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Single molecule fluorescence studies of surface-adsorbed fibronectin

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Abstract

The conformation of the extracellular matrix protein fibronectin plays a critical role in regulating cell function, including cell adhesion and migration. While average conformations of large ensembles of adhesion proteins have been previously measured, cells may sensitively respond to conformational outliers. We therefore applied both single molecule imaging and spectroscopy techniques to map a range of conformational states of individual fibronectin molecules adsorbed to glass, as well as to measure their conformational fluctuations in time. Single-step photobleaching experiments confirmed single molecule sensitivity. Single molecule spectra showed fluctuations in the peak wavelength, both over a spatial ensemble of molecules and in a single molecule over time, most likely indicating the different conformational states fibronectin can assume upon surface adsorption. Single-pair fluorescence resonance energy transfer (FRET) revealed that a fraction of fibronectin molecules existed in conformations that allowed for energy transfer between the labeled cysteine residues of the two dimeric arms folded upon each other, and that fluctuations occurred in the FRET efficiency. Fluorescence polarization experiments identified two possible sources of FRET fluctuations: changes in fluorophore orientation and conformational fluctuations of fibronectin over a time scale of seconds.

Keywords: Single molecule fluorescence; Fibronectin; RGD; Protein adsorption; Structural fluctuations; Biomaterials; Cell adhesion.

1. Introduction

Single molecule spectroscopy techniques have made it possible to probe the dynamics of structural changes in individual proteins and nucleic acids, such as blinking and switching behavior of single molecules of green fluorescent protein [1], catalysis by and folding of individual ribozyme molecules [2], the infection pathway of an adenovirus [3] and the dynamics of the chaperon–cochaperonin interaction [4]. Fluorescence resonance energy transfer (FRET) has become a common technique to probe molecular level distances and their spatial–temporal variations [5,6]. Single molecule FRET studies thus have the potential to map the range of conformations of proteins adsorbed to model biomaterial surfaces.

Upon implantation, most biomaterials quickly become coated with a layer of proteins which dictate cellular responses to the implant, often leading to either inflammation or fibrosis [7]. The conformation of proteins on surfaces plays a key role in controlling cell behavior including cell migration, differentiation, and spreading [8–10]. Moreover, just a few proteins in non-physiological conformations may be capable of invoking adverse cellular responses. Therefore in a population of adsorbed proteins, the mean conformation may not be as relevant as the conformation of proteins in the outlying segments of the conformational distribution. For this reason, it is critical to go beyond ensemble measurements of protein conformations on surfaces.

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Fibronectin (Fn) is an important extracellular matrix protein that adsorbs to biomaterial surfaces and mediates cell adhesion [11]. Moreover, surface-adsorbed Fn also regulates various cell-signaling pathways that are important in determining a host's response to a biomaterial. For example, macrophage adhesion to Fn leads to the formation of foreign-body giant cells (FBGCs), and the FBGC formation depends on the relative orientation of Fn's main cell adhesion domains, RGD and PHSRN [12]. The spatial distribution of Fn or of its recognitions sites further impacts cellular responses. For instance, cell migration can be supported at lower average RGD densities if the RGD is presented to cells in clusters [13], beads coated with trimers of $FnIII_{7\text{-}10}$ but not with the monomer alone can bind tightly to cell surfaces [14], and nanopatterns made from single RGD-peptides can support cell adhesion only at a minimum RGD-peptide spacing [15,16].

A variety of techniques have been employed to examine Fn conformation, including XPS, ToF SIMS [17], ESR [18], antibody binding [19], and FRET [20]. Ensemble measurements have revealed that Fn is compact in solution and partially opens up upon surface adsorption in a manner such that the average adsorbed Fn conformation is dependent on the surface chemistry of the substrate [9,10,21–25]. In cell culture, Fn becomes highly extended and even partially unfolded when incorporated into matrix fibrils [26,27]. Resolution of single Fn molecules on surfaces has been attempted by atomic force microscopy (AFM) [22,28] and electron microscopy [23]. These experiments have indicated that surface-adsorbed Fn can exist in several conformations. Mirroring the ensemble results, Fn conformation at a single molecule level also appears to depend on surface chemistry, with Fn being more extended on hydrophilic than on hydrophobic surfaces [22]. In order to gain further static and dynamic information about the conformation of surface-adsorbed Fn, we applied spatially resolved fluorescence spectroscopy, FRET, and other imaging modes to probe Fn on surfaces in physiological buffer. We show that fluorescently labeled Fn exhibits spectral shifts over time, polarization changes and dynamic variations in FRET efficiency, all of which indicate that the protein is not trapped in a single conformation after adsorption to the surface. Our findings have implications for the structure of Fn on a surface and the presentation of its molecular recognition sites.

2. Materials and methods

2.1. Fluorescence labeling of fibronectin's buried cysteine residues

Human plasma Fn was obtained from Gibco Life Technologies (Carlsbad, CA) or Chemicon International (Temencula, CA). In contrast to our previous work [26], the Fn dimer here was labeled on one or two of its four free sulfhydryl groups on the modules $FnIII_7$ and $FnIII_{15}$ (Fig. 1a) with cysteine reactive fluorophores (Molecular Probes, Eugene, OR) following standard labeling protocols provided by Molecular Probes. For single molecule imaging, spectroscopy and polarization measurements, single-labeled Fn (Fn-D) was obtained by denaturing the protein in 4 M guanidium hydrochloride (Sigma-Aldrich, St Louis, MO) and incubating it with a 10–20-fold molar excess of the fluorophore Alexa 488 maleimide. Separation of Fn-D from the free fluorophore was done either by size-exclusion chromatography (Sephadex G-25 gel filtration column, Amersham Pharmacia) or dialysis (Slide-A-Lyzer, Pierce). Refolding of the protein occurred upon the separation of Fn-D, since the GdnHCl was also removed from the solution, by both chromatography and dialysis. For FRET experiments, the denatured protein was incubated with a 10–20-fold molar excess of both Alexa 488 (donor) and Alexa 594 (acceptor), yielding double-labeled Fn (Fn-D/A). Since the fluorophore reactivity depends on several factors including the type of fluorophore, the manufacture batch of the protein and the fluorophore and the amount of time the fluorophore is incubated with the protein, we empirically varied these conditions and only used batches where Fn was labeled on average with either only a single donor (Fn-D), or one donor and one acceptor (Fn-D/A). The average number of donors and acceptors per dimer was determined by measuring the absorbance of the labeled protein in solution at 280, 496 and 594 nm. By measuring the photobleaching characteristics of Fn-D molecules, we calculated that about 7% of molecules ($n = 234$) were labeled with more than one donor. We were not able to determine exactly which of the cysteines shown in Fig. 1a were labeled, and therefore all our conclusions were made taking this uncertainty into account. All analysis was performed on proteins that were labeled with either single donor molecules or a single donor–acceptor pair, as determined by their photobleaching characteristics. We performed our single molecule experiments with several different batches of labeled protein and the results did not depend on the particular batch used.

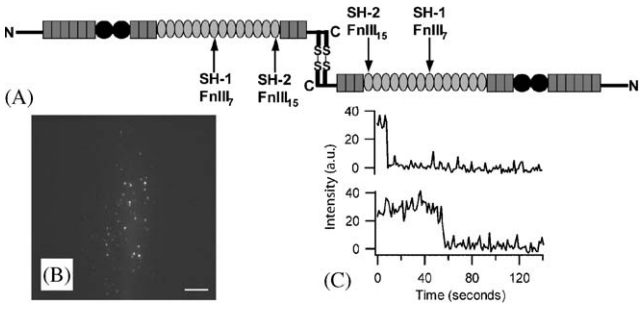

Fig. 1. Single molecule imaging of labeled fibronectin adsorbed to quartz. (a) Schematic diagram of fibronectin indicating the modular composition and the position of SH groups available for site-specific photolabeling. (b) Image of several single molecules of Fn labeled with Alexa 488 (Fn-D) after a 500 ms exposure. Scale bar is 20 μm. (c) Fluorescence intensity as a function of time from two different Fn-D molecules. The molecules emit at a constant intensity until single step photobleaching occurs, indicating the presence of only one fluorophore per protein.