FOCAL ADHESIONS, CONTRACTILITY, AND SIGNALING

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ABSTRACT

Focal adhesions are sites of tight adhesion to the underlying extracellular matrix developed by cells in culture. They provide a structural link between the actin cytoskeleton and the extracellular matrix and are regions of signal transduction that relate to growth control. The assembly of focal adhesions is regulated by the GTP-binding protein Rho. Rho stimulates contractility which, in cells that are tightly adherent to the substrate, generates isometric tension. In turn, this leads to the bundling of actin filaments and the aggregation of integrins (extracellular matrix receptors) in the plane of the membrane. The aggregation of integrins activates the focal adhesion kinase and leads to the assembly of a multicomponent signaling complex.

INTRODUCTION

The adhesive interactions between a cell and its surrounding extracellular matrix (ECM) regulate its morphology, migratory properties, growth, and differentiation. One model system for studying adhesion to ECM is the focal adhesion (FA). FAs are specialized sites of adhesion developed by many cells in culture. They consist of aggregated ECM receptors (integrins) that span the plasma membrane, interacting on the outside with ECM components and on the inside with bundles of actin filaments (stress fibers). Many proteins have been identified in FAs, particularly at their cytoplasmic face. Some of these proteins have predominantly a structural role, whereas others are involved in signal transduction.

The field of FA research has grown considerably since one of us participated in writing a review on FAs for this series (Burridge et al 1988). In large part, this growth reflects the increasing interest in integrin-mediated signaling and the widely recognized effect integrin-mediated adhesion has on many aspects of cell behavior. Several excellent recent reviews cover various areas of integrin-mediated signal transduction and FA research (Adams & Watt 1993, Juliano & Haskill 1993, Hemler et al 1994, Pavalko & Otey 1994, Clark & Brugge 1995, Yamada & Miyamoto 1995, Ridley 1995, Richardson & Parsons 1995), including two in Volume 11 of this series (Jockusch et al 1995, Schwartz et al 1995). Our intention here is to avoid repetition of these reviews. Thus we focus on one topic generally neglected: the relationship between contractility/isometric tension and the formation of FAs. We believe that this is an area critical to understanding not only the assembly of these structures but also the signaling that occurs within them. In this context, we discuss the low molecular weight GTP-binding protein Rho, which stimulates the formation of FAs and stress fibers and whose mode of action has generated considerable interest.

STRUCTURAL ORGANIZATION OF FOCAL ADHESIONS

Many of the structural details of FAs have been reviewed recently (Jockusch et al 1995). Structural proteins in FAs are listed in Table 1. Several appear to be

Table 1 Structural components of focal adhesions

Transmembrane	Cytoplasmic		
1. integrins	4. actin	10. zyxin	16. aciculin*
2. syndecan IV	5. α-actinin	11. CRP	17. dystrophin*
3. dystroglycan*	6. filamin	12. radixin	18. syntrophin*
	7. vinculin	13. fimbrin	19. utrophin*
	8. talin	14. profilin	
	9. tensin	15. VASP	

Components marked with an asterisk are found only in the FAs of specialized cells types. 1. reviewed by Hynes 1992; 2. Woods & Couchman 1994; 3. Belkin & Smalheiser 1996; 4. Geiger 1979; 5. Lazarides & Burridge 1975, Wehland et al 1976; 6. Mittal et al 1987, Pavalko et al 1989; 7. Geiger 1979, Burridge & Feramisco 1980; 8. Burridge & Connell 1983; 9. Wilkins et al 1986, Wilkins et al 1987, Bockholt et al 1992; 10. Beckerle 1986; 11. Sadler et al 1992; 12. Sato et al 1992; 13. Bretscher 1981; 14. listed here because of the binding to VASP; Reinhard et al 1995a; 15. Reinhard et al 1992; 16. Belkin et al 1994; 17, 18. Kramarcy & Sealock 1990; 19. Belkin & Burridge 1995.

universal FA constituents, whereas others are cell-type specific (e.g. dystrophin, aciculin, etc). It has not been determined whether some of these components have structural or signaling functions.

Integrins and Other Transmembrane Components

The major transmembrane components in FAs are integrins, a large family of transmembrane heterodimers (Hynes 1992). Integrins are classified into several families according to their β subunits. Most β chains can pair with multiple α subunits, and a few α subunits pair with more than one β subunit (Hynes 1992). The α and β subunits each span the membrane once and have large extracellular and short intracellular domains (with the exception of the β_4 cytoplasmic domain, which is 1000 amino acids long and interacts with the intermediate filament cytoskeleton rather than with microfilaments). Most of the integrins involved in formation of focal adhesions are members of the β_1 or β_3 families. Pairing different α subunits with these β chains generates receptors for many different ECM proteins. A major requirement for FA development is that the ECM provides a ligand for integrins expressed on the cell surface. The type of integrin found within focal adhesions is generally dictated by the ECM to which cells adhere (Singer et al 1988, Dejana et al 1988, Fath et al 1989). However, a situation has been described in which a laminin-binding integrin, $\alpha_6\beta_1$, not known to interact with fibronectin, was detected in the FAs of cells adhering to fibronectin (Cattelino et al 1995). For cells grown in serum, vitronectin is typically the ECM protein adsorbed to the glass or plastic substratum, and cells expressing the vitronectin receptor $\alpha_v\beta_3$ will develop FAs containing this integrin. Probably the most frequently studied FAs are those

made by cells plated on surfaces coated with fibronectin. These FAs involve the major fibronectin receptor $\alpha_5\beta_1$.

Both the vitronectin receptor $\alpha_v\beta_3$ and the fibronectin receptor $\alpha_5\beta_1$ recognize the sequence RGD within their respective ligands (Hynes 1992; Ruoslahti, this volume). Massia & Hubbell studied the adhesion of cells plated directly onto RGD peptides covalently attached to substrates. At very low densities, cells attach but do not spread. At higher densities, when the spacing between peptides reaches $\approx$ 440 nm, cells spread but do not form FAs. However, at still higher densities of RGD peptides, with a spacing of $\approx$ 140 nm or less, spreading is accompanied by development of FAs (Massia & Hubbell 1991). This requirement for a high density of integrin ligands suggests that the interacting integrins need to be clustered in order for FAs to form.

The development of FAs has been studied in cells adhering to cell-binding fragments of fibronectin that contain the RGD sequence. Whereas some investigators observed FAs on these domains of fibronectin (Singer et al 1987), others noted their failure to form (Beyth & Culp 1984, Izzard et al 1986, Woods et al 1986) or that the FAs were small and abnormal (Streeter & Rees 1987). Woods and coworkers found that assembly of FAs on the cell-binding fragment of fibronectin was stimulated by addition of a proteoglycan-binding domain of fibronectin (Woods et al 1986). Alternatively, the cells could be stimulated with phorbol esters to activate protein kinase C (PKC) (Woods & Couchman 1992). The reason why FA formation on the cell-binding fragment of fibronectin requires this additional stimulation has been a puzzle, given that simple clustering of integrins with antibodies seems to mimic their formation. A possible explanation has been suggested by Hotchin & Hall (1995) who have found that active Rho is required for FAs to form on intact fibronectin. In some situations, PKC stimulation has been reported to activate Rho (Hall 1994), which raises a question about the state of Rho in the experiments examining FA formation on fibronectin fragments. Was Rho relatively inactive in the cells that failed to form FAs on fibronectin fragments, and did it become activated by the proteoglycan-binding domains of fibronectin or by PKC stimulation? The role of Rho in clustering integrins into FAs is discussed below.

A transmembrane proteoglycan, syndecan 4, has been identified in the FAs of many different cell types adhering to various ECM proteins (Woods & Couchman 1994). In serum-starved cells, syndecan 4 is absent from FAs but can be recruited by stimulating with serum or by activating PKC (Baciu & Goetinck 1995). When cells are plated on fibronectin in the presence of serum, the localization of syndecan 4 to FAs increases with time and is more associated with mature FAs rather than with newly forming ones at the leading edge (Baciu & Goetinck 1995). The late recruitment of syndecan 4 to FAs suggests that it

is not involved in the early signaling events associated with FA formation. It will be important to investigate the interactions of the syndecan cytoplasmic domain with structural and signaling components.

Dystroglycan (cranin, laminin-binding protein 120) (Smalheiser & Schwartz 1987, Douville et al 1988, Ibraghimov-Beskrovnaya et al 1992, 1993, Gee et al 1993, Smalheiser & Kim 1995) is a transmembrane receptor for laminin, unrelated to integrins that has been identified in the FAs of several cell types (Belkin & Smalheiser 1996). It has been studied mainly in the context of skeletal muscle, where it binds dystrophin and is in a complex with several dystrophin-associated glycoproteins (Ibraghimov-Beskrovnaya et al 1992, Ervasti & Campbell 1993a,b). However, dystroglycan is quite widely distributed (Smalheiser & Schwartz 1987, Ibraghimov-Beskrovnaya et al 1993). It remains to be determined whether its presence in FAs is due to a direct association with some of the major structural proteins of FAs, such as talin, vinculin and α-actinin, or whether it is associated indirectly via dystrophin or utrophin which, in turn, may be interacting with other components at these sites.

Integrin Cytoplasmic Domains

The cytoplasmic domains of integrins are critical for FA formation. Many of their structural features and interactions have been reviewed by Sastry & Horwitz (1993). The role of the cytoplasmic domains in targeting β_1 and β_3 integrins to FAs has been studied using mutations, deletions, and chimeric constructs. Deletion of the β_1 cytoplasmic domain blocks FA localization of integrin heterodimers (Solowska et al 1989, Marcantonio et al 1990, Hayashi et al 1990), whereas chimeric constructs containing the β_1 cytoplasmic domain fused to unrelated receptors target to FAs (Geiger et al 1992b, LaFlamme et al 1992). Consistent with this, deletion of α cytoplasmic domains drives integrin heterodimers to FAs (Briesewitz et al 1993, Ylanne et al 1993). These results indicate that the β subunit cytoplasmic domain is responsible for targeting an integrin to FAs, whereas the α subunit cytoplasmic sequence inhibits FA localization. Ligand binding relieves this inhibition (LaFlamme et al 1992). The relationship of the α and β subunit cytoplasmic domains has not been fully resolved, but in the nonligand-bound state, the two cytoplasmic domains appear to be in a close proximity (Briesewitz et al 1995). Following ligand binding to the extracellular domain, a conformational change is transmitted to the cytoplasmic domains, allowing the β cytoplasmic region to interact with focal adhesion components. Deletion of the α cytoplasmic domain or expression of the β cytoplasmic domain on its own as a chimeric protein appears to mimic the ligand-bound state.

A key question is whether ligand occupancy increases the affinity of interaction between integrins and cytoskeletal proteins. Miyamoto and colleagues compared the effects of integrin clustering with ligand occupancy (Miyamoto et al 1995a). Integrins were aggregated on the surface of cells using beads coated with non-inhibitory antibodies or with antibodies that block integrin function. Integrin clustering by non-inhibitory antibodies stimulates the colocalization of tensin and focal adhesion kinase (FAK), but not talin, vinculin, α-actinin, or actin. Clustering integrins with inhibitory antibodies (which mimic ligand binding), or with non-inhibitory antibodies in the presence of soluble ligands, stimulates co-clustering of talin, vinculin, α-actinin, and actin. These results argue that, while clustering alone will recruit some proteins, additional proteins are recruited in response to the combination of clustering and ligand occupancy. It may be that ligand occupancy is itself sufficient to induce this association between integrins and these cytoskeletal proteins, but this is difficult to resolve experimentally. Earlier work by this group, in which soluble ligands were seen to drive integrins into FAs formed by other integrins, strongly supports the idea that integrin occupancy is itself sufficient to induce cytoskeletal association with integrins (LaFlamme et al 1992).

Alternatively spliced cytoplasmic domains have been identified for both β_1 and β_3 integrin subunits. These differ in their ability to support FAs or target to these structures. In humans, four alternatively spliced isoforms of β_1 have been identified and are referred to as β_{1A} and β_{1B} (Balzac et al 1993, 1994), β_{1C} (Languino & Ruoslahti 1992), and β_{1D} (van der Flier et al 1995, Zhidkova et al 1995, Belkin et al 1996). Recent genomic analysis of the mouse β_1 integrin gene located only the exons for β_{1A} and β_{1D} (Baudoin et al 1996), which raises the possibility that the β_{1B} and β_{1C} variants emerged late in the evolution of the β_1 integrin family. β_{1A} is the predominant and original isoform identified. In humans, β_{1B} and β_{1C} appear to be relatively minor forms and are not found in FAs.

β_{1B} may be involved in regulation of adhesion and migration (Balzac et al 1993, 1994), whereas β_{1C} exerts a negative effect on growth (Meredith et al 1995, Fornaro et al 1995) (discussed below). In contrast to the low levels of expression of β_{1B} and β_{1C}, the major β_1 integrin isoform in striated muscles is β_{1D} (van der Flier et al 1995, Zhidkova et al 1995, Belkin et al 1996), which is found at all sites of actin filament-membrane attachment (Belkin et al 1996). Unlike the other isoforms, β_{1D} and β_{1A} share significant sequence homology in their alternatively spliced regions. β_{1D} is found in FAs, both in cultured myotubes and when transfected into nonmuscle cells (Belkin et al 1996). It has been speculated that β_{1D} interacts not only with the structural proteins of FAs but also with the specialized cytoskeletal proteins that line the

muscle sarcolemma (Belkin et al 1996). β_1 integrins appear to associate with the dystrophin-containing lattice in muscle development (Lakonishok et al 1992). The forces transmitted across the sarcolemma of striated muscles are much greater than those experienced by the plasma membranes of most other cells. These high forces may have generated the need for a unique integrin isoform to provide stronger attachments to the membrane.

Cytoplasmic Proteins

Recently, the number of proteins identified at the FA cytoplasmic face has expanded greatly (Table 1). So too have the number of interactions (Table 2). It has become difficult, if not impossible, to represent this complexity adequately (Figure 1). Several components, as well as interactions, have not been included. The diagram conveys neither the dynamic nature of many of the interactions nor the stoichiometry of the components. The relative abundance of FA constituents varies considerably; FAK, paxillin, tensin, zyxin, and many signaling components are minor compared with vinculin and talin. We are confident that more FA proteins will be discovered and that the complexity of interactions will increase further. Space limitations restrict our discussion to a few topics relevant to the assembly of these structures. More information about FA structural proteins is given elsewhere (Jockusch et al 1995).

One striking observation is that a large number of FA proteins bind actin. This large number hints at redundancy in the linkage of actin filaments to integrins (and possibly to other transmembrane components in FAs). Some of the actin-binding proteins may function at different stages in the life of a FA. It has been suggested, for example, that talin and vinculin may be involved in FA formation, whereas α-actinin may be more important in maintaining or stabilizing microfilament attachment in mature FAs (DePasquale & Izzard 1987, 1991, Pavalko & Burridge 1991, Nuckolls et al 1992). Multiple linkages may also facilitate actin polymerization, permitting addition of actin monomers while

Table 2 Interactions between structural proteins in focal adhesions

	Integrin	Actin	α-actinin	Fimbrin	Profilin	Radixin	Talin	Tensin	VASP	Vinculin	Zyxin
Integrin	—	—	1	—	—	—	2	—	—	—	—
Actin	—	—	3	4	5	6	7	8	9	10	—
α-actinin	1	4	—	—	—	—	—	—	—	11	12
VASP	—	9	—	—	13	—	—	—	—	—	14
Vinculin	—	10	—	—	—	—	15	16	—	—	—

1. Otey et al 1990; 2. Horwitz et al 1986; 3. Burridge & Feramisco 1980; 4. Bretscher 1981; 5. Lassing & Lindberg 1985; 6. Tsukita & Hieda 1989; 7. Muguruma et al 1990, Kaufmann et al 1991; 8. Wilkins et al 1987, Lo et al 1994; 9. Reinhard et al 1992; 10. Menkel et al 1994, Johnson & Craig 1995a; 11. Belkin & Koteliansky 1987, Wachsstock et al 1987; 12. Crawford et al 1992; 13. Reinhard et al 1995a; 14. Reinhard et al 1995b; 15. Burridge & Mangeat 1984; 16. Wilkins et al 1987.

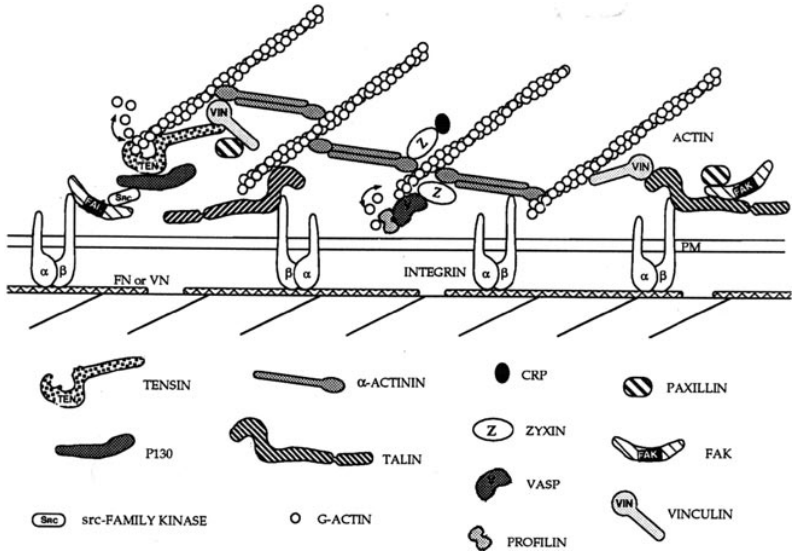

Figure 1 A focal adhesion. The model shows some of the interactions determined in vitro for proteins within focal adhesions formed on a surface coated with fibronectin (FN) or vitronectin (VN). For the sake of clarity, so that linking proteins can be visualized, integrins are shown dispersed rather than clustered. They are linked to actin microfilaments via talin, vinculin and/or α-actinin. Actin polymerization at these sites may involve either tensin or profilin, which interacts with VASP, a zyxin-binding protein. A few of the signaling components (FAK, paxillin, p130^{cas}, src family kinases) have also been included; however, several FA proteins and many of the interactions have been omitted. It should be noted that the diagram misrepresents the stoichiometry of components in focal adhesions, i.e. some such as vinculin and talin appear to be many times more abundant than others, such as FAK, paxillin, tensin, and zyxin.

simultaneously maintaining attachment to the membrane. It is noteworthy that some FA proteins, including tensin and talin, have multiple actin-binding sites (Lo et al 1994; L Hemmings et al, manuscript submitted). These may also permit subunit addition while maintaining attachment, as has been suggested for insertin, a fragment of tensin (Weigt et al 1992, Ruhnau et al 1989). Additionally, multiple binding sites may cross-link actin filaments and thus stabilize attachments to the membrane.

The attachment of actin filaments to integrins in FAs remains poorly understood. Both talin and α-actinin were shown to bind to integrins in vitro with relatively low affinity (Horwitz et al 1986, Otey et al 1990). A talin-binding site within the β_{1A} cytoplasmic domain corresponding to residues 780–789 was mapped using synthetic peptides (Tapley et al 1989). Work from this laboratory,

however, indicates that sequences along most of the length of the cytoplasmic domain contribute to talin binding (K Simon, unpublished observations). In the case of α-actinin, peptide-binding studies indicate that two regions in the cytoplasmic domain are involved, residues 768–778 and 785–794 (Otey et al 1993). It has been difficult to demonstrate that these interactions also occur in vivo. Working with neutrophils, Pavalko and coworkers demonstrated coimmunoprecipitation of α-actinin and β_2 integrin (Pavalko & LaRoche 1993). In an attempt to get at the in vivo associations of proteins with integrin cytoplasmic domains, Lewis & Schwartz (1995) transfected full-length or truncated chicken β_1 integrins into mouse fibroblasts and examined the colocalization of proteins with these integrins after they had been clustered with antibody-coated beads. This provided a way to map regions of the β_1 cytoplasmic domain responsible for recruiting specific proteins. Deletion of the last 13 residues at the C-terminus inhibits colocalization of talin, actin, and FAK with clustered integrins. Interestingly, α-actinin continues to codistribute with integrins; this is blocked by deletion of an additional 15 residues. These experiments support the idea that talin binding to integrins is required for attachment of actin and that, on its own, α-actinin is insufficient to recruit actin filaments to clustered integrins.

The localization of α-actinin to FAs has been controversial. Although it is usually listed as a FA component, it is often not detected in FAs by immunofluorescence or electron microscopy even though it is prominent along the associated stress fibers (Zigmond et al 1979, Chen & Singer 1982, Samuelsson et al 1993). However, when labeled α-actinin is microinjected, it readily targets to FAs (Feramisco 1979, McKenna et al 1985, Sanger et al 1987). Pavalko and coworkers provided evidence that the inability to detect α-actinin in FAs by immunofluorescence results from inaccessibility of the α-actinin to antibodies (Pavalko et al 1995). FAs are dense structures and this may limit antibody accessibility.

Vinculin is one of the most abundant FA proteins, interacting with talin, α-actinin, tensin, and paxillin. For many years, the reported interaction of vinculin with actin was controversial. The demonstration by Jockusch's group that a vinculin tail domain construct binds actin (Menkel et al 1994) was difficult to reconcile with the inability of several groups to find significant actin binding with intact vinculin (e.g. Evans et al 1984, Otto 1986). A breakthrough came with the discovery by Johnson & Craig of an intramolecular, head-tail interaction within vinculin (Johnson & Craig 1994). This interaction masks binding sites for both talin and actin in vinculin's head and tail domains, respectively (Johnson & Craig 1994, 1995a). It seems likely that exposure of these sites is an important event in the assembly of FAs. It is notable that vinculin binds acidic phospholipids (Niggli et al 1986, Isenberg 1991), in particular PIP_2

(Fukami et al 1994), and that the binding site for PIP_2 is located in the hinge region between the head and tail domains (Johnson & Craig 1995b). These observations prompted examination of the effects of lipids, specifically PIP_2, on the conformation of vinculin. It was shown that the head-tail interaction of vinculin could be dissociated by acidic phospholipids and most effectively by PIP_2 (Weekes et al 1996, Gilmore & Burridge 1996). This exposed the binding sites for actin and talin, and a potential PKC phosphorylation site in the tail of vinculin. Vinculin immunoprecipitated from adherent cells contained PIP_2, but not other phospholipids, consistent with PIP_2 being the physiological effector of the conformational change (Gilmore & Burridge 1996).

Significantly, PIP_2 levels are elevated in response to adhesion in a Rho-dependent manner (Chong et al 1994). These observations are discussed in more detail below in the context of FA assembly.

Zyxin is one of the less abundant proteins present in FAs and adherens junctions (Beckerle 1986). This 82-kDa protein, expressed most prominently in fibroblasts and muscle cells, contains a proline-rich stretch at the C-terminus, involved in binding α-actinin (Crawford et al 1992), and three N-terminal LIM domains (Sadler et al 1992). LIM domains, each composed of two histidine- and cysteine-rich zinc fingers, mediate specific protein-protein interactions (Schmeichel & Beckerle 1994). They have been identified in a number of proteins, some of which are transcription factors, whereas others, such as the cysteine-rich proteins (CRP), are made up almost entirely of LIM domains. Zyxin does not contain an obvious nuclear targeting sequence, but truncation of the proline-rich N-terminus induces nuclear localization of zyxin (Nix & Beckerle 1995). In addition, a 44-amino acid sequence within the proline-rich region localizes zyxin to FAs. This raises the exciting possibility that zyxin shuttles between FAs and the nucleus in response to other factors interacting with this domain. The first LIM domain of zyxin binds to CRP, which is also localized in FAs (Sadler et al 1992, Crawford et al 1994). Expression of CRP proteins is tightly regulated during myogenesis and these proteins have been shown to potentiate muscle differentiation.

Another interesting interaction occurs between zyxin and VASP (M Reinhard et al 1995). VASP (vasodilator-stimulated phosphoprotein) was originally identified in platelets (Halbrugge & Walter 1989) as a major substrate of the cAMP-dependent protein kinase (PKA) and the cGMP-dependent protein kinase (PKG), both of which are stimulated by agents that inhibit platelet activation. Subsequently, VASP was identified in fibroblast FAs and along stress fibers, as well as in membrane ruffles (Reinhard et al 1992, Haffner et al 1995). VASP contains a polyproline-rich sequence that binds profilin (M Reinhard et al 1995), thus indicating a possible function in the control of actin polymerization.

This idea has received strong support from studies on the movement of *Listeria monocytogenes. Listeria* moves inside host cells by rapid polymerization of actin filaments at one pole of the bacterium (Pollard 1995). Both profilin and VASP have been identified at this site (Theriot et al 1994, Chakraborty et al 1995). VASP binds to the *Listeria* protein ActA (Pistor et al 1995), which is responsible for bacterial motility, and suggests that VASP provides a bridge between the bacterium and the microfilament system. Some sequence homology exists between ActA and zyxin, and these proteins even cross react immunologically (R Goldsteyn & E Friederich personal communication). These observations suggest that zyxin, VASP, and profilin function as a complex, regulating the nucleation of actin polymerization at FAs and equivalent sites in cells. How important actin polymerization is in a stable FA is not clear. It is probably more important in the initial stages of FA development, during the assembly of stress fibers and FAs. This may explain why profilin is generally not detected in mature FAs (Buss et al 1992). An unanswered question concerns the role of VASP phosphorylation by the cyclic nucleotide-dependent protein kinases.

CONTRACTILITY: INFLUENCE ON THE FORMATION OF STRESS FIBERS AND FOCAL ADHESIONS

It had been suggested that stress fibers contract isometrically and that this isometric tension contributes to their assembly (Heath & Dunn 1978, Burridge 1981). Numerous observations contributed to this hypothesis. For example, stress fibers contain many of the contractile proteins found in muscle, including actin and myosin II, and these are arranged in a sarcomeric type of organization (Weber & Groesche-Stewart 1974, Lazarides & Burridge 1975, Gordon 1978, Kreis & Birchmeier 1980). Although this pattern is suggestive of a contractile function, stress fibers are rarely seen to shorten in living cells. However, they are potentially contractile, as revealed in permeabilized cells exposed to ATP (Isenberg et al 1976, Kreis & Birchmeier 1980, Crowley & Horwitz 1995). It was argued that shortening usually cannot occur because of the strong adhesion at FAs to a rigid substratum, which generates isometric tension. A visible manifestation of tension exerted on the underlying substrate is seen when cells are grown on flexible silicone rubber surfaces. Fibroblasts and other cells that have prominent stress fibers visibly wrinkle these substrates (Harris et al 1980). Detachment of cells from the rubber results in the rapid loss of wrinkles. Evidence that isometric tension can generate bundles of microfilaments comes from studies using cytoplasmic extracts of *Physarum* induced to contract under isotonic or isometric conditions (Fleischer & Wohlfarth-Bottermann 1975).

Isometric contraction generates dense arrays of microfilaments, reminiscent of stress fibers. These structures rapidly disassemble upon the release of tension, just as stress fibers disassemble when cells are detached from a substrate. In contrast, when the cytoplasmic extracts are stimulated to contract under isotonic conditions, shortening is not accompanied by the development of large bundles of microfilaments (Fleischer & Wohlfarth-Bottermann 1975).

Much additional evidence supports the idea that isometric tension contributes to the formation of stress fibers. For example, fibroblasts contract collagen gels in which they are suspended. Free-floating gels may be contracted over several days to as little as 10% of their original size, and the fibroblasts in these gels lack stress fibers. If the gels are anchored, isometric tension is generated and the fibroblasts develop prominent stress fibers (Mochitate et al 1991, Tomasek et al 1992, Tomasek & Haaksma 1991, Grinnell 1994, Halliday & Tomasek 1995). Upon release of tension in anchored gels, there is a rapid contraction of the gel followed by disassembly of the stress fibers (Mochitate et al 1991, Tomasek et al 1992, Grinnell 1994). Application of tension to cells in culture also stimulates the formation of stress fibers (Franke et al 1984). When tension is applied to a localized region of the cell surface, a bundle of actin filaments is induced immediately subjacent to the site of applied tension (Kolega 1986).

Danowski demonstrated that when contractility is stimulated in fibroblasts by microtubule depolymerization, formation of stress fibers is induced (Danowski 1989). Under these conditions, FAs are also stimulated to form (B Geiger & A Bershadsky, personal communication; M Chrzanowska-Wodnicka, unpublished observations). How microtubule depolymerization stimulates contractility has not been established. One set of models has invoked "tensegrity" theories, in which microfilaments are viewed as tension-generating elements and microtubules as compression-resisting struts (Ingber 1993, Buxbaum & Heidemann 1988). The collapse of the compression-resisting elements, the microtubules, should lead to increased contractility. However, an alternative interpretation has been suggested by Kolodney & Elson, who have demonstrated enhanced myosin light chain (MLC) phosphorylation in response to microtubule depolymerization. This suggests that microtubule depolymerization directly activates actomyosin contractility, rather than simply removing an opposing structural element (Kolodney & Elson 1995).

Agents that inhibit contractility promote stress fiber and FA disassembly. For example, stress fibers are disrupted by microinjection of antibodies that bind to the myosin rod domain and inhibit myosin filament assembly (Honer et al 1988). Butanedione monoxime (BDM) inhibits myosin ATPase activity (Higuchi & Takemori 1989, McKillop et al 1994, Cramer & Mitchison 1995), decreases fibroblast contractility, and leads to the reversible disassembly of

both stress fibers and FAs (Chrzanowska-Wodnicka & Burridge 1996). It has been long known that high cAMP levels disrupt stress fibers in many cell types. Pursuing the basis for this, Lamb and coworkers related the effects of high cAMP to decreased MLC phosphorylation (Lamb et al 1988). Elevated PKA activity disrupts stress fibers and leads to phosphorylation of the MLC kinase (MLCK), which has been correlated with decreased activity in vitro. MLCK is a key protein regulating nonmuscle actin-myosin interaction and contractility (see below). Further evidence for a role for MLCK in stress fiber stability was obtained by microinjection of antibodies against the MLCK into cells. The antibodies immunoprecipitated MLCK and led to the disassembly of stress fibers (Lamb et al 1988). The FAs were not examined in these experiments, but in a separate study, elevation of cAMP in the same cell type disrupted FAs (Turner et al 1989). Pharmacological agents that inhibit MLCK also inhibit contractility and lead to stress fiber and FA disassembly (Volberg et al 1994, Chrzanowska-Wodnicka & Burridge 1996). MLC phosphorylation can also be modulated by protein phosphatases. Elevating the level of protein phosphatase 1 (PP1) in fibroblasts by microinjection decreases MLC phosphorylation, which leads to a rapid loss of stress fibers (Fernandez et al 1990).

Taken together, this large body of evidence supports the idea that isometric tension induced by contractility drives the formation of stress fibers and FAs. Conversely, these structures disassemble when contractility is inhibited or tension is released. Some agents that stimulate formation or disassembly of FAs may exert their primary effects on contractility. For this reason, and because nonmuscle contractility can be modulated in many different ways, we discuss the regulation of contractility in the next section.

Regulation of Contractility

Much of what is known about the regulation of vertebrate nonmuscle contractility is derived from work on vertebrate smooth muscle. Many, but not all, of the regulatory components are present in both systems. In smooth muscle and nonmuscle cells, the dominant regulatory system involves phosphorylation of the myosin regulatory light chains (20 kDa) by a MLCK (Figure 2). MLCK phosphorylates both Ser-19 and Thr-18 (Sellers 1991, Tan et al 1992). Ser-19 is the major site of phosphorylation induced by agonists that stimulate light chain phosphorylation. Thr-18 is phosphorylated at a slower rate but becomes phosphorylated under conditions of maximal stimulation. The phosphorylation of the regulatory light chain increases the actin-activated ATPase activity of both smooth and nonmuscle myosin II (Adelstein & Conti 1975) and is required for myosin-driven actin filament movement in in vitro motility assays (Sellers 1991, Tan et al 1992). This regulation of myosin motor function may be the primary role of this light chain phosphorylation.

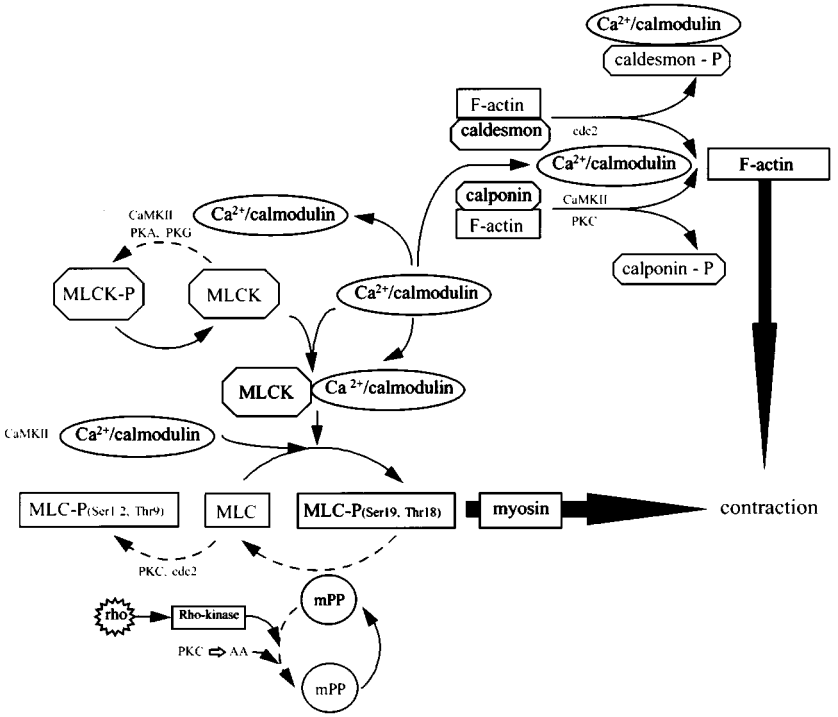

Figure 2 Regulation of contractility in smooth muscle and nonmuscle cells. The interaction of myosin with actin is regulated by phosphorylation of the regulatory myosin light chains (MLC) as well as by the actin-binding proteins, caldesmon and calponin. Active proteins are shaded, inactive are blank. MLC phosphorylation on Ser-19 and Thr-18 is catalyzed by the MLCK, which is activated by binding Ca^{2+}/calmodulin. The same sites on MLCs can also be phosphorylated by CaM kinase II (CaMKII). MLCK is inhibited by phosphorylation by PKA, PKG, or CaM kinase II. MLC dephosphorylation occurs by a myosin phosphatase (mPP), which is inhibited in smooth muscle by activated Rho-stimulates Rho-kinase, which phosphorylates mPP, thereby inhibiting it activity (Kimura et al 1996). The myosin phosphatase is also inhibited by arachidonic acid, which may be generated by activation of PKC. Inhibitory phosphorylation of MLCs occurs primarily on Ser-1 and Ser-2, but also on Thr-9 in vitro, and is catalyzed by PKC and the mitotic kinase $p34^{cdc2}$ (cdc2). The actin-binding proteins, caldesmon and calponin, inhibit the interaction of myosin with actin in the absence of Ca^{2+}. In the presence of Ca^{2+}, the inhibition is relieved either by phosphorylation of caldesmon or calponin, or by binding Ca^{2+}/calmodulin. Phosphorylation of caldesmon by $p34^{cdc2}$ also releases caldesmon from actin.

Regulatory light chain phosphorylation also induces a conformational change in the myosin monomer (Craig et al 1983, Citi & Kendrick-Jones 1986, Ikebe & Reardon 1990) that might be involved in control of thick filament assembly. Nonmuscle myosin II can exist in two conformational states. In the first, the myosin rod is folded back on itself and interacts with the myosin heads. The second conformation is elongated, resembling the classical conformation of isolated skeletal muscle myosin (Citi & Kendrick-Jones 1986). In the folded conformation, myosin cannot assemble into filaments. The equilibrium between the two conformational states is shifted by phosphorylation of Ser-19. This favors the extended conformation and promotes assembly of myosin filaments. Phosphorylation of Thr-18 stabilizes the extended conformation and may also enhance filament formation (Ikebe 1989, Ikebe et al 1988). In lower organisms, such as *Dictyostelium*, phosphorylation of the heavy chains inhibits myosin filament assembly (Tan et al 1992). In vertebrate smooth and nonmuscle cells, heavy chain phosphorylation also occurs. Whether this phosphorylation regulates myosin filament assembly has not been determined, although it seems a likely possibility.

The MLCK from smooth muscle and nonmuscle cells is regulated by Ca^{2+}/calmodulin binding. In the resting state, in the absence of intracellular calcium, an auto-inhibitory domain blocks the kinase domain within MLCK (Tan et al 1992). Elevation of intracellular calcium results in calmodulin binding to MLCK. This causes a conformational shift of the auto-inhibitory domain, which activates the kinase. The interaction of MLCK with calmodulin is additionally regulated by phosphorylation. PKA, PKG, and CaM kinase II can all phosphorylate MLCK at a site that decreases its affinity for Ca^{2+}/calmodulin, thus inhibiting MLCK activity (Nishikawa et al 1984, Tansey et al 1994). A novel isoform of MLCK (embryonic MLCK) has recently been identified in nonmuscle cells (Gallagher et al 1995). This is the predominant or only MLCK expressed by some cells, whereas elsewhere this MLCK is coexpressed with the smooth muscle isoform. This embryonic MLCK is regulated by calcium, but little else is known about its regulation. The MLCK from *Dictyostelium* does not have a Ca^{2+}/calmodulin binding site, and its regulation remains to be elucidated (Tan et al 1992).

Dephosphorylation of MLCs counteracts the effect of their phosphorylation and provides a target for regulating myosin/actin interaction. The predominant myosin phosphatase (mPP), an isoform of PP1, consists of three subunits: a 37-kDa catalytic subunit, a 130-kDa subunit that targets the phosphatase to myosin, and a 20-kDa subunit (Alessi et al 1992, Shimizu et al 1994, Shirazi et al 1994). Dissociation of the catalytic subunit from the other components decreases its activity and is one mechanism by which agonists such as arachidonic acid may

stimulate contractility (Gong et al 1992, Somlyo & Somlyo 1994). Another mechanism for regulating mPP activity is by phosphorylation of the 130-kDa myosin-binding subunit (Trinkle-Mulcahy et al 1995, Ichikawa et al 1996). In permeabilized smooth muscle cells, ATP-γ-S promoted contractility and elevated MLC phosphorylation at low calcium concentrations. This was due to decreased mPP activity and correlated with thiophosphorylation of the 130-kDa subunit of mPP (Trinkle-Mulcahy et al 1995). Studying purified mPP, Ichikawa and coworkers (1996) demonstrated the presence of a kinase associated with the phosphatase. This kinase phosphorylated the 130-kDa subunit, decreasing phosphatase activity. Kaibuchi and colleagues have shown that activated Rho stimulates Rho-kinase to phosphorylate the 130-kDa subunit of mPP, thereby inhibiting mPP activity (Kimura et al 1996) (see below).

The regulatory light chains of smooth and nonmuscle myosins are substrates for other kinases in addition to MLCK. CaM kinase II can phosphorylate Ser-19, the same site that is phosphorylated by MLCK (Tan et al 1992). However, because the affinity of CaM kinase II for Ca^{2+}/calmodulin is 100-fold less than that of MLCK, it may not be relevant under most conditions. PKC phosphorylates the regulatory light chain on three sites in vitro, Ser-1, Ser-2, and Thr-9, and the two serines have also been observed to be phosphorylated in vivo (Tan et al 1992). Phosphorylation at these sites decreases the actin-activated ATPase of myosin previously phosphorylated by MLCK and impairs myosin filament stability (reviewed in Tan et al 1992). The action of PKC is essentially antagonistic to MLCK with regard to myosin activity and contractility. $p34^{cdc2}$ also phosphorylates the regulatory light chains on Ser-1 and Ser-2 (Satterwhite et al 1992). The phosphorylation on Ser-1 and Ser-2 is high in mitotically arrested cells and remains high during mitosis until anaphase, when the contractile ring begins to develop. At this point there is decreased phosphorylation on Ser-1 and Ser-2 and increased phosphorylation on Ser-19 (Satterwhite et al 1992, Yamakita et al 1994).

These results have led to the hypothesis that light chain phosphorylation by $p34^{cdc2}$ inhibits contractility during mitosis until the onset of cytokinesis, at which point contractility is important for the contractile ring and the development of the cleavage furrow. The loss of stress fibers and FAs in mitotic cells is striking and correlates well with decreased contractility induced by $p34^{cdc2}$. This also supports the idea that the presence of stress fibers and FAs depends on contractility and that inhibition of contractility leads to disassembly of these structures.

Although light chain phosphorylation is a major regulatory target for smooth muscle and nonmuscle contractility, other regulatory proteins have also been identified (Figure 2). Caldesmon and calponin, two calcium-regulated proteins

found on smooth muscle thin filaments, inhibit the actin-activated myosin ATPase in vitro in the absence of calcium. Nonmuscle cells contain a lower molecular weight isoform of caldesmon, which lacks a central domain of the smooth muscle caldesmon. Although the nonmuscle form of caldesmon is significantly smaller, its properties appear to be similar to the smooth muscle form. Caldesmons bind both actin and myosin (Matsumura & Yamashiro 1993). Together with tropomyosin, caldesmon binding to actin filaments increases their stability and resistance to severing by gelsolin in vitro (Ishikawa et al 1989a,b). In reconstituted systems, caldesmon has been shown to inhibit tropomyosin-stimulated, actin-activated myosin ATPase (Yamashiro et al 1995). This effect of caldesmon results from its binding to actin and is lost in the presence of Ca^{2+} and calmodulin, which release caldesmon from the actin-myosin complex and permit actin-myosin interaction. Caldesmon is phosphorylated by several serine/threonine kinases in vitro (Matsumura & Yamashiro 1993), and the phosphorylation of caldesmon has been reported to affect its interactions with the actomyosin system. In particular, phosphorylation of caldesmon by $p34^{cdc2}$ may be one of the events regulating reorganization of the actin cytoskeleton during mitosis (Yamashiro et al 1990, 1991). Phosphorylated caldesmon dissociates from microfilaments, which may promote microfilament severing by gelsolin, thereby contributing to the mitotic disassembly of stress fibers.

Calponin is a 35-kDa protein found primarily in smooth muscle, although it is reported also to be also in some nonmuscle cells (Takeuchi et al 1991). It regulates myosin ATPase activity in a phosphorylation-dependent fashion. When not phosphorylated, it binds to actin and inhibits the Mg ATPase activity of myosin in vitro. Phosphorylation of calponin by PKC or the Ca^{2+}/calmodulin-dependent protein kinase II relieves this inhibition (Winder & Walsh 1990). Calponin phosphorylation increases in parallel with MLC phosphorylation as smooth muscle is induced to contract by activating with carbachol or with okadaic acid, which inhibits the intrinsic phosphatases, consistent with a role of calponin phosphorylation in regulating the contractility of smooth muscle (Winder & Walsh 1990).

RHO

A major regulator of FAs and stress fibers is the small GTP-binding protein Rho (Hall 1994, Takai et al 1995), which is a member of the ras superfamily of proteins. In mammalian cells, it belongs to a subfamily that consists of Rho, rac, cdc42, TC10, and rhoG. Each of these proteins has several closely related isoforms (Hall 1994). In addition to other functions, these Rho-related proteins regulate the organization of the actin cytoskeleton. Whereas Rho regulates FAs and stress fibers (Ridley & Hall 1992), Rac promotes membrane

ruffling (lamellipodia) (Ridley et al 1992), and cdc42 promotes the extension of microspikes (filopodia) (Nobes & Hall 1995). Here we concentrate on the effects and mechanism of Rho (Table 3).

The functions of Rho have been studied using two powerful tools: The first is the *Clostridium botulinum* C3 exotransferase (C3), which ADP-ribosylates and inactivates Rho (Narumiya et al 1988, Sekine et al 1989, Aktories & Hall 1989). The second is a recombinant, constitutively activated Rho, which is generated by mutating glycine-14 within rho's effector domain to valine (Val-14rho). This mutation is equivalent to the Val-12 activating mutation in ras, which decreases GTPase activity. The disruption of stress fibers by the C3 exotransferase first indicated a function for Rho in the regulation of actin filaments (Chardin et al 1989). This work was extended by Paterson and colleagues who introduced constitutively activated Rho into cells, stimulating their development of a contracted morphology (Paterson et al 1990). Microinjection of activated Rho into quiescent mouse fibroblasts lacking stress fibers and FAs rapidly induces these structures to form (Ridley & Hall 1992). The quiescent fibroblast system has

Table 3 Effects of Rho

	Cell type	References
Cytoskeletal effects		
Regulation of cell morphology	Vero cells	Chardin et al 1989
	Swiss 3T3 fibroblasts	Paterson et al 1990
Induction of stress fibers	Swiss 3T3 fibroblasts,	Ridley & Hall 1992
	mast cells,	Price et al 1995
	MDCK epithelia	Ridley et al 1995
Induction of actin polymerization	Mast cells	Norman et al 1994
Regulation of cell motility	Neutrophils,	Takaishi et al 1993
	mouse 308R	Takaishi et al 1994
	Swiss 3T3 cells	Stasia et al 1991
Inhibition of SF/HGF-induced motility	MDCK epithelia	Ridley et al 1995
Cell division,	*Xenopus* embryos,	Kishi et al 1993
cleavage furrow formation	sand dollar eggs	Mabuchi et al 1993
Enhancement of contractility	Smooth muscle	Hirata et al 1992
Ca^{+2} sensitization	Smooth muscle	Kokubu et al 1995
Neurite retraction and cell rounding	Neurons	Jalink et al 1994
MLC phosphorylation	Smooth muscle	Noda et al 1995
Regulation of integrins		
Inhibition of integrin-dependent adhesion to fibronectin	Monocytes	Aepfelbacher 1995
Integrin-dependent aggregation	Platelets,	Morii et al 1992
	B lymphocytes	Tominaga et al 1993
Chemoattractant-induced adhesion	Lymphocytes	Laudanna et al 1996

been employed by Rozengurt and colleagues to study the actions of growth factors and neuropeptides. In addition to inducing the reformation of stress fibers and FAs, these agents stimulate the tyrosine phosphorylation of several FA proteins, including FAK, paxillin, and $p130^{cas}$ (Zachary et al 1992, 1993, Kumagai et al 1993, Seckl & Rozengurt 1993, Seufferlein & Rozengurt 1994, 1995). Several of these agents (LPA, bombesin, endothelin, thrombin) mediate their effects via Rho (Ridley & Hall 1992, Jalink et al 1994, Rankin et al 1994). These factors act on receptors that are coupled to heterotrimeric G proteins, including $G_{\alpha 12}$ and $G_{\alpha 13}$ (Buhl et al 1995). The pathway(s) from these to Rho have not been elucidated, although there is evidence for a tyrosine kinase (PTK) upstream of Rho (Nobes et al 1995). Ultimately, these agents must converge to promote the active form of Rho with GTP bound.

Like other G proteins, Rho is active when it has GTP bound. The intrinsic GTPase activity of Rho hydrolyzes the bound GTP, rendering Rho inactive. The cycling between the GTP-bound and GDP-bound forms is directly regulated by several proteins. For the ras family of proteins, the low intrinsic rate of GTP hydrolysis is enhanced by GTPase-activating proteins (GAPs), whereas the exchange of GDP for GTP is enhanced by guanine nucleotide exchange factors (GEFs). Guanine nucleotide dissociation inhibitors (GDIs) slow the rate of GDP dissociation and thereby lock the G protein into the inactive state (Hall 1994). GEFs activate G proteins, whereas GAPs will turn them off, and GDIs maintain them in the inactive state. Several GAPs for Rho have been identified (Ridley 1995). The first, rhoGAP, has a higher activity for cdc42 (Ridley et al 1993). A second GAP for Rho, p190 (Lancaster et al 1994), prevents the formation of stress fibers and FAs when microinjected into cells (Ridley et al 1993). Recently, another form of p190 was discovered (Burbelo et al 1995). An unconventional myosin has been described with rhoGAP activity in the tail domain (J Reinhard et al 1995). One GEF for Rho is the product of the *Dbl* oncogene. The region of Dbl responsible for GEF activity (Dbl homology domain) has been identified in several other proteins including Vav, Ect2, Lbc, Ost, Tim, and Tiam (Ridley 1995), some of which may have GEF activity for Rho or other Rho family members. A rhoGDI has been identified that acts on Rho, Rac, and cdc42. Introduction of rhoGDI into cells inhibits both Rho- and rac-dependent reorganization of actin (Nishiyama et al 1994). The regulation of Rho and related proteins is clearly complex. Adding to this complexity is evidence that various members of the ras superfamily may interact via their GAPs, GEFs, and GDIs. For example, p120 RasGAP interacts with p190 RhoGAP (Settleman et al 1992). Much remains to be learned about the interplay of ras family regulatory proteins.

Downstream Targets of Rho

Several potential targets for Rho have been identified. Cell adhesion regulates the level of PIP_2. Cells put into suspension demonstrate decreased levels of PIP_2. This limits the response of suspended cells to growth factors that exert their growth stimulating effects via PIP_2 hydrolysis (McNamee et al 1993). PIP_2 synthesis from its precursor PIP, catalyzed by PIP 5-kinase, is stimulated by activated Rho (Chong et al 1994), and recently an interaction between Rho and one PIP 5-kinase isoform has been identified (Ren et al 1996, Kimura et al 1996). PIP_2 acts on several cytoskeletal proteins, promoting actin polymerization and enhancing FA formation (see below).

By analogy with ras, which activates the raf/MEK/MAP kinase pathway (Vojtek & Cooper 1995), it was expected that Rho would initiate a kinase cascade. The Rho family members, Rac and cdc42, have been shown to activate kinase pathways that parallel the ras/MAP kinase pathway (Coso et al 1995, Minden et al 1995). In addition, Rho was shown to activate one of the endpoints of these Rac and cdc42 pathways (Hill et al 1995). During the past year, two distinct serine/threonine protein kinases that interact with Rho have been identified by several groups. The first was detected initially using GTP-Rho to screen a rat brain expression library (Leung et al 1995). The sequence of this Rho-binding kinase (ROKα) appears to be the truncated form of a bovine brain kinase (Rho-kinase) retained on a GST-Rho affinity column (Matsui et al 1996). A similar kinase was isolated from human platelets using labeled GTP-Rho in ligand overlay assays, cloned and given the name $p160^{ROCK}$ (Rho-associated, coiled-coiled-containing protein kinase) (Ishizaki et al 1996). $P160^{ROCK}$ has significant sequence homology with ROKα/Rho-kinase with the differences possibly reflecting tissue isoforms or species variation. This family of kinases shares homology particularly in its kinase domain with the myotonic dystrophy kinase. Experiments with the purified Rho-kinase indicate that it has little if any kinase activity for many cytoskeletal proteins, with the striking exception of the myosin-binding subunit of the myosin phosphatase (Matsui et al 1996, Kimura et al 1996). As discussed below, this suggests an important link between Rho pathways and contractility, because phosphorylation of this subunit of the myosin phosphatase inhibits the phosphatase activity (Trinkle-Mulcahy et al 1995, Ichikawa et al 1996, Kimura et al 1996). A different serine/threonine protein kinase, PKN, that interacts with Rho was also identified by two of the above groups (Watanabe et al 1996, Amano et al 1996). In addition, the yeast two-hybrid system was used to identify rhophilin, a protein sharing homology with PKN but lacking kinase activity (Watanabe et al 1995). The discovery of these Rho-stimulated serine/threonine kinases is exciting. Each may be the first step in distinct kinase cascades initiated by activated Rho.

Rho may also regulate the affinity of certain integrins for their ligands. Some integrins require activation for binding to their ligands, a phenomenon known as inside-out signaling (for details, see Ginsberg et al 1993, Schwartz et al 1995). The platelet integrin $\alpha_{IIb}\beta_3$ is held in an inactive conformation until platelets are stimulated by thrombin or other agents that trigger blood clot formation. Following platelet stimulation, activation of $\alpha_{IIb}\beta_3$ is a late event leading to platelet aggregation. This is blocked by the C3 exotransferase (Morii et al 1992). The inhibition of platelet aggregation by C3 may reflect rho's action on the cytoskeleton, or on the activation of $\alpha_{IIb}\beta_3$ because both are involved in platelet aggregation. Members of the leukocyte β_2 integrin family similarly require activation. Cell adhesion by $\alpha_L\beta_2$ is blocked by C3 (Tominaga et al 1993). This inhibition differs from the effects of cytochalasin, indicating that the action of Rho cannot be accounted for simply by its effects on the cytoskeleton and suggesting direct effects on integrin activation. The use of C3 has implicated Rho in the activation of β_1 and β_2 integrins in response to chemoattractants (Laudanna et al 1996). The original observations that C3 causes rounding up of cells are also consistent with C3 decreasing adhesion by blocking an effect of Rho on integrins (Chardin et al 1989, Paterson et al 1990). Together, these results suggest that Rho may activate integrins, increasing their interaction with ECM ligands, although the mechanism is far from clear.

Rho and Contractility

Rho is a potent stimulator of stress fibers and focal adhesions. As discussed earlier, contractility and isometric tension also contribute to stress fiber and FA formation. This leads to the question: Does Rho induce formation of these structures by stimulating contractility? Several lines of evidence support this idea. Many of the agents that activate Rho, such as LPA, endothelin, bombesin, and thrombin, are vasoconstrictors, which stimulate smooth muscle contraction. Direct evidence for an effect of Rho on contraction comes from studies with permeabilized smooth muscle. In this system, introduction of activated Rho enhances contractility at given calcium concentrations and lowers the calcium requirement for contractility (Hirata et al 1992). Rho has been implicated in several other contractile events (Table 3). For example, microinjection of C3 inhibits the development of the contractile ring, a transient bundle of microfilaments responsible for generating the cleavage furrow and cytokinesis (Mabuchi et al 1993, Kishi et al 1993). Some Rho-activating agents appear to stimulate contraction of cells other than smooth muscle. For example, thrombin stimulates neurite retraction (Jalink & Moolenaar 1992, Suidan et al 1992) and induces contraction of fibroblasts and endothelial cells (Giuliano & Taylor 1990, Goeckeler & Wysolmerski 1995). LPA stimulates contraction of chicken embryo fibroblasts and human endothelial cells (Kolodney & Elson 1993), as well

as neurite retraction (Jalink et al 1994). The contractions induced by LPA and thrombin are Rho-mediated based on their inhibition by C3 (Jalink et al 1994). Induction of stress fibers and FAs by LPA treatment of quiescent Balb/c 3T3 cells is accompanied by an increase in contractility (Chrzanowska-Wodnicka & Burridge 1996). Several agents that inhibit actin-myosin interaction block the LPA-induced formation of stress fibers and FAs, and inhibit the LPA-stimulated tyrosine phosphorylation of FA components. These inhibitors also block FA and stress fiber assembly induced by microinjected Rho, indicating that contractility is downstream of Rho in the LPA pathway (Chrzanowska-Wodnicka & Burridge 1996).

How might Rho stimulate contractility? LPA and thrombin stimulate MLC phosphorylation (Kolodney & Elson 1993, Goeckeler & Wysolmerski 1995, Chrzanowska-Wodnicka & Burridge 1996). This increase in MLC phosphorylation appears to precede the appearance of stress fibers and FAs, which suggests that it is an early event in the assembly of these structures rather than a consequence of their formation (Chrzanowska-Wodnicka & Burridge 1996). MLC phosphorylation could result from stimulation of MLCK or inhibition of the MLC phosphatase. Several studies on smooth muscle point to an inhibition of phosphatase activity by Rho. In smooth muscle, it has been known for some time that excitatory agonists stimulate higher MLC phosphorylation than that induced by KCl depolarization, suggesting that these agonists potentiate phosphorylation not only by elevating intracellular calcium, but also by an additional mechanism (Somlyo & Somlyo 1994). Using permeabilized smooth muscle preparations, MLC phosphorylation and contractility are enhanced at fixed calcium concentrations by GTPγS, which suggests the involvement of a G protein (Kitazawa et al 1991). In this system, GTPγS inhibits MLC dephosphorylation, consistent with inhibition of a phosphatase (Kitazawa et al 1991). C3 blocks the GTPγS-enhanced phosphorylation of MLCs, implicating Rho in this process (Noda et al 1995). In addition, C3 promotes the dephosphorylation of MLCs in permeabilized smooth muscle, further suggesting that Rho inhibits the MLC phosphatase (Noda et al 1995).

Two recent observations suggest a pathway by which Rho may inactivate the MLC phosphatase (mPP). First, there is the discovery that phosphorylation of the 130-kDa-subunit of mPP inhibits its activity (Trinkle-Mulcahy et al 1995, Ichikawa et al 1996, Kimura et al 1996). Second, there is the identification of the Rho-stimulated serine/threonine kinases (Leung et al 1995, Amano et al 1996, Watanabe et al 1996, Ishizaki et al 1996, Matsui et al 1996). One of these, Rho-kinase, phosphorylates the 130-kDa subunit of mPP in vitro (Matsui et al 1996, Kimura et al 1996). It will be important to establish whether Rho-kinase alone or a Rho-activated kinase cascade is involved in the inactivation of mPP in

vivo. Potential pathways by which Rho may regulate MLC phosphorylation and other cytoskeletal events such as actin polymerization are indicated in Figure 3.

A MODEL FOR FOCAL ADHESION ASSEMBLY

Based on the observations discussed above, we have proposed a model for how contractility can promote the assembly of FAs (Figure 4) (Chrzanowska-Wodnicka & Burridge 1996). In this model, cells in suspension have integrins dispersed over the cell surface (*A*). Clustering the integrins with antibodies (*B*) coclusters FAK and stimulates tyrosine phosphorylation. In the absence of ligand binding, the integrins are not associated with actin filaments. Cells adhering to ECM via integrins are shown in panels *C* and *D*. In *C*, the cells are quiescent. The integrins mediating adhesion to the underlying ECM are distributed over the ventral surface and are not clustered into FAs. However, because the integrins are bound to their ECM ligands, they are coupled physically to actin microfilaments via proteins such as talin or α-actinin. The actin filaments associated with these integrins are under little or no tension, because the myosin II is in its inactive conformation. In *D*, the cells have been stimulated to contract by agents that activate Rho. Rho activation leads to MLC phosphorylation. This turns on myosin function and also possibly induces a conformational change in the myosin, promoting myosin filament assembly. The resulting force generation will align the actin filaments. Alignment will also be promoted by the bundling action of the myosin filaments, which are very effective at cross-linking F-actin. The tension generated will be transmitted to the integrins in the membrane, leading to their aggregation. This clustering of integrins is a cornerstone in the assembly of FAs. Certainly, clustering of integrins from the outside, in combination with ligand occupancy, induces colocalization of many FA proteins (Miyamoto et al 1995a,b). In earlier models, it was assumed that simply plating cells on ECM proteins leads to the clustering of integrins in FAs. However, this does not occur if Rho is inactive (Hotchin & Hall 1995), which implies that the clustering is driven in some way from the inside rather than from interactions with the ECM on the outside. We have noted that integrins mediating adhesion will aggregate into FAs when contractility is stimulated and, conversely, will disperse from FAs when contractility is inhibited (Chrzanowska-Wodnicka & Burridge 1996). The generally low affinity of integrins for their ECM ligands (Hynes 1992) should result in relatively rapid rates of dissociation. These will permit remodeling of integrin-ECM interactions in response to tension.

In the formation of FAs induced by activated Rho, we envisage a cooperativity between the clustering of integrins driven by contractility and the effects of elevated PIP_2 (Figure 3). Activated Rho stimulates PIP 5-kinase (Chong

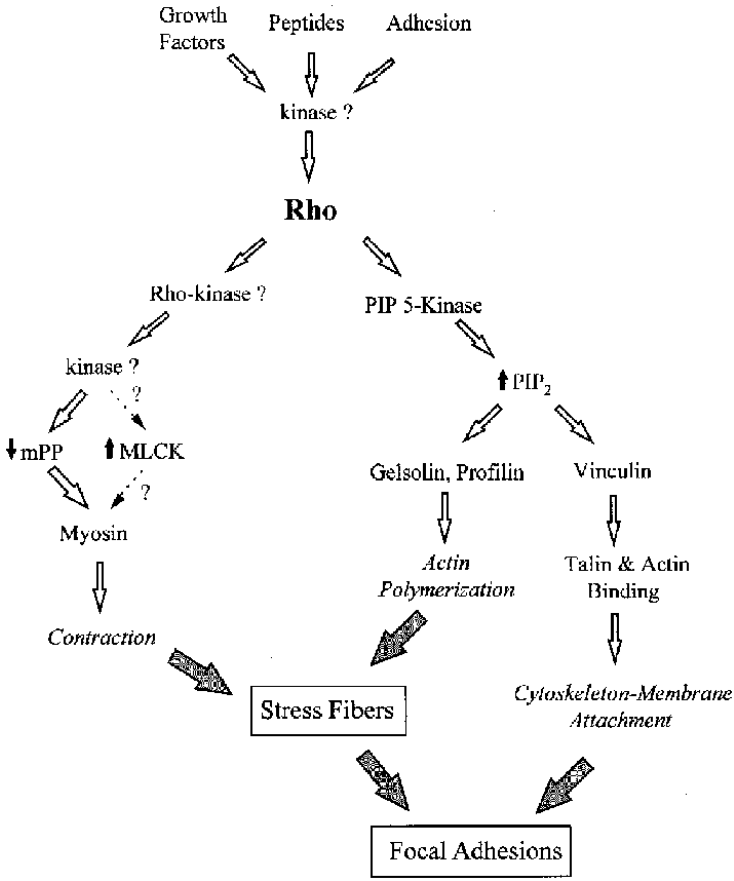

Figure 3 Pathways by which Rho regulates the actin cytoskeleton. Activation of Rho by growth factors, peptides, or adhesion is proposed to regulate the organization of the actin cytoskeleton via two synergistic pathways. In one, MLC phosphorylation is elevated via a kinase cascade that inhibits the myosin phosphatase (mPP). Stimulation of the MLCK (dotted line) may also occur, but there is no evidence for this. Light chain phosphorylation stimulates contractility. In turn, this leads to the bundling of actin filaments into stress fibers and clustering of integrins into focal adhesions. In the second, complementary pathway, Rho stimulates PIP 5-kinase, elevating PIP_2 levels. PIP_2 dissociates gelsolin and profilin from actin, promoting actin polymerization. PIP_2 also binds to vinculin, causing a conformational change that exposes binding sites for talin and actin, enhancing focal adhesion assembly.

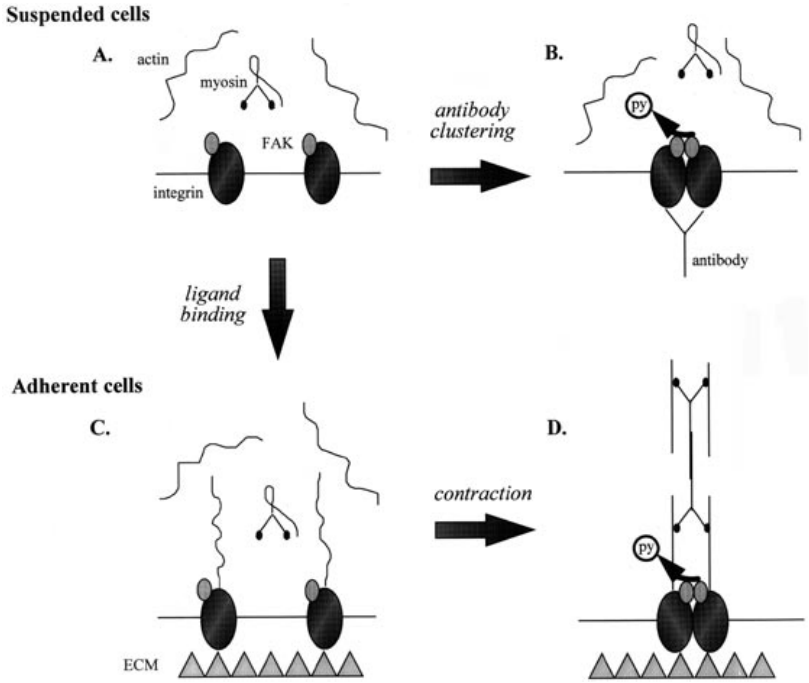

Figure 4 Model for contraction-induced formation of focal adhesions and stress fibers. In suspended cells (*A*), integrins are dispersed over the cell surface and not attached to actin filaments. Integrins can be clustered from the outside with antibodies (*B*), inducing FAK phosphorylation, but not attachment to actin, without ligand binding to the integrins. Adherent cells are shown in (*C*) and (*D*). Their integrins are bound to ECM ligands and because of this ligand occupancy become attached to actin filaments. However, in (*C*) the cells are quiescent, Rho is inactive, and the myosin is in the nonfunctional conformation. In (*D*) Rho activation stimulates contractility by elevating MLC phosphorylation. This leads to myosin filament formation and a force-generating interaction with actin. The tension exerted on the actin filaments bundles them into stress fibers and clusters the integrins to which they are attached. In turn, the integrin clustering stimulates FAK activity, triggering the signaling events associated with tyrosine phosphorylation in FAs.

et al 1994) and elevated PIP_2 stimulates dissociation of both profilin and gelsolin from actin, thereby promoting actin polymerization (Lassing & Lindberg 1985, Janmey & Stossel 1987). In addition, PIP_2 affects the conformation of vinculin, dissociating the intramolecular head/tail interaction, thereby exposing the talin- and actin-binding sites (Gilmore & Burridge 1996). PIP_2 has also been reported to enhance the binding of α-actinin to actin (Fukami et al 1992). Together these actions enhance stress fiber and FA formation. Inhibiting either side of the pathway illustrated in Figure 3 prevents assembly of FAs. Sequestering PIP_2 by microinjection of antibodies against PIP_2 into quiescent cells blocks the formation of FAs and stress fibers induced by Rho (Gilmore & Burridge 1996). Similarly, blocking contractility by a variety of agents with different modes of action inhibits the Rho-mediated assembly of stress fibers and FAs (Chrzanowska-Wodnicka & Burridge 1996).

FA assembly is accompanied by recruitment of signaling components. Evidence has been presented that this recruitment depends on tyrosine phosphorylation (Miyamoto et al 1995b). Signaling in FAs is further discussed below, but some points are relevant here in the context of assembly. One of the most prominent signaling events within FAs is the tyrosine phosphorylation that involves FAK. FAK may associate directly or indirectly with integrins and becomes activated as a result of integrin aggregation (Kornberg et al 1991, Miyamoto et al 1995a). The model predicts that anything promoting contractility and the consequent aggregation of integrins will stimulate FAK activation (if FAK is associated with the clustered integrins), which provides an explanation for the stimulation of FAK by the diverse group of agents that act via Rho. It is generally envisaged that autophosphorylation of FAK recruits src family kinases that, in turn, phosphorylate other components, generating binding sites for signaling proteins with SH2 domains. A multicomponent signaling complex is assembled that depends on the initial activation of FAK (Schaller & Parsons 1994, 1995, Miyamoto et al 1995b, Yamada & Miyamoto 1995, Richardson & Parsons 1995, Schwartz et al 1995). A surprising but consistent observation has been that disruption of the actin cytoskeleton, for example by treatment with cytochalasin D, inhibits the phosphorylation of FAK (Pelletier et al 1992, Haimovich et al 1993, Bockholt & Burridge 1993, Sinnett-Smith et al 1993, Seufferlein & Rozengurt 1994, Shattil et al 1994) as well as various downstream signaling events (Chen et al 1994). In the proposed model, FAK activation depends on the clustering of integrins from the inside by contractility. Disruption of the actin cytoskeleton would consequently block integrin aggregation driven by contractility and would account for the inhibition of FAK activity. Similarly, the model offers a potential explanation for the biphasic effects of PDGF on FAK phosphorylation. At low concentrations, PDGF stimulates FAK

phosphorylation, but at higher concentrations it disrupts stress fibers and FAK phosphorylation is inhibited (Rankin & Rozengurt 1994).

This model also offers an explanation for the observation that cells could spread on a low density of integrin ligand but required higher densities in order to form FAs (Massia & Hubbell 1991). At low-ligand density, clustering the integrins would result in their dissociation from ligand. In the absence of bound extracellular ligands, the integrins would likely detach from the cytoskeleton. Therefore, a sufficiently high density of ligand on the substrate would be needed for the aggregated integrins to remain bound.

Does this model for FA formation also account for their assembly as suspended cells are plated on an ECM or during cell migration? We suspect that many aspects of the model are still relevant. Evidence shows that active Rho is required for the formation of FAs when cells are plated on ECM substrates (Hotchin & Hall 1995). FAs appear to form spontaneously when many cells are plated on ECM, but for most cells, Rho remains in an active state for several hours even in the absence of serum. When cells are plated on ECM substrates, inhibitors of contractility block the formation of FAs or result in very small FAs that appear not to have matured into their normal organizational state (M Chrzanowska-Wodnicka & K Burridge, unpublished observations). These results are consistent with a Rho-mediated contractile event contributing to the formation of FAs in cells spreading on ECM proteins.

Tyrosine Phosphorylation and Focal Adhesion Assembly

Several studies have shown that PTK inhibitors block the formation of FAs and stress fibers induced either by adhesion to an ECM-coated surface (Burridge et al 1992, Romer et al 1992, 1994) or by Rho activation (Seckl & Rozengurt 1993, Chrzanowska-Wodnicka & Burridge 1994, Ridley & Hall 1994). Because the major sites of tyrosine phosphorylation in cultured fibroblasts are FAs and because these inhibitors decrease the tyrosine phosphorylation of FA proteins such as FAK and paxillin, the interpretation of these data was that tyrosine phosphorylation of FA components contributes to the assembly of these structures (Burridge et al 1992, Romer et al 1994). However, several lines of evidence indicate that neither the kinase inhibited in these studies nor its substrates reside in FAs. Wilson and coworkers provided evidence that FAK is not required for the formation of FAs in vascular smooth muscle cells (Wilson et al 1995). Similarly, when transformation by temperature-sensitive mutants of v-src was shut off, FAs reassembled in the absence of tyrosine-phosphorylated FAK or paxillin (Fincham et al 1995). Cells from FAK^- mice reveal prominent FAs that contain phosphotyrosine (Ilic et al 1995). Because FAK appears to be the major PTK in FAs in normal cells, this result is unexpected. In the absence of FAK, possibly other kinases are recruited to these structures, or the level or

activity of src family kinases in FAs may increase. Alternatively, a homologue of FAK, such as CAKβ/PYK2/RAFTK (Avraham et al 1995, Lev et al 1995, Sasaki et al 1995) may be expressed in these cells, partially compensating for the lack of FAK. If there is compensation by another kinase, it is incomplete because FAK$^-$ mice die as embryos (Ilic et al 1995). The prominent phosphotyrosine in the FAs of the FAK$^-$ cells differs from results obtained using microinjection of a C-terminal FAK construct that contains the FAT sequence but lacks the kinase domain (Gilmore & Romer 1996). This FAK construct displaces endogenous FAK from FAs and abolishes detectable phosphotyrosine at these sites. Significantly, FAs can still be assembled in these cells, but they lack detectable FAK and phosphotyrosine. These experiments suggest that FAK is not required for FA assembly or maintenance, although it appears to be important for other signaling pathways (Gilmore & Romer 1996).

Inhibition of FAK tyrosine phosphorylation has also been observed in cells overexpressing pp41/43^{FRNK} (FRNK), the naturally expressed C-terminal domain of FAK, which lacks the kinase domain (Richardson & Parsons 1996). Overexpression of FRNK also diminished the tyrosine phosphorylation of paxillin and tensin. These cells could still form focal adhesions but at a slower rate. Cell spreading on a fibronectin substrate was significantly retarded (Richardson & Parsons 1996).

These lines of evidence indicate that although tyrosine phosphorylation and FAK activity recruit signaling components to FAs, FAK does not have a role in the assembly of the structural components. If this is the case, where do the PTK inhibitors act to block the formation of FAs and stress fibers? Evidence indicates that they act both upstream and downstream of Rho activation (Ridley & Hall 1994, Chrzanowska-Wodnicka & Burridge 1994, Nobes et al 1995). In the model proposed for FA assembly (Figure 3), Rho initiates a kinase cascade that results in MLC phosphorylation, thereby stimulating contractility. We suspect that one of the kinases regulating contractility may be blocked by PTK inhibitors. Alternatively, a tyrosine kinase may be involved in Rho-stimulated PIP_2 synthesis.

SIGNALING IN FOCAL ADHESIONS

Integrin-mediated cell adhesion triggers tyrosine phosphorylation, ion fluxes, and lipid metabolism (Clark & Brugge 1995, Schwartz et al 1995). Together or individually, these affect many downstream pathways, influencing events such as gene expression, progression through the cell cycle, and apoptosis. Some of the integrin-mediated signal transduction pathways, such as tyrosine phosphorylation, have been shown to be initiated from FAs, whereas others may precede FA formation and be early responses to integrin occupancy or clustering. The

Table 4 Signaling proteins at the cytoplasmic face of focal adhesions

Tyrosine kinases	Heterotrimeric G proteins	Proteases
1. FAK	6. $\gamma 5$	10. calpain II
2. src		
3. csk	Adapters	Others
4. fyn	7. paxillin	11. PI3K
	8. p130^{cas}	12. LIP1
	9. Grb2	
Serine kinases		
5. PKC α, δ		

1. Schaller et al 1992; 2. Kaplan et al 1994; 3. Bergman et al 1995; 4. included because of its interaction with FAK; Cobb et al 1994; 5. Jaken et al 1989, Barry & Critchley 1994; 6. Hansen et al 1994; 7. Turner et al 1990; 8. Petch et al 1995; 9. M Kinch, unpublished observation; 10. Beckerle et al 1987; 11. Geiger et al 1992a; 12. Serra-Pages et al 1995.

idea that FAs are major sites of signal transduction is supported by the identification of multiple signaling proteins in FAs (Table 4). Numerous interactions occur between these and other signaling components involved in receptor PTK signaling pathways (Table 5). Using beads coated with antibodies or integrin ligands, Miyamoto and colleagues have identified a striking list of signaling proteins that associate with clustered integrins in a tyrosine phosphorylation-dependent manner (Miyamoto et al 1995b). Many have not been seen in FAs, but the bead technique may allow easier visualization of components that associate transiently with integrins. Alternatively, some of these components may be involved with endocytosis, a process also dependent on tyrosine phosphorylation (Shimo et al 1993, Salamero et al 1995). In the following discussion, we concentrate on signaling involving tyrosine phosphorylation.

Tyrosine Phosphorylation

Early work on tyrosine phosphorylation in FAs has been reviewed (Burridge et al 1988). A link between integrins and tyrosine phosphorylation was first demonstrated in platelets, where thrombin was shown to trigger tyrosine phosphorylation in an integrin-dependent fashion (Ferrell & Martin 1989, Golden & Brugge 1989). These observations were followed by studies with cells in culture, where it was also shown that integrin clustering or integrin-mediated adhesion stimulate tyrosine phosphorylation of a small set of proteins (Guan et al 1991, Kornberg et al 1991, Burridge et al 1992). Coincident with these studies was the discovery of a cytoplasmic PTK that localizes to FAs, the FA kinase (pp125^{FAK} or FAK) (Schaller et al 1992, Hanks et al 1992). The discovery of FAK galvanized the field, and FAK was shown to be the most prominent of the tyrosine-phosphorylated proteins in cells responding to integrin clustering

Table 5 Interactions between signaling proteins and focal adhesion components

	Structural components														
	Integrin	Vinculin	Talin	α-actinin	FAK	src	fyn	csk	Tensin	Paxillin	p130	PI-3 K	C3G	crk	GRB2
FAK	1	—	2	—	—	3	4	5	—	6	7	8	—	—	9
src	—	—	—	—	3	—	—	10	—	11	12	13	—	—	—
fyn	—	—	—	—	4	—	—	14	—	—	15	16	—	—	—
csk	—	—	—	—	5	10	14	—	—	17	—	—	—	—	—
Tensin	—	—	—	—	—	—	—	—	—	—	18	—	—	—	—
Paxillin	19	20	—	—	6	11	—	17	—	—	—	—	—	21	—
p130	—	—	—	—	7	12	15	—	18	—	—	—	22	23	—
PI-3-K	—	—	—	24	8	13	16	—	—	—	—	—	—	—	—
C3G	—	—	—	—	—	—	—	—	—	—	22	—	—	25	—
crk	—	—	—	—	—	—	—	—	—	21	23	—	25	—	—
GRB2	—	—	—	—	9	—	—	—	—	—	—	—	—	—	—
Endonexin	26	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Ilk	27	—	—	—	—	—	—	—	—	—	—	—	—	—	—

1. Schaller et al 1995; 2. Chen et al 1995; 3. Cobb et al 1994; Xing et al 1994; 4. Cobb et al 1994; 5. Birge et al 1993; 6. Turner & Miller 1994, Hildebrand et al 1995; 7. Polte & Hanks 1995; 8. Chen & Guan 1994b, Guinebault et al 1995; 9. Schlaepfer et al 1994; 10. Sabe et al 1994; 11. Weng et al 1993; 12. Reynolds et al 1989; 13. Fukui & Hanafusa 1989, Pleiman et al 1994; 14. reviewed in Sabe et al 1994; 15. L Petch, unpublished data; 16. Prasad et al 1993, Pleiman et al 1994; 17. Sabe et al 1994, Schaller & Parsons 1995; 18. Lo et al 1994; 19. Schaller et al 1995; 20. Turner et al 1990; 21. Birge et al 1993, Schaller & Parsons 1995; 22. Vuori et al 1996; 23. Birge et al 1993; 24. Shibasaki et al 1994; 25. Matsuda et al 1994, Tanaka et al 1994; 26. Shattil et al 1995; 27. Hannigan et al 1996.

or integrin-mediated adhesion (Burridge et al 1992, Hanks et al 1992, Guan & Shalloway 1992, Kornberg et al 1992, Lipfert et al 1992). Several other FA proteins were identified as also becoming tyrosine-phosphorylated in response to integrin-mediated adhesion. These included paxillin (Burridge et al 1992), tensin (Bockholt & Burridge 1993), PI 3-kinase (Chen & Guan 1994a), and $p130^{cas}$ (Petch et al 1995, Vuori & Ruoslahti 1995, Nojima et al 1995). Notably, the more major proteins in FAs—talin, vinculin, integrins—contain little or no phosphotyrosine. This contrasts with the situation in src-transformed cells in which low levels of phosphotyrosine are detected in these proteins.

FAK is a cytoplasmic PTK with three domains. The central catalytic domain separates an N-terminal domain, shown to bind to $\beta 1$ integrin cytoplasmic domain peptides (Schaller et al 1995), and a C-terminal domain, which contains a FA-targeting (FAT) sequence (Hildebrand et al 1993). This latter domain contains binding sites for at least two FA proteins, paxillin and talin (Turner & Miller 1994, Chen et al 1995, Hildebrand et al 1995, Tachibana et al 1995). Lewis & Schwartz (1995) found that association of FAK with clustered integrins in vivo depended on the most C-terminal 13 residues of the β_1 cytoplasmic domain. This was also the sequence required for co-clustering talin and paxillin with integrins, possibly indicating that one or other might contribute to FA targeting. In addition, FAK constructs containing the paxillin-binding sequence target to FAs, but fail to do so if this binding sequence is disrupted (Tachibana et al 1995). In these experiments, the interaction with talin was not examined, but it might also contribute to targeting FAK to FAs. These results support the idea that the interaction of FAK with paxillin or talin targets it to FAs. However, Miyamoto and colleagues found that simple aggregation of integrins was sufficient to co-cluster FAK but not talin or paxillin (Miyamoto et al 1995a). Co-clustering of talin required both aggregation and ligand occupancy, whereas co-clustering of paxillin required tyrosine phosphorylation as well.

The significance of the interaction of the N-terminal domain with integrin peptides is not obvious because this region does not appear to be involved in targeting FAK to FAs. Possibly this interaction functions in the activation of FAK, but most of the evidence indicates that clustering of integrins triggers FAK activation (Kornberg et al 1991, Defilippi et al 1994) and that ligand occupancy is not involved (Miyamoto et al 1995a). However, clustering of one integrin with an activating antibody failed to induce tyrosine phosphorylation (Pelletier et al 1995). Integrin aggregation may dimerize FAK, and thereby stimulate transphosphorylation and activation. This would be consistent with the model for activation of receptor PTKs (Ullrich & Schlessinger 1990).

For many receptor PTKs, ligand binding triggers autophosphorylation. The resulting phosphotyrosines in the receptor cytoplasmic domain bind SH2

domain-containing proteins. A similar situation is found for FAK. Several sites of tyrosine phosphorylation have been identified that recruit specific proteins via their SH2 domains (Schaller et al 1994, Schlaepfer et al 1994). The major site of tyrosine phosphorylation in FAK is Tyr-397, which arises through autophosphorylation (Schaller et al 1994). This generates a binding site for the SH2 domains of $pp60^{src}$ and $pp59^{fyn}$, both of which have been shown to complex with FAK in cells (Cobb et al 1994, Schlaepfer et al 1994, Xing et al 1994). These kinases phosphorylate additional sites in FAK and may stimulate FAK activity (Schlaepfer et al 1994, Calalb et al 1995). One of these sites, Tyr-925, complexes with the adapter protein Grb2 (Schlaepfer et al 1994). In normal cells, the association of Grb2 with FAK is dependent on integrin-mediated adhesion, but in v-src-transformed cells, FAK is constitutively tyrosine-phosphorylated and bound to Grb2. In these src-transformed cells, Grb2 is further associated with Sos, a Ras GTP/GDP exchange factor that activates the MAP kinase pathway (Schlaepfer et al 1994). This pathway may also operate in normal cells because MAP kinase activation has been observed in cells plated onto fibronectin in the absence of growth factors (Chen et al 1994, Schlaepfer et al 1994, Morino et al 1995, Zhu & Assoian 1995), and antibody-clustering of $\alpha_2\beta_1$ integrins in lymphocytes elevates the level of Ras-GTP (Kapron-Bras et al 1993). However, MAP kinase activation may be triggered by other signaling cascades initiated by integrin-mediated adhesion.

The association of $pp60^{src}$ and $pp59^{fyn}$ with FAK raises the question of which PTK is responsible for the tyrosine phosphorylation of specific FA proteins. Examining cells from transgenic mice that had had their *src*, *fyn*, or *yes* genes individually knocked out revealed that the patterns of tyrosine phosphorylation upon adhesion to fibronectin resembled that in control cells, with the exception of $p130^{cas}$ (Bockholt & Burridge 1995). The tyrosine phosphorylation level of $p130^{cas}$ was reduced in the fyn^- and yes^- cells, and to a particularly low level in the src^- cells, suggesting that its phosphorylation is dependent on src and to a lesser extent on the other src family kinases. Although many of their characteristics resemble normal cells, the src^- cells spread more slowly on fibronectin (Kaplan et al 1995). Besides FAK, one of the major tyrosine-phosphorylated proteins in FAs is paxillin (Turner 1994). Paxillin binds FAK and is a substrate for FAK in vitro and in vivo (Bellis et al 1995, Hildebrand et al 1995, Schaller & Parsons 1995). Autophosphorylation of FAK on Tyr-397 is required for paxillin phosphorylation (Schaller & Parsons 1995), which suggests that src or fyn may be involved, given that these two kinases bind to this site. Paxillin is a substrate in vitro not only for FAK, but also for src and csk (the kinase that regulates src activity by phosphorylation of its C-terminal tyrosine) (Schaller & Parsons 1995). All three kinases phosphorylate sites in vitro that are also

phosphorylated in vivo (Schaller & Parsons 1995). These authors suggest a model whereby cell adhesion results in FAK autophosphorylation. This phosphorylation recruits src/fyn or csk by binding to the SH2 domains of these kinases. In turn, these phosphorylate paxillin on multiple sites. The absence of an obvious decrease in paxillin tyrosine phosphorylation in the src$^-$ cells may reflect redundancy in the system with fyn or csk replacing src (Bockholt & Burridge 1995).

Csk is an important regulator of the src family kinases. It inhibits their activity by phosphorylating the C-terminal tyrosine residue. This C-terminal phosphotyrosine then participates in an intramolecular association with its own SH2 domain, leading to a steric inhibition of the kinase domain (Cooper & Howell 1993). Overexpression of csk leads to its detection within FAs (Bergman et al 1995), suggesting that it is normally present but below the level of detection. Deletion of Csk results in src that is not phosphorylated on its C-terminal tyrosine (Tyr-527), and src now becomes detected in focal adhesions (Kaplan et al 1994). Similarly, mutating tyr-527 to phenylalanine, which prevents phosphorylation, results in src becoming prominently associated with FAs. Interestingly, the targeting of src to FAs occurs in the absence of the kinase domain and requires an intact SH3 domain (Kaplan et al 1994). The SH3 domain has been shown previously to bind to paxillin (Weng et al 1993). This may be the interaction that targets src to FAs, whereas the src SH2 interaction with FAK may activate src by displacing the intramolecular association between the SH2 domain and Tyr-527. A src construct lacking the kinase domain, but with intact SH2 and SH3 domains, elevates phosphotyrosine in FAs, particularly in FAK (Kaplan et al 1994). The mechanism for this is uncertain, but it may reflect the src SH2 domain protecting tyrosine-phosphorylated residues from tyrosine phosphatases. In csk$^-$ cells the level of phosphotyrosine in paxillin is increased, consistent with src family kinases being responsible (Nada et al 1994, Thomas et al 1995). The relationship of src and fyn has been investigated in the csk$^-$ background using double knockouts (Thomas et al 1995). These authors observed a decrease in the total level of phosphotyrosine in the src$^-$csk$^-$ cells but not the fyn$^-$csk$^-$ cells, suggesting that src is more critical than fyn to the elevation of phosphotyrosine in the csk$^-$ cells. However, the elevated phosphotyrosine in paxillin was partially decreased by the introduction of either the *src*$^-$ or *fyn*$^-$ mutation, consistent with both these kinases having a role in phosphorylating paxillin.

Another major tyrosine-phosphorylated protein in FAs is p130^{cas} (Petch et al 1995). The data from the src$^-$ cells suggest that much of the tyrosine phosphorylation of this protein is also from the activity of src (Bockholt & Burridge 1995). Like paxillin, this is a protein that interacts with other signaling proteins

(Table 5). The sequence of p130^{cas} reveals that it contains one SH3 domain and numerous tyrosines within the consensus-binding sites for the SH2 domains of src and crk (Sakai et al 1994). Both paxillin and p130^{cas} bind the adapter protein crk, which consists of SH2 and SH3 domains (Birge et al 1992, 1993, Schaller & Parsons 1995). The latter mediates interaction with two guanine nucleotide exchange factors for ras, Sos and C3G (Matsuda et al 1994, Tanaka et al 1994). The multiple interactions of paxillin and p130^{cas} with other signaling proteins has led to the general view that these proteins may be equivalent to the IRS-1 protein in insulin-signaling pathways. IRS-1 becomes tyrosine-phosphorylated on multiple sites in response to insulin binding to the insulin receptor. This provides a scaffold for downstream signaling components to bind via their SH2 domains. Similarly, following their tyrosine phosphorylation, paxillin and p130^{cas} may also act as scaffolds, recruiting other signaling proteins into a complex that activates separate or overlapping signaling pathways.

Very little is known about tyrosine phosphatases (PTPs) in FAs. The transmembrane PTP, LAR has been detected in the FAs of some cells, together with an interacting cytoplasmic protein, LIP (Serra-Pages et al 1995). However, in several cell types LAR has not been detectable in FAs, indicating that other PTPs may be present (G Schneider & K Burridge, unpublished observations).

Does Integrin-Mediated Adhesion Activate Rho?

The formation of stress fibers and FAs requires activated Rho (Ridley & Hall 1992). Cells plated on an ECM in the absence of growth factors also develop FAs, raising the possibility that integrin-mediated adhesion activates Rho, thus providing the stimulus for FA formation. Supporting this idea was the demonstration that PIP_2 levels increase when cells adhere to ECM McNamee et al 1993) and that this depended on Rho (Chong et al 1994). These results indicate that integrin engagement activates Rho. However, this has been challenged by the experiments of Hotchin & Hall (1995), who found that quiescent cells, with low levels of active Rho failed to form FAs or stress fibers when plated on fibronectin substrates for 30–45 min. Under these conditions, FAs formed rapidly upon Rho activation. Similar experiments were performed by Barry and coworkers who also observed a failure to form FAs and stress fibers 30 min after plating quiescent cells on fibronectin (Barry et al 1996). However, in this study FAs and stress fibers were seen 3 h after adhesion to fibronectin, indicating that this interaction does activate Rho, albeit inefficiently or with a delayed time course.

The activation of Rho in response to integrin-mediated adhesion may involve an arachidonic acid/leukotriene pathway. The spreading of cells on ECM substrates stimulates and requires activation of PKC (Chun & Jacobson 1992, 1993,

Vuori & Ruoslahti 1993) and release of arachidonic acid (Chun & Jacobson 1992). Blocking the metabolism of arachidonic acid to leukotrienes prevents HeLa cells spreading on a collagen matrix, and this block can be partially overcome by added leukotrienes (Chun & Jacobson 1992). Notably, leukotrienes have been reported to stimulate Rho (Peppelenbosch et al 1995). Together these results suggest a pathway in which leukotrienes may link integrin-mediated adhesion to Rho activation. Leukotriene synthesis and stimulation of Rho also provide a connection between Rac and Rho in cells responding to EGF (Peppelenbosch et al 1995). The activation of Rac is associated with extensive membrane ruffling (Ridley et al 1992), and this phenotype resembles the behavior of cells freshly plated on ECM. Indeed, the behavior of cells spreading on ECM resembles cells responding sequentially to cdc42, Rac, and finally Rho. Initial adhesion is associated with production of filopodia (the cdc42 phenotype) (Albrecht-Buehler 1976), followed by lamellipodia (the Rac phenotype) and, finally, when cells have become well spread, the development of stress fibers and FAs (the Rho phenotype). A hierarchy has been demonstrated previously, with cdc42 upstream of rac, which is upstream of Rho (Nobes & Hall 1995). It is conceivable, therefore, that this is also the pathway from integrin-mediated adhesion to activation of Rho, going via cdc42, Rac, and the synthesis of leukotrienes.

An alternative pathway by which Rho may become activated during adhesion involves Vav, a possible Rho GEF. In platelets, Vav becomes tyrosine-phosphorylated upon platelet activation by thrombin or collagen (Cichowski et al 1996). The stimulation by collagen occurs via the integrin $\alpha_2\beta_1$, indicating that in some circumstances Vav regulation is integrin-dependent. Vav binds the FA protein zyxin, contains one SH2 domain, two SH3 domains, a pleckstrin homology domain, and a dbl homology domain. The latter is associated with guanine nucleotide exchange. Whether Vav acts on Rho or another ras-related protein is controversial, but the phenotype of cells transformed by Vav suggests that it acts directly or indirectly on Rho (Khosravi-Far et al 1994). Vav is normally confined to hematopoietic cells, but a widely distributed isoform, Vav2, has been identified (Henske et al 1995). Vav itself becomes phosphorylated on serine and tyrosine residues and is probably involved in several signaling cascades (Gulbins et al 1993, Uddin et al 1995, Matsuguchi et al 1995). If Vav2 becomes phosphorylated by integrin engagement, this would suggest one way by which adhesion might activate Rho. Because Rho enhances adhesion and spreading, the activation of Rho by adhesion suggests a positive feedback loop. Positive feedback loops have previously been suggested to occur during adhesion and spreading in the context of PKC activation (Chun & Jacobson 1993).

Focal Adhesions and Growth Control

It has been long known that most normal cells in culture are anchorage dependent, i.e. they require attachment to a substrate in order to go through the cell cycle (Stoker et al 1968). The requirement for anchorage is abolished by many different oncogenes. Suspension of normal cells arrests not only DNA synthesis, but also inhibits RNA and protein synthesis (Otsuka & Moskowitz 1975, Benecke et al 1978, 1980, Farmer et al 1978, Ben-Ze'ev et al 1980). Cell cycle progression is blocked in G_1 by suspension (Otsuka & Moskowitz 1975). Clearly with normal cells, adhesion-dependent signals regulate many activities and permit cells to respond to growth factors. Several integrin-mediated signaling pathways have been implicated. Elevated intracellular pH has been associated with growth of normal cells, and this is regulated by integrins via the Na^+/H^+ antiporter (Schwartz et al 1989, 1990, 1991a,b, Ingber et al 1990). Integrins have also been linked to elevated intracellular Ca^{2+} (Pelletier et al 1992, Leavesley et al 1993, Schwartz et al 1993, Schwartz 1993, Schwartz & Denninghoff 1994). In endothelial cells, the integrin-dependent elevation in intracellular Ca^{2+} involves an integrin-associated protein (IAP) (Schwartz et al 1993). The mechanism by which integrins elevate intracellular Ca^{2+} has not been resolved, but intracellular Ca^{2+} has been associated with many mitogenic signals and pathways. Intracellular Ca^{2+} levels are frequently elevated by release from intracellular stores via a pathway that involves PIP_2 hydrolysis and the generation of inositol trisphosphate (IP_3) (Berridge 1993). As discussed above, PIP_2 levels are elevated by adhesion in a Rho-dependent manner (McNamee et al 1993, Chong et al 1994). One pathway from growth factor receptors involves activation of phospholipase C, which hydrolyzes PIP_2 to generate IP_3 and diacyl glycerol. These act as second messengers elevating intracellular Ca^{2+} and activating PKC. The decrease in PIP_2 that occurs with suspension of cells deprives PLC of its substrate and has been correlated with the inability of cells in suspension to respond to growth factors, even though the growth factors bind to their receptor PTKs (McNamee et al 1993).

Many lines of evidence point to a collaboration between the signals emanating from FAs and growth control. For example, in collagen gels, cells show high proliferation rates if the gel is anchored so that the cells generate isometric tension and develop stress fibers. In contrast, cells in free-floating gels do not develop isometric tension or stress fibers, and the cells become arrested for DNA synthesis and proliferation (Lin & Grinnell 1993, Grinnell 1994). With adherent cells in culture, several mitogens stimulate tyrosine phosphorylation of the same repertoire of FA proteins (FAK, paxillin, $p130^{cas}$, tensin) (Zachary et al 1992, 1993, Kumagai et al 1993, Sinnett-Smith et al 1993, Hordijk et al 1994, Seufferlein & Rozengurt 1994). Most, if not all, of these mitogens stimulate

Rho, contractility, and the formation of FAs in quiescent cells. Introduction of activated Rho into quiescent cells is itself sufficient to stimulate progression through the cell cycle and DNA synthesis (Olson et al 1995).

Adhesion to ECM activates the MAP kinase pathway, which is also triggered by many growth factors. MAP kinase activation may be via Grb2 binding to phosphorylated Tyr-925 in FAK (Schlaepfer et al 1994), but other adapter proteins interact with FA proteins, such as paxillin and $p130^{cas}$ (Table 5) and may provide parallel routes to MAP kinase activation. Adhesion alone is an insufficient stimulus to activate the MAP kinase pathway if Rho is not active (Hotchin & Hall 1995). It is not clear why growth factors and adhesion to ECM should both stimulate MAP kinase activation. The experimental paradigm of plating suspended cells on ECM substrates such as fibronectin is clearly artificial. One can imagine, however, that an equivalent situation is encountered normally when circulating cells adhere to a damaged blood vessel wall or when cells respread after mitosis. Similarly, during migration, the formation of new adhesions may trigger localized activation of MAP kinase pathways.

Is FAK involved in the synergy between integrin and growth factor signaling? Analysis of the role of FAK in growth control has not been possible with the FAK^- cells because they were transfected with activated p53 to promote growth (Ilic et al 1995). However, in an experimental model discussed above, FAK function was analyzed using microinjection of a C-terminal construct to displace endogenous FAK from FAs (Gilmore & Romer 1996). Displacement of FAK from FAs significantly decreased the number of cells entering DNA synthesis in response to stimulation with serum. This finding supports the idea that signaling from FAK contributes to and may be necessary for normal cells to respond to growth factors and to display anchorage-dependent growth. A similar conclusion was reached in experiments where epithelial cells were transfected with constitutively active FAK. This rendered the epithelial cells anchorage independent and even made them tumorigenic (Frisch et al 1996). In the model for FA formation discussed above, FAK activation results from integrin clustering induced by contractility within the microfilament system. This model predicts that disruption of the cytoskeleton should prevent FAK activation, which would resemble loss of adhesion in terms of growth control. This indeed is the case. Disruption of the cytoskeleton with cytochalasin mimics the effect of cell suspension, blocking cells that are anchorage dependent in G_1 (Bohmer et al 1996).

Direct interactions between integrins and growth factor-signaling components have been identified. In response to insulin stimulation, IRS-1 associates with the vitronectin receptor, $\alpha_v\beta_3$. Insulin stimulates growth of cells adhering to vitronectin more effectively than it stimulates cells adhering to other ECM proteins (Vuori & Ruoslahti 1994). The FGF receptor *flg* has been identified

in FA complexes isolated on fibronectin-coated beads (Plopper et al 1995). In addition, numerous signaling components associated with receptor PTKs also associate with clustered integrins (Miyamoto et al 1995b).

The interplay between integrins and growth factors is illustrated by the growth and differentiation of myoblasts. Sastry and colleagues have found that ectopic expression of the α_5 integrin subunit inhibits differentiation, promoting proliferation. In contrast, ectopic expression of α_6 promotes differentiation and inhibits proliferation in this system (Sastry et al 1996). Chimeric constructs, in which the cytoplasmic domains were truncated or swapped, demonstrated that these effects depended on the α subunit cytoplasmic domains. The ectopic expression of α_5 changed the response of myoblasts to specific growth factors. For example, normally both TGFβ and bFGF inhibit proliferation and stimulate differentiation, but following transfection with the α_5 subunit, these growth factors inhibited differentiation. However, only bFGF stimulated proliferation, whereas TGFβ now induced apoptosis (Sastry et al 1996). Ectopic expression of α_5 in other situations also affects growth characteristics. For example, expression of α_5 in tumor cells deficient in this integrin subunit has been correlated with restoration of a normal phenotype and anchorage-dependent growth (Giancotti & Ruoslahti 1990). These effects of α_5 expression on tumor cell growth are complex. Ectopic expression of α_5 inhibits anchorage-independent growth, but it promotes growth on an appropriate ECM substrate (Varner et al 1995). These findings imply that a negative growth signal is transmitted by the unengaged $\alpha_5\beta_1$ integrin, but a positive proliferation signal is triggered upon ligand binding to this integrin. Clearly, different α subunits generate distinct signals, although very little is known about their nature. Integrin cytoplasmic domains also generate growth-related signals as evidenced by the inhibition of cell cycle progression and proliferation induced by ectopic expression of the β_{1C} alternatively spliced cytoplasmic isoform (Meredith et al 1995, Fornaro et al 1995). β_{1C} has been identified in platelets, megakaryocytes, and some other blood cells (Languino & Ruoslahti 1992). It is absent from growing endothelial cells but is induced when endothelial cells are growth arrested by exposure to tumor necrosis factor α (Fornaro et al 1995).

The expression of α_5 integrin in transformed cells restores more normal growth characteristics and the formation of FAs. As mentioned above, in normal cells the ability to form FAs correlates with anchorage-dependent growth, and disruption of FAs leads to cell cycle arrest. However, a paradoxical situation is seen in cells in which the levels of vinculin or α-actinin are decreased, for example, by antisense cDNA constructs. Depressing the level of either protein results in decreased FAs, increased motility, and anchorage-independent growth (Rodriguez Fernandez et al 1992, 1993, Gluck et al 1993, Gluck & Ben-Ze'ev

1994). Cells with decreased levels of vinculin or α-actinin form tumors when injected into nude mice. Moreover, several transformed cells or tumors show decreased expression of vinculin or α-actinin. Elevating the levels of these proteins restores a more normal phenotype, including anchorage-dependent growth and suppression of tumorigenicity (Gluck et al 1993, Rodriguez Fernandez et al 1992). A possible explanation for these observations relates to the fact that both vinculin and α-actinin are major PIP_2-binding proteins (Fukami et al 1994). Levels of PIP_2 are reduced when normal cells are suspended, and this has been correlated with growth arrest of cells in suspension (McNamee et al 1993). If vinculin and α-actinin are major sinks for cytoplasmic PIP_2, it is conceivable that reducing their levels increases PIP_2 availability to hydrolysis by PLC and thus permits suspended cells to respond to growth factor stimulation by PIP_2 hydrolysis. It will be important to determine whether vinculin and α-actinin buffer PIP_2 levels. If incorporation of vinculin and α-actinin into FAs releases their bound PIP_2, this would also increase the PIP_2 available for hydrolysis and provide another link between focal adhesions and mitogenic pathways.

Apoptosis

Cooperativity between integrin-mediated adhesion and growth factors is also seen in relation to apoptosis. Loss of adhesion blocks progression through the cell cycle for normal anchorage-dependent cells. But more than that, for some cells it also induces apoptosis (programmed cell death), particularly in the absence of growth factors (Meredith et al 1993, Frisch & Francis 1994, Re et al 1994). Engagement of different integrins prevents apoptosis in different cell types. Thus the α_5 integrin subunit increases survival of CHO cells adhering to fibronectin in the absence of growth factors (Zhang et al 1995). Similarly, in muscle cells expressing ectopic α_5, survival is increased in the presence of bFGF, whereas untransfected cells become apoptotic (Sastry et al 1996). β_1 integrins prevent apoptosis in mammary epithelial cells adhering to basement membranes (Boudreau et al 1995). In contrast, disruption of $\alpha_v\beta_3$ interactions promote apoptosis in colon carcinoma cells, endothelial cells, and melanoma cells (Bates et al 1994, Brooks et al 1994, Montgomery et al 1994). The term *anoikis* (Greek for homelessness) has been coined to describe the apoptosis that results from loss of normal adhesion to ECM (Frisch & Francis 1994).

Is there a role for FAK in integrin-mediated survival versus apoptosis? Meredith and coworkers demonstrated that apoptosis of suspended cells could be prevented by elevating tyrosine phosphorylation, consistent with, but not proving, a role for FAK in this process (Meredith et al 1993). The involvement of FAK in apoptosis/survival has been approached recently in several ways. Reducing the level of FAK in several tumor cell lines with antisense oligonucleotides induced detachment from the substrate and a high incidence

of apoptosis (Xu et al 1996). Interestingly, normal cells did not respond by detachment or apoptosis, which led these investigators to propose targeting FAK with antisense oligonucleotides as a potential strategy for treating tumors. However, others have found that perturbations of FAK in normal cells lead to apoptosis. Apoptosis was induced in chicken embryo fibroblasts by microinjection of either an antibody against FAK or integrin cytoplasmic domain peptides that correspond to the region shown to bind FAK in vitro (J Hungerford et al, manuscript submitted). Epithelial cell lines that go into apoptosis upon suspension were rescued by transfection with constitutively activated forms of FAK (Frisch et al 1996). Rescue from apoptosis did not occur if the cells were transfected with a kinase-dead mutant of FAK or with a mutant in which the major autophosphorylation site in FAK had been deleted. Together these results provide strong support for the idea that a major function for FAK is to signal to a cell that it is in contact with ECM. In the absence of this signal, cells will go into apoptosis. With tumor cells, growth becomes anchorage independent owing to activation of oncogenes that short circuit the FAK pathway. It is interesting that many invasive tumors display elevated expression of FAK, which may contribute to their anchorage-independent growth (Owens et al 1995).

TELEOLOGY

Why do cells in culture form FAs? The existence of FAs and stress fibers is closely correlated. They have little to do with cell migration; they are absent from many migratory cells and prominent in the least motile cells (Burridge 1981). In nonmuscle tissues, large bundles of actin filaments, which are contractile, are common in two circumstances: cytokinesis and wound contracture (Gabbiani et al 1973). We suspect that the origin of stress fibers and FAs reflects the response of cells in culture to an apparent wound environment. Many of the agents that activate Rho are released by platelets or other cells in response to wounding (e.g. LPA, thrombin, endothelin). Some of these factors are present in serum; thus cells cultured with serum respond as if in a wound. These factors stimulate Rho-mediated contraction. However, because the underlying substrate is rigid and the adhesions to it are strong, contraction generates isometric tension, leading to the development of stress fibers and FAs. For most tissues, wounding is also a potent mitogenic signal. Cells closing a wound proliferate and normally this ceases once the wound has been repaired. A parallel situation is seen experimentally with cells in collagen gels. If contraction of the gel is physically restricted, the cells develop isometric tension and prominent stress fibers (Tomasek et al 1992, Grinnell 1994). Under these conditions, the cells respond to mitogens by proliferation. Following release of tension, stress fiber disassembly occurs and is accompanied by a return to quiescence and a loss of responsiveness to growth factors (Grinnell 1994). Similarly, when

normal cells in culture are detached from a substrate or are prevented from spreading and generating tension, they become refractory to growth factors. Not only does the formation of FAs reflect the response of cells in culture to a wound environment, but the signaling at FAs may also relate to this wound response. The clustering of integrins stimulated by contractility activates FAK and triggers signaling cascades that synergize with growth factors. Together these responses combine in tissues to close a wound both by contraction and cell proliferation. In tissue culture, they contribute to the appearance of stress fibers and FAs and to anchorage-dependent growth.

PERSPECTIVES FOR THE FUTURE

The last few years have witnessed considerable progress in the field of focal adhesion research. Particularly rapid advances have been made identifying downstream pathways from Rho. Because of this, it seems likely that the steps from the activation of Rho to the assembly of focal adhesions will be elucidated in the near future. Many of the structural interactions that occur in FAs may take longer to resolve. We anticipate that FAs will continue to serve as a useful model for studying the signaling that is initiated in response to adhesion to ECM. Many of the presumptive signaling components in FAs, such as paxillin and $p130^{cas}$, have yet to be assigned a function. Experimental strategies aimed at determining their functions are being actively pursued. Perhaps the greater challenge will be to unravel the web of signaling pathways that emerge from FAs. The interactions between integrin-mediated signals and the signals generated in response to growth factors promise to be fertile grounds, relevant to understanding not only anchorage-dependent growth, but also differentiation and apoptosis.

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