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The structure of cell-matrix adhesions: the new frontier

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Abstract

Adhesions between the cell and the extracellular matrix (ECM) are mechanosensitive multi-protein assemblies that transmit force across the cell membrane and regulate biochemical signals in response to the chemical and mechanical environment. These combined functions in force transduction, signaling and mechanosensing contribute to cellular phenotypes that span development, homeostasis and disease. These adhesions form, mature and disassemble in response to actin organization and physical forces that originate from endogenous myosin activity or external forces by the extracellular matrix. Despite advances in our understanding of the protein composition, interactions and regulation, our understanding of matrix adhesion structure and organization, how forces affect this organization, and how these changes dictate specific signaling events is limited. Insights across multiple structural levels are acutely needed to elucidate adhesion structure and ultimately the molecular basis of signaling and mechanotransduction. Here we describe the challenges and recent advances and prospects for unraveling the structure of cell-matrix adhesions and their response to force.

Cell-matrix adhesions are a collection of discrete entities

Cell matrix adhesions were first identified over 40 years ago [1]. Their complex structure and diverse function, however, took a while to unfold. They were first observed as discrete, focal regions in close apposition to the substratum using interference reflection microscopy. A decade later, correlative light and conventional electron microscopy showed actin filament bundles terminating or emanating from these adhesions revealing a connection between the ECM and the actin cytoskeleton [2]. Antibodies raised against molecules purified from chicken gizzard smooth muscle, e.g., α-actinin, vinculin, and talin, localized specifically to these adhesion sites, thus ushering the molecular era of adhesion research [3-7]. Subsequently, a plethora of other adhesion components have been identified by their localization to adhesions [8]. They include specific ECM components, like fibronectin, the transmembrane integrin receptors that link cytoplasmic actin to the matrix, and a large number of molecules involved in signal transduction. This structural complexity is reflected in the diverse functions mediated by adhesions, e.g., cytoskeletal organization and contraction, regulation of proliferation, cell survival, gene expression, protrusion (migration), and adhesion [9,10].

The heterogeneity of adhesions

The pivotal role that adhesions play in such diverse cellular functions makes them prime targets for structural analysis with a goal of unraveling the relations between these functions and their underlying structural organization. However, high resolution structure-analysis of adhesions is highly challenging due to the large number and size of components, diversity of adhesion types and variety of adhesion mediated functions. Furthermore, the inherent transient nature of some adhesions, which can form and mature over a long time period and then can also disassemble, produces a continuum of states that results in a heterogeneity in adhesion types. When this heterogeneity is combined with an incomplete characterization of what cellular outputs are generated by which adhesions, the result is incomplete and potentially misleading structure-function relationships.

Nearly 200 different molecules have been associated with adhesions [8], and new evidence suggests there are more [11]. Force, phosphorylation, conformation change, or association with other molecules regulates the activities of many of these molecules; one consequence of this regulation is to unmask new domains and create new binding sites [12,13]. While the catalog of core adhesion components is likely in place, their stoichiometries, associations, individual structures and relative positions in adhesions are not. Structural analyses of adhesions are further challenged by the variety of different morphologies and signaling properties. This diversity likely reflects the presence of different components, changes in stoichiometry, activation states, and modes of interactions, all of which leads to heterogeneity that complicates efforts studying the fine structure of adhesions.

Some adhesion archetypes

Four archetypal adhesion structures have been described, however: nascent adhesions, focal complexes, focal adhesions, and fibrillar adhesions. In migrating fibroblasts, nascent adhesions are small (< 0.25 μm), short-lived (~ 1 min), and myosin II independent structures that are restricted to the lamellipodium; they require actin polymerization for their formation [12,13]. Nascent adhesions can mature to larger (~ 1 μm in diameter), dot-like focal complexes that reside at the lamellipodium–lamellum interface. Their formation is myosin II-dependent, and they are prominent when Rac is continually activated. They persist for at least several minutes. Focal complexes can mature into larger, elongated focal adhesions, which can be up to several microns long and reside at the ends of actin bundles (stress fibers). Focal adhesions can persist even longer than focal complexes, with half-lives up to 20 mins. Fibrillar adhesions are very large, stable adhesions that are associated with large actin bundles and fibrillar fibronectin. The current evidence suggests that focal adhesions activate Rho, which in turn promotes the formation of focal and fibrillar adhesions; whereas focal complexes and nascent adhesions can activate Rac, which promotes their formation. This establishes a feedback loop that forms and maintains these adhesions[12].

There is strong evidence that physical forces contribute to adhesion maturation and perhaps even the properties of specific adhesions within a class. For example, rigid substrata or application of high forces favors formation of large focal adhesions whereas soft substrata or inhibition of actomyosin contractility favors formation of focal complexes and nascent adhesions [14-16]. Several models for this force-sensitivity have been proposed [14,17,18], and initial studies reveal possible mechanisms. However, systematic studies on how force affects adhesion structure, composition, and signaling are largely lacking. Furthermore, attempts to visualize adhesions in living cells in 3D with resolution similar to that routinely used in 2D has been challenging [19]. However, three recent publications demonstrate the existence of discrete adhesions in cells migrating in 3D collagen gels [20], or cell-free matrices [21]. It appears that background fluorescence, high pliability, matrix fiber structure,

and expression of key components impact on successfully visualizing adhesions in 3D environments.

The challenges of determining the structure of adhesions – merging different resolutions

Determining the structure of adhesions will require the linking of several scales, from light microscopy, for overall morphology and function, to high-resolution analyses of individual molecules and their interactions. Even at light resolution (~200nm), the overall shape, potential subunit structure, and heterogeneity within an adhesion including the Z dimension, i.e., the connection between actin and the integrin, has not been firmly ascertained. High resolution structures of the molecules that comprise adhesions and the interaction interfaces among them reside at the other resolution extreme. Since many adhesion components are large with multi-domain domains that are difficult to express using conventional protocols, their structural characterization is a significant challenge. Furthermore actin is intimately associated with adhesions, and therefore, its structure and higher order organization need to be determined and incorporated. The latter is also a nontrivial structural challenge. High resolution structures of adhesions are further complicated by the activation-induced states of key adhesion-associated molecules [13]. Integrins, for example, exist in different conformations that may reflect different functional states [22]. Key signaling molecules like FAK are thought to reside in a phosphorylation-dependent, activated conformation [12]. Finally, some adhesion molecules, e.g., p130Cas and talin, are reportedly conformationally regulated by force [23]. All of this underscores the potential structural heterogeneity among adhesions.

Commonly, such complexity in cell biological systems has been tackled by a reductionist approach in which the corresponding system is broken down into small isolated pieces so that the most relevant parts of the system and interactions among them can be identified and studied. The reductionist agenda assumes that knowing the behavior of the participating molecules is sufficient for providing a mechanistic description of the behavior of the system. While the development of an integrated system model requires in-depth understanding of all system components, this knowledge becomes most meaningful and useful when it is related to all components working as an ensemble within a living cell. Furthermore, the plethora of potential interactions among adhesion components requires direct observation of nearest neighbors in situ.

The challenge of conjoining structure and function in a dynamic system

The major challenge in addressing these structural problems is how to link specific cellular outputs to ultrastructure both statistically and dynamically. For example, the dendritic network model of directed cell migration suggests that the leading edge is pushed forward by an array of actin filaments with a characteristic branched morphology produced by the Arp2/3 actin nucleation complex [24]. Much of this model is derived from analyses of actin polymerization and imaging of single filament assembly in vitro and from two-dimensional electron microscopic images of actin networks in detergent extracted, chemically fixed, dehydrated cells. By extrapolation, it is generally assumed that regions with increased branching activity and filament density would be associated with faster protrusion. While a large part of the cell migration literature is based on this assumption, such a structure function relationship has been inferred but not yet been shown rigorously in vivo. Furthermore, recent studies challenge the view. Quantitative Fluorescent Speckle Microscopy (qFSM), a live cell imaging modality delivering maps of the rates of filament turnover and motion with submicron and second scale resolution [25], using epithelial cells show that protrusion may be driven by two partially overlapping, yet differentially regulated

actin networks [26-28]. Molecular and functional analyses of the relationship between edge movement, assembly, and contraction forces suggest that forward motion of the cell edge at the onset of a protrusion cycle may be initiated by an Arp 2/3 independent elongation of the actin in the lamella. This is followed by a second, Arp2/3-mediated actin branching in the lamellopodium (branched network), which reinforces cytoskeleton expansion against pressures from plasma membrane and extracellular environment [29]. While intriguing, this model as well as the others is still under scrutiny [30,31].[32,33]

Elucidating the relative position of adhesion molecules by subresolution light microscopy

The resolution of conventional light microscopy has been recently expanded by PALM and interferometric PALM (iPALM) technologies, which are continuously being developed into super-resolution imaging approaches [34,35]. These imaging technologies overcome some of the resolution limitations inherent to light microscopy. High resolution can be achieved either at the sub-nanometer level for few molecules or, more commonly, in the range of tenths of nanometers for structural reconstructions involving thousands to millions of fluorescently-labeled objects. This resolution is seen using any of these related approaches, e.g., PALM, FPALM, STORM, dSTORM, and PALMIRA [36]. At present, the major limitation of this approach is the long image acquisition times required for stochastic sampling. This necessitates chemical fixation, which may perturb structure and can inhibit fluorescence, and for now, precludes following dynamic processes. Furthermore, for accurate structural analyses at the single molecule scale, PALM requires expression of fluorescent fusion proteins, which may also interfere with the native ultrastructure. However, recent studies using super-resolution microscopy have revealed some major insights of the overall features of adhesions. Most importantly, adhesion components are layered in the Z dimension with actin and α-actinin well away from the substratum, talin bridging from actin to integrin and signaling molecules associated with the integrin domain, providing the first glimpse on the three-dimensional (3D) characterization of these sites [37].

Bridging function with structure using correlative light and electron microscopy

In principle, by mining structural variations originating from micro-, meso- and macro-scales, electron microscopy (EM) could serve as the gold standard for adhesion structure. Towards this end, the approach is to seamlessly tie spatial and functional data derived from live cell imaging (via light microscopy) with information derived from high-resolution transmission electron cryo-microscopy (cryo-EM) and cryo-tomography (cryo-ET). Live-cell fluorescence imaging, using genetically (or chemically) labeled proteins, can be used to track dynamically a set of proteins via multiplexing approaches (Figure 1). This provides the means to follow simultaneously multiple processes and thereby derive the hierarchy and kinetics between activities and their constituents. Cryo-EM or cryo-ET provides the ability to determine, in a fully hydrated state and in situ, the three-dimensional (3D) structures of the underlying large, dynamic macromolecular assemblies that govern these processes through structural adaptations. Thus, correlative Light and Electron Microscopy (cLEM) is an emerging approach aimed at establishing methods and technologies for systematically and quantitatively determining structure-function correlates in a physiologically relevant environment (recent reviews [38-40]).

Bridging atomic and molecular resolution using Cryo-EM and Cryo-ET

Cryo-electron tomography (cryo-ET), when combined with computational motif extraction, alignment, classification and averaging, can provide information about these multi-protein

assemblies in situ at 3-4 nm resolution. This hybrid approach also allows correlating high-resolution structural information available from *in vitro* studies of adhesion components to the *in situ* characterization from whole cells. In principle this approach can provide molecular-level information (3-4nm) on protein interfaces and detect conformational changes in response to stimuli such as force. Cryo-ET is unique in its ability to provide this range of resolution in all directions (x,y and z) under fully hydrated conditions.

The ability to generate 3D volumes of assemblies *in situ* allows filaments or networks to be followed. The consequence is that branching, crosslinking, or overlapping arrangements, which might seem similar in a 2D projection of a single planar section, can be faithfully recognized and followed within the volume. The elegant work of Medalia, et al. [41], for example, demonstrates that cryo-ET of intact Dictyostelium discoideum cells could reveal the connections of the actin-filament network with the plasma membrane as well as possible actin filament branches. Similar views of branched networks have been seen in higher eukaryotic cells [42,43]. In contrast, cryoEM and CryoET imaging of lamellipodia by Small et al [33,44] suggest that actin filaments are almost exclusively un-branched. Re-analysis of the Small data [60,61,52)] show the presence of branches in the raw volumes of the primary data. In addition, the branch junctions were of shape similar to cyro-ET structures of Arp2/3 complex mediated branched junctions formed in vitro [62]. The precise number of these branches is still unclear .These contradictions reveal the limits of the methodology and the care that must be taken in analyzing and interpreting this kind of EM data.

This raises the question of whether cLEM technology can be used to provide a quantitative correlation between structure and functional outputs? The answer is ‘not yet’, In the actin cytoskeleton example, the putative spatial overlap of distinct actin networks and the dimensions of the filaments (10nm) in the volume of the leading edge of a cell will probably be recognizable only by the application of high-resolution image processing tools to the 3D tomographic volumes, supported by sophisticated image segmentation and topology classification algorithms [45]. Visual inspection of cyro-ET projection images is insufficient (see Fig 1 [38,46,47]). Furthermore, over-expression of tagged proteins, dynamic heterogeneity, adaptability of simple preparations to accommodate the various imaging technologies, and lack of robust, rigorous and systematic imaging and analysis approaches can lead to identification of erroneous features within the 3D volumes. Thus, it is essential that the features in question can be unequivocally correlated with known proteins or protein assemblies either computationally, by biochemical labeling, or by mutagenesis. Recently, it was suggested that membrane-cytoskeleton interactions at focal adhesions are mediated through particles located at the cell membrane and attached to actin fibers [17]. This observation is intriguing. However, their identification in terms of protein assemblies is not yet known.

Furthermore, at least, two roadblocks still need to be overcome prior to embarking into systematic analysis of spatiotemporally transient structure-function relationships of matrix adhesions using cryo-ET: i) Temporal latency and spatial imprecision in the alignment derived by the need for sequential in situ LM and EM imaging; ii) Low throughput which requires narrowing views to a small subset of a much broader distribution of structures. However, the technology is advancing fast in an effort to overcome these obstacles. Temporal latency is being conceptually addressed by establishing quantitative high-resolution light microscopy approaches that allow identification of functional outputs at the length and time scales of well-defined structural inputs. For increasing the throughput, Cryo-ET must be streamlined into an automated, truly high-throughput workflow to meet the requirements for quantitative structural analyses of transient cellular processes. These new experimental and imaging technologies would allow acquisition of a sufficient number of views to cover the spectrum of structural instances with distinct functional outputs. Once

these technologies mature and are truly integrated, quantitative practices for marrying these imaging procedures seamlessly over their scale gap will need to be put into practice.

Adhesion structure using isolated components

Unlike cytoplasmic organelles, like ribosomes, no one has reported a pure preparation of adhesions. Two features of adhesions may provide the means to simplify the structure challenge. One is that the adhesion appears to have two functionally separable activities that may be structurally separable as well. They are the integrin-actin linkage, which is thought to be comprised of a small set of molecules, e.g., integrin, talin, vinculin, α–actinin, and actin [12]. It seems that this linkage could be studied in isolation using a reconstituted system. The other activity is signaling. This is likely mediated by discrete, transient molecular complexes, suggesting that their structures could be similarly studied as isolated complexes. Indeed recent exploratory attempts to determine in vitro reconstituted, mini cell-matrix adhesions, using key structural components or signaling assemblies have been reported. Taylor, et al. [48,49] used a two component model system comprised of domains from vinculin and α-actinin to reconstitute a cytoplasmic face of a focal adhesion. Reidl, et al. shown recently that members of the novel SH2-containing protein (NSP) and Crk-associated substrate (Cas) protein families form multi-domain signaling platforms that mediate cell migration and invasion [50]. An interesting feature of this system is an enzyme-to-adaptor structural conversion that enables high-affinity, yet promiscuous and highly dynamic interactions. This results in a modular signaling platform of individual patterns of signaling domains, phosphorylation and binding motifs. Both of these studies not only provide a proof of concept for feasibility; they also highlight the potential of such approach for retrieving structural details of other complexes and signaling modules in which the underlying assembly and 3D organization are guided by spatiotemporally transient interactions.

Major progress has been made on the high-resolution structures of some key individual molecules; e.g., integrin, talin, vinculin, paxillin, and p130Cas [12,51,52]. Some data have also been collected on the interactions and functions of selected components, especially the integrin–talin–vinculin–actin linkage [53-55], and the interaction of paxillin with regulators of Rac activation [56].

The effect of force on adhesion structure

Several recent studies have provided a glimpse into the forces and molecular domains generated and sensed by adhesions [22,57,58]. These studies provide initial molecular insights into force-induced activation, binding partners, dissociation constants, rough spatial localization, and atomic structures of implicated domains. However, how adhesion composition and ultrastructure depend on force and signaling inputs is largely an exciting unaddressed challenge.

Conclusion and perspective

Adhesion research has advanced enormously since the initial discovery and characterization of adhesions several decades ago. During this time, their functional complexity has evolved to include their roles in linking actin with the ECM and serving as signaling centers that regulate proliferation, gene expression, cell survival, and cytoskeletal dynamics and organization. The next major challenge is to unravel the structure of adhesions. This is a formidable undertaking due to the molecular complexity and the diversity of adhesion types and functions. However, it may be possible to functionally dissect adhesions into component processes, e.g. the ECM – actin linkage and signaling. Furthermore, the actin-ECM linkage is mediated by a relatively small number of molecules, and adhesion generated signals

appear to emerge from discrete complexes, or modules. Finally, it is likely that both the integrin-actin linkage and signaling complexes can be purified or reconstituted, and therefore their structures can be studied in isolation. In the end, however, matrix adhesions will need to be studied in cells, the only environment that would allow tying their structural organization with dynamics and force. It now appears that the technologies for doing this are either in place or on the horizon. Sub-resolution light microscopy and cryoEM, tomography, and computational feature identification are among the technologies that promise major insights into adhesion structure. While the challenge is significant, the important and impact will be enormous.

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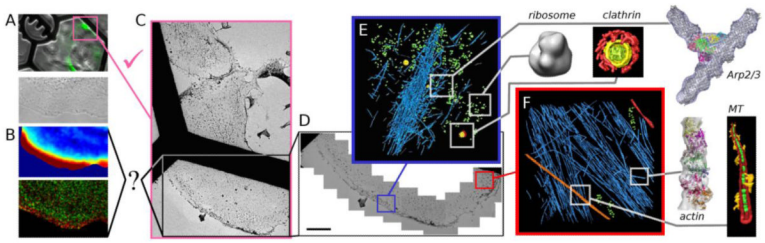

Figure 1. Strategy for correlating and integrating structures over scales

Multi-resolution workflow: Cells are grown on EM amenable substrates with: (A) fluorescence used to localize the regions of interest (B-D, bar in D = 2μm). This correlation approach also allows correlating dynamic information obtained by live-cell imaging, and (B) the underlying structure. (E) Surface representation of a cryo-tomogram from a region in the lamellipodium (blue in D). (F). Surface representation of a cryo-tomogram from a region in the lamella (red in D). The actin-network morphology appears markedly different. Ribosomes, clathrin, actin filaments, microtubules and Arp2/3-mediated actin branches can be readily identified in the reconstructions. We use the structural information of these assemblies previously obtained from reconstituted systems to aid analysis of the extracted motifs (for example [59-61]). Data kindly provided by Karen L.Anderson, Violaine Delorme, Wendy Ochoa, Matthias Eibauer, Florian Beck, Stephan Nickell and Niels Volkmann.