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Cell Shape Modulations and Cell Surface-Nucleus Connections: Prerequisites for Cell Migration

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Functional Phases in Cell Attachment and Spreading

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Abstract

There are many types of cellular motility and inherent in each motile process is a series of steps that must be performed for the cell to accomplish the desired function. Underlying each motility step is a set of proteins that must be activated in the right place for the correct period. In the case of cell attachment and spreading, cells perform several tasks that appear to occur in series rather than in parallel, which results in the cell transitioning between a number of distinct phases. For each phase, there are significant differences in protein activities, which belie differences in function for each of the phases. In the isotropic mode of attachment and spreading, we observed four distinct phases: suspension, early spreading, contractile spreading and fully spread. Suspension cells often exhibit a basal level of motility, in which they extend and retract large finger-like projections presumably to explore the environment. In early isotropic spreading, cells have committed to spreading on the surface and there is stimulated actin assembly with relatively little contraction of the assembled filaments. Over a very short period, cells transition to contractile spreading that is characterized by periodic contractions that test the rigidity of the surface. When the cell is fully spread, extension activity is significantly decreased and focal complexes start to assemble near the cell periphery. Transitions between the phases occur quite rapidly with dramatic changes in the activity of many cellular components. The highly reproducible characteristics of behavior in each of the phases indicates that the cells have only a few modes of spreading behavior and comparisons between cellular activities should be made between cells in the same phase.

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1.1 Introduction

The process of cell attachment and spreading onto a matrix-coated surface has been studied at many different levels. At a basic level, the binding of integrins to the matrix molecules underlies the process because anti-integrin antibodies or RGD-peptides block cell spreading or cause cells to be released from a surface. Blocking actin filament assembly blocks spreading but allows weak adhesion. A number of treatments such as calpain inhibition also inhibit spreading indicating that many proteins are involved directly or indirectly in the process [1]. Sorting out the functional roles of the various proteins is difficult because of the coordination between different activities and the redundancy in different functions. Recent quantitative analyses of spreading have revealed several distinct steps or phases in the process that represent new aspects of the process that were not evident previously.

With the development of rapid digital methods to analyze images, it is now possible to re-examine the behavior of cells at a submicron level on a relatively continuous basis. This review considers several recent studies of cellular motile behavior primarily using total internal reflection fluorescence (TIRF) and differential interference contrast (DIC) microscopy. The findings are consistent with observations using other microscopic methods but provide new insights into the motile process. Although it is useful justification for studying a protein to say that without it an animal will die, a tissue will be malformed or a cell will not undergo a process, the understanding of the cellular process involves a much more detailed description. We need to borrow logic from the engineering of complex systems to come up with a complete description. Part of the process of design engineering is to describe the functional requirements for a given process and the dependent parameters [2]. Several aspects of this paradigm are not particularly suited for the dynamics of biological systems. However, it is a useful exercise to define all of the requirements for a given process to be accomplished, e.g. energy source, protein synthesis, ion movement, filament assembly or motor activity. Those requirements can only be achieved by satisfying certain dependent parameters. In the case of energy, there typically must be an ATP source that is linked in some way to ATP hydrolysis. This sort of accounting often seems mundane or trivial; however, it provides the basis for putting the understanding of cell function in context. Otherwise, there is no obvious way to organize the numerous cell functions and the various interactions that they have into a cohesive description.

1.2
Fibroblast Spreading on Matrices

The process of fibroblast spreading on an extracellular matrix is necessary for fibroblast survival and is a process that can be easily quantified using total internal reflection fluorescence (TIRF) microscopy. TIRF microscopy can be used to follow the regions of close contact (< 200 nm) between the glass surface and the cells. Teleological arguments favor the idea that fibroblasts have a major role in generating force on collagen and other matrices during wound repair and the maintenance of tendons or other connective tissues. If a given cell cannot generate force, then it is in the wrong place in the body and should die, which explains the rapid apoptosis on soft or non-adherent substrata [3]. Upon detailed analysis, spreading for fibroblasts involves the generation of physical force and it is force on matrix ligands in addition to not matrix binding that is required for survival and growth.

1.3
Summary of Spreading Process

The initial steps of cell spreading involve the binding of matrix ligands and activation of edge extension by progression through a series of distinct phases of cell behavior (see list below [22]). However, even in the absence of a substrate or hormone signals, cells exhibit motility that is characterized by the extension and retraction of actin-rich processes. An explanation for this basal phase of motility is that the cell is continually probing its environment (Fig. 1.1). If the environment has a matrix mole-

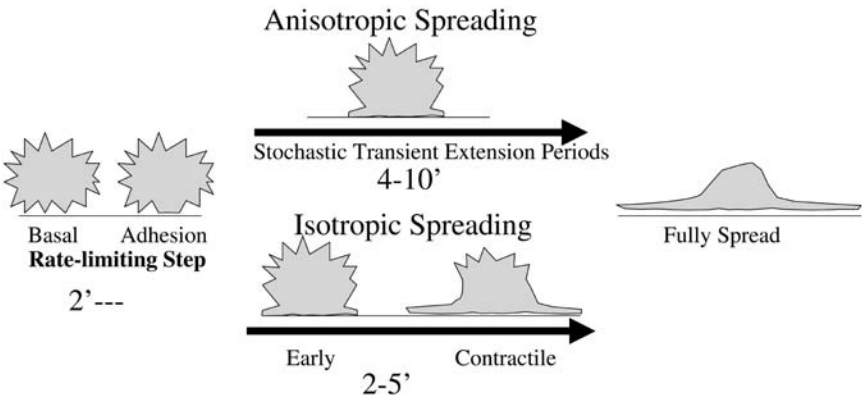

Fig. 1.1 Phases of fibroblast spreading. The distinct phases of cell spreading are shown for cells adhering to a fibronectin coated surface (adapted from [5, 6]).

cule and that matrix attaches to a rigid substrate, then the cell can generate force on the matrix, which activates further spreading. There are two distinct modes of spreading, isotropic and anisotropic. In the case of anisotropic spreading, there are multiple extension and retraction events that are limited to small regions of the edge. In the case of isotropic spreading, most of the edge extends rapidly for 1–2 min. Since the isotropic spreading is favored in serum-starved cells, it may be similar to the type of motility stimulated by epidermal growth factor [4]. After an initial period of extension with little retraction, then the activation of contraction will stimulate multiple cycles of extension and contraction that test the rigidity of the substratum. Gradually the extension activity decreases until the cell is fully spread and focal complexes start to form. In the majority of cases, the cell waits until it is fully spread before it polarizes and starts to move in a given direction.

1.3.1 Steps in Cell Spreading

Phases and Phase Transitions in Spreading (Functional Roles)

1) Basal motility of suspended cells
2) Initial attachment (cell recognizes and binds to surface without spreading)
3) Transition to spreading (integrate signal from surface to activate spreading)
4) Spreading (two different early spreading phases are seen, isotropic and anisotropic)
   (a) Anisotropic spreading (series of spreading and contractile events that test the surface rigidity often involving filopodia. Continues until cell is spread)
   (b) Isotropic spreading (nearly continuous spreading of whole edge initially. Favored in serum starvation)
      (i) Early isotropic spreading (rapid spreading to get a large contact area)
      (ii) Transition to contractile spreading (significant contact area, about 700 square microns, start of testing of environment stiffness)
      (iii) Contractile spreading (periodic contractions to test stiffness)
5) Transition to fully spread form (surface area to volume ratio, signal from surface, and low level of motility).

1.3.1.1 Basal Motility Phase (Cells in Suspension)

When cells are in suspension, they can be quiescent but often they exhibit motility by extending large finger-like processes for 5–10 μ. This ba-

sal motility appeared normal in that the rate of extension was approximately the same as during edge extension on a matrix-coated surface and the time of extension was typically less than a minute. If the extended processes bound to extracellular matrix (ECM) ligands, the cell would try to spread on the ECM-coated surface as described below. Alternatively, cell motility was stimulated by hormone activation, which increased activity for 1–2 min. In both states (basal and enhanced), motility involved cycles of extension and retraction that lasted 20–60 s.

1.3.1.2 **Adhesion to the Surface**

When cells settle onto a matrix-coated surface, they probe the surface before they extend a small lamella on the surface. During the time lag, the cell touches the surface multiple times and possibly requires an internal activation event to adhere. Even centrifugation of some cells onto the surface does not result in immediate adhesion. When cells are in a suspended state, the process of forming a stable adhesion is still slow and does not occur with each touch of the cell to the surface even at high fibronectin concentrations on the surface.

1.3.1.3 **Initiation of Actin Assembly and Spreading (Rate-limiting Step)**

As the cell starts to spread, the initial lamella originates from the cortical (actin-rich) region at the periphery of the cell. Because there is no organized myosin component in such peripheral regions, the centripetal, inward, movement of the actin is limited. Rather, we see the "aging" of the filaments in the central region. The actin assembly near the glass surface is dependent upon signals from the matrix binding to stimulate further assembly (although this is not totally clear, because it is difficult to catch cells at this very early stage). In normal gravity, the initiation of spreading is the rate-limiting step and we find dramatic differences in the average time for initiation of spreading dependent upon the concentration of matrix ligand on the glass. Our findings indicate that the concentration of matrix molecules on the surface is inversely related to the time lag before the start of spreading [5]. Two logical explanations for this phenomenon are either, (1) the cell requires the accumulation of X number of binding events before it will spread or (2) the probability of a critical activation event is dependent upon the concentration of matrix (i.e. with a higher concentration, the average time until an event occurs is less). Specific modeling of this under defined integrin and matrix conditions may yield interesting aspects of the mechanism.

1.3.1.4 Continued Spreading

Differences between isotropic and anisotropic spreading (Stages 4a & b)
At this stage, the extension of lamellipodia is essentially continuous in isotropic and discontinuous in anisotropic cells. Although there are oscillations in the rate of extension of the leading edge in later stages of isotropic spreading, the assembly of actin filaments is essentially continuous. In anisotropic spreading, large areas of the cell edge are quiescent, showing neither extension or rearward movement of actin. Further, when the edge does extend, there is rearward transport of actin at early stages. From the earliest stages, there may be a myosin network associated with the lamellipodial actin in anisotropic spreading, which results in greater contraction in the anisotropic case [5]. Inhibition of integrin binding will rapidly block further motility in all cases, indicating that there needs to be continuous input from the integrin binding.

As the actin filaments in the central region of the initial contact zone are disassembled, the central region of the cytoplasm or endoplasm will start to spread on the surface. Initially, the endoplasm is approximately spherical. When the edge of that sphere comes into contact with peripheral regions that are spread on the matrix surface, there must be a breakdown of the endoplasm–ectoplasm boundary and a spreading of the endoplasm on the substrate. We can see from the behavior of the GFP-α-actinin in central regions that there is a phase of little or no contraction when this region expands to fill in behind the spreading wave of lamellipodial actin.

Isotropic activation of and local periodic cycles of contraction (Stage 4b.ii)
When the endoplasm–ectoplasm boundary reforms in the region adjacent to the glass surface, the contractile machinery reforms at the periphery of the endoplasm. This increases the rate of centripetal movement and logically the force on the substrate is increased. In the area under the center of the cell, there are foci of contraction, which appear as local contractions. The pattern of alpha actinin fluorescence in TIRF undergoes a concerted contraction that appears to signal the formation of a continuous cytoskeletal network to transmit tension from one side of the cell to the other. Once the network is formed the cell is poised to develop force at its periphery and test the rigidity of the substrate. We don't know the composition of the structural elements of the network but fundamentally it is needed for the generation of force on the substrate.

For the cell to extend further, the cell must normally find and test a rigid surface. Contractions pull on the matrix molecules and if they are attached to a rigid substrate, force is generated rapidly and locally. Either rapid or local force generation is interpreted as the signal that the surface is rigid and the cell should extend further on the surface. If a rigidity signal is generated by a contraction, the cell also appears to generate

a signal to test the surface again for its rigidity. Thus, we found a periodic cycle of contractions on rigid surfaces. In this cycle, the first contraction not only causes further extension but also generates a factor that activates the next contraction. The period between the contractions is related to the time needed for the actin to move from the cell edge to the back of the lamellipodia (about 25 s to travel 1.5 μm) [6]. When the actin reaches the end of the lamellipodium, it disassembles and releases the agent that then activates the next round of contractions. In this manner a cyclic contraction pattern can be generated, which will repeatedly test the rigidity of the substrate before the cell extends further.

1.3.1.5 **Transition to Fully Spread State**

For normal spreading, we believe that the cell volume and surface area are constant. As cells transition from the nearly spherical form in suspension to a highly spread form, the excess membrane area is depleted and cell ultimately must stop spreading. In the quantitative analyses of spreading, there is a general decrease in spreading activity over time as the spread area asymptotically approaches the maximal spread area. Often the cells will not polarize until they are maximally spread and polarization then is accompanied by a slight decrease in area. We don't understand what triggers cessation of spreading or sets the final spread area.

1.3.2
Binding to Rigid Matrices Causes Strengthening of Cytoskeleton–Integrin Linkages

An important part of motility is the dynamics of the linkages between the liganded integrins and the cytoskeleton. When force applied to those contacts to enable the cell to move the contact or to move itself, there are characteristic changes that involve activation of growth and signaling to the cell nucleus [7]. As lamellipodia extend to new regions of a surface, new matrix molecules bind to integrins that are at the leading edge. When fibronectin or other ECM ligands bind to integrins at an extending leading edge, there is rapid attachment of the integrins to the cytoskeleton [8]. Preferential attachment to the cytoskeleton at the leading edge is an indication that a specialized set of proteins is concentrated there [9–11]. As the actin in the leading edge moves rearward, there are a number of events that are triggered by the generation of force on the integrin–cytoskeleton bonds. We have a partial list of the components involved and have developed a working model (Fig. 1.2); however, many critical questions remain, including the identification of the roles that many other proteins play in the process.

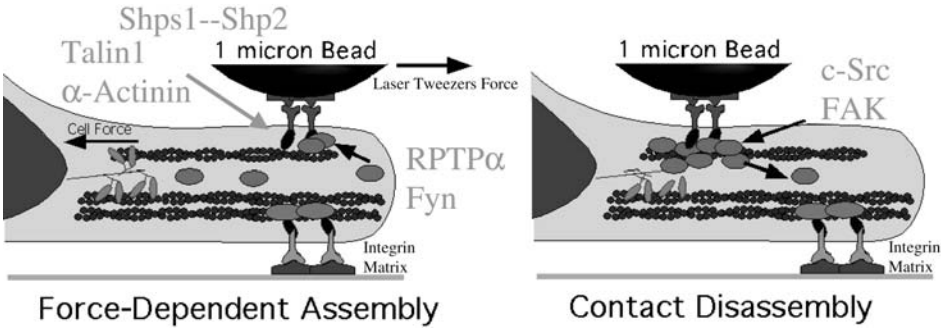

Fig. 1.2 Model for integrin–cytoskeleton linkage strengthening. The force-dependent strengthening of the linkages between fibronectin–integrin complexes and the actin-rich cytoskeleton are summarized. This is only a partial list of the components involved but these appear to have critical roles in the process. (This figure also appears with the color plates.)

1.3.2.1 Initial Binding of Fibronectin Multimers at the Leading Edge and Over Actin Cables

Using fibronectin and anti-integrin antibody coated beads, the initial events in $\alpha_v\beta_3$ and possibly $\alpha_5\beta_1$ integrin binding to the cytoskeleton have been defined. Cross-linking of the integrins by multiple fibronectin ligands is a critical first step and the minimum aggregate is a trimer rather than a dimer [10]. Ligand binding and cross-linking initially results in edge binding [9]; and then after 3–5 min, the aggregates accumulate over focal contact points [10]. The steps in between are poorly understood but earlier studies of fibronectin bead binding suggest two models: (1) diffusion-trapping model or (2) directed transport model. In the diffusion-trapping model, the finding that fibronectin beads can diffuse when bound behind the leading edge makes it possible for those liganded integrins to diffuse to the focal contact sites where linkage proteins would be concentrated and would trap those complexes. In a directed transport model, the focal contacts would be considered as contractile foci and the actin network to which the liganded integrins were attached would be drawn to such foci. Once aggregated at such points, additional proteins of the focal contacts would stabilize the liganded integrins in those regions. Smaller foci would be more likely to dissipate and would end up in larger foci.

1.3.2.2 Force-dependent Activation of the ECM–Integrin Complexes

There is considerable evidence that the rearward movement of actin from the leading edge results in force-dependent activation of signaling

processes locally that are clearly involved in whole cell responses to adhesion. At leading lamellipodia the responses are different than at the retracting tails of cells. Based upon several recent papers, there is a relatively complex working model for the events that follow from $\alpha_v\beta_3$ integrin binding to fibronectin or vitronectin. Initially, binding of a fibronectin trimer with proper spacing will engage talin1 binding and become linked to the cytoskeleton. The initial linkages can be easily broken with 2 pN of force but will eventually become strengthened. In parallel, there appears to be activation of Src family kinases by RPTPα, which is at the leading edge in complex with $\alpha_v\beta_3$ integrin during the first 30 min of spreading [12]. As beads are pulled rearward against a laser tweezers force, the linkages to the cytoskeleton are strengthened [13]. Deletion of either talin1 [14], RPTPα [12] or Shp2 [15] prevents the strengthening of the cytoskeleton linkages, indicating that the process involves many cellular components.

Our working model for strengthening is that fibronectin binding to $\alpha_v\beta_3$ causes attachment to the cytoskeleton either through talin1 or other components. Force generation on the complex activates RPTPα, which then activates several Src family kinases, including Fyn. Fyn is localized to early focal complexes under liganded integrins and catalyzes the strengthening of the linkages. We speculate that Fyn indirectly causes many focal complex proteins to assemble at the site including paxillin, vinculin and alpha actinin. However, strengthening correlates most strongly with the binding of alpha actinin and not with paxillin [15]. Alpha actinin binding depends upon local FAK inactivation by Shp2 which is in turn downstream of Shps1 [15]. A scaffolding role is postulated for talin1, since its deletion blocks strengthening but does not block activation of Src family kinases or spreading [14]. A major gap in this model involves the steps leading from Fyn activation to the assembly of focal complex components. Finally, we know that the strengthening often only lasts for a period of 1–2 min in the absence of continued force application [16] and the steps leading to stable focal contact formation have not been analyzed in this way, although many of these proteins are thought to have roles in stable focal contacts.

1.3.2.3 **Additional Steps in the Spreading Process**

For the membrane to assemble adjacent to the glass surface, the local integrin binding of ligand must cause activation of actin assembly and the subsequent machinery. One model that fits with many observations is that PLC is activated locally which in turn recruits PKC that causes MARCKS to leave the membrane, revealing more PIP2 than was cleaved. The activation of SFKs by RPTPs is early in the process and may be critical for subsequent steps. Another early step is the activation of GEFs (Dbl family members) that produce active Cdc42, Rac, and Rho for sub-

sequent steps [17]. For example, the activation of WASP and Arp2/3 must follow for actin polymerization. In addition, the activation of PAKs leading to activation of LIM kinase and cofilin for filament disassembly should occur simultaneously. An additional factor that needs activation is a myosin that will draw actin filaments in the lamellipodium rearward. It is obvious that many of these different protein complexes need to be controlled in parallel and the important issue for the future is how can there be such a seamless communication between them.

1.3.3 MTs and Motility

MT activation of Rac1 in a positive feedback through APC [18] and Asef and Rac1 acts through PAK1 to activate motility [19]. Many labs have focused on the role of mDia in the feedback between microtubule polymerization and motility [20, 21]. When we have depolymerized microtubules prior to spreading, spreading has proceeded normally. Thus, we believe that the role for microtubule-dependent motility is primarily in polarization.

1.3.4 Conclusion

Recent quantitative analyses of cell spreading and motility have revealed that the cells have a complicated but characteristic pattern of motility. It is useful to categorize the process in terms of motility phases that occur in a serial fashion for specific motile processes. Both physical feedback from rigidity sensing and the strengthening of integrin-cytoskeleton linkages are important for cell viability and other critical aspects of cell function such as morphology. Future detailed analyses at the quantitative level holds the prospect of defining the protein complexes that accomplish specific functions and the ways that they communicate with other functional complexes.

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