was broken or unbinding of lectins from GLA took place at the end of the plateau is a debatable point, but since experiment with covalent crosslinkers, where unbinding of membrane proteins from the AFM probe could be excluded, gave similar value of tether termination for Type I curves, we conclude that tether was severed. This conclusion gives an estimate for the anchoring force of GLA as larger than ~70 pN in average which is in agreement with the estimate given by Bell [31].

Type II curves are more like those commonly observed in tether formation characterized by the force peaks corresponding to unbinding of membrane components from the cytoskeleton. We can therefore conclude that the difference in Type I and II curves is in the degree of cytoskeletal interaction of the proteins used to pull out tethers from the RBC surface. One unique feature observed in our experiment for Type II curves was that the force peaks were observed not only in the beginning of the tether formation but also toward the end of the plateau. Such an increase of force toward the end of the plateau has been previously observed and attributed to the gradual depletion of phospholipids reservoir by Raucher and Sheetz [32]. Both the plateau force and the nonlinear increase of the force in their observation were ~20–30% of our observation. Although this hypothesis is an attractive one to explain the simultaneous disappearance of the plateau force and the force peak, it is not likely to apply to our case because tether elongations to similar lengths were observed without the nonlinear increase of the force when WGA was used as the tether leading lectin.

An alternative model is the one given in Fig. 2b. The lectin molecule on the AFM tip pulls out a membrane protein in association with membrane lipids as well as a half broken cytoskeletal components which has a rubber like elastic property. At the beginning of tether formation, the membrane protein-cytoskeleton bonds are mostly

destroyed with one or multiple force peaks at the initial stage of tether formation, and further elongation of the tether is accompanied by elongation of the component(s) of the half broken cytoskeletal structure. This model seems to be a good representation of what is happening at the molecular level but it still needs to explain why the two events, i.e., tether breakdown and unbinding of cytoskeletal components take place simultaneously unless fortuitous coincidence of the two events is assumed.

Discussion

In this paper, it has been clearly shown that interaction of GLA with two lectins, WGA, and PVL, was strong enough to pull out lipid tethers up to a few micrometers with a stationary plateau of the tensile force of ~70 pN which was abruptly terminated. The force profile of the entire sequence of the event was designated as Type I force curves. Since the same range of force was obtained when the covalent crosslinkers were used to ensure extraction of membrane proteins, we concluded that the tether was broken at the end of the ~70 pN plateau leaving a glycophorin molecule or its complex with lipid on the AFM probe in most of the cases. A finite probability of unbinding of lectin-GLA pairs should be reserved, though, especially when WGA was used, because the dissociation constant of WGA to GLA is 175 nM, 40× larger than that of PVL (4.3 nM) [33].

From the force value of ~70 pN (although not an equilibrium force), we estimated the radius of the tether to be about ~22 nm. A larger force observed on Balb 3T3 cells in our previous work may be attributed to different values of bending modulus between RBC and Balb 3T3 cells [34].

Force extension curves obtained with Con A as a functional molecule on the AFM probe were classified into two groups, Type I and Type II. We proposed that the former curves were from Band 3 proteins free of association with the cytoskeletal components and the latter curves from those in association with.

The above proposal was supported by the lack of Type II class of force extension curves in the result of experiments performed on heat treated deglycosylated RBC where spectrin, the major components of RBC cytoskeletal structure, was denatured, and therefore linkage between Band 3 and cytoskeletal structure was broken. Results on the deglycosylated RBC confirmed that Type I and II were two major types of force extension curves that could be obtained from the RBC surface.

It is true that membrane proteins including the three major ones addressed in this paper are not exclusively either with or without linkage to the cytoskeletal structures. Moreover, the linkage state of each protein has been reported to be changeable according to its ligand binding status. We proposed in this paper that the linkage status of the membrane proteins to the cytoskeletal structure is reflected in the qualitative feature of the force extension curves. Should this proposal be confirmed in several other systems, it would be a useful method to study locally and transiently changing linkage status of specific membrane proteins to the cytoskeletal structures by using lectins, antibodies, and other ligands with specificity towards particular membrane proteins.

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