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# Mitogenic Signal Transduction by Integrin- and Growth Factor Receptor-mediated Pathways

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**Engagement of cells with the extracellular matrix (ECM) proteins is crucial for various biological processes, including cell adhesion, spreading, proliferation, differentiation, migration, apoptosis, and gene induction, contributing to maintenance of tissue integrity, embryogenesis, wound healing, and the metastasis of tumor cells (Hynes, 2002b; Juliano, 2002). The engagement involves cell adhesion mediated by integrins, a large family of cell adhesion receptors that are transmembrane glycoproteins which bind to ECM or to counter-receptors on neighbor cells. In this review, the molecular basis of signaling mediated by integrins and their collaboration with growth factor receptors will be discussed, based on recent observations. Although other cell adhesion receptors including cadherins, selectins, syndecans, and the immunoglobulin superfamily of cell adhesion molecules (IgCAMs) can play important roles or be involved in these processes, we suggest readers refer to recent outstanding reviews on them (Barclay, 2003; Brummendorf and Lemmon 2001; Panicker *et al.* 2003).**

**Keywords:** Actin; Cell Adhesion; Erk; FAK; GTPases; Integrins; Receptors; Signaling.

## Integrins

Integrins are heterodimeric molecules composed of an α and a β subunit, with a long extracellular domain binding to ECM, a transmembrane domain, and a short cytoplasmic domain associating with the actin cytoskeleton and affiliated proteins (Burridge and Chrzanowska-Wodnicka, 1996; Hynes and Lander, 1992). At least 18 distinct α subunits and 8 or more β subunits of the integrin receptor family members have been cloned so far in mammals, leading to generation of 24 or so distinct αβ heterodimeric receptors (Giancotti, 2000). Many integrin α and β subunits have the ability to associate with more than one partner, resulting in a redundancy of integrin subtype heterodimerization; for example, the β1 subunit can dimerize with about 12 α subunits and the α6 subunit can dimerize with β1 and β4 subunits. In addition to this redundancy in forming heterodimers, integrins have another version of redundancy in ligand binding. Most integrin heterodimers recognize several ECM proteins, whereas integrin α5β1 binds only fibronectin (Wehrle-Haller and Imhof, 2003).

Major ECM proteins include fibronectin, laminin, vitronectin, and collagen. When a cell adheres to ECM or is seeded onto a dish precoated with a specific ECM, it adheres to the ECM through specific sites within the cell comprised of integrin-rich adhesion structures known as focal adhesions or focal contacts (Burridge and Chrzanowska-Wodnicka, 1996). Via interactions of integrins with both ECM and intracellular proteins simultaneously, adherent cells are able to associate with proper binding partners and to transduce bi-directional signals (Coppolino and Dedhar, 2000; Liddington and Ginsberg, 2002). Therefore, integrins are important component not only for the structure and architecture of tissues but also for signal transduction leading to regulation of many biological functions in a cell. Integrins are connected to intracellular proteins that include diverse signaling molecules enriched at focal adhesion (or contacts) and are also linked to the actin cytoskeleton (Brakebusch and Fassler, 2003). This linear

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Abbreviaitons: ECM, extracellular matrix; Erk, extracellular signal-regulated kinase; FAK, focal adhesion kinase; RAK, receptor tyrosine kinase.

linkage from the ECM outside of a cell to the actin cytoskeleton via integrin receptors is essential for an efficient signaling connection for cells to respond to extracellular cues. Integrin receptors can transduce signals alone (direct signaling by engagement via integrins on the surface of a cell) or collaboratively with other membrane receptors such as growth factor stimulation of receptor tyrosine kinases (RTKs) in an anchored cell (co-signaling).

## Signaling by integrins

Interaction of integrins with ECMs at focal contacts or adhesions leads to clustering of integrins and recruitments of signaling molecules and actin filaments to the cytoplasmic domain of integrins (Hynes, 2002a). Cell adhesion to ECM alone can activate diverse intracellular signaling molecules, including focal adhesion kinase (FAK), Erk and c-Jun kinase (JNKs) MAP kinases, and Rho GTPase family members including RhoA, Rac1, and CDC42 (Juliano, 2002). These important molecules have been shown to regulate cell adhesion, spreading, proliferation, survival (or apoptosis), and morphological changes of diverse cell types including epithelial cells, endothelial cells, fibroblasts, and other mesenchymal cell types.

**Integrin signaling for FAK activation** More than 10 years ago, cell adhesion via integrin engagement (to ECM) was shown to result in enhanced intracellular protein tyrosine phosphorylation (Kornberg *et al*., 1991). One of major species among the phospho-tyrosine proteins activated by cell adhesion was shown to be a non-receptor tyrosine kinase, known as FAK (Schaller *et al*., 1992). In a focal adhesion, integrins are linked to actin filaments by virtue of a number of signaling and structural molecules including FAK, c-Src, PI-3-Kinase, RhoGAP, paxillin, talin, p130CAS, integrin-linked kinase (ILK), and phosphorylated Caveolin-1 (Schoenwaelder and Burridge, 1999). The protein complex resulting from these interactions can serve as a structural and functional link between integrins and the actin-containing cytoskeleton (Fig. 1).

FAK is a non-receptor tyrosine kinase that locates predominantly in focal adhesions of adherent cells. It shares little sequence homology with other tyrosine kinases outside its kinase domain, and lacks transmembrane domain(s), lipid modification or acylation sites, and SH2 and SH3 domains. It contains three domains; a central kinase domain, an N-terminal domain binding to other proteins, and a COOH-terminal domain with two proline-rich sequence motifs and a region required for focal adhesion targeting called the "FAT" sequence (Schaller *et al*., 1995). FAK is phosphorylated on many tyrosine residues and activated upon integrin-mediated adhesion, and dephosphorylated when cells are detached (Schaller, 1996). FAK was shown to bind β1 integrin cytoplasmic

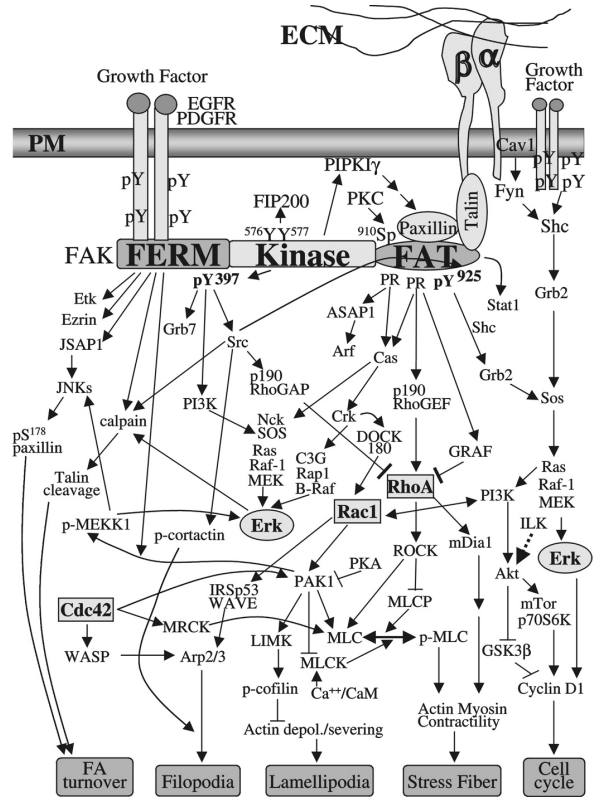


**Fig. 1.** Intracellular signaling pathways regulated by either direct integrin-mediated cell adhesion alone or collaboration with growth factor receptors-mediated pathways, leading to cell cycle progression and/or morphological maintenance for migration.

domain peptides (Schaller *et al*., 1995), but further studies showed that integrin binding region of FAK is not required for targeting to focal adhesions and the FAK binding region of β3 integrin is not needed for FAK activation, indicating an indirect interaction of integrins with FAK at focal adhesions (Shen and Schaller, 1999; Tahiliani *et al*., 1997). This may be likely since FAK binds to talin and paxillin, which interact with integrins (Chen *et al*., 1995; Hildebrand *et al*., 1995). Recently it was also shown that c-Src-dependent phosphorylation of FAK $Tyr^{861}$ outside of the FAT domain promoted the association of FAK with an avβ5 integrin signaling complex after stimulation of endothelial cells with VEGF (Eliceiri *et al*., 2002). However, how activation of FAK happens is not clearly revealed yet, although FAK has been extensively being studied.

FAK is autophosphorylated predominantly on $Tyr^{397}$ in response to integrin activation, and/or is phosphorylated at Tyr576/577 by c-Src leading to enhancement of catalytic activity of FAK (Schaller, 2001). In addition, double mutation at FAK residues 578 and 581 (Lys → Glu) in the kinase activation loop rendered it super active, allowing a maximally enhanced adhesion-dependent phosphorylation of paxillin, a FAK target (Gabarra-Niecko *et al*., 2002). This supports the idea that conformational changes within

the FAK kinase domain can also be important for maximal activation (Nowakowski *et al*., 2002). Phosphorylated $Tyr^{397}$ conforms to the consensus binding for SH2 domains for c-Src (Schaller and Parsons, 1994), p85 regulatory subunit of PI-3-Kinase and Grb7 (Shen *et al*., 2002). It was also shown that Src interaction with FAK leads to phosphorylation of FAK at several sites including $Tyr^{407}$, $Tyr^{576}$, $Tyr^{577}$, $Tyr^{861}$, and $Tyr^{925}$, which then results in the adaptor protein Grb2 binding to FAK at residue $Tyr^{925}$ (Calalb *et al*., 1996). Grb2 binding to FAK has been proposed as a possible link between FAK tyrosine phosphorylation and the integrin-mediated activation of MAPK (Schlaepfer *et al*., 1999). Interestingly, this Grb2 binding to phosphorylated $Tyr^{925}$ of FAK appears to dislocate FAK from focal contacts (Katz *et al*., 2003). In addition to interaction with talin and paxillin, a proline-rich domain close to the C-terminus of FAK recruits SH3-domain containing proteins such as p130CAS and the Rho-GAP protein GRAF (Hildebrand *et al*., 1995; Polte and Hanks, 1995). Therefore, it is likely that FAK, Src, $p130^{CAS}$ and paxillin form a signaling complex at cell adhesion sites, whose assembly is normally initiated by autophosphorylation of FAK (Shen and Guan, 2001). On the other hand, FIP200 binds FAK kinase domain, resulting in inhibition of FAK catalytic activity (Abbi *et al*., 2002), and phosphorylation of FAK on $Ser^{910}$, by either protein kinase C or Erk2 following cell stimulation or during mitogenesis, appears to block paxillin binding to the FAK FAT domain, indicating a feedback loop regulating FAK signaling at focal contacts (Hunger-Glaser *et al*., 2003). Although it is not fully understood, dephoshorylation of FAK has been shown be via tyrosine phosphatases such as PTEN, PTP1B, SHP-2, PTP-PEST, and LMW-PTP (Angers-Loustau *et al*., 1999; Liu *et al*., 1998; Rigacci *et al*., 2002; Tamura *et al*., 1998; Vadlamudi *et al*., 2002; Yu *et al*., 1998). The dephosphorylation of FAK tyrosines appears to lead to reduced cell spreading, number and size of focal adhesions, and migration. On the other hand, FAK was shown to form a complex with Calpain 2 and p42Erk, thus taking a novel and kinase-independent role as a protease-targeting adaptor protein, leading to focal adhesion turn over during transformation and migration (Carragher *et al*., 2003).

FAK activation in numerous studies has been shown important in cell adhesion and spreading (Abbi and Guan, 2002), migration and invasion (Hsia *et al*., 2003; Vuori and Ruoslahti, 1999), and cell survival (Frisch *et al*., 1996a). In addition, evidence was accumulated showing that cell adhesion-dependent FAK activity is important for proliferation and cell cycle progression (Gilmore and Romer, 1996; Oktay *et al*., 1999; Zhao *et al*., 1998). Interestingly, via formation of a signal complex including c-Src/ FAK/p130Cas/Crk, FAK has been shown to activate the c-Jun N-terminal kinase (JNK) (Tanaka *et al*., 1997). FAK transduces signals to activate JNK as well as Erk1/2 (Igishi *et al*., 1999), which have been implicated in transcriptional activation of cyclin D1 (Lavoie *et al*., 1996; Wisdom *et al*., 1999). Multiple signaling connections from FAK to Erk1/2 are shown to be important for cell proliferative activity (Danen and Yamada, 2001). Interestingly, JNK phosphorylation of $Ser^{178}$ of paxillin regulated cell migration, indicating a role of the phosphorylation in focal adhesion turnover (Huang *et al*., 2003).

**Direct integrin signaling for activation of MAP kinase cascade** In addition to FAK, MAP kinases including Erk1/2, c-Jun kinase (JNKs), and p38 MAPK are activated by integrin-mediated cell adhesion (Danen and Yamada, 2001). Activation of MAPK (or Erk1/2) pathway by cell adhesion provides a common route leading to transcriptional regulation of certain genes, which are critical for cell growth and differentiation (Giancotti and Ruoslahti, 1999). However, the intriguing aspects of their activation mechanisms have not been yet fully explained.

Integrin-mediated activation of Erk1/2 appears due to several plausible mechanisms (Fig. 2). One mechanism involves Src-mediated phosphorylation of FAK $Tyr^{925}$, as explained above. FAK autophosphorylates $Tyr^{397}$, creating a docking site for the Src homology 2 (SH2) domain of Src or Fyn (Schlaepfer *et al*., 1994). Src phosphorylates FAK at $Tyr^{925}$, creating a binding site for the signaling complex including adaptor protein Grb2 and Ras GTP-exchange factor mSOS (Schlaepfer *et al*., 1994). Grb2 can also indirectly link FAK phosphorylation at $Tyr^{925}$ to Erk1 activation, via formation of a complex with Shc that is phosphorylated by FAK and c-Src (Schlaepfer *et al*., 1998). In addition, $p130^{CAS}$ interacts with FAK through its SH3 domain and is then phosphorylated by c-Src, leading to recruiting of Crk. Crk associates with Sos or C3G, the GEF for Rap-1, resulting in subsequent activation of B-Raf (Barberis *et al*., 2000; Vuori *et al*., 1996). These interactions between signaling or structural molecules link FAK to signaling pathways that modify the organization of cytoskeleton and Erk1/2 cascade (Danen and Yamada, 2001). On the other hand, integrin-mediated Erk1/2 activation can also take place FAK-independently (Lin *et al*., 1997b). Being kinetically different from slow and sustained FAK-mediated Erk1/2 activation, Shc might be responsible for the initial high-level activation of Erk1/2 through a complex formation with Shc/Fyn/Cav-1 upon cell adhesion (Wary *et al*., 1998). Certain α integrin subunits such as integrin α1, α5, and αv, bind to the membrane protein caveolin-1, through their external and transmembrane domains. Then recruiting of Fyn and Shc to Cav-1 results in phosphorylation of Shc $Tyr^{317}$, leading to recruiting of the Grb2/Sos complex for Ras/Erk1/2 cascade activation (Wary *et al*., 1998). Recently it was reported that the Shc- and FAK-mediated Erk1/2 activations upon cell adhesion take place independently and function in a parallel fashion, and that FAK might en-

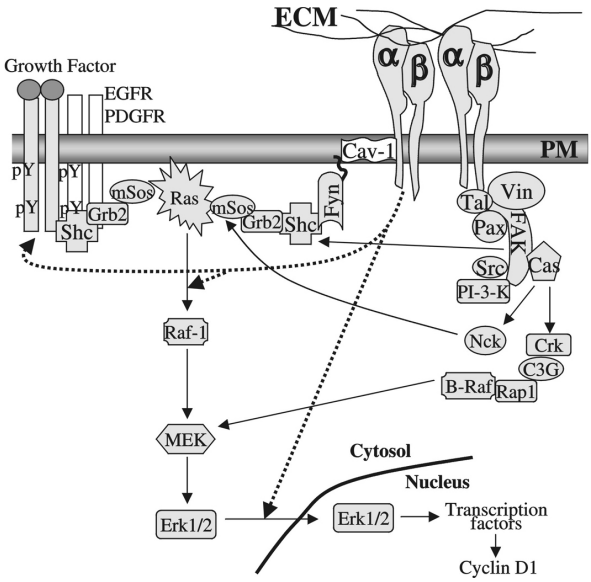


**Fig. 2.** Erk1/2 activation leading to cyclin D1 expression by collaborative cross-talks between integrins and growth factor receptors. Among diverse mechanisms for Erk1/2 activation, two mechanisms through either Caveolin-1 (Cav-1)/Shc or FAK have been shown. Dotted lines indicate the steps of growth factor receptor-mediated signaling pathway that integrin-mediated cell adhesion can regulate.

hance and prolong integrin-mediated activation of Erk1/2 through $p130^{CAS}$, CrkII, and Rap1 in cells expressing B-Raf (Barberis *et al*., 2000). This suggestion is consistent with another observation that the C3G-CrkII complex (upstream of B-Raf) plays a negative role in anchorage-dependent regulation of Erk1/2 activation, in a manner inversely dependent on overexpression of B-Raf (Buensuceso and O'Toole, 2000). Therefore, these indicate that the expression levels of Raf-1 and/or B-Raf, depending on cell types or signaling contexts, determine the kinetic aspects of integrin signaling to Erk1/2.

Another possible mechanism for integrin-stimulated Erk1/2 activation involves PI-3-K, which may activate Raf-1 through activation of PKC isoforms (King *et al*., 1997; Miranti *et al*., 1999; Naranatt *et al*., 2003). These studies including Shc-mediated or PKC-mediated pathways appear not to involve FAK in the activation of Erk1/2. In one study, overexpression of a constitutively activated form of FAK failed to activate Erk1/2, and in addition overexpression of a dominant-negative form of FAK had no effect on Erk1/2 activation (Lin *et al*., 1997b).

The models described above include the activation of Ras in the integrin-mediated activation of Erk1/2, supported by studies using a Ras dominant-negative (Ras N17) to block the adhesion activation of Erk1/2 (Clark and Hynes, 1996). Other studies, however, indicate a Ras-independent activation of Erk1/2. When the N-terminal domain of Raf-1 was used as a specific dominant-negative inhibitor of Ras signaling, the activation of Erk1/2 was not affected (Chen *et al*., 1996). In addition, another previous study showed that a Raf-1 mutant that does not bind Ras could be activated via integrin-mediated adhesion, also supporting a Ras-independent activation of Erk1/2 (Howe and Juliano, 1998). Recently it was reported that cell adhesion onto fibronectin resulted in calmodulin-dependent kinase II (CaMKII) activation, which interacts with and activates Raf-1 and thus activates Erk1/2, indicating a new mechanism of integrin-mediated Erk1/2 activation through intracellular $Ca^{2+}$ signaling (Illario *et al*., 2003).

In addition to Erk1/2, JNKs and p38 MAPK are activated by integrin-mediated cell adhesion. The integrin-mediated JNK activation is dependent on FAK and involved in cell survival (Almeida *et al*., 2000), cell cycle progression (Oktay *et al*., 1999), or cell spreading (Takino *et al*., 2002). On the other hand, p38 is activated by integrins for regulation of collagen gene expression (Ivaska *et al*., 1999), cell motility (Klekotka *et al*., 2001), or functional expression of integrin αv subunit and urokinase plasminogen activator (uPA) (Chen *et al*., 2001).

The physiological significance of integrin-mediated activation of MAP kinases is not clear, since the activity of Erk1/2 mediated by cell adhesion alone is not sufficient for the cell cycle traverse. Adhesion-mediated Erk1/2 activation shows an increase of several folds, peaks around 30 min after adhesion (or replating onto specified ECMs), and declines with a residual activity and a slow kinetics; in contrast, growth factor mediated Erk1/2 activation peaks shortly after treatment and declines to background with a faster kinetics (Roovers and Assoian, 2000). Therefore, it is clear that additional signals provided by growth factors though integrin co-signaling with receptor tyrosine kinases (RTKs) are still needed for cell proliferation. In addition, morphological rearrangement modulated by integrin-mediated activation of small GTPase family members and/or actin cytoskeletal integrity has also been shown to be importantly involved in G1/S cell cycle transition (Danen and Yamada, 2001; Huang and Ingber, 2002; Mettouchi *et al*., 2001; Renshaw *et al*., 1996).

**Connection of integrins to actin filaments** A major function of integrins is to link extracellular matrix proteins to intracellular actin filaments, by virtue of interaction of integrin cytoplasmic tails with actin binding protein such as talin, filamin, integrin-linked kinase (ILK), and α-actinin (Pozzi and Zent, 2003) (Fig. 3). Through these interactions, integrins can play a critical role in regulating actin filament reorganization and in transducing the physical forces of the cytoskeleton to the ECM via bi-directional signal transduction (Coppolino and Dedhar, 2000). ECM engagement of integrin results in conformational changes of integrins leading to straightening of the integrin β1 cytoplasmic tail, and the changes allow the

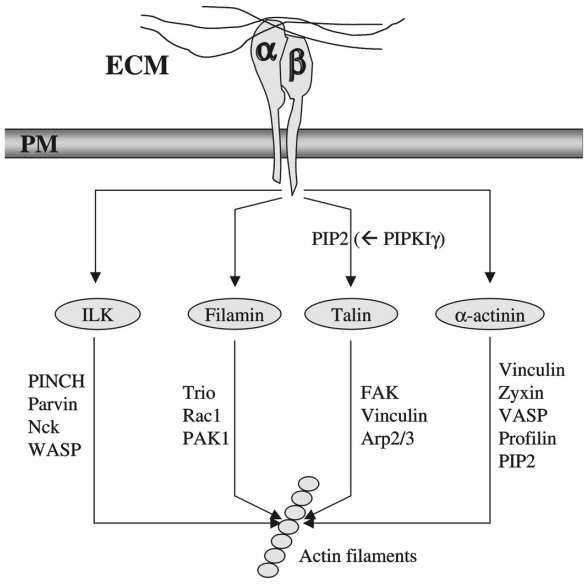


**Fig. 3.** Connections of integrins to actin filaments. Along with each mediator connecting integrins to actin filaments, other molecules involved in the connection via complex formation or regulation of the complex formation have also been shown.

tail (its conserved NPxY motif) to bind talin (Horwitz *et al*., 1986). In reverse, binding of talin to the tail triggers conformational changes of integrins, leading to modulation of integrin affinity for ECM during inside-out signaling (Tadokoro *et al*., 2003). The binding of talin with integrin may be targeted by proteases such as calpain, which is shown to bind Erk1/2 and FAK, as is acting as an adaptor protein during the focal adhesion turnover and motility where actin cytoskeletal rearrangement occurs actively (Carragher *et al*., 2003; Cuevas *et al*., 2003). Therefore, the formation of cell adhesion complexes consisting of ECM, integrins, and FA molecules including actin, assures cell adhesion to ECM as well as targeted location of actin filaments and signaling molecules, and thus is critical for cell growth, survival, migration, and cell polarity. During migration, it is clear that the interaction of integrins with actin filaments takes place dynamically and differentially; formation of a complex consisting of ECM, integrins, and actin cytoskeleton and then initiation of actin reorganization promotes different types of membrane protrusions at the leading edge of a migrating cell, but the linkage is later dissolved via detachment of integrin from ECM and the cytoskeleton at the rear of the cell (Ballestrem *et al*., 2001; Laukaitis *et al*., 2001). As for signal transduction, actin-binding proteins bound to integrins recruit signaling molecules and function as platforms anchoring both kinase and phosphatases, for example integrin-linked kinase (ILK). In addition to talin and ILK explained above, other molecules mediating the integrin linkage to the cytoskeleton include α-actinin and filamin.

Talin is known to interact with integrin, FAK, phosphatidylinositol (4,5) bisphosphate (PIP2), phosphatidylinositol phosphate kinase type Iγ (PIPKIγ), and actin. Talin-associated PIPKIγ produces PIP2, which binds talin and also vinculin that interacts with talin, α-actinin, and VASP, facilitating the formation of FAs (Brakebusch and Fassler, 2003). When vinculin binds talin, its interaction to actin is facilitated and probably requires dissociation of PIP2 due to overlapping binding sites (Gilmore and Burridge, 1996; Steimle *et al*., 1999). Furthermore, vinculin can recruit the Arp2/3 complex to polymerize actin monomers and thus initiate actin nucleation (DeMali *et al*., 2002).

ILK is a Ser/Thr kinase that binds to β integrin subunits, and is suggested to function in diverse integrin signaling pathways (Dedhar, 2000; Sakai *et al*., 2003). Several experiments by Dedhar and colleagues suggested that ILK was shown to phosphorylate $Ser^{473}$ of PKB/Akt (Delcommenne *et al*., 1998; Persad *et al*., 2001), leading to integrin-mediated cell proliferation and survival, although the $Ser^{473}$ may be indirectly or autophosphorylated as reported in other studies (Lynch *et al*., 1999; Toker and Newton, 2000). Another ILK target is GSK-3β, whose phosphorylation leads to stabilization and increase of β-catenin in nucleus and eventual increase in cyclin D and Myc expression for cell growth (Novak *et al*., 1998; White *et al*., 2001). In addition to the C-terminal domain with putative kinase activity and paxillin binding ability (Nikolopoulos and Turner, 2001), ILK is composed of an N-terminal region with 4 ankyrin repeats and a pleckstrin homology (PH) domain, which is shown to bind the double zinc finger domain (LIM)-only adaptor proteins PINCH-1 and PINCH-2 (Braun *et al*., 2003; Zhang *et al*., 2002), which bind an adaptor protein, Nck (Tu *et al*., 1998; 2001). Nck has SH2 and SH3 domains, so through their interactions it can bind EGFR or PDGFR (via SH2 domain) and actin-regulatory proteins such as WASP (Wiskot-Aldrich Syndrome protein), DOCK180 (180 kDa protein downstream of Crk), and PAK1 (p21-activated kinase) via its SH3 domain, leading to dynamic reorganization of actin. Another group of proteins binding to ILK includes α-parvin (actopaxin or CH-ILKBP, calponin homology-ILK binding protein; (Nikolopoulos and Turner, 2000; Tu *et al*., 2001), β-parvin (affixin; Olski *et al*., 2001; Yamaji *et al*., 2001), and γ-parvin. The complex consisting of ILK-PINCH-parvin was shown to important for cell adhesion, spreading, FA assembly and stress fiber formation in several cell culture systems (Tu *et al*., 2001). However, paxillin appears to be required for the complex to be targeted to FAs and F-actin interaction through vinculin (Nikolopoulos and Turner, 2002).

α-actinin links tails of integrins to actin fibrils. It also binds to several cytoplasmic molecules including vinculin, zyxin, Erk1/2, MEKK1, protein kinase N (PKN), and the p85 regulatory domain of PI3-K. The protein-protein in-

teractions among α-actinin, actin, PKN, and PI3-K can be regulated also by PIP2, but interaction between integrins and α-actinin is by PIP3 (Brakebusch and Fassler, 2003). In its role as a scaffolding protein to support these protein complexes, α-actinin can be phosphorylated by FAK and thereby its association with talin decreases (Izaguirre *et al*., 2001).

Filamins link actin filaments in parallel bundles or orthogonal networks, so the type of actin organization depends on the ratio of actin to filamin. Binding partners of filamin include integrin, Rho GTPases, MEKK, and guanine nucleotide exchange factors (GEFs). In addition to involvement in regulation of cell surface levels of integrins, filamin controls integrin function involved in membrane protrusion, cell polarization, and migration (Calderwood *et al*., 2001). Filamin also provides a platform for recruiting of signaling molecules and thus stimulates cytoskeletal rearrangement; it can bind to Trio, a GEF for Rac1, RhoA, and RhoG, and facilitate activation of Rac1, which in turn activates PAK1 (Bellanger *et al*., 2000). In addition, filamin can also activate PAK1 independent of Rac1, and this linkage appears to important for membrane ruffling activity via the PAK1-mediated phosphorylation of filamin probably at $Ser^{2152}$ (Vadlamudi *et al*., 2002).

**Integrin-mediated regulation of Rho family GTPases**

In addition to activation of FAK and Ras/Raf-1/MEK/MAPK (Erk) pathway, integrin-mediated cell adhesion stimulates Rho family members including RhoA, Rac1, and CDC42 (Fig. 1). After ECM engagement, integrins transduce intracellular signaling cascades that regulate formation, turnover, and linkage of actin filaments. During early adhesion, cells develop membrane protrusions, where integrin-mediated adhesions are associated with the development of tension, activation of Rac1 and Cdc42, and actin polymerization. The later adhesion/spreading phase involves RhoA activation and increased contractility. In this aspect, integrin-mediated activation of Rho family GTPases is critically important in actin rearrangements. It is well known that integrin-mediated activation of RhoA, Rac1, or CDC42 rearranges the actin cytoskeletal network, leading to morphological features of stress fiber formation/focal adhesion turnover, lamellipodia, or filopodia formation, respectively (Hall, 1998). By virtue of regulation of actin cytoskeleton by small GTPases, they influence diverse key cellular processes (DeMali *et al*., 2003; Etienne-Manneville and Hall, 2002).

The mechanisms of how integrin-mediated cell adhesion regulates activities of the GTPases and how the GTPases regulate actin cytoskeleton are being actively studied. Rho family GTPases bound to GTP are active, but GDP-bound forms are inactive. This switch between active and inactive forms is catalyzed by guanine nucleotide exchange factors (GEFs) leading to activation and by GTPaes-activating proteins (GAPs) stimulating their intrinsic GTPase activities. In response to integrin interaction with ECM, several non-receptor tyrosine kinases including FAK and c-Src are activated, by uncertain mechanisms. These tyrosine kinases phosphorylate other downstream molecules including GEFs (e.g., Vav) and adaptor protein complexes, such as Paxillin and PKL, or p130CAS, Crk, and ELMO that bind GEFs like Pix or DOCK180. These phosphorylations and formation of protein complexes lead to activation of Rac1 (Malliri and Collard, 2003). In addition to this GEF-mediated Rac1 activation upon cell engagement, integrin-mediated Rac1 activation can involve targeting of the GTP-bound protein to the adhesion site. It was previously reported that a certain portion of active Rac1 is sequestered by RhoGDI, and that integrin engagement brings the RhoGDI-bound Rac-GTP to the plasma membrane where active Rac1 is released from RhoGDI and encounters its effectors (Del Pozo *et al*., 2002).

On the other hand, in response to cell adhesion to fibronectin, RhoA is initially inactivated and then activated (Ren *et al*., 1999). During this response, non-integrin receptors such as syndecan-4 as well as integrin β1 were shown to contribute to the initial decrease (O'Connor *et al*., 2000; Saoncella *et al*., 1999). However, other integrins even including β1 subunit have also been observed to contribute to the later activation (Danen *et al*., 2002; Leyton *et al*., 2001; Miao *et al*., 2002), indicating that the RhoA response to cell adhesion may reflect its dependency on cell types and available expression of numerous RhoGEFs.

The GTPases regulate various cellular functions involved in both normal and pathological cell behaviors. This involvement of GTPases is through their ability to interact with diverse downstream targets. Rac1 and Cdc42 were shown to transduce signals to actin-associated proteins (i.e., IQGAPs, WASP, WAVE, etc), lipid kinases such as PI3-K, phospholipases such as PLC-β and PLD, serine/threonine kinases including MEKK, MRCK (myotonic dystrophy kinase-related Cdc42-binding kinase), PAK, and $p70^{S6K}$, and tyrosine kinase such as Ack (activated-Cdc42-associated tyrosine kinase) (Cotteret and Chernoff, 2002). RhoA also signals to ROCK and mDia (mammalian Diaphanous-related forming protein) (Frame and Brunton, 2002). Both ROCK stimulated by RhoA, and p21-activated kinase (PAK1) stimulated by Rac-1, can phosphorylate and thereby activate LIM kinase, which in turn phosphorylates and inactivates cofilin (an actin depolymerizing protein), thus leading to promotion of actin filament assembly (Ridley, 2001). On the other hand, activation of Rac1 and CDC42 can be linked to actin polymerization, though mediation by WASP, WAVE adaptor proteins, and the Arp2/3 complex in forming actin filaments (Ridley, 2001; Takenawa and Miki, 2001). During branching of actin filaments via the Arp2/3 complex, c-

Src-mediated activation of cortactin is required for the bridging of the Arp2/3 complex to actin filaments (Weaver *et al*., 2002). It is now clear that activation or deactivation of Rho GTPase family members when cells adhere to ECMs is a key event for localized control of actin filament assembly, contractility, and cell motility.

In addition to their roles in actin filament reorganization, Rho GTPases have been shown to be important for cell growth, cell cycle progression and differentiation (Danen and Yamada, 2001; Moon and Zheng, 2003). Interestingly, activated GTPases are shown to regulate cell cycle progression, by modulating the levels of the G1 phase cyclin, cyclin D, transcriptionally or translationally, or the induction timing of the cyclin D during the G1 phase (Danen and Yamada, 2001; Mettouchi *et al*., 2001; Renshaw *et al*., 1996; Welsh *et al*., 2001). Rac1 activation leads to an enhanced transcription of cyclin D (Page *et al*., 1999), via NF-κB transcription activity in fibroblast cells (Joyce *et al*., 1999), or alternatively regulates cyclin D level at the translational level following activation of Shc and FAK, through mediation of SOS and PI3-K (Mettouchi *et al*., 2001). In addition, Rho is involved in the accumulation of cyclin D protein by fibronectin and at the same time reduction of $p21^{Cip/Waf}$ was observed (Danen *et al*., 2000). Interestingly, Welsh *et al*. (2001) reported that RhoA inhibited an alternative Rac/Cdc42-dependent pathway, which resulted in a strikingly early G1-phase expression of cyclin D1. Cyclin D1 was induced in mid-G1 phase because a Rho switch couples its expression to sustained ERK activity rather than Rac-1 and Cdc42, allowing the correct timing of cyclin D1 expression in G1 phase. Evidences have also been reported that an intact actin cytoskeletal network integrity is also critical for G1/S transition; demolishing of the network by a pharmacological reagent inhibited G1/S transition (Huang and Ingber, 2002). On the other hand, Rho family GTPases, GEFs, and effectors were shown to involved in cell growth regulation (Bishop and Hall, 2000). For example, RhoGAP has been shown to be involved in cell growth and differentiation, by virtue of affecting central spindle formation in the cytokinesis step of cell division (Mishima *et al*., 2002), and its signaling linkage to RhoA-ROCK-insulin receptor substrate (IRS)-CREB (CRE-binding factor) (Sordella *et al*., 2002). Recently, it was reported that disruption of stress fiber formation by inhibition of MLCK blocked sustained Erk1/2 signaling, leading to G1-arrest, whereas comparable disruption of the cytoskeleton by inhibition of ROCK blocked Erk1/2 signaling but led to a more rapid progression through G1 phase by virtues of cyclin D1 induction and Cdk4 activation via Rac1/Cdc42 (Roovers and Assoian, 2003). This study indicates that ROCK-dependent stress fiber formation is required for sustained Erk1/2 signaling and mid-G1 cyclin D1 induction, but not for Cdk4 or Cdk2 activation, so that G1 cell cycle progression does not require stress fiber formation as long as Rac1/Cdc42 signaling is available for cyclin D1 induction. However, it was also reported that inhibition of Rho prevents induction of cyclin D1 (Hansen and Albrecht, 1999). Therefore, whether the Rac1/Cdc42 signaling is available to induce cyclin D1, may be a factor to decide how Rho and/or ROCK signaling affects cyclin D1 induction.

Hyperactivity of PAK, a major downstream effector of Rac1/Cdc42, appears to contribute cell proliferation in human breast cancer-derived cell lines and certain cancers (Mira *et al*., 2000; Obermeier *et al*., 1998). PAK was also shown to regulate Raf-1 activation via phosphorylation of Ser338 and thus Erk1/2 activation during growth factor and integrin stimulation (Chaudhary *et al*., 2000; Zang *et al*., 2002). Very recently, the stimulatory effects of PAK1 on cyclin D1 expression in mammary epithelial and cancer cells has been shown dependent on NFκB pathways, in terms of stimulated cyclin D1 promoter activity, increased levels of cyclin D1 mRNA and protein, and nuclear accumulation of cyclin D1, which were blocked via inhibitory approaches including an autoinhibitory peptide or short interference RNA (Balasenthil *et al*., 2004).

While Rho GTPases and effectors are involved in cell cycle progression and cell growth through regulation of cyclin levels, not only integrin-mediated activation of GTPases but also mitogenic stimulation of signal activation (e.g., Erk1/2) will be required, because integrin-mediated activation of signaling alone is not sufficient for cell cycle traverse.

## Integrins signaling collaborative with growth factor receptors

Most cells are anchorage-dependent for growth, meaning that proliferation does not occur unless the cells are attached to ECMs by integrins. The presence of soluble growth factors such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) is not sufficient for cell proliferation; additional signaling from integrin-mediated cell adhesion is also necessary. By virtue of collaborative signaling by integrins with other cell surface receptors, such as receptor tyrosine kinases (RTKs) or G-protein coupled receptors (GPCRs), integrin-mediated cell anchorage has been observed to have profound effects, in terms of amplitude, duration, and/or spatial aspects on signaling processes. The consequence of this collaborative signaling will be progression of G1 and G1-S transition, since mitogen- and cell adhesion-dependent signal activities including Erk1/2 activation, result in sequential accumulation of cyclins (D1, E, and A), and activation of CDKs (cyclin-dependent kinases) leading to Rb phosphorylation and thereby de-repression of E2F transcription factor (Danen and Yamada, 2001). This relationship lasts until the completion of Rb phosphorylation and inhi-

bition of E2F activity by cyclin A-Cdk2 complex, when cells have passed the restriction point and are mitogen-independent (Pardee, 1989).

There are several known intracellular signaling molecules that are activated synergistically by both integrins and growth factor receptors. They include the Ras/Raf-1/MEK/Erk1/2 pathway, Rho family GTPases, PI-3-K, ribosomal S6 kinase (RSK), Jun amino-terminal kinase (JNK), FAK and Paxillin, and $p130^{CAS}$ [reviewed in (Comoglio *et al*., 2003; Giancotti and Tarone, 2003; Schwartz and Ginsberg, 2002; Yamada and Even-Ram, 2002)]. Cell adhesion and intact cytoskeletal network integrity (as explained earlier) are important for cell growth as well as for architectural aspects of cell-cell or cell-ECM contacts. Evidence has been accumulated to show that integrin-mediated signaling can influence RTK-initiated signaling including the Ras/Raf1/MEK/Erks cascade (Giancotti and Tarone, 2003; Yamada and Even-Ram, 2002). So far, diverse evidences of mechanisms underlying adhesion-dependent Erk1/2 activation by growth factors have been accumulated (Danen and Yamada, 2001; Howe *et al*., 2002).

**Regulation of Ras/Raf-1/MEK/Erks cascade by integrin co-signaling with growth factor receptors** Evidence of dependency of growth factor-mediated cell proliferation, in cultured model systems, on specific integrin-mediated adhesion include stimulation of cell growth with EGF, PDGF, insulin, and VEGF as explained earlier (Eliceiri, 2001). Growth factors trigger signals leading to cell proliferation via the Ras/Erks cascade and induction of cyclin D during early to mid G1 phase (Albanese *et al*., 1995; Lavoie *et al*., 1996; Sherr, 1994). Whereas growth factor-mediated signaling activates Erk1/2 in a transient and relatively modest manner, adhesion-mediated signaling causes a weak activation of Erk1/2. Therefore, activation of Erk1/2 is combined by both integrin- and growth factor receptors-mediated signaling pathways for transcription of cyclin D (Roovers *et al*., 1999). So far, evidence of several mechanisms underlying adhesion-dependent Erk1/2 activation by growth factors have been accumulated (Danen and Yamada, 2001; Howe *et al*., 2002).

Growth factor binding to their receptors results in clustering of the receptors and autophosphorylation of tyrosine residues in the cytoplasmic tails of the receptors, which leads to formation of binding sites for diverse SH2-domain containing adaptor proteins such as Shc and/or Grb2, and to activation of the Ras/Raf1/MEK/Erks cascade (Olayioye *et al*., 2000; Riese and Stern, 1998; Schlessinger, 2002). Adhesion dependency of Erk1/2 activation by growth factor indicates that additional signaling via integrin-mediated adhesion must impinge in the growth factor-mediated signaling for cells to maintain biological functions such as proliferation. Anchorage-dependent regulation of the Ras/Raf-1/MEK/Erks cascade appears clearly dependent on the actin-cytoskeleton, since disruption of the focal adhesions and stress fibers by cytochalasin D treatment at a lower concentration did not affect Erk1/2 activation by growth factors (Aplin *et al*., 1999a). However, in the same study Cdc42-dependent promotion of cortical actin assembly was shown important for the Erk1/2 activation, indicating that integrin-mediated cell adhesion and subsequent actin cytoskeleton organization can regulate upstream to downstream coupling of Ras/Raf-1/MEK/Erks intracellular signaling cascade. It was also reported that cell adhesion could regulate trafficking of activated Erk1/2 from cytosol to nucleus (Aplin *et al*., 2001). Thus, in suspension cells, or in cells treated with cytochalasin D, the normal nucleus translocation of activated Erk1/2 did not occur because of disruption of the cytoskeleton. Therefore, even enforced activation of Erk1/2 by active Raf-1 or MEK did not allow translocation of active Erk1/2 to nucleus and phosphorylation of its target, Elk-1 (Aplin *et al*., 2001).

**Cross-talks between integrins- and growth factor receptors** The collaboration between signaling by integrin engagement and signaling by growth factors is required to promote efficient activation (and autophosphorylation) of RTKs, such as the EGF, PDGF, and FGF receptors (Juliano, 2002; Yamada and Even-Ram, 2002). The mechanisms of activation of growth factor receptors-mediated signaling in collaboration with integrins were shown to follow the typical paradigm for activation of growth factors-mediated signaling. That is, although transmembrane integrin and growth factor receptors are induced to cluster by matrix proteins, intracellular signaling mediated by growth factor receptors, including Erk1/2 activation, depends on binding of the growth factor (Miyamoto *et al*., 1996). A study showed that human foreskin fibroblasts on vitronectin, a ligand for the $\alpha_v\beta3$ integrin, display enhanced growth and MAPK activation in response to PDGFRβ, compared to cells cultured on non-$\alpha_v\beta3$ ligands (Schneller *et al*., 1997). Further, an association between a highly phosphorylated fraction of the PDGFRβ and $\alpha_v\beta3$ integrin was promoted by PDGF, indicating a direct effect of ECM on the RTK signaling pathway. It was previously reported that integrin $\alpha5\beta1$, but not $\alpha2\beta1$, expression in normal rat intestinal epithelial cells allows preferential survival of cells during serum deprivation through differential activation of PKB/Akt, but not Erk1/2 (Lee and Juliano, 2000), and that this effect was possibly via the differential activation of EGFR signaling to the PI-3-K/PKB survival pathway, but equal activation of Erk1/2. This occurred through differential formation of signaling complexes including activated EGFR, ErbB3, p85 regulatory domain of PI3-K, and integrin $\alpha5$ subunit (Lee and Juliano, 2002). On the other hand, the association may result in dephosphorylation of the tyrosine residue of

PDGFR, where Ras-GAP binds, thus inhibiting of Ras-GAP and potentiating Ras signaling for mitogenic stimulation (DeMali *et al*., 1999). Therefore, it is possible that the intensity and duration of EGFR transactivation and subsequent MAPK (Erk1/2) (or other downstream effectors) activation presumably via a specific assemblage of integrin subtypes and growth factor receptor types represent a decision point between cell proliferation and alternative pathways such as cell survival.

**Cross-talks downstream of integrins- and growth factor receptors** Collaboration exists between integrins and growth factor receptors at the receptors level, as explained above. In addition, collaboration takes places downstream of the receptors. Integration between the ECM- and EGFR-derived signaling pathways lies downstream of EGFR autophosphorylation and at, or upstream of, Raf-1 activation (Moghal and Sternberg, 1999). In cases where integrin signaling regulates the coupling between upstream and downstream components in the Ras/Raf-1/MEK/Erks cascade, the regulation steps were diverse, such as from Ras to Raf-1 (Lin *et al*., 1997a), from Raf-1 to MEK (Renshaw *et al*., 1997), upstream of Raf-1 (Le Gall *et al*., 1998), association of RasGAP to RTK (i.e., upstream of Ras) (DeMali *et al*., 1999), and nuclear translocation of activated Erk1/2 (Aplin *et al*., 2001). On the other hand, growth factor stimulation has been shown to result in activation of signaling molecules downstream of integrin, at the level of integrin subunit itself (Mariotti *et al*., 2001; Trusolino *et al*., 2001), FAK (Ivankovic-Dikic *et al*., 2000; Sieg *et al*., 2000), or Src kinases (Eliceiri *et al*., 2002; Mariotti *et al*., 2001). This diversity may be due to differences in cell types and signaling properties.

Presumably indirect interaction between EGFR or PDGFR and integrins via a complex including FAK (*N*-terminus binding to RTKs), paxillin (binding to α4 integrin subunit), and talin (binding to integrin β1 subunit) has also been reported leading to activation of Erk1/2 (Renshaw *et al*., 1999). Recently, it was shown that integrins $\alpha_v\beta3$ and αvβ5 differentially regulate the Ras-Raf1-Mek-Erk1/2 pathway (Hood *et al*., 2003). In this study, VEGF- and αvβ5-mediated Erk1/2 activation appears to involve c-Src and to regulate upstream of Ras, whereas bFGF- and $\alpha_v\beta3$-mediated Erk1/2 activation seems to involve FAK and PAK1-mediated phosphorylation of Raf-1 $Ser^{338}$ and to regulate downstream of Ras but upstream of Raf-1. Therefore, it appears obvious that formation of direct or indirect complexes between specific RTKs and the integrins subtypes could lead to enhanced opportunities for RTK dimerization and cross-phosphorylation of specific signaling pathways depending on the cells' needs.

**Integrin co-signaling with growth factor receptors without growth factors lignading** There are several examples that integrin mediated regulation of RTK activation occurs in the absence of growth factors including ones with PDGFβ receptor (Sundberg and Rubin, 1996), Met (Wang *et al*., 1996), EGFR (Moro *et al*., 1998), and VEGFR (Wang *et al*., 2001). In the system of EGFR, integrins and growth factor receptors are clustered by integrin interaction with its ECM and form a signaling complex that is dependent on c-Src. This complex recruits $p130^{CAS}$ and Crk, but not FAK, resulting in EGFR phosphorylation (at tyrosine residues different from those by EGF ligand binding) and thus Erk1/2 activation even without EGF (Moro *et al*., 1998; 2002). Importantly, Moro *et al*. (1998) showed that adhesion-induced EGFR activation does not induce cell proliferation, but was shown to protect EGFR-transfected NIH3T3 cells from serum deprivation-mediated apoptosis. Also in the study by Trusolino L and Comoglio PM (Trusolino *et al*., 2001), introduction of an activating mutation or overexpression of growth factor receptor could induce tyrosine phosphorylation of β4 integrin subunit even without its ECM binding.

## Concluding remarks

The interaction of cells via integrins to extracellular matrix proteins has profound effects on diverse cellular processes. Integrins seem to provide not only structural bridges between extracellular matrix proteins and intracellular actin cytoskeleton, but also signal transduction, either by integrins alone or in collaboration with other receptors such as those for growth factor. Signaling relays via protein-protein interactions and actin cytoskeleton rearrangements are important for tight regulation, leading to proper maintenance of cellular functions such as cell proliferation. Therefore, understanding the various process regulated by integrins may have critical impacts on approaches for the cure of human diseases driven by abnormal proliferation such as cancer.

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