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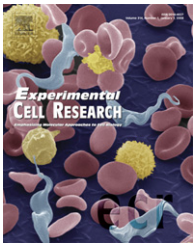

Research Article

Lamellipodia nucleation by filopodia depends on integrin occupancy and downstream Rac1 signaling

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ABSTRACT

Time-lapse video-microscopy unambiguously shows that fibroblast filopodia are the scaffold of lamellipodia nucleation that allows anisotropic cell spreading. This process was dissected into elementary stages by monitoring cell adhesion on micropatterned extracellular matrix arrays of various pitches. Adhesion structures are stabilized by contact with the adhesive plots and subsequently converted into lamellipodia-like extensions starting at the filopodia tips. This mechanism progressively leads to full cell spreading. Stable expression of the dominant-negative Rac1 N17 impairs this change in membrane extension mode and stops cell spreading on matrix arrays. Similar expression of the dominant-negative Cdc42 N17 impairs cell spreading on homogenous and structured substrate, suggesting that filopodia extension is a prerequisite for cell spreading in this model. The differential polarity of the nucleation of lamellipodial structures by filopodia on homogenous and structured surfaces starting from the cell body and of filopodia tip, respectively, suggested that this process is triggered by areas that are in contact with extracellular matrix proteins for longer times. Consistent with this view, wild-type cells cannot spread on microarrays made of function blocking or neutral anti-β_1 integrin antibodies. However, stable expression of a constitutively active Rac1 mutant rescues the cell ability to spread on these integrin microarrays. Thereby, lamellipodia nucleation by filopodia requires integrin occupancy by matrix substrate and downstream Rac1 signaling.

Introduction

Cell adhesion to extracellular matrix plays a key role in cell physiological processes including motility, anoikis and proliferation. These processes are essential for embryonic development, wound healing or inflammation. Although it usually takes more than 1 h for fibroblast cells to achieve spreading with mature focal adhesions, the initial steps of this process are much faster [1,2]. The early stages of cell spreading involve two types of membrane protrusions: actin-rich veils named

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Abbreviations: FN7–10, fragment of fibronectin comprising type III domains 7 to 10; FITC, fluorescein isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate; α-MEM, minimal essential medium with alpha modification; DMEM, Dulbecco's modified essential medium; EDTA, ethylene diamine tetra acetic acid; PBS, phosphate-buffered saline

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lamellipodia and finger-like projections called filopodia. Initial steps of spreading are thought to share molecular processes with membrane extensions at the leading edge of motile cells that are fundamental for cell guidance [1,3,4]. Increasing knowledge has been accumulated about the molecular regulation of actin dynamics including the control of lamellipodia and filopodia extensions [5,6] by the G protein Rac1 and Cdc42, respectively, the nucleation of microfilaments by the Arp2/3 complex [7–9] and the anti-capping activity of the Ena/VASP protein family [10,11]. However, little is known about the role of cell adhesion onto extracellular matrix although a recent report indicated the presence of integrins in filopodia playing a major role in cell adhesion [12]. In membrane protrusions, it has also been suggested that integrins might be activated but still unliganded [13]. Indeed, the interplay between lamellipodia and filopodia during cell spreading and motility is still controversial. The classical view is that the primary function of filopodia is to sample the immediate environment and to transduce signals that trigger the extension of lamellipodia in a distinct process [14,15]. The choice between one and the other mode in actin polymerization would be dependent on the capping protein at the barbed end [16]. Alternatively, it was proposed that merging of convergent lamellipodia would result in the bundling of actin filament that would initiate filopodia formation [17]. However, nucleation of membrane veils (that are very similar in structure to lamellipodia) from filopodia originated from the growth cone has recently been described [18]. The lack of experimental evidence that could strengthen one hypothesis to the other is mostly due to the highly dynamic nature of cell membrane protrusions. Indeed, the speed of lamella protrusion and retraction is about 0.4 μm/s [19], whereas the speed of filopodia extension at the growth cone ranges from 0.1 to 0.2 μm/s [20]. Until the recent progress of optical microscopy, this high speed associated with the small size of the structures made measurements and time-lapse video uneasy. Moreover, filopodia are very thin structures that are barely visible with classical microscopy techniques. Finally, the highly irregular shape of the cells and the cycles of extensions and retractions of the membrane have blurred the observation of possible rules that may govern cell spreading although careful microscopy analyses suggest that such rules do exist [17,21].

Constraining cell adhesion to micrometric adhesive islands separated by anti-adhesive surfaces creates reproducible geometric cell shapes and controls cell physiology. Pioneering studies from the groups of Donald Ingber and Georges Whitesides have shown that constraining cell shape using micrometer-sized extracellular matrix islands resulted in the regulation of proliferation and apoptosis [22,23]. Simplifying cell geometry helps observations of the sequential steps and discloses important rules of cell behavior and cytoskeleton dynamics. For instance, it was shown that cells cultured on square matrix islands preferentially extended lamellipodia from their corners [24] or that spatial distribution of the matrix has a role in determining the orientation of the division axis of HeLa cells [25].

Here, we have analyzed fibroblast spreading onto fibronectin fragment by fast video-microscopy. Movies unambiguously indicated that filopodia nucleates lamellipodia on their sides. This nucleation starts from the cell body and moves to the filopodia tips. To dissect this process into discrete stages, we used micrometric matrix protein arrays to follow adhesion of NIH 3T3 cells. With such patterns, the anchorage points of subcellular size are spatially defined and limited in number. Conversely to the commonly used large patterned adhesive surfaces that restrain the cell spreading to predetermined geometrical shapes [23,26], matrix protein microarrays allow a higher degree of freedom since the cells can choose between numbers of elementary shapes that may depend on the constraints of actin cytoskeleton assembly. Moreover, the processes of membrane extensions and cell attachment to the extracellular matrix are spatiotemporally disconnected. Time-lapse video-microscopy unambiguously shows that fibroblasts spreading on microstructured matrix protein arrays primarily use filopodia to reach new adhesive surfaces. At the contact of adhesive surfaces, these structures are stabilized and subsequently nucleate lamellipodia-like extensions that lead to full spreading. We have shown that this process depends on integrin occupancy and downstream Rac1 signaling.

Materials and methods

Cell culture

NIH 3T3 fibroblasts were cultured in α-MEM (Gibco-InVitrogen, Oxon, UK) supplemented with 10% inactivated fetal calf serum, penicillin and streptomycin and were harvested with trypsin/EDTA. Cells were plated with 60,000 cells in 2 ml on microstructured arrays (area of 440 mm^2) in 30-mm Petri dishes and were left to spread for 4 h. For cells stably expressing GFP-RacV12 or GFP-RacN17, cells at 50% confluence were starved in α-MEM without fetal calf serum overnight before plating on patterned substrates in α-MEM complemented with serum.

Immunostaining

Cells were fixed in PBS with 3% paraformaldehyde supplemented with 2% sucrose and were permeabilized with 0.2% Triton X-100 in PBS. Nonspecific sites were blocked with PBS and 10% goat serum for at least 45 min at 37 °C. Monoclonal anti-vinculin (clone VIN-11-5, Sigma, l'Isle d'Abeau, France) or anti-Rac1 (clone 102 Transduction Laboratories, Becton Dickinson, Le Pont de Claix, France) were used for focal adhesions and lamellipodia staining, respectively. Tetramethyl rhodamine isothiocyanate (TRITC)–phalloidin (Sigma-Aldich) was used for F-actin visualization. Cells were observed with an Olympus Provis AX70 microscope (Olympus division Bio, Rungis, France) with a 60× planapo objective (N.A. 1.4).

Retroviral cell infections

The coding cDNAs for Rac1 N17, Rac1 V12, Cdc42 N17 and Cdc42 V12 cloned into the pBabe GFPpuro vector were a gift from Dr C. Gauthier-Rouvière (CRBM Montpellier France). Phoenix cells grown in DMEM in 10 cm Petri dishes at 30–40% confluence were transfected with pBabe Rac1 or Cdc42 vectors using EXGEN 500 (Euromedex, Souffelweyersheim, France) according to the manufacturer's recommendations. After 24 h, the medium was removed and replaced by 5 ml of