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published in

*From Computational Biophysics to Systems Biology (CBSB07),
Proceedings of the NIC Workshop 2007,*
Ulrich H. E. Hansmann, Jan Meinke, Sandipan Mohanty,
Olav Zimmermann (Editors),
John von Neumann Institute for Computing, Jülich,
NIC Series, Vol. 36, ISBN 978-3-9810843-2-0, pp. 159-163, 2007.

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Role of Filopodia in Adhesion Formation During Migration of Epithelial Cells

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Filopodia are a characteristic feature of many motile cells, such as keratinocytes and other epithelial cells. Filopodia are believed to act as guiding cues to direct cell migration, but how their specific functionality is implemented still remains elusive. Using live cell imaging and immunolabeling we show that filopodia are sites of adhesion formation during cell migration. Cell-substrate adhesions formed by migrating epithelial cells strongly depend on filopodia, showing a spatial and temporal correlation between filopodia dynamics and adhesion formation. We observed that: 1) the formation of adhesions depends on the persistence of filopodia. 2) Adhesion sites within a certain filopodium contain early adhesion markers such as vinculin or vasp. 3) Adhesions formed within filopodia are additionally defined by markers for mature adhesions such as tensin or zyxin. 4) In the nascent lamellipodium the filopodial adhesions grow in size whereas no obvious change in composition takes place. Our results implicate an essential role for filopodia in guiding cell migration by directing the formation of new cell-substrate adhesions.

1 Introduction

Cell adhesion is one of the most essential processes for proper cell function and movement of cells. For example, formation of multicellular organisms or the locomotion of cells of the immune system is impossible without cell adhesion and movement. The adhesion itself depends on protein complexes called focal adhesion sites¹. These micrometer-sized adhesion sites form a connection between the extracellular matrix surrounding the cell and the actin cytoskeleton. Focal adhesions are characterized by a specific set of proteins such as integrins, spanning the plasma membrane, regulatory kinases or proteins like vinculin, zyxin or VASP, bridging the integrins to actin fibers. Latest adhesion site formation models imply for moving cells a formation of focal adhesion sites closely behind the leading edge of the cell's lamellipodia (Fig. 1)^{2,3}. Over time the cell moves over the freshly formed adhesion sites transducing them to the rear site of the cell where release takes place. Formation and release times are highly dynamic in the range of seconds to minutes. In these models filopodia, i.e. finger like protrusion emanating from the lamellipodium, are mostly ignored although they are present in almost any motile cell. Instead it is assumed that filopodia mainly sense the environmental conditions without affecting adhesion site formation⁴.

Our experiments show for the first time an essential role of filopodia in the life cycle of adhesion sites. Filopodia determine the location of almost all focal adhesion sites upon cell movement and are the first place of complete adhesion site formation. Such site becomes only increased in size when reach by the lamellipodium and therefore visible by classical microscopic techniques.

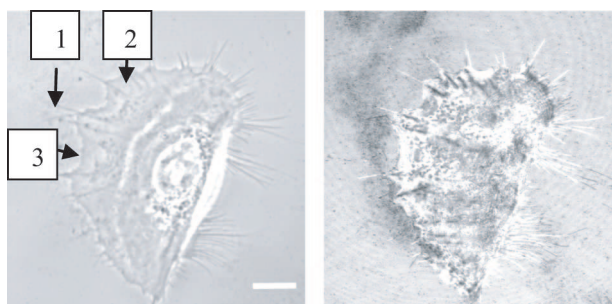


Figure 1. Keratinocyte motility was induced by EGF and cells were analyzed by phase contrast (left) and RICM (right). Note that focal adhesion sites appear as black lines in RICM and describe prolongations of filopodia. 1 = filopodium, 2 = lamellipodium, 3 = leading edge. Scale bar = 10 μm .

2 Experimental

Cell analyses were performed using primary human foreskin keratinocytes. Cell biological procedures were performed according to standard protocols. Cells were either analyzed directly by phase contrast microscopy or reflection interference contrast microscopy (RICM) or transfected with GFP-fusion proteins and analyzed by fluorescence microscopy. RICM allows the determination of distances between surface and adhered cell. The darker the signal is, the smaller the distance. Alternatively, cells were fixed and proteins were stained by immunolabelling.

3 Results and Discussion

In order to analyze filopodia function in focal adhesion site formation, keratinocytes were incubated for one day and subsequently activated by addition of epidermal growth factor (EGF). EGF stimulates cell motility of keratinocytes. Individual moving cells were analyzed by phase contrast and simultaneously by RICM. These data indicated a formation of focal adhesion sites right behind adhered filopodia (Fig. 1). Additionally, the shape of these adhesion sites resembled the one of filopodia.

The given data indicate a role of filopodia in focal adhesion site formation. Such hypothesis was analyzed in more detail. As filopodia are continuously formed "spikes" sensing the substrate conditions in the direction of movement they can either attach to the substrate to form temporarily stable connections or become retracted if no connection could be formed. By analyzing the leading edge over time we could determine that focal adhesion sites were formed only at positions of stable adhered filopodia. Areas of the leading edge characterized by filopodia unable to attach to the substrate could not respond with the formation of focal adhesion sites (Fig. 2).

The formation of focal adhesion sites always behind stably attached filopodia could be explained by two adhesion models. First, filopodia could sense the substrate. Their stable adhesion could then function as signal in order to form focal adhesion sites right behind the filopodia. The alternative would be the formation of small adhesion sites already in the filopodia. In this case the lamellipodium would just function as enlargement factor as

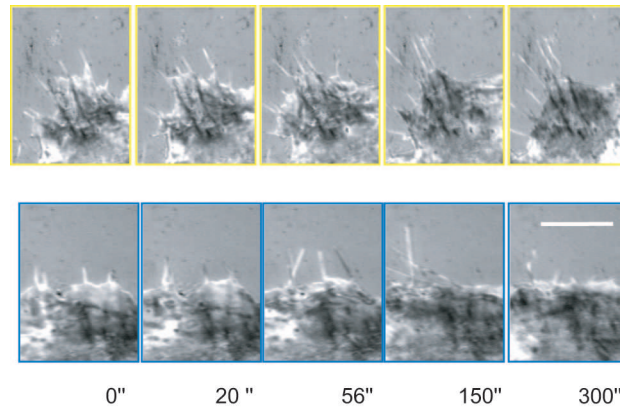


Figure 2. Motile keratinocytes were analyzed by RICM in areas of stable (top) and unstable (bottom) filopodia formation over time. Focal adhesion sites were only detected at positions of stable filopodia. Scale bar = 5 μm .

soon as it has reached the filopodia adhesion site. These models were tested by analyzing the protein localization of focal adhesion site proteins in detail as the latter model fully depends on a localization of these proteins already in the filopodia. Therefore various adhesion site specific proteins were analyzed in fixed samples by immunofluorescence experiments (Fig. 3).

The data pointed out that every focal adhesion site specific protein analyzed (in total 8) could be detected in stably adhered filopodia. Even proteins believed to be markers for old focal adhesions, as zyxin, were present.

When these proteins were analyzed in living cells using GFP-fusions over time, localizations of all proteins in filopodia could be confirmed. In addition, upon cell movement filopodia signals became overgrown by the lamellipodia leading edge. At that moment filopodia adhesion sites grew in size and became classical focal adhesion sites within a few seconds (Fig. 4). Adhesion site formation at other sites than filopodia adhesions were barely detected.

As given results identified filopodia as origin for focal adhesion site formation, we were interested how adhesion behaviour would change upon inhibition of filopodia formation. Such formation depends on the second messenger PI(4,5)P₂ finally triggering filopodia outgrowth via Cdc42 and WASP. Sequestering PI(4,5)P₂ by neomycin sulphate we were able to fully block filopodia formation. Such block neither affected actin filament organization nor cell polarization but induced formation of small dot like adhesion structures (Fig. 5). Additionally, these adhesion structures were clustered and displaced into the lamella compared to untreated cells. Therefore the neomycin experiments argue for a direct influence of filopodia in early adhesion site formation right at the leading edge. As soon as filopodia are blocked in their formation, localization and form of adhesion structures resemble those known from cells naturally characterized by the absence of filopodia. If such morphological switch affects migration efficiency needs to be shown.

In summary, the given data strongly argue for a new adhesion model for motile cells. Sites of first contact to newly explored substrates are filopodia adhesions. Whenever these

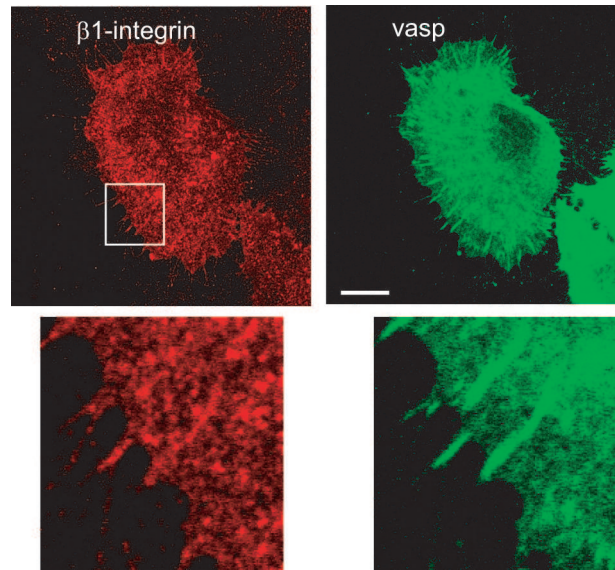


Figure 3. Motile keratinocytes were fixed and immunofluorescently labelled using antibodies against focal adhesion site specific proteins β 1 integrin or vasp. Note that both proteins can be found in the filopodia extensions. Scale bar = 10 μ m.

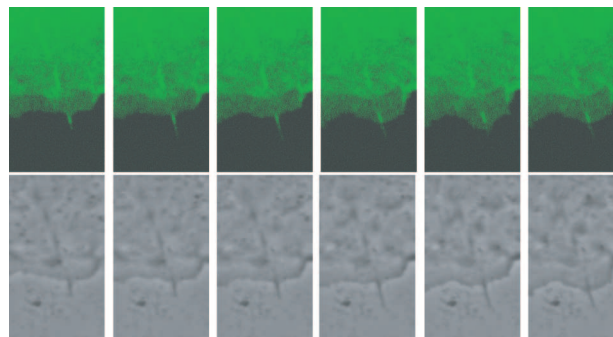


Figure 4. GFP-VASP transfected keratinocytes were simultaneously analyzed in fluorescence (upper row) and phase contrast (lower row). Note that the filopodia staining becomes a focal adhesion site over time. Image intervals = 10 s.

adhesions can not be built up under regular conditions or are unstable, no subsequent focal adhesion site will be formed and movement in that direction is blocked. In contrast, whenever a filopodia stably contacts the substrate, such a contact resembles a fully developed, yet very small adhesion site. When reached by the lamellipodium, such site is just built up in size. Therefore, filopodia are the key factors for movement, the direction of movement and finally for the formation and localization of focal adhesion sites in motile cells.

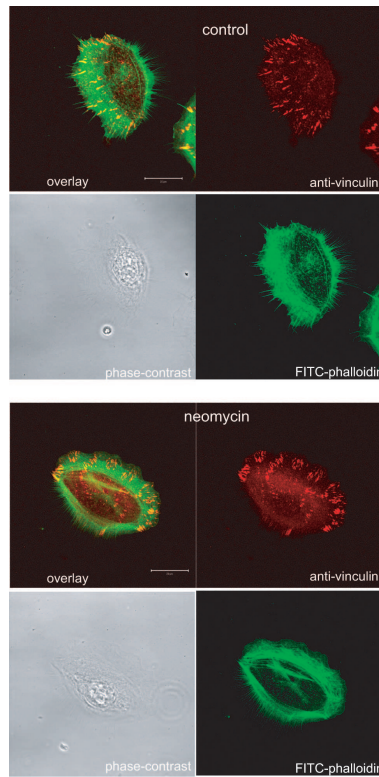


Figure 5. Keratinocytes were stimulated with eGF and analyzed for adhesion site formation in the absence (control) or presence of 10 mM Neomycin. Subsequently, cells were fixed and analyzed for vinculin (red) and actin (green). Cell morphology was additionally determined in phase contrast. Scale bar = 10 μ m.

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