CHAPTER

6

Protein Networks in Integrin-Mediated Adhesions

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OUTLINE

Summary

In this chapter we address the molecular complexity of integrin-mediated adhesions to the extracellular matrix. We constructed, by means of a systematic literature search, a comprehensive "adhesome" network, consisting of the diverse components of integrin-mediated adhesions and the multitude of interactions between them. Here, we present the network at several distinct levels. These range from an examination of the entire network, via a survey of specific families of components, to the characterization of functional subnets, then to individual protein entourages and, finally, to specific domains of adhesome constituents. We

then discuss how these domains can be modified by signaling molecules, each of which can act as a "switch" that "turns on" or "turns off" the molecular interactions within the adhesion structure. By deconstructing the entire adhesome network into functional subnetworks, we gain insight into the structural and functional organization of integrin adhesions.

Definitions

Integrin adhesome   Defined (Zaidel-Bar et al., 2007) as the collective of molecules participating in the formation, stabilization and signaling activity of all types on integrin-mediated adhesions, including focal adhesions, focal complexes, fibrillar adhesions and podosomes (Geiger et al., 2001). In this article, we address their molecular complexity.

Intrinsic components of the adhesome   Defined as molecules physically associated with adhesions (based on morphological criteria).

Associated components   Defined as molecules that are not stable residents of adhesion sites, but have been shown to interact with the intrinsic adhesome components **and** affect their function.

Interactions between adhesome constituents Can be non-directional *binding interactions*, as well as directional *signaling interactions* between specific signaling molecules and their downstream molecular targets. These interactions were operationally defined as *activating interactions* (e.g. phosphorylation, G-protein, the GTPase modulator, GEF) and *inhibitory interactions* (e.g. dephosphorylation, proteolysis, the GTPase modulator, GAP). Additional details are provided below.

INTRODUCTION

Interactions of living cells with each other and with the extracellular matrix are mediated by a variety of structurally and molecularly defined adhesion sites. These membrane-bound compartments consist of multimolecular complexes containing specific adhesion receptors, cytoskeletal components and interconnecting adapter proteins. In addition to these scaffolding elements, adhesion sites host a wide variety of signaling molecules that regulate their formation and turnover and participate in adhesion-mediated signaling events (Burgeson and Christiano, 1997; Burridge et al., 1988; Hynes, 1992; Simon and Goodenough, 1998; Stevenson and Keon, 1998; Yap et al., 1997).

Current thinking supports the view that adhesion structures serve, not only as means to link cells physically into functional tissues and organs, but also as a means by which cells learn about the nature of their environment, thereby triggering diverse responses that affect cell growth, viability, differentiation, morphogenesis and migration (Danen and Sonnenberg, 2003; Gumbiner, 2005; Kaverina et al., 2002; Watt, 2002). It appears that, via these adhesions, cells sense several features of their neighborhood, including the molecular composition of the matrix, its geometry and its physical properties (e.g. rigidity). This information is then integrated and translated into specific adhesion-mediated signaling events, that drive physiological cellular responses (Geiger and Bershadsky, 2002). Particularly relevant in this context are recent findings indicating that adhesion sites are highly mechanosensitive, and respond to both external and internal forces by altering their assembly dynamics and signaling activity (Katsumi et al., 2004; Zaidel-Bar et al., 2005). The molecular mechanisms underlying such responses are still poorly understood (Bershadsky et al., 2003), yet they appear to involve forced changes in the conformation of specific adhesion-associated molecules, which leads to their phosphorylation and "switching-on" their signaling activity (Geiger, 2006; Sawada et al., 2006).

In recent years, much effort has been invested in deciphering the composition and molecular architecture of integrin adhesions, and the complex interactions between their numerous

constituents (Lo, 2006; Zamir and Geiger, 2001). Our current understanding of the molecular structure of diverse adhesion sites and of their signaling responses is primarily based on a combination of cellular localization data yielded by immunofluorescence microscopy, and biochemical studies, mostly consisting of protein-binding data. These data revealed that adhesions formed via a single class of receptors (integrin-mediated adhesions) can be structurally and functionally heterogeneous, consisting of at least four types of adhesion sites: focal complexes, focal adhesions, fibrillar adhesions and podosomes. Each of these structures can play distinct roles in the initiation of adhesions, stress fiber formation, matrix fibrillogenesis and matrix degradation (Geiger et al., 2001; Linder and Aepfelbacher, 2003; Nobes and Hall, 1995; Zamir et al., 2000). Although all these structures are similar in molecular composition, they differ greatly in such features as subcellular localization, morphology and dynamics, which are of critical functional importance.

PROBLEM

Overall, most studies of adhesion sites have focused on the in-depth characterization of individual proteins, and the signaling pathways affecting their activity (e.g. Brown and Turner, 2004; Legate et al., 2006)]. However, attempts to assign specific biological functions to individual molecules have proven to be difficult, probably because of the enormous complexity of adhesion sites and their diversity. Deciphering the function of particular proteins is further complicated by the tendency of adhesion components to function as multi-protein modules (Hartwell et al., 1999). Recent inventories of adhesion proteins (Lo, 2006; Zamir and Geiger, 2001) were an important prerequisite for system approaches, but analysis of the adhesome at the network level is still lacking.

SOLUTION

A Holistic View of the Adhesome

Adhesion sites, like many other cellular systems, can be studied in a "bottom-up" approach, "reconstructing" multimolecular function units from their individual constituents, or "top-down," starting with the complex, unperturbed structure. During the past few years, knowledge of the molecular underpinnings of the "integrin adhesome" and its constituents has greatly expanded. As a result, we are now able to combine bottom-up and top-down views, and construct an all-inclusive, mostly protein-based model of this "global molecular network." By subsequently dividing the adhesome into smaller modules, or "subnets," we can then explore the inner workings of this complex molecular ensemble.

Constructing the Adhesome Network

The definition of the integrin adhesome, its intrinsic and associated components and the nature of links and interactions between them are admittedly artificial, yet they enable us to distinguish between these molecules and the interactions between them in a clear and convenient manner. That said, many of the "associated" proteins may in fact reside, under certain conditions or for a limited period of time, in the adhesome proper, and could simply be evading detection. Conversely, some of the "intrinsic" proteins might reside only temporarily in the adhesion site, carrying out their tasks and then leaving.

The actual data used to compile the list of components of the integrin adhesome, and our description of the relationships between them, were derived from virtually thousands of publications that have appeared to date in the scientific literature. Our search, based on the criteria outlined above, indicated that the number of "intrinsic components" of this adhesome is close

to 90, and the number of "associated components" that interact with the endogenous molecules and affect their activity and fate is over 60 (Zaidel-Bar et al., 2007). It should be emphasized that in this survey we refrained, insofar as was possible, from validating or questioning statements made in the different publications, or from overemphasizing data that received more attention or appeared to be more extensively substantiated. It is, perhaps, inevitable that certain components or interactions have escaped our notice. For this, we apologize.

The vast body of literature concerning cell adhesion documents more than 680 direct interactions between the various components of the adhesome. More than half of these represent direct binding between components; the others are directional "signaling interactions" that can either activate (i.e., positive input) or inhibit (i.e., negative input) the target molecule. Given that the same modification may activate certain molecular functions and inhibit others, we will refer here to specific modifications as "activating" or "inhibitory" according to the type of modification rather than the actual activity of the target (see below). In such instances, the signaling molecules consist of enzymes such as kinases, phosphatases, proteases, or Rho-family GTPases and their modulators (GEFs and GAPs). On average, each adhesome component can interact with about nine other components, making the adhesome a highly interconnected network (Fig. 6.1). However, the distribution of interactions is highly non-uniform: many proteins have few connections and a few have many, as is characteristic of biological networks (Barabasi and Oltvai, 2004).

Critical examination of the crude global network comprising the adhesome is a far from trivial task, and identification of the internal order among its components is hardly straightforward. It is important to bear in mind that not all components are always present in any given cell or adhesion, and all signaling pathways linking them are not active at all times (see below). To gain a deeper understanding of the functional interactions within the global network, one needs to examine the network more specifically, at the level of its segmentation into simpler structural or functional subnets.

Functional Families of the Adhesome

Functional annotation of the adhesome resulted in the classification of 18 families of components, based on their primary biological activity (Fig. 6.2). The three largest families consist of adapter, adhesion/receptor and actin-associated proteins. The main function of the adapter family is to form a robust structural scaffold, which anchors several signaling molecules, thus facilitating and directing their activity. The adhesion/receptor family contains, in addition to integrins, a dozen or so other transmembrane molecules, all of which are associated with, and affect, integrin-mediated interactions (Humphries et al., 2005). The actin-associated components are involved in linking the adhesion site to the cytoskeleton, and for their mechanical coupling. Most other families of adhesome components consist of enzymes, which regulate the formation and turnover of the adhesion complex, and participate in the generation, amplification and transduction of adhesion-mediated environmental cues. These include molecules such as protein and lipid kinases or phosphatases, in addition to Rho-family GTPases and their modulators, GEFs and GAPs. Notably, among the adhesome components, there also exist a single protease (calpain) and a single E3-ligase (Cbl), and a small, yet intriguing, group of proteins that are involved in RNA or DNA regulation and presumably shuttle between the nucleus and the adhesion site. Another small group of proteins, the function of which in integrin adhesions remains unclear, consists of three ion channels. Moreover, in addition to the approximately 150 intrinsic and associated proteins, the adhesome also contains three non-protein components: the
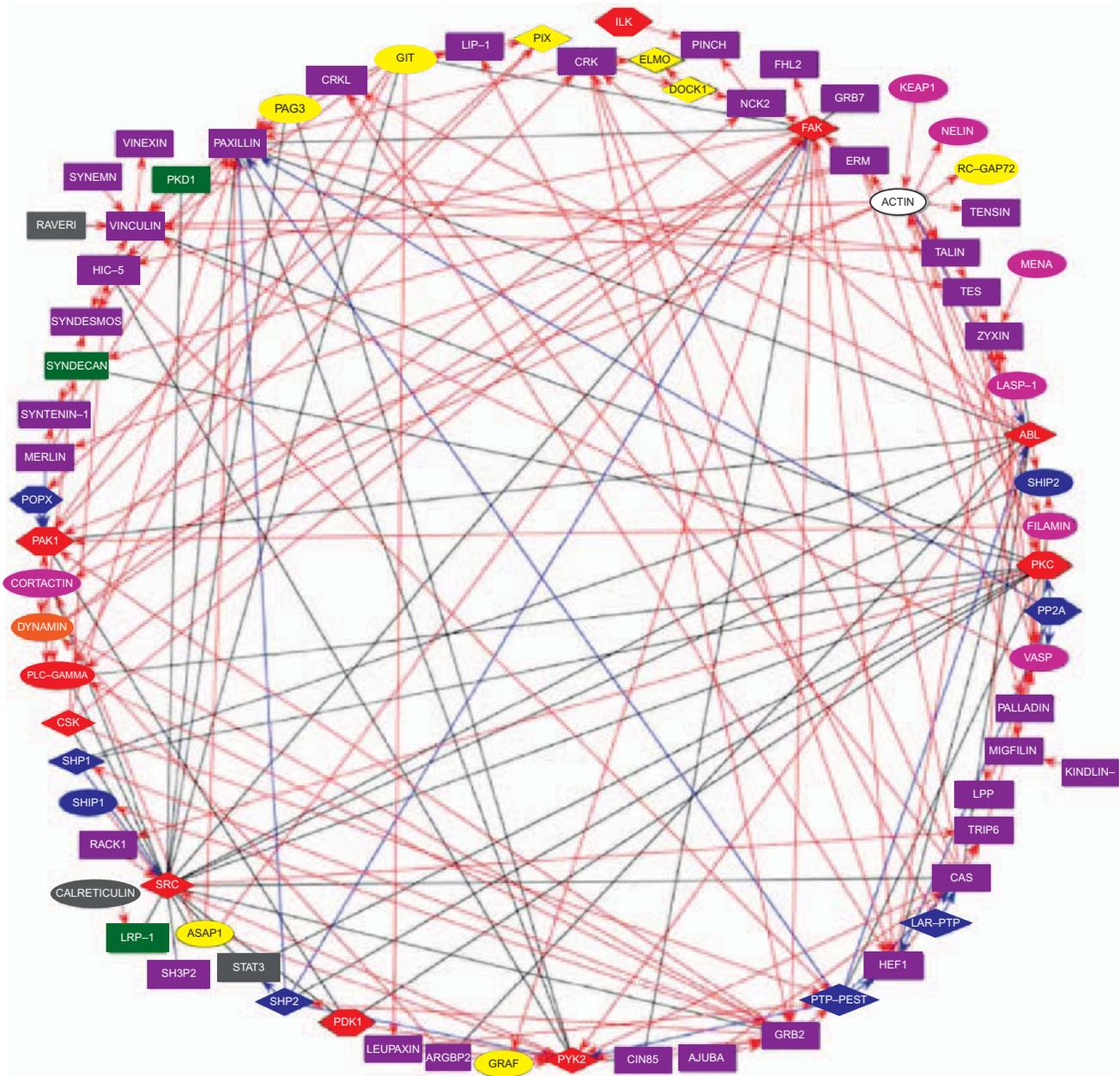
FIGURE 6.1   The Adhesome Network.
Illustration of the interactions between the intrinsic components of the adhesome. Directional interactions are depicted by arrows, red for positive inputs and blue for negative inputs; black lines denote binding interactions. The shape and color of each protein symbolizes its function, according to the scheme presented in Figure 6.2.

lipid derivatives phosphatidylinositol bisphosphate (PIP2) and phosphatidylinositol triphosphate (PIP3) and calcium ions.

As the number of proteins in each family varies greatly, so, too, does the number of interactions each family has with other proteins, indicating that their tendency to interact depends on the protein's specific function. For example, each component of the 46-member family of adapter proteins interacts, on average, with seven different proteins, whereas the phosphatidylinositol (PtdIns) kinase family, with only three members, takes part in an average of 16 interactions per protein (Fig. 6.2).

Protein type	Example symbol	Number of proteins in adhesome	Number of interactions (range)
Adapter	TENSIN	46	7 (1–35)
Adhesion/Receptor	INTEGRIN-B	14	6 (1–33)
Actin regulation	VASP	12	6 (1–16)
S/T kinase	ILK	11	9 (4–36)
Tyrosine kinase	FAK	9	22 (12–48)
GEF	VAV	9	7 (3–18)
Tyrosine phosphatase	SHP1	9	8 (1–16)
GAP	GRAF	9	6 (2–10)
GTPase	ARF1	5	11 (4–17)
RNA/DNA regulation	STAT3	4	3 (1–7)
PtdIns kinase	P13K	3	16 (6–24)
PtdIns phosphatase	SHIP1	3	6 (5–6)
S/T phosphatase	POPX	3	4 (1–8)
Chaperone	HSP27	3	2 (2–2)
Ion-channel	HERG	3	2 (1–3)
Actin	ACTIN	1	23
E3-ligase	CBL	1	25
Cysteine-protease	CALPAIN	1	21

FIGURE 6.2 Classification of Adhesome Components.
Classification of 18 families of adhesome components, based on their primary biological activity.

Interrelationships Between the Functional Protein Families

Are there ground rules that determine the nature of the cross-talk between the protein families of the adhesome? If the probability that any two given proteins will interact with each other is independent of their function, we would expect the number of interactions between any two functional families of proteins to be simply proportional to the total number of interactions of their family members. Significantly higher or lower numbers of reported interactions between the components of two functional families, as compared with the calculated random interaction value, would indicate that the two are specifically "connected" or "non-connected," respectively. Indeed, using a hypergeometric test, we detected pairs of protein families that had a high tendency to interact (P value $<T$) or not to interact (P value $>1 - T$). (We used $T = 4 \times 10^{-3}$, taking into account a false discovery rate of 20% for multiple-hypothesis testing.) Our results, illustrated in Figure 6.3, indicate that the interactions between certain protein families are favored, whereas interactions between other protein families are strongly discouraged. Not surprisingly, actin regulators regulate actin, adhesion/receptor
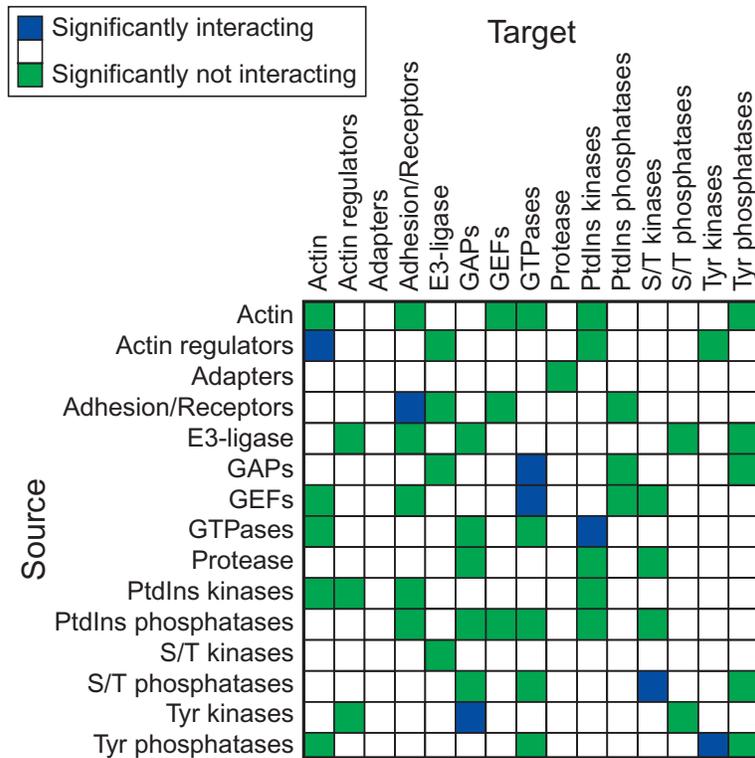
FIGURE 6.3   Interaction Between Functional Protein Families.
The probability that any two families of proteins will interact was calculated according to their total number of interactions, compared with the actual number of interactions between them (see text for details). Families that tend to interact significantly more than expected are marked by a blue square at their intersection in the matrix; families that interact significantly less than expected—i.e., tend not to interact—are marked by a green square. Note that the matrix is not symmetrical, because interactions are directional between a source (on the left) and target family (top). PtdIns, phosphatidylinositol; Tyr, tyrosine.

proteins interact with each other, and GAPs and GEFs modulate the activity of GTPases. Less obvious, *a priori*, are our discoveries concerning the enzyme protein families: GTPases, for example, tend to regulate PtdIns kinases, tyrosine kinases regulate GAPs, and serine/threonine (S/T) and tyrosine phosphatases dephosphorylate S/T and tyrosine kinases, respectively.

The strong tendency of certain protein families not to interact is also a matter of interest. As already indicated, adhesion receptors do not interact with actin, and GTPases do not interact with themselves. Interestingly, the E3 ligase (Cbl) does not target actin regulators or adhesion/receptors, nor does it target GAPs or S/T or tyrosine phosphatases. Furthermore, the protease (calpain) does not target GAPs or PtdIns kinases, and tyrosine kinases do not phosphorylate actin

regulators. It should be emphasized that these calculations reflect general tendencies of cross-family interactions; thus a few of the components in non-interacting families may, in fact, interact; similarly, not all proteins in significantly interacting families necessarily interact.

Resolving Functional Subnets

Another approach for resolving functionally relevant modules within the integrin adhesome is the bottom-up construction of functional subnets, based on a specific type of protein modification. For example, one could focus on all the reported phosphorylation events (e.g. on S/T or on tyrosine) within the adhesome, mark the targets of such events, define the kinases and

phosphatases involved and determine which proteins regulate their activity. Using the interaction database of the adhesome, one could also construct subnets for different regulatory pathways, involving, for example, proteases, GTPases, actin regulators and the like (Zaidel-Bar et al., 2007). Such subnets are of a considerably simpler nature than that of the global network of the adhesome, and can be more readily used by both experimentalists and modelers.

As an example, take the case of a "lipid subnet" that contains two main effector molecules, PIP2 and PIP3 (Fig. 6.4). The formation of PIP2 and PIP3 is regulated by PtdIns kinases and phosphatases, which in turn are primarily regulated by GTPases and tyrosine, as well as S/T kinases. The lipids themselves are mostly involved in the regulation of the actin-binding capabilities of adapter proteins (e.g. talin or vinculin), and in the control of actin bundling and cross-linking proteins such as filamin and alpha-actinin. In some cases, the lipids activate the effects on actin; in others, they inhibit such activity. PIP2 and/or PIP3 also regulate the S/T kinases of the adhesome, although, interestingly enough, they have no effect on tyrosine kinases. PIP2 and PIP3 do not have common substrates within the adhesion sites, with the exception of AKT. Naturally, one may proceed further "upstream" of these functional subnets, and extend the spectrum of regulatory components to additional hierarchical levels, or to interconnections with other subnets, inevitably rendering the subnet much more comprehensive—but, on the other hand, less specific. We consider functional subnets to be useful tools in planning perturbation experiments, using drugs or specific short interfering RNA (siRNA) to determine how different effector molecules modulate the cellular function of choice.
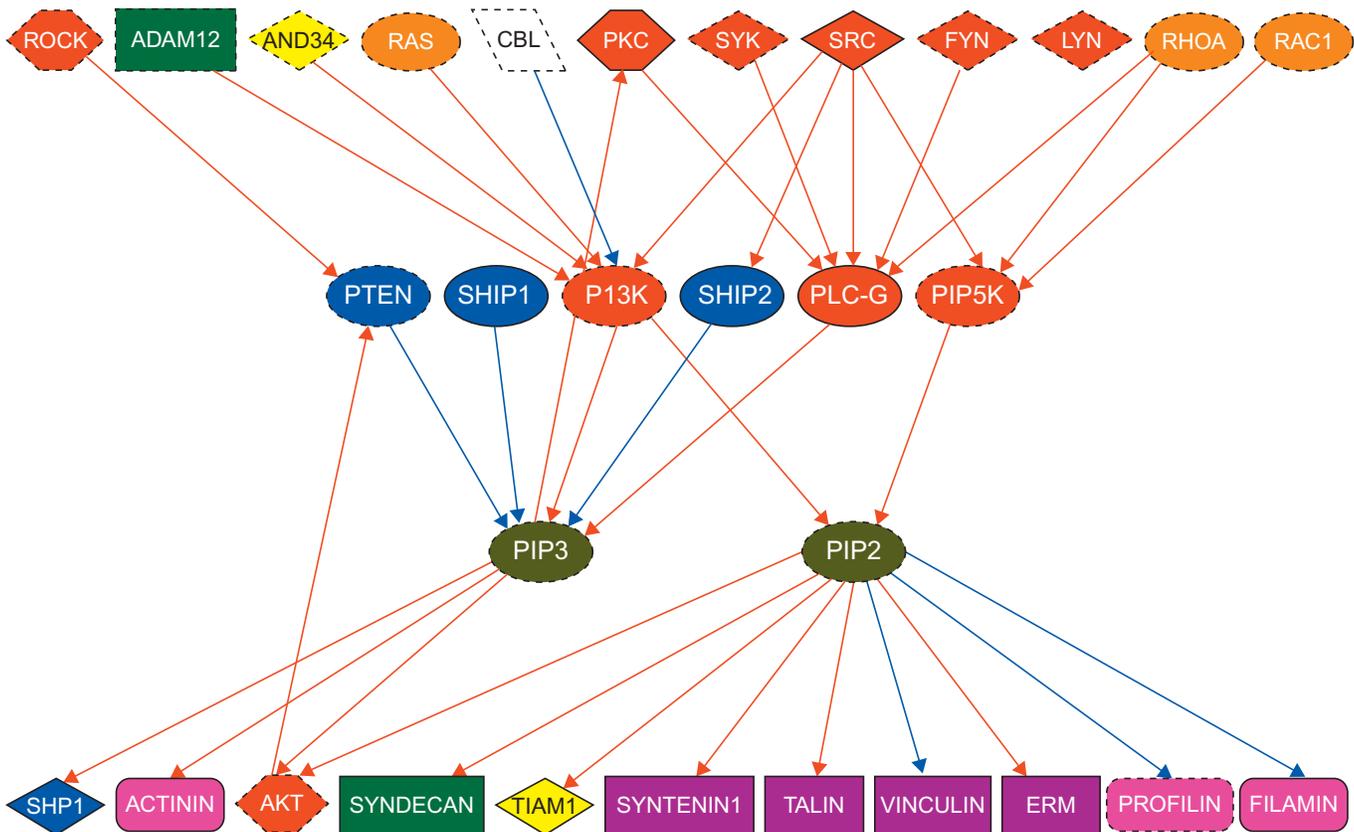

FIGURE 6.4　Lipid Subnet.
The lipid derivatives PIP2 and PIP3, their targets and their regulators, two hierarchical levels up. Only directional interactions are depicted: red for activating and blue for inhibiting. See text for further discussion.

Identifying Molecular Entourages

Zooming in on individual adhesome components can provide additional functional insights into the direct effectors and targets of key adhesome components. For example, one could examine the individual protein connections of actin (Fig. 6.5A) and of protein kinase C (Fig. 6.5B). What can we learn from such schemes? As mentioned above in the section on interrelationships, actin interacts with actin regulators and adapter proteins. However, it also clearly interacts with certain kinases, and in some cases (Abl, PLD1) it inhibits their enzymatic activity. Another example, namely protein kinase C (PKC) (all isoforms combined), reveals that these enzymes not only bind to adapter proteins and actin, but also regulate tyrosine kinases and phosphatases. Moreover, several, very different signaling pathways (including lipids, Rho GTPases, tyrosine and S/T phosphorylation) converge on their regulation. Such information can also be useful in the of planning perturbation experiments (e.g. siRNA suppression of gene expression, or the use of specific inhibitors).

Protein Domains and Switchable Links Enriched in the Adhesome Net

A search of the adhesome for specific protein domains reveals several domains that are significantly enriched within the adhesome, as compared with their presence in the entire human proteome (Table 6.1). These include the Pleckstrin homology (PH) and FERM domains, both of which target proteins to the plasma membrane, and the Calponin homology (CH) domain, which is an F-actin-binding motif. The importance of tyrosine phosphorylation for the regulation of protein–protein interactions is manifested by the enrichment of the tyrosine kinase and phosphatase domains, in addition to the Src homology 2 (SH2) domain, all of which mediate interactions with phosphorylated tyrosine residues. Protein–protein interactions, which are a hallmark of the adhesome, are also mediated by Src homology 3 (SH3), FERM and LIM domains, which mediate binding among specific proteins.

It is noteworthy that, within the adhesome, many of the links connecting the various protein components are not constitutive, but rather can be switched on or switched off by corresponding signaling components. The proteins talin and Crk demonstrate these functional transitions (Fig. 6.6). Both proteins can exist in two conformational states: a folded, inactive state, in which some of the binding domains are unavailable for interaction; and an active, extended form, in which most or all of the binding domains are exposed (Critchley, 2005; Feller, 2001). Crk, for example, is locked in a folded state by an interaction between its C-terminal SH2 domain, and phosphorylated tyrosine 221, located close to its N-terminus. This tyrosine residue is phosphorylated by Abl, which can also bind to Crk in the folded conformation. Dephosphorylation of Crk by phosphotyrosine phosphatase (PTP)-1B opens up the protein, rendering its SH2 domain available for binding to one of several phosphoproteins, including paxillin, cas and cortactin. This binding depends on the phosphorylation states of these proteins, which, in turn, are regulated by specific tyrosine kinases (e.g. Src, FAK, Abl) and phosphatases (e.g. PTP-PEST, SHP2). At the same time, the SH3 domain of Crk can bind to one of several GEFs for Rac, Rho or Ras, or to Abl or JNK (Feller, 2001).

It is likely that, following the same principle, unlocking of the pivotal adapter protein talin has major effects on its activity. In its conceived folded conformation, talin would still be able to bind PIP5K, which, when localized to the membrane, catalyzes the formation of PIP2. Following PIP2 binding, talin would undergo a conformational change, revealing binding sites for actin, vinculin and integrin, which are essential for its localization in the adhesion site and for integrin activation (Critchley, 2005). The binding of talin to PIP5K, integrin or layilin, through its FERM domain, is mutually exclusive, whereas its binding to FAK and actin can co-exist with any of the
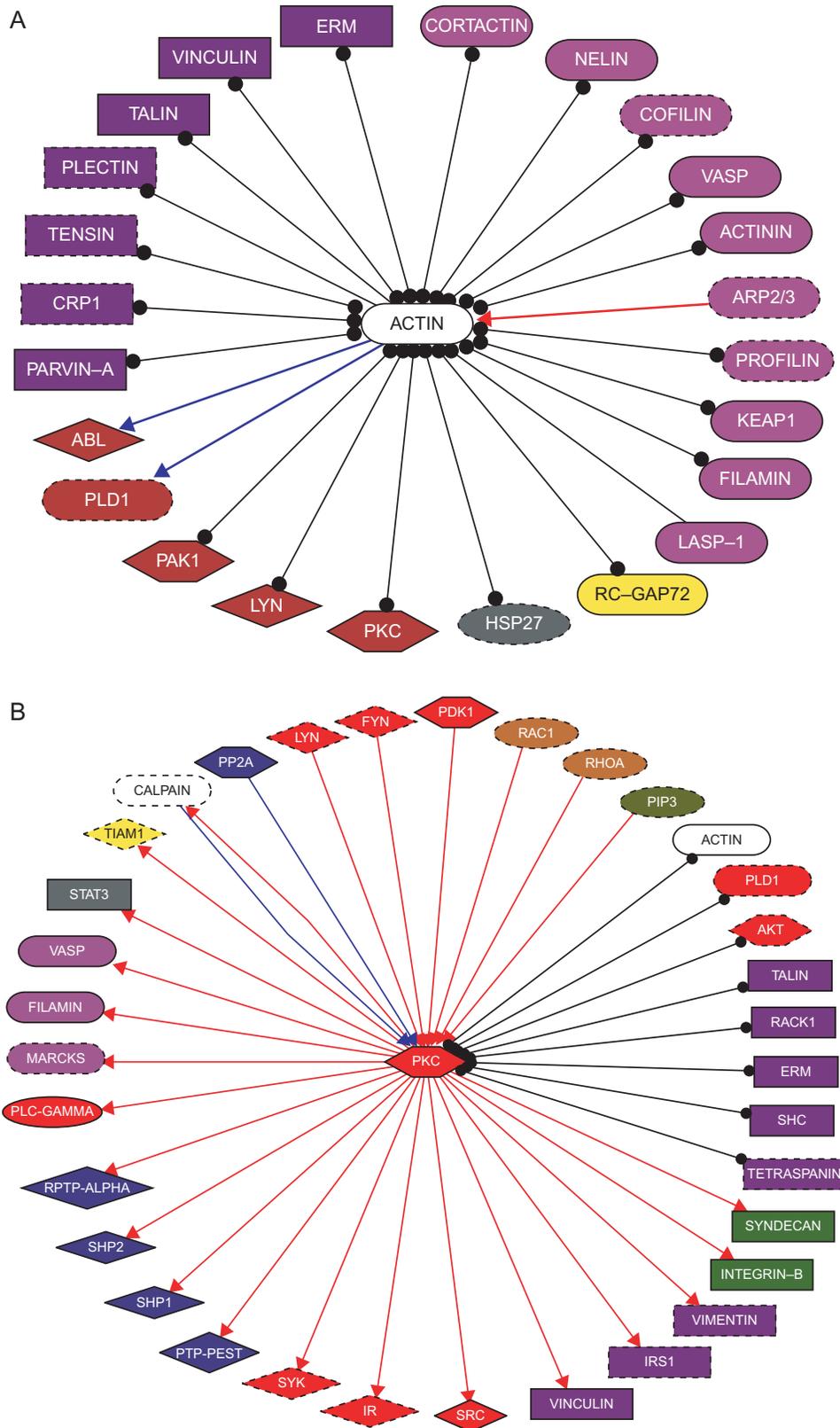
FIGURE 6.5   Protein Entourages of Actin and PKC.

Depicting the direct partners of a single component, in this case (A) actin or (B) protein kinase C (PKC). As in Figure 6.1, directional interactions are depicted by arrows, red for positive inputs and blue for negative inputs; black lines denote binding interactions.

TABLE 6.1   Domains Associated with Adhesome Components

Domain	Function	P-value enrichment in:	
		Intrinsic Adhesome proteins	Associated Adhesome proteins
SH3 (Src homology 3) IPR001452	Mediates specific protein–protein interactions, by binding to PXXP-containing sequence motifs in target proteins	1.01E-10[***]	0.02170[***]
PH (Pleckstrin homology) IPR011993	Recognizes phosphoinositide headgroups and can target its host protein to the plasma membrane through its association with phosphoinositides	6.64E-05[***]	0.19098
SH2 (Src homology 2) IPR000980	Interacts with high affinity to phosphotyrosine-containing target peptides in a fashion that differs from one SH2 domain to another, and is strictly phosphorylation-dependent	1.42E-09[***]	0.00055[***]
LIM IPR001781	A zinc-binding, cysteine-rich motif consisting of two tandemly repeated zinc fingers. Appears to mediate protein–protein interactions	7.66E-09[***]	0.26090
Tyrosine kinase IPR008266	This signature contains the active site aspartate residue, which is specific to tyrosine protein kinases	0.02917[***]	0.00388[***]
Tyrosine phosphatase IPR000242	Tyrosine-specific protein phosphatase	0.00973[***]	0.00113[***]
FERM IPR009065	Responsible for PIP2-regulated membrane binding of proteins, and is also postulated to mediate PIP2-dependent protein–protein interactions	2.63E-07[***]	1
CH (Calponin homology) IPR001715	Two CH domains in tandem form an F-actin binding region and cross-link actin filaments into bundles and networks. A subset of CH domains act as regulatory domains or protein–protein interaction scaffolds to modulate the activity of proteins in which they are present	0.00293[***]	0.21320

The eight domains that appear in adhesome proteins significantly more than in the annotated human genome. This was calculated as follows: the number of times a domain appears in intrinsic or associated adhesome proteins was compared with the number of times the same domain appears in the 12 600 human genes in the Uniprot database

PIP2, phosphatidylinositol bisphosphate.

[***]Domain regarded as significantly enriched (P value less than 0.0292, which corresponds to a false discovery rate of 10%).

aforementioned interactions. Talin can also be cleaved by calpain (Critchley, 2005).

All in all, the enzymatic and binding activity of almost every protein in the adhesome is regulated by other components. For example, the binding of adapters containing SH2 domains (e.g. Grb2, Nck2, Shc) to phosphoproteins (e.g. Cas, HEF1, Gab1) is regulated by tyrosine kinases and phosphatases (e.g. Src, Csk, Shp2, RPTP-alpha), which in turn are regulated by S/T kinases (e.g. PKA, PKC), which themselves can be activated by Rho GTPases. Rho GTPases also activate PtdIns kinases, the products of which, PIP2 and PIP3, affect the actin-binding capabilities of proteins (e.g. vinculin, filamin, ERM) and regulate the enzymatic activity of kinases and phosphatases (e.g. AKT, SHP1). On the whole, more than half of the scaffold
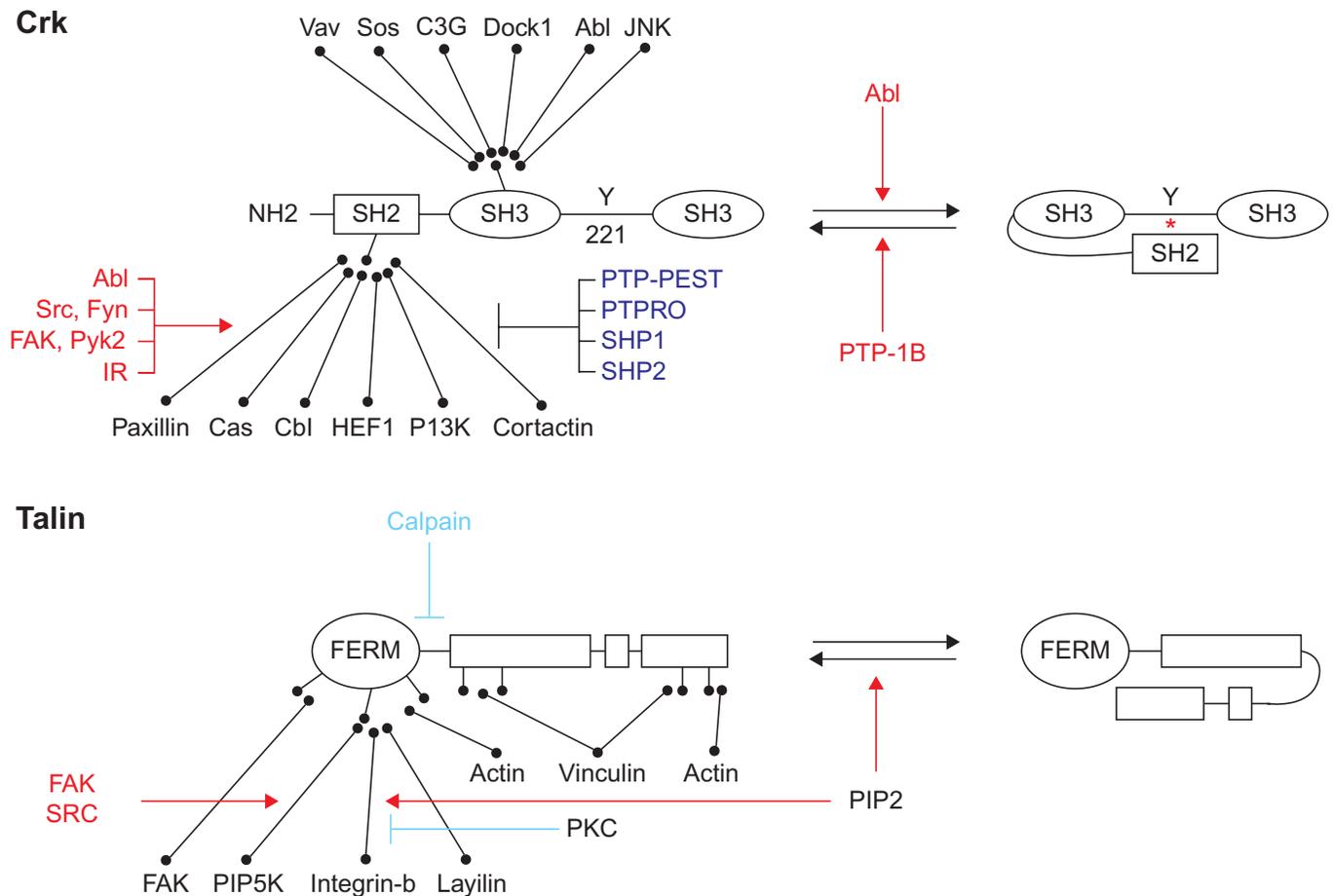
FIGURE 6.6   Protein Domains and Switchable Links of Crk and Talin.
The domains and potential interactions of two adapter proteins, Crk and talin, are presented. Many of their potential interactions can be turned on or off by regulatory modifications ("switches"), which are depicted in color (red = positive, blue = negative).

proteins in the adhesome are subjected to modification by their signaling partners, suggesting that such modifications can play a critical role in regulating the formation and turnover of the adhesion machinery.

CONCLUSION

In this chapter, we have explored the literature available on the molecular constituents of integrin adhesions and their interactions, in order to construct a global molecular network. consisting of more than 150 protein components and 680 links, defined as the integrin adhesome.

Our objective was to divide the adhesome into simpler functional subnets and model their possible interactions at several hierarchical levels. In the adhesome, these interactions are dominated by links that can be switched on or switched off by associated signaling elements. This modular view of the adhesome reveals novel design principles that govern the molecular and functional architecture of these adhesions, and open up new possibilities for their controlled perturbation. We propose that the dissection of complex molecular circuits into functional, interacting units might serve as a general tool for understanding the integrative activity of complex biological networks.

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