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Regulation of cadherin-based epithelial cell adhesion by endocytosis

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1. ABSTRACT

Regulation of cadherin—based adhesion is an important but poorly studied question. Little is known which mechanisms control the size, number, strength, dynamics, and motility of adherens junctions. Cadherin endocytosis has been suggested to be one such mechanism. Recent studies have revealed several pathways of cadherin endocytosis, each of which appears to have specific functions. These pathways are surprisingly similar to those responsible for endocytosis of growth factor receptors. They could mediate the uptake of cadherin from both the inside and outside of adherens junctions thereby controlling different parameters of cell-cell adhesion structures.

2. HORIZONTALS AND VERTICALS OF CADHERIN ADHESION

Adherens junctions are some of the most prominent cell-cell adhesion structures to be found in any of our cells. They not only interconnect the cells into highly organized tissues, but also serve as platforms for forming other intercellular junctions. Yet adherens junctions are not static structures. Recent progress in live cell imaging shows that adherens junctions are in constant, directional motion (1). This observation suggests that adherens junctions are linked to some kinds of intracellular motors. It also suggests that the adhesive bonds interconnecting the two halves of the junction are strong enough to sustain the

stress induced by this motion, which is unlikely to be completely synchronized in two neighboring cells. Adding even more complexity to this model, numerous FRAP (fluorescence recovery after photobleaching) experiments have shown that individual cadherin molecules continuously move in and out of adherens junctions (2-4). This exchange of cadherin has been traditionally regarded as a lateral (horizontal) diffusion of cadherin molecules (5). Recent progress in understanding cadherin traffic (reviewed recently in 6, 7) suggests, however, that the lateral diffusion might not be a primary mechanism of the continuous remodeling of adherens junctions. Instead, cadherin endocytosis, which emerged as a vertical dimension of cadherin junction dynamics, may play the leading role in this process.

The endocytosis of growth factor receptors has been shown to regulate many parameters of downstream receptor signaling. To fulfill this regulatory function, two types of endocytosis execute the receptor uptake from the cell surface. Constitutive but slow-rate endocytosis, often mediated by AP2-clathrin interactions, regulates the overall amounts of the receptors on the cell surface. Once receptors are activated, they are rapidly internalized by alternative endocytic mechanisms. The latter process, which in many cases is stimulated by receptor monoubiquitination, determines the duration of growth factor signaling. Molecular mechanisms of cadherin endocytosis are not as well studied as those for growth factor receptors. Even less is known about which cadherin pool, engaged or unengaged in adhesion at the moment of endocytosis, is subject to internalization. Recent data, however, shows that cadherin endocytosis and endocytosis of growth factor receptors share many of the same critical molecules. In this review we briefly discuss the emerging mechanisms and functions of cadherin internalization.

3. INTERRELATED ARRAY OF CADHERIN INTERNALIZATION PATHWAYS

Available data show that cadherin constitutive endocytosis is mediated by a clathrin-dependent pathway (8, 9). One of the suggested mechanisms that couple cadherin with the clathrin endocytic machinery is a binding of the adaptor complex AP2 to the dileucine motif located at the juxtamembrane region of E-cadherin. A point mutation of this motif was shown to inhibit cadherin internalization in MDCK cells (10). The authors proposed a simple model suggesting that this motif becomes accessible for AP2 only once E-cadherin dissociates from one of its major intracellular partner p120ctn. Therefore, p120ctn, in agreement with earlier works of other authors (9, 11, 12), regulates the cell surface retention and lifetime of E-cadherin. However, the work of the Stow group did not support the role of dileucine motif in cadherin endocytosis; their biochemical and live-imaging experiments suggested that this motif is required for Rab11-dependent targeting of E-cadherin to the basolateral membrane (13, 14). In either case, however, the dileuicine motif cannot be the sole motif responsible for constitutive cadherin endocytosis. First, VE-cadherin, which like E-cadherin is constitutively endocytosed by clathrin under the regulation of p120ctn, does not posses diluicine motif (9). Second, intracellular regions lacking diluicine motif of another classic cadherin, N-cadherin, were able to mediate an efficient constitutive endocytosis (15). Third, proteasome inhibitors blocked p120ctn-controlled cadherin degradation (11). Since proteasome inhibitors rapidly deplete the intracellular pool of free ubiquitin (16, 17), this observation suggests that cadherin ubiquitination plays an important role for selecting p120ctn-unbound cadherin for the degradation pathway.

Another possible form of constitutive cadherin endocytosis is macropinocytosis. This form of internalization is proposed to be specific for a cadherin pool unengaged in adhesion at the moment of endocytosis (18). It could be responsible for the formation of large intracellular vesicles found in keratinocytes in low calcium media or after disruption of the cadherin junction by a cadherin-specific antibody (19). Available data could not exclude, however, that this form of endocytosis uptakes E-cadherin only upon E-cadherin overexpression or after the application of some specific external stimuli (see below).

Various growth factors, such as TGFbeta (20); EGF (21, 22), HGF (23, 24), FGF (25) were shown to increase the rate of cadherin internalization that in some cells resulted in the complete disintegration of adherens junctions. This rapid event might be a critical cause of the epithelial-mesenchimal transition. And again, different endocytic pathways have been suggested to mediate this regulated endocytosis. For example microinjection of the constitutively active Rac1 mutant destroyed adherens junctions and delivered cadherin (as well as other cell surface receptors, like transferrin receptor) to the large intracellular vesicles (19). It is possible that this effect is caused by intensive macropinocytosis, the activation of which compensates a deficiency of the clathrin-mediated endocytosis that is blocked by Rac1 hyperactivation. In good agreement with this hypothesis, it was recently shown that the activation of EGF receptors dramatically increased cadherin macropinocytosis in MCF-7 cells and that this increase depended on Rac1 activation (26). Alternatively, some authors interpreted the EGF-induced increase in cadherin internalization as activation of the caveolin-dependent endocytosis (21). In favor to this idea, they showed that cadherin associates with caveolin1 in A-431 cells and cholesterol-sequestering reagents, like beta-cyclodextrin or filipin blocked cadherin internalization (21).

Another pathway potentially regulating the level of cadherin on the cell surface is cadherin monoubiquitination and its subsequent internalization via clathrin-dependent endocytosis. Rapid cadherin internalization and dissolution of adherens junctions was shown to accompany the activation of ts-v-Src mutant in MDCK cells resulting in cell-cell contact disruption and in the recruitment of cadherin into recycling endosomes (27-29). Upon Src activation, two tyrosines of E-cadherin (Y755 and Y756) are phosporylated, producing a binding site for E3 ubiquitin-ligase Hakai. After binding, this ligase ubiquitinates E-cadherin-catenin complex that eventually

facilitates cadherin internalization (28) and targeting for lysosomal degradation (30).

Finally, some pool of surface-located cadherin molecules apparently associates with growth factor receptors such as EGF receptor, ErbB-1 (31, 32) and HGF receptor, c-Met (33, 34). E-cadherin apparently directly interacts with both of them via its extracellular domain (32, 35). These interactions are very likely to be critical for targeting c-Met to the basolateral surface of epithelial cells where they co-localize with E-cadherin-catenin complex. Interestingly, endocytosis of these two proteins, c-Met and E-cadherin, was markedly accelerated by the same stimuli such as HGF, low calcium media, and TPA (36). These data suggest that internalizations of these two proteins from the cell surface are somehow coordinated. There are several obvious mechanisms for such coordination. First, if indeed the same mechanisms are responsible for the internalization of activated growth factor receptors and cadherin, then activation of this mechanism by any stimuli (for example by growth factors) inevitably would result in accelerated internalization of both proteins. Another possibility is that both proteins are coendocytosed because of their physical interactions (37). Finally, it was found that in some cells the formation of adherens junctions seems to activate EGF receptors via EGF-independent activation (32, 38 see however 39). Another work suggests the opposite: adherens junctions downregulate ErbB-1 activity (40). Nevertheless, modulation of ErbB-1 activity by cadherin may modulate the rate and mechanisms of ErbB-1 endocytic pathways, which, in turn, by coendocytosis, modulate E-cadherin internalization. Clearly, a lot of work is needed to fully understand the relationships between growth factor receptors and cadherin endocytosis.

Altogether the data described above emphasize the robustness of the cadherin internalization process, which can be controlled by multiple independent mechanisms. Importantly, cadherin endocytic mechanisms and their regulation are, at least in general, similar to those for growth factor receptors.

4. ENDOCYTIC DISRUPTION OF ADHERENS JUNCTIONS IS ONE OF POSSIBLE MECHANISMS FOR JUNCTION DYNAMICS

Cadherin uptake from the cell surface may influence cadherin adhesion by two different nonexclusive mechanisms: endocytosis from outside and inside the adherens junctions. By the first mechanism, endocytosis would uptake cadherin molecules unengaged in adhesion and, in theory, could control the level of free cadherin on the cell surface. Indeed macropinocitosis of free cadherin was demonstrated in the experiments with preconfluent epithelial cells (8, 18). Free cadherin was also proposed to undergo endocytosis through a clathrin-dependent pathway (41, 42). Furthermore the latter two works envisioned a complex Rac/Cdc42/nectin-dependent signaling pathway inhibiting cadherin endocytosis once it is recruited into nascent adherens junctions. By this mechanism, cadherin engaged in adhesion becomes inaccessible to endocytic machinery. Nevertheless, some processes should mediate an efficient and apparently regulated removal of cadherin from cell-cell contacts. The lack of such mechanism(s) would obviously result in unlimited expansion of adherens junctions. Below we summarize the data indicating that one such mechanism is cadherin endocytosis.

The clearest evidence for internalization of cadherin from the junctions have been obtained using a calcium-switch model. The calcium-dependency of cadherin-based adhesion is a very well characterized phenomenon. The cadherin ectodomain, in the absence of calcium ions, immediately changes its conformation; it becomes accessible for trypsin digestion and cysteine-specific biotinylation (43, 44). Junctional proteins, including cadherin, are then rapidly internalized (45). The detailed molecular mechanisms underlining this remarkable and long-known effect are not completely understood. The critical and unresolved question is whether cadherin first loose their adhesive bonds and then are internalized or, vice versa, they are first internalized, forcefully breaking apart cadherin adhesive bonds. The first possibility is widely accepted and is based on the analysis of cadherin-cadherin interactions in different *in vitro* assays (reviewed in 44, 46).

However, a number of experimental data performed on both cellular and molecular levels indicate that the mechanism of cell-cell contact disintegration in low calcium media is far more complex. The possibility that cadherin adhesion bonds themselves are calcium-independent has been clearly suggested by crystallography works, which showed that cadherin adhesion interface does not include calcium-binding sites (47). The maintenance of cadherin adhesion bonds in low calcium has been further supported by direct biochemical experiments. We showed that cadherin adhesive dimers, which can be extracted from cells by nonionic detergents, are surprisingly stable in low calcium. The same dimers immediately dissociate, however, upon calcium depletion in living cells (48). Notably, this dissociation of cadherin adhesive dimers by calcium deficiency in living cells can be blocked by many maneuvers that inactivate cadherin endocytosis, such as hypertonic media, low temperature or ATP depletion (49). Thus, in living cells as well as solution, cadherin adhesive dimers are stable in low calcium. Their dissociation *in vivo* requires some active processes; one candidate for this is endocytosis. This conclusion, however, is in sharp disagreement with *in vitro* binding experiments, which demonstrate that cadherin-cadherin interactions are calcium-dependent. Detailed comparison of cadherin dimer formation on the cell surface and on the surface of agarose beads provided a clue for understanding this discrepancy (50): cadherin dimers, which formed at these two conditions, are structurally different. Cadherin dimers formed on the agarose surface immediately dissociate at calcium concentrations below 100 mkM threshold. By contrast, on the cell surface calcium was needed only for adhesive dimer production but not for their maintenance. We proposed that cells have a special mechanism, lacking *in vitro*, which mediates the assembly of such calcium-independent cadherin adhesive dimers. Taken together, biochemical and molecular examinations of cadherin-cadherin interactions suggest that just the depletion of

calcium ions is not sufficient to dissolute adherens junctions.

The complex cellular mechanisms responsible for cell-cell dissociation in low calcium are also evident from experiments with living cells. It was shown that several compounds, very different on first glance, could protect cell-cell contacts from degradation by low calcium. These compounds include relatively unspecific protein kinase inhibitors, such as H7 (51) or staurosporine (52), casein kinase 1 inhibitor IC261 (53), the microtubule-specific drug nocodazole (54, 55), and agents that block actin-myosin contractility (51, 56). Importantly, these drugs did not protect cadherin from low calcium-induced changes in the cadherin ectodomain conformation (51, 52). Furthermore these drugs are cell-type specific. For example, only keratinocytes but not other cells tested acquired nocodazole-induced calcium-independent adhesion in Kee and Stainert (54) experiments. The fact that the processes underlying calcium-independent cadherin adhesion are redundant underlines the complexity of the entire process. Several possibilities have been suggested. Actin filament contractility, activated by low calcium, was suspected by Citi *et al* (51) and Ivanov *et al* (56). However, it is not clear what triggers such highly organized actin-based contractility upon calcium depletion. Some evidence points to cadherin endocytosis. Ivanov *et al.* (57) showed that different drugs that inactivate, while not very specifically, clathrin-dependent endocytosis markedly stabilized cadherin adhesion in low calcium. Interestingly, the stabilizing effect of kinase inhibitors on cell-cell contacts in low calcium also could be based on the inhibition of endocytosis. Some protein kinase C (PKC) isozymes apparently are required for cadherin internalizations (58). Therefore the kinase inhibitor H7 potentially could block cell-cell dissociation in low calcium by inhibiting PKC and subsequent downregulation of cadherin internalization. Inactivation of cadherin endocytosis by staurosporine, which also inhibits PKC, was experimentally demonstrated (52). Finally, nocodazole may also inactivate cell-cell contact dissociation through the inhibition of endocytosis; it was shown that this agent inhibits SV40, Listeria and some other pathogen entry into cells (59, 60). Notably, nocodazole induced the protection effect independently to RhoA activation and stress fiber formation (61). Altogether, the stability of adherens junctions at low calcium at conditions that inactivate endocytosis supports the data obtained in the biochemical and structural works described above: some active processes are needed for cadherin junction disintegration, and one such process is the endocytic uptake of cadherin molecules directly from the adherens junctions.

Does cadherin uptake from adherens junctions occur at the standard calcium concentration? Clear indications that this is the case have been found in our recent work (49). Using immunoelectron microscopy we showed the presence of endocytic invaginations consuming E-cadherin directly from cell-cell contacts, including from well-organized adherens junctions in confluent cultures of A-431 and HaCat cells. Furthermore, two different approaches, hypertonic shock and ATP depletion, inhibiting practically all forms of endocytosis, resulted in the rapid accumulation of adhesive dimers in cells and led to a profound increase in cadherin cell-cell contact staining. These data suggest that cadherin endocytosis may be essential to counterbalance a continuous assembly of cadherin in adherens junctions under physiological calcium concentration. Importantly, cadherin endocytosis activated by growth factors or v-Src during epithelial to mesenhimal transition may also proceed within adherens junctions. At least Warren and Nelson in 1987 reported an appearance of numerous clathrin-coated pits in the region of adherens junction in the v-Src-transformed MDCK cells (27).

In summary, recent studies have revealed several forms of cadherin endocytosis, each of which has specific functions and mechanisms. First, cadherin is constitutively endocytosed and recycles back to the plasma membrane. This form of endocytosis is perhaps similar to the constitutive endocytosis of growth factor receptors and is mediated through a classic clathrin-mediated pathway or macropinocytosis. Whether these processes distinguish free from junctional cadherin is not clear, but apparently they both regulate the total level of cadherin on the cell surface. Deficiency of p120ctn in the cadherin-catenin complex might target cadherin to another endocytic pathway, which eventually leads to cadherin degradation in lysosomes. In different cell types or along tumor progression different forms of cadherin endocytosis may be dominant. Second, by specific internalization of the junctional cadherin, cells may regulate the strength and dynamics of existing contacts. This type of endocytosis may be analogues to the internalization of the growth factor receptors upon their activation. The similarity in mechanisms of these two processes may explain why the stimulation of EGF or HGF receptors activates cadherin endocytosis. The interrelation of these two processes might be very important during carcinogenesis. Further detailed studies of cadherin endocytosis on molecular levels are needed to fully assess the contribution of cadherin endocytosis in cell-cell junction remodeling.

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