Hierarchical assembly of cell–matrix adhesion complexes

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Abstract

The adhesion of cells to the extracellular matrix is a dynamic process, mediated by a series of cell-surface and matrix-associated molecules that interact with each other in a spatially and temporally regulated manner. These interactions play a major role in tissue formation, cellular migration and the induction of adhesion-mediated transmembrane signals. In this paper, we show that the formation of matrix adhesions is a hierarchical process, consisting of several sequential molecular events. One of the earliest steps in surface recognition is mediated, in some cells, by a 1 μm-thick cell-surface hyaluronan coat, which precedes the establishment of stable, cytoskeleton-associated adhesions. The earliest forms of these integrin-mediated contacts are dot-shaped FXs (focal complexes), which are formed under the protrusive lamellipodium of migrating cells. These adhesions recruit, sequentially, different anchor proteins that are involved in binding the actin cytoskeleton to the membrane. Conspicuous in its absence from FXs is zyxin, which is recruited to these sites only on retraction of the leading edge and the transformation of the FXs into a focal adhesion. Continuing application of force to focal adhesions results in the formation of fibrillar adhesions and reorganization of the extracellular matrix. The formation of these adhesions depends on actomyosin contractility and matrix pliability.

Introduction

Cell adhesion to the ECM (extracellular matrix) plays key roles in the assembly of cells into functional multicellular organisms. At the same time, such adhesions are involved in transmembranal signalling processes that regulate cell behaviour and fate. Adhesive interactions occur via a variety of molecular systems. These include different integrin receptors that bind to ECM molecules via their extracellular domains and interact via their cytoplasmic moieties with the actin cytoskeleton [1,2]. Adhesive interactions are mediated by a network of 'anchor proteins', some of which directly mediate the linkage between the actin and the membrane, whereas others play a regulatory role (reviewed in [3–5]). Adhesion is also mediated via a variety of membrane- or matrix-bound glycosaminoglycan molecules [6,7]. Integrin-mediated adhesions are molecularly heterogeneous, appearing in different forms such as 'classical' FAs (focal adhesions), FBs (fibrillar adhesions) and FXs (focal complexes). Each one of these has a typical morphology and molecular composition (see [8] and Figure 1). In motile cells, the spatially and temporally regulated formation and dissociation of matrix adhesions play a central role in the motile process.

In this paper, we will consider the molecular steps involved in cell–matrix adhesion, including hyaluronan-mediated attachment and the sequential formation of FX, FA and FB. We will discuss the mechanisms underlying the formation of each of these adhesion sites and the interdependence between them.

Key words: cell adhesion, fibrillar adhesion, focal adhesion, focal complex.

Abbreviations used: ECM, extracellular matrix; ESEM, environmental scanning electron microscopy; FA, focal adhesion; FB, fibrillar adhesion; FX, focal complex; GFP, green fluorescent protein.

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Hyaluronan-mediated adhesions: the first encounters

ECM consists of a rich variety of macromolecules that are recognized by a comparably large variety of cell-surface receptors. Whereas integrins and their ligands appear to play a major role in the formation of cell–matrix adhesion, other adhesive systems have also been described, including matrix- and membrane-bound glycosaminoglycans. Recently, we have shown that cell-associated hyaluronan plays a central role in mediating early stages in the attachment of cells to external surfaces [9–13]. In a series of studies on cell adhesion to molecularly defined crystal surfaces and to conventional cell adhesion substrates (glass and tissue culture dishes), it was found that rapid (time scale of seconds) attachment of A6 epithelial cells was mediated by cell-associated hyaluronan [13]. This glycosaminoglycan is a large linear polymer of [D-*N*-acetylglucosamine-β-D-glucuronate] that can be associated either with the ECM or with the plasma membrane. Its role in surface adhesion was demonstrated by the drastic inhibition of the rapid attachment to compatible crystal surfaces, observed after treating the cells with hyaluronidase. Subsequent addition of pure hyaluronan to the hyaluronidase-treated cells or to the matrix could restore adhesion. Interestingly, the presence of hyaluronan on both the surfaces inhibited cell adhesion [13].

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Figure 1 | Immunofluorescence microscopy of integrin-mediated adhesion structures

Porcine aortic endothelial (**a**, **c**) and rat embryonic fibroblast (**b**) cells cultured on glass coverslips were fixed and double-labelled with phalloidin-FITC (green) to visualize the actin cytoskeleton and a prominent component of the adhesion plaque. Highly phosphorylated FX at the leading edge are seen by labelling for PY (**a**), vinculin staining reveals large FA at the ends of thick actin cables (**b**) and tensin labelling (**c**) emphasizes the long streaks of FB in central regions of the cell. Scale bar, 5 μm; insets enlarged ×3.

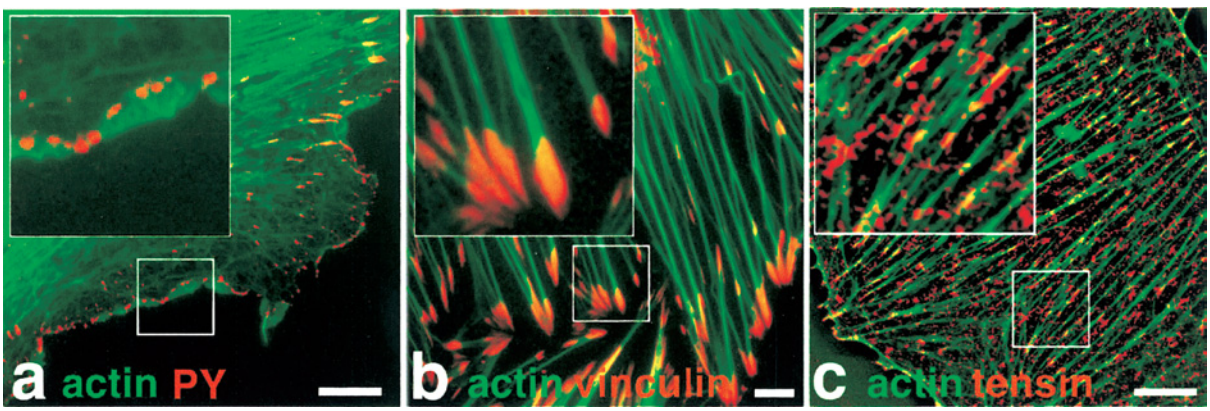
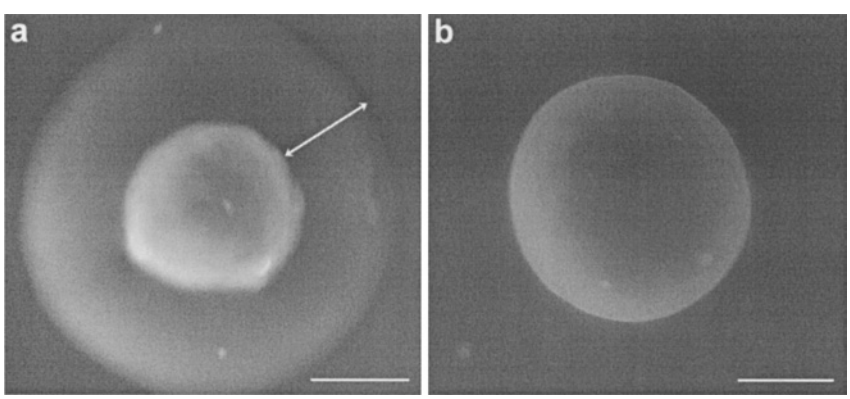


Figure 2 | Visualization of hyaluronan pericellular coat using ESEM

Chondrocytes were fixed and incubated with uranyl acetate in suspension, then examined using ESEM. (**a**) Untreated cells have 4.4 ± 0.7-μm-thick halos around them (arrow). (**b**) Hyaluronidase-treated cells are not surrounded by such halos. Scale bar, 5 μm.



To visualize hyaluronan associated with the cell surface, we have chosen to examine chondrocytes, which are known to produce large amounts of this glycosaminoglycan, using ESEM (environmental scanning electron microscopy). Cells were fixed in suspension, incubated with uranyl acetate, and deposited on glass coverslips. A 4.4 ± 0.7 μm wide, sharply defined halo was seen around the cells (Figure 2a). Treatment with hyaluronidase destroyed this halo (Figure 2b). Using a particle exclusion assay, we then reconstructed the three-dimensional features of the cell-bound hyaluronan coat. This was performed by immersing rhodamine-labelled chondrocytes in a suspension of 0.4 μm silica beads, coated with fluorescein. Three-dimensional microscopic examination indicated that the beads are excluded from a several-μm-thick zone surrounding the entire cell [9], whereas hyaluronidase-treated chondrocytes lack this coat.

It was further demonstrated that the initial phase of matrix adhesion of chondrocytes is hyaluronan-dependent, similar to what was observed for A6 cells. Cells were allowed to adhere to glass surfaces for 10–25 min, and then subjected to flow exerting shear force of 6.5 dyn/cm^2. Cell movements were recorded by time-lapse phase-contrast microscopy. It was found that chondrocytes drifted under flow by 43.10 ± 10.79 μm before detaching from the surface. Using biotinylated hyaluronan-binding protein and streptavidin CY3 labelling, hyaluronan-rich 'footprints' were visualized attached to the substrate behind them. In contrast, hyaluronidase-treated cells were washed away immediately after applying the flow [9]. These results indicate that chondrocytes establish, initially, 'soft contacts' with the surface through a hyaluronan-based coat. The surface adhesion, mediated by the hyaluronan coat, occurs within seconds after the cell first encounters the external surface. This was

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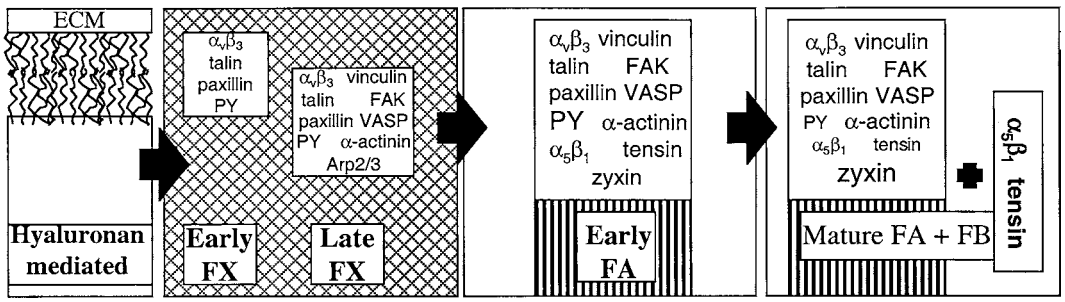
Figure 3 | Evolution of matrix adhesion

This scheme depicts temporal stages in the formation and reorganization of cell–matrix adhesions, starting with hyaluronan-mediated attachment (left) to FXs, FAs and FBs. The characteristic molecular components of each form of adhesion are listed (see Discussion and conclusion section and [8,9,14] for details).



demonstrated by studies in which A6 epithelial cells were trapped in laser tweezers and brought to the proximity of a hyaluronan-binding surface [the {011} faces of calcium-(*R*,*R*)-tartrate tetrahydrate crystals]. It was found that as soon as the cell approached the crystal surface it was essentially immobilized, and could not be detached from the crystal by the laser tweezers. The time required for the establishment of this strong attachment was, apparently, <1 s (E. Zimmerman, L. Addadi, B. Geiger and M. Elbaum, unpublished work).

Formation of FX during protrusion of the leading edge

The hyaluronan-mediated adhesion described above appears to be rather transient, and is replaced, within a few tens of seconds-to-minutes by integrin-containing contacts. To study early stages in integrin-based adhesion, we have examined the formation of FXs under the advancing leading edge (Figure 1a). The experimental system used in the present paper consisted of pig aortic endothelial cells, migrating into an '*in vitro* wound', introduced into the confluent monolayer. Monitoring the migration of the cells into the wound by phase-contrast microscopy, followed by immunofluorescence microscopy for various FA proteins, enabled us to determine the molecular composition of the newly formed FX. Comparison between leading edge dynamics and FX composition indicated that the incorporation of the different components into nascent adhesion sites is a hierarchical process. The earliest observed molecules were $\alpha v\beta 3$-integrin and phosphotyrosine, closely followed by talin and paxillin. Later, vinculin and α-actinin entered the developing FX, along with FAK and VASP. Thus the exact composition of an FX depends on its age, which inversely correlates with the rate of protrusion of the near-by leading edge. Interestingly, two components of FA, zyxin and tensin, were absent from FX, regardless of their age [14].

Retraction-induced transformation of FX into FA

Time-lapse video microscopy of migrating cells, expressing fluorescent derivatives of FA proteins [e.g. GFP (green fluorescent protein)–paxillin], revealed that during the forward movement of the lamellipodium, FX, with typical area of 0.25 μm^2, are formed and persist for a few minutes, until the leading edge further advances and new FX are formed in front of them [14–16]. These cycles of FX formation and dissociation persist as long as the lamellipodium advances. When the lamella retracts, or even stops protruding, many FX disappear, whereas a subset of these adhesions starts growing and transforms into definitive FA (Figure 1b) [14]. This transformation is not manifested just by a growth in size of the adhesion site, but also by changes in its molecular composition (Figure 3). Thus the formation of FA is accompanied by recruitment of zyxin to the membrane and the concomitant assembly of an actin bundle. This transition apparently depends on actomyosin-driven contractility, which applies force at cell–matrix adhesions. This is supported by the observation that myosin light-chain kinase inhibitors enhance lamellipodial protrusions, induce an accumulation of FX close to the cell's edge and block FA formation. The notion that local mechanical forces activate the growth of FA is in line with experiments in which local forces were directly applied to small matrix adhesions of serum-starved cells using a micropipette. It was found that this perturbation induced a rapid growth of the adhesion site [17] and that this growth was dependent on the presence of an active form of Rho-A in the cells. The development of FA was further shown to depend on two downstream targets of Rho, namely Rho kinase, which activates the cellular contractile machinery (these forces were 'replaced' by the pipette pulling in the experiment described above) and mDia, which is involved in the regulation of both actin and tubulin dynamics [17]. The nature of the 'mechanosensor' responsible for the force-activated assembly of FA is still poorly understood (see [18]).

Force-dependent matrix fibrillogenesis and FB formation

FAs seem to be rather robust structures, yet they are, in fact, highly dynamic structures. This feature is manifested at different levels. It was shown long ago, using FRAP (fluorescence

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recovery after photobleaching), that components of the submembrane plaque, such as actin, vinculin and α-actinin maintain a dynamic equilibrium between their FA-associated and cytoplasmic pools [19,20]. Moreover, FAs often appear to 'migrate' centripetally, relative to the substratum, due to a polar extension of the adhesion in the direction of the attached stress fibre, and dissociation at the other end [21]. Careful examination of the molecular heterogeneity of adhesion sites in cultured fibroblasts revealed an additional dynamic process, characterized by the formation of a new type of adhesions, termed 'fibrillar adhesions' (FB), where $\alpha5\beta1$ integrin associates with fibronectin fibrils [22]. FBs differ from FAs in their characteristic morphology, consisting of elongated fibrils or array of dots, and their distribution in more central areas under the cells (Figure 1c). As indicated in Figure 3, FBs differ from classical FAs in the primary integrin receptors present in them ($\alpha5\beta1$ compared with $\alpha v\beta3$), as well as in the composition of the submembrane plaque. FAs contain high levels of phosphotyrosine and such proteins as paxillin and vinculin, but display only low levels of tensin. FBs, on the other hand, contain relatively high levels of tensin and little or no phosphotyrosine [22].

Time-lapse video microscopy of cells expressing GFP–tensin revealed a continuous centripetal flow of tensin from FAs to FBs, suggesting that the latter adhesions are derived from FAs. Addition of kinase inhibitors that block actomyosin contractility, such as H-7, ML-7 or Y-76342, inhibit the formation and translocation of these FBs [23]. It was further shown that the assembly of FB depends on the pliability of the ECM, suggesting a role for FB in matrix reorganization [24]. It was also shown that c-Src-null cells fail to form FB, indicating that this tyrosine kinase is involved in this process [25]. The mechanism underlying the effect of tyrosine phosphorylation on FA turnover and FB formation is still unclear.

Discussion and conclusion

In this paper, we have reviewed several temporal and interdependent steps in the adhesion of cells to solid surfaces. We showed that the adhesive interaction is a highly dynamic process, which involves a concerted and highly co-ordinated action of several molecular systems. The most extensively characterized family of matrix adhesions are those that utilize integrin as the primary 'adhesion receptor'. The 'prototypic' form of these adhesions are FAs that are large, robust and relatively stable adhesion sites, consisting of numerous 'structural' and 'signalling' molecules that participate in the assembly of this cytoskeleton-bound adhesive complex, and in its multiple regulatory effects on cell behaviour and fate.

We chose to emphasize here the involvement of a pre-integrin adhesion system, based on the surface recognition and attachment, mediated by a membrane-bound hyaluronan coat. We describe here studies that demonstrate the presence of a thick gel-like layer, surrounding a wide variety of cells (Figure 2). The thickness of this coat can vary from approx. one to several micrometres, depending on the cell type. The pericellular coat consists primarily of hyaluronan, as deduced from its sensitivity to hyaluronidase treatment, and mediates rapid surface attachment (time scale of seconds or less). The need for such a pre-integrin tethering system emerges also from a rough calculation of the probability of integrins to participate in the formation of the very first interactions between a cell and the external ECM. A rough estimate of the fraction of the cell surface occupied by integrin receptors in the proper orientation to bind their ECM ligands, and of the fraction of the external surface occupied by the RGD-binding site, leads to the conclusion that the probability of a successful integrin–RGD encounter, precursor to the formation of stable adhesion, is extremely low. An ideal mechanism for rapid cell tethering should involve molecules that (i) can bind specifically to the ECM, (ii) are highly prominent at the cell surface and (iii) are flexible enough to adapt to the irregular geometry of the matrix. The hyaluronan coat appears to be a good candidate to perform such activity. It has several partner ECM molecules (e.g. chondroitin sulphate, heparan sulphate and aggrecan), and was shown to react in a highly stereospecific manner with well-defined surfaces. The long, linear chains of hyaluronan form a continuous gel-like layer on the membrane, which can efficiently interact with the ECM. The exact organization of hyaluronan in this coat is still unclear, and might vary from one cell type to the other. Thus when applying shear flow to chondrocytes, attached to a glass surface via the hyaluronan coat, they drift, leaving behind hyaluronan tracks, whereas the hyaluronan-dependent attachment of A6 epithelial cells is insensitive to such flow [9]. We propose that this difference may be attributed to the presence of entangled multilayers of hyaluronan on the chondrocytes, in contrast with a single, membrane-bound layer in the epithelial cells. This hypothesis is currently being investigated. It should be emphasized that irrespective of the exact structure of the hyaluronan coat, such a thick, membrane-bound layer probably affects not only adhesive interactions, but also essentially any interaction with external molecules, such as growth- or motility-regulating factors.

The transition from hyaluronan- to integrin-mediated adhesion involves a significant change in the range of membrane-to-substrate distance, from a few micrometres to approx. 15 nm, which is the reported extracellular gap characteristic of FAs. How this transition occurs is still unclear. Is the hyaluronan gel condensed, degraded, reshuffled laterally or endocytosed? These possibilities are currently being explored.

The three forms of integrin-mediated adhesions described in the present paper are molecularly distinct and functionally interdependent structures (Figure 3). FXs are the main precursors of FA, and are formed in the vicinity of the advancing lamella of motile cells. These small adhesions (typical area of approx. 0.25 μm^2) are short-lived (usually just a few minutes) and are stationary relative to the substrate. Their specific contribution to cell motility is not fully understood, although they might be involved in tethering

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the 'rear end' of the actin meshwork at the leading edge of the cell to the solid substrate, thus directing the force produced by the polymerizing filaments to produce primarily a forward protrusion of the lamellipodium. As shown by us and others [14,26,27], the assembly of these small adhesions is a hierarchical molecular process whereby proteins are sequentially recruited to the newly formed FX.

The transformation of FX into FA requires a new input, namely mechanical force, which apparently plays a central role in regulating the dynamic reorganization of integrin-mediated adhesions at large. It was shown that retraction of the leading edge due to internal contractile forces or external perturbation leads to growth of the adhesion site and (for FX) recruitment of zyxin. It is proposed that these matrix adhesions act as 'mechanosensors', which respond to the local application by expanding and recruiting additional components. The mechanism of action of these sensors is not clear yet, but it appears to be activated directly by applying force to the transmembrane protein complex that form the adhesion site, rather than the local activation of stress-responsive channels, since an 'assembly signal' could be produced in detergent-extracted cells by stretching the matrix on which they grow [28]. Attempts to characterize the nature of the mechanosensor implicate different signalling systems in its action. Thus it was demonstrated that the activation of Rho-A is essential for FA growth, and that two of its downstream targets are involved, namely Rho-activated kinase (which triggers actomyosin contractility), and mDia (which is involved in polymerization dynamics of both actin and tubulin) [17]. Additional signalling systems that might be associated with the stimulation of FA assembly involve protein tyrosine phosphatases [29,30].

Finally, mechanical force appears to be also involved in FA turnover and the formation of FB. These adhesions were shown to coincide with fibronectin fibrils and contain relatively high levels of tensin. Time-lapse video microscopy of cells expressing GFP-tensin demonstrated that FB emerge from FA and translocate centripetally. This segregation of the adhesion sites is driven by actomyosin forces, and can be blocked by myosin light-chain kinase or Rho-kinase inhibitors [23]. It appears that contractile forces, when applied to adhesion sites, may have two distinct responses, depending on the physical properties of the matrix. Pulling on an adhesion to a rigid matrix leads to the growth of the adhesion site, whereas pulling on an adhesion to a soft matrix can lead to the translocation of the receptor, along with the attached ECM. The formation of FB can thus be involved in ECM fibrillogenesis.

In conclusion, the adhesion to external surfaces indeed appears to be a multistage process consisting of several temporal stages, starting with hyaluronan-mediated attachment and developing into the different forms of integrin- and cytoskeleton-mediated contacts. The development of these adhesion sites is regulated by Rho-family G-proteins and by mechanical forces, directly applied to the adhesion sites.

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