Transmembrane cooperative linkage in cellular adhesion

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Various dynamic cellular activities require precise regulation of extracellular adhesion. Here we propose a simple thermodynamic model that does not depend on affinity regulation of transmembrane adhesion molecules but, rather, is based on the principles of collision-limited reactions. We show that the number of transmembrane adhesion molecules forming extracellular bonds depends on the degree of cytoskeletal damping of their lateral mobility (translational entropy) within the plane of the plasma membrane. This type of transmembrane cooperativity between cytoskeletal linkage and the number of extracellular bonds does not require high affinities to the cytoskeleton (micromolar range) and will be particularly effective at low extracellular affinities of adhesion molecules (millimolar range).

Introduction

Cellular differentiation and overall tissue organization require that cells undergo physical contacts with the surrounding extracellular matrix (cell-to-substrate adhesion) or with neighboring cells (cell-to-cell adhesion). Cell adhesion is mediated by a variety of different monomeric or dimeric membrane-bound cell adhesion molecules. On the cytoplasmic side most adhesion molecules, such as classical cadherins, integrins, dystroglycan, CD44 or neuronal adhesion molecule (NCAM) are connected via a chain of adaptor molecules to actin filaments, intermediate filaments or to the spectrin meshwork. This cytoskeletal anchorage appears to be important for strengthening of adhesion (Hynes and Zhao, 2000; Gumbiner, 2000; Hynes, 1999; Giancotti, 1997).

Adhesive contacts must be able to assemble and dissociate to allow dynamic cellular behavior to occur such as cell movement and remodeling of cellular shape. These processes are critically involved in important physiological and pathological aspects such as wound healing, tumor metastasis or paracellular transport of macromolecules and cells through endothelial or epithelial barriers. Formation and dissociation of adhesive bonds implies mechanisms that are able to modulate the adhesive strength of adhesion molecules to their extracellular receptors. It is presently not well understood how such an inside-out regulation of adhesion might occur.

A currently favored model for regulation of adhesion that does not require conformational changes of the adhesion molecules implies cytoskeleton-mediated assembly and disassembly of clusters of adhesion molecules (Kusumi et al., 1999; Gumbiner, 2000). It is assumed that clustering increases and disassembly of clusters decreases adhesive strength assuming that a local high density of bonds will confer higher adhesive strength than the same number of bonds dispersed over a greater cell surface area. Different aspects of the association of the cytoskeleton to adhesion molecules and receptors have been treated mathematically (Brandts and Jacobson, 1983; Lauffenburger and Linderman, 1993; Orsello et al., 2001). These aspects include, prevention of mechanical extraction of adhesion molecules, control of membrane flexibility, clustering-mediated improvement of multivalent extracellular interactions and shaping of contact areas.

In the present study we have addressed a thermodynamic aspect that has not been adequately been dealt with in previous studies and which appears to be of particular importance in view of the surprisingly low binding affinity of cadherin-type adhesion molecules determined previously (Baumgartner et al., 2000a/b). The model we wish to propose implies a transmembrane cooperative linkage mechanism by which the number of adhesion molecules (per unit area of membrane surface) forming extracellular bonds and hence the overall cellular adhesive strength can be significantly modulated by the degree of cytoskeletal linkage of their cytoplasmic domains. In this model the strength of cellular adhesion is controlled by the lateral mobility of adhesion molecules in the plane of the lipid bilayer, or, in other words by their two-dimensional transla-

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tional entropy. The only property of adhesion molecules, essential for this thermodynamic process is their ability to undergo simultaneous physical attachment to ligands at both sides of the plasma membrane (i.e. at the inner side to the cytoskeleton and at the outer side to extracellular ligands) that is strong enough to effectively reduce the translational entropy of the adhesion molecules. Transmembrane cooperativity defined in this way is physical in nature and does not require transmembrane conformational changes or ligand-induced oligomerization that, if occurring, would further modify this process.

Theory

Kinetic background

Collision-limited reaction. Adhesion molecules studied in the present approach are assumed to be capable of diffusing freely in the plane of the plasma membrane and to form non-covalent bonds with either ligands of the extracellular matrix (cell-to-substrate contact) or with other adhesion molecules or ligands present in the plasma membrane of neighboring cells (cell-to-cell contact). Extracellular matrix molecules serving as cellular attachment sites are considered to be non-diffusible such as the matrix molecules adsorbed to the surface of a culture dish. On their intracellular side, these adhesion molecules are assumed to be able to form non-covalent bonds to the membrane-associated cytoskeleton which is assumed to be non diffusible (for situations in which binding of cytoskeletal components underlies three-dimensional diffusion see Results).

Bond formation to either extracellular ligands or to the cytoskeleton can be described as a collision-limited reaction that must be conceptually separated in two steps (Bell, 1978). In the first step two molecules X and Y (both or only one of which is assumed to be freely diffusible) encounter each other, i.e. they approach each other by diffusion at a specific distance \( r_{XY} \) (encounter radius) to form an encounter complex \( XY \). In the second reaction step, molecules X and Y undergo non-covalent binding to form the complex \( B_{XY} \). The total reaction of reactants X and Y can be written as

\[
X + Y \rightleftharpoons XY \rightleftharpoons B_{XY} \tag{1}
\]

The equilibrium constant \( K \) is the ratio of bonds and encounter complexes. \( K_{XY} \) is the equilibrium constant for the encounter step that depends on the encounter radius \( r_{XY} \) of the interacting molecules (critical distance between two molecules required for binding) and can be calculated in membranes using Fick's first law of diffusion to be

\[
K_{XY} = \pi \cdot r_{XY}^2 \cdot N_A \tag{2}
\]

with \( N_A \) denoting Avogadro's number. Assuming \( r_{XY} \) to be 1 nm this yields \( K_{XY} \approx 2 \cdot 10^6 \) mol\(^{-1}\)m\(^2\). Eq. (2) is still valid if only one of the reactants is freely diffusible whereas the other reactant remains in an immobile non-diffusible position. Because the encounter complex \( XY \) is a short living transient state that is quantitatively negligible in comparison to the bound \( B_{XY} \) and unbound \( XY \) reactants, the reactions can be simplified to

\[
X + Y \rightleftharpoons B_{XY} \tag{3}
\]

Life times of bonds in the presence of external forces. In the presence of an external force \( f \) (force per unit area), the corresponding equilibrium constant \( K(f) \) underlying the formation of an adhesive bond, e.g. between an adhesion molecule and its extracellular ligand can be described according to Bell (1978) to be

\[
K(f) = K(0) \cdot \exp \left[ -\frac{\gamma f}{k_B T N_A} \right] \tag{4}
\]

Here \( K(0) \) denotes the equilibrium constant for the bond formation in the absence of an external force; \( \gamma \) is the specific length of the bond, \( k_B \) is the Boltzmann's constant, \( T \) is the absolute temperature and \( N_A \) is the number of adhesion molecules per unit area cell surface forming extracellular bonds (corresponds to \( B_{XY} \) in Eq. 1, i.e. the area concentration).

Model systems

Based on these two theoretical assumptions, it is possible to calculate \( N_A \) in the presence and absence of intracellular bonds to the cytoskeleton assuming the simplest case that one adhesion molecule has a single extracellular binding site for a ligand and a single intracellular binding site for the cytoskeleton.

Cell-to-substrate adhesion. The first model (Fig. 1a) illustrates different steps of collision-limited reactions underlying the formation of a transmembrane ternary complex consisting of one adhesion molecule bound extracellularly to an immobile substrate binding site (S) and connected with its intracellular portion to a binding site on a preformed cytoskeleton.

The corresponding kinetic scheme for a collision-limited reaction (Fig. 1b) is based on the assumption that the distance between the area of the plasma membrane interacting with the substrate is close enough and remains in this state long enough to allow binding of an adhesion molecule to the substrate, and, at the same time, to establish intracellular bonds to binding sites of the cytoskeleton. Many of these binding sites are assumed to be opposite to the substrate binding site to allow simultaneous intracellular and extracellular binding.

At step 3 of Figs. 1a and b an adhesion molecule \( A \) is freely diffusible in the plane of the plasma membrane and will reach, controlled by the equilibrium constant \( K_{AS} \), a cytoskeleton binding site (step 4) to which it binds driven by the equilibrium constant \( K_{f} \). At step 6 the cytoskeleton-bound adhesion molecule \( B_{AC} \) formed at step 4 has undergone extracellular binding (controlled by the force-dependent equilibrium constant \( K_{f}(f) \)) to form an intra- and extracellularly bound ternary complex \( (B_{ASC}) \). On the left side (step 2) the adhesion molecule has diffused to and bound to the cytoskeleton with no matching extracellular binding site (\( B_{AS} \)). Likewise, at step 1 the substrate binding site does not match with an intracellular cytoskeleton binding site (\( B_{AC} \)). Steps 1 and 2 were included in the kinetic scheme in order to allow calculation of situations in which a fraction of cytoskeleton binding sites is modified (blocked or made available) by signalling pathways affecting the stage of polymerization and/or phosphorylation of components of the cytoskeleton (Angst et al., 2001), or, in which extracellular binding sites become masked or unmasked (by anti-adhesive molecules or proteases) (Blobel, 2000; Murphy and Gavrilovic, 1999). The total number of adhesion molecules transmitting adhesive force per unit area of cell surface \( (N_A) \) at various values of \( K_{f}(0) \) \( (K_f \) at zero force) and \( K_f \) can be calculated as follows:

Calculation of this model is based on an equilibrium approximation of the effect of steady force. This requires the time constants for assembly and disassembly of the cytoskeleton itself to be at least one or two orders of magnitude greater than the time constants for association and dissociation of the adhesion molecules with the cytoskeleton and the substrate, respectively. The steady state equations including the mass conservation can be solved numerically by using the method of fix-point iteration. However, an analytical approach is possible if the situation is considered in which all substrate binding sites match with cytoskeleton binding sites and vice versa (\( S^* \) and \( C^* \) are 0) i.e. each bound adhesion molecule should be capable of becoming linked to the cytoskeleton. In this case we can obtain the analytical relationship between external force \( f \) and the number of free adhesion molecules \( A \). Denoting the total concentration (bound and unbound) of adhesion molecules \( A_{tot} \) we obtain the relation of \( A \) and \( f \) to be
substrate binding sites, PM plasma membrane, A adhesion molecules, lower parts of (respectively. A more detailed view on the contact area is shown in the lower parts of (a) and (c). C denotes cytoskeleton binding sites, S substrate binding sites, PM plasma membrane, A adhesion molecules, CStot the total concentration of matching substrate- and cytoskeleton binding sites (see Appendix). The rather unconventional description f(A) (force as a function of concentration of bonds) is chosen for mathematical simplicity. Typical examples will be shown below.

By using the additional assumption that the number of substrate binding sites is significantly higher (e.g. one order of magnitude) than the number of adhesion molecules (as one would expect from a culture dish coated with adhesive proteins or peptides), one can obtain an analytical solution for the number of adhesive bonds per unit area \( N_0 \) in dependence on the force \( f \). Under these assumptions the concentration of free substrate and cytoskeleton binding sites \( CS \) is approximately equal to the total concentration of substrate binding sites leading to

\[
f = -\frac{k_BT}{\gamma} \cdot \{ A_{\text{ro}} - A \cdot \{ 1 - K_a \cdot K_{af} \cdot (A_{\text{ro}} - CS_{\text{ro}} - A) \} \}.
\]

with \( CS_{\text{ro}} \) denoting the total concentration of matching substrate- and cytoskeleton binding sites (see Appendix). The rather unconventional description \( f(A) \) (force as a function of concentration of bonds) is chosen for mathematical simplicity. Typical examples will be shown below.

Another interesting parameter is the detachment force, i.e. the force causing complete detachment of the cells from the substrate. Mathematically, the detachment force \( f_d \) is defined as the force where the slope of \( N_0(f) \) approaches \( -\infty \). This can be calculated to be

\[
f_d = \frac{A_{\text{ro}} \cdot k_BT}{\gamma} \cdot W\left[ \frac{K_{af} \cdot CS \cdot K_a(0)(1 + K_a)}{e^{-1} \cdot (1 + K_{af} \cdot CS \cdot K_a)} \right]
\]

with \( W(x) \) denoting Lamberts W-function which satisfies the relation \( W(x) \cdot \exp(W(x)) = x \) (Corless et al. 1996). For zero force this yields

\[
N_0(f = 0) = \frac{A_{\text{ro}} \cdot K_{af} \cdot K_a(0) \cdot CS \cdot (1 + K_a)}{1 + K_{af} \cdot CS \cdot K_a(0)(1 + K_a)}
\]

and \( B_{XY} \) a bond between component \( X \) and \( Y \). S' and C' denote substrate or cytoskeleton binding sites lacking corresponding opposing binding sites in either the cytoskeleton or substrate, respectively. \( K_{af} \) : equilibrium constant for the diffusion step; \( K_a \) : external binding constant of adhesion molecules; \( K_c \) : binding constant of adhesion molecules to the cytoskeleton.

Cell-to-cell adhesion. In the second model (Fig. 1c) two cells adhere to each other on their extracellular side by adhesion molecules, both of which are freely diffusible in the plane of the interacting plasma membranes. In this case the interacting adhesion molecules (A) are assumed to be identical monovalently binding molecules (homotypic binding). However, this model also applies for heterotypic binding if the
two interacting molecules are different molecules but possess a single binding site for each other and if the affinities to the cytoskeleton are the same. As outlined above for substrate binding (Fig. 1a), the kinetic scheme for cell-to-cell adhesion underlies the same rationale. The bound adhesive (trans-interacting) complex between two adhesion molecules (AA) is considered diffusible unless cytoskeletal linkage of at least one of the interacting adhesion molecules occurs.

The steady state solutions for the resulting kinetic model (Fig. 1d) can be solved numerically only. However, by analyzing the two extreme cases of no cytoskeleton binding and complete cytoskeleton binding it is possible to obtain an estimate of the maximum amplification of the extracellular binding strength. In the first case only the situation is considered in which no binding of the adhesion molecule to the cytoskeleton can occur. Solving the law of mass conservation in a similar way as outlined above for cell-to-substrate adhesion the following relation is obtained:

\[ f = -k_BT \cdot \ln \left( \frac{A_{tot} - A}{A^2 K_c(0) K_{dif}} \right) \cdot (A_{tot} - A) \]  

The detachment force \( f_d \) can be determined by finding the value for \( f \) where \( \partial f / \partial A = 0 \) yielding

\[ f_d(K_c = 0) = -k_BT \cdot \ln \left( \frac{A_{tot}[B(g) + 1]}{B(g) - 1} \cdot \frac{1}{g} \right) \]  

with \( g = 4 A_{tot} K_c(0) K_{dif} \) and \( B(g) \) denoting the function that fulfills \( g \cdot \exp[B(g)] - B(g)^2 + 1 = 0 \). The introduction of this transcendental function is necessary because the denominator of the logarithm in Eq. 11 contains an \( A^2 \)-term. However, if \( g \leq 1/4 \) we can apply a series approximation of \( B(g) \) yielding \( f_d \) to be

\[ f_d(K_c = 0) = -k_BT \cdot \ln \left( \frac{A_{tot}}{A_{tot} - A} \right) \cdot \frac{A_{tot} \cdot A}{g \cdot (3 + g)} \]  

In the second extreme case in which all adhesion molecules are linked to the cytoskeleton, a direct solution of Eq. 4 is possible

\[ f = -k_BT \cdot \frac{N_c}{(A_{tot} - A) \cdot K_{dif}} \cdot N_c \]  

where \( A_{tot} \) denotes the total concentration of adhesion molecules having access to an adhesion molecule on the neighboring cell. From this relation the detachment force can be calculated to be

\[ f_d(K_c = \infty) = \frac{k_BT}{\gamma} \cdot \frac{A_{tot} \cdot W[K_c(0) \cdot \text{e}^{-1}]}{A_{tot}} \]  

Similar to the cell-to-substrate scenario we can define the ratio

\[ \text{mc} := \frac{f_d(K_c = \infty)}{f_d(K_c = 0)} \]  

as a measure for the maximal possible transmembrane cooperativity (gain in adhesive strength by cytoskeletal linkage).

**Results**

In a first attempt we wanted to determine the influence of cytoskeletal linkage on the number of substrates-bound adhesion molecule \( N_c \) per unit area of the cell surface contacting the substrate. The two-dimensional concentration of adhesion molecules was assumed to be \( 10^9 \) mol/m², which corresponds to \( 10^5 \) adhesion molecules/\( \mu \text{m}^2 \) membrane area (equivalent to \( 10^6 \) adhesion molecules on an average cell surface of \( 1000 \mu \text{m}^2 \)). This value is in the range of the concentration of integrins in the plasma membrane of activated platelets (5 – 8 · 10⁴ molecules per activated platelet with a total surface of 50 \( \mu \text{m}^2 \) (Holmsen, 1990; Eigenthaler and Shattil, 1996)). Moreover, the realistic assumption was made that the concentration of substrate binding sites (e.g. on a protein-coated culture dish) is significantly higher (order of magnitude) than the concentration of adhesion molecules in the plasma membrane of the cells spreading on the substrate (see remarks preceding Eq. 1). For each substrate binding site, one matching binding site should be available on the cytoskeleton, i.e. each bound adhesion molecule should be able to associate with the cytoskeleton. The equilibrium constant of the substrate binding step \( K_c(0) \) of the encountered molecules at zero force was assumed in this case to be \( K_c(0) = 10^5 \), which means that the ratio between bound complexes \( C_{xy} \) and unbound encounter complexes \( (XY) \) is 99:1.

In these models we assumed the encounter reaction between adhesion molecules and the cytoskeleton to underly two-dimensional diffusion (preassembled subplasmalemmal cytoskeleton) for mathematical simplicity. The general outcome of this model remains basically the same, if three-dimensionally diffusing components of the cytoskeleton (e.g. adaptor proteins), were taken into account by numerical simulations (data not shown).

Figure 2 shows that cytoskeletal linkage causes a considerable increase in external bonds \( N_e \) at zero external (distracting) force. If no binding to the cytoskeleton occurs \( K_c = 0 \), only 20% of adhesion molecules will be in a substrate-bound state to transmit adhesive force. If, however, affinity to the cytoskeleton is in the range of the substrate affinity \( K_c = 10^2 \), already 85%...
of adhesion molecules will be kept in a substrate-bound (adhesive) state. Further increase of cytoskeletal affinity has only relative little impact on $N_0$ (about 100% bound to the substrate at $K_c = 10^6$). However, this rather small (15%) difference of $N_0$ between $K_c = 10^2$ and $K_c = 10^6$ has a strong non-linear impact on the detachment force which increases three-fold at the same time, reaching 75 Pa at $K_c = 10^6$.

The functional significance of cytoskeletal binding on $N_0$ and, thus, on the detachment force is shown in Fig. 3 using Eq. 8. This diagram shows that $K_c$ values of $10^2 - 10^4$ ($K_D$ values of cytoskeleton-to-membrane associations are generally $\leq 10^{-5}$ M; (Kreis and Vale, 1999a, b) are already sufficient to exert the maximal possible increase in detachment force. Significant gain in adhesive strength (i.e. transmembrane cooperativity) by cytoskeletal immobilization (Fig. 4) will be only possible at rather low substrate binding affinities, i.e. at $K_a(0) \leq 10^4$ in order to increase binding strength above one order of magnitude. Moreover, if the two-dimensional concentration of adhesion molecules is assumed to be one order of magnitude lower ($10^{-10}$ mol/m$^2$ = 100 molecules/µm$^2$) than considered in the calculations shown in Figs. 2–4, even an increase in $K_a(0)$ to $10^6$ would benefit from cytoskeletal binding by increasing binding strength above tenfold (data not shown). On the other hand, trapping of adhesion molecules at small junctional areas may result in local concentration above 1000 molecules/µm$^2$ ($10^{-8}$ mol/m$^2$), which is already sufficient to exert the maximal possible increase in detachment force.

For simplicity, calculation of transmembrane cooperativity was based on the assumption that interaction between adhesion molecules and the cytoskeleton is not affected by force. However, if we assume these cytoskeletal bonds to underlie a similar decay of affinity during force action as considered for the external adhesive bonds (Bell, 1978), transmembrane cooperativity can only be solved numerically. As one could expect, dependency of transmembrane cooperativity on force-exposed $K_c$ still remains qualitatively largely unchanged but is shifted towards $K_a$ values of about one order of magnitude higher (data not shown).

Figure 5 illustrates the results obtained for transmembrane cooperativity of adhesion molecules mediating cell-to-cell adhesion (Eq. 14). The gain in detachment resistance by maximal cytoplasmic immobilization compared to no binding to the cytoskeleton is most pronounced at low external binding constant, but even at $K_a(0) = 10^2$ cooperativity is still close to tenfold and, hence, slightly above the gain calculated for substrate adhesion at $K_a(0) = 10^4$ (Fig. 4). At an assumed two dimensional concentration of $10^{-10}$ mol/m$^2$ (instead of $10^{-8}$ mol/m$^2$ used in Fig. 5) even a tenfold higher equilibrium concentration of 100 molecules/µm$^2$, $K_a(0)$ would be required for significant transmembrane cooperativity (data not shown).
such an inhibitory signalling pathway. The resulting detachment forces were calculated in dependence on the cytoskeletal affinity ($K_C$) of the remaining (unblocked) adhesion molecules. Adhesion molecules on the interacting neighboring cell were assumed to be maximally bound to the cytoskeleton ($r_{AA} = 1\mu m$; $K_{AA} = K_{AA} = 10^{-9} mol/m^2$; $K_A(0) = 10^3$).

A large variety of adhesion molecules has been shown to associate with their cytoplasmic domains to adaptor molecules that link them to the actin-, spectrin- or intermediate filament-based cytoskeleton (Hynes and Zhao, 2000; Gumbiner, 2000; Hynes, 1999; Giancotti, 1997; Kreis and Vale, 1999a, b). One functional aspect of such linkage is to transmit external forces acting on the cell directly to the cytoskeletal framework thereby preventing the plasma membrane from mechanical injury. This mechanical aspect is obvious from blistering diseases of the skin, caused by genetic defects of intermediate filaments and associated junctional adaptor molecules (Nievers et al., 1999; McGrath, 1999; Fuchs and Cleveland, 1998). Moreover, linkage of the cytoskeleton to adhesion contacts allows the generation of contractile force against the cellular environment, which is an important requirement for cellular migration (Galbraith and Sheetz, 1998).

In the present study we have addressed another important aspect of cytoskeletal linkage of adhesion molecules, namely the possible role of the cytoskeleton in regulating cellular adhesion by a transmembrane cooperative linkage mechanism. Rapid changes in adhesion, i.e. formation and dissociation of adhesive contacts, are necessary for dynamic cellular behavior to occur, such as cell migration, tissue repair, and morphogenesis. These dynamic events require mechanisms by which cells can quickly control the number and position of extracellular adhesive bonds. Except for some members of the integrin family that can change their external affinities by an inside-out signalling mechanism (assumed to be created by changes in the mode of cis-interaction between $\alpha$- and $\beta$-chains (Eigenthaler and Shattil, 1996; Hughes and Pfaff, 1998; Bazzoni and Hemler, 1998), all other adhesion molecules have so far not been shown to undergo transmembrane conformational regulation of their affinity (Chothia and Jones, 1997). Therefore, regulation of adhesive affinity of most adhesion molecules must occur by some kind of transmembrane cooperativity. The basic question underlying the present study was to find out whether cytoskeleton-mediated quenching or damping of the translational entropy (lateral mobility) of adhesion molecules would be sufficient to allow regulation of adhesion and, in particular, whether such a regulation could occur at reasonable levels of affinity.

The main outcome of the present study is that an efficient regulation of the number of extracellular bonds (by a factor of $10^0$) at two-dimensional concentrations of adhesion molecules of $10^2$ – $10^3$ molecules/$\mu m^2$ will only occur at rather low external affinities of adhesion molecules ($K_C(0) \approx 10^{-2}$ – $10^{-4}$). As a matter of fact, recent studies on the affinity constants of two different adhesion molecules namely VE-cadherin (Baumgartner et al., 2000a, b) and CD2/CD48 (Pierr et al., 1996) indicate low-affinity binding in the millimolar range. These low-affinity adhesion molecules, therefore, would be ideal candidates for regulation of the number of their extracellular bonds by cytoskeletal cooperativity.

However even adhesion molecules with higher external affinities (micromolar range) that would not significantly benefit from cytoskeletal immobilization according to our model, should be taken into account for some degree of transmembrane regulation by cytoskeletal cooperativity. This is because the plasma membrane is a flexible oscillating layer that does not provide precise (optimal) distances for maximal interaction between adhesive molecules and their extracellular ligands (as assumed in the idealized models treated in this study). Steady changes of the distance between the interacting surfaces will shift the average two-dimensional affinity of adhesion molecules to lower values than those measured under optimized conditions in vitro. In this context, the cytoskeleton associated with adhesion molecules may serve to reduce (damp) membrane oscillations thereby improving the probability of rebinding after dissociation of bonds thus increasing the two-dimensional affinity of adhesion molecules.

### Discussion

A large variety of adhesion molecules has been shown to associate with their cytoplasmic domains to adaptor molecules that link them to the actin-, spectrin- or intermediate filament-based cytoskeleton (Hynes and Zhao, 2000; Gumbiner, 2000; Hynes, 1999; Giancotti, 1997; Kreis and Vale, 1999a, b). One functional aspect of such linkage is to transmit external forces acting on the cell directly to the cytoskeletal framework thereby preventing the plasma membrane from mechanical injury. This mechanical aspect is obvious from blistering diseases of the skin, caused by genetic defects of intermediate filaments and associated junctional adaptor molecules (Nievers et al., 1999; McGrath, 1999; Fuchs and Cleveland, 1998). Moreover, linkage of the cytoskeleton to adhesion contacts allows the generation of contractile force against the cellular environment, which is an important requirement for cellular migration (Galbraith and Sheetz, 1998).

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![Fig. 6. Simulation of the consequences of inhibitory signalling pathways causing decreasing fractions of cell-to-cell adhesion molecules available for cytoskeletal interactions. The resulting detachment forces were calculated in dependence on the cytoskeletal affinity ($K_C$) of the remaining (unblocked) adhesion molecules. Adhesion molecules on the interacting neighboring cell were assumed to be maximally bound to the cytoskeleton ($r_{AA} = 1\mu m$; $K_{AA} = K_{AA} = 10^{-9} mol/m^2$; $K_A(0) = 10^3$).](image-url)
A further outcome of the present study is that an increase in the number of extracellular bonds per unit area ($N_b$) created by cytoskeletal immobilization has a strong non-linear impact on the force required for detachment of the interacting surfaces (detachment force). The example given in Fig. 2 shows that the rather small increase in $N_b$ from 85% to 100% caused by cytoskeletal binding at $K_c$ values of $10^3$ and $10^4$, respectively, results in a three-fold increase in the adhesive strength. This striking non-linear increase in adhesive strength is directly related to the phenomenon that a distracting force will cause an exponential decrease in the binding affinity (Bell, 1978). During such stress-induced decay of affinity, cytoskeletal cooperativity will become increasingly efficient in that it improves rebinding of dissociated molecules that would otherwise disappear by lateral diffusion. As outlined above and shown in Fig. 3, cytoskeletal cooperativity is maximally efficient at low extracellular binding affinities. Consequently, cytoskeletal binding will become particularly important for adhesion molecules under mechanical stress conditions.

In the cellular model systems treated in this study, we assumed random formation of adhesion contacts rather than preferential concentration of adhesion molecules at specialized membrane domains. Whereas many adhesion molecules display rather uniform distribution throughout the attached cell surface such as the nonclassical LI-cadherin along the lateral cell surface of the intestinal epithelium (Berndorff et al., 1994), classical cadherins and integrins have been shown to be concentrated at circumscribed adhesion sites, termed focal substrate contact, hemidesmosome, desmosome, and adherens junction, respectively (Gumbiner, 2000; Hynes and Zhao, 2000; Fuchs and Cleveland, 1998). On the cytoplasmic side, these junctions are associated with bundles of actin filaments (focal contacts, adherens junctions) or intermediate filaments (hemidesmosomes, desmosomes). It is generally assumed that clustering of adhesion molecules at these specialized junctional membrane sites provides higher adhesive strength as compared to the same number of adhesion molecules dispersed over a larger membrane area. However, this simple assumption is only correct if a distracting force is acting asymmetrically at the edge of the contact zone (wedge- or zip-per-like mode of separation). This mode of separation is particularly important for several types of cell-to-substrate contacts such as leukocytes or endothelial cells adhering to the vascular wall while exposed to the shear force of blood flow. Clusters of adhesion molecules will provide focal sites of higher resistance under these conditions. This has been elegantly demonstrated by experiments in which Chinese hamster ovary (CHO) cells were transfected with a fusion protein consisting of Xenopus C-cadherin in which the cytoplasmic domain has been replaced by three tandem repeats of the FK506 binding protein that contains three binding sites for the cell-permeant bivalent crosslinker FK1012 (Yap et al., 1997). After FK1012-induced cross-linking and clustering of cadherins, cellular adhesion on cadherin surface under fluid shear stress was significantly increased.

However, in most circumstances separating forces act more or less perpendicularly to the adhesive surface between cells. This is not only true for cell-to-cell adhesion in solid tissues such as liver or brain but also for adhesion between virtually all simple epithelia lining the gastrointestinal tube or the tubules of the kidney. In these tissues clustering of adhesion molecules will not per se improve adhesion strength between cells.

In a static system, such as two plates fixed together by a given number of bonds (e.g. rivets) it is only the number of bonds, but not their distribution that directly correlates with resistance to a distracting force. Clustering of these bonds at a small area will not change the unit force acting on a single bond (total force divided by total number of bonds) and will not increase resistance against detachment. This view is also valid for a dynamic system in which bonds (adhesion molecules) are able to associate and dissociate freely and change their position by lateral diffusion. The only benefit of clusters of adhesion molecules in such a dynamic system (without cytoskeletal linkage) is to delay the time course of detachment, because the higher local concentration of adhesion molecules within a cluster will increase the probability of collision and rebinding after bond dissociation. However, at the margins of clusters, adhesion molecules face extremely unfavorable conditions for rebinding, because the concentration of adhesion molecules in the remaining membrane area is significantly lower (zero if we consider all molecules clustered) as compared to conditions in which adhesion molecules are assumed to be randomly distributed. Consequently, clusters will disassemble rapidly by lateral diffusion unless adhesion molecules are constrained by linkage to the cytoskeleton. On the other hand, immobilization of adhesion molecules by cytoskeletal linkage would precisely meet the conditions discussed above for a static system in which clusters of immobilized adsive bonds will not delay the time course of detachment, and, accordingly, will not provide overall higher adhesive resistance towards perpendicularly acting distractive forces as compared to the same number of randomly distributed adhesion molecules bound to the cytoskeleton.

In the light of these considerations, the biological sense of clustering of adhesion molecules at junctions between cells of solid tissues or simple epithelia exposed to perpendicularly acting forces must include other aspects. One aspect favoring clustered distribution of adhesion molecules would be to cause effective damping of plasmalemmal oscillations at sites of clustering. This would allow trans-interaction of adhesion molecules at optimal encounter distance kept constant by local high bond density. Cooperative binding (increase in avidity) by oligomerization of multivalently binding adhesion molecules (Shapiro et al., 1995) would be a further aspect favoring clustering. But for most adhesion molecules including cadherins cooperative interaction has not been convincingly demonstrated yet.

In conclusion, transmembrane cooperativity of extracellular adhesion by linkage of adhesion molecules to the cytoskeleton would be a powerful mechanism by which cells would be able to control and modify extracellular adhesion. This mechanism would be most effective at millimolar extracellular affinities of adhesion molecules and requires affinities to the cytoskeleton at the micromolar level in order to obtain maximal transmembrane cooperativity. Clustering of adhesion molecules would further improve extracellular adhesion towards shear-forces (high local bond density), but may also assist adhesion by improvement of the two-dimensional affinity by damping of plasmalemmal oscillations.

**Appendix**

For the kinetic scheme describing cell-to-substrate adhesion depicted in Figure 1b, the law of mass conservation yields a system of nonlinear equations:
\[ A_{eq} = A \cdot \left[ 1 + K_{A} \cdot \left( C_{S} - \left( 1 + K_{C} \cdot (S + C) \right) + K_{C} \cdot (C + S) \right) \right] \] (17)

\[ C_{S,eq} = C_S \cdot \left[ 1 + A \cdot K_{A} \cdot \left( K_{A} + K_{C} + K_{A} \cdot K_{C} \right) \right] \] (18)

\[ S_{eq} = S \cdot \left[ 1 + A \cdot K_{A} \cdot C_{stot} = C \cdot \left( 1 + A \cdot K_{A} \cdot C \right) \right] \] (19)

Here the equilibrium constant \( K_{A} \) is force dependent as described in Eq. 4 i.e.

\[ K_{A}(f) = K_{A}(0) \cdot \exp \left[ \gamma \cdot f \cdot N \right] \] (20)

with the number of bonds

\[ N_f = B_{Ad} + B_{Ac} + B_{Ac} = A \cdot K_{Ad} \cdot K_{A} \cdot \left( S + C \cdot (1 + K_{C}) \right) \] (21)

Unfortunately, this system of nonlinear equations could not be solved analytically to obtain an expression for \( N_f \) as a function of the applied force \( f \) and the total concentrations \( A_{eq}, C_{S,eq}, S_{eq} \), and \( C_{S,eq} \). However, assuming \( S \) and \( C \) to be negligible, the above equations can be solved to find \( f \) as a function of the concentration of free adhesion molecules \( (A) \) by simply substituting \( K_{A}(f) \) from Eq. (20) and \( C_S \) from Eq. (18) into Eq. 17 and taking the logarithm yielding Eq. 5.

Unfortunately Eq. 5 contains a term \( A \cdot \ln(C_{1} + C_{3}A + A^{2})/(C_{1}A + C_{3}A^{2}) \) which cannot be inverted easily. However, if the number of binding sites is significantly higher than the number of adhesion molecules, i.e. the term \( A \cdot K_{Ad} \cdot K_{A} \cdot K_{C} \cdot K_{C} \) in Eq. 18 is much smaller than 1, \( C_{S} \) in Eq. 17 can be approximated by \( C_{S,eq} \) leading to the equations

\[ A_{eq} = A \cdot \left[ 1 + K_{Ad} \cdot K_{A} \cdot \left( C_{S,eq} \cdot (1 + K_{C}) \right) + K_{C} \cdot C_{S,eq} \right] \] (22)

\[ = A \cdot K_{Ad} \cdot K_{A} \cdot \left( C_{S,eq} \cdot (1 + K_{C}) \right) \]

After substituting \( K_{A}(f) \) according to Eq. 20 and taking the logarithm over these equations contain terms like \( A \cdot \ln(C_{1} + A)/(C_{1} + C_{3}A) \) which can be inverted by using Lamberts W-function \( W(x) \). This function satisfies \( W(x) \cdot \exp(W(x)) = x \) (Corless et al., 1996) leading to Eq. 6.

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