Membrane and acto-myosin tension promote clustering of adhesion proteins

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Physicists have studied the aggregation of adhesive proteins, giving a central role to the elastic properties of membranes, whereas cell biologists have put the emphasis on the cytoskeleton. However, there is a dramatic lack of experimental studies probing both contributions on cellular systems. Here, we tested both mechanisms on living cells. We compared, for the same cell line, the growth of cadherin-GFP patterns on recombinant cadherin-coated surfaces, with the growth of vinculin-GFP patterns on extracellular matrix protein-coated surfaces by using evanescent wave microscopy. In our setup, cadherins are not linked to actin, whereas vinculins are. This property allows us to compare formation of clusters with proteins linked or not to the cytoskeleton and thus study the role of membrane versus cytoskeleton in protein aggregation. Strikingly, the motifs we obtained on both surfaces share common features: they are both elongated and located at the cell edges. We showed that a local force application can impose cell symmetry breaking in both cases. However, the origin of the force is different as demonstrated by drug treatment (butanedione monoxime) and hypotonic swelling. Cadherins aggregate when membrane tension is increased, whereas vinculins (cytoplasmic proteins of focal contacts) aggregate when acto-myosin stress fibers are pulling. We propose a mechanism by which membrane tension is localized at cell edges, imposing flattening of membrane and enabling aggregation of cadherins by diffusion. In contrast, cytoplasmic proteins of focal contacts aggregate by opening cryptic sites in focal contacts under acto-myosin contractility.

Cell adhesion has been approached theoretically by the study of membrane-mediated protein aggregation (1–3). Entropic fluctuations and elastic properties of membranes were predicted to promote clustering of proteins. Experimentally, the principle of membranes inducing aggregation was tentatively probed on vesicles coated with proteins or beads (4, 5). Although there was no evidence for a contribution of membrane in protein aggregation in cells, the above mechanisms were suggested to be involved in cell adhesion. In contrast, adhesive formation in cells was shown by cell biologists to be intimately connected with actin cytoskeleton organization. The transmembrane protein integrin binds to extracellular matrix (ECM) proteins. This phenomenon leads to the assembly of a molecular complex, named focal contact, which is connected to acto-myosin stress fibers. The transmembrane protein cadherin binds to cadherins on neighboring cells, promoting the formation of adherens junctions, also connected to the actin cytoskeleton (for a review, see refs. 6 and 7). For focal contacts, acto-myosin force was shown to promote their growth. For adherens junctions, actin polymerization was shown to be the driving force for their assembly (8). The possible importance of the implication of the membrane was usually neglected. To take into account both contributions, we designed an experiment on living cells. We studied adhesion of a cell on substrates covered with either ECM proteins or cadherin. To follow the adhesive patterns in both cases, we transfected the same cell line with constructs encoding a C-terminal vinculin GFP fusion for ECM surfaces and a C-terminal cadherin GFP fusion for cadherin surfaces. In our setup, integrin was connected to actin through a molecular complex, but cadherin was not. Because these events occur in the vicinity of the surface, we mounted an evanescent wave microscope that enabled us to image the cadherin surface clusters. In our study, only cadherin–cadherin interactions were observed, without adherens junction assembly. So the cadherin cluster under study is different from a mature adherens junction and should be viewed as a pattern formed by molecules free to diffuse in a membrane interacting with the grafted partners on the surface. Moreover, cadherin patterns seem to behave as glue, escaping cell regulation. This type of aggregation is highly interesting: it behaves as a passive system while being in a real cell and it enables the study of the role of membrane in the aggregation process. For both integrin–ECM and cadherin–cadherin interactions, elongated patterns were formed at the cell periphery along the tension lines. However, we show in this study that the origin of the clustering is different for ECM and cadherin surfaces. The former is caused by acto-myosin contractility, whereas the latter is promoted by an increase in membrane tension.

Materials and Methods

Cell Culture and Protocol for Cell Detachment Before Replating on Cadherin Substrate. We used EAhy cells that form adherens junctions and focal contacts. This line was derived by fusing human umbilical vein endothelial cells with the permanent human cell line A549 (9). Cells were maintained in DMEM (41965-039, GIBCO) supplemented with 10% bovine calf serum (SH30072.03, HyClone). They were transfected by using ExGen 500 (ETI0250, Euromedex, Mundolsheim, France) with constructs encoding a C-terminal vascular endothelial (VE)-cadherin GFP fusion, a truncated C-terminal VE-cadherin GFP fusion (10), and a C-terminal vinculin GFP fusion (11) following the protocol of the manufacturer. For experiments on cadherin surfaces, cells were grown in standard conditions until confluence. EAhy cells were incubated for 1 min with 0.25% trypsin/1 mM EDTA (25200-072, GIBCO) before centrifugation. They were washed once with DMEM with 0.1% trypsin inhibitor (T9003, Sigma) and replated in L15 medium (21083-027, GIBCO) on cadherin surfaces. For experiments with ECM proteins, cells were grown on coverslips with serum and serum-starved for 3 h before the experiment. Incubation with serum leads to a coating of ECM proteins (among which fibronectin and vitronectin). We alternatively grafted fibronectin (F1145, Sigma) on surfaces following the protocols of ref. 12.

Force Application and Drug Treatment. Glass capillaries (GC100F-10, Clark Electromedical Instruments, Pangbourne, U.K.) were pulled with a micropipette puller (caP-97, Sutter Instruments, Novato, CA), yielding micropipettes with 1 nN/m spring constant.

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Abbreviations: VE, vascular endothelial; ECM, extracellular matrix; BDM, butanedione monoxime; RICM, reflection interference contrast microscopy.

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coated with 1
washed three times with distilled water. Finally, coverslips were
Sigma) for 20 min and charged with 100 mM nickel sulfate
were incubated with 1
0.08% glutaraldehyde (G-7526, Sigma) in distilled water for 1 h
bottomed Petri dishes). The coverslips were then incubated with
were mounted on observation chambers (no. 1 coverslip-
heated for 10 min at 100
Sigma) at 30 mM for 30 min and with latrunculin A (L12370,
were incubated with 1
–
2230
Swelling was made by exchanging L15 medium for L15 diluted in
water (between 30% and 70%). The results obtained with drugs
and force application were reproduced on at least four different
cells.

Fixation and Staining. Cells were fixed with 3% paraformaldehyde (P6148, Sigma) in PBS for 20 min, permeabilized with 0.5%
Triton X-100 in the same solution of paraformaldehyde for 3
min, and washed twice for 5 min in PBS. Cells were then
incubated with primary antibodies [anti-β-catenin (C2206, Sigma), anti-p120 (610133, Transduction Laboratories, Lexington,
anti-paxillin (610193, Transduction Laboratories), anti-cadherin (C1821, Sigma), and anti-fibronectin (F3648, Sigma)] for 45 min. They were washed three times for 5 min in
PBS, incubated for 45 min with the appropriate secondary antibody [Cy3 (Jackson ImmunoResearch), 115-165-146 (mouse),
111-166-047 (rabbit); Alexa Fluor 488 (Molecular Probes),
A11045 (mouse), A11046 (rabbit); Alexa Fluor 555 (Molecular Probes), A11029 (mouse), A 11034 (rabbit)] or with fluores-
cently labeled phalloidin (A12379 and A22283, Molecular
Probes) for actin staining. They were finally washed three times
in PBS. The coverslips were then mounted with Mowiol
(32,459-0, Aldrich).

Preparation of Cadherin Surfaces. To guarantee a proper orienta-
tion of the cadherin molecule on the surface, we used a
recombinant cadherin expressed in
Valently on glass surfaces, inspired by Noren
Cadherins could therefore be properly oriented on Ni-
functionalized glass surfaces. The protocol for grafting Ni co-

Results
Adhesive Protein Patterns. On ECM surfaces, we observed normal
focal contacts (Fig. 1 a–d). They were located at the cell
periphery and elongated (≈4 μm long). Stress fiber ends were
colocalized with these patterns (Fig. 1 d). They contained ty-
rosine phosphorylated proteins, vinculin, and paxillin but not
cadherin (Fig. 1a). On cadherin surfaces, the individual cadherin
patterns shared common features with focal contacts on ECM
surfaces: they are elongated structures localized at the cell edges
(compare Fig. 1 c and e for length and location). However,
cadherin patterns were sometimes so close that they could also
completely cover the cell borders. In contrast to focal contacts,
they were not associated with tensed stress fibers (Fig. 1 d and h). Cadherin patterns are stable for at least 24 h. We checked that cellular cadherins were irreversibly bound to the surface cadherins by performing fluorescence recovery after photobleaching experiments: the bleached motif did not reappear within 2 h (see Fig. 7, which is published as supporting information on the PNAS web site). Note that the formation of cadherin patterns is not associated with cell spreading: they do appear only ∼5 h after plating (see Fig. 8, which is published as supporting information on the PNAS web site).

The Role of Actin on Cadherin Surfaces. Actin is not required for the formation of cadherin patterns. Two different experiments led us to this conclusion. First, we used cells expressing a truncated cadherin GFP that was not able to bind actin through catenins. We observed the same type of streak patterns with these truncated cadherins (see Fig. 9, which is published as supporting information on the PNAS web site). Thus actin is not necessary for the appearance of the motifs. But more strikingly, when latrunculin A (a drug that depolymerizes actin) was added, motifs already formed remain unchanged (Fig. 10, which is published as supporting information on the PNAS web site). From these two experiments, we concluded that actin is not involved in the formation of cadherin structures. We also stained for the other proteins normally present in adherens junctions: p120, β-catenin, and α-catenin. They were absent from the patterns, confirming that actin was not connected to cadherins through these complexes.

Real Cadherin–Cadherin Interactions. In evanescent wave microscopy, ∼200-nm thickness is excited above the coverslip surface. So the fluorescent signal can come from cadherins that are not really bound to grafted cadherins. We thus dissolved the membrane by adding 0.1% SDS or 1% Triton X-100. We observed no disappearance of cadherin patterns. We concluded that GFP cadherins were really linked to surface cadherins.

Force. Both vinculin and cadherin patterns share a common feature: symmetry breaking. We thus checked whether an externally applied force could generate these patterns. We applied the force locally at the cell edges centripetally, as in ref. 13. As expected for ECM surfaces, force promoted the growth of focal contacts (Fig. 2 a–e) at the cell periphery. The same kind of protein recruitment was observed on cadherin surfaces (Fig. 2 f–i), but the extension of pipette motion had to be larger. As a consequence, membrane was pulled and the motifs growth could be observed on the cell edges expected to be under tension. The motif dynamics is entirely defined by its area, density, and axis ratio, the contact being viewed as an ellipse. For vinculin motifs, the contacts grow continuously from the cell edge to the inside, the density of the motifs being constant (Fig. 2d). As opposed to focal contact formation, there is no directionality in the growth of cadherin patterns, as their final shape is formed in a few seconds (see area and axis ratio curves, Fig. 2j). The patterns then densify, recruiting new GFP cadherins (see density curve, Fig. 2f).

From this experiment, we conclude that applying a force on cells adhering on a surface with two different ligand-receptor proteins entails a recruitment of adhesive proteins. However, we have shown (see above) that whereas focal contacts were associated with acto-myosin stress fibers, cadherin motifs were not bound to the cytoskeleton. We propose two different mechanisms for force-promoted recruitment upon force application that can be extended to natural conditions. Focal contacts grow through local force application, mimicking acto-myosin action. In contrast, on cadherin surfaces, the application of the force with the pipette provoked an increase in membrane tension and hence cadherin recruitment: cadherin patterns grow because an increase in membrane tension brings the membrane closer to the substrate and allows further interactions between adhesive partners. We checked this hypothesis by acting specifically on acto-myosin contractility and membrane tension.

Acto-Myosin Contractility. When contractility was inhibited by addition of BDM, cadherin contacts remained the same (Fig. 3 d–f). This finding is in sharp contrast with ECM surfaces: after addition of BDM, focal contacts completely disappeared (Fig. 3 a–c). These results show that acto-myosin contractility is necessary for focal contacts but not for cadherin patterns.

Swelling. We increased the membrane tension through a hypotonic swelling (16). We observed the appearance of cadherin motifs (Fig. 4 e–h). This phenomenon was similar to the one observed with the application of the force. We performed the same experiment on ECM surfaces: we did not see any vinculin recruitment, and moreover focal contacts sometimes disappeared (Fig. 4 a–d). This experiment proves that membrane tension is the key parameter for cadherin recruitment.
Membrane Flattening and Cadherin Recruitment by Diffusion. We proposed above a mechanism of cadherin recruitment upon an increase in membrane tension. We view the increase in membrane tension as the means to force the membrane to be in close contact with the cadherin surface. Tension per se is not the motor of aggregation but it leads indirectly to the aggregation. If flattening is the key phenomenon, upon growth of cadherin contacts, two distinct regions should be observed for the membrane-to-surface distance as revealed by RICM (17). In the contact location, the image should be dark; in the vicinity of this region, the overall RICM background should become whiter because of the expected escape of the tensed membrane. This is indeed what we observed when we performed a swelling experiment acquiring GFP VE-cadherin total internal-reflection fluorescence and RICM images (see Fig. 5). To show that diffusion is responsible for recruitment, we give the following estimate. The motif is formed on a typical 100-s time scale. We performed a fluorescence recovery after photobleaching experiment in our system. The extracted cadherin diffusion coefficient as measured with evanescent wave microscopy is 0.005 μm²/s. Such a value is lower than the diffusion coefficient of free cadherin \( D = 0.04 \) μm²/s for Adams et al. (18) and \( D = 0.02 \) μm²/s for Sako et al. (19). This finding is not surprising because cadherins in our system are diffusing in the proximity of the surface. With this value, we can estimate the time required to fill a 2-μm² area by pure diffusion: we obtain the expected 100-s time scale.

### Discussion

The nature of the mechanism that leads to protein aggregation is very different between cadherin and vinculin. The cadherin motifs are irreversibly formed (no drugs were able to remove the contacts) in contrast to vinculin motifs that could be removed by adding BDM, for example. For cadherin, it is a passive response of the cell. We propose that the cell does not have any control on the fate of cadherin, because the adhesive structures are

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**Fig. 3.** Inhibiting contractility using BDM entails disappearance of focal contacts but not of cadherin patterns on a cadherin surface. Contacts revealed by GFP vinculin, before addition of BDM (a) and 30 min after (b) are shown. Contacts revealed by GFP VE-cadherin before addition of BDM (d) and 30 min after (e) are shown. Typical evolution of vinculin (c) and cadherin (f) motif density when BDM is added (time 0).

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**Fig. 4.** Increasing membrane tension through hypotonic swelling entails recruitment of cadherin on cells adhering on a cadherin surface but not recruitment of vinculin on cells adhering on a ECM surface. GFP vinculin images before swelling (a and b) and 50 min after swelling (c). (d and e) Zoomed images of the area depicted by the rectangle in a. GFP VE-cadherin images before swelling (e and f) and 1 h after swelling (g) are shown. (f and g) Zoomed pictures of the area depicted by the rectangle in e. Typical evolution of vinculin (d) and cadherin (h) motif density during hypotonic choc. The arrows indicate the time for change in medium. The percentage refers to the distilled water proportion in the medium.
lacking the proteins implicated in the formation of adherens junctions. In contrast, in the case of vinculin, the cell can regulate the assembly of adhesive structures via a cytoplasmic protein complex. Such a regulation can also exist with cadherins as shown by experiments using beads covered with cadherins (20–23). In this setup, cadherins are not just behaving as glue. Indeed, artificial adherens junctions are assembled around the beads, linking cadherin to the cytoskeleton and activating signaling pathways such as the rac GTPase one.

There could be several explanations for the nonformation of mature adherens junctions in our system. In adherens junctions between two cells, the plane of contact is a symmetry plane. In our system, we have broken this symmetry: cadherins on the surface are not mobile and the surface is infinitely stiff. Moreover, we grafted monomers of VE cad1–4 HIS on the surface, then enabling the recruitment of cadherin by the surface. Dashed lines represent the actin cortex that is connected to the membrane. Arrows indicate the position of the cadherin-coated surfaces. Noren et al. (14) showed in particular that Rac was activated by C-cadherin engagement. In addition, Kovacs et al. (25) plated CHO cells transfected with E-cadherin on Fn-E-cadherin-coated surfaces and showed that cadherin engagement can recruit Arp2/3 to nascent adhesive contacts. Although all of these effects actually show a role for cadherin homophilic interaction in cell signaling, there was no clear evidence that catenins and actin colocalized with cadherin clusters on surfaces. Some immunofluorescence images of clusters were shown. However, no experiment proving that these clusters were really bound to the surface was produced, as we did with the Triton experiment. The signaling response on surfaces thus can not necessarily be compared directly with the signaling pathway linked to real adherens junctions.

Adhesive junctions can be divided into three parts: the ligand–receptor interaction, the cytoplasmic adhesive complex, and the actin cytoskeleton. We compared in this study aggregation of the ligand proteins in the case of cadherin surfaces and aggregation of the cytoplasmic part in the case of ECM surfaces. We have shown that the cytoplasmic proteins of focal contacts aggregation is controlled by acto-myosin contractility. In contrast, cadherin aggregation is controlled by membrane tension. Note that even in the case of focal contacts we do not exclude that integrins are recruited in the same fashion as cadherins. Indeed, Tsuruta et al. (26) have shown that integrin and cytoplasmic proteins of focal contacts (CPFC) do not behave in the same way when actin is depolymerized, leading in particular to acto-myosin contractility inhibition: integrin motifs remained the same, whereas cadherin disappeared. So care should be taken not to extend CPFC assembly properties to integrin.

Integrin-ECM binding is not sufficient for assembly of the three parts of focal contacts mentioned above. Focal contacts act as mechanosensors, i.e., growing when submitted to a force (13, 27, 28). The tension exerted by the cytoskeleton could reveal new binding sites for cytoplasmic proteins of the adhesive complex. As mentioned above, our measured diffusion coefficient is consistent with this scenario. We do not state here that membrane tension induces an attractive interaction between cadherins, but that it only mechanically brings the membrane in close proximity with the surface, then enabling the recruitment of cadherin by diffusion.

Typical lengths of elongated contacts and time scales of their formations should be compared in both systems: (i) in both cases, aggregates appear at the cell periphery; (ii) symmetry is broken; (iii) both have a comparable finite length of $\approx 4 \mu m$; (iv) the density of patterns can be much higher on cadherin surfaces, leading even to continuous cadherin areas (see Fig. 4g); and (v)
the total recruitment time scale is a few hundred of seconds for both contacts. In the case of cadherins, concerning $i$, the cell senses membrane tension at its periphery. The length of the contacts is determined by the screening length of the force $\lambda$, which would explain $iii$. This length can be estimated by taking results obtained for ribbon lying on an elastic layer: the screening length

$$\lambda = \sqrt{\frac{Eh}{\mu_a}}$$

where $E$ is the cytoskeleton Young modulus, $h$ is the cytoskeleton thickness, $h_0$ is the cadherin coating thickness, and $\mu_a$ is the shear modulus of this coating (29). We estimated $\lambda$ by taking $E \approx 100 \mu_0$ (F. Chamaraux and B. Fourcade, personal communication), $h = 200$ nm (N. Morone and A. Kusumi, personal communication), $h_0 = 50$ nm, taking into account the grafting chemistry, and obtained $\lambda \approx 1\mu$m. Note that this estimate should be taken only as good indications for the length, because the $E$ over $\mu_a$ ratio could be smaller. Concerning $ii$, the symmetry breaking is caused by the direction of the force when membrane tension is increased. In the case of ECM surfaces, as stated above, integrin could be recruited with the same mechanism as cadherin, i.e., by membrane tension. However, as shown by experiments with BDM, acto-mysin force is still required for the maturation of focal contacts. The localization at the cell periphery and the symmetry breaking can be explained by the distribution and direction of stress fibers in the cell. Along the same line, the finite length of focal contacts is determined by the acto-mysin contractility (28). The 100-s time scale may be caused by a diffusion limited phenomenon in both cases. However, for vinculin, it could also be related to the time scale corresponding to the opening of cryptic site. An explanation for cadherin pattern high density requires further investigation.

It is worthwhile to ask whether the tension role observed here could be important in the first stages of adherens junctions formation. It has been established that cadherins were recruited as a consequence of actin polymerization (8). Indeed, in our case, we showed that actin was not playing this key role. However, tension could also be involved in adherens junctions formation. The corresponding observation would be as follows: between neighboring cells, cadherins would accumulate at cell edges, for example, because of membrane tension thus provoking symmetry breaking. This was indeed observed in Adams et al. (18) (see their figure 5). Moreover, we propose that in this latter case as actin was connected to cadherins it was responsible for the increase in membrane tension. The actin cytoskeleton in natural adherens junctions would play the role of our pipette in our setup.

From all of these results, one can conclude that forces definitively play a role in cells for protein clustering. We demonstrated that in contrast to focal contacts the force transduction for cadherins was not mediated by the cytoskeleton but by the membrane. Changes in the elastic properties of the membrane are certainly regulated by the cells. It has been shown, for example, that a shear stress applied on a cell, could modify membrane tension (30). It would be worthwhile to characterize these membrane-related pathways to the same extent as for the cytoskeleton.

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