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Abstract

The dynamic regulation of cell-matrix adhesion is essential for tissue homeostasis and architecture, thus numerous pathologies are linked to altered cell-extracellular matrix (ECM) interaction and ECM scaffold. The molecular machinery involved in cell-matrix adhesion is complex and involves both sensory as well as matrix-remodelling functions. In this review, we focus on how protein conformation controls the organization and dynamics of cell-matrix adhesion. The conformational changes in various adhesion machinery components are described, including examples from ECM as well as cytoplasmic proteins. The discussed mechanisms involved in the regulation of protein conformation include mechanical stress, post-translational modifications and allosteric ligand-binding. We emphasize the potential role of intrinsically disordered protein regions in these processes and discuss the role of protein networks and co-operative protein interactions during the formation and consolidation of cell-matrix adhesion and extracellular scaffolds.

1. Introduction

Cell-matrix adhesion is essential for homeostasis and physiological function of the majority of cells existing in a multicellular organism. In order to fulfill their multiple functions, cells embedded within a tissue need to thoroughly control and constantly adjust their cell-matrix linkage. For example, cell motility and ECM remodeling, processes critical for development [1] as well as wound healing [2] and immune surveillance [3] are all enabled by the dynamic control of cell-matrix adhesions. Many different cytoplasmic and transmembrane proteins associate in dynamic ways, in order to maintain the structural integrity, as well as the signaling capacity of cell-matrix adhesions [4]. Therefore, in addition to the investigation of protein-protein and protein-lipid interactions that can be analyzed under equilibrium conditions, it is important to analyze the dynamics of the cellular adhesion machinery.

One possibility to control cell-matrix adhesion is via mechanical tension [5]. The association with the contractile actin cytoskeleton on the one hand, and the mechanical resistance of the extracellular matrix scaffold on the other hand, puts cell-matrix adhesion machineries under tension that efficiently controls the structural cohesion and cellular signaling capacity. Since proteins are generally studied under equilibrium conditions and often in the absence of other molecular components or their biological environment, it is not easy to understand how factors such as force-mediated reversible unfolding of proteins are involved in establishing the mechanical link between the ECM and the cytoskeleton.

The aim of this review is to discuss the role of conformational changes in proteins as a regulator of cell-matrix adhesions. We would like to show by a couple of examples how the conformational state of proteins, reflected by inter-domain junctions as well as non-structured protein regions, are

used to create functional cell-matrix adhesions. Specifically, we will discuss the factors that control protein conformation in cell-matrix adhesions, as well as the mechanism or energy needed to drive the conformational change or allostery, by binding to an adapter protein or extracellular ligand. In addition, the mechanisms that revert proteins to their original state after the dissociation of interactions or after release of mechanical tension will be discussed. For example, integrins can be activated by the divalent cation Mn^{2+}, which results in a shift of the conformational equilibrium of the receptor [6], leading to increased affinity for extracellular ligands [7] and causes reversible clustering and association with intracellular adapter proteins [8, 9]. In addition to such allosteric regulation, recent data also revealed several examples of dynamic changes in protein conformation and signaling that is controlled by mechanical tension: applying mechanical tension on proteins modifies or stabilizes conformations that favors new interactions, such as in catch-bonds, and enables post-translational modifications, e.g. due to the exposure of cryptic sites. These changes enable novel protein functions. Finally, we discuss the importance of unstructured regions in protein function in the context of cell-matrix adhesions.

2. Conformational changes within ECM proteins

The extracellular matrix (ECM) has important roles in tissue by providing attachment sites for cells, but also by acting as a physical scaffold with specific mechanical features. Some ECM proteins such as elastin or fibronectin are highly elastic, but also resilient, making tissue capable of deforming in a reversible way. Other proteins, such as collagen, form ECM fibers or networks that contribute to the mechanical stiffness of the tissue. On the other hand, the highly flexible and compliant glycocalyx provides a reservoir for growth factors as well as preventing cell interactions while allowing cushioning and pressure resistance.

A significant portion of ECM proteins are multimodular and oligomeric, providing thus multivalent binding sites for cells and enabling cross-linking with other ECM proteins, such as fibronectin. Certain ECM proteins have established a capacity to polymerize and self-assemble spontaneously into fiber or network-like assemblies (laminin and collagen). Other ECM molecules are secreted as inactive molten globular structures and need to be shaped by cells into a 3D scaffold (e.g. fibronectin). Examples of ECM proteins are shown in Table 1, which also includes information concerning pathologies associated with these proteins, highlighting the critical importance of ECM proteins for proper tissue functionality.

What is the role of conformational changes in ECM proteins during cellular adhesion? A significant portion of the elasticity observed in the ECM is associated with different types of carbohydrates and polysaccharides, which do not have distinct conformational states. Dynamic structures and short-term, low-affinity interactions are characteristic for cell-carbohydrate interactions, such as leukocyte rolling mediated by selectins [10]. Alternatively, hyaluronic acid, mediating interactions to receptors such as CD44, exert synergistic functions with RGD-mediated integrin adhesion, allowing spreading of cells under very soft culture substrates, or provoke epithelial to mesenchymal transformation [11, 12]. Other proteins such as von Willebrand factor (vWF) form extremely long protein polymers and work like fishing lines to capture platelets under high shear forces by using catch bonds that are influenced by O-glycosylation of the protein [13]. Due to their large size ECM proteins are complicated to understand and to study. Notably, structural studies of ECM proteins have revealed that even the largest proteins are composed of smaller subunits with well-defined structure, like beads on a string. Whether this design provides a specific function is not clear. It has been proposed that multi-modularity makes it possible for one protein to generate a pattern of different responses and thus act as sophisticated environmental sensor [14]. While domain rearrangement may occur under low tensile stresses, domain unfolding can occur at higher stresses, thus changing the properties of such ECM proteins in response to different levels of tension.

An example of a heavily characterized, large ECM protein is fibronectin (FN). This multimodular 440-kDa protein has two major conformational states. Soluble FN, which is a major protein component of blood plasma, is thought to have globular structure. Cellular FN contains two additional alternatively spliced domains that create new binding and interaction sites with cells leading to an insoluble form of FN. Insoluble FN assembles into FN fibers, which is an important constituent of vessels and connective tissues. The conversion from the soluble to insoluble form is an excellent example of how conformational changes in sub-domains drive the biological process of fibrillogenesis. It is thought that simultaneous interaction of the integrin receptor with the cytoskeleton and FN facilitates this transition by applying mechanical force to FN and unmasking binding sites for adjacent FN molecules, thus leading to polymerization and initiation of fibril formation [15, 16].

Several studies have focused on FN conformation when embedded in ECM. FN fibrils are remodeled and reorganized by living cells. However, how the remodeling happens at the molecular level remains debated. Two models have been presented to explain the stretching of FN fibers. The first model involves unfolding of individual FN domains. This model is built on findings made for individual FN domains unfolded by atomic force microscopy (AFM) [17, 18], optical tweezers [19] and steered molecular dynamics [20, 21]. Further experimental validation for this model has been acquired by determining the distances between fluorescent probes attached along the FN molecule by fluorescence resonance energy transfer (FRET) [22, 23]. These studies suggest that FN, when embedded in fibrils, gains length via unfolding of individual FN domains, and the mechanical properties of each of these domains control the sequence of unfolding events. The other model involves global conformational rearrangement of FN during the stretching of FN fibrils. In this model, FN molecules are embedded or associate with fibrils in a partially closed conformation, and the extension of the fiber leads to opening and stretching of the FN protein witout the need for unfolding of individual domains [24].

To approach the controversy between the two models, Lemmon et al. recently utilized a cysteine labeling approach to study the exposure of residues buried in the 3D-structure of FN type III modules [24]. With the help of experimentally determined structures of FN domains, they introduced buried cysteine residues into type III modules by mutagenesis and then measured the solvent exposure of those cysteines by binding to a fluorescently labeled cysteine-reactive probe in FN embedded into ECM in cell culture. Another set of measurements was performed for the individual type III modules in solution. These experiments revealed that some of the domains were labeled even in the absence of mechanical stress, proposing dynamic equilibrium between folded and (partially) unfolded states. Of those domains which were not labeled when studied in solution as isolated subunits, only domains III-6 and III-12 became cysteine-labeled when embedded in FN fibrils. Therefore the main conclusion of this study was that domain unfolding alone is not sufficient to explain the great extendibility of FN fibrils [25]. Another aspect of this finding, the importance of (partially) unfolded protein regions for cellular function, is discussed later in this article.

Another recent study utilized a mathematical model to investigate the FN conformation within fibrils [26]. In this approach, the findings made by Lemmon and coworkers [24] were utilized to build a model, where some of the FN modules were partially unfolded in the absence of mechanical stress, while a majority of the modules were compact. In this model, the main contribution to fiber extension was by the unfolding of the FN domains and a good match to experimental data for artificial FN fibrils [25] was obtained.

Although the discussion concerning the details of the FN conformation in ECM fibrils may continue, it is apparent that dynamic conformational changes are playing an important role in ECM biology. For example, FN and one of its extracellular binding partners, tenascin, which both evolved in chordates have critical functions in a pressurized vascular system [27]. The specific features of FN allow the creation of a malleable ECM that also captures information about the physical strain

exerted on the protein scaffold surrounding cells. Thus, cells receive signals and survey their environment by being attached to FN-containing fibers, while being able to respond to and remodel the organization of the ECM. A defect in this delicate cross-talk, e.g. linked to the absence of integrin adapter proteins, can cause vascular deformations and pathological conditions [28] (see below).

Conformational changes in ECM proteins have been studied intensively. Table 1 lists some examples of ECM components and how their function is regulated by conformation. The list aims not to be complete, but rather shows examples of conformationally regulated proteins.

Table 1 Conformational regulation of ECM proteins. A list of selected proteins with indicated structural organization, functions and pathological implications is provided.

Molecule	Structural organization	Functions	Conformational flexibility	Diseases associated
Fibronectin (FN)	Multimodular disulphide-linked dimer	Cellular attachment, fiber formation, ECM cross-linker, growth factor binding reservoir	Transformation from globular to fibrillar, domain unfolding, cryptic binding sites	Cavernous vessels malformations [28], glomerulopathy with fibronectin deposits (GFND) [29]
Elastin	Repetitive, crosslinked	Elastic fibers, ECM binding	Reversible stretching [30, 31]	Supravalvular aortic stenosis (SVAS) and Williams-Beuren syndrome (WBS) [32]
Collagens	Repetitive, crosslinked	Fibrils, strength	Local stretching [33]	Several diseases such as Alport syndrome [34], tumors [35] and Stickler syndrome [36]
Tenascin	Disulphide-linked hexamer	Cross-linking	Domain unfolding [37]	Vascular disease [38]
vWF	Multimodular dimer	Bridging molecule, platelet adhesion	Stretching [39]	vWD (bleeding diathesis)
Fibrillin	Repetitive EGF motifs	Elastic fibrils, tensile strength	Reversible stretching, Ca^{2+} binding associated with conformation [40, 41]	Marfan syndrome [42]

One fascinating layer of conformational regulation is induced in ECM proteins by post-translational modifications (PTMs). In addition to cross-linking, which is one of the best known PTMs to stabilize or alter the mechanical properties of ECM proteins [43], proteolytic cleavage as well as differential glycosylation needs to be considered. Cross-linking of collagen fibers increases the mechanical stiffness of stromal tissue, which in turn induces focal adhesion formation and enhanced signaling through focal adhesion kinase potentially leading to dedifferentiation or carcinogenesis [35, 44]. In contrast to cross-linking, proteolytic cleavage of ECM proteins is critical for tissue remodeling and for enhanced cell invasion. In contrast, small integrin-binding fragments of collagen IV have been shown to have anti-metastatic functions *in vivo* [45]. Glycosylation modifies the molecular surface of extracellular proteins, which can affect protein oligomerization, binding and interaction with cell surface receptors, such as in the case of vitronectin. Decrease in vitronectin glycosylation leads to increased size of the vitronectin oligomers, simultaneously augmenting binding to collagen[46]. Alternatively, conformational flexibility can be modified by O-glycosylation, such as in the case of vWF, where this modification results in changes in platelet capturing under shear stress[13].

3. Integrin conformational regulation as an example of allosterically regulated receptors

Integrin receptors are prototypic allosterically controlled proteins. Conformational changes of the extracellular, transmembrane and cytoplasmic domains are provoked by binding of extracellular ligands and cytoplasmic adapter proteins, which have coined the terms “outside-in” and “inside-out” conformational activation. However, in order to form a stable link between the extracellular matrix and the actin cytoskeleton, integrins need to simultaneously contact extracellular ligands and cytoplasmic adapters. In addition to the influence of ligand-binding and di-valent cations on the conformational equilibrium of micelle embedded integrins [6], application of tensional forces along

the ECM-actin axis induce a catch-bond behavior [47], thus further stabilizing integrin-ligand interaction and force bearing. Although still incompletely understood, the catch-bond mechanism is likely connected to an approximately 200-fold increase in ligand-binding affinity due to hybrid-domain swing-out [7], and this open conformation is in turn stabilized by tensional forces acting on the β-integrin subunit. The continuum of different ligand binding states and respective modification of MIDAS and ADMIDAS ion coordination in the β-I domain allows understanding of how ligand binding to the integrin head domain influences affinities at multiple levels [7] (Fig. 1). For example, a primary contact of the Asp residue of the RGD-ligand with the MIDAS cation is further stabilized by the interaction of Arg with the β-propeller of the α-subunit. In the case of FN-binding to the α5β1 integrin, additional interactions between the synergy site residue R1376 of FN and of D154 of the integrin α-subunit enhance the on-rate of binding [48, 49] (Fig. 1). In addition, studies with the C-terminal peptide of fibrinogen show interaction also with the ADMIDAS cation, which prevents its interaction with the carbonyl oxygen of Met335 of the β6-α7-loop to stabilize the extended, open conformation of integrins [7, 50] (Fig. 1). Based on this example, it is possible that other extracellular integrin ligands also target the ADMIDAS cation to stabilize the ligand-bound conformation. This could be the case in FN, in which glutamic acid 1462 of the 10th type III repeat is located in close proximity of ADMIDAS (Fig 1D). Consistent with such a model are EM images of the α5β1/FN 7-10 complex, showing interactions with the 10th but not with the synergy site-containing 9th type-III repeat of FN [49]. Such differential interactions will have consequences under tension, as the distance between inter-domain binding sites are much more sensitive to mechanical stress than binding sites located within the same FN-type-III domain. Thus initial diffusion-driven binding involving the synergy site, would be replaced by a tension driven switch in FN-integrin interaction [51], thereby matching the stress resistance of individual type-III domains of FN of 5-10 pN to the strength of the integrin catch-bond of 20-30 pN.

Figure 1 Hytönen and Wehrle-Haller

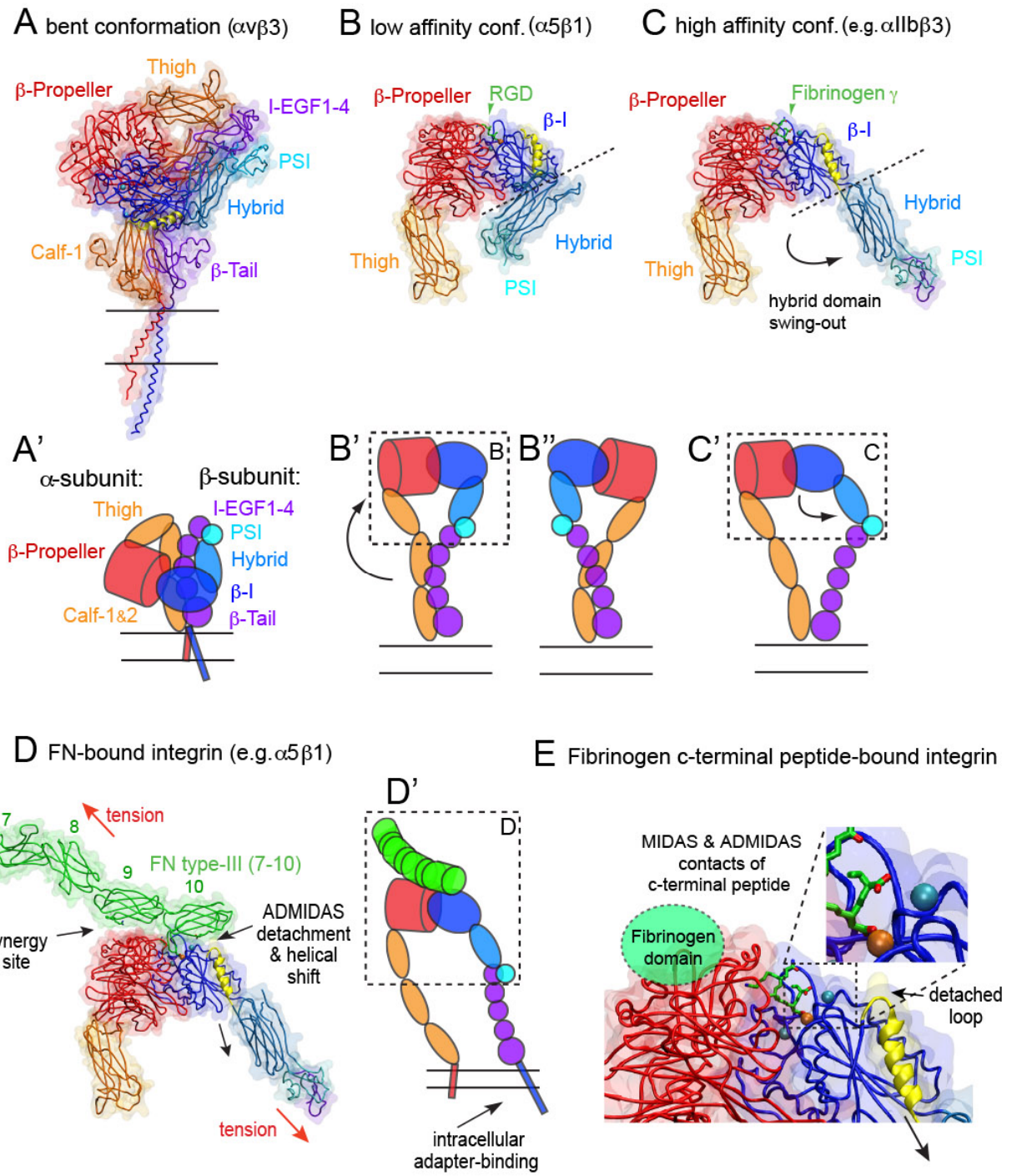

Figure 1 Allosteric protein: Conformational regulation of integrin

The heterodimeric αβ integrin receptor consisting of an α-subunit (red hues) and β-subunit (blue hues) can adopt different conformations, depending on the binding of allosteric ligands or interacting adapter molecules. **A**, αvβ3 integrin displaying the bent conformation, in which the ligand-binding site is facing the plasma membrane and the transmembrane and cytoplasmic domains are clasped, thereby hiding the membrane proximal talin-binding site in the cytoplasmic tail. **B**, the extended, closed conformation can be induced in αIIbβ3 by the addition of Mn^{2+} to the medium. Although the extracellular domain is extended, the transmembrane domain and cytoplasmic tails are proposed to be clasped [6]. Although the integrin is in the low affinity conformation, ligands can bind via an acidic amino acid to the MIDAS Mg^{2+} ion in the β-subunit and an arginine or lysine to the β-propeller of the α-subunit. In response to ligand binding, the Mg^{2+} coordination of the MIDAS and ADMIDAS Ca^{2+} ion is switching to the high affinity form (affinity increase about 200 fold), which involves detachment of the ADMIDAS ion from the carbonyl oxygen of Met335 located in the β6-α7 loop (yellow) leading to the movement of the α7-helix (yellow) and the swing-out of the hybrid domain [7]. **C**, the extended, high-affinity conformation is caused by hybrid domain swing out, which provokes further opening of the integrin legs and potential unclasping or dissociation of the transmembrane and cytoplasmic clasp [6, 52]. **D**, model of an open, high affinity form of α5β1 integrin, bound to the cell-binding fragment of FN, consisting of type III domains 7-10. Please note the synergy site interactions between the α-subunit (D154) and FN type III repeat 9 (R1379) [48]. **E**, C-terminal fibrinogen peptide bound to the open and extended form of αIIbβ3 [50]. The peptide contacts the α-subunit with a lysine and the ADMIDAS ion with the free carboxy terminus, thereby stabilizing the open conformation of the integrin. In addition to the peptide, additional fibrinogen interactions with the β-propeller are proposed to contribute to fibrinogen binding [50]. Note also the proximity of the ADMIDAS ion in the α5β1/FN

complex to a glutamic acid in the RGD-peptide containing domain in FN in D. Panel A was prepared using the integrin model published by the group of Springer [53]. The integrin structure in panel B corresponds to α5β1 (PDB 3VI4), containing bound RGD peptide [48]. Panel C is a chimeric structure created by joining the α5-chain from PDB 3VI4 (B) and β3-chain and ligand from PDB 2VDO (αIIbβ3, E)[50]. The chimeric structure (panel C) was obtained by aligning the two β-propeller domains using Swiss PDB viewer (http://www.expasy.org/spdbv/) [54]. Panel D has been created by positioning the FN fragment (PDB 1FNF [55]) according to the RGD peptide in PDB 3VI4 (panel B) and then slightly tilted to approach the synergy site. Then the β-chain was replaced by the one from PDB 2VDO. The structure in panel E corresponds to αIIbβ3, PDB 2VDO. The structural presentations are prepared using VMD 1.9.1 and the schematic drawings with Adobe Illustrator.

Although it is quite intuitive to propose that the binding affinity of extracellular integrin ligand should correlate with the ability to induce stable cell-matrix adhesion, this model is not always supported by experimental data. While Mn^{2+} induces a 100-fold increase in LFA-1 affinity for soluble ICAM-1 and enhanced cell adhesion, the depletion of intracellular energy stores blocks cell adhesion on immobilized ICAM-1, but not high affinity binding of soluble ligand. This proposes that energy-dependent post-ligand binding events are also key factors for the establishment of integrin-mediated cell-matrix adhesions [56]. Potential mechanisms to enhance the cell-matrix adhesion are to increase the binding strength of individual integrin-bonds (e.g. tension-mediated affinity regulation), or alternatively, to increase the number of integrins per adhesion site via integrin clustering (valency regulation) [57]. Recent experiments suggest that an increase in integrin activation measured by the oligomeric ligand mimetic IgM antibody PAC-1 is not due to a higher integrin affinity, but rather due to the capacity of integrin receptors to cluster after talin-

mediated activation [58]. In turn, any process that stimulates integrin clustering should contribute to an increase in integrin-valency and thus cause enhanced cell-matrix adhesions. Interestingly, intracellular adapter proteins such as talin and kindlin that are involved in integrin conformational activation [59, 60], are also critical to induce integrin clustering in cells plated on immobilized integrin ligands [8, 9, 61]. Importantly, stable integrin clustering can only be seen on immobilized ligands, again supporting the notion that talin and kindlin mediated conformational changes require the presence of ligand. This is also corroborated by studies with individual nano-disc embedded αIIbβ3 integrins, of which the extended conformation increased from 10 to 20% in the presence of talin head and to at least 40% in the presence of the fibrin ligand [62], indicating the essential role of extracellular ligand in stabilizing integrin activation and/or in assisting integrin clustering by favoring the reciprocal association of cytoplasmic adapter proteins.

Initial ideas to explain integrin clustering by transmembrane-domain interactions [63] have not been confirmed [64]. Although the disruption of the transmembrane association leads to conformational activation and integrin clustering, this is not mediated by intra-membrane associations. In fact deletion of tetraspanin CD151, which normally associates with α3β1 integrin in adhering podocytes, leads to reduced cell adhesion and pathological integrin clustering, suggesting that other multi-transmembrane proteins modulate integrin behavior and potentially also integrin traffic in cells [65]. In the absence of such transmembrane modulators, integrin clustering could also be the consequence of a ligand-mediated integrin-capturing process caused by a glycocalyx-mediated repulsion of the plasma membrane [66]. Alternatively, integrin clustering could also be caused by phase separations in cholesterol containing membranes and specific lipid compositions [67], as integrin adapters such as talin and kindlin associate with PIP2 and PIP3 lipids, respectively [9, 68]. Accordingly, the acute removal of PIP2-lipids from the plasma membrane induces the rapid dispersal of integrin clusters [8], strongly suggesting that acidic phospholipids play a key role in the organization and clustering of integrins in the plasma membrane. In addition, many other focal

adhesion adapter proteins show the ability to bind to PIP2 enriched membranes or to become conformationally activated by interaction with the membrane (see Figure 2). Therefore, the PIP2 containing membrane creates a 2D scaffold onto which cytoplasmic adapters are captured to form integrin-containing cell-matrix adhesions. This mechanism of recruitment has the consequence that relatively low-affinity protein-protein or protein-peptide interactions will be enhanced by several orders of magnitude simply by confining and concentrating the binding partners to a very thin, principally 2D reaction volume [69]. The interaction with membrane might also be thermodynamically important, because many of the intracellular proteins involved in cell-matrix adhesion complexes have a positively charged surface patch, which will orient the protein in respect to the other protein partners, thus contributing to the entropy component of the binding process (Figure 2).

Figure 2 Hytönen and Wehrle-Haller

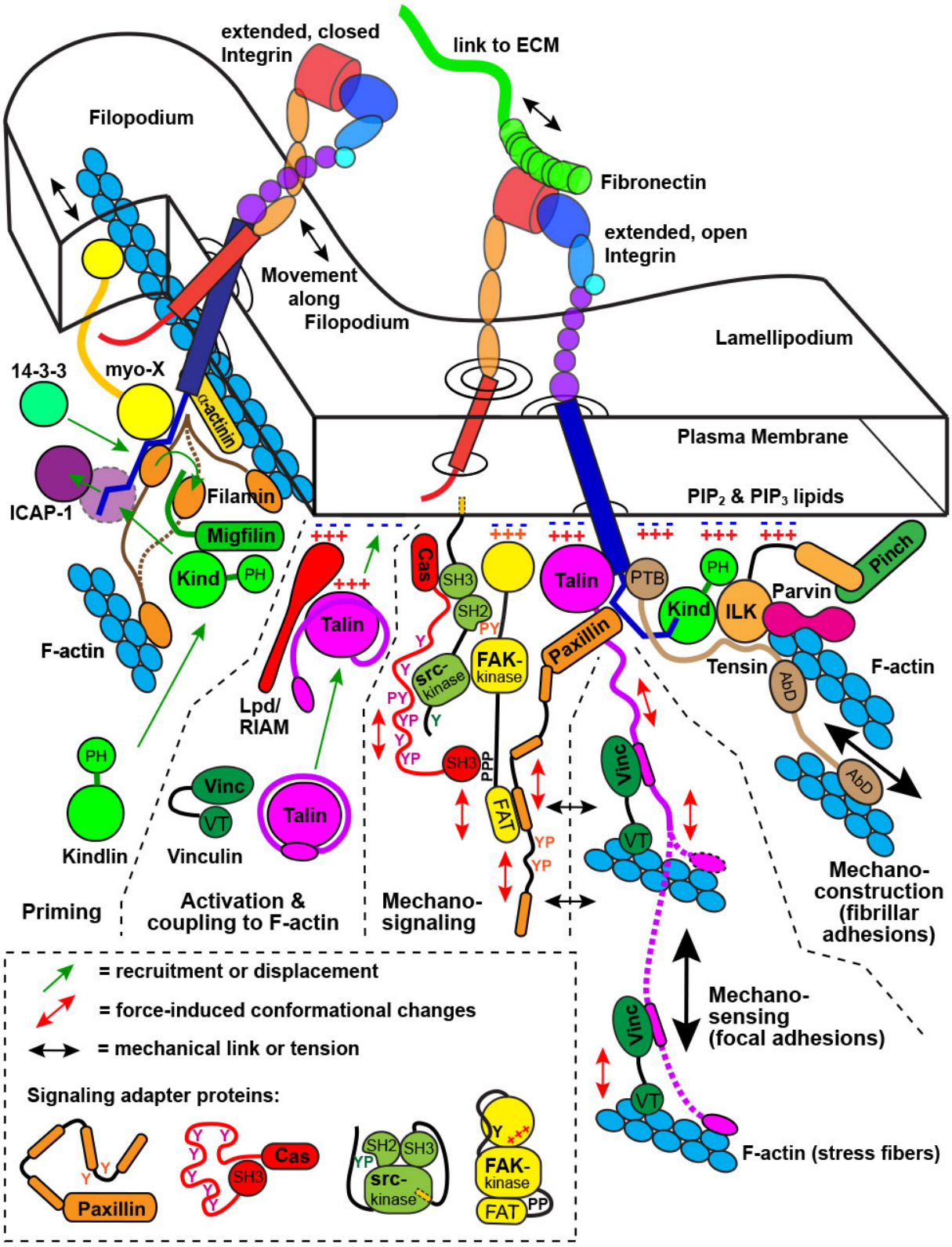

Figure 2: Assembly of cell-matrix adhesions via synergistic and co-operative binding modes

Multidimensional representation of the formation of cell-matrix adhesion complex, highlighting the functional characteristics of the integrin-associated intracellular proteins. The left hand side shows integrin/adapter interactions that are proposed to prime the integrin and to present it along the shaft of filopodia in an extended and ligand responsive state. Interactions with myosin-X at the proximal NPXY motif [70], filamin at the inter-NxxY region [71] and ICAP binding to the distal NxxY motif of β1-integrin prevents its precocious activation [72, 73]. Phosphorylation mediated 14-3-3 binding to the inter-NxxY region [74], as well as migfilin-mediated recruitment of kindlin and simultaneous interference with filamin binding prepares the integrin for interaction with talin [75, 76]. Allosteric activation of talin is considered a key step in stabilizing the ligand-bound, open conformation of the integrin. The release of the auto-inhibited form of talin into an integrin-bound form involves multiple steps, including the presence of kindlin [59, 77], unclasping of the head-tail interaction by the activity of lamellipodin/RIAM [78, 79], acidic phospholipids [9, 80], as well as binding of the C-terminus of talin to the head-domain of vinculin and F-actin [81-83]. In response to tension on the vinculin and F-actin bound talin-C-terminus and to extracellular opposing forces, the talin rod domain is unfolding, allowing the recruitment of additional vinculin proteins via exposing vinculin binding site helices, thereby assuring F-actin linkages over a large range of tensional forces [84, 85]. Despite the recruitment of talin to activated, ligand-bound and clustered integrins, signaling proteins such as FAK and paxillin require the presence of tension and linkage to the actin cytoskeleton of the integrin/talin complex [8]. Although not further characterized, it requires vinculin and F-actin binding sites in the c-terminal rod domain of talin [86], suggesting the existence of a functional link between paxillin and FAK that is sensitive to tension-mediated conformational changes of talin. We propose that binding of the N-terminal FERM domain of FAK to acidic phospholipids, as well as the mechanical extension of the FAT domain, leads to the tension-mediated activation of FAK, as well as exposure of phospho-tyrosines and proline-rich domains. This enables further interactions and

molecular extension and exposure of signaling sites in response to mechanical tension. In contrast to the talin-mediated slip-bond acting in a range of 2pN [87], the mechanical stretching of FN molecules to induce FN fibrillogenesis requires forces >5pN [17], involving acto/myosin-interaction, the ILK/pinch/parvin complex and recruitment of tensin [88, 89]. Furthermore, α-actinin, which moves together with non-clustered integrins prior to adhesion formation [90], is involved in stabilizing the mechanical resistance of the integrin/F-actin bond, thus enabling focal adhesion maturation and a dynamic response to the physical state of the extracellular matrix [91, 92].

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4. Intracellular proteins and conformational regulation

Numerous intracellular proteins are involved in cell-matrix adhesions (Figure 2 shows some key components of cell-matrix adhesions, some of them discussed below more thoroughly). The regulation of this highly complicated process requires extensive control. Conformational changes regulating the activity and interactions within intracellular proteins are numerous. Therefore, we focus here on two main themes: We discuss the conformational regulation within intracellular signaling proteins, and the processes associated with mechanosensing in focal adhesions.

Protein phosphorylation is a highly important component of cellular signaling. Phosphorylation is involved in virtually all cellular functions, and therefore it is essential to regulate the activity of the proteins responsible for phosphorylation, i.e. kinases. The conformational changes associated with activation of Ser/Thr and Tyr kinases are thoroughly reviewed by Huse and Kuriyan [93]. In addition to conformational changes in kinases, access to their target sites may also be subject to conformational control. For example, tension applied to a protein domain may expose a

phosphorylation site, which in turn recruits phospho-protein specific adapter proteins and downstream signaling thereby converting mechanical force into a biochemical signal.

Cell-matrix adhesions are cellular substructures involved in contact between cells and their surrounding tissue. Among different types of adhesion structures [15] focal adhesions are the best studied and contain numerous protein components – close to 200 different proteins have been collated into an "*adhesome*" network [94] while about 1000 proteins have been identified by proteomics analyses of purified focal adhesions [95-97]. Focal adhesions are highly dynamic in structure and composition, and capable of responding to mechanical signals. On the one hand they act as mechanical anchors, whilst on the other hand they act as force-sensing tools to constantly probe the cellular environment and to adapt the cell-matrix accordingly. For example, when cells are cultured on stiff substrate, large focal adhesions are formed. In contrast, cells on soft substrate have sparse and small focal adhesions. Neither the mechanisms of this force-sensing, nor the proteins or molecules responsible for this feedback system are completely understood.

The following paragraphs aim to shed light on these topics by summarizing the findings related to conformational changes taking place in selected intracellular proteins.

c-Src

Phosphorylation appears to be one mechanism controlling the conformation and activity of kinases. Src kinase, an important player in controlling the signaling associated with cell-matrix interaction, maintains its inactive state via auto-inhibition. The activity of Src is regulated by phosphorylation on Tyr416 located in the activation loop, rendering the kinase active. In contrast, Tyr527 when phosphorylated by the c-terminal Src kinase (Csk) binds to the N-terminal SH2-domain of Src forming an auto-inhibited complex. Membrane-detachment of Src and further stabilization of the

auto-inhibited form can be induced by the N-terminal binding of myristate, which serves as a membrane anchor in the active kinase, to a binding pocket in the c-terminal kinase lobe [98] (Figure 2). In the case of c-Abl, allosteric inhibitors to the myristate binding pocket efficiently returns the mutationally activated kinase into the inactive, drugable conformation [99]. In contrast to compounds or residues that stabilize the inactive conformation, recent computational study suggests that phosphorylation at Tyr416 "locks" the Src kinase domain in the active conformation [100]

Cas

Crk-associated substrate (Cas, also called p130CAS) is a substrate of Src kinase localized mainly in focal adhesions. Cas has important roles in cellular signaling [101]. The mechanosensory role of Cas was originally proposed by Tamada et al. [102], who found stretch-dependent tyrosine phosphorylation of Cas by Src family kinases (SFKs) taking place in cytoskeletons prepared by ripping away the rest of the cell by detergents. To further examine this model, Sawada et al. [103] prepared terminally biotinylated Cas substrate domain (CasSD), and attached it on avidinylated latex membrane. The mechanical stretching applied via the extension of the membrane resulted in increased tyrosine phosphorylation of CasSD as measured via phospho-specific antibody [103](see also Figure 2).

Both termini of Cas have been found to be important for the localization of Cas to focal adhesions [104]. The amino-terminal SH3 domain of Cas is known to bind proline-rich motifs in numerous proteins, including FAK [105], PTP-PEST [106] and C3G [107], to name a few. In v-Src (kinase analogous to Src from Rous sarcoma virus) transformed, invasive cells, phosphorylation of the proline-binding pocket of the SH3 motif has been observed, which reduces FAK association and displaces Cas from focal adhesions. In contrast, cell invasion is reduced and Cas is hyper-phosphorylated if the SH3 domain cannot be phosphorylated [108]. Similarly, Cas-SH3 interaction with the hinge region of vinculin enhances focal adhesion size and cellular traction forces, which are lost when the Cas-binding site in vinculin is mutated [109]. This suggests that mechanical coupling of Cas to FAK and

vinculin contributes to a positive feedback loop reinforcing cell adhesion and traction forces in cells exposed to mechanical stresses.

Talin

Talin is a large cytoplasmic protein directly interacting with integrin β-tails via its N-terminal head domain and with actin via its C-terminal rod domain (for review, see [110]). The importance of talin for cellular adhesion has been confirmed by knockout and silencing studies [111, 112]. There are two conformationally controlled processes associated with talin: i) release of head-rod auto-inhibition associated with integrin binding [113] (Figure 2) and ii) stretching of the rod domain, potentially exposing a number of vinculin-binding sites [84, 85] (Figure 2).

A recent study suggests that talin adopts a donut-shaped structure in its auto-inhibited state [114] (Figure 2). In this configuration, the integrin-binding sites in the talin head are buried. The R9 rod domain appears to be responsible for rod-head interaction [114]. Disrupting this interaction increases the amount of talin associated with the cytoskeleton [115]. However, it was found that further manipulation of talin was required to promote the association of talin with plasma membrane, namely disruption of the contact between the F1-F2 domains in the talin head and the R1 and R2 domains in the talin rod [115]. Despite several studies focusing on this topic, including those which have proposed a role for PIPkinase-γ and acidic phospholipids in driving the opening of talin (see, for example [9, 116]), the regulation of talin activation still requires more attention.

The second level of conformational regulation of talin is associated with mechanical tension within the cell. Super-resolution light microscopy confirmed localization of the talin head at the level of the plasma membrane, while its C-terminal rod-domain was associated with the F-actin backbone of focal adhesions [117]. By using double-tagged talin, N-terminally with EGFP and C-terminally with mCherry, Margadant *et al*. [118] were able to determine the length of individual talin molecules embedded in cellular adhesions. In the absence of molecular tension (in the presence of myosin

inhibitor), the length of talin was 50-60 nm. Elongation to 90-250 nm was observed in the direction of actin flow in non-treated cells. Interestingly, overexpression of vinculin head domain increased the average length of talin to about 400 nm and also suppressed the fluctuation in talin length [118]. Stretching of talin has been studied using both computational and experimental methods. Lee et al. [119] proposed rotational movement of vinculin-binding site 1 (VBS1) in talin due to mechanical stress applied on a talin rod fragment in steered molecular dynamics simulations performed in an implicit water model. Hytönen et al. [85] utilized similar technology in explicit water, and found that mechanical stress first disrupted the interfaces between alpha-helix bundles and then made it possible for water to penetrate into the talin-helical bundle, exposing the VBS sequences [85]. An experimental model utilizing a terminally tagged talin rod fragment and magnetic tweezers was utilized by del Rio et al [84], who were able to measure an increase in binding of fluorescently labeled vinculin head domains to stretched talin molecules. Therefore it appears that the talin rod domain can be mechanically elongated in living cells and this may open novel binding sites for vinculin (Figure 2). Vinculin seems to be able to contribute to the conformational regulation of talin by inhibiting the extension of talin [118]. Therefore, the talin-vinculin pair may be considered as a potential force sensor in living cells, where the amount of vinculin bound to talin helps cells to adjust to tensional fluctuations along the integrin-talin-actin axis.

Vinculin

As discussed above, vinculin associates with talin via its head- and interacts with actin via its tail-domain. It has numerous other binding partners, many of those interacting with the hinge domain. Head-tail interaction of vinculin causes auto-inhibition and decreases binding to talin [120] (Figure 2). Applying mutations to this interface was found efficient in increasing talin-vinculin colocalization when these proteins were artificially targeted to mitochondria using ActA tag [121]. Based on FRAP experiments, the head domain of vinculin was also found to incorporate more tightly into focal adhesions and slowed down the dynamics of talin as well vinculin [81].

Vinculin, once integrated into adhesion complexes, appears to stabilize the adhesions. In order to analyze if vinculin experiences mechanical tension, a FRET-based molecular sensor was constructed by Grashoff et al. [122] This sensor consists of mTFP1-venus fluorescent protein pair connected via an elastic $(GPGGA)_8$ linker, which was placed between the vinculin head and tail domains. Using this setup, they demonstrated that vinculin experiences mechanical tension and the extension of vinculin could be measured in living cells via this sensor.

What is the biological importance of mechanical tension applied to vinculin? Does mechanical stress expose novel binding sites in vinculin? These are questions to be answered by forthcoming studies.

α-actinin

α-actinin, similarly to talin, consists mainly of α-helices. α-actinin is an antiparallel homodimer, which crosslinks actin filaments [123]. It consists of an N-terminal calponin-homology (CH) domain, followed by a rod domain made of four α-helical spectrin repeats, ending with a C-terminal head domain, which has homology to calmodulin. CryoEM analysis has revealed two different conformations for the CH domains, where one is to be found in an open conformation at one end of the molecule, whilst the other is to be found in a closed conformation at the other end [124] The authors propose that this conformational flexibility would enable α-actinin to crosslink actin fibers with a range of different angles. α-actinin was one of the first found proteins to bind to the cytoplasmic tails of β-integrins [125]. Studies with β2-integrin tails however, showed only a very weak interaction, which was enhanced by neutrophil activation [126]. Responsible for this effect is an auto-inhibition by the distal part of the β2-integrin tail, which was reverted by a surprisingly large panel of point-mutations, suggesting a subtle equilibrium between a non-structured, binding competent and a closed, auto-inhibited configuration of the β2-integrin tail [127]. In contrast to the β2-tail, the β1-integrin tail binds α-actinin even in non-attached cells [127], which nicely explains its co-

diffusion outside of focal adhesions [90] and the recruitment of α-actinin to membrane anchored β1-tails, allowing the analysis of the complex by CryoEM [128] (Figure 2). How α-actinin is exchanged with talin during integrin activation is not yet understood, but several studies show a critical role of α-actinin in the maturation of focal adhesions [91, 92]. Similar to talin, α-actinin is also proposed to be involved in vinculin-mediated mechanosensing. While vinculin can bind to a 2D-lattice of α-actinin in the absence of force [129], applying a constant velocity pulling force on the α-actinin anti-parallel dimer enables the liberation of an α-helix in silico [130], which has been crystalized together with the vinculin head domain [131]. Thus α-actinin takes a key position in presenting integrins at the cell surface in an actin-scaffold dependent manner, nicely demonstrating how different sets of integrin adapter proteins control the function of these receptors at the plasma membrane.

Filamin

Filamins (filamin A, filamin B and filamin C in vertebrates) are homodimeric proteins interacting with actin via an N-terminal actin-binding domain and harboring 24 immunoglobulin-like domains, of which the last one is responsible for dimerization [132]. Filamins are flexible proteins found associated with filopodia and exhibiting an integrin binding domain. By binding to integrins, filamins are competing/collaborating with other integrin binding proteins for integrin recruitment [71, 76] (Figure 2). Filamins are involved in cellular sensing and mechanoprotection and are multi-tasking proteins, interacting with more than 90 proteins [133]

Filamins are also subject to conformational regulation. Lad et al. crystallized three-domain fragments of human filamin A (IgFLNa19-21). They found that the N-terminus of IgFLNa20 forms a beta-strand that associates with the CD face of IgFLNa21 occupying the integrin binding site. Further, they demonstrated that disruption of this auto-inhibition enhances integrin-binding of filamin A [134] (Figure 2). This property of filamin was further examined by Pentikäinen et al., who

found it possible to displace the A-strand from IgFLN20 bound to the IgFLN21 by applying external forces in MD simulation, low enough not to disturb the folding of the globular filamin domains [135]. Since a similar behavior was also observed for FLNa18-19 pair, it is possible that small amounts of mechanical strains perceived by actin-bound filamin is enabling integrin-binding and priming at sites of cell protrusions.

5. The role of intrinsically disordered protein regions in cell-matrix adhesion

Traditionally, structural biology has been focused to determine protein architecture with the highest possible resolution. X-ray crystallography is the most often used method in structural determination, and it relies on the formation of well-organized crystals with high similarity between individual proteins in the protein crystals. Proteins, however, are not only composed of well-structured polypeptide chains. It has been found that significant portions of protein sequences are without a distinct structure. Some proteins have been found to be completely unstructured and are thus called intrinsically disordered proteins (IDPs) [136] It is not obvious why entire IDPs and proteins with intrinsically disordered protein regions (IDPRs) exist. Do these segments just connect different domains of a protein or do they have specific functions, as for example in p130Cas, where the analysis by AFM and magnetic tweezers suggested intrinsically disordered domains [137]

When examinating the structures of cell-matrix adhesion associated intracellular adapter proteins, several contain IDPRs (Table 2). Furthermore, IDPRs appear to be located in regions known to be key for the biological function of these proteins.

In focal adhesion kinase (FAK), the activation loop (A-loop) located in the catalytic domain represents an IDPR when the protein is in the auto-inhibited state, making contact with the adjacent FERM domain. Upon kinase activation the A-loop becomes phosphorylated adopting a distinct

conformational state, detectable in the active form of the kinase [138] This finding is consistent with observations at a proteomic level where IDPRs are enriched with phosphorylation sites, potentially serving to convert disordered protein regions into structured regions [139].

Table 2 Intrinsically disordered protein regions (IDPRs) involved in cellular adhesion.

Protein	Residues	PDB ID	Structural context	(Proposed) function	Reference
Talin-1	134-172	3IVF	Head F1 loop	Lipid binding	[140]
Vinculin	844-876	1TR2	Neck	Protein interactions	[141]
FAK	376-393	2AL6	FERM domain loop	Linker	[142]
FAK	569-583[b]	2IJM	Catalytic domain (A-loop)	Phosphorylated, adjacent to the active site	No associated publication (2IJM)
Predicted					
Kindlins	~150-250[a]		F1 loop	Lipid binding	[143]
Paxillin	inter LD-domains		Connectors	Phosphorylation and proline-rich	
FAK	677-912		Connector	Phosphorylation and proline-rich	
Cas			Connector	Phosphorylation	

[a]NMR analysis (^{1}H-^{15}N heteronuclear single quantum coherence spectrum) of the isolated mouse kindlin-1 sequence stretch 141–249 revealed substantially disordered conformation

[b]A distinct conformation for the loop has been observed in the activated (phosphorylated) form of FAK kinase domain [138]

In vinculin, the IDPR is located in the neck, which is the sequence between head and tail domains and associated with numerous interactions between vinculin and other proteins [144] Similarly,

integrin cytoplasmic domains have been found to be highly flexible, however they can adopt specific structures when associated with specific, but often competing binding partners [145]. Because of their flexibility IDPRs appear to be ideally suited for protein interactions. Interestingly, Iakoucheva et al. found that IDPs have an important role in cellular signaling, regulation and cancer [146]. In addition, many proteins involved in cell-matrix adhesions contain PEST sequences (enriched in proline (P), glutamic acid (E), serine (S) and threonine (T)) that have the signature of IDPRs [147]. In additions to potential protein or lipid binding interactors, PEST sequences are considered to be signals for protein degradation. Due to the unstructured nature of IDPRs, mechanical stretching could convert them from a molten globular state into elongated structures easily recognized by various peptide recognition motifs.

Thus, IDPRs should also be discussed in the context of molecular recognition. Since the introduction of the "Lock and Key" hypothesis by Emil Fischer (1894), our view of molecular interactions has evolved and the "Induced Fit" model has been developed to explain molecular recognition as a stepwise process of conformational changes of the binding partners leading to complex formation [148]. Another theory is called "Conformational Selection", which proposes that proteins exist as an ensemble of conformations in dynamic equilibriums, and the binding event changes the equilibrium by sequestering certain conformations to the bound state [149]. While both of these models have been developed for the study of molecular recognition in solution, cell-matrix adhesions provide a unique example where protein-protein complexes are assembling on the molecular surface of a 2D plasma membrane (see Figure 2). Interactions with acidic phospholipids has been proposed to be the driving force for the formation of a positively charged α-helix in the highly charged, but unstructured F1-loop in talin [150]. In addition to an induced fit, interaction with acidic phospholipids may also reduce the dynamic equilibrium of a peptide to better match the recognition site of a binding partner. The role of the plasma membrane is illustrated in two cases: While the interaction between the membrane proximal integrin binding site in talin contributes only slightly to the binding affinity of integrin

peptides in solution [151], such an interaction is readily detected when integrin/talin association is analyzed in a cellular model containing a physiological membrane [9]. Talin and kindlin cooperate to induce integrin activation in intact cells [59, 77]. In contrast, in a binding assay in the absence of membranes these two proteins bind independently and without synergy to the integrin cytoplasmic tail [152].

Another aspect related to IDPRs is connected to binding thermodynamics. If the binding reaction involves loss of entropy, the overall free energy of the binding is less favorable for the binding. This means lower affinity. For cellular mechanisms requiring active regulation, it would be very important to maintain relatively low affinity, but still ensure specificity. IDPRs appear to be a perfect solution for this need [136, 153] . Recent study has illustrated the potential of IDPRs in allosteric regulation of molecular interactions in adenoviral protein E1A [154]. It was found that both positive and negative cooperativity occurs between the interacting proteins in this molecular complex, depending on the available interaction sites on E1A. In the light of these findings, the cytoplasmic domain of integrin β-chain appears to be an exciting candidate for such allosteric regulation (Figure 2).

6. Sequential binding vs. co-operative binding: from pairwise interactions to systems biology

The process leading to complex cellular substructures such as focal adhesions is fascinating and has led to concept of an adhesome [94]. However, the majority of the information concerning molecular interactions has been collected from experiments capable of only recognizing pairwise interactions. In the light of the complexity of cell-matrix adhesions (see figure 2), is it sufficient to restrict our experiments to this low level of complexity? Could all biological processes be explained by defining the interactions between individual components and then analyzing the resulting data using systems biology tools? The answer to this question may likely be a “no”, especially considering not

only protein-protein, but also protein-lipid interactions critical for cell-matrix adhesions. In the presence of a charged membrane, a low-affinity binding interaction in solution can increase in affinity by several orders of magnitudes [69].

Thus, the integrin cytoplasmic domain is a good example of a molecular hub, where several molecules compete and require spatial and temporal orchestration for binding. While talin is competing with filamin and α-actinin for access to integrins, (Figure 2), kindlin itself competes with ICAP-1 to gain access to integrins [155]. While kindlin alone cannot activate integrins, talin in turn requires kindlin for integrin activation and platelet spreading [156]. Such cooperation could occur by lipid-binding of both adapter proteins [157], but may still require physical interactions between talin and kindlin, in order to have a binding advantage over other adapter proteins that possess similar lipid-interaction capacities. In addition to these equilibrium interactions, it is also likely that mechanical tension along the ligand-integrin-talin-actin axis is modifying the affinity of binding interactions. Alternatively this could lead to the exposure of cryptic binding sites for which the talin-vinculin association is a good example [84, 85]. This seemingly chaotic process, however, leads to biologically functional assemblies of intracellular proteins. To understand this process better, we need to approach the question with more complete models and apply computational biology and more sophisticated bioinformatics tools to explore the multi-dimensional universum of cell-matrix adhesions.

7. Turnover of cell-matrix adhesions; proteolytic versus signal-mediated disassembly

Diamonds are forever, however cell-matrix adhesions are dynamic in nature and are associated with cellular behavior such as migration and wound-healing. Therefore, this last chapter discusses the mechanisms and conformational regulation involved in adhesion disassembly [145].

Proteolytic processing of focal adhesion proteins has been found to be an integral component of the process of focal adhesion disassembly. Proteolytic cleavage of talin at the head to tail junction as well as close to the F-actin binding domain in the tail by Ca^{2+} mediated calpain-2 proteolysis is critical for the turnover of focal adhesions [158, 159]. Besides talin, calpain-2 cleaves also FAK, which proteolytic cleavage is also critical for dynamic remodeling of focal adhesions [160]. To ensure selectivity of cleavage, it is likely that the respective cleavage sites of these proteins are only exposed in the context of cell matrix-adhesions and influenced by tension or post-translational modifications occurring during focal adhesion maturation [161, 162]. In contrast to talin where proteolytic cleavage represents a safety valve to prevent cell immobilization, cleavage of paxillin between the LD1 and LD2 motifs has the reverse effect. While paxillin cleavage slows down adhesion turnover, proteolytic stabilization of paxillin, for example observed in invasive tumors, enhances the turnover of cell-matrix adhesions [163]. While the mechanism of this regulation is not yet understood, the paxillin cleavage site maps to a region in paxillin involved in phosphorylation-dependent control of FAK association and activity in nascent adhesions [164].

In addition to proteolytic cleavage, Src tyrosine kinase mediated phosphorylation of FAK has been linked to enhanced focal adhesion turnover [165]. Phosphorylation of Tyr957 is required for adhesion turnover and is located in the focal adhesion targeting domain (FAT) of FAK. Thus, the FAT domain appears to play a critical role in orchestrating focal adhesion turnover and talin-cleavage, since this domain interacts with paxillin LD-motifs as well as the talin-head. Specifically, the latter interaction appears to be critical for focal adhesion turnover and talin processing [166].

In addition to recruiting critical integrin adapter proteins to sites of cell-matrix adhesions, acidic phospholipids have also been involved in recruiting the clathrin-mediated endocytosis process, which depends on adapter proteins, exhibiting β-integrin tail interactions that target the same sites

as the talin head [145, 167-169]. Thus the control over the cell-matrix adhesion assembly-disassembly process could be controlled by the local concentration of acidic phospholipids [145, 170].

In the context of adhesion disassembly, one needs to keep in mind that under 2D cell culture conditions the cells are able to sense many more extracellular ligands than are required for cell spreading and adhesion [171]. Furthermore, in a 3D or 1D culture system, the equilibrium between adhesion assembly and disassembly appears to be more dynamic than in 2D [172], controlled by cellular contractility [173], and more responsive to differential integrin adapter signaling [174]. However at this point, the amount of mechanistic data is still limited.

To this point, it is likely that the majority of mechanisms involved in the assembly of cell-matrix adhesions are reversible and are also involved during the disassembly phase. However, under physiological stress or when high adhesion dynamics are required, cells may bypass slow reversible mechanisms and utilize shortcuts that require proteolytic cleavage and destruction of cell-matrix components. It is clear that we require further research to understand these processes in more detail.

Conclusions

The control of cellular processes requires not only adjustment of the concentration of individual components via regulated gene expression, but heavily exploits differential protein conformations. By focusing on mechanisms that regulate cell-matrix adhesion, we have illustrated how fine-tuning of protein-protein and protein-lipid interactions can have an impact on the intracellular architecture and extracellular scaffold.

By gaining more structural information and details on conformational changes, we will eventually be able to identify and to therapeutically control critical molecular switches associated with cell-

matrix adhesions. New technologies, such as the collection of structural information from nano/microcrystals or by using x-ray free-electron lasers is a promising approach [175]. Combining this new technology with NMR may provide us with tools to extend our understanding how proteins use their stable as well as flexible domains to control extensive conformational rearrangements occurring in cell-matrix adhesions.

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