Multiple Membrane Tethers Probed by Atomic Force Microscopy

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ABSTRACT Using the atomic force microscope to locally probe the cell membrane, we observed the formation of multiple tethers (thin nanotubes, each requiring a similar pulling force) as reproducible features within force profiles recorded on individual cells. Forces obtained with Chinese hamster ovary cells, a malignant human brain tumor cell line, and human endothelial cells (EA hy926) were found to be 28 ± 10 pN, 29 ± 9 pN, and 29 ± 10 pN, respectively, independent of the nature of attachment to the cantilever. The rather large variation of the tether pulling forces measured at several locations on individual cells points to the existence of heterogeneity in the membrane properties of a morphologically homogeneous cell. Measurement of the summary lengths of the simultaneously extracted tethers provides a measure of the size of the available membrane reservoir through which co-existing tethers are associated. As expected, partial disruption of the actin cytoskeleton and removal of the hyaluronan backbone of the glycocalyx were observed to result in a marked decrease (30–50%) in the magnitude and a significant sharpening of the force distribution indicating reduced heterogeneity of membrane properties. Taken together, our results demonstrate the ability of the plasma membrane to locally produce multiple interdependent tethers—a process that could play an important role in the mechanical association of cells with their environment.

INTRODUCTION

The plasma membrane of mammalian cells is a highly dynamic structure and its biomechanical properties are vital to the regulation of many cellular functions, such as adhesion, migration, signaling, and morphology (1). One of the most dynamic processes within these membranes is the formation of tethers or thin nanotubes. These structures have been implicated in cell-cell adhesion (2) and recent studies suggest they might also provide a pathway for intracellular and intercellular communication (3–7).

In vivo, tethers form during the primary adhesion and rolling motion of activated leukocytes on vascular endothelial cells or platelets along the walls of blood vessels (2,8,9). Hence, tether formation corresponds to the initial event leading to the extravasation of activated white blood cells at the sites of inflammatory reactions (10). In these systems, membrane tethers originate from pre-existing microvilli through specific selectin/glycoprotein bond formation between cells under hemodynamic conditions (11).

Membrane nanotubes have also been observed between liposomes and have been shown to readily form in red blood cells (12,13), neutrophils (14), neurons (15), fibroblasts (16,17), as well as epithelial (18) and endothelial cells (19). Several experimental methods have been used to characterize the mechanical properties of membrane tethers, such as micropipette aspiration assays (12,13,20–23) and optical tweezers (15,24,25). In these experiments, tethers are observed in force-versus-distance curves as well-defined plateaus occurring at constant force. The presence of plateaus can be understood in terms of a membrane reservoir being gradually depleted upon pulling on the bilayer (16). These studies also revealed that tether length (i.e., available membrane reservoir) and tether formation force are influenced by the various components of the cytoskeleton. On the intracellular side, the membrane is connected to the cytoskeleton through a variety of proteins and other complexes (26,27) and this association has been proposed to play a major role in cell membrane cohesion. The influence of cytoskeletal integrity on the force needed to form tethers has been investigated earlier (28). These experiments demonstrated that the disruption of the cytoskeleton leads to a decrease of the force required to extract and elongate tethers. On the extracellular side, the cell membrane is covered by a glycosaminoglycan and proteoglycan network, the glycocalyx. Whether or not the glycocalyx influences the properties of membrane tether formation has not been explored. Another important question concerns the possible heterogeneity in the interaction of the cytoskeleton/glycocalyx with the membrane over a morphologically homogeneous cellular surface.

Tether formation in cell motility and cellular adhesion is likely to involve the simultaneous formation of multiple tethers. To our knowledge, the tether pulling experiments performed until now have primarily addressed the formation of single tethers. One recent study explored dual tether extraction using the micropipette aspiration technique. Here the tethers were observed not in force-elongation profiles, but rather through the analysis of the dependence of the pulling force on the growth velocities of the tethers (29).

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Other recent work has demonstrated that multiple membrane tethers can be formed in a minimal system composed of a giant unilamellar vesicle, kinesin-coated beads, microtubules, and ATP as energy source (3,5,30). Beyond these examples, little is known about the behavior of multiple, simultaneously existing tethers in real cells, and their coupling with the overall membrane reservoir or their association with each other. Whether or not multiple membrane tethers can be simultaneously extracted from the membranes of living cells is still a matter of controversy. Indeed, multiple tethers extracted from close locations along the membrane surface are expected to rapidly coalesce. In a recent theoretical article, Derényi et al. (31) predicted that, in the absence of pinning forces, multiple membrane tethers coalesce smoothly. However, this study also points out that in real cells, membrane heterogeneities or coupling to the cytoskeleton may prevent tether fusion.

To extract multiple tethers, a large initial adhesion force has to be overcome. For this, a force transducer with the ability to measure a broad range of forces (such as those encountered in specific and nonspecific cellular adhesion events) is needed. The atomic force microscope (AFM) (32) has proven to be a powerful tool for single molecular investigations, to observe biological structures and to study intramolecular and intermolecular interactions (for reviews on the subject, see (33,34)). Recently, a variety of biologically relevant binding forces have been characterized by force spectroscopy including the rupture force of a covalent bond (35), unfolding forces in individual biomolecules (36–40), rupture forces between various ligands and receptors (41,42), unbinding forces of cadherins (43), and cell-cell interaction forces (44,45). The ability of AFM cantilevers to detect a large range of forces (picoNewtons to nanoNewtons) provides the opportunity to simultaneously monitor the formation of individual or multiple tethers and to bring new insight into the behavior of multipally extracted tethers. In this study, we used the AFM to extract multiple tethers, using a variety of cells with different morphology and origin, including Chinese hamster ovary (CHO) cells, a malignant human brain tumor cell line (HB), and endothelial cells. These studies were aimed at demonstrating that formation of multiple membrane nanotubes is a ubiquitous phenomenon, largely independent of particular cell type. In particular, we show that multiple tether formation can be induced locally through contact of the AFM cantilever with the cell membrane, and that the tethers extracted are interdependent. By measuring the contributions of both the cytoskeleton and the glycocalyx to the tether forces, we bring new insights into the mechanism of membrane tether formation and the behavior of multiple, simultaneously extracted tethers.

MATERIALS AND METHODS

Cell culture and treatments

Chinese hamster ovary cells (CHO-K1 cells, American Type Culture Collection, Manassas, VA) and the human brain tumor cell line (HB) (46) were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% penicillin/streptomycin mixture (Sigma-Aldrich) following standard procedures. The human endothelial cells EA hy926 were a generous gift of Dr. C-J S. Edgell (47), and were maintained in HAM'S F-12 supplemented with 20% fetal bovine serum (Sigma-Aldrich). Cells were plated on glass coverslips (Pierce Biotechnology, Rockford, IL), placed in 35-mm plastic petri dishes (Techno Plastic Products, Trasadingen, Switzerland), or plated directly in petri dishes, and cultured at 37°C in a 5% CO_2 incubator typically for 24 h. In cytoskeletal disruption experiments, the cells were in regular medium supplemented with latrunculin A (Sigma), a specific actin polymerization inhibitor (48–50) at various concentrations (0.1, 0.2, 0.5, and 1.0 μM) for 30 min before the measurement. The importance of the glycocalyx in the fomation of membrane nanotubes was studied through one of its major components, the glycosaminoglycan hyaluronan (51). The removal of hyaluronan from the surface of substrate-attached cells was achieved by a 30-min incubation in the presence of hyaluronidase (500 IU/ml) (Sigma-Aldrich) in serum- and polysaccharide-free medium. For surface modification assays, cantilevers (Veeco, Santa Barbara, CA) and glass coverslips (Pierce Biotechnology) were put in the 0.1 mg/ml Poly-L-Lysine solution (Sigma) for 15 min, then rinsed with Milli-Q water (Millipore, Billerica, MA) and air-dried. The effect of a collagen-coated surface was measured by incubation of cantilevers in a type I collagen solution (1 mg/ml; Sigma) at 4°C for 60 min, then rinsing with Milli-Q water and air-drying in a laminar flow hood.

Force spectroscopy measurements

Our in-house-built force measurement device, based on the design and operation of an AFM, was attached to the stage of an inverted optical microscope (Olympus IX70, Olympus America, Melville, NY). This arrangement allowed for precise positioning of the cantilever on the area of interest along the cell membrane. Soft silicon nitride cantilevers (Veeco, Santa Barbara, CA) were cleaned in 70% ethanol, rinsed in Milli-Q water, and then sterilized with UV light for 15 min. Each cantilever was calibrated after a given experiment using thermal noise amplitude analysis (52,53). The measured spring constants were between 8 and 11 mN/m, in agreement with the nominal spring constant of 10 mN/m.

Cells were placed under the force device in CO_2-independent medium (Invitrogen, Carlsbad, CA) containing 2% fetal bovine serum at room temperature. A typical experiment was performed as follows: the cantilever was moved toward the surface until contact with the cell membrane (observed from the deflection of the cantilever) was established (Fig. 1). Contact was maintained for 2–30 s, and then the cantilever was retracted from the cell surface (Fig. 1 *B*). A typical retraction resulted in a series of rupturelike discontinuities in force, as shown schematically in Fig. 1 *B*. Loading rates were maintained between 3 and 5 μm/s. Force elongation profiles were recorded using a number of cells from each cell type, with each cell subjected to multiple retraction experiments. Several hundred discrete events were used for data analysis for each of the three cell lines.

Visualization of membrane nanotubes with quantum dot-labeled cells

Endothelial cells (EA hy926) were washed three times in phosphate-buffered saline (Fisher Scientific, Pittsburgh, PA) to remove culture medium followed by incubation with sulfo-NHS-biotin (Sigma, St. Louis, MO) at a concentration of 100 μg/ml for 15 min at room temperature. Subsequently, cells were washed four times in HAM'S F-12 cell culture medium (Wisent, St. Bruno, Canada) and incubated with streptavidin-conjugated Q-dots (Quantum Dot, Hayward, CA) with a fluorescence maximum at 605 nm. Finally, cells were rinsed an additional three times to remove unbound Q-dots and micrographs were obtained with an inverted epifluorescence microscope (Axiovert 200, Zeiss, Thornwood, NY) on which the force spectrometer was mounted. Images were taken (40× objective) with an ME2 CCD camera (Finger Lakes Instrumentation, Lima, NY) at a resolution of